PARTIAL PURIFICATION AND CHARACTERIZATION OF DIHYDRODIPICOLINIC ACID SYNTHETASE FROM SPORULATING BACILLUS MEGATERIUM KM

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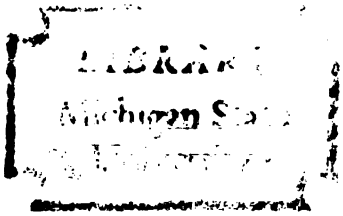
THESIS



This is to certify that the

thesis entitled

Partial Purification and Characterization of
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R. V. Lechowich
Major professor

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ABSTRACT

PARTIAL PURIFICATION AND CHARACTERIZATION OF DIHYDRODIPICOLINIC ACID SYNTHETASE FROM SPORULATING BACILLUS MEGATERIUM KM

By

Francis H. Webster

Sporulating cells of Bacillus megaterium Km ATCC 13632 produced in a 0.4% glucose, 0.2% casamino acids, and GB minerals medium buffered at pH 7.0 were used as the source of dihydrodipicolinic acid synthetase. A synchronous culture technique employing three 10% v/v transfers was used in the production of sporulating cells. The culture was harvested when 60% of the cells contained spore forms. Dihydrodipicolinic acid synthetase was purified 150-fold by ammonium sulfate fractionation at pH 7.5, heating at 45 C at pH 6.0, ammonium sulfate fractionation at pH 6.0, and subsequent DEAE cellulose chromatography. During the final stage of the purification procedure the enzyme exhibited sensitivity to refrigeration temperatures. The enzyme had a pH optimum at 7.65 in imidazole buffer. The apparent Km values were 4.6×10^{-4} and 5.0×10^{-4} for β-aspartyl semialdehyde and pyruvate, respectively. All attempts to demonstrate cofactor requirements were unsuccessful. Sulfhydryl inhibiting reagents and lysine

did not inhibit the enzymatic reaction. The enzyme exhibited maximum thermal resistance at pH 10.5. The thermal stability of the enzyme at 75 C was increased more than 1800-fold by the addition of 0.3 molar pyruvate. The E_a was 67,300 cal per mole for the thermal denaturation of the enzyme. At 60 C the $\Delta F\dagger$, $\Delta H\dagger$, and $\Delta S\dagger$ for the thermal denaturation of the enzyme were 66,700, and 22,250 cal per mole and 133 cal per mole per degree, respectively.

PARTIAL PURIFICATION AND CHARACTERIZATION OF DIHYDRODIPICOLINIC ACID SYNTHETASE FROM SPORULATING *BACILLUS MEGATERIUM* KM

By

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DEDICATION

To Charlene and Bobby

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INTRODUCTION

The problems of food preservation were of primary concern to mankind long before the advent of modern technological processes. Even in the twentieth century seasonal production of various commodities and extreme time and distance factors complicate the task of presenting a high quality and nutrious product while maintaining appropriate esthetic qualities. Food preservation has three basic principles; prevention or delay of self decomposition, prevention or delay of microbial decomposition, and prevention of damage because of insects, animals, and mechanical causes (21). Asepsis is a very important principle; however, due to natural microbial contaminants other methods must be applied to each product in order to control microbial growth. Removing microorganisms, hindering the growth and activity of microorganisms, or killing of the microorganisms are the general techniques employed in controlling microbial decomposition of food products. The type of raw material, economic considerations, and the esthetic and nutritional quality of the final product dictate the methods used for processing the food material. Destruction of

microorganisms by high temperature treatments is one of the classical and most widely practiced methods of food preservation. Thermal processes are first directed toward elimination of public health hazards and secondly are oriented by economic considerations. Cells and spores of microorganisms vary widely in their resistance to high temperatures. The most heat resistant forms are the bacterial endospores. The heat resistance of the various species ranges from a fraction of a minute to greater than 20 hr at 100 C (21). The heat resistance of the spores of any one species is dependent upon the nutrient source, temperature, pH, mineral content, and the oxidation-reduction potential of the growth medium. Conditions during heating and subculture are also important in determining heat resistance. The exact nature of the heat resistance mechanism is not known; however, maximum heat resistance is dependent upon the production of "normal" stable spores. Normal spores contain approximately 4 to 15% dipicolinic acid (DPA) on a dry weight basis (26). Spores which have a significantly lowered DPA content are heat sensitive and may germinate spontaneously. Dipicolinic acid is not the sole determining criterion for heat resistance, but certain minimum levels appear to be essential for the spores to exhibit normal thermal stability properties. The biosynthetic pathway of this unique compound was only recently partly

resolved (1). The enzymes responsible for DPA synthesis in sporulating organisms had not been isolated and characterized previously. This study was designed to isolate and characterize dihydrodipicolinic acid synthetase, the initial enzyme in the biosynthetic pathway, in order to partially resolve the biochemical events involved in the production of this unusual biological compound. Bacillus megaterium KM was used as the enzyme source in this study. This organism was chosen because it sporulated well under a variety of conditions and thereby provided a model system which had sufficient flexibility to accommodate necessary experimental manipulations. It is believed that the findings obtained using this microorganism might be applied to other sporulating bacilli, including food spoilage microorganisms.

LITERATURE REVIEW

Dipicolinic acid (DPA) or 2,6-dicarboxypyridine is a unique biological compound found within the spores of all species of Bacillus and Clostridium analyzed and in spores of Sporosarcina ureae. The only other reported occurrence of DPA is as a metabolic byproduct of Penicillium citreo-viride (51). Dipicolinic acid is not incorporated into the cellular contents of P. citreo-viride, however.

Halvorson and Howitt (26) listed the following characteristics of DPA: (1) strong chelating agent, (2) occurs only in bacterial endospores, (3) present in high concentrations (4 to 15% of the dry weight) in the spore, (4) synthesized just prior to the formation of the heat resistant spore, and (5) is completely lost during germination.

Dipicolinic acid was first isolated and identified in 1953 (56). Since that time, considerable research effort has been directed toward characterization of the biological function of DPA, identification of the in vivo chemical state of DPA, demonstration of the location of DPA within the endospore, and determination of the biosynthetic pathway of DPA.

Relationship of Dipicolinic Acid to the Physiological and Morphological Processes of Sporulation

The sporulation process of bacterial species belonging to the family Bacillaceae results in the formation of a single spore within a sporangium derived from a single vegetative cell. This spore is resistant to heat, radiation, desiccation, staining, and various other physical and chemical treatments. The spore is also highly refractile to light, due to the high refractive index of the spore components, as evidenced by phase contrast microscopy. The spore is an essentially dormant state in the life cycle of spore-forming bacteria wherein the spore remains viable for an indefinite period.

The sporulation process has been divided into seven stages (17, 65). Several studies have correlated the seven stages of sporulation with the attainment of the various chemical and physical properties characteristic of the spore (17, 65, 80). The terminology suggested by Murrell (45) will be used to describe the sporulation process. The end of the exponential growth phase is arbitrarily defined as the initiation of stage 1 (preseptation) of the sporulation process. Stage 2 (septation) is characterized by invagination of the plasma membrane to form the spore septum. The appearance of refractile forespores commences late in stage 3 (protoplast development) and is completed during stage 4 (cortex formation).

Calcium uptake and DPA synthesis are initiated during stage 4 and completed by the end of stage 5 (spore coat formation). Graphical representation of Ca++ uptake and DPA synthesis describes the parallel nature of these phenomena. The development of heat resistance begins in stage 5 and is completed during stage 6 (maturation). The initial appearance of heat resistant forms follows DPA synthesis by about 2 hr. Lysis of the sporangia followed by the release of the mature spores into the medium occur during stage 7.

Vinter (73) followed the formation of disulfide (S-S)-rich structures and calcium accumulation in sporulating Bacillus megaterium. Formation of S-S rich structures occurred prior to calcium uptake and at the same time as radiation resistance was detected in the sporulating culture. Therefore, acquisition of radiation resistance and the formation of S-S structures probably occur in stage 3.

Location of Dipicolinic Acid in the Spore

The cortex and protoplast have been suggested as possible locations of DPA in the spore. Dipicolinic acid synthesis and Ca++ uptake at the time of cortex formation suggest that DPA and Ca++ become incorporated in the cortex (58). Mayall and Robinow (43) concluded that the cortex was the probable location of these materials due

to the rapid release of Ca++ and DPA and the concurrent cytological disappearance of the cortex during germination.

However, microincineration studies (38, 71) have indicated that the major ash components of the spore are located in the protoplast. Assuming Ca++ is complexed with DPA in the spores, DPA would then be located in the protoplast. The experiments of Donnellan and Setlow (14) support the results of the microincineration studies. Similar unidentified photoproducts were observed in hydrolyzed deoxyribonucleic acid from ultraviolet irradiated spores and in irradiated DNA previously dried in the presence of Ca-DPA or metal salts.

Chemical State of Dipicolinic Acid in the Spore

The chemical state of DPA in the bacterial spore has not been conclusively resolved. Dipicolinic acid is usually extracted as a calcium dipicolinate (Ca-DPA) chelate (58). However, the extraction of various other metal DPA chelates from the spore has been reported (67). The metal chelate of DPA found in the spore is dependent upon the divalent metal cations supplied in the growth medium (20, 67). Dipicolinic acid and Ca++ have been postulated to occur in a 1:1 molar ratio in the chelate (61); however, the Ca++:DPA ratio is not always close to unity (46). "Whether the divergence results from partial replacement of Ca++ by other metals, analytical errors,

or unknown factors is not known" (46). Windle and Sacks (76) studied the electron paramagnetic resonance spectra of spores produced in a Mn++ rich medium. Spectra similar to that of the manganese dipicolinate chelate were observed in spores prior to but not after germination and autoclaving. Baily, Karp, and Sacks (2) examined the ultraviolet spectra of dry spores embedded in potassium bromide. Utilization of reference pellets containing only the analogous spore coat fractions enabled them to produce spectra which contained the three characteristic peaks of Ca-DPA. They concluded that some type of Ca-DPA chelate accounted for at least part of the Ca++ and DPA present in the spore.

Dipicolinic acid has been observed in combination with amino acids in spore extracts. Young (79) proposed that DPA could be complexed with proteins and/or nucleic acids in the spore, thus providing a means of protein or nucleic acid stabilization. Isolation of the monoethyl ester of DPA has been reported (30, 54), but it accounted for only 1% of the total DPA extracted. However, sufficient ethyl groups were demonstrated to account for the possibility of an esterfied form of DPA in the spore. These investigators concluded that DPA may exist in an esterfied form in the spore and the extraction conditions could have caused hydrolysis of the ester linkage.

Functions of Dipicolinic Acid

Numerous studies have led to several theories concerning the intrinsic biological function of DPA. Halvorson and Howitt (26) classified the proposed functions of DPA as specific or nonspecific depending upon the mode of action of DPA. The thermal resistance of bacterial spores was proposed to be the result of a nonspecific stabilization of protein by removal of water. Calcium accumulation, stimulation of electron transport, enzyme stabilization, maintenance or dormant enzyme systems and stimulation of germination were listed as possible specific functions of DPA.

Thermal Resistance

Thermal resistance is acquired after DPA synthesis and Ca++ accumulation and is concurrently lost with Ca++ and DPA during the germination process. The relationship between Ca++, DPA and thermal resistance has been extensively studied (3, 10, 26, 68). Several investigators (3, 7, 26) have demonstrated that spores deficient in Ca++ have reduced heat resistance. Also, spores produced in a medium deficient in Ca++ or other divalent metal cations had less than normal DPA concentrations (3, 68).

Church and Halvorson (9) reported a direct relationship between heat resistance and DPA content in spores of *Bacillus cereus* T made deficient in DPA by alterations

of the growth medium. A similar correlation was reported for B. cereus T spores produced endotrophically in the presence of varied amounts of Ca++ (3). Wooley and Collier (78) reported that there appeared to be a positive relationship between Ca++, DPA, and thermal resistance in Clostridium roseum.

Conversely, spores of Cl. roseum produced in the presence of L-alanine contained reduced levels of DPA but were more heat resistant than DPA-rich spores produced in a medium lacking added L-alanine (7).

Lechowich and Ordal (39) and Levinson et al. (40) found no obvious agreement between DPA and thermal resistance and calcium and thermal resistance. However, their results suggested a positive agreement between the Ca:DPA ratio and thermal resistance.

Murrell and Warth (47) correlated DPA content with heat resistance for twenty different species of Bacillus and reported a 700-fold variation in heat resistance. The DPA content of the spores varied from 5 to 13.5% in a random manner with no apparent relationship to the heat resistance.

Wise et al. (77) reported the isolation of a B. cereus mutant which produced spores devoid of DPA. The mutant spores were stable at 70 C, while the wild type exhibited thermal stability at 95 C. In contrast, the vegetative cells were easily destroyed at 55 to 60 C.

Addition of DPA to the sporulation medium permitted the mutant to incorporate a portion of the DPA, and the resultant spores were completely stable at 80 C.

Calcium Accumulation

Since the synthesis of DPA precedes the formation of heat stable spores, follows the appearance of spore forms, and occurs during the same stage of sporulation as Ca++ accumulation, Halvorson and Howitt (26) proposed that DPA might be necessary for Ca++ accumulation.

In contrast, Black et al. (3) suggested Ca++ might be involved in spores produced endotrophically in a calcium deficient medium supports this hypothesis (3). Foerster and Foster (20) concluded that whether "accumulation of divalent metal cations is the cause of efficient DPA biosynthesis or the result of it is still a matter of conjecture."

Maintenance of Dormancy

Reiman (61) proposed that DPA and calcium are bound in a chelate involving Ca-DPA and protein (spore structure and/or spore enzymes) which could be responsible for the maintenance of dormancy. The enzyme systems in this case would be reversibly inactivated by complexing with the Ca-DPA chelate. A similar proposal was put forth by Halvorson and Howitt (26). These proposals were based on the observation that a number of enzyme systems which

are inactive in the dormant spore are activated by germination or physical and chemical treatments which bring about the release of DPA (27, 29). However, in low-DPA heat sensitive spores, the dormancy of the glucose oxidation system is unchanged (26).

Stabilization of Spore Enzymes

The demonstration that Ca-DPA complexed with amino acids and possibly proteins (79) led Halvorson and Howitt (26) to propose that Ca-DPA-protein complexes could be a vital factor in the stabilization of spore enzymes.

Mishiro and Ochi (44) reported that DPA protected serum albumin from heat denaturation. The heat stability of Bacillus subtilis spore glucose dehydrogenase was observed to be enhanced by 0.5 M DPA (24). Vary and Halvorson (72) found 0.001 M DPA conferred heat stability upon a purified "initiator" protein. Conversely, Powell (57) reported that DPA failed to protect adenine deaminase against thermal inactivation.

Stimulation of Germination

Initially, Reiman and Ordal (62) reported that Ca++ and DPA in equimolar quantities could induce germination. Keynan and Halvorson (36) reported on the effect of Ca-DPA germination of B. cereus T. They suggested that Ca-DPA added exogenously acted as endogenously released DPA and that it had two functions: (1) to drive the

L-alanine dehydrogenase reaction and (2) to induce metabolically dependent germination, independent of the L-alanine trigger mechanism.

Correlation of DPA content to germination was investigated in a related study (37). When the DPA content of B. cereus T was reduced from 7.5% to 2 or 3%, the spores germinated spontaneously after heat activation and were sluggish in response to all germination stimulants except Ca-DPA.

Jaye and Ordal (33) studied the effects of various metal-DPA chelates upon germination. They concluded that the initiating event in germination induced by metal dipicolinates was physiochemical in nature due to the high threshold levels required to initiate germination and the lack of specificity for the divalent metal cation. A similar conclusion was reached by Keynan et al. (35).

Foerster and Foster (19) investigated the response of spores to various germination stimulants. Because of the efficient substitution and even superiority of various anions and cations for the metal salts of DPA, they concluded that the effect of DPA on germination was as a nonspecific ion rather than a specific chelation phenomenon.

Stimulation of Electron Transport

Harrell (28) reported that DPA stimulated the glucose oxidation system of Bacillus spores. In a preliminary

study, Halvorson et al. (25) found that DPA stimulated the nicotinamide adenine dinucleotide ($NADH_2$) cytochrome C reductase and diaphorase of spores and vegetative cells. More recently Doi and Halvorson (13) demonstrated that DPA stimulated the soluble $NADH_2$ oxidase of spores by serving as a cofactor for the enzyme. Since the soluble oxidase previously had been found to be the primary pathway of electron transport in spores (12), these authors concluded that DPA could play an important role in dormancy and morphogenesis. In contrast, Pepper and Costilow (52) reported that DPA had no effect upon the soluble $NADH_2$ oxidase of Bacillus popilliae.

Biosynthesis of DPA

Several pathways for biosynthesis of DPA have been proposed. The initial theory was that DPA, a heterocyclic nitrogen-containing compound, might be formed through ring closure of the corresponding aliphatic compound. Perry and Foster (53) investigated the possibility of a direct conversion of diaminopimelic acid (DAP) to DPA. However, labeling studies revealed that only 4% of the DPA carbon was derived from DAP and that a significant amount of labeled carbon was observed in a number of amino acids. Powell and Strange (59) demonstrated that extracts of sporulating cells and whole cells were unable to synthesize DPA from DAP. Martin and Foster (42) conducted isotope competition experiments which indicated

that DAP was not a key intermediate in the formation of DPA. Their labeling experiments demonstrated a high rate of conversion of glutamate, aspartate, alanine, proline, and serine to DPA. They found exogenously added CO_2 comprised less than 5% of the DPA carbon and concluded that this amount was insignificant. Analysis of the labeling pattern existing in the pyridine and carboxy moieties indicated that DPA was formed from a $C_3 + C_4$ condensation. Aspartate and pyruvate or alanine and oxalacetate or their derivatives were proposed to be involved in the condensation reaction. The studies of Hodson and Foster (31) and Kanie and Foster (34) were consistent with the $C_3 + C_4$ condensation proposed by Martin and Foster (42).

Powell and Strange (59) observed the nonenzymatic formation of DPA from diketopimelic acid and ammonia in the presence of nonspecific bacterial homogenates or quinones. These authors were unable to detect the precusor, diketopimelate, in the cell free extracts. However, Tannenbaum and Kaneko (70) reported the isolation of diketopimelate from Penicillium citreo-viride.

Dihydrodipicolinate, formed by the condensation of β-aspartyl semialdehyde and pyruvate, was demonstrated by Yugari and Gilvarg (82) to be an intermediate in the lysine biosynthetic pathway. This important discovery indicated that DPA could be formed by a modification of the lysine pathway. Bach and Gilvarg (1) demonstrated

the synthesis of DPA from pyruvate and β-aspartyl semialdehyde in cell-free extracts of sporulating B. megaterium. Chasin and Szulmajster (8) investigated the formation of DPA in cell-free extracts of sporulating B. subtilis and B. megaterium. Purified dihydrodipicolinic acid synthetase from Escherichia coli was added to cell-free extracts of the sporulating Bacillus. Both enzymatic and nonenzymatic syntheses of DPA were detected. The nonenzymatic synthesis was stimulated by Mn++ and completely inhibited by 5×10^{-3} M ethylenediaminetetraacetic acid (EDTA). The enzymatic synthesis was found to require NAD+ or FAD as a cofactor.

The above results indicate that the synthesis of DPA proceeds via the following reactions:

Pyruvate + β-aspartyl semialdehyde

Dihydrodipicolinic acid

Dihydrodipicolinic acid + NAD or FAD

Dipicolinic acid + $NADH_2$ or $FADH_2$

Enzyme Characteristics

Dihydrodipicolinic acid synthetase has been isolated and characterized (69, 82), but at the present time DPA dehydrogenase has been demonstrated only in crude cell-free extracts. E. coli dihydrodipicolinic acid synthetase had a pH optimum of 8.4 in tris buffer (82). The Michaelis-Menten constants were 1.3×10^{-4} and 2.5×10^{-4} for β-aspartyl

semialdehyde and pyruvate, respectively. No cofactor requirements were detected.

MATERIALS AND METHODS

Protein Analysis

The methods of Warburg and Christian (74) and Lowry *et al.* (41) were used to determine protein concentration. The Warburg and Christian analysis was conducted without modification. The technique of Lowry *et al.* was modified in two instances. The Folin phenol reagent was diluted 1:1 (v/v) with distilled water and separate stock solutions of sodium tartrate and cupric sulfate were prepared and then mixed just prior to use. A standard curve, using bovine serum albumin as a reference protein, was prepared over a concentration range of 15 to 150 µg per assay. The abosrbance at 520 mµ was determined in a Beckman DBG spectrophotometer (Beckman Instruments, Fullerton, California).

Preparation of DEAE Cellulose

Diethylaminoethyl (DEAE) cellulose columns were prepared according to the methods of Petersen and Sober (55). The columns were repacked with fresh DEAE cellulose after each chromatographic separation.

Preparation of Bio-Gel Columns

Bio-Gel P-2 and P-200 (Bio-Rad Laboratory, Richmond, California) columns were prepared according to the methods of Flodin (18). After the column was packed at least three column volumes of buffer were allowed to pass through the gel to assure proper equilibration and to obtain a constant height of the packed material. The packed columns were checked with blue dextran 2000 for uniformity of packing and to determine void volumes. The columns were washed free of residual materials after use with several volumes of buffer.

Dipicolinic Acid Analysis

Dipicolinic acid analyses were conducted by a modification of the Janssen, Lund and Anderson colorimetric technique (32). A sufficient amount of sporulating cells to obtain a positive reading or 5 to 15 mg (dry weight) of spores in 15 to 20 ml of distilled water were added to a 25 ml volumetric flask. The flask was loosely capped and heated 1 hr in an autoclave at 121 C. After heating, the flask was cooled and 4 ml of a 4% trichloracetic acid solution was added to precipitate interferring materials. The flasks were placed at 5 C for 1 hr to allow complete precipitation. The contents of the flask were brought to volume by the addition of distilled water. The precipitate was removed by centrifugation at 10,000xg for 20 min. The supernatant fluid was decanted and an

aliquot was used for the colorimetric analysis as described by Janssen et al. (32).

Determination of Percent Sporulation

Culture samples were observed with an American Optical microscope (American Optical Co., Buffalo, New York) with phase contrast optics at 1000 x mignification. All phase dark and phase bright spore forms were counted as positive indices of sporulation. Percent sporulation was computed according to the following equation:

$$\frac{\text{Cells containing spore forms}}{\text{Total number cells counted}} \times 100 = \text{Percent sporulation}$$

Preparation of β-Aspartyl Semialdehyde

β-Aspartyl semialdehyde (BAS) was prepared by ozonalysis of dl-allyglycine (Nutritional Biochemical Co., Cleveland, Ohio) and purified by chromatography on Dowex 50 (hydrogen form) as described by Black and Wright (4). The purified product was stored at -20 C.

Preparation of Homoserine Dehydrogenase

Growth of Saccharomyces Cerevisiae

Saccharomyces cerevisiae cells were grown in a 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1.0% glucose medium. Three liter Fernbach flasks containing 1 liter of medium were inoculated with 5 ml of an 18 hr culture. The culture was incubated at room temperature

on a New Brunswick model V rotary shaker (New Brunswick Scientific, New Brunswick, New Jersey) set at 200 RPM. The cultures were harvested at 18 to 24 hr by centrifugation at 5000xg for 15 min. The cells were suspended in 0.01 M potassium phosphate pH 6.5, washed twice, and stored at -20 C.

Breakage of Yeast Cells

Thirty milliliters of yeast cells and 20 ml of 0.10 to 0.11 mm (dia) glass beads (Bronwill Scientific, Rochester, New York) were placed in a 75 ml mechanical homogenizer flask and cooled to 5 C. The cells were homogenized for 90 sec in a Braun MSK cell homogenizer (Bronwill Scientific, Rochester, New York) operating at 4000 oscillations per minute. The flask was cooled by liquid carbon dioxide (CO_2) during homogenization. The cell extract was decanted and the glass beads were washed with 30 ml of 0.01 M potassium phosphate pH 6.5 to recover more enzyme. The above procedure was repeated several times until a sufficient quantity of crude extract was obtained. Cell debris was removed by centrifugation at 20,000xg for 30 min at a temperature of 3 C.

Purification of Homoserine Dehydrogenase

Homoserine dehydrogenase was purified according to the procedure of Black and Wright (5) with one exception. The final purification step, chromatography on calcium

phosphate was omitted because at this point in the procedure the enzyme was free from all interferring oxidase and dehydrogenase activities. The enzyme was stored at -20 C.

Quantitation of β-Aspartyl Semialdehyde

Homoserine dehydrogenase was used to quantitate BAS. The reaction mixture contained in a 3 ml volume:

200 µM potassium phosphate, pH 7.0

0.2 µM $NADH_2$

0.05 to 0.15 µM BAS

Homoserine dehydrogenase

A reference cuvette was prepared identically except the $NADH_2$ was omitted. The reaction was allowed to go to completion and the change in abosrbance at 340 mµ was recorded. Since there is a 1:1 relationship between moles of $NADH_2$ oxidized and moles of BAS reduced, the BAS concentration can be calculated according to the following formula:

Change in absorbance at 340 mµ = ELC

E = Molar extinction coefficient for $NADH_2$

L = Light path in centimeters

C = Concentration in moles

Production of Sporulating Cells of B. Megaterium KM and Determination of the Relationship Between Sporulation and Dipicolinic Acid Synthesis

B. megaterium KM ATCC 13632, obtained from the laboratory of Dr. Z. J. Ordal, Department of Food Science, University of Illinois, was used in this study as the enzyme source. The organism was grown in a modification of the chemically defined sporulation medium (CDGS) of Nakata (48). The medium contained, per liter of distilled water: 0.0005 g $FeSO_4 \cdot 7H_2O$; 0.005 g $CuSO_4 \cdot 5H_2O$; 0.005g $ZnSO_4 \cdot 7H_2O$; 0.5g $MnSO_4 \cdot H_2O$; 0.2g $MgSO_4$; 2.0g $(NH_4)_2SO_4$; 0.08g $CaCl_2 \cdot 2H_2O$; 4.0g glucose; and 2.0g casamino acids. The medium was buffered at pH 7.0 with 0.1 M potassium phosphate. The glucose, casamino acids, and $CaCl_2$ were prepared and sterilized individually and added to the medium at the time of inoculation. The mineral solution (G salts) containing the first six salts at 100x concentration was prepared separately, filter sterilized, and 10 ml added to 1 liter of medium at the time of inoculation.

A stock suspension of B. megaterium KM spores was heat shocked for 20 min at 80 C and inoculated into the growth medium. A synchronous culture technique was employed with 10% (v/v) transfers at 5, 4, and 3 hr, respectively. Throughout the first two transfers the culture was incubated at 35 C in a New Brunswick Model G25 incubator rotary shaker at 200 RPM. The final

transfer was into 30 liters of medium in a 50-liter Ferma Cell fermenter (New Brunswick Scientific, New Brunswick, New Jersey). The temperature was maintained at 35 C, agitation was 200 RPM, and aeration was 1.5 CFM. Every 45 min, starting at 8 hr after the final transfer, a 50 ml sample was taken for DPA analysis and a small aliquot was reserved for the determination of percent sporulation. The samples for DPA analysis were concentrated by centrifugation at 10,000xg for 20 min. The supernatant was decanted and discarded. The pellet was suspended in 15 ml of distilled water and analyzed as previously described. Percent sporulation and DPA concentration were correlated graphically in order to determine the appropriate time to harvest the culture for enzyme extraction.

Preparation of Cells for Enzyme Extraction

The B. megaterium KM culture was inoculated and grown as previously described. When the percent sporulation had reached a minimum of 60%, the culture was cooled to 10 C with the cooling system on the fermenter. The cells were then harvested by continuous centrifugation at 30,000xg at 3 C. After harvesting, the cells were resuspended in 0.025 M potassium phosphate at pH 7.0, washed twice, and stored at -20 C.

Preparation of Cell Free Extract

The cells were broken in a Eppenbach Colloid mill (Gifford-Wood Co., Hudson, New York) by a slight modification of the technique of O'Conner et al. (49). Four hundred milliliters of cells and 200 ml of 0.10 to 0.11 mm glass beads were added to the colloid mill and cooled to 5 C by liquid CO_2. The cells were then homogenized for 12 min at a clearance of 0.0030 in. at a rheostat setting of 110. During the homogenization of the cells the temperature was maintained at less than 15 C by controlling the liquid CO_2 flow rate. The cell homogenate was decanted after breakage was completed, and the glass beads were washed with 100 ml of 0.025 M potassium phosphate buffer at pH 7.0 to recover more enzyme. The supernatant fluids were pooled and the debris was removed by centrifugation at 25,000xg for 30 min at 3 C. The cell free extract was decanted and adjusted to pH 7.5 with 0.2N sodium hydroxide (NaOH). This fraction was termed the crude extract.

Purification of Dihydrodipicolinic Acid Synthetase

Ammonium Sulfate Fractionation

The extract was maintained at 3 C in an ice bath for the initial and the subsequent fractionations with ammonium sulfate ($(NH_4)_2SO_4$). The extract was gently mixed on a magnetic stirrer throughout the $(NH_4)_2SO_4$

fractionations. The extract was brought to 54% saturation by slowly adding solid $(NH_4)_2SO_4$. After all of the salt was added, the extract was agitated for 30 min. The inactive precipitate was removed by centrifugation at 20,000xg for 15 min at 3 C. The supernatant fluid was then brought to 66% of saturation by the addition of more solid $(NH_4)_2SO_4$ and then allowed to mix for 30 min. The active precipitate was recovered by centrifugation at 20,000xg for 15 min at 3 C. The supernatant fluid was discarded and the precipitate was suspended in 50 ml of 0.1 M potassium phosphate buffer at pH 6.0 which was 0.005 M with respect to EDTA.

Heating at 45 C

The resulting fraction was heated with gentle agitation at 45 C in a constant temperature water bath for 15 min. The extract was then immediately cooled in an ice bath and the inactive precipitate removed by centrifugation at 20,000xg for 15 min at 3 C.

Second Ammonium Sulfate Fractionation

Solid $(NH_4)_2SO_4$ was slowly added to the extract until 35% of saturation was reached. The extract was stirred for 30 min and the inactive precipitate was removed by centrifugation at 20,000xg for 15 min at 3 C. Next, solid $(NH_4)_2SO_4$ was added to the 50% saturation level. The extract was stirred for 30 min and the

active precipitate was collected by centrifugation at 20,000xg for 15 min at 3 C. The pellet was resuspended in 10 ml of 0.1 M potassium phosphate at pH 7.5 which was 0.005 M with respect to EDTA.

Desalting of Extract for DEAE Cellulose Chromatography

The sample was applied to a 33x2.2 (i.d.) cm Bio-Gel P-2 column. The column was eluted with 0.1 M potassium phosphate pH 7.5 which was 0.005 M with respect to EDTA. The column was operated at a flow rate of 3 ml per min and the effluent was monitored with an ultraviolet absorption meter at 254 mμ (Gilson Medical Electronics, Middleton, Wisconsin). The initial active peak was collected and the remaining fractions were discarded.

DEAE Cellulose Chromatography

The active fraction from the Bio-Gel P-2 column was applied to a 21x2.2 (i.d.) cm DEAE cellulose column equilibrated with a 0.1 M potassium phosphate buffer which was 0.005 M with respect to EDTA. The column separation was carried out at room temperature. The column was eluted with successive 100 ml volumes of the same buffer which contained 0.2, 0.3, and 0.4 M potassium chloride respectively. One 3.6 ml fraction was collected each min and analyzed for protein and enzymatic activity. The most active fractions were pooled and adjusted to

pH 6.5 with 1N HCl. The preparation was then stored at -20 C.

Bio-Gel P-200 Chromatography

This purification step was used to prepare large quantities of partially purified enzyme for routine analysis. The active material from the second $(NH_4)_2SO_4$ fractionation was applied to a 36x4 (i.d.) cm Bio-Gel P-200 column equilibrated with a 0.1 M potassium phosphate buffer pH 7.5 also containing 0.005 M EDTA. The column separation was carried out at room temperature. The column was operated at a flow rate of 1 ml per min and 5.3 ml fractions were collected. The fractions were analyzed for protein by the method of Warburg and Christian (74) and enzymatic activity. The most active fractions were combined and adjusted to pH 6.5 with 1N HCl. The enzyme was divided into 3 ml fractions to avoid loss of enzymatic activity due to repeated freezing and thawing and stored at -20 C.

Dihydrodipicolinic Acid Synthetase Assay

The enzymatic assay used in this study was derived from the 270 mμ assay of Yugari and Gilvarg (82). The reaction mixture contained in a 3 ml volume:

250 μM imidazole pH 7.5

20 μM pyruvate

5 μM BAS

and condensing enzyme

A blank sample was prepared in an identical manner except the BAS was omitted. All assays were conducted at 35 C unless otherwise stated. Since the reaction had a lag period of approximately 7 min before assuming a linear rate, the enzyme was diluted to obtain a change in absorbance which was less than 0.70 in 15 min. All reaction components except BAS were mixed and equilibrated at the desired temperature. The BAS was neutralized with 1N sodium bicarbonate ($NaHCO_3$) and then added to start the reaction. The reactions were followed at 270 mμ in a Beckman DBG spectrophotometer and the change in absorbance was recorded with a 10 in. Beckman recorder. The temperature was controlled by circulation of water from a constant temperature water bath through the jacketed sample compartment. An absorbance change of 0.01 absorbancy units per min was defined as 1 unit of enzyme activity.

Characterization of Dihydrodipicolinic Acid Synthetase

Effect of Temperature on Reaction Rate

DEAE cellulose purified enzyme and standard assay procedures were used to obtain reaction rates over a temperature range of 25 to 45 C.

Effect of pH of Reaction Rate

DEAE cellulose purified enzyme and standard assay procedures were used to determine reaction rates over

the pH range of 7.0 to 8.0. The imidazole buffer was prepared at 0.2 pH unit intervals throughout the test range. Since possible changes in pH due to improper neutralization of the acidic component existed, the pH of each assay mixture was determined immediately after the addition of BAS.

Effect of Enzyme Concentration on Reaction Rates

Appropriate dilutions of DEAE cellulose purified enzyme, within the prescribed absorbance range limitations, were tested for linearity of response. Standard assay procedures were employed.

Determination of the Absorbance Spectrum of the Reaction Product

The BAS concentration in the reaction mixture was reduced to 1 μM to maintain the total change in absorbance for spectrum determinations within a readable range on the spectrophotometer. Diluted DEAE cellulose purified enzyme was used in this experiment. The reaction was started and allowed to go to completion. Change in absorbance over a range of 240 to 300 mμ was determined at 5 mμ intervals.

Determination of the Michaelis-Menten Constant

The BAS concentration was held at constant levels and the pyruvate concentration in the reaction mixture was varied from 1 to 30 μM in determining the Michaelis-Menten constant (Km) of pyruvate. Similarly, the pyruvate concentration was held at constant levels and the BAS concentration in the reaction mixture was varied from 1 to 10 μM in determining the Km for BAS. The enzyme purified on DEAE cellulose and standard assay procedures were employed in these determinations. The change in absorbancy between 7.5 and 10 min was used as the rate value due to the initial lag period of the reaction.

Effect of Sulfhydryl-Inhibiting Reagents Upon Enzymatic Activity

One tenth and 0.2 ml volumes of 0.1 M iodoacetate, 0.1 M iodoacetamide, or 0.0005 M para-chloromecuribenzoate (PCMB) were added to the standard reaction mixture and incubated for 10 min before starting the reaction. Assays without added inhibiting reagents were run as controls for each experiment.

Effect of Sulfhydryl-Reducing Reagents Upon the Reaction Rate

One tenth milliliter of 0.1 M β-mercaptoethanol or 0.1 M cysteine was added to the standard reaction mixture and allowed to incubate for 10 min before starting the

reaction. The enzyme purified on DEAE cellulose and standard assay procedures were employed in determining the reaction rates. Assays without added reducing reagents were run as controls for each experiment.

Effect of Sulfhydryl-Reducing Reagents on the Absorbance Spectrum of the Reaction Product

The analysis was conducted, with one exception, as previously described in determination of the absorbance spectrum of the reaction product. One tenth milliliter of 0.1 M β-mercaptoethanol was added to the reaction mixture.

Effect of Lysine on the Reaction Rate

One tenth and 0.2 ml volumes of 0.2 M lysine were added to the standard reaction mixture and incubated for 10 min before starting the reaction. Assays without added lysine were run as controls for each experiment.

Determination of Thermal Inactivation Characteristics

Effect of pH on Thermal Inactivation at 55 C

Three buffer systems were used to determine the effects of pH on thermal inactivation of the enzyme; 0.1 M potassium phosphate from pH 6.5 to 8.0, 0.1 M boric acid - NaOH from pH 8.0 to 9.0, and 0.1 M glycine -

NaOH from pH 9.0 to 11.5. Five tenths milliliter of diluted enzyme purified on Bio-Gel P-200 was added to 4.5 ml of the appropriate buffer and the pH was adjusted with 1N NaOH. Five tenths milliliter aliquots were added to 13x100 mm screw-cap test tubes. The tubes were capped and submerged in a 55 C constant temperature water bath. The tubes were gently agitated to assure rapid equilibration to temperature during the first 30 sec of the heating period. The tubes were immediately cooled in an ice bath after heating for the appropriate time interval. The pH of the heated enzyme was readjusted prior to analysis in all thermal inactivation experiments above pH 8.0.

Effect of Temperature on the Thermal Inactivation Rate at pH 7.0

Five tenths milliliter of diluted enzyme purified on Bio-Gel P-200 was added to 4.5 ml of 0.1 M potassium phosphate at pH 7.0. The pH was checked and, if necessary, adjusted with 1N NaOH. Five tenths milliliter aliquots were dispensed into 13x100 mm screw-cap test tubes and treated as described in the section on the effect of pH on thermal inactivation rates.

Effect of Pyruvate on the Thermal Inactivation Rate at 75 C

Appropriate volumes of 0.5 M pyruvate, prepared in 0.1 M potassium phosphate at pH 7.0 were added to 0.5 ml of diluted enzyme purified on Bio-Gel P-200, and the total volume was adjusted to 5 ml with 0.1 M potassium phosphate at pH 7.0. The pH was checked and, if necessary, adjusted with 1N NaOH. Five tenths milliliter aliquots were dispensed into 13x100 mm screw-cap test tubes and treated as described in the section on the effect of temperature on the thermal inactivation rate. Final pyruvate concentration ranged from 0.03 to 0.3 M.

Calculations

Velocity Constant

The velocity constant (K) for the thermal inactivation process was calculated according to the following equation:

$$K = \frac{1}{(t_2 - t_1)} \ln \frac{C_1}{C_2}$$

where C_1 and C_2 are enzyme concentrations at times t_1 and t_2, respectively.

Activation Energy

The Arrhenius equation relating the velocity constant K to absolute temperature is:

$$2.3 \log K = B - \frac{E_A}{R_T}$$

where E_A is the activation energy, R is the gas constant, T is the absolute temperature, and B is an intergration constant. The activation energy is determined by plotting log K versus the reciprocal of the absolute temperature and determining the slope of the line. The slope is equal to $E_A/2.3R$.

Thermal inactivation of the enzyme was assumed to be an activation process and was treated according to the Eyring theory of absolute reaction rates (16). A somewhat rigid enzyme structure is assumed, which is rapidly denatured when the stabilizing bonds are broken.

The free energy change ($\Delta F^{\dagger}$) due to the formation of the activated complex is

$$\Delta F^{\dagger} = RT \ln \frac{K'T}{Kh}$$

where R is the gas constant, T is the absolute temperature, K is the inactivation constant, h is Planck's constant, and K' is the Boltzman constant.

The enthalpy change ($\Delta H^{\dagger}$) due to the formation of the activated complex is

$$\Delta H^{\dagger} = E_a - RT$$

where E_a is the activation energy derived from the Arrhenius plot. The entropy change ($\Delta S^{\dagger}$) is

$$\Delta F^{\dagger} = \Delta H^{\dagger} - T\Delta S^{\dagger}$$

using the previously determined values of $\Delta F^{\dagger}$ and $\Delta H^{\dagger}$ at absolute temperature T.

RESULTS

Initial experiments were designed to determine the optimum time to harvest sporulating cells to be used as the source of enzyme. Figure 1 illustrates the relationship between percent sporulation and DPA synthesis. Sporulation commenced 9.5 hr after the final transfer and was essentially complete by the end of 13 hr. Dipicolinic acid synthesis followed the sporulation process by about 1 hr and was completed at 14 hr. Spore crops produced in this manner contained approximately 10% DPA on a dry weight basis. The data indicated a minimum of 60% of the cells contained spore forms before DPA synthesis assumed a linear rate. Therefore, when producing cells for enzyme extraction, the culture was not harvested until a minimum of 60% of the cells contained spore forms.

Table 1 presents the results of a typical dihydrodipicolinic acid synthetase purification experiment. The percent yield column has been adjusted to reflect samples reserved for protein and enzymatic analysis. Two different enzymatic preparations were prepared by variation of the final step in the purification procedure. Fractionation by DEAE cellulose chromatography resulted in a

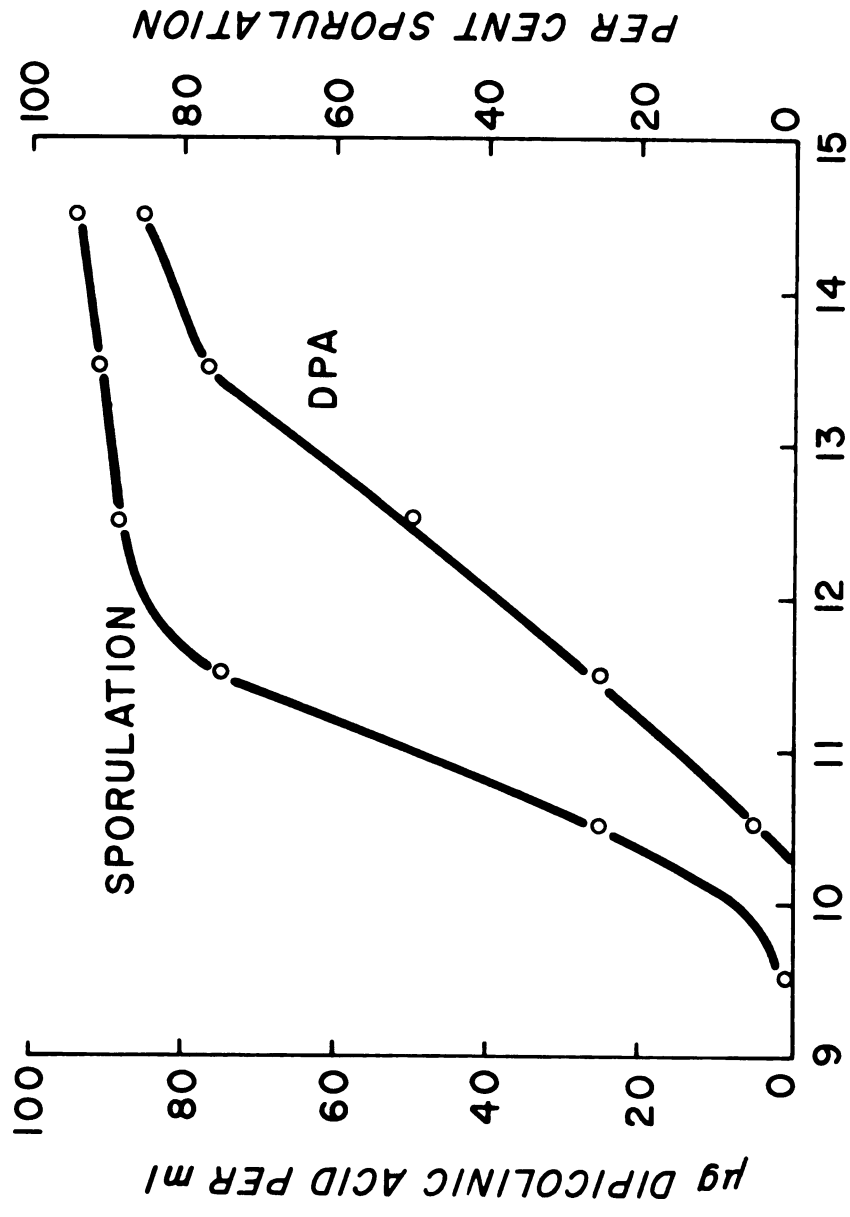

Figure 1. Progress of sporulation and dipicolinic acid synthesis in an active culture of Bacillus megaterium Km.

Table 1. Purification procedures and typical results for dihydrodipicolinic acid synthetase from sporulating Bacillus megaterium KM.

Procedure	Vol	Activity	Protein	Specific Activity	Purification	Yield
	ml	units/ml	mg/ml	units/mg		%
CRUDE EXTRACT	250	45	8.1	5.7	1	100
$(NH_4)_2 SO_4$ (54-66%)	60	133	7.0	18.5	3.27	68
45°C 15 min	54	133	3.5	37.0	6.54	68
$(NH_4)_2 SO_4$ (35-50%)	11	460	9.5	48.5	8.54	53
1 DEAE	30	31.4	0.038	861	151.60	10.6
2 P-200	55	66.7	0.24	268	55	33

152-fold purification with retention of 11% of the original activity. Bio-Gel P-200 chromatography produced a 55-fold purification while retaining 33% of the original activity. The enzyme exhibited cold sensitivity during the column chromatography separations, therefore the columns were maintained at room temperature. The enzyme preparations were stabilized to storage at -20 C with a minimum loss of activity by adjusting the pH to 6.5.

The DEAE cellulose elution profile is presented in Figure 2. Upon elution from the column the enzyme was essentially free from nucleic acids according to the Warburg and Christian technique. Tubes 42 to 50 contained the majority of the enzymatic activity and were retained for subsequent experimentation.

Figure 3 represents the Bio-Gel P-200 elution profile. The relative R_f value (elution volume/void volume) was 1.85 which indicated the enzyme had entered the gel to a great extent. The preparation was estimated to contain approximately 8 to 10% nucleic acids as contaminants using the Warburg and Christian technique. Tubes 46 to 57 contained the majority of the enzymatic activity and were retained for subsequent use in the thermal inactivation experiments.

Figure 4 demonstrates the effect of enzyme concentration on reaction rate. The reaction is shown to be

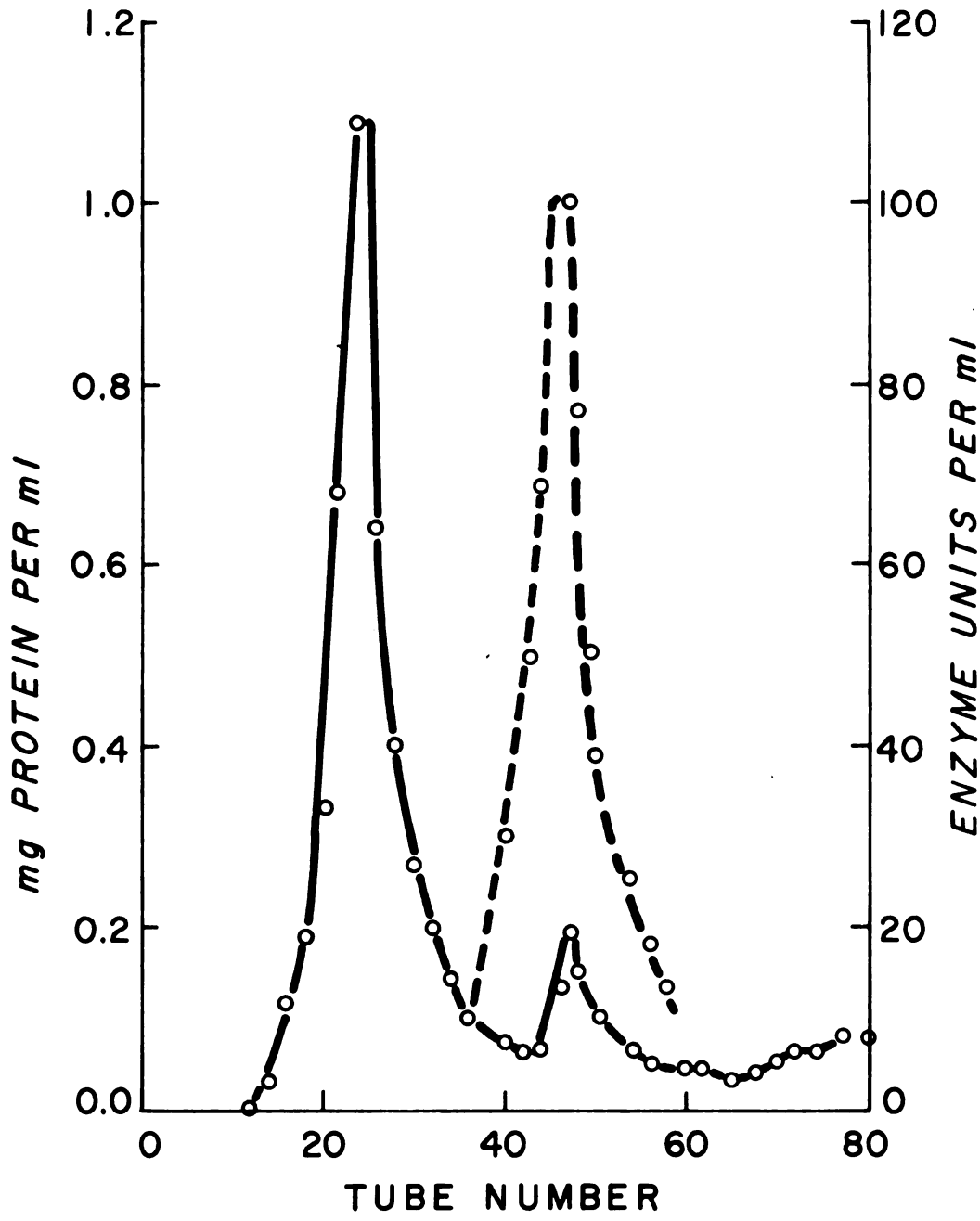

Figure 2. Elution profiles of protein and dihydrodipicolinic acid synthetase activity from a DEAE cellulose column eluted with a step gradient of potassium chloride in 0.1 M potassium phosphate buffer at pH 7.5.

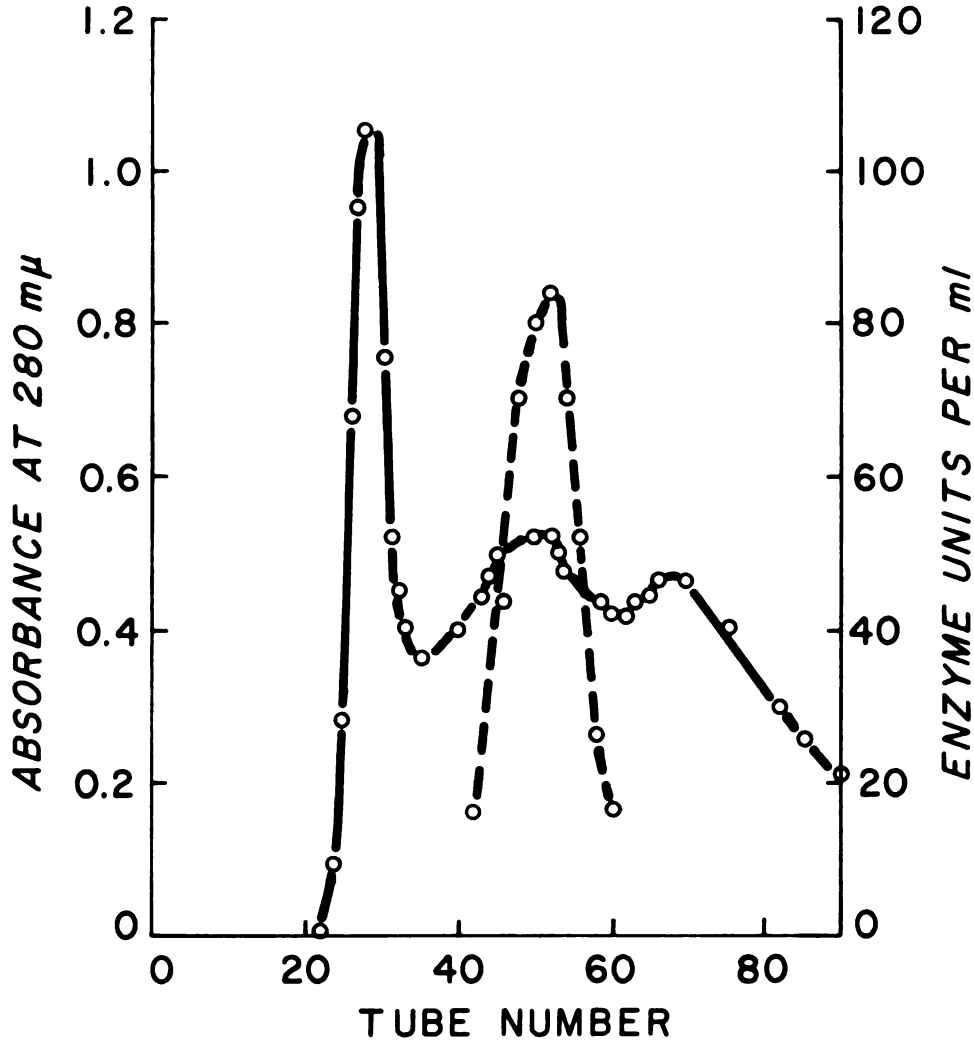

Figure 3. Elution profile of 280 mμ absorbing materials and dihydrodipicolinic acid synthetase activity from a Bio-Gel P-200 column eluted with 0.1 M potassium phosphate buffer at pH 7.5.

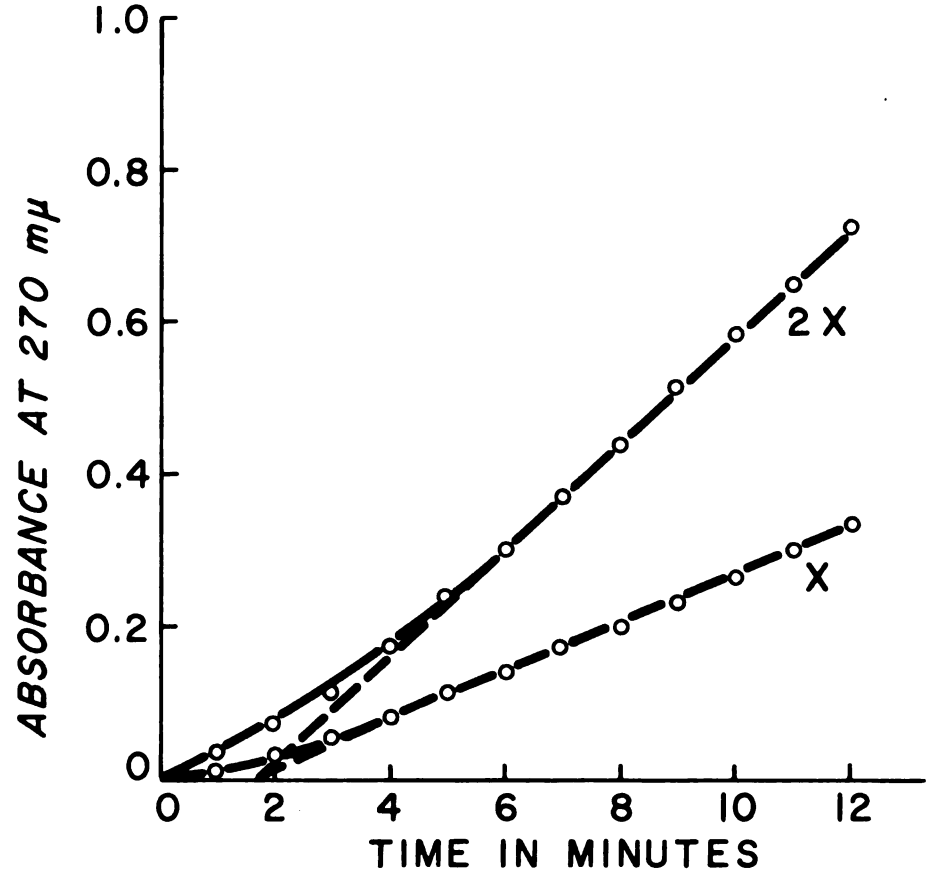

Figure 4. Relationship of enzyme concentration to reaction rate for 150-fold purified dihydrodipicolinic acid synthetase.

dependent upon enzyme concentration within the given absorbancy range and time. The initial lag period of the reaction is clearly depicted.

Figure 5 describes the effect of pH in the range of 7.0 to 7.85 on enzymatic activity. The enzyme exhibited a relatively symmetrical pH vs. activity curve with an optimum at pH 7.65.

The effect of temperature on the reaction rate is presented in Figure 6. The enzymatic activity increased with temperature from 25 to 45 C. The temperature coefficient (Q_{10}) was 2.83 from 25 to 35 C and 2.53 from 35 to 45 C. A seven-fold difference in reaction rates between 25 and 45 C was observed.

Figure 7 illustrates the 1/s vs. 1/v plots for the determination of the Michaelis-Menten constants for BAS and pyruvate. Values of 4.6×10^{-4} and 5.0×10^{-4} were obtained for BAS and pyruvate, respectively.

The absorbance spectrum of the reaction product between 240 and 300 mμ is presented in Figure 8. The reaction product exhibited a broad symmetrical absorbance spectrum with an absorbance maximum at 265 mμ. Addition of β-mercaptoethanol or cysteine to the reaction mixture produced an apparent stimulation of the enzyme as evidenced by an immediate increase in the reaction rate. However, addition of iodoacetate, iodoacetamide, or para-chloromercuribenzoate (PCMB) to the reaction failed

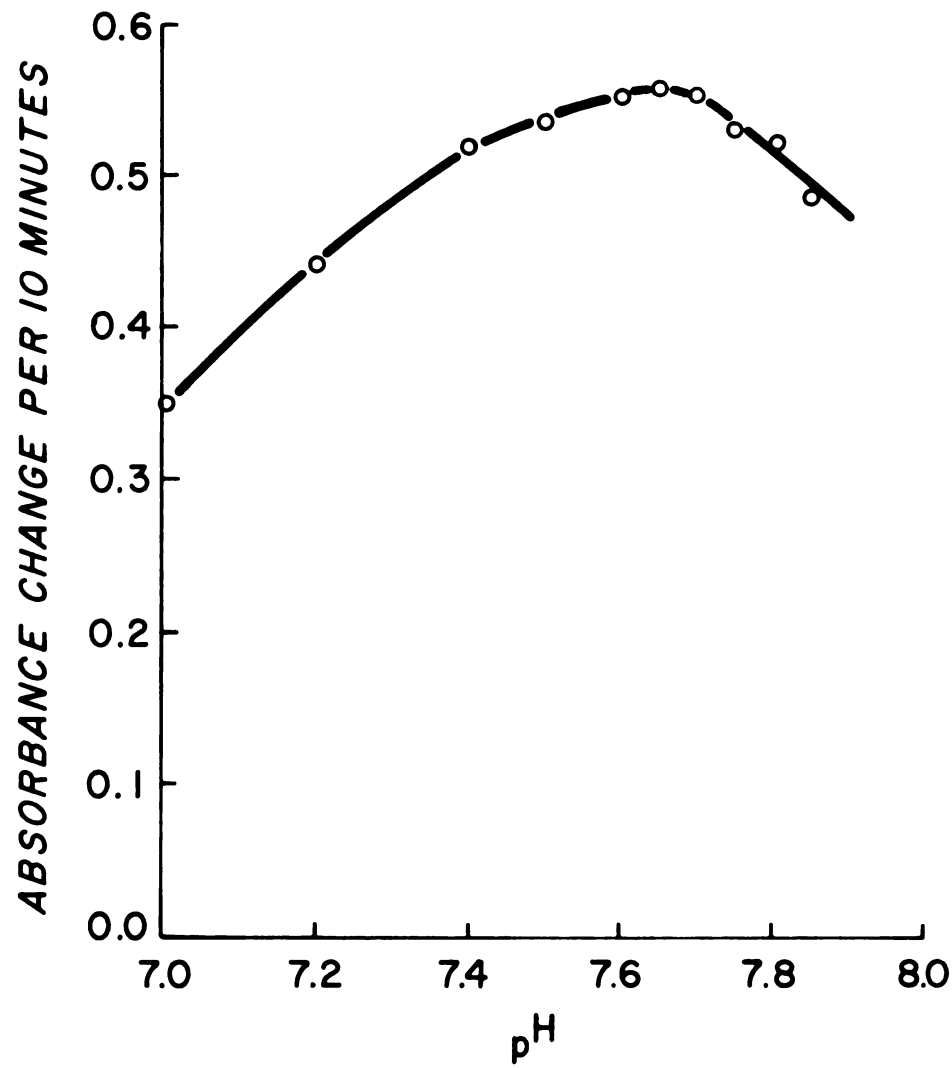

Figure 5. Effect of pH on reaction rate of 150-fold purified dihydrodipicolinic acid synthetase in imidazole buffer.

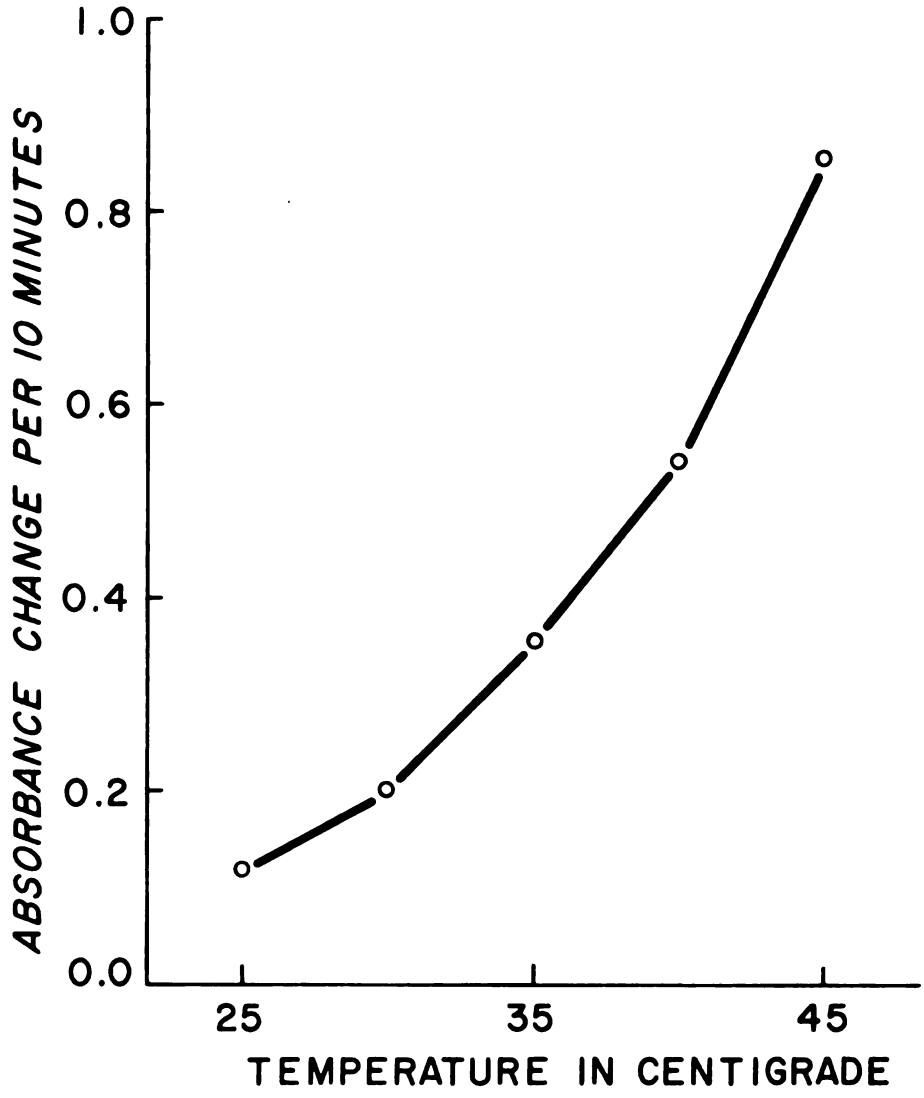

Figure 6. Effect of temperature on the reaction velocity of 150-fold purified dihydrodipicolinic acid synthetase.

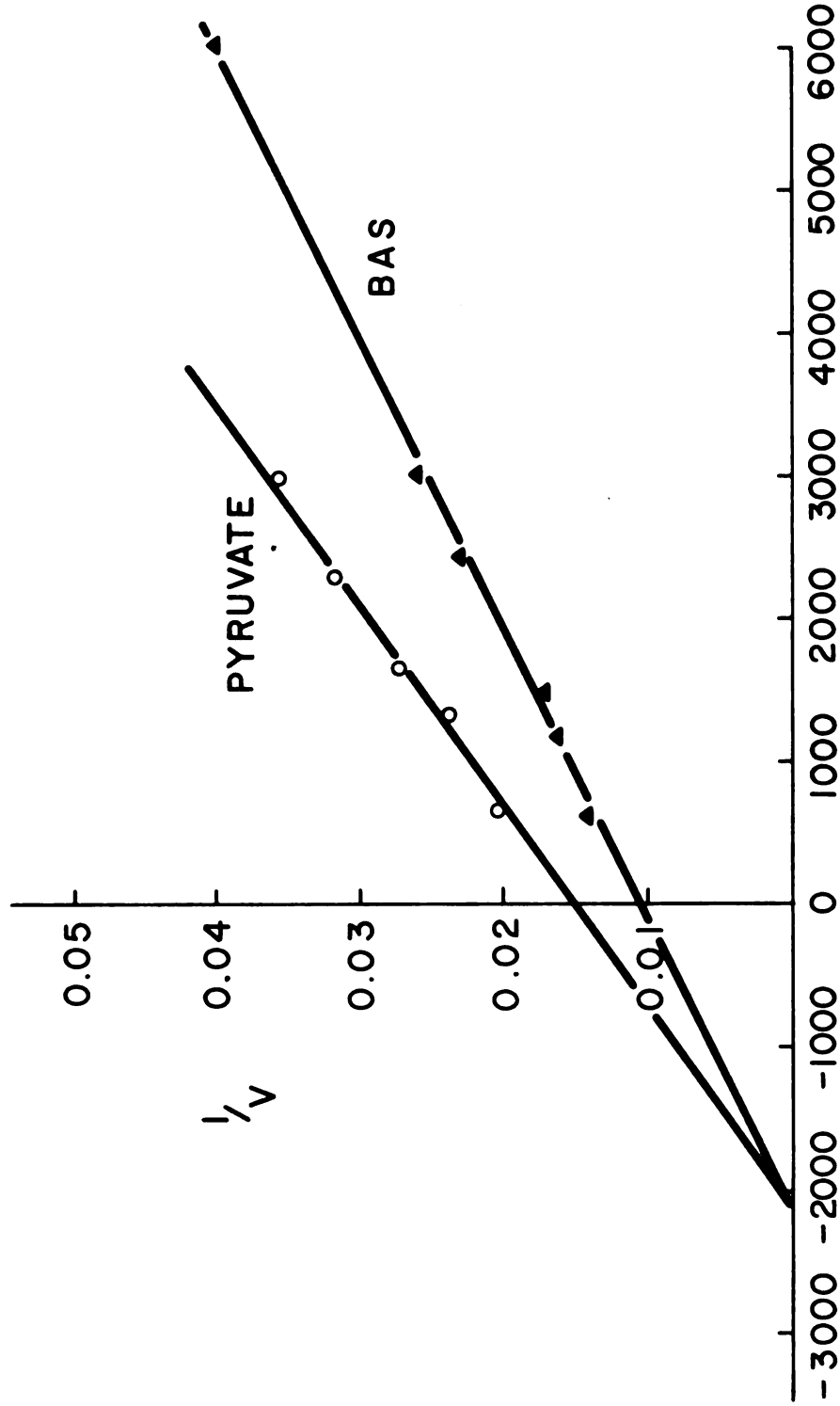

Figure 7. Effect of various concentrations of pyruvate and β-aspartyl semialdehyde (BAS) on the reaction velocity of 150-fold purified dihydrodipicolinic acid synthetase.

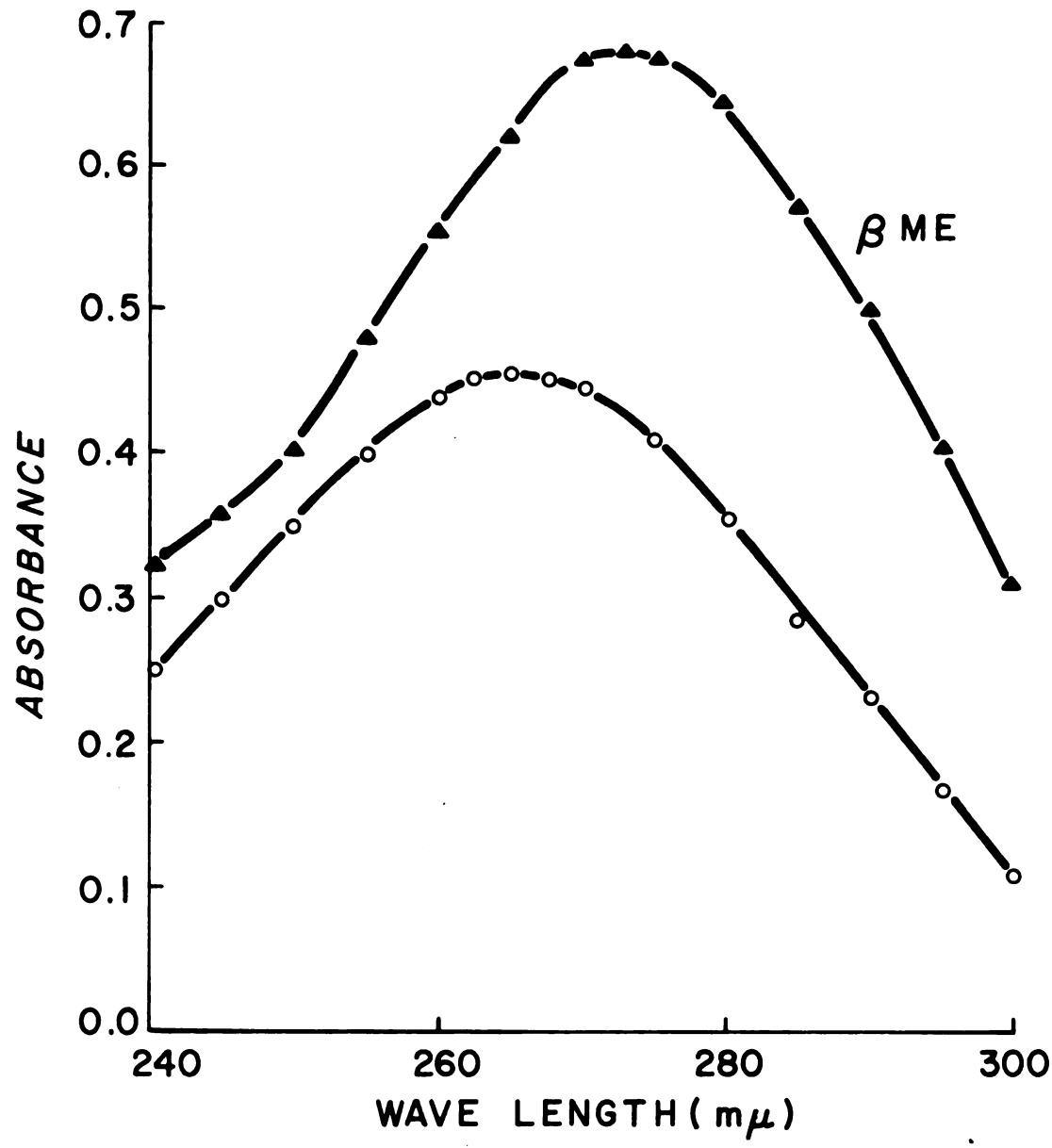

Figure 8. Effect of β-mercaptoethanol on the absorption spectrum of the 270 mμ assay product of 150-fold purified dihydrodipicolinic acid synthetase.

to produce any inhibition. In an attempt to resolve these differences, the spectrum of the reaction product was determined with and without added β-mercaptoethanol. Figure 8 represents the results of these determinations. In the presence of β-mercaptoethanol the reaction product had a broad symmetrical absorbance spectrum with a maximum absorbance at 273 mμ in contrast to the maximum absorbance at 265 mμ without added β-mercaptoethanol. Also, the intensity of the absorption maximum is almost 50% greater in the presence of β-mercaptoethanol.

Experiments conducted to test the effect of lysine on the reaction indicated that lysine had no effect. Addition of lysine to a final concentration of 0.013 M failed to produce any inhibition of the reaction.

The effect of pH on thermal inactivation of the enzyme at 55 C in 0.1 M potassium phosphate from pH 6.5 to 8.0 is summarized in Figure 9. First-order reaction kinetics were observed in all instances. As the pH increased from 6.5 to 8.0, the half-life of the enzyme increased from 5.3 to 20 min.

Figure 10 demonstrates the rates of thermal inactivation at 55 C in 0.1 M boric acid-NaOH from pH 8.0 to 9.0. Thermal inactivation of the enzyme followed first-order reaction kinetics. The 55 C half-life increased from 5.8 min at pH 8.0 to 75 min at pH 9.0. Figures 11 and 12 describe the rates of thermal

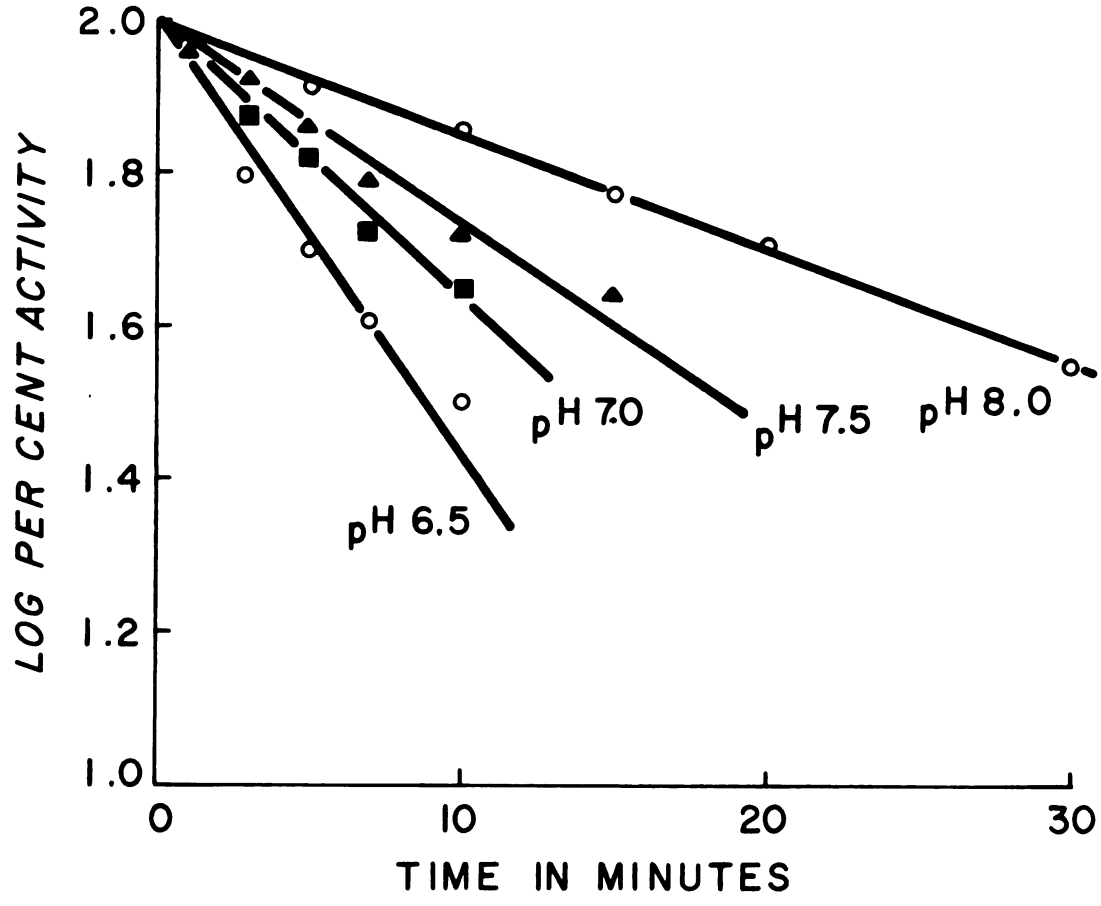

Figure 9. Time required for thermal inactivation of 55-fold purified dihydrodipicolinic acid synthetase in 0.1 M potassium phosphate buffer over the pH range of 6.5 to 8.0 at 55 C.

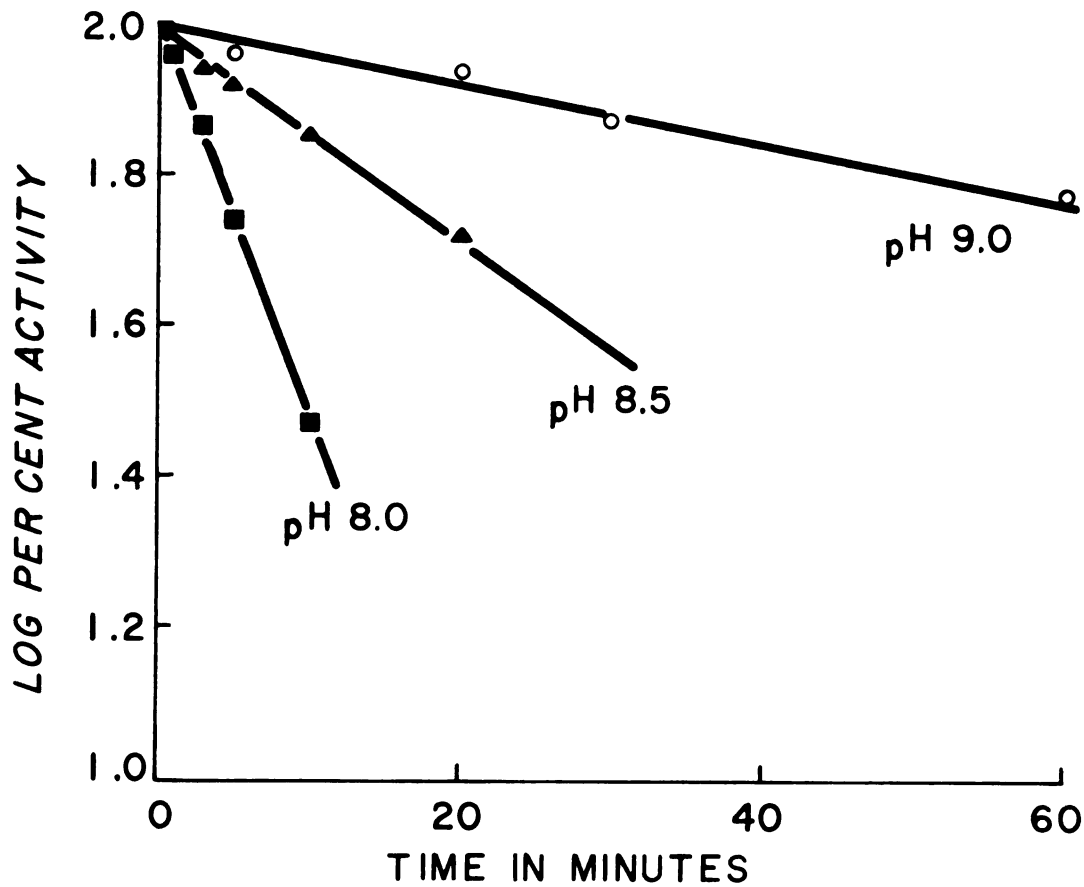

Figure 10. Time required for thermal inactivation of 55-fold purified dihydrodipicolinic acid synthetase in 0.1 M boric acid-sodium hydroxide buffer over the pH range of 8.0 to 9.0 at 55 C.

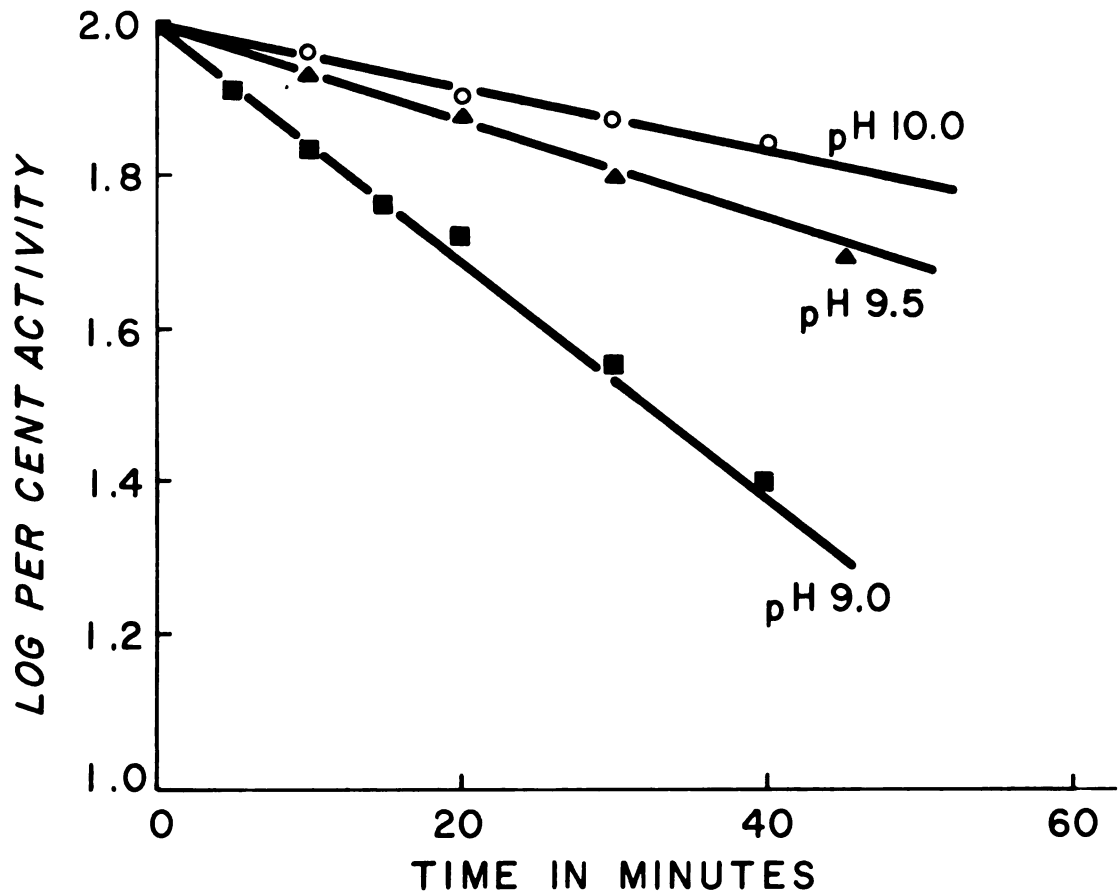

Figure 11. Time required for thermal inactivation of 55-fold purified dihydrodipicolinic acid synthetase in 0.1 M glycine-sodium hydroxide buffer over the pH range of 9.0 to 10.0 at 55 C.

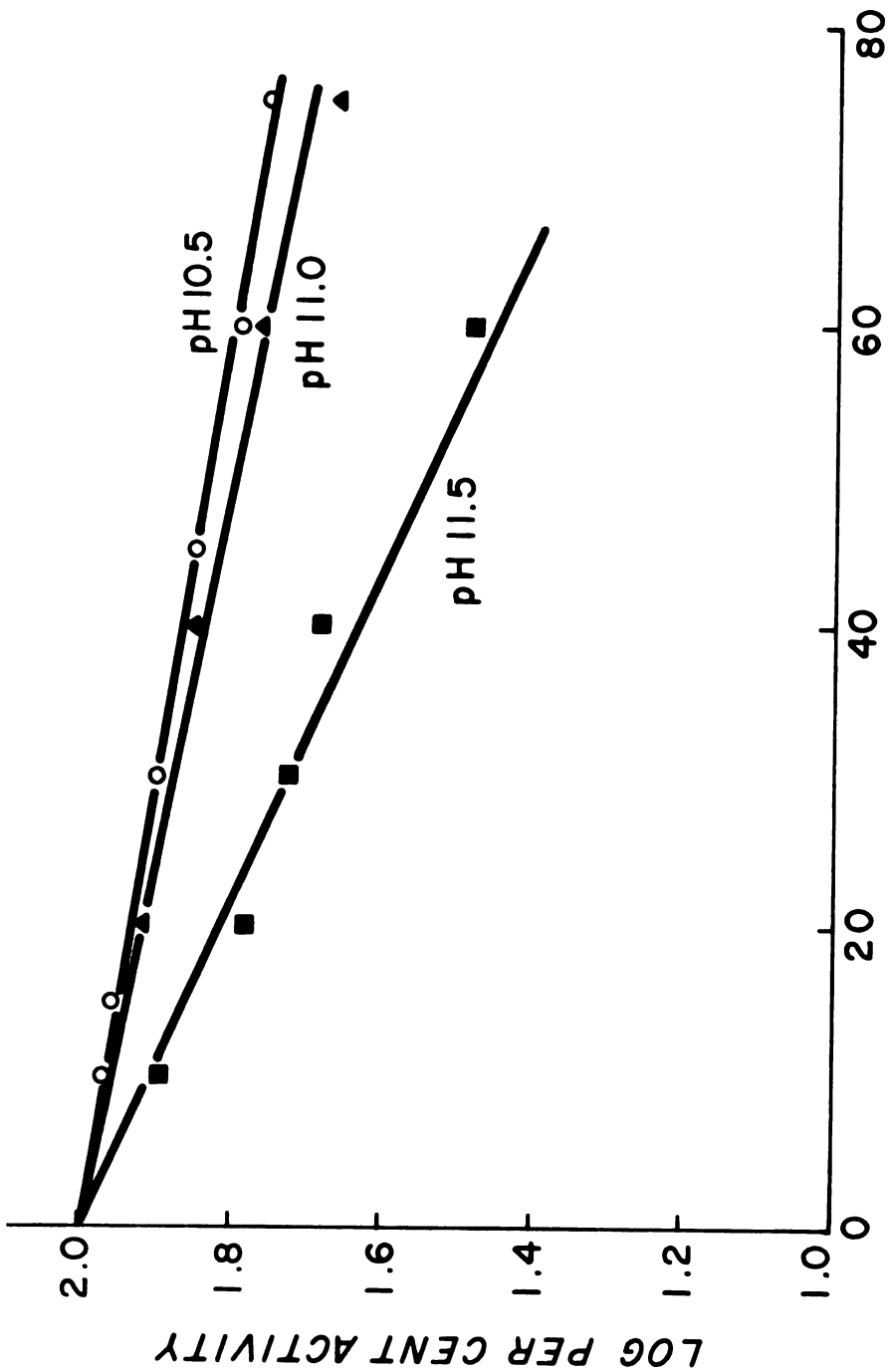

Figure 12. Time required for thermal inactivation of 55-fold purified dihydrodipicolinic acid synthetase in 0.1 M glycine-sodium hydroxide buffer over the pH range of 10.5 to 11.5 at 55 C.

inactivation at 55 C in 0.1 M glycine-NaOH from pH 9.0 to 11.5. The half-life increased from 19.5 min at pH 9.0 to 89 min at pH 10.5 and then decreased to 33.5 min at pH 11.5. In all cases the first-order reaction kinetics were observed.

The relationship of pH to the thermal inactivation constant (K) is shown in Figure 13. Also, the effect of the buffer system on the inactivation constant at similar pH and the rate of change with varying pH is clearly indicated. A point of maximum thermal stability was found at pH 10.5. The slopes of the curves for the phosphate and borate buffers were 0.4 and 1.1, respectively. The initial slope of the curve for the glycine buffer was 0.6 while above pH 11.0 the slope was approximately 0.7.

Figure 14 demonstrates the effect of temperature on the thermal inactivation rate in 0.1 M potassium phosphate pH 7.0. The loss of enzyme activity followed first-order reaction kinetics in all instances. Half-lives of 45, 8.5, and 1.9 min were observed at 50, 55, and 60 C, respectively. A Z value of 7.3 C was determined from these data. The Z value is the temperature change, in degrees centigrade, required to effect a ten-fold change in the rate of thermal inactivation.

An Arrhenius plot of the thermal inactivation data at pH 7.0 is presented in Figure 15. The E_a was 67,300 cal per mole for the thermal denaturation of the enzyme.

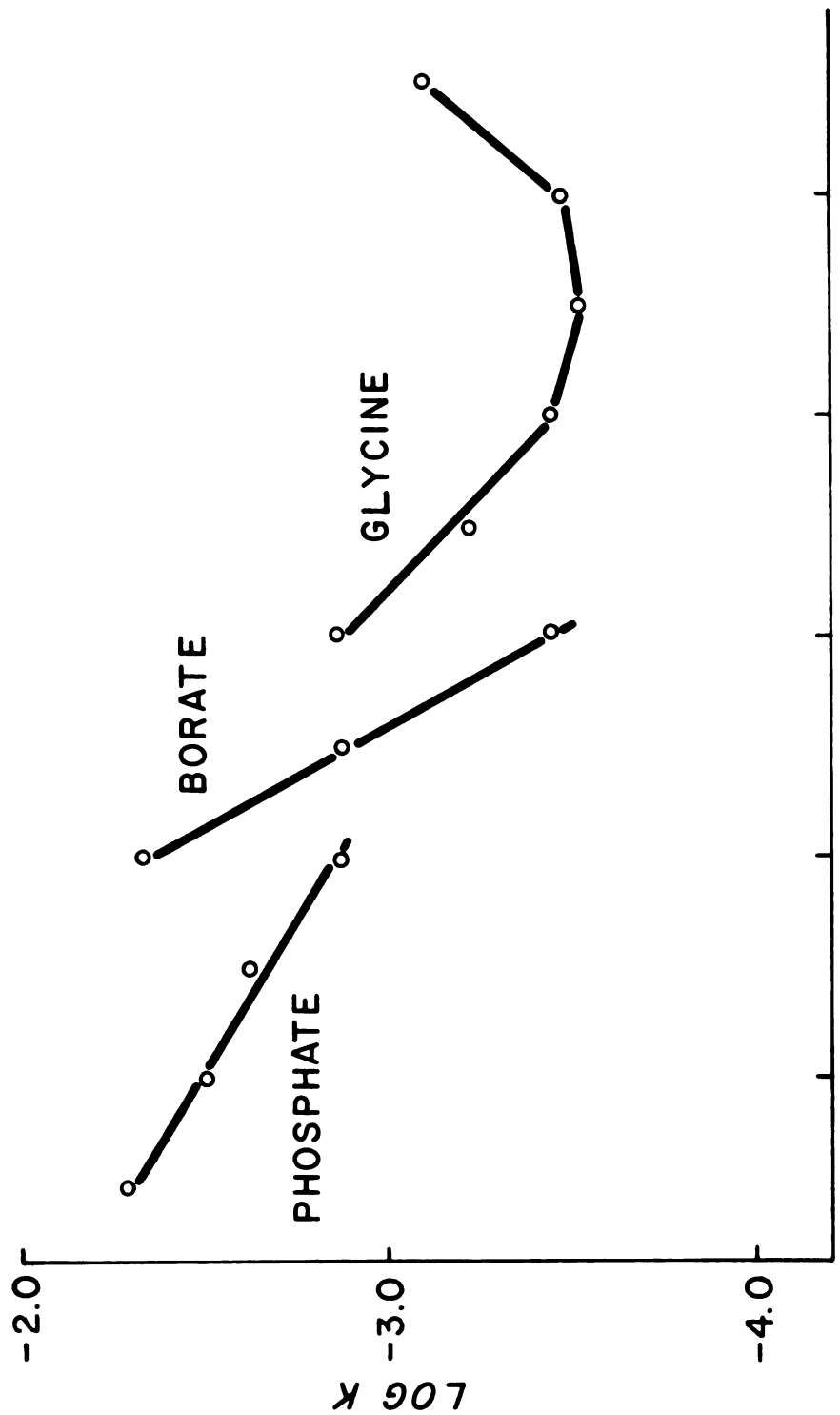

Figure 13. Relationship of pH to 55 C thermal inactivation rate constants of 55-fold purified dihydrodipicolinic acid synthetase.

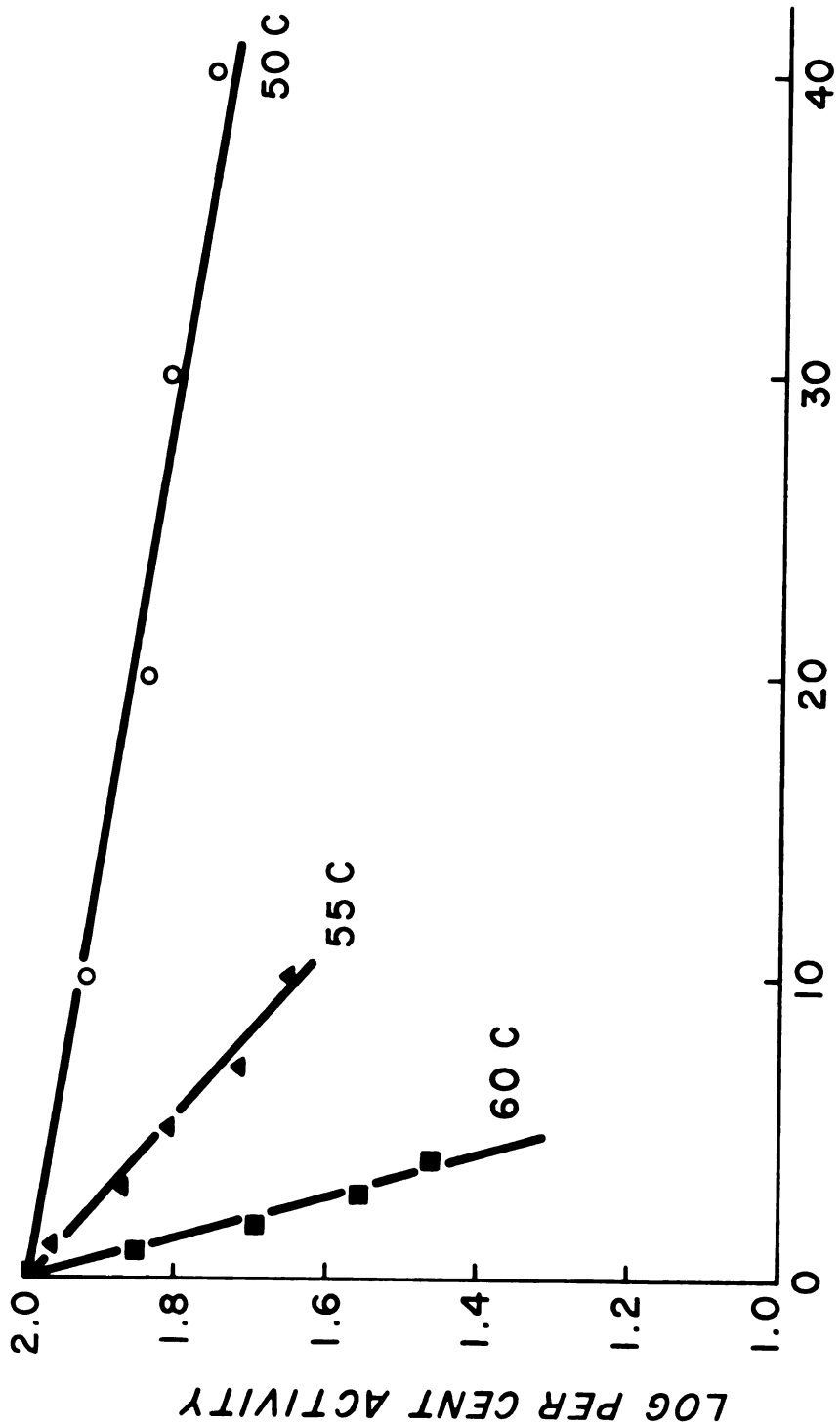

Figure 14. Time required for thermal inactivation of 55-fold purified dihydrodipicolinic acid synthetase in 0.1 M potassium phosphate buffer at pH 7.0 over the temperature range of 50 to 60 C.

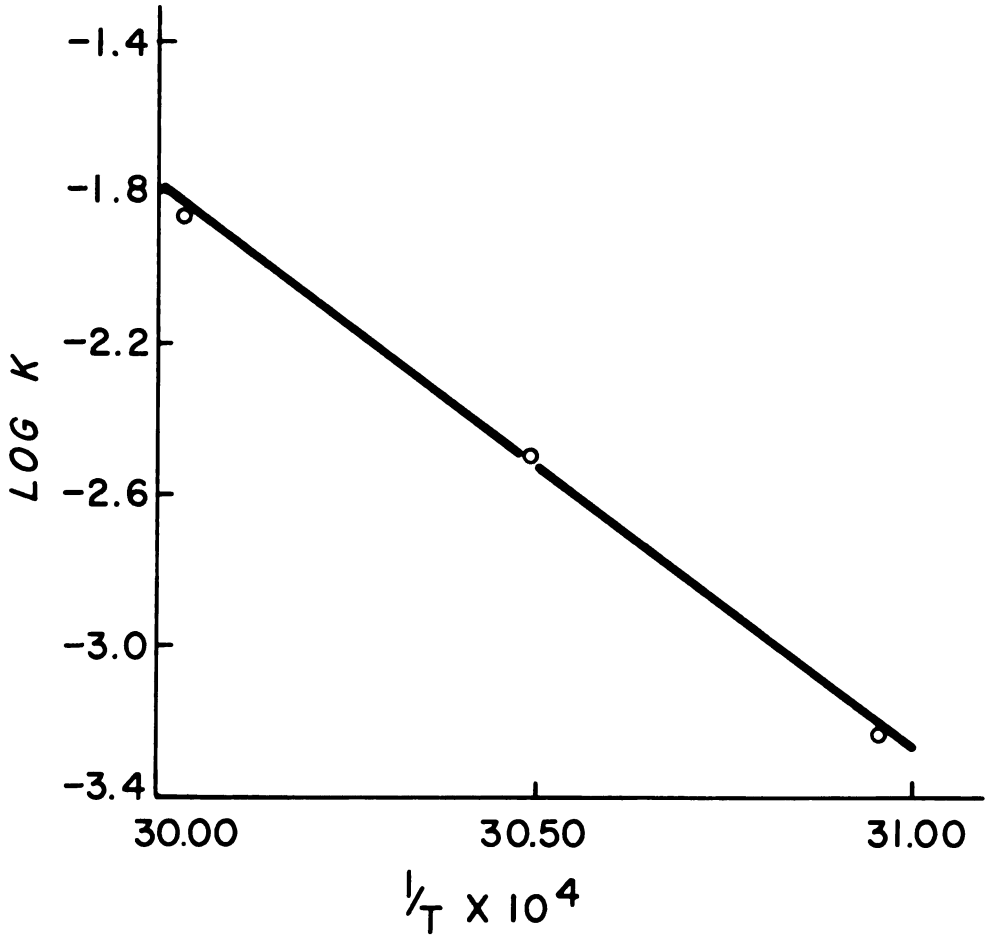

Figure 15. Effect of temperature on thermal inactivation rate constants of 55-fold purified dihydrodipicolinic acid synthetase in 0.1 M potassium phosphate buffer at pH 7.0.

At 60 C, the $\Delta F^{\dagger}$, $\Delta H^{\dagger}$, and $\Delta S^{\dagger}$ values were 22,250 and 66,700 cal per mole and 133 cal per mole per deg, respectively.

The effect of pyruvate on thermal inactivation at 75 C in 0.1 M potassium phosphate at pH 7.0 is reported in Figure 16. First-order reaction kinetics were observed. As the pyruvate concentration was increased from 0.03 to 0.3 M, the half-life at 75 C increased from 2.7 to 18.3 min.

Figure 17 illustrates the effect of pyruvate concentration on the thermal inactivation constant. The slope of the log-log plot is 0.95.

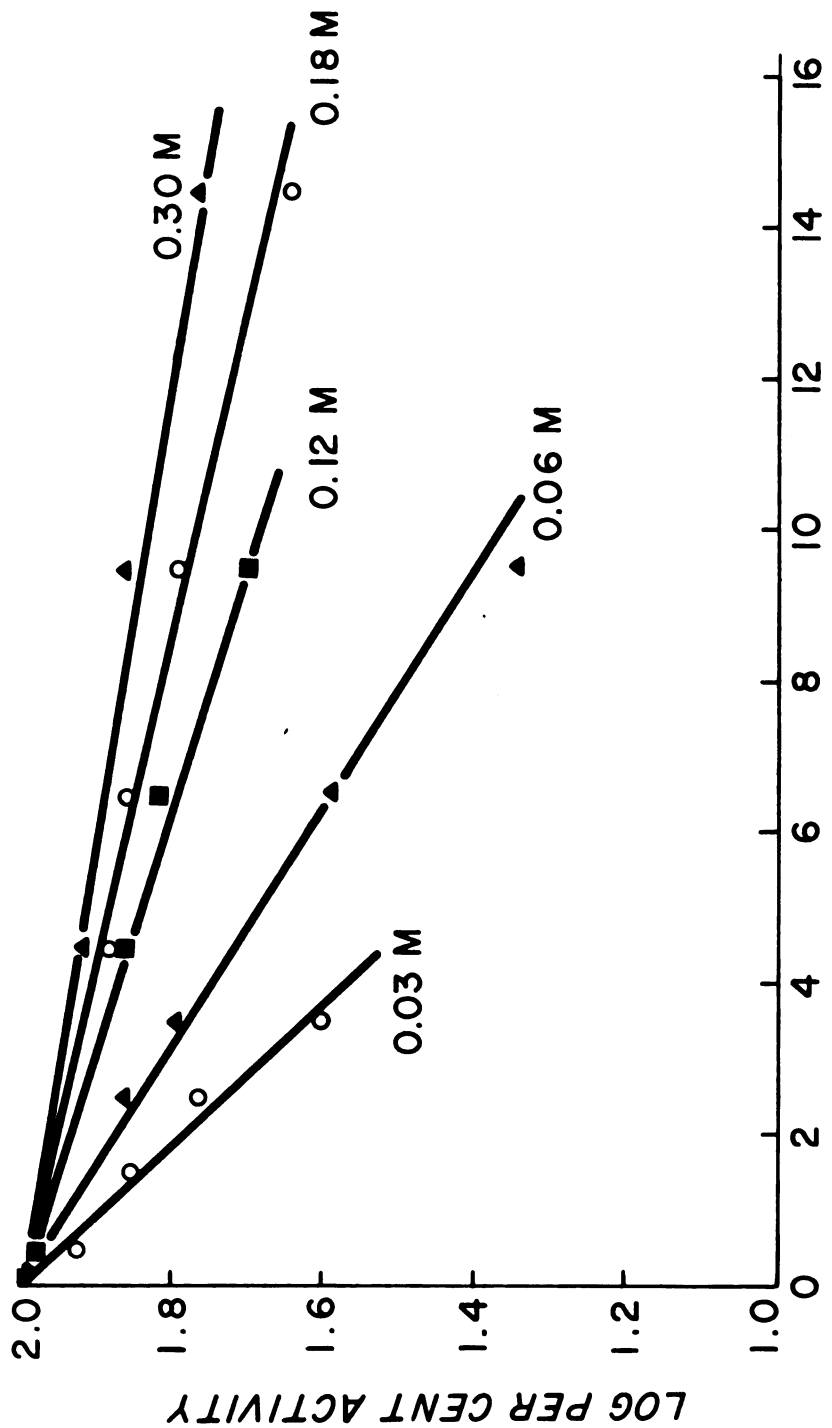

Figure 16. Time required for thermal inactivation of 55-fold purified dihydrodipicolinic acid synthetase in 0.1 M potassium phosphate buffer at pH 7.0 at 75 C in the presence of 0.03 to 0.3 M pyruvate.

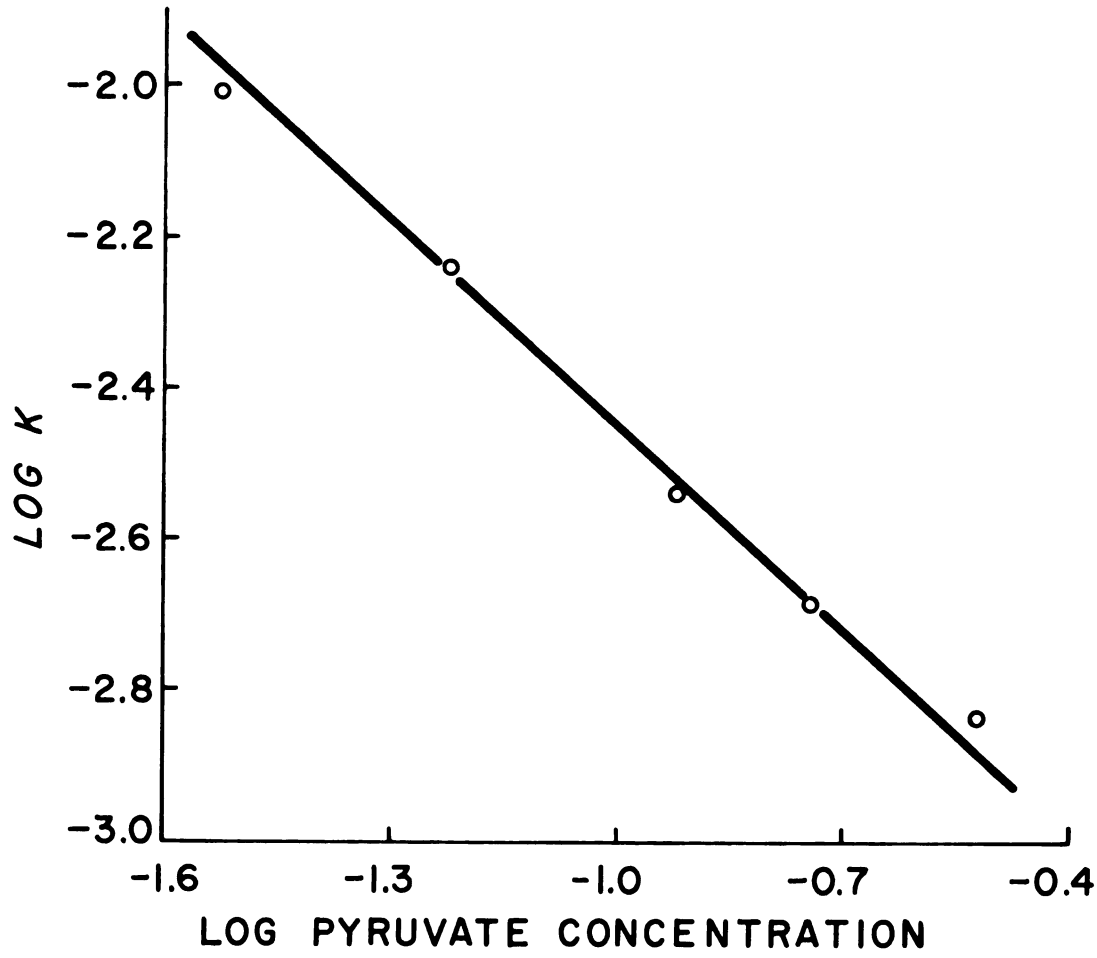

Figure 17. Effect of pyruvate concentration on the thermal inactivation rate constants of 55-fold purified dihydrodipicolinic acid synthetase at 75 C in 0.1 M potassium phosphate buffer at pH 7.0.

DISCUSSION

Dihydrodipicolinic acid synthetase catalyzes a very complex condensation reaction wherein a carbon-carbon bond is formed and an oxygen on the C-4 of β-aspartyl semi-aldehyde (BAS) is eliminated. The results of Yugari and Gilvarg (82) strongly suggested that a single enzyme was required to carry out this conversion. Similarly, throughout the fractionation procedures of this study there was no apparent indication that two separate enzymatic components were required to conduct the condensation. This conclusion is supported by the observation that the formation of δ-amino levulinic acid which involves formation of a carbon-carbon bond accompanied by double bond formation appears to be catalyzed by a single enzyme (22).

Dihydrodipicolinic acid synthetase from Escherichia coli was reported to be inhibited by lysine (82). Feedback inhibition of this reaction has great significance as a biological control mechanism. E. coli has been demonstrated to possess two aspartyl kinases, one under feedback control by lysine and the other controlled by threonine (81). As pointed out by Yugari and Gilvarg (82), the diminished production of aspartyl phosphate

and BAS in response to exogenous lysine would be a meaningless and possibly harmful mechanism if there were no means for shunting these intermediates toward synthesis of threonine and methionine. The inhibition of the condensing reaction provides this biological control mechanism. However, the results of this study are in direct opposition to this control mechanism. A final lysine concentration of 0.013 M failed to exhibit any indication of inhibiting sporulating B. megaterium KM dihydrodipicolinic acid synthetase. The quantity of lysine added was sufficient to detect feedback inhibition; therefore, it is concluded that this enzyme does not serve as a biological control mechanism in B. megaterium KM. Similarly, lysine was reported to exhibit no inhibition of dihydrodipicolinic acid synthetase from Bacillus lichenformis (69). Grandgenett and Stahly (23) reported that lysine inhibited diaminopimelic acid (DAP) decarboxylase in cell free extracts of B. lichenformis. Metabolic regulation at the DAP decarboxylase step provides a logical control mechanism for sporulating organisms. Inhibition at the dihydrodipicolinic acid synthetase step would not allow normal DPA synthesis and would result in the formation of unstable spores. Also, a large amount of DAP is present in the cortical material of bacterial spores indicating that the control mechanism should exist after the synthesis of DAP to allow normal spore development.

The apparent enhancement of enzymatic activity by sulfhydryl reducing agents does not correlate with the lack of inhibition by PCMB, iodoacetamide, and iodoacetate. Examination of the reaction product's absorption spectrum revealed the spectrum was shifted slightly to the higher ultraviolet region with an absorption maximum at 273 mμ and that the absorption intensity was increased by about 50%. Sulfhydryl, hydroxyl, amino, and some of the halogen group have been classified as auxochromes (15). Auxochromes are groups that do not in themselves show selective absorption above 200 mμ but when attached to a given chromophoric system, usually cause a shift in the absorption to a longer wavelength with an increased intensity of the absorption peak. It was concluded that the sulfhydryl reagents were involved in an auxochromic effect rather than the enzymatic reaction resulting in an apparent stimulation of the reaction due to an increase in the extinction coefficient of the reaction product.

During the purification procedure and ensuing studies no cofactor requirement was detected. Since extensive dialysis, DEAE chromatography, and the presence of EDTA in the buffer system failed to ellicit any cofactor requirement, it was concluded that a metal or biochemical cofactor was not involved in the enzymatic reaction unless it was tightly bound to the enzyme structure.

The pH optimum of the dihydrodipicolinic acid synthetase at pH 7.65 is in contrast to the optimum value of pH 8.4 reported by other researchers (69, 82). However, variations in the enzyme between different organisms or the fact that different buffer systems were used for these determinations could easily explain the discrepancies observed.

The apparent KM values of 4.6×10^{-4} and 5.0×10^{-4} are in reasonably good agreement with the values of 1.3×10^{-4} and 2.5×10^{-4} reported by Yugari and Gilvarg (82). However, Stahly (69) reported an inhibition of the enzyme by high concentrations of BAS in the presence of low concentrations of pyruvate. As the BAS concentration was increased, a resulting increase in the apparent KM of pyruvate was observed while V_{max} remained constant. A similar phenomenon was not observed for the KM of BAS. The results of this study neither confirm nor invalidate these findings. Since the assay did not allow the determination of the initial reaction rate, the values reported are considered as apparent KM values.

The enzyme was observed to exhibit cold-sensitivity after the second ammonium sulfate fractionation. Stability at 4 C was observed in 30% saturated ammonium sulfate solutions. The enzyme could be stabilized to storage at -20 C by adjusting the pH to 6.5. Inactivation of the enzyme at 4 C did not exhibit any indication of being

a reversible phenomenon. Shukuya and Schwert (66) reported that glutamic acid decarboxylase was not stable at 0 C but was stable at 25 C. This enzyme was stabilized by the addition of pyridoxal phosphate (a cofactor) and high ionic strength solutions. Several investigators (6, 50, 60) have demonstrated that serum lipoproteins are cold-sensitive. The possibility of a dissociated cofactor or the enzyme being a lipoprotein are strictly conjectural at this time. As previously stated, all attempts to demonstrate a cofactor requirement were unsuccessful. Until large quantities of the enzyme can be prepared in a more highly purified state, meaningful studies on the composition of the enzyme cannot be conducted.

Examination of the thermal inactivation data reveals the complexity of these phenomena. The inactivation of enzymes is nearly always due to the denaturation of the enzyme protein (11). A strong effect of pH is characteristic of protein denaturation. Generally, a zone of maximum stability, not necessarily including the isoelectric point, is observed; and the rates of inactivation increase on the acid and alkaline sides of the zone of maximum stability. Several other factors such as ionic strength, protein concentration, and the protective action of substrates and other substances also influence thermal inactivation rates. The effect of pH

on thermal inactivation rates was clearly demonstrated in this study. A region of maximum stability was observed at pH 10.5 with decreasing stability on either side of this value. An optimum thermal stability at this pH appears to be unique to this particular enzyme. The literature surveyed contained no reference to a protein with an optimum stability at such a highly basic pH. Although this phenomenon is an obvious reflection of the chemical and physical properties of the enzyme, sufficient data are not available to provide a realistic interpretation of these properties.

The variation of the half-lives at similar pH values in different buffer systems reflects the influence of ionic strength and suspending medium upon thermal inactivation rates. No attempt was made to control ionic strength, but it was relatively constant for the individual buffer systems. The relationship between the inactivation constant and pH further describes the complexity of thermal inactivation. Sadoff et al. (64) have suggested that the slope of log K versus pH describes the number of protons exchanged with the environment. The slopes of this plot for the phosphate and glycine buffers were approximately 0.5 while that of the boric acid buffer was about 1.0. Specific ion effects and variations in ionic strength could easily account for the variations in half-life at constant pH. However, the reasons for

the differences in the relationship between log K and pH are not obvious.

The ability of substrate to stabilize the enzyme is clearly demonstrated in the studies conducted at 75 C. In the absence of pyruvate, the half-life at 75 C in pH 7.0 phosphate buffer was calculated to be 0.01 min. The stability was increased 270-fold by 0.03 M pyruvate and 1850-fold by 0.3 M pyruvate. The slope of log K versus log of pyruvate concentration was approximately 1.0 indicating that one pyruvate molecule was bound at each active site on the enzyme.

The E_a values for the inactivation of enzymes and the denaturation of proteins are extremely high, ranging from about 40,000 to 100,000 cal per mole (75). The high heat of activation is due to exceptionally high positive entropies of activation. This has been interpreted as indicating the breaking of a large number of weak bonds, such as hydrogen bonds (11). The results of this study are in agreement with those conclusions. An extremely high E_a, 67,300 cal per mole, was accompanied by a high positive entropy, 133 cal per mole per deg, indicating the breakage of a large number of weak bonds. The $\Delta F^{\dagger}$ for thermal denaturation of almost all proteins is approximately 25 + 5K cal per mole (63). The $\Delta F^{\dagger}$ of 22,250 cal per mole observed for dihydrodipicolinic acid synthetase satisfies this parameter.

LITERATURE CITED

1. Bach, M. L. and C. Gilvarg. 1966. Biosynthesis of dipicolinic acid in sporulating Bacillus megaterium. J. Biol. Chem. 241:4563-4566.

2. Bailey, G. F., S. Karp, and L. E. Sacks. 1965. Ultraviolet absorption spectra of dry spores. J. Bacteriol. 89:984-987.

3. Black, S. H., T. Hashimoto and P. Gerhardt. 1960. Calcium reversal of the heat susceptability and dipicolinate deficiency of spores formed endotrophically in water. Can. J. Microbiol. 6:213-224.

4. Black, S. and N. G. Wright. 1955. Aspartic β-semialdehyde dehydrogenase and aspartic β-semialdehyde. J. Biol. Chem. 213:39-50.

5. Black, S. and N. G. Wright. 1955. Homoserine dehydrogenase. J. Biol. Chem. 213:51-60.

6. Bornstein, J. 1953. Insulin-reversible inhibition of glucose utilization by serum lipoprotein fractions. J. Biol. Chem. 205:513-519.

7. Byrne, A. F., T. H. Burton, and R. B. Koch. 1960. Relation of dipicolinic acid content of anaerobic bacterial endospores to their heat resistance. J. Bacteriol. 80:139-140.

8. Chasin, L. A. and J. Szulmajster. 1967. Biosynthesis of dipicolinic acid in Bacillus subtilis. Biochem. Biophys. Res. Comm. 29:648-654.

9. Church, B. D., and H. Halvorson. 1959. Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. Nature 183:124-125.

10. Curran, H. R., B. C. Brunstetter, and A. T. Myers. 1943. Spectrochemical analysis of vegetative cells and spores of bacteria. J. Bacteriol. 45:485-494.

11. Dixon, M. and E. C. Webb. 1964. Enzymes, 2nd Ed., Academic Press, New York, p. 146-166.

12. Doi, R. H. and H. Halvorson. 1961. Comparison of electron transport systems in vegetative cells and spores of Bacillus cereus. J. Bacteriol. 81:51-58.

13. Doi, R. H. and H. Halvorson. 1961. Mechanism of dipicolinic acid stimulation of the soluble reduced diphosphopyridine nucleotide oxidase of spores. J. Bacteriol. 81:642-648.

14. Donnellan, J. E. and R. B. Setlow. 1965. Thymine photoproducts but not thymine dimers found in ultraviolet-irradiated bacterial spores. Science 149:308.

15. Dyer, J. R. 1965. Application of Absorption Spectroscopy of Organic Compounds, Prentice Hall, Inc., Englewood Cliffs, p. 10-11.

16. Eyring, H. 1935. The activated complex and the absolute rate of chemical reactions. J. Chem. Phys. 3:107.

17. Fitz-James, P. C. 1963. In "Mecanismes de Regulation des Activities Cellularires Chez les Microorganisms." p. 529. J. Senez (ed.), Gordon and Breach, New York.

18. Flodin, P. 1962. Dextran gels and their application in gel filtration. Dissertation. Uppsala, A. B. Pharmacia, Uppsala, Sweden.

19. Foerster, H. F. and J. W. Foster. 1966. Response of spores to combinations of germinative compounds. J. Bacteriol. 91:1168-1177.

20. Foerster, H. F. and J. W. Foster. 1966. Endotrophic calcium, strontium, and barium spores of Bacillus megaterium and Bacillus cereus. J. Bacteriol. 91: 1333-1345.

21. Frazier, W. C. 1967. Food Microbiology. McGraw-Hill Book Co., New York, p. 74-108.

22. Gibson, K. D., A. Neuberger, and J. J. Scott. 1955. The purification and properties of δ-aminolevulinic acid dehydrase. Biochem. J. 61:618-629.

23. Grangenett, D. and D. P. Stahly. 1968. Studies on diamininopimelic acid decarboxylase in Bacillus licheniformis. Bacteriol. Proc. 68:141.

24. Hachisuka, Y., K. Tochikubo, Y. Yokoi, and T. Murachi. 1967. The action of dipicolinic acid and its chemical analoges on the heat stability of glucose dehydrogenase of Bacillus subtilis spores. J. Biochem. 61:659-661.

25. Halvorson, H., R. Doi, and B. D. Church. 1958. Dormancy of bacterial endospores: regulation of electron transport by dipicolinic acid. Proc. Natl. Acad. Sci. 44:1171-1180.

26. Halvorson, H. and C. Howitt. 1961. The role of DPA in bacterial spores. p. 149-164. In H. O. Halvorson (ed.), Spores II. Burgess Publishing Co., Minneapolis.

27. Halvorson, H. O. and B. D. Church. 1957. Biochemistry of spores of aerobic bacilli with special reference to germination. Bacteriol. Rev., 21:112-131.

28. Harrell, W. K. 1958. Stimulation of glucose oxidation in extracts of bacterial spores by dipicolinic acid. Can. J. Microbiol. 4:393-398.

29. Harrell, W. K. and E. Mantini. 1957. Studies on dipicolinic acid in the spores of Bacillus cereus var. terminalis. Can. J. Microbiol. 3:735-739.

30. Hodson, P. H. and J. W. Foster. 1965. Methyl dipicolinate monoester from spores of Bacillus subtilus var. niger. J. Bacteriol., 90:1503.

31. Hodson, P. H. and J. W. Foster. 1966. Dipicolinic acid synthesis in Penicillium citreo-viride. J. Bacteriol. 91:562-569.

32. Janssen, F. W., A. J. Lund, and L. E. Anderson. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science, 127:26-27.

33. Jaye, M. and Z. J. Ordal. 1965. Germination of spores of Bacillus megaterium with divalent metal-dipicolinate chelates. J. Bacteriol., 89:1617-1618.

34. Kanie, M., S. Fujimoto, and J. W. Foster. 1966. Chemical degradation of dipicolinic acid-C^{14} and its application in biosynthesis by Penicillium citreo-viride, 91:570-577.

35. Keynan, A., Z. Evenchick, H. O. Halvorson, and J. W. Hastings. 1964. Activation of bacterial endospores. J. Bacteriol., 88:313-318.

36. Keynan, A. and H. O. Halvorson. 1962. Calcium dipicolinate induced germination of Bacillus cereus spores. J. Bacteriol., 83:100-105.

37. Keynan, A., W. G. Murrell, and H. O. Halvorson. 1962. Germination properties of spores with low dipicolinic acid content. J. Bacteriol., 83:395-399.

38. Knaysi, G. 1965. Further observations on the spodogram of Bacillus cereus endospores. J. Bacteriol., 90:453-455.

39. Lechowich, R. V. and Z. J. Ordal. 1960. The influence of the sporulation temperature on the thermal resistance and chemical composition of endospores. Bacterial Proc. 44-45.

40. Levinson, H. S., M. T. Hyatt, and F. E. Moore. 1961. Dependence of the heat resistance of bacterial spores on the calcium: dipicolinic acid ratio. Biochem. Biophys. Res. Comm., 5:417-421.

41. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193:265-275.

42. Martin, H. H. and J. W. Foster. 1958. Biosynthesis of dipicolinic acid in spores of Bacillus megaterium. J. Bacteriol., 76:167-178.

43. Mayall, B. H. and C. F. Robinow. 1960. Observations with the electron microscope on the organization of the cortex of resting and germinating spores of B. megaterium. J. Appl. Bacteriol., 20:333-341.

44. Mishiro, Y. and M. Ochi. 1966. Effect of dipicolinate on the heat denaturation of proteins. Nature, 211:1190.

45. Murrell, W. G. 1961. In G. G. Meynell and H. Gooder (ed.), Microbial Reaction to Environment. Symp. Soc. Gen. Microbiol., 11:100. Cambridge University Press, Cambridge, England.

46. Murrell, W. G. 1967. The biochemistry of the bacterial endospore. p. 133-251. In A. H. Rose and J. F. Wilsinson (ed.), Advances in Microbial Physiology, Vol. 1, Academic Press, New York.

47. Murrell, W. G. and A. A. Warth. 1965. Composition and heat resistance of bacterial spores. p. 1-23. In L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.

48. Nakata, H. M. 1964. Organic nutrients required for growth and sporulation of Bacillus cereus. J. Bacteriol., 88:1522.

49. O'Conner, R. J., R. H. Doi, and H. Halvorson. 1960. Preparation of bacterial spore extracts by use of the Eppenbach colloid mill. Can. J. Micro., 6:233-235.

50. Oncley, J. L., F. R. N. Gurd, and M. J. Melin. 1950. Preparation and properties of serum and plasma proteins. XXV. Composition and properties of human serum β-lipoprotein. J. Am. Chem. Soc., 72:458-465.

51. Ooyama, J., N. Nakamura, and O. Tanabe. 1960 Biosynthesis of dipicolinic acid by a Penicillium sp. Bull. Agr. Chem. Soc. Japan, 24:743-744.

52. Pepper, R. E. and R. N. Costilow. 1965. Electron transport in Bacillus popilliae. J. Bacteriol., 89: 271-276.

53. Perry, J. J. and J. W. Foster. 1955. Studies on the biosynthesis of dipicolinic acid in Bacillus cereus var. mycoides. J. Bacteriol., 69:337-346.

54. Perry, J. J. and J. W. Foster. 1956. Monoethyl ester of dipicolinic acid from bacterial spores. J. Bacteriol., 72:295-301.

55. Peterson, E. A. and H. A. Sober. 1956. Chromatography of proteins. I. Cellulose ion exchange abosrbants. J. Am. Chem. Soc., 78:751-755.

56. Powell, J. F. 1953. Isolation of dipicolinic acid (pyridine 2:6 dicarboxylic acid) from spores of B. megaterium. Biochem. J., 54:210-211.

57. Powell, J. F. 1957. Biochemical changes occurring during spore germination in Bacillus species. J. Appl. Bacteriol., 20:349-359.

58. Powell, J. F. and R. E. Strange. 1956. Biochemical changes occurring during sporulation in Bacillus species. Biochem. J., 63:661-668.

59. Powell, J. F. and R. E. Strange. 1969. Synthesis of dipicolinic acid from α, ε-diketopimelic acid. Nature, 184:878-880.

60. Pullman, M. E., H. S. Penefsky, A. Patta, and E. Racker. 1960. Partial resolution of the enzymes catalyzing oxidative phosphorylation. I. Purification and properties of soluble dinitrophenol-stimulated adenosine triphosphate. J. Biol. Chem., 235:3322-3327.

61. Reiman, H. 1961. Germination of bacteria by chelating agents. p. 24-48. In H. O. Halvorson (ed.), Spores II. Burgess Publishing Co., Minneapolis.

62. Reiman, H. and Z. J. Ordal. 1961. Germination of bacterial endospores with calcium and dipicolinic acid. Science, 113:1703-1704.

63. Sadoff, H. L. Spore Enzymes. In G. W. Gould, H. Hurst, and J. Z. Wolf (ed.), The Bacterial Spore. Academic Press, London. In press.

64. Sadoff, H. L., J. A. Bach, and J. W. Kools. 1965. Significance of multiple forms of glucose dehydrogenase in relation to heat resistance. p. 97-110. In L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.

65. Schaeffer, P., H. Ionesco, A. Ryter, and G. Balossa. 1963. In Mecanismes de Regulation des Activities Cellulaires chez les Microorganisms. J. Senez (ed.), p. 553. Gordon and Breach, New York.

66. Shukuya, R. and G. W. Schwert. 1960. Glutamic acid decarboxylase. III. The inactivation of the enzyme at low temperatures. J. Biol. Chem., 235:1658-1662.

67. Slepecky, R. A. 1961. Multiple functions of dipicolinic acid. p. 171-179. In H. O. Halvorson (ed.), Spores II. Burgess Publishing Company, Minneapolis.

68. Slepecky, R. and J. W. Foster. 1959. Alterations in the metal content of spores of Bacillus megaterium and the effects on some spore properties. J. Bacteriol., 78:117-123.

69. Stahly, D. P. 1968. Properties of Bacillus licheniformis dihydrodipicolinic acid synthetase. Bacteriol. Proc., 68:141.

70. Tannenbaum, S. W. and K. Kaneko. 1964. Biosynthesis of dipicolinic acid and of lysine in Penicillium citreo-viride. Biochemistry, 3:1314-1322.

71. Thomas, R. S. 1964. Ultrastructure localization of mineral matter in bacterial spores by micro-incineration. J. Cell. Biol., 23:113-133.

72. Vary, J. C. and H. O. Halvorson. 1968. Initiation of bacterial spore germination. J. Bacteriol., 95:1327-1334.

73. Vinter, V. 1960. The synthesis of specific calcium and cystine containing structures in sporulating cells of bacilli. Folia. Microbiol., 5:217-230.

74. Warburg, O. and W. Christian. 1942. Isolierung and kristallisation des garungsferments Enolase. Biochem. Z., 310:384-421.

75. White, A., P. Handler, and E. L. Smith. 1959. Principles of Biochemistry. McGraw-Hill, New York. p. 231.

76. Windle, J. J. and L. E. Sacks. 1963. Electron paramagnetic resonance of manganese (II) and copper (II) in spores. Biochem. et Biophys. Acta, 66: 173-179.

77. Wise, J., A. Swanson, and H. O. Halvorson. 1967. Dipicolinic acid-less mutants of Bacillus cereus. J. Bacteriol., 94:2075-2076.

78. Wooley, B. C. and R. E. Collier. 1965. Changes in thermoresistance of Clostridium roseum as related to the intracellular content of calcium and dipicolinic acid. Can. J. Microbiol., 11:279-285.

79. Young, I. E. 1959. A relationship between the free amino acid pool, dipicolinic acid, and calcium from resting spores of Bacillus megaterium. Can. J. Microbiol., 5:197-202.

80. Young, I. E. and J. C. Fitz-James. 1959. Chemical and morphological studies of bacterial spore formation. I. The formation of spores in Bacillus cereus. J. Biophys. Biochem. Cytol., 6:467-498.

81. Yugari, Y. and C. Gilvarg. 1962. Coordinated end-product inhibition in lysine synthesis in Escherichia coli. Biochem. et Biophys. Acta, 62:612-617.

82. Yugari. Y. and C. Gilvarg. 1965. The condensation step in diaminopimelate synthesis. J. Biol. Chem., 240:4710-4715.

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