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Partial Purification and Characterization of Dihydrodipicolinic Acid Synthetase from Sporulating *Bacillus megaterium*[1]

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Sporulaton of *Bacillus megaterium* Km (ATCC 13632) was synchronized by a technique employing three 10% transfers. The culture was harvested when 60% of the cells contained spore forms. Dihydrodipicolinic acid synthetase was purified 150-fold by ammonium sulfate fractionation at *p*H 6.5, heating for 15 min at 45 C at *p*H 6.0, ammonium sulfate fractionation at *p*H 6.0, and subsequent chromatography on diethylaminoethyl cellulose. During the final stage of the purification procedure, the enzyme exhibited sensitivity to refrigeration temperatures. The enzyme had a *p*H optimum of 7.65 in imidazole buffer. The apparent K_m values were 4.6×10^{-4} and 5.0×10^{-4} M 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 maximal thermal resistance at *p*H 10.5. The thermal stability of the enzyme at 75 C was increased more than 1,800-fold by the addition of 0.3 M pyruvate. The E_a was 67,300 cal/mole for the thermal denaturation of the enzyme. At 60 C, the ΔF, ΔH, and ΔS values for the thermal denaturation of the enzyme were 22,250, 66,700, and 133 cal per mole per degree, respectively.

Initial experiments by Martin and Foster (15) on the biosynthesis of dipicolinic acid (DPA) indicated that aspartate and pyruvate or alanine and oxalacetate, or their derivatives were the logical precursors of DPA. The studies of Hodson and Foster (11) and Kanie et al. (13) were consistent with this proposal. Recently, dihydrodipicolinic acid, formed by the condensation of pyruvate and β-aspartyl semialdehyde (BAS), was demonstrated by Yugari and Gilvarg (27) to be an intermediate in the lysine biosynthetic pathway. This indicated that DPA could be formed by a modification of the lysine synthetic pathway. Bach and Gilvarg (1) demonstrated the synthesis of DPA from pyruvate and BAS in cell-free extracts of sporulating *Bacillus megaterium*. Chasin and Szulmajster (5) 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 an enzymatic and a nonenzymatic synthesis of DPA were detected. The nonenzymatic synthesis was stimulated by Mn^{++} and completely inhibited by 0.005 M ethylenediaminetetraacetic acid (EDTA). The enzymatic synthesis was found to require nicotinamide adenine dinucleotide (NADH) or flavine adenine dinucleotide (FAD) as a cofactor. This study was undertaken to isolate and characterize dihydrodipicolinic acid synthetase from sporulating organisms.

MATERIALS AND METHODS

Organism and growth conditions. *B. megaterium* Km (ATCC 13632), obtained from the laboratory of Z. J. Ordal, 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 of Nakata (16). The medium contained (in grams per liter of distilled water): $FeSO_4 \cdot 7H_2O$, 0.0005; $CuSO_4 \cdot 5H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.005; $MnSO_4 \cdot H_2O$, 0.05; $MgSO_4$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.08; $(NH_4)_2SO_4$, 2.0; glucose, 4.0; Casamino Acids, 2.0. The medium was buffered at *p*H 7.0 with 0.1 M potassium phosphate. The glucose and Casamino Acids were prepared and sterilized individually and added to the

[1] Journal Article 4752 of the Michigan Agricultural Experiment Station. A preliminary report of part of this study was presented at the 68th Annual Meeting of the American Society for Microbiology at Detroit, Mich., 5-10 May 1968. This work was presented in a thesis by the senior author in partial fulfillment of the requirements of Michigan State University for the Ph.D. degree.

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medium at the time of inoculation. The mineral solution was prepared separately, filter sterilized, and added to the medium at the time of inoculation.

Preparation of materials. The β-aspartyl semialdehyde was prepared by the ozonolysis procedure of Black and Wright (2) using DL-allyl glycine (Nutritional Biochemicals Co., Cleveland, Ohio). Homoserine dehydrogenase purified from *Saccharomyces cereviseae* by the method of Black and Wright (3) was used to measure the product of ozonolysis.

Diethylaminoethyl (DEAE) cellulose columns were prepared by the method of Peterson and Sober (19).

Bio-Gel P-200 and P-2 (Bio-Rad Laboratories, Richmond, Calif.) were prepared according to the method of Flodin (9).

Dihydrodipicolinic acid synthetase assay. The enzymatic assay used in this study was derived from the assay of Yugari and Gilvarg (27). The reaction mixture contained 250 μmoles of imidazole (*p*H 7.6), 20 μmoles of pyruvate, 5 μmoles of BAS, and condensing enzyme in a 3-ml volume. A blank sample was prepared in an identical manner except that the BAS was omitted. Assays were conducted at 35 C, unless otherwise stated. Since the reaction had a lag period of approximately 3 to 5 min before assuming a linear rate, the enzyme was diluted to obtain a change in absorbancy 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 1 M $NaHCO_3$ and then added to start the reaction. The reactions were followed at 270 nm in a DBG spectrophotometer (Beckman Instruments Co., Inc., Fullerton, Calif.) and the change in absorbancy was recorded with a 25.4 cm recorder (Beckman Instruments Co., Inc.). The temperature was controlled by circulation of water from a constant-temperature water bath through the jacketed sample compartment. An absorbancy change of 0.01 per min was defined as 1 unit of enzyme activity.

Dipicolinic acid analysis. Dipicolinic acid analyses were conducted by a modification of the Janssen, Lund, and Anderson colorimetric technique (12), in which the heating time was increased to 1 hr and trichloroacetic acid was substituted for acetic acid.

Determination of sporulation. Samples of cultures were observed by using phase-contrast microscope optics at a magnification of 1,000. Both dark and bright spore forms were counted.

Protein analysis. The methods of Warburg and Christian (24) and Lowry et al. (14) were used to determine protein concentration; bovine serum albumin was used as a standard.

Determination of the relationship between sporulation and dipicolinic acid synthesis. 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 an incubator rotary shaker (model G25, New Brunswick Scientific Co., New Brunswick, N.J.) at 200 rev/min. The final transfer was into 30 liters of medium in a 50-liter Ferma-Cell fermentor (New Brunswick Scientific Co.). The temperature was maintained at 35 C; agitation was 200 rev/min, and aeration was 1.5 ft^3/min. Every 45 min, starting at 8 hr after the final transfer, a 50-ml sample was taken for DPA analysis and a small sample was reserved for the determination of sporulation. The samples for DPA analysis were concentrated by centrifugation at 10,000 × *g* for 20 min. The supernatant fluid was decanted and discarded. The pellet was suspended in 15 ml of distilled water and analyzed as previously described. Per cent sporulation and DPA concentration were correlated graphically to determine the appropriate time to harvest the culture for enzyme extraction.

Preparation of cell-free extract. When the sporulation had reached a minimum of 60%, the culture was cooled to 10 C in about 1 hr with the cooling system on the fermentor. The cells were then harvested by continuous centrifugation at 30,000 × *g* at 3 C. After harvesting, the cells were resuspended in 0.025 M potassium phosphate at *p*H 7.0, washed twice, and stored at −20 C. Thawed cells were broken in an Eppenbach Colloid mill (Gifford-Wood Co., Hudson, N.Y.) by a slight modification of the technique of O'Connor et al. (17). Cells (400 ml) and glass beads (200 ml; 0.10 to 0.11 mm) were added to the colloid mill and the contents were cooled to 5 C by liquid CO_2. The cells were homogenized for 12 min at a clearance of 0.003 inch at a rheostat setting of 110. During 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 *p*H 7.0 to recover additional enzyme. The supernatant fluids were pooled and the debris was removed by centrifugation at 25,000 × *g* for 30 min at 3 C. The cell-free extract was decanted and adjusted to *p*H 7.5 with 0.2 N NaOH. This fraction was termed the crude extract.

Purification of dihydrodipicolinic acid synthetase. The extract was maintained at 3 C for the initial and subsequent fractionations with $(NH_4)_2SO_4$. The extract was brought to 54% saturation by adding $(NH_4)_2SO_4$ and was agitated for 30 min. The inactive precipitate was removed by centrifugation at 20,000 × *g* for 15 min at 3 C. The supernatant fluid was then brought to 66% of saturation of $(NH_4)_2SO_4$ and mixed for 30 min. The active precipitate was recovered by centrifugation at 20,000 × *g* 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 *p*H 6.0, which was 0.005 M with respect to EDTA. The resulting fraction was heated with agitation at 45 C for 15 min and immediately cooled to 3 C; the inactive precipitate was removed by centrifugation at 20,000 × *g* for 15 min at 3 C. Solid $(NH_4)_2SO_4$ was added to the extract until 35% of saturation was reached; the mixture was stirred for 30 min and the inactive precipitate was removed by centrifugation at 20,000 × *g* for 15 min at 3 C. Next, solid $(NH_4)_2SO_4$ was added to obtain 50% saturation; the mixture was stirred for 30 min and the active

precipitate was collected by centrifugation at 20,000 × *g* for 15 min at 3 C. The pellet was resuspended in 10 ml of 0.1 M potassium phosphate (*p*H 7.5), which was 0.005 M with respect to EDTA. The sample was applied to a Bio-Gel P-2 column (33 by 2.2 cm, inside diameter). The column was eluted with 0.1 M potassium phosphate (*p*H 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 nm (Gilson Medical Electronics, Middleton, Wis.). The active fraction from the Bio-Gel P-2 column was applied to a DEAE cellulose column (21 by 2.2 cm, inside diameter) equilibrated at room temperature with a 0.1 M potassium phosphate buffer, which was 0.005 M with respect to EDTA. 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 minute and analyzed for protein and enzymatic activity. The most active fractions that eluted with 0.3 M KCl were applied to a Bio-Gel P-200 column (36 by 4 cm, inside diameter) equilibrated at room temperature with a 0.1 M potassium phosphate buffer (*p*H 7.5) also containing 0.005 M EDTA. The column was operated at a flow rate of 1 ml/min, and 5.3-ml fractions were analyzed for protein by the method of Warburg and Christian (24) and for enzymatic activity. The most active fractions were combined and adjusted to *p*H 6.5 with 1 N HCl. The enzyme was divided into 3-ml fractions to avoid loss of enzymatic activity due to repeated freezing and thawing and was stored at −20 C.

Determination of thermal inactivation characteristics. Three buffer systems were used to determine thermal inactivation of the enzyme: 0.1 M potassium phosphate from *p*H 6.5 to 8.0, 0.1 M boric acid-NaOH from *p*H 8.0 to 9.0, and 0.1 M glycine-NaOH from *p*H 9.0 to 11.5. Diluted enzyme (0.5 ml) purified on a Bio-Gel P-200 column was added to 4.5 ml of the appropriate buffer and the *p*H was adjusted with 1 N NaOH. Samples (0.5 ml) were added to screw-cap test tubes (13 by 100 mm); the tubes were capped and submerged in a constant-temperature water bath which maintained the temperature at ±1.0 C. 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. The *p*H of the heated enzyme was readjusted prior to analysis in all thermal inactivation experiments above *p*H 8.0. Parameters of thermal inactivation of the enzyme were calculated by using the Eyring theory of absolute reaction rates (8).

RESULTS

Initial experiments were designed to determine the optimal time to harvest sporulating cells to be used as the source of enzyme. 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 that 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 purification of dihydrodipicolinic acid synthetase. Two different enzymatic preparations were made by variation of the final step in the purification procedure. Fractionation by DEAE cellulose chromatography resulted in a 152-fold purification with recovery of 11% of the original activity. Bio-Gel P-200 chromatography produced a 55-fold purification with recovery of 33% of the original activity. The enzyme exhibited cold sensitivity; therefore, the columns were maintained at room temperature. The enzyme preparations were stabilized to storage at −20 C with a minimal loss of activity by adjusting the *p*H to 6.5.

The elution profile from DEAE cellulose is presented in Fig. 1. Upon elution with 0.3 M KCl, the enzyme was essentially free from nucleic acids

TABLE 1. *Purification procedures and typical results for dihydrodipicolinic acid synthetase from sporulating B. megaterium Km*

Prccedure	Vol (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg)	Purification	Per cent yield[a]
Crude extract	250	45	8.1	5.7	1	100
$(NH_4)_2SO_4$ (54 to 66%)	60	133	7.0	18.5	3.27	68
45 C, 15 min	54	133	3.5	37.0	6.54	68
$(NH_4)_2SO_4$ (35 to 50%)	11	460	9.5	48.5	8.54	53
DEAE[b]	30	31.4	0.038	861	151.60	10.6
P-200[c]	55[c]	66.7	0.24	268	55	33

[a] Adjusted to reflect samples reserved for protein and enzymatic analyses.
[b] Enzyme was chromatographically purified by using DEAE cellulose.
[c] Enzyme was chromatographically purified by using Bio-Gel P-200.

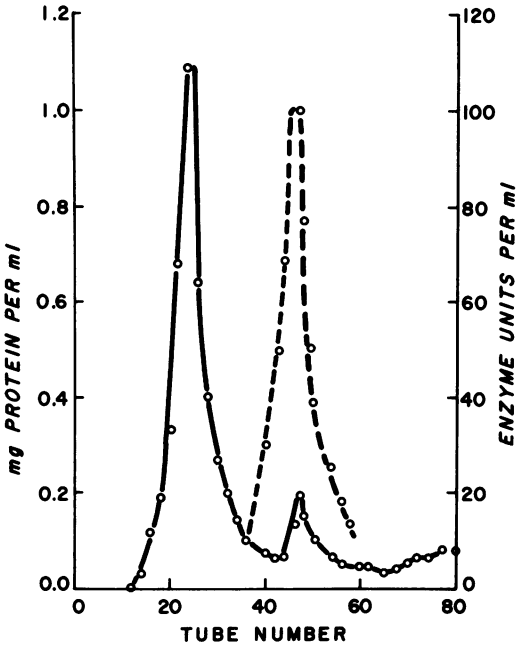

FIG. 1. *Elution profiles of protein and dihydrodipicolinic acid synthetase activity from a DEAE cellulose column eluted with a step gradient of potassium chloride in 0.1 M potassium phosphate buffer at pH 7.5. Symbols: solid line, protein concentration; broken line, enzymatic activity.*

by use of the Warburg and Christian technique (24). Tubes 42 to 50 contained the majority of the enzymatic activity and were retained for subsequent experimentation.

Figure 2 represents the Bio-Gel P-200 elution profile. The relative R_F value (elution volume/void volume) was 1.85, which indicated that the enzyme had entered the gel to a great extent. The preparation was estimated to contain approximately 8 to 10% nucleic acids as contaminants. Tubes 46 to 57 contained the majority of the enzymatic activity and were retained for subsequent use in thermal inactivation experiments.

Figure 3 demonstrates the effect of enzyme concentration on reaction rate. The reaction is shown to be dependent upon enzyme concentration within the given absorbancy range and time. The initial lag period of the reaction is clearly depicted.

The effect of *p*H on enzymatic activity was examined by using imidazole buffer in the *p*H range of 7.0 to 8.4 at *p*H unit intervals of 0.2. The enzyme exhibited a relatively symmetrical *p*H versus activity curve with an optimum at *p*H 7.65.

The K_m constants were determined for BAS and pyruvate. The BAS concentration was kept constant, and the pyruvate concentration was varied from 1 to 30 μM to determine the K_m of pyruvate. Similarly, the pyruvate concentration was kept constant, and the BAS concentration was varied from 1 to 10 μM to determine the K_m for BAS. The change in absorbancy between 7.5

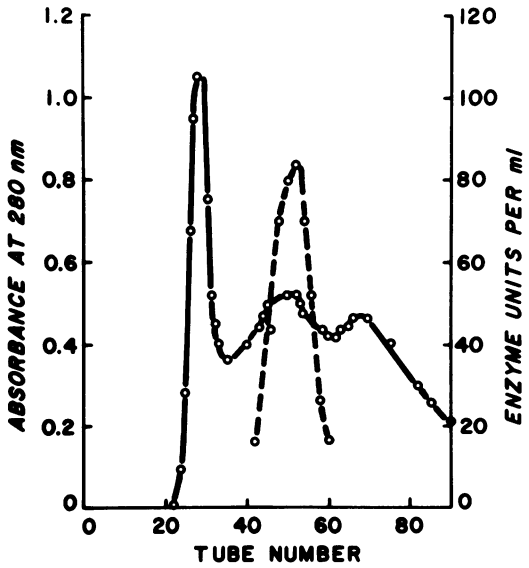

FIG. 2. *Elution profile of 280-nm absorbing materials and dihydrodipicolinic acid synthetase activity from a Bio-Gel P-200 column eluted with 0.1 M potassium phosphate buffer at pH 7.5. Symbols: solid line, protein concentration; broken line, enzymatic activity.*

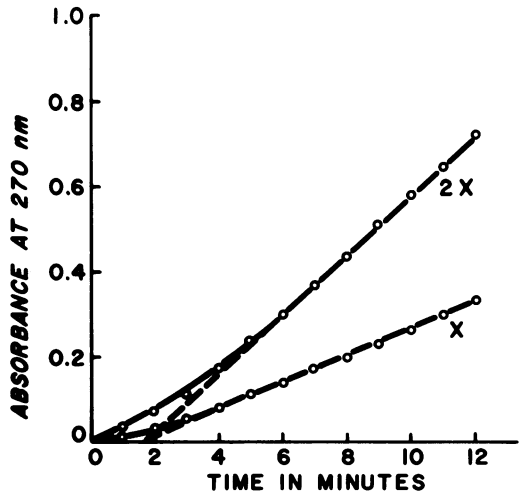

FIG. 3. *Relationship of enzyme concentration to reaction rate for 150-fold purified dihydrodipicolinic acid synthetase. Appropriate dilutions of DEAE cellulose-purified enzyme, within the prescribed absorbance range limitations, were tested for linearity of response. Standard assay procedures were employed.*

and 10 min was determined by standard assay procedures. This time period was selected due to the initial lag period of the reaction. The K_m values were found to be 4.6×10^{-4} and 5.0×10^{-4} M for BAS and pyruvate, respectively.

The absorbancy of the reaction product between 240 and 300 nm is presented in Fig. 4. The reaction product exhibited broad symmetrical absorption with a maximum at 265 nm. 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 parachloromercuribenzoate (PCMB) to the reaction failed 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. In the presence of β-mercaptoethanol the reaction product had a broad symmetrical absorbance spectrum with a maximum absorbance at 273 nm in contrast to the maximum absorbance at 265 nm without added β-mercaptoethanol. Also, the intensity of the absorption maximum is almost 50% greater in the presence of β-mercaptoethanol.

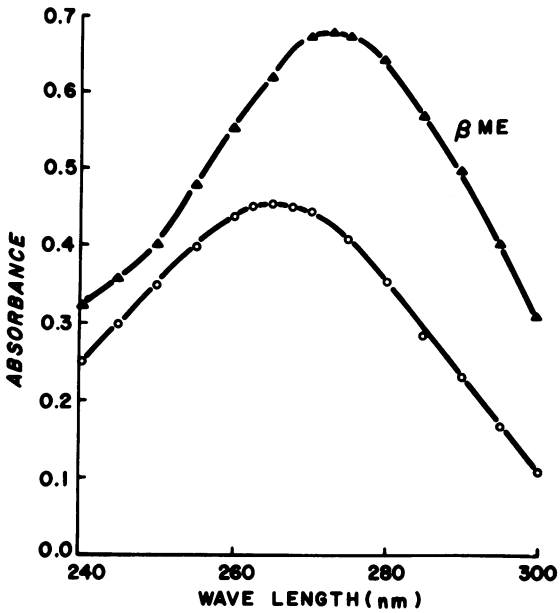

FIG. 4. *Effect of β-mercaptoethanol on the absorption spectrum of the 270-nm assay product of 150-fold purified dihydrodipicolinic acid synthetase. The BAS concentration in the reaction mixture was reduced to 1 μM to maintain a measurable total change in absorbance for spectrum determinations. To determine the effects of sulfhydryl reducing agents, 0.1 ml of 0.1 M β-mercaptoethanol was added to the reaction mixture and the reaction was allowed to go to completion. Change in absorbancy over a range of 240 to 300 nm was determined at 5-nm intervals. Symbols: △, with added β-mercaptoethanol; ○, no β-mercaptoethanol added.*

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 *p*H on thermal inactivation of the enzyme at 55 C in 0.1 M potassium phosphate from *p*H 6.5 to 8.0 is summarized in Fig. 5A. First-order reaction kinetics were observed in all instances. As the *p*H increased from 6.5 to 8.0, the half-lives (time for 50% inactivation) of the enzyme increased from 5.3 to 20 min.

Figure 5B demonstrates the rates of thermal inactivation at 55 C in 0.1 M boric acid-NaOH from *p*H 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 *p*H 8.0 to 75 min at *p*H 9.0. Figures 5C and 5D describe the rates of thermal inactivation at 55 C in 0.1 M glycine-NaOH from *p*H 9.0 to 11.5. The half-life increased from 19.5 min at *p*H 9.0 to 89 min at *p*H 10.5 and then decreased to 33.5 min at *p*H 11.5. In all cases, first-order reaction kinetics were observed.

The relationship of *p*H to the thermal inactivation constant (K) is shown in Figure 6. Also, the effect of the buffer system on the inactivation constant at similar *p*H and the rate of change with varying *p*H are clearly indicated. A point of maximal thermal stability was found at *p*H 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, whereas above *p*H 11.0 the slope was approximately +0.7.

Figure 7 demonstrates the effect of temperature on the rate of thermal inactivation in 0.1 M potassium phosphate (*p*H 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.

An Arrhenius plot of the thermal inactivation data at *p*H 7.0 is presented in Figure 8. The E_a was 67,300 cal/mole for the thermal denaturation of the enzyme. At 60 C, the ΔF, ΔH, and ΔS values were 22,250, 66,700, and 133 cal permole per degree, respectively.

The effect of pyruvate on thermal inactivation at 75 C in 0.1 M potassium phosphate at *p*H 7.0 is reported in Figure 9. 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.

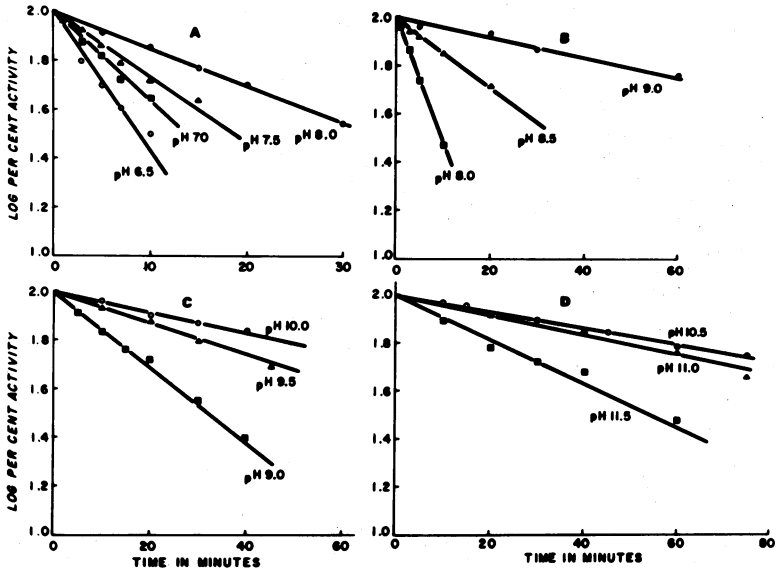

FIG. 5. *Time required for thermal inact vation at 55 C of 55-fold purified dihydrodipicolinic acid synthetase (A) in 0.1 M potassium phosphate buffer over the pH range of 6.5 to 8.0, (B) in 0.1 M boric acid-sodium hydroxide buffer over the pH range of 8.0 to 9.0, (C) in 0.1 M glycine-sodium hydroxide buffer over the pH range of 9.0 to 10.0, and (D) in 0.1 M glycine-sodium hydroxide buffer over the pH range of 10.5 to 11.5.*

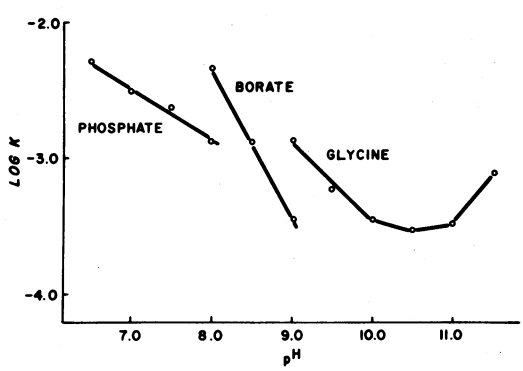

FIG. 6. *Relationship of pH to 55 C thermal inactivation rate constants of 55-fold purified dihydrodipicolinic acid synthetase. The thermal inactivation rate constant (K) is plotted on the ordinate as $\log_{10}K$ which is obtained from the equation, $K = (1/t_2 - t_1)\ln(C_1/C_2)$, where C_1 and C_2 are enzyme concentrations at times t_1 and t_2, respectively.*

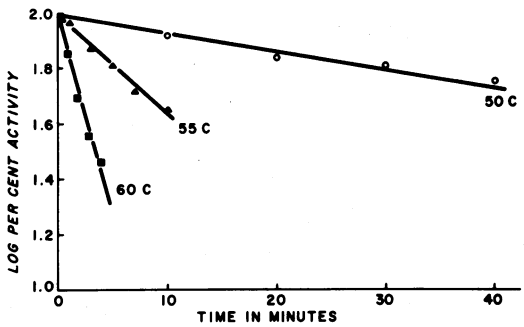

FIG. 7. *Time required for thermal inactivation of 55-fold purified dihydrodipicolinic acid synthetase in 0.1 M potassium phosphate buffer at pH 7.0 over the temperature range of 50 to 60 C.*

DISCUSSION

Dihydrodipicolinic acid synthetase catalyzes a complex condensation reaction wherein a carbon-carbon bond is formed and an oxygen on the C-4 of BAS is eliminated. The results of Yugari and Gilvarg (27) 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-aminolevulinic acid, which involves formation of a carbon-carbon bond accompanied by double-bond formation, appears to be catalyzed by a single enzyme (10).

Dihydrodipicolinic acid synthetase from *E. coli* was reported to be inhibited by lysine (27). Feedback inhibition of this reaction has significance as a biological control mechanism. *E. coli* has been demonstrated to possess two aspartyl kinases, one under feedback control by lysine, one other controlled by threonine (26), and a third which has not been reported to be subject to

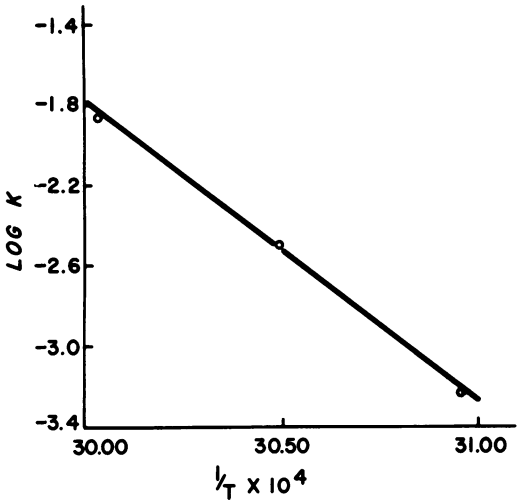

FIG. 8. *Effect of temperature on thermal inactivation rate constants of 55-fold purified dihydrodipicolinic acid synthetase in 0.1 M potassium phosphate buffer at pH 7.0. Values given on the abscissa are reciprocals of the absolute temperature which have been multiplied by 10^4.*

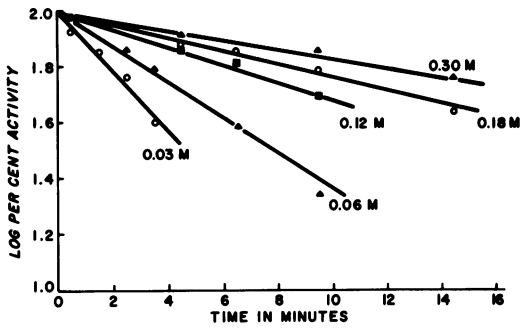

FIG. 9. *Time required for thermal inactivation of 55-fold purified dihydrodipicolinic acid synthetase in 0.1 M potassium phosphate buffer at pH 7.0 at 75 C in the presence of 0.03 to 0.3 M pyruvate. Appropriate volumes of 0.5 M pyruvate, prepared in 0.1 M potassium phosphate at pH 7.0, were added to 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 measured and adjusted, if necessary. Samples (0.5 ml) were dispensed into screw-cap test tubes (13 by 100 mm) and treated as described.*

feedback control (23). As pointed out by Yugari and Gilvarg (27), 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 did not inhibit sporulating *B. megaterium* Km dihydrodipicolinic acid synthetase. Since the quantity of lysine added was sufficient to detect feedback inhibition, it is concluded that this enzyme does not serve as a biological control mechanism in *B. megaterium* Km. Similarly, *B. licheniformis* dihydrodipicolinic acid synthetase was not inhibited by lysine (D. P. Stahly, Bacteriol. Proc., p. 141, 1968). D. Grandgenett and D. P. Stahly (Bacteriol. Proc., p. 141, 1968) reported that lysine inhibited diaminopimelic acid (DAP) decarboxylase in cell-free extracts of *B. licheniformis*. 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 absorption spectrum of the reaction product revealed the spectrum was shifted slightly to the higher ultraviolet region with an absorption maximum at 273 nm and that the absorption intensity was increased by about 50%. Sulfhydryl, hydroxyl, amino, and some of the halogen group have been classified as auxochromes (7). Auxochromes are groups that do not in themselves show selective absorption above 200 nm, 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 elicit 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 optimal *p*H of 7.65 for the dihydrodipicolinic acid synthetase is in contrast to the optimal *p*H of 8.4 reported by others (27; D. P. Stahly, Bacteriol. Proc., p. 141, 1968). However, enzyme variations between different organisms or the fact that different buffer systems were used for these determinations could account for the observed discrepancies.

The apparent K_m values of 4.6×10^{-4} and 5.0×10^{-4} M for pyruvate and BAS, respectively, are in reasonably good agreement with the values of 1.3×10^{-4} and 2.5×10^{-4} M reported by Yugari and Gilvarg (27). However, D. P. Stahly (Bacteriol. Proc., p. 141, 1968) 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 K_m of pyruvate was observed, whereas V_{max} remained constant. A similar phenomenon was not observed for the K_m 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 to be apparent K_m 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 *p*H to 6.5. Inactivation of the enzyme at 4 C did not exhibit any indication of being a reversible phenomenon. Shukuya and Schwert (22) 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 (4, 18, 20) have demonstrated that serum lipoproteins are cold sensitive. The possibility of a dissociated cofactor or of the enzyme being a lipoprotein is strictly conjectural at this time. As previously stated, all attempts to demonstrate a cofactor requirement were unsuccessful.

Thermal inactivation of the enzyme is complex. The inactivation of enzymes is nearly always due to the denaturation of enzyme protein (6). A strong effect of *p*H 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 *p*H on thermal inactivation rates was clearly demonstrated in this study. A region of maximum stability was observed at *p*H 10.5, with decreasing stability on either side of this value. An optimal thermal stability at this *p*H appears to be unique to this particular enzyme. The literature surveyed contained no reference to a protein with an optimal stability at such a highly basic *p*H. 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 *p*H values in different buffer systems reflects the influence of ionic strength and suspending medium upon the rate of thermal inactivation. No attempt was made to control ionic strength, but it varied only slightly for the individual buffer systems. The relationship between the inactivation constant and *p*H further describes the complexity of thermal inactivation. Sadoff et al. (21) suggested that the slope of log K versus *p*H describes the number of protons exchanged with the environment. The slopes of this plot for the phosphate and glycine buffers were approximately 0.5, whereas 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 *p*H. However, the reasons for the differences in the relationship between log K and *p*H 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 *p*H 7.0 phosphate buffer was calculated to be 0.01 min. The stability was increased 270-fold by 0.03 M pyruvate and 1,850-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 protein are extremely high, ranging from about 40,000 to 100,000 cal/mole (25). 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 (6). The results of this study are in agreement with those conclusions. An extremely high E_a, 67,300 cal/mole, was accompanied by a high positive entropy, 133 cal per mole per degree, indicating the breakage of a large number of weak bonds. The ΔF for thermal denaturation of almost all proteins is approximately 25 ± 5 kcal per mole (20*a*). The

ΔF of 22,250 cal/mole observed for dihydrodipicolinic acid synthetase is in this range.

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