The Mechanism of End Product Inhibition of Serine Biosynthesis

1. PURIFICATION AND KINETICS OF PHOSPHOGLYCERATE DEHYDROGENASE*

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SUMMARY

Phosphoglycerate dehydrogenase was purified from extracts of Escherichia coli by conventional methods and the enzyme was crystallized from ammonium sulfate solution. The crystalline preparation appeared homogenous in the ultracentrifuge and upon zone electrophoresis.

The reduction of hydroxypyruvate-P proceeded faster than the oxidation of phosphoglycerate (PGA) and the latter reaction was inhibited by DPNH. The equilibrium value measured in the direction of PGA oxidation was $7 \times 10^{-12}$ M at pH 7.5 and 25°C.

L-Serine in the range of 1 to 10 $\mu$M inhibited the PGA dehydrogenase-catalyzed oxidation of PGA and reduction of hydroxypyruvate-P. Maximum inhibition was about 95%.

The dose-response curves for serine were sigmoid-shaped and when converted to Hill plots gave slopes that approached 2. L-Serine was a noncompetitive inhibitor with respect to hydroxypyruvate-P, an uncompetitive inhibitor with respect to PGA and DPNH, and gave "mixed" type inhibition with respect to DPN. The substrates gave regular Michaelis-Menton kinetics.

Analogues of DPNH functioned as coenzymes but TPNH had only 7.5% the activity of DPNH.

The initial reaction is the oxidation of the glycolytic intermediate 3-phosphoglycerate (I) to hydroxypyruvate-P (II) followed by transamination to serine phosphate (III) and dephosphorylation to serine (IV). This sequence appears to be the only functional pathway in E. coli since mutant strains that lack the capacity to carry it out possess a nutritional requirement for serine (1, 2). Evidence that this pathway is subject to regulation by the end product came initially from the work of Roberts et al. (3), who showed with growing cells that an exogenous supply of serine restricted endogenous synthesis from glucose. This observation was followed by experiments with extracts of E. coli showing that serine inhibited the conversion of 3-phosphoglycerate to serine phosphate. Subsequent kinetic studies implicated the first enzyme PGA dehydrogenase as the point of inhibition.

(1) Umbarger and Umbarger obtained similar results with extracts of Salmonella typhimurium and E. coli (2, 4).

The enzymatic reactions used by Escherichia coli to synthesize serine are shown below.

\[
\begin{align*}
\text{COO}^- & \quad \text{COO}^- & \quad \text{COO}^- & \quad \text{COO}^- \\
\text{HCOH} & \quad \text{C-O} & \quad \text{H}_2\text{NCH} & \quad \text{H}_2\text{NCH} \\
\text{H}_2\text{COPO}_3^- & \quad \text{H}_2\text{COPO}_3^- & \quad \text{H}_2\text{COPO}_3^- & \quad \text{H}_2\text{COH} \\
\text{I} & \quad \text{II} & \quad \text{III} & \quad \text{IV}
\end{align*}
\]

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1 The abbreviation used is: PGA, 3-phosphoglycerate.
Described (5, 6). During the hydrolysis the amount of material that acted as substrate for PGA dehydrogenase increased for 4 days. In the hydrolysate at that time the ratio of moles of enzymatically oxidizable DPNH to moles of phosphate was 0.81. Chromatograms showed inorganic phosphate and two phosphate esters. Analysis of the phosphate esters showed that the major component made up 90% of the mixture and was enzymatically active. The minor component was not a substrate for the enzyme and appears to be a breakdown product of hydroxyprolyl-P. After hydrolysis of the ketal, hydroxyprolyl-P was stored as the free acid at -20°C. DPN and DPNH were purchased from Pabst. Amino acids and other organic chemicals were obtained from commercial sources. DEAE-Sephadex and Sephadex G-25, G-150, and G-200 were purchased from Pharmacia. Dithiothreitol was obtained from Calbiochem.

**Instruments**

Absorbance was measured in a Zeiss M4Q II spectrophotometer. Fluorescence was measured in an Aminco/Bowman fluorometer fitted with a 416-992 xenon lamp and constant temperature cell compartment. Wave length calibration was performed with a Pen-Ray mercury lamp. Ultra centrifugation was performed in a Spinco model E ultracentrifuge. Zone electrophoresis was carried out on cellulose polyacetate strips in a Gelman electrophoresis chamber, model 1170.

**Enzyme Assay**

Enzyme activity was measured by following the appearance or disappearance of DPNH. When hydroxyprolyl-P was the substrate DPNH disappearance was measured spectrophotometrically or fluorometrically. When PGA was the substrate DPNH appearance was measured fluorometrically. The conditions used to assay DPNH disappearance spectrophotometrically were similar to those previously described (6). The reaction mixture contained 10 μM Tris buffer (pH 8.8), 0.2 mM DPNH, 0.36 mM hydroxyprolyl-P, 0.1 mM dithiothreitol, and enzyme (volume, 0.5 ml). The reaction was initiated by the addition of enzyme or hydroxyprolyl-P. Absorbance at 340 μM was measured at 15-sec intervals and the change observed between 15 and 45 sec was used to calculate enzyme activity. The maximum change utilized to calculate enzyme activity was 0.200 optical density unit/30 sec. One unit of enzyme activity is defined as that amount of enzyme that catalyzes the disappearance of 1 mmole of DPNH per min at 25°C. Specific activity is expressed as units per mg of protein. To measure DPNH fluorometrically the reaction mixture was activated at 340 μM and the change in emission at 465 μM was followed. Standard DPNH solutions were used to calibrate the instrument. To assay DPNH disappearance the reaction mixture contained 20 mM phosphate or Tris buffer at the desired pH, 0.02 mM DPNH, 0.18 mM hydroxyprolyl-P, 0.1 mM dithiothreitol, and enzyme in a final volume of 1.0 ml. The reaction was started with either enzyme or substrate and proceeded at 25°C. To assay DPNH appearance the reaction mixture contained 20 mM phosphate buffer (pH 7.50), 1 mM DPN, 10 mM PGA, 5 mM hydrazine sulfate neutralized to pH 7.5, 0.1 mM dithiothreitol, and enzyme. The reaction was started by the addition of enzyme or PGA. The change in fluorescence was followed manually at set time intervals or continuously by means of a strip recorder.

**Analytical Procedures**

Protein was determined in the unfractionated extracts by the method of Lowry et al. (7) and in fractionated material by absorbance measurements at 280 μM. Phosphate was assayed by the method of Bartlett (8).

**Enzyme Purification**

Growth of Bacteria and Preparation of Bacterial Extracts—The organism used as a source of enzyme was E. coli strain B which has been maintained in our laboratory for several years. The growth medium and conditions used to obtain bacterial growth have been described (1). The glucose concentration used was 2 mg per ml and bacteria were harvested after they reached the stationary phase of growth. Harvesting was performed by centrifugation and the cells were then washed with cold medium lacking glucose. The cells were disrupted by grinding in a chilled mortar with twice their weight of alumina (Alcoa A301). The disrupted cells and alumina were extracted with cold Tris-chloride buffer (50 mM, pH 7.5) containing 2 mM dithiothreitol; 5 ml of buffer were used per g (wet weight) of cells. Debris and alumina were removed by centrifugation at 4000 × g (all centrifugations were performed in a Sorval RC-2 at 0-2°C). After removal of the supernatant fluid the pellet was re-extracted with fresh buffer (2 ml per g of cells) and recentrifuged. The supernatant fluids were pooled. In a typical preparation 30 liters of culture were processed. The yield of cells was 130 g and about 800 ml of unfractionated extract were obtained. The unfractionated extract was immediately subjected to the following procedure.

Streptomycin and Ammonium Sulfate Precipitation—The nucleic acids were precipitated from the supernatant fluid by the slow addition of 200 ml of 5% (w/v) streptomycin sulfate. Constant stirring was maintained during the addition and the solution was kept cold by placing them in an ice bath. The precipitate was removed by centrifugation and washed with a brief treatment in a blender with 200 ml of 50 mM Tris-Cl buffer, pH 7.5, containing 2 mM dithiothreitol and 1.5% (w/v) streptomycin sulfate. The precipitate was removed by centrifugation and the combined supernatant fluid (1200) were added 700 g of solid ammonium sulfate. The precipitate that formed was stored at 4°C in the ammonium sulfate solution. Enzyme activity was stable when the preparation was stored in this form and the material obtained from separate preparations could be pooled. It was convenient to precipitate the enzyme with ammonium sulfate after each step in the purification. The precipitations were performed by adding solid ammonium sulfate to give a final concentration of 75%.

Ammonium Sulfate Fractionation—The ammonium sulfate precipitates prepared from three batches of cells (total volume was 90 liters of bacterial culture) were pooled and removed from the ammonium sulfate solution by a 15-min centrifugation at 6000 × g. The precipitate was dissolved in 400 ml of 10 mM Tris-Cl, pH 7.5, containing 2 mM dithiothreitol (Buffer A). The volume of the solution and the absorbance at 280 μM were recorded. From the difference between the volume of the protein solution obtained and the amount of buffer used, an estimate was made of the quantity of ammonium sulfate in the precipitate. Additional Buffer A was added to give the solution a final volume of...
of 2 liters and an absorbance of about 8 at 280 nm. To this solution were added, with stirring, 528 g of solid ammonium sulfate. The precipitate that formed at this salt concentration (40% saturation) was removed by centrifugation and an additional 228 g of ammonium sulfate were added to the supernatant fluid to bring the salt concentration to 55% saturation. The precipitate that formed was collected by centrifugation and dissolved in 150 ml of Buffer A. The enzyme activity and protein concentration of this solution were measured; then solid ammonium sulfate was added to precipitate the protein. The resulting suspension was stored at 4\°.

**Acetone Precipitation**—The protein precipitated by ammonium sulfate was collected by centrifugation and dissolved in 1.5 liters of Buffer A. The resulting solution which had an absorbance of about 4 at 280 nm was cooled in ice water and 1.5 liters of acetone were added. The acetone was precooled to -20\° and was added with mechanical stirring at such a rate that the temperature remained between 3\° and 7\°. The precipitate that formed was rapidly collected by centrifugation (4000 × g) and then extracted sequentially with 300 and 240 ml of Buffer A. The soluble protein was then precipitated with ammonium sulfate.

**DEAE-Sephadex Chromatography**—Half of the precipitated protein was dissolved in 30 ml of buffer and salt was removed from the solution by passage through a column of Sephadex G-25. This solution was then added to a column, 2.3 × 30 cm, containing 3.5 g of DEAE-Sephadex A-50. After washing with Buffer A the column was developed with a 600-ml linear gradient of ammonium sulfate in Buffer A. Fig. 1 presents a typical elution profile showing protein concentration and enzyme activity. Fractions with a high specific activity were pooled and the protein was precipitated by the addition of ammonium sulfate.

**Gel Filtration on Sephadex G-150**—The ammonium sulfate precipitates from two DEAE-Sephadex columns were pooled and dissolved in 7 ml of 0.01 M potassium phosphate buffer, pH 7.5, containing 2 mM dithiothreitol (Buffer B). This solution was placed on the top of a column of Sephadex G-150, 1.5 × 330 or × 220 cm. The column was developed with Buffer B.

**RESULTS**

**Purity of Crystalline Enzyme**—The specific activity of the enzyme achieved after the second crystallization was not altered upon recrystallization (Table I). Recrystallization of the enzyme to constant specific activity is strong evidence that the preparation is free of contaminating proteins. Analytical ultracentrifugation (Fig. 4) and zone electrophoresis (Fig. 5) failed to detect inhomogeneity in the crystalline preparation. The $s_{20, w}$ of the enzyme when measured at 6 mg per ml was 7 and the mobility at pH 7.5 was 0.85 relative to crystalline bovine serum albumin. Interpretation of electrophoresis patterns obtained at high pH values was complicated by dissociation of the enzyme into subunits.

**Rate Measurements of Forward and Reverse Reactions**—The enzymic reaction was assayed by the fluorometric method in the

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**Fig. 2. Sephadex G-150 gel filtration.** For this fractionation a column of 1.5 × 330 cm was used with a bed volume of 380 ml. The flow rate was about 10 ml per hour. Fractions of 6.5 ml were collected.

**Crystallization of Enzyme**—The protein precipitate obtained from the Sephadex G-150 column was dissolved in a small volume of Buffer B and a solution of saturated ammonium sulfate was added dropwise with stirring until a faint opalescence was observed. The opalescent solution was placed at 4\° overnight. When the solution was stirred the next day, it showed a sheen that indicated the presence of crystals. Examination of this solution under the microscope showed the presence of rhombic crystals (Fig. 3). The crystals were collected by centrifugation and dissolved in Buffer B and the crystallization procedure was repeated. A third crystallization was then performed. A summary of the purification procedure is given in Table I. By use of this procedure, crystalline enzyme was obtained from E. coli grown in the laboratory and from cells purchased from a commercial source (Grain Processing Company). The crystalline preparation from commercial cells possessed a specific activity of 50% that obtained from cells grown in the laboratory. Whether the lower activity reflects differences in growth conditions or manipulations of the cells is unknown.
direction of oxidation of PGA (forward reaction) and in the direction of hydroxypyruvate-P reduction (reverse reaction). The results (Fig. 6) show that the reverse reaction was faster than the forward reaction. The ratio of the rates of the reverse to the forward reaction was 70 at 25°C and 40 at 37°C. These data were obtained at pH 7.5 with the enzyme saturated with substrate and coenzyme. The turnover number for hydroxypyruvate-P reduction calculated from the specific activity of the crystalline enzyme (Table I) and a molecular weight of 165,000 was 1,100 moles per mole per min. The equilibrium constant

$$K_{eq} = \frac{[HPAP][DPNH][H^+]}{[PGA][DPN]}$$

where HPAP is hydroxypyruvate-P, was obtained by following the reaction in the forward direction. The values were $6 \times 10^{-11} \text{ M}$ at 25°C and $7 \times 10^{-11} \text{ M}$ at 37°C.

Because of the greater speed and accuracy of the measurements the majority of kinetic experiments was performed by assaying enzyme activity in the direction of DPNH oxidation.

**Effect of pH**—When initial rates (measured between 0 and 45 sec) of DPNH oxidation were determined at different pH values, the activity showed a maximum around pH 8.5 (Fig. 7). However, if the assays were continued for longer periods of time, the reaction at pH 8.5 failed to go to completion (Fig. 8). Preincubation of enzyme at pH 5.5, 7.5, 8.5, and 9.5 followed by assays at pH 7.5 showed that at pH 5.5 and 7.5 enzyme activity remained but at pH 8.5 and 9.5 it was irreversibly lost. Tris or phosphate buffers were used interchangeably in the pH range 7.0 to 8.5 with the same effect on enzyme activity.

**Factors Affecting Forward Reaction**—In previous studies of serine synthesis it was reported that pyruvate stimulated the conversion of PGA to serine-P (1). The dependence of this reaction on pyruvate concentration is shown in Table II. This effect was specific and other carbonyl compounds such as hydroxypyruvate, acetaldehyde, and dihydroxyacetone-P did not replace pyruvate. Alternative explanations for this stimulation

**Table I**

**Summary of purification**

Enzyme activity was measured at pH 8.8 and 25°C by following spectrophotometrically the disappearance of DPNH. An enzyme unit is equal to 1 nanomole of DPNH oxidized per min at 25°C under standard assay conditions. The protein concentration of the crude extract was measured by the procedure of Lowry et al. (7); that of the other fractions was calculated from the absorbance at 280 nm with a $E_{280}$ of 0.7 to convert absorbance measurements to protein concentrations.

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Protein</th>
<th>Enzyme units</th>
<th>Specific activity</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>35,000</td>
<td>630,000</td>
<td>18.1</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin and (NH₄)₂SO₄ precipitation</td>
<td>22,400</td>
<td>485,000</td>
<td>21.5</td>
<td>76</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>9,600</td>
<td>485,000</td>
<td>50.5</td>
<td>76</td>
</tr>
<tr>
<td>Acetone fractionation</td>
<td>2,050</td>
<td>420,000</td>
<td>205.0</td>
<td>67</td>
</tr>
<tr>
<td>DEAE-Sephadeck chromatography</td>
<td>215</td>
<td>223,000</td>
<td>1,500.0</td>
<td>51</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>91</td>
<td>275,000</td>
<td>3,000.0</td>
<td>44</td>
</tr>
<tr>
<td>First crystallization</td>
<td>42.5</td>
<td>238,000</td>
<td>5,600.0</td>
<td>38</td>
</tr>
<tr>
<td>Second crystallization</td>
<td>33.5</td>
<td>225,000</td>
<td>6,700.0</td>
<td>36</td>
</tr>
<tr>
<td>Third crystallization</td>
<td>33.0</td>
<td>220,000</td>
<td>6,650.0</td>
<td>35</td>
</tr>
</tbody>
</table>

Fig. 4. Sedimentation pattern obtained in the analytical centrifuge. Centrifugation was performed in a double sector cell and pictures were taken with bar angle of 65°.

Fig. 3. Crystals of PGA dehydrogenase. The photograph was taken with a phase contrast microscope. × 800.
were that pyruvate in conjunction with lactate dehydrogenase in the enzyme preparation removed DPNH formed during PGA oxidation, or that pyruvate interacted directly with the PGA dehydrogenase to increase enzyme activity. The effect of 20

![Zone electrophoresis](image)

**Fig. 5.** Zone electrophoresis. Electrophoresis was performed on 6-inch polyacetate strips (Sephapore III, Gellman Instrument Company, Ann Arbor, Michigan). The potassium phosphate buffer contained 0.1 mM dithiothreitol and was chilled to 4°C prior to the electrophoresis run. Protein, 30μg, was applied and after electrophoresis, for the times indicated, the protein was stained with Procion blue.

![Fluorometric assay](image)

**Fig. 6.** Fluorometric assay of the forward and reverse reaction. The assay was performed at pH 7.5 and 25°C. The concentration of hydroxyprimary-P and PGA was 0.18 mM and 5 mM, respectively. The concentration of both DPN and DPNH was 20 uM. These concentrations of hydroxyprimary-P and DPNH saturated the enzyme and the concentration of PGA and DPN used exceeded their K values. The same enzyme concentration was used in both reactions. O—O, 3-phosphoglycerate + DPN → hydroxyprimary-P + DPNH; •—•, hydroxyprimary-P + DPNH → 3-phosphoglycerate + DPN.

**Fig. 7.** The effect of pH on enzyme activity. Enzyme activity was assayed by following spectrophotometrically the initial rate of DPNH disappearance. ○, Tris; •, phosphate.

![Time course](image)

**Fig. 8.** Time course of DPNH disappearance at pH 7.5 and 8.8. The reaction was run in phosphate buffer and was followed spectrophotometrically.

**TABLE II**

**Effect of pyruvate concentration on conversion of 3-phosphoglycerate to serine-P**

The conditions for assaying the conversion of 1C-PGA to 1C-serine phosphate and the method for obtaining the enzyme preparation were previously described (1).

<table>
<thead>
<tr>
<th>Pyruvate in incubation (μM)</th>
<th>Serine-P formed (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>108</td>
</tr>
<tr>
<td>3</td>
<td>1140</td>
</tr>
<tr>
<td>6</td>
<td>1500</td>
</tr>
<tr>
<td>12</td>
<td>2004</td>
</tr>
<tr>
<td>30</td>
<td>2724</td>
</tr>
<tr>
<td>40</td>
<td>2712</td>
</tr>
</tbody>
</table>

μM pyruvate was tested on the crystalline PGA dehydrogenase and, as shown in Fig. 9, it inhibited rather than stimulated the oxidation of PGA. DPNH also inhibited the forward reaction with 6.5 × 10^-2 M, causing 50% inhibition. These data indicate
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3-PGA + DPN → HPAP + DPNH

COMPLETE

HYDRAZINE

6.5 x 10^-6 M DPNH

(HPAP + HYDRAZINE + PYRUVATE)

1.5 x 10^-6 M DPNH

Fig. 9. Factors affecting PGA oxidation. The reaction was run at 25° in 80 mM phosphate buffer, pH 7.5, with 5 mM PGA, 20 μM DPN, and 8 mM dithiothreitol. When present, hydrazine was at a concentration of 10 mM. HPAP, hydroxypyruvate-P.

Fig. 10. Serine inhibition at pH 8.8. The standard spectrophotometric assay conditions were used to follow DPNH disappearance except that L-serine was added at the molar concentrations indicated.

Fig. 11. Serine inhibition at pH 7.5. The standard fluorometric assay conditions were used to follow DPNH disappearance. L-Serine was added at the molar concentration indicated.

Fig. 12. Serine inhibition of PGA oxidation. Assays were performed at 37° with the standard conditions described under "Materials and Methods" except that L-serine was added at the molar concentration indicated.

that, rather than activating the PGA dehydrogenase, pyruvate probably stimulated the synthesis of serine-P by acting in conjunction with lactic dehydrogenase to remove DPNH. A lactic dehydrogenase has been reported in extracts of E. coli which could in the presence of pyruvate oxidize DPNH and thereby stimulate serine-P synthesis (9).

In the assay of PGA dehydrogenase shown in Fig. 9, hydrazine increased the extent of the reaction probably by trapping the hydroxypyruvate-P formed. Serine-P transaminase and glutamate function to remove hydroxypyruvate-P in partially fractionated extracts (1) and measurements of the equilibrium constant for the transaminase show that serine-P synthesis is favored. It appears that in the growing cell PGA oxidation is pulled by the second step in the pathway and by the removal of DPNH.

Effect of Serine Concentration—The observation that PGA dehydrogenase was inhibited by serine provided an enzymatic explanation for feedback inhibition of serine biosynthesis (1, 2). Therefore, a detailed kinetic study of the inhibition was made to probe its mechanism. The majority of the experiments used DPNH disappearance to assay enzyme activity and assays were performed at pH 8.8, the pH optimum, and at pH 7.5, at which the enzyme was stable. Fig. 7 shows that serine inhibited at all pH values at which activity was detected but that inhibition was somewhat stronger at lower pH values. The curves describing the inhibition of hydroxypyruvate-P reduction as a function of serine concentration were sigmoid (Figs. 10 and 11), and confirmed that at pH 7.5 serine was a more effective inhibitor than at pH 8.5. At pH 7.5 5 μM serine produced 50% inhibition. Serine inhibition of PGA oxidation depended on serine concentration, as shown in Fig. 12. These data correspond to those obtained for serine inhibition of the conversion of PGA to serine-P (1). The curve was sigmoid with 50% inhibition occurring at 75 μM.

While the serine concentration required to inhibit the enzyme was greater when the reaction was followed in the forward rather than the reverse direction, maximum inhibition was greater than 95% in both cases and the dose-response curves had the same shape. Use of the modified Hill equation de-

2 Unpublished observations.
scribed by Monod, Changeux, and Jacob converted these curves to linear plots (Figs. 10, 11, and 12) with slopes (n) that approached 2. In the equation as shown (Figs. 10, 11, and 12) v was the reaction rate in the presence of serine, V was the rate in the absence of serine and I was the concentration of serine. That the slopes of the Hill plots were close to 2 indicates the presence on the enzyme of at least two interacting binding sites for serine. Fluorometric titrations detect only two serine sites.

**Table III**

**Inhibition by amino acids**

Activity was measured spectrophotometrically at pH 8.8 by following DPNH disappearance.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Serine</td>
<td>$5 \times 10^{-3}$</td>
<td>30</td>
</tr>
<tr>
<td>Glycine</td>
<td>1</td>
<td>47</td>
</tr>
<tr>
<td>L-Allothreonine</td>
<td>1</td>
<td>43</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>L-Homoserine</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

**Table IV**

**Inhibition by amino acids in conjunction with serine**

The assay conditions were those described in the legend to Table III. All assays were performed with L-serine at a concentration of 5 μM. The second amino acid was present at the concentration indicated.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Concentration</th>
<th>Inhibition</th>
<th>Observed</th>
<th>Calculated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Serine + glycine</td>
<td>1</td>
<td>89</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>L-Serine + L-allothreonine</td>
<td>1</td>
<td>72</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>L-Serine + L-alanine</td>
<td>2.5</td>
<td>66</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>L-Serine + L-threonine</td>
<td>2.0</td>
<td>66</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>L-Serine + L-homoserine</td>
<td>4.0</td>
<td>66</td>
<td>69</td>
<td></td>
</tr>
</tbody>
</table>

* These values were the sum of the inhibition produced by each amino acid alone.

**Fig. 13.** The effect of hydroxypyruvate-P (HPAP) concentration on the enzyme velocity and serine inhibition. Disappearance of DPNH was followed fluorometrically. At pH 8.8 the HPAP concentration was 20 μM and at pH 7.5 was 20 μM. The hydroxypyruvate-concentration (moles per liter) was altered as shown. **SER**, serine.

**Fig. 14.** The effect of DPNH concentration on the enzyme velocity and serine inhibition. At pH 8.8 the standard fluorometric assay was used to follow the disappearance of DPNH except that the DPNH concentration was varied. At pH 7.5 the reaction was followed in an Eppendorf photofluorometer (made available through the courtesy of Dr. R. Esterbrook). The assay mixture contained in a final volume of 2.5 ml, 0.1 M phosphate buffer, 0.1 mM dithiothreitol, 70 μM hydroxypyruvate-P, and various molar concentrations of DPNH. The data obtained at pH 7.5 are shown in the inset.

**Fig. 15.** The effect of PGA concentration on the enzyme velocity and serine inhibition. The standard assay for the appearance of DPNH was used except that the PGA concentration (moles per liter) was altered and the reaction was run at 37°. The reaction was started with PGA and was followed for 2.5 min.

This observation, taken together with the kinetic data, suggests extensive interaction between these sites.

**Specificity of Amino Acid Inhibition**—In the earlier study several amino acids inhibited the PGA dehydrogenase to a limited extent (1). Therefore, the ability of amino acids to inhibit the crystalline enzyme was tested at a number of con-
The standard conditions for measuring DPNH disappearance were used except that the pyridine nucleotide analogues replaced DPNH. To measure the disappearance of acetylpyridine-DPNH the mixture was activated at 360 nm (adsorption maximum for this compound), and the emission at 465 nm was recorded. The data shown were obtained with a nucleotide concentration of 20 μM and assays at other concentrations showed that this concentration effectively saturated the enzyme.

**TABLE V**

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPNH</td>
<td>100</td>
</tr>
<tr>
<td>TPNH</td>
<td>7.5</td>
</tr>
<tr>
<td>3-Acetylpyridine-DPNH</td>
<td>45</td>
</tr>
<tr>
<td>Deamino-DPNH</td>
<td>112</td>
</tr>
</tbody>
</table>

centrations alone and in conjunction with serine. At a concentration of 10 μM at which serine inhibited 75%, no other amino acids inhibited and the concentration of the amino acids had to be increased 100-fold before inhibition was observed. Quantitatively the inhibition by serine was specific. Some of the data obtained are shown in Tables III and IV. Glycine and l-allothreonine were, after serine, the most effective inhibitors, and the inhibition produced when two amino acids were present in the assay was approximately the sum of the inhibition produced when the amino acids were tested separately.

**Effect of Substrate Concentration on Nature of Serine Inhibition**—The investigation of serine inhibition was carried out at several substrate and coenzyme concentrations. The results of experiments in which hydroxypyruvate-P concentrations were varied in the assay (Fig. 13) showed that inhibition by serine was non-competitive. The $K_m$ for hydroxypyruvate-P was smaller by an order of magnitude at pH 7.5 than that found at pH 8.5. At both pH values Michaelis-Menten kinetics was observed. These data were obtained with the enzyme saturated with DPNH.

When the DPNH concentration was altered (Fig. 14) at saturating levels of hydroxypyruvate-P, Michaelis-Menten kinetics was again observed and the $K_m$ value at pH 7.5 was lower than that of pH 8.8. At pH 8.8, serine was an uncompetitive inhibitor altering both $V_{max}$ and $K_m$, and preliminary measurements indicate the same type of inhibition at pH 7.5 (Fig. 14). No indication was found of interaction between the binding sites for coenzyme or substrate.

The forward reaction, PGA oxidation, was studied at pH 7.5 and 37°. Under these conditions, the $K_m$ for PGA was 1.1 mM (Fig. 15) and the inhibition by serine was uncompetitive. The $K_m$ for DPN was 8 μM (Fig. 16) and inhibition by serine was of the "mixed" type (1) with both $K_m$ and $V_{max}$ altered by serine. These data agree with those reported previously for the coupled assay (1).

**Specificity of Pyridine Nucleotide Coenzymes—Analouges of DPNH** were tested at a variety of concentrations and the relative activities show (Table V) that, at levels that saturate the enzyme, deamino-DPNH was as effective a coenzyme as DPNH while enzyme activity with 3-acetylpyridine-DPNH was 40% of maximum. TPNH showed a limited amount of activity. This activity was not due to contaminating DPNH, since increasing the TPNH concentration failed to increase the activity. With all of the coenzyme analogues inhibition by serine was observed.

**DISCUSSION**

The Carnegie Institution monograph, *Studies of Biosynthesis in Escherichia coli* (3), contains quantitative data that assist in correlating properties of the purified PGA dehydrogenase with its activity in the bacterial cell. Such correlations appear justified in the case of the enzyme described in this paper, since the organism from which it was obtained, *E. coli* strain B, and the growth medium were the same as used by the Carnegie group. From the data on the yield of crystalline enzyme (Table I) it was calculated that 400 g (wet weight) of cells contained 115 mg of PGA dehydrogenase. This corresponds to 0.7 μmole of the enzyme and if 75% of the wet weight of a bacterial pellet was taken as intracellular water the enzyme concentration in the cell was approximately 2.3 μM. Many of the kinetic experiments described in this paper and most of the studies in the accompanying papers (12, 13) were performed in the range of 0.1 to 1.0 μM. With the use of the data on the composition of the cell and the rate of growth, the minimum cellular biosynthetic capacity for serine was calculated (3, 14). This calculated value was higher than the activity of the crystalline enzyme measured at 37° in the direction of PGA oxidation. However, the activity shown by the partially fractionated extracts for serine phosphate synthesis approximated the cell's biosynthetic capacity (1). It appears that the oxidation of PGA was faster when coupled to the serine phosphate transaminase and DPNH was removed by lactic dehydrogenase than when activity was measured under the conditions used to assay the isolated enzyme.

It can also be calculated from the data in Table I that the PGA dehydrogenase constitutes about 0.25% of the soluble protein in the cell. When the number of metabolic reactions in the cell is considered this value is high. The presence of large quantities of PGA dehydrogenase could reflect a need to offset a low turnover number or a failure to regulate synthesis. Attempts to show regulation of dehydrogenase synthesis by repression were unsuccessful (14).
The inhibition of PGA dehydrogenase by L-serine explains the observation made in growing bacteria that exogenous serine limited endogenous synthesis (3, 14). A general model has been proposed by Monod, Wyman, and Changeux (15) to explain the properties of regulatory enzymes and within the framework of this model two types of systems have been recognized (K and V) which are distinguished on the basis of kinetic properties. The properties of the PGA dehydrogenase most closely resemble a V system. The measurements of velocity as a function of substrate concentration showed regular Michaelis kinetics for all four substrates. These results indicate that, while the enzyme molecule may have more than one binding site for a substrate, the sites do not interact. Serine produced an effect on the $K_m$ values of PGA and the coenzymes, but the primary kinetic consequence of serine binding appears to be a reduction in $V_{\max}$ and whether the alterations in $K_m$ values were a consequence of this effect or due to changes in dissociation constants has not been determined.

The sigmoid shaped dose response curves show that serine binds to PGA dehydrogenase at interacting sites. These kinetic measurements are confirmed by direct binding studies described in the accompanying paper (12), which also show that the binding of serine alters the conformation of PGA dehydrogenase. The reduction in $V_{\max}$ probably reflects this change in protein structure. Aside from the effects of serine on the $K_m$ values of PGA and the pyridine nucleotides, the PGA dehydrogenase meets the requirements of the V type allosteric enzyme described by Monod et al. (15). However, its properties could be accounted for by other models such as those described by Koshland, Nemethy, and Filmer (16), and our kinetic experiments are not extensive enough to distinguish between the various models suggested.

Other amino acids inhibited the PGA dehydrogenase but the concentrations required to get a specific level of inhibition were much higher than the serine level needed. All of the amino acids tested in conjunction with serine either had no effect or increased the inhibition to a value close to the sum of the inhibition produced by both amino acids separately. Those assays were performed in the range in which inhibition was approximately linear with serine concentration (10 to 80% inhibition) and the results indicate that when amino acids inhibit PGA dehydrogenase they do so by occupying the same site as serine. It is unlikely in view of the concentrations required that amino acids other than serine and glycine play a physiologically important role in regulating serine synthesis. On these grounds the inhibition is specific. However, comparison of the inhibition produced by the different amino acids allows certain properties of the serine-binding site to be deduced. In this sense the amino acids were used as serine analogues. Glycine was a better inhibitor than alanine even though the latter is smaller than serine. It therefore appears that the inhibitor site is not rigid but adapts to the ligand. From the inhibition produced by the 4-carbon hydroxy amino acids, L-allothreonine, L-threonine, and L-homoserine, we conclude that the most effective compound had the hydroxyl group in an L-erythro relationship to the a-amino group. Presumably this is the configuration taken by serine on the enzyme. This approach could be extended to get detailed information on the contribution to binding of the carboxyl, amino, and hydroxymethyl group of serine.

PGA dehydrogenase is readily reversible and the equilibrium favors hydroxy pyruvate-P reduction. However, subsequent reactions in the pathway together with the removal of DPNH would effectively make the reaction in the cell irreversible and justify the inhibition acting at this point. In some tissues the PGA dehydrogenase is not sensitive to serine inhibition (6, 17-19), and the possibility exists that these tissues have a high enough DPNH concentration to make the dehydrogenase reaction reversible. The DPNH concentration in tissues which lack serine inhibition of PGA dehydrogenase is a subject that requires further investigation.

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