Purification and Properties of an NADP-specific 6-Phosphogluconate Dehydrogenase from Streptococcus faecalis

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A procedure is described for the purification of 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase (decarboxylating) EC 1.1.1.44) from cell extracts of Streptococcus faecalis. A 180-fold purification was achieved with an over-all yield of about 12% and an average specific activity of 14. The enzyme was homogeneous as determined by polyacrylamide gel electrophoresis, immunoelectrophoresis, and sedimentation equilibrium studies. Its weight average molecular weight, as measured by sedimentation equilibrium, was 108,000 ± 3,600. Other methods employed for molecular weight determinations gave values that ranged between 106,000 and 115,000. An analysis of the enzyme by sodium dodecyl sulfate polyacrylamide gel electrophoresis showed it to be a dimer composed of subunits having equal molecular weights. The amino acid composition of the streptococcal enzyme is reported. The apparent \( K_m \) values for NADP and 6-phosphogluconate were calculated from kinetic data and found to be 0.015 mM and 0.024 mM, respectively. Kinetic studies also indicated that the binding of one substrate did not affect the apparent affinity of the enzyme for the other substrate.

Glucose-grown cells of Streptococcus faecalis possess the enzymes of at least the oxidative portion of the hexose-monophosphate pathway (1) as well as all of the enzymes of the Embden-Meyerhof pathway. This organism, therefore, has the potential to metabolize glucose by either of these two pathways. It was established from labeling studies, however, that virtually all of the glucose degraded proceeded through the Embden-Meyerhof pathway with the stoichiometric accumulation of lactate (2, 3). In searching for a biochemical explanation for this preferential use of the Embden-Meyerhof pathway, it was found that the glycolytic intermediate, Fru-1,6-P₂, acted as a modifier for a key enzyme from each of the pathways. This ligand was a specific and absolutely required activator for the lactate dehydrogenase (4) and a potent and specific inhibitor of the 6-phosphogluconate dehydrogenase (5). The potential physiological significance of these interactions in partitioning glucose carbon between the hexose-monophosphate and Embden-Meyerhof pathways in S. faecalis has been discussed (5, 6).

Modulation of 6-phosphogluconate dehydrogenase activity by Fru-1,6-P₂ may be a regulatory mechanism of rather broad biological significance. Carter and Parr (7) originally found that this enzyme from rat liver, human white cells, and certain microorganisms (including a strain of S. faecalis) was sensitive to Fru-1,6-P₂, and Gumaa and McLean (8) reported that the 6-phosphogluconate dehydrogenase from Krebs ascites cells was also inhibited by Fru-1,6-P₂. Sensitivity of the sheep liver enzyme to Fru-1,6-P₂ was reported by Dyson and D'Orazio (9, 10) and inhibition of the Neurospora 6-phosphogluconate dehydrogenase by Fru-1,6-P₂ was reported by Scott and Abramsky (11).

A series of studies have been initiated in this laboratory that are designed to gain insight into the mechanism by which Fru-1,6-P₂ acts to modulate the activity of the streptococcal 6-phosphogluconate dehydrogenase. A prerequisite for such an investigation was the purification of the enzyme and a characterization of its basic properties. This communication reports the purification of the S. faecalis enzyme to a state of homogeneity and describes certain of its physical, chemical, and kinetic properties. In a subsequent publication we will describe some of the regulatory properties of the enzyme with special emphasis given to the Fru-1,6-P₂-mediated inhibition.

**EXPERIMENTAL PROCEDURE**

**Materials**

Reagents were obtained from the following sources: disodium 6-phosphogluconate, NADP, D-ribulose 5-phosphate (75% pure), and Trizma (2-Amino-2-hydroxymethyl-1,3-propanediol) base from Sigma: acrylamide, bisacrylamide, N,N,N'-tetramethylenediamine, 6093

* C. W. Parr, personal communication.
and sodium dodecyl sulfate from Eastman; DTNB from Aldrich; ovalbumin, aldolase, chymotrypsinogen A, and ribonuclease A from Pharmacia; glyceraldehyde-3-phosphate dehydrogenase and diithio- threitol from Calbiochem; ultrapure guanidine from Schwarz-Mann; and bovine serum albumin monomer and guinea pig liver transglutaminase were generous gifts from Dr. S. L. Chung, Laboratory of Biochemistry, National Institute of Dental Research.

Cellulose phosphate (Sigma, coarse mesh) was washed successively prior to use with 0.25 M NaOH, distilled water, 0.20 M HCl, distilled water, and then equilibrated with 10 mM Tris-acetate buffer, pH 6.0, containing 5.0 mM MgCl₂, DEAE-cellulose (Whatman, DE52; microgranular, preswollen) was used directly after removal of the "fines" and subsequent equilibration with 50 mM Tris-HCl buffer, pH 7.5, containing 1.0 mM 2-mercaptoethanol.

**Methods**

**Cultivation of Organism**—Streptococcus faecalis ATCC 2792 (formerly designated as strain MR) (5, 12) was grown in a complex medium containing the following per liter: dextrose, 5 g; yeast extract (Difco), 2.5 g; tryptone (Difco), 2.5 g; and dextrose, 5 g (added as a separate sterile solution). The final pH of the medium was 7.5. A 20-liter culture incubated at 37°C for 8 hours was used to inoculate 400 liters of medium in a fermentor. The large culture was incubated anaerobically (under an atmosphere of helium) at 37°C for 24 hours, and then harvested from the stationary phase of growth, washed 3 times with 50 mM Tris-HCl buffer, pH 7.5, and stored as a wet cell paste at -80°C until further use. The average cell yield from a 400-liter culture was about 600 g wet weight under the above conditions. For smaller runs, the organism was cultivated in 20-liter carboys filled completely with medium.

**Protein**—Protein concentration in crude, cell-free extracts was determined by the modified biuret method (13) and for the more purified fractions the method of Lowry (14) was employed. Bovine serum albumin was used as a standard for both methods.

**Enzyme Assay**—The 6-phosphogluconate dehydrogenase activity was measured at room temperature in a Gilford model 2400 recording spectrophotometer. The standard assay mixture contained the following components: a final volume of 1.0 ml, 50.0 mM Tris-HCl buffer, pH 7.5; 0.125 mM Tris-6-phosphogluconate; and 0.25 mM NADP. Reactions were initiated by the addition of enzyme and followed by measuring the initial increase in absorbance at 340 nm with time. The reaction was linear with time for 30 to 90 s and the reaction rate was a linear function of protein concentration within the ranges employed. An enzyme unit is that amount of enzyme which catalyzes the reduction of 1 μmol of NADP/min. Specific activity is expressed as enzyme units per mg of protein.

**Polyacrylamide Gel Electrophoresis**—Analytical polyacrylamide gel electrophoresis was carried out as described by Jovin et al. (15). The Tris glycine system of Ornstein and Davis (17) was used without modification. Protein in the gel columns was stained with a 0.05% solution of Coomassie blue and excess dye was removed by bathing the columns in 10% trichloroacetic acid overnight (18). The staining of gels for enzyme activity is described in the legend of Fig. 1.

Preparative polyacrylamide gel electrophoresis was conducted with the apparatus manufactured by the Buchler Instruments Co. (Poly-Prep), which incorporates the original design of Jovin et al. (15). The procedures employed for preparing the anionic gel column as well as the upper and lower buffers were as described in the Buchler preparative polyacrylamide gel electrophoresis instruction manual (pH 9.3 system).

**Preparation of 6-Phosphogluconate Dehydrogenase Antiserum**—Two 6-month-old male New Zealand white rabbits received 0.75 ml intradermal injections of an emulsion containing the following: 0.37 ml of Freunds complete adjuvant; 0.38 ml of 0.85% NaCl in 20 mM sodium phosphate buffer, pH 7.2; 10 μg of methylated bovine serum albumin; and 80 μg of pure streptococcal 6-phosphogluconate dehydrogenase (Step 5 fraction, Table I). The rabbits were given four such intradermal injections over an interval of 21 days. Control rabbits were immunized with an emulsion of a mixture of ovalbumin, aldolase, chymotrypsinogen A, and ribonuclease A from Pharmacia; glyceraldehyde-3-phosphate dehydrogenase and diithiothreitol from Calbiochem; ultrapure guanidine from Schwarz-Mann; and bovine serum albumin monomer and guinea pig liver transglutaminase. Antisera were prepared in rabbits using a method which resulted in a high antibody titer. The antiserum was divided into 1-ml portions and stored at -20°C until used.

**Immuno-electrophoresis**—Immuno-electrophoresis was carried out using immunodiffusion equipment (LKB products) according to the manufacturer's instructions (LKB brochure No. 6800-EO1), except that agarose was substituted for noble agar in the preparation of the gel matrix. Excess antiserum was removed by washing the slides with 0.1% NaCl and the immune precipitates were stained with 0.1% Amido black. Controls were run using pooled serum taken from the two rabbits prior to the immunization regimen.

**Amino Acid Analysis**—Amino acid analyses were performed on the homogeneous enzyme (Step 5 fraction, Table I) which had been dialyzed for 48 hours against four changes of deionized water. Samples were hydrolyzed in 6 N HCl at 110°C for 24, 48, and 72 hours. The amino acid analyses were conducted with a Hitachi-Perkin Elmer KLA-3B amino acid analyzer utilizing the zinc-ligand system under the following conditions: buffer flow rates of 90 ml/hour, unlabeled flow rate of 45 ml/hour, and a column temperature of 55°C. Single buffers of pH 5.14 and 4.12 were used for the basic and neutral acidic columns, respectively. Sample addition (200 μl of enzyme in buffer at pH 3.12) was made with a Hitachi-Perkin Elmer Autosampler. Time for complete analyses was 2 hours and 24 min. Calculations were performed by the half-height-ordinate method. Half-cystine was determined as cysteic acid after performic acid oxidation according to the method of Moore (19). Tyrosine and tryptophan were determined on unhydrolyzed samples by the spectrophotometric method of Edelhoch (20). The amino acid analysis data were corrected and calculated by a program developed at the National Institute of Dental Research by Dr. Karl Piez using an IBM 360 computer.

**Estimation of Sulphhydryl Groups**—Enzyme sulphydryl groups were estimated by the procedure of Eilman (21) in 50 mM Tris-HCl buffer, pH 7.5, at 25°C in the presence of 4 mM guanidine. The yellow color that developed after the addition of DTNB (0.23 mM) was measured at 412 nm, and the number of sulphydryl groups was calculated using an extinction coefficient of 13,600 M⁻¹ cm⁻¹.

**Molecular Weight Determination by Sedimentation Equilibrium**—The purified enzyme was dialyzed against four changes of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM 2-mercaptoethanol and 0.1 mM KCl for at least 24 hours before the sedimentation analysis. Sedimentation equilibrium studies were done using the short column, high speed method of Yphantis (22). The experiments were conducted at 5°C in a Spinco model E analytical ultracentrifuge (at 14,900 rpm) equipped with Rayleigh interferometric optics. Fluorocarbon FC-43 (Beckman) was used as a base for the solution column. Rayleigh fringes were measured on a Nikon profile projecter model 6C. The data were fit to Equation 1 by means of an interactive curve-fitting program (MLAB) developed at the National Institutes of Health (23) and run on a PDP-10 digital computer, assuming equal variance for fringe displacements greater than 100 μm.

\[ \frac{d \ln (Y_n - Y_0)}{d r^2} = \frac{M \left(1 - \frac{1}{r}ight)^2}{2 \rho T} \]  (1)

The term \((Y_n - Y_0)\) is the fringe displacement in micrometers, \(\omega\) is the angular velocity in radians per second, \(M\) is the molecular weight, \(\rho\) is the partial specific volume, \(p\) is the solution density, and \(r\) is the distance from the center of rotation in centimeters.

**Analysis of Kinetic Data**—The procedure used in the analyses of kinetic data was, in general, that of Cleland (24). Reciprocal velocities were plotted graphically first against the reciprocals of the substrate concentrations and these plots were linear in all cases. The data were fitted to Equation 2, assuming equal variance for the initial velocities.

\[ v = \frac{VA}{K_a + K_B + K_A + AB} \]  (2)

The curve-fitting program (MLAB) was utilized for all fits. The slopes (K/V) and intercepts (l/V) obtained from fits to Equation 2 were plotted graphically against the reciprocal of the nonvaried substrate or coenzyme concentration for the initial velocity experiments. Final estimates of kinetic constant were made by fitting the data points to the appropriate over-all equation. Data for the initial velocity of the reaction were found to conform to Equation 3.

\[ v = \frac{VA}{K_a K_B + K_B K_A + AB} \]  (3)

The points of the double reciprocal plots shown (Figs. 6 and 7) are the experimental values and the lines drawn through these points are those calculated from fits to Equation 3 by the least squares method.
RESULTS

Enzyme Purification

All steps of the purification procedure described below were carried out at 0 5°C unless otherwise specified.

Step 1. Preparation of Cell Extract—The frozen cells (150 g wet weight) were suspended and allowed to thaw in 3 times their volume of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM 2-mercaptoethanol. The cell suspension was disrupted by treatment for 30 min in a Branson 185-watt Sonifier. Unbroken cells and cellular debris were removed by centrifugation at 37,000 x g for 30 min, and the supernatant fluid was collected by decantation. Smaller particulate material was then removed by a second centrifugation at 105,000 x g for 90 min.

Step 2. Acid Precipitation—The 105,000 x g supernatant fluid (about 500 ml) was dialyzed overnight against 14 liters of 50 mM sodium acetate buffer, pH 4.5. The precipitate which formed was collected by centrifugation at 15,000 x g for 45 min and resuspended in about 500 ml of 50 mM Tris-acetate buffer, pH 6.0. Sodium hydroxide (0.1 n) was added slowly to the turbid suspension with stirring until the pH stabilized at 6.0. Additional Tris-acetate buffer, pH 6.0, was then added to bring the total volume to about 1 liter. At this point the turbid suspension clarified and MgCl₂ was added to give a final concentration of 5.0 mM.

Step 3. Cellulose Phosphate Column Chromatography—The enzyme solution from Step 2 was applied to a cellulose phosphate column (6 x 36 cm) previously equilibrated with 10 mM Tris-acetate buffer, pH 6.0, containing 5 mM MgCl₂. The column was then washed with the same buffer until the absorbance of the eluate at 280 nm was less than 0.1. A linear gradient extending between zero and 1.0 M KCl in a total volume of 2 liters of 10 mM Tris-acetate buffer, pH 6.0, containing 5 mM MgCl₂ was next passed through the column with a peristaltic action pump and 20-ml effluent fractions were collected at a rate of one fraction every 3 to 4 min. The enzyme was eluted at approximately 0.55 M KCl and those fractions having a specific activity greater than 2.0 were pooled. This solution was dialyzed overnight against several changes of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM 2-mercaptoethanol and 5 mM MgCl₂. Prompt dialysis of the enzyme after its elution from the cellulose phosphate column was necessary because considerable inactivation occurred in the presence of high salt concentrations and because of the absence of a sulfhydryl reducing agent. Magnesium chloride was included in all buffers for this chromatographic step because it facilitated binding of the enzyme to the cellulose phosphate column. It is also important to note that the presence of 2-mercaptoethanol or EDTA in these buffers prevented binding of the enzyme to the cellulose phosphate.

Step 4. DEAE-cellulose Column Chromatography—The dialyzed Step 3 fraction was applied to a DEAE-cellulose column (2.5 x 30 cm) and was eluted with a linear gradient extending between 0 and 0.5 M KCl in 1 liter of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM 2-mercaptoethanol. Effluent fractions of 6.0 ml each were collected at a rate of one fraction every 3 min and the enzyme was eluted at approximately 0.22 M KCl. Those peak fractions that had a specific activity greater than 4.0 were pooled and then immediately concentrated to about 20 ml in an Amicon ultrafiltration cell (model 401) equipped with a UM-10 membrane.

Step 5. Preparative Polyacrylamide Gel Electrophoresis—An anionic polyacrylamide gel column was prepared as described under "Methods." The resolving gel portion of the column was 6.0 cm long and the concentrating gel portion was 1.0 cm in length. A current of 37 ma was applied to the column for 1 hour prior to the application of the Step 4 fraction. As much as 125 mg of protein from the Step 4 fraction (about 19 ml) could be layered onto the column. A current of 37 ma (approximately 160 to 180 volts) was applied to the column after the sample addition, and the elution buffer (0.1 M Tris-HCl buffer, pH 8.1, containing 10 mM dithiothreitol) was pumped through the elution chamber at a constant rate of 0.8 ml/min. Fractions of 3.7 ml each were collected. Enzyme activity generally began to appear in the effluent fractions after about 13 hours. In order to determine which fractions were to be pooled, samples from those fractions that had a specific activity of about 14 were placed on each of two analytical polyacrylamide gel columns (0.8 x 10.0 cm). After electrophoresis, one column was stained for protein using Coomassie blue (18) and the other was stained for enzyme activity as described for Fig. 1. Those fractions that contained a single protein and corresponding activity band were pooled, concentrated in an Amicon ultrafiltration cell as described previously, and then dialyzed overnight against 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM dithiothreitol. The total recovery of enzyme activity from the preparative polyacrylamide gel column was consistently greater than 96%.

A summary of the results from a typical purification procedure is given in Table I. By this procedure, the enzyme was obtained in a yield of greater than 12% with an over-all purification of about 180-fold. Unless otherwise noted, the Step 5 fraction (Table I) was used in all of the studies that follow.

General Properties

Stability and pH Optimum—The purified enzyme was stored at 5°C in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM dithiothreitol and 20% glycerol. Under these conditions, it was completely stable over at least a 2-week period. It was quite unstable, however, when stored in the absence of a sulfhydryl reducing agent. The enzyme also lost greater than 50% of its activity after a single freezing and thawing regardless of whether a sulfhydryl reducing agent was present or not. For routine studies the dithiothreitol and glycerol were removed from a portion of the enzyme by passage through a Sephadex G-50 column, which was previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The Sephadex G-50 effluent enzyme fraction was stable for 10 to 12 hours.

The enzyme had a rather broad pH optimum that extended between pH 7.5 and 8.0 under standard assay conditions with

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Total enzyme recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>27,694</td>
<td>2230</td>
<td>0.08</td>
<td>100</td>
</tr>
<tr>
<td>2. Acid precipitate</td>
<td>20,400</td>
<td>1876</td>
<td>0.09</td>
<td>84.1</td>
</tr>
<tr>
<td>3. Cellulose phosphate chromatography</td>
<td>297.8</td>
<td>640</td>
<td>2.15</td>
<td>28.7</td>
</tr>
<tr>
<td>4. DEAE-cellulose chromatography</td>
<td>87.2</td>
<td>408</td>
<td>4.68</td>
<td>18.3</td>
</tr>
<tr>
<td>5. Preparative polyacrylamide gel electrophoresis</td>
<td>18.8</td>
<td>272</td>
<td>14.47</td>
<td>12.2</td>
</tr>
</tbody>
</table>
saturating levels of substrate and coenzyme. The activity at pH 7.0 and 8.5 was about 74 and 69%, respectively, of that at pH 7.8. It showed no activity when tested for glucose-6-phosphate dehydrogenase (25) or aldolase (26).

Effect of Metals—The Streptococcus faecalis enzyme was neither dependent upon nor stimulated by Mg²⁺, Mn²⁺, or Ca²⁺ in the concentration range of 1.0 to 10.0 mM. Identical results were obtained with the chloride or the sulfate salts of these cations. Concentrations of Mg²⁺ in excess of 50.0 mM, however, were decidedly inhibitory. EDTA had no effect on enzyme activity when included in the standard assay at concentrations up to 50.0 mM.

Criteria of Purity

The purified enzyme was subjected to electrophoresis on standard analytical polyacrylamide gel columns. The results in Fig. 1 show it to be homogeneous as indicated by the presence of a single protein and corresponding enzyme activity band. The purified 6-phosphogluconate dehydrogenase also migrated as a single protein species when analyzed by immunoelectrophoresis using antiserum prepared against the purified enzyme (Fig. 2). Additional evidence for the homogeneity of the enzyme came from sedimentation equilibrium studies, which will be discussed later.

Chemical and Physical Properties

Absorption Spectrum—The 6-phosphogluconate dehydrogenase displayed a typical protein absorption spectrum in the ultraviolet region with a maximum extinction at 280 nm and a ratio of absorbances at 280 and 260 nm of 1.7. The E₁%₀,₅₀ calculated from the nitrogen content of the dry, ash-free protein (14.4% nitrogen) was 12.8.

Amino Acid Composition—An analysis of three samples of the enzyme hydrolyzed in 6 N HCl was carried out as described under “Methods.” The results are given in Table II. The data shown are the mean of values obtained from the 24-, 48-, and 72-hour hydrolysates, respectively. Determination of half-cystine residues as cysteic acid after performic acid oxidation showed 5.1 mol of half-cystine/108,000 g of enzyme (see the

![Fig. 2. Immunoelectrophoresis of the purified 6-phosphogluconate dehydrogenase from Streptococcus faecalis. Approximately 2 μl (5 µg of protein) of the Step 5 enzyme (Table I) was added to the upper sample well and a current of 25 ma was then passed through the gel matrix for 75 min. Thirty microliters of antiserum were placed in the trough and the slide was stored in a high humidity chamber overnight at room temperature. Excess antiserum was removed and the immune precipitate was stained with Amido black as described under “Methods.”](http://www.jbc.org/)

**TABLE II**

| Amino acid composition of 6-phosphogluconate dehydrogenase
|---|---|---|
| The values shown were corrected for destruction of serine, threonine, and tyrosine and for the rate of release of valine and isoleucine from 24-, 48-, and 72-hour hydrolysates. The value for half-cystine was determined as cysteic acid after performic acid oxidation. Tryptophan and tyrosine were determined spectrophotometrically on unhydrolyzed samples as described under “Methods.” The data have been normalized to a molecular weight of 108,000.
| Amino acid | Amino acid residues per 108,000 | Nearest integer |
| Aspartic acid | 85.2 | 85 |
| Threonine | 49.1 | 49 |
| Serine | 51.3 | 51 |
| Glutamic acid | 137.2 | 137 |
| Proline | 35.6 | 36 |
| Glycine | 85.5 | 86 |
| Alanine | 94.1 | 94 |
| Half-cystine | 5.1 | 5 |
| Valine | 53.4 | 53 |
| Methionine | 26.2 | 26 |
| Isoleucine | 65.7 | 66 |
| Leucine | 80.0 | 80 |
| Tyrosine | 42.6 | 43 |
| Phenylalanine | 35.9 | 36 |
| Histidine | 10.9 | 11 |
| Lysine | 68.5 | 69 |
| Arginine | 37.0 | 37 |
| Tryptophan | 17.4 | 17 |
next section for molecular weight determinations). When the enzyme was analyzed in 4.0 M guanidine for sulfhydryl groups by the colorimetric DTNB procedure of Ellman (21), a value of 4.9 mol of -SH/108,000 g of enzyme was obtained. This is in good agreement with the results obtained from the amino acid analysis. The native enzyme, on the other hand, reacted rather slowly with DTNB. As shown in Fig. 3, approximately 3 mol of DTNB reacted per mol of enzyme after 30 min incubation and this was accompanied by a 55% loss of activity.

**Molecular Weight**—The molecular weight of the 6-phosphogluconate dehydrogenase was determined by the high speed sedimentation equilibrium technique of Yphantis (22). Three different protein concentrations (0.033, 0.046, and 0.065%) were used in these studies and plots of the ln of the fringe displacements as a function of the radius squared yielded straight lines for each protein concentration analyzed. One such plot for a protein concentration of 0.046% is shown in Fig. 4. These data further indicated that the enzyme preparation was homogeneous. A partial specific volume of 0.736 cm³/g was estimated from the amino acid composition (Table II) and the specific volumes of the amino acids. An apparent weight average molecular weight of 108,000 ± 3,600 was calculated from the three separate protein concentrations as described under “Methods” (Equation 1).

![Fig. 3. Effect of reaction of 6-phosphogluconate dehydrogenase with DTNB on catalytic activity.](image)

Fig. 3. Effect of reaction of 6-phosphogluconate dehydrogenase with DTNB on catalytic activity. The Step 5 enzyme (Table I) (11.8 µg of protein) was incubated at room temperature in 45 mM Tris-Cl buffer, pH 7.5, containing 0.235 mM DTNB. At the times indicated, samples were removed from the reaction mixture and assayed for 6-phosphogluconate dehydrogenase activity (O—O) as well as following the change in absorbance at 412 nm. The moles of DTNB reacting per mol of enzyme (O—O) were calculated using an extinction coefficient of 13,600 at 412 nm for the colored anion of 5-thio-2-nitrobenzoic acid (or its thioquinone form), an $E_{412}^\text{molar}$ for the enzyme of 12.8, and assuming a molecular weight of 108,000.

Other methods employed for estimating the molecular weight of the enzyme included (a) polyacrylamide gel electrophoresis with varying gel concentrations as described by Hedrick and Smith (27), and (b) chromatography of the enzyme on a 6% agarose column (Bio-Gel A-5m) equilibrated with 10 mM Tris-acetate buffer, pH 6.0, containing 0.3 M NaCl and 1.0 mM EDTA. Bovine serum albumin monomer was used as a protein standard for both methods. The former method gave a molecular weight of 106,000, which is in good agreement with the value obtained from the sedimentation equilibrium studies. The latter method gave a slightly higher value of 115,000. This higher value might possibly be explained by an interaction of the enzyme with EDTA, which resulted in a change in the shape of the protein. EDTA does interact with the *S. faecalis* 6-phosphogluconate dehydrogenase in some manner, for it has been shown to completely desensitize the enzyme to Fru-1,6-P₂ inhibition without affecting catalytic activity (3, 28).

**Subunit Composition**—The subunit composition of the 6-phosphogluconate dehydrogenase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (29). The sodium dodecyl sulfate-treated enzyme, which was catalytically inactive, migrated as a single protein species and by employing suitable standards the molecular weight of this species was estimated by interpolation to be approximately 52,000 (Fig. 5). Thus, the native enzyme appears to exist as a dimer composed of monomers having the same molecular weight. Whether or not the monomers are identical with respect to their primary structure, however, cannot be ascertained unequivocally from the data at hand. There is, in fact, some preliminary indirect evidence, which suggests that the monomers may not be identical. It was observed consistently that storage of the pure enzyme in the absence of a sulfhydryl reducing agent (dithiothreitol or 2-mercaptoethanol), resulted.
in a significant decrease in specific activity. When such preparations were subjected to analytical polyacrylamide gel electrophoresis as described for Fig. 1, three additional protein and corresponding enzyme activity bands were observed. Two of these bands (major) trailed the native enzyme band, while a third band (minor) preceded it. This was apparently not due to an aggregation phenomenon as was found for the sheep liver (30) and Neurospora crassa 6-phosphogluconate dehydrogenases (11), since an analysis of the enzyme preparation by sucrose density gradient centrifugation revealed but a single protein species whose molecular weight was the same as that of the native enzyme. Moreover, when these enzyme preparations were analyzed in polyacrylamide gels of varying concentrations according to the method of Hedrick and Smith (27), plots of the log of the relative mobility of the bands versus gel concentration gave parallel rather than nonparallel lines. These results also indicated that the multiple bands were of identical or similar molecular weights and further, that the observed differences in electrophoretic mobility were due to charge differences of the various protein species (27). The addition of dithiothreitol to some of these preparations caused a reversion of the multiple bands to a single protein and corresponding activity band. This was not a consistent observation, however, and a complete explanation of this phenomenon must await more detailed studies.

Kinetics—Initial velocity experiments were conducted to determine the relative affinity of the purified enzyme for its substrate and coenzyme. Data for the double reciprocal plots are shown in Figs. 6 and 7. The intersecting patterns observed indicate that the presence of one substrate did not affect the affinity of the enzyme for the varied substrate. The \( K_m \) calculated for NADP was 0.015 mM and that for \( \alpha \)-glucose 6-phosphate was 0.024 mM. The purified enzyme was highly specific for NADP, with no activity being observed when NAD served as the coenzyme.

The reverse reaction (NADPH oxidation in the presence of \( \alpha \)-ribulose 5-phosphate and \( \text{CO}_2 \)) could be shown with the S. faecalis enzyme, but only to a very limited extent. With a reaction mixture containing enzyme, 50 mM Tris-HCl buffer, pH 7.5, 5 mM \( \alpha \)-ribulose 5-phosphate, 20 mM NaHCO\(_3\) saturated with gaseous \( \text{CO}_2 \), and 50 \( \mu \)M NADPH, the rate of NADPH oxidation was less than 25% of the rate of the forward reaction and even this slow reaction could be sustained for only a very limited time. This may have been due either to product inhibition (NADP or 6-phosphogluconate) or to the depletion of gaseous \( \text{CO}_2 \), which has been suggested to be the required substrate for the backward reaction catalyzed by the sheep liver enzyme (31). Various modifications of the assay, such as changes in pH, addition of EDTA or divalent cations, did not affect the rate of the reverse reaction catalyzed by the S. faecalis enzyme.

**DISCUSSION**

The procedure developed for purifying the 6-phosphogluconate dehydrogenase from S. faecalis yielded a homogeneous preparation that was quite stable to storage. The enzyme was neither inhibited by metal-complexing agents (28), nor was its activity stimulated by divalent cations.

Although homogeneous preparations of this enzyme have been obtained from Candida utilis (32), sheep liver (30, 33), rat liver (34), N. crassa (11), and human erythrocytes (35), this is to our knowledge the first report of a pure 6-phosphogluconate dehydrogenase from a bacterial source. Some of the physical and kinetic properties of the pure enzymes from these diverse sources are summarized in Table III. It is to be stressed that absolute comparisons of the properties listed is not possible, since the methods of analysis and assay conditions were not the same. Nevertheless, it may be seen that all of the enzymes, regardless of their sources, have very similar molecular weights and all are dimers composed of monomeric subunits of equal molecular weight. Furthermore, with the possible exception of

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**Fig. 5.** Molecular weight of the subunits of 6-phosphogluconate dehydrogenase as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The subunit molecular weights of the protein standards was plotted on a logarithmic scale against their relative migration distance (mobility). The polyacrylamide gel concentration was 10% and the concentration of sodium dodecyl sulfate was 0.1%.

**Fig. 6.** Double reciprocal plots of 6-phosphogluconate dehydrogenase activity at various concentrations of NADP as a function of 6-phosphogluconate concentration. Each assay contained 14.4 \( \mu \)g of enzyme protein and the concentrations of coenzyme and substrate were varied as indicated.

**Fig. 7.** Double reciprocal plots of 6-phosphogluconate dehydrogenase activity at various concentrations of 6-phosphogluconate (6-PG) as a function of NADP concentration. Each assay contained 14.4 \( \mu \)g of enzyme protein and the concentrations of substrate and coenzyme were varied as indicated.
the sheep liver enzyme, all have similar apparent \( K_m \) values for their substrate and coenzyme. The amino acid composition of the bacterial, yeast, and mammalian enzymes does differ, however, in several respects. Most notably the S. faecalis enzyme (Table II) and the C. utilis enzymes (types I and II) (32) have a much higher glutamic acid content and a much lower cysteine content than does the sheep liver (30) or human erythrocyte enzyme (38).

Another property that the S. faecalis 6-phosphogluconate dehydrogenase shares with the enzyme from several other sources is its sensitivity to reagents that interact with sulfhydryl groups. DTNB, for example, caused a 55% loss of activity after interacting with a calculated 3 of the 5 cysteine residues present in the molecule (Fig. 3). Recently, we have found that p-hydroxymercuribenzoate is an even more effective inhibitor of the streptococcal enzyme and 6-phosphogluconate, but not NADP, protects against this inhibition (39). A similar protection by substrate against the inhibitory effect of sulfhydryl reagents has been reported for the enzyme from several other sources (30, 32) have a much higher glutamic acid content and a much lower cysteine content than does the sheep liver (30) or human erythrocyte enzyme (38).

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