The results presented here are part of a study of pentose synthesis in *A. faecalis*. The mechanism of pentose synthesis is of interest since the organism can synthesize pentose from acetate, but cannot use hexoses for growth (1). In the course of the investigation into the enzymes associated with this synthesis, glyceraldehyde phosphate dehydrogenase activity, with either triphosphopyridine nucleotide or diphosphopyridine nucleotide as coenzyme, was observed. The activity of the enzyme with triphosphopyridine nucleotide as coenzyme suggested that this enzyme was different from the yeast and muscle dehydrogenases, but similar to the plant triphosphopyridine nucleotide-linked enzyme. This paper describes the purification of the enzyme with triphosphopyridine nucleotide as coenzyme, was observed. The activity of the enzyme with triphosphopyridine nucleotide as coenzyme suggested that this enzyme was different from the yeast and muscle dehydrogenases, but similar to the plant triphosphopyridine nucleotide-linked enzyme. This paper describes the purification and properties of the glyceraldehyde 3-phosphate dehydrogenase from *A. faecalis*.

**Experimental**

**Chemicals**—Fructose-DP was purchased from the Sigma Chemical Company and was purified (2). TPN and DPN (95 per cent pure), aldolase (5 times crystallized), glutathione reductase, alcohol dehydrogenase, and oxidized glutathione were also purchased from the Sigma Chemical Company. DEAE-cellulose was obtained from the Eastman Kodak Company. D-Glyceraldehyde 3-phosphate, 95 to 100 per cent pure when assayed enzymatically (3), was a gift from Dr. Leo Hall of the Department of Biochemistry at the University of Virginia. Calcium phosphate gel was prepared according to the procedure of Keilin and Hartree (4).

**Assay for Glyceraldehyde-3-P Dehydrogenase**—The assay system used to test for enzymatic activity during the purification of the enzyme contained 10 μmoles of fructose-DP, 51 μmoles of Na₂H₂AsO₄, 0.6 μmoles of cysteine, 51 μmoles of glycine, 0.5 μmoles of TPN or DPN, 100 μg. of aldolase, the enzyme preparation to be tested, and water to 3 ml. in a quartz spectrophotometer cuvette (5). The final pH was 7.8. Before the addition of the aldolase, the system was incubated for 5 minutes. There was no change in optical density at 340 μm on the Beckman DU spectrophotometer during this time. After the addition of the aldolase, the increase in optical density at 340 μm was recorded every 15 seconds for a period of at least 2 minutes as an indication of glyceraldehyde-3-P dehydrogenase activity.

The characteristics of the purified enzyme were studied with glyceraldehyde-3-P as substrate. The specificity assay systems used are outlined under the appropriate sections in "Results." In each case, after 2½ minutes of incubation, the reaction was initiated with the addition of substrate.

A unit of enzyme is that amount of enzyme which caused an increase in optical density at 340 μm of 0.001 per minute. Specific activity is expressed as units per mg. of protein. Protein concentration was determined according to Warburg and Christian (6).

**Growth of Cells and Preparation of Crude Extracts**— Cultures of *A. faecalis* were grown in 1 liter amounts in Fernbach flasks at 28-30° on a rotary action flask shaker (New Brunswick Scientific Company, model V) at 770 oscillations per minute for 18 hours in medium, at pH 8.5, containing 0.1 per cent NH₄Cl, 0.1 per cent KH₂PO₄, 0.05 per cent MgSO₄·7H₂O, 0.01 per cent yeast extract, 0.6 per cent sodium acetate, and 1 per cent mixture of metal salts (7).

The cells were collected by centrifugation at 3° and washed once with 0.001 M EDTA, pH 8. Twelve grams wet weight of bacteria (from 3 1. of culture) were suspended in 65 ml. of 0.001 M EDTA, pH 8, and disrupted by sonic oscillation for 15 minutes in a 10 kc. Raytheon sonic oscillator. The material was centrifuged for 30 minutes at 20,000 × g. The cells were resuspended in 60 ml. of 0.001 M EDTA, again disrupted by sonic oscillation, and centrifuged. The supernatant fluids were pooled. The extract was diluted with 0.001 M EDTA to a concentration of about 10 mg. protein per ml. All of the purification procedures were performed below 5°.

**Purification of Glyceraldehyde-3-P Dehydrogenase**—Ammonium sulfate (3.75 M, pH 8) was added with stirring to the crude extract to a final concentration of 1 M. The precipitate was removed by centrifugation for 30 minutes at 20,000 × g and discarded. To the supernatant fluid, 0.2 volume of proline (2 per cent solution, pH 5) was added. The solution was dialyzed for 12 hours against 12 l. of 0.001 M EDTA, pH 8. After removal of the precipitated nucleic acids by centrifugation, the supernatant fluid was adjusted to a final concentration of 2.3 M with solid (NH₄)₂SO₄ and to pH 8 with 5 M NH₄OH, and was stirred for 30 minutes. After centrifugation, the supernatant fluid was brought to a final concentration of 3.0 M with solid (NH₄)₂SO₄ and the pH was adjusted to 8 as before. The
Chromatography of the enzyme preparation on DEAE-cellulose. The open area refers to optical density at 280 nm, and the shaded area refers to units of activity with TPN as coenzyme. The assay system is described in "Experimental."

**Table I**

Purification of glyceraldehyde 3-phosphate dehydrogenase

<table>
<thead>
<tr>
<th>Step</th>
<th>Units per ml</th>
<th>Specific activity</th>
<th>Total units</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>330</td>
<td>40</td>
<td>66,000</td>
<td></td>
</tr>
<tr>
<td>Protamine treatment</td>
<td>150</td>
<td>50</td>
<td>57,400</td>
<td>5,600</td>
</tr>
<tr>
<td>3 M (NH₄)₂SO₄ precipitation</td>
<td>5,800</td>
<td>320</td>
<td>58,000</td>
<td>6,000</td>
</tr>
<tr>
<td>Dialysis</td>
<td>3,500</td>
<td>270</td>
<td>51,800</td>
<td>5,600</td>
</tr>
<tr>
<td>Ca₃(PO₄)₂ gel treatment</td>
<td>1,800</td>
<td>950</td>
<td>46,800</td>
<td>4,500</td>
</tr>
<tr>
<td>Dialysis</td>
<td>1,400</td>
<td>900</td>
<td>35,100</td>
<td>3,600</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>270</td>
<td>5,800</td>
<td>34,500</td>
<td>3,500</td>
</tr>
</tbody>
</table>

Material was centrifuged. The supernatant fluid was discarded and the precipitate was resuspended in 10 ml of 0.001 M EDTA, pH 8. The enzyme preparation was dialyzed for 24 hours against 6 l of 0.005 M potassium phosphate buffer containing 0.005 M cysteine, pH 8. After dialysis the enzyme preparation was 0.08 M with respect to (NH₄)₂SO₄.

The material was thawed after storage overnight at -20°C, and an equal volume of 0.0001 M cysteine, pH 8, was added. After adsorption of 26 ml of enzyme (150 mg protein) with 41.6 ml of Ca₃(PO₄)₂ gel (6.8 mg dry weight per ml), the material was centrifuged. The supernatant fluid was discarded. The gel was extracted with stirring for 15 minutes with 26 ml of 0.1 M potassium phosphate buffer, pH 7.5. After centrifugation, the gel was discarded. The supernatant fluid was dialyzed for 2 hours against 6 l of 0.005 M phosphate buffer containing 0.003 M cysteine, pH 8.

Chromatography of Enzyme Preparation—The procedure used was similar to that described by Ginsburg (9). An aqueous slurry of 3 gm of DEAE-cellulose was packed with 1 pound pressure into a glass column 2 x 20 cm. The column was washed with 200 ml of 0.2 M potassium phosphate buffer, pH 7.5, containing 0.0001 M cysteine, followed by washings with 200 ml of water. The sample was adsorbed onto the column. The adsorbed enzyme was eluted from the column with 0.005 M potassium phosphate buffer containing increasing amounts of NaCl. A reservoir and two mixing flasks were used to give a linear increase from 0 to 0.2 M NaCl going through the column. Approximately 13 ml fractions were collected. Samples were assayed for protein concentration and glyceraldehyde-3-P dehydrogenase activity with both TPN and DPN. The elution pattern is shown in Fig. 1. The results of the purification procedure are outlined in Table I.

Because of the presence of cysteine in the assay system, a linear rate of enzymatic activity was not obtained with glyceraldehyde-3-P as substrate. This observation was probably due to the decrease in the amount of available glyceraldehyde-3-P by its interaction with cysteine. When fructose-DP and aldolase were used as a continuous source of glyceraldehyde-3-P, the effect of cysteine was not apparent. Because of the disappearance of substrate by other than enzymatic means, measurements of optimum substrate and coenzyme concentrations were not accurate using the cysteine-requiring enzyme.

It was observed that if all reagents were prepared in glass-

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distilled water, the enzyme did not require cysteine through the steps of purification involving (NH₄)₂SO₄ precipitations. However, even with this precaution, treatment with Ca₃(PO₄)₂ gel and cellulose chromatography gave poor yields, and resulted in a cysteine requirement of the enzyme. The degree of purity of the glyceraldehyde-3-P dehydrogenase was, therefore, sacrificed for a cysteine-free system with which to study the characteristics of the enzyme. The purification of the enzyme was the same as described above, through the (NH₄)₂SO₄ precipitations. The enzyme was dialyzed for 2½ hours against 6 l. of 0.005 M potassium phosphate buffer, pH 8.

To remove triosephosphate isomerase, the preparation (20 ml., containing 120 mg. of protein) was treated for 10 minutes with 14 ml. of Ca₃(PO₄)₂ gel (6.8 mg. per ml.) and centrifuged. The gel was discarded. The supernatant fluid, free from triosephosphate isomerase and containing 445 units of glyceraldehyde-3-P dehydrogenase activity per mg. of protein, was used for the described studies.

RESULTS

pH Optimum—The enzyme has a pH optimum of 9.0 (Fig. 2). Potassium phosphate buffer was used to study the activity at pH 6 to 7; glycylglycine, at pH 7 to 9; sodium barbital, at pH 8.5 to 9.5; and glycine, at pH 9 to 10.5. Arsenate solutions were adjusted to the desired pH before addition to the reaction mixtures.

Glutathione Requirement—Although the TPN-enzyme had maximum activity in the absence of glutathione with fructose-DP and aldolase, it was only half as active with glyceraldehyde-3-P as substrate. The final concentration of glutathione required in the latter system for maximum activity was about 0.0017 M (Fig. 3).

As mentioned in “Experimental,” a linear rate of enzymatic activity could not be observed in the presence of glutathione or cysteine. Therefore, although maximum activity was obtained with glutathione present, the studies of enzymatic activity were performed in the absence of glutathione.

Arsenate Requirement—For maximum activity of the glyceraldehyde-3-P dehydrogenase, arsenate was required in a final concentration of 0.014 M (Fig. 4). Phosphate did not fully replace the requirement for arsenate since maximum enzymatic activity was 80 per cent of that with arsenate. Furthermore, 3.7 times more phosphate than arsenate was required to attain maximum activity (Fig. 4).

Substrate Concentration—When the enzyme was assayed with either DPN or TPN as coenzyme, the point of maximum enzymatic activity was reached in both cases at a final concentration of 0.0003 M glyceraldehyde-3-P (Fig. 5). The relative rates of activity with DPN and TPN as shown in Fig. 5 are not indicative of the enzyme-coenzyme affinity, since more enzyme...
was used in the assay with DPN than in that with TPN, and DPN was not supplied in optimum amounts.

**Coenzyme Requirement**—In the crude extract a very active DPNH oxidase (1) prevented detection of glyceraldehyde-3-P dehydrogenase activity with DPN as coenzyme. After the protamine treatment, there was no DPNH oxidase activity, and activity with DPN was measurable. It was observed throughout the purification procedures (Table 1) that enzymatic activity with TPN was about 10 times greater than with DPN under the conditions of the assay. Between 0.01 and 0.013 μmole of TPN per ml. of assay mixture was required for maximum activity, whereas about 1.1 μmoles of DPN were required (Fig. 6). With an increase in the amount of DPN beyond 1.1 μmoles, enzymatic activity was inhibited.

**Inhibition with Iodoacetate**—To study the sensitivity of the dehydrogenase to iodoacetate, an aliquot of the enzyme preparation (before gel treatment) was incubated with iodoacetate and fructose-1,6-P for 24 minutes before initiation of the reaction with aldolase. The enzyme (60 μg. of protein) was inhibited 50 per cent by a final iodoacetate concentration of $9 \times 10^{-5}$ M, and was completely inhibited by a final concentration of $1.8 \times 10^{-3}$ M. The inhibition was not reversed by the addition of 1 to 10 μmoles of glutathione.

**Characteristics of Activity**—The possibility of contamination of one coenzyme with the other, and the possibility of enzymatic conversion of one coenzyme to the other were eliminated by the following assays. Glyceraldehyde-3-P dehydrogenase from A. faecalis was incubated with TPN and limiting substrate until the reaction ceased (OD$_{340 \text{ mp}} = 0.520$). The subsequent addition of oxidized glutathione and glutathione reductase (TPNH specific) decreased the optical density within 1 minute to 0.050. When acetaldehyde and alcohol dehydrogenase (DPNH specific) were substituted for the oxidized glutathione and glutathione reductase, no change in optical density was observed. However, with DPN as hydrogen acceptor, the addition of acetaldehyde and alcohol dehydrogenase caused a rapid decrease in optical density, whereas the addition of the oxidized glutathione and glutathione reductase had no effect.

**DISCUSSION**

A glyceraldehyde-3-P dehydrogenase has been purified from A. faecalis. The enzyme is similar to the muscle and yeast dehydrogenases with respect to: (a) the requirement for arsenate or phosphate for activity, (b) the irreversible inhibition of enzymatic activity with iodoacetate, and (c) the requirement for a sulfhydryl compound for full activity (10, 11). The pH optimum of 9.0 is the same as that for the muscle enzyme and somewhat higher than that for the yeast enzyme. The enzyme differs, however, from the muscle and yeast enzymes because of its activity with TPN as coenzyme. Three different glyceraldehyde-3-P dehydrogenases have been reported in plants, namely a DPN-linked enzyme similar to that of muscle and yeast and two TPN-linked enzymes, one of which does not require phosphate or arsenate for activity (12-14). The enzyme from A. faecalis is not identical to either of the TPN-linked plant enzymes because it is active with both DPN and TPN and because it requires arsenate or phosphate for activity. Therefore, the enzyme appears to be of a different type, unlike the muscle, yeast, or plant enzymes.

The observation that the ratio of enzymatic activity with TPN and DPN as coenzymes remained constant throughout the
purification of the enzyme suggests that one enzyme is responsible for both activities. Further evidence for this is the fact that maximum activity was reached at the same concentration of glyceraldehyde-3-P in both the TPN and DPN systems (Fig. 5). Because the enzyme requires about 100 times more DPN than TPN for equal and maximum activity, it is postulated that TPN is the "true" coenzyme for the enzyme.

SUMMARY

1. A glyceraldehyde phosphate dehydrogenase has been purified from Alcaligenes faecalis. This enzyme appears to be different from the muscle, yeast, and plant glyceraldehyde 3-phosphate dehydrogenases.

2. The pH optimum of the enzyme is 9.0. Arsenate or phosphate is required for enzymatic activity. Glutathione is required for maximum activity. Iodoacetate irreversibly inhibits the enzyme.

3. The enzyme requires triphosphopyridine nucleotide (TPN) or diphosphopyridine nucleotide (DPN) as coenzyme, but needs 100 times more DPN than TPN for maximum activity.

4. On the basis of the constant ratio of activity with TPN and DPN throughout purification and the almost identical substrate concentration curves with TPN or DPN as coenzyme, it is postulated that glyceraldehyde 3-phosphate dehydrogenase activity with both DPN and TPN is due to one enzyme in A. faecalis.

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Glyceraldehyde Phosphate Dehydrogenase Activity with Triphosphopyridine Nucleotide and with Diphosphopyridine Nucleotide
Faith N. Brenneman and Wesley A. Volk


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