Tartaric Acid Metabolism

VIII. CRYSTALLINE TARTRONIC SEMIALDEHYDE REDUCTASE

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SUMMARY

Tartronic semialdehyde reductase from Pseudomonas putida has been purified and crystallized. As calculated from hydrodynamic measurements, the active enzyme has a molecular weight of 104,000 and appears to be composed of four subunits, each of which has a molecular weight of approximately 26,000. The enzyme catalyzes the reversible reduction of tartronic semialdehyde to D-glycerate in the presence of DPNH or TPNH. Glyoxylate and lactate are not substrates. The equilibrium constant of 6 x 10^{-11} has been calculated when DPNH formation is measured. The reaction is anion sensitive.

Both Pseudomonas putida and Pseudomonas acidovorans have been observed to possess a group of enzymes catalyzing the conversion of meso- and L(+)-tartrate to D-glycerate (1-5). Oxaloglutarate, the β-keto acid intermediate in this pathway, readily undergoes a cation-catalyzed, nonenzymatic decarboxylation (Reaction I) to hydroxypyruvate and tartronic semialdehyde (6), each of which can be reduced to D-glycerate by specific reductases present in both organisms (1, 5). Although the physiological role of these enzymes in the metabolism of the tartaric acids remains unknown, tartronic semialdehyde reductase (Reaction II) does participate in the pathway leading from glyoxylate to glycerate (7) and has been crystallized from P. putida during these studies differs in several respects from that previously isolated from P. cacao (8) and further characterization of the former enzyme has revealed a subunit structure. This report presents the purification, crystallization, and properties of the tartronic semialdehyde reductase from P. putida.

MATERIALS AND METHODS

With the exception of tartronic semialdehyde (1), malonic semialdehyde (9), and succinic semialdehyde (10) which were prepared as described elsewhere, all chemicals were obtained commercially.

Standard Enzyme Assay—Enzyme activity was followed spectrophotometrically by measuring the decrease in absorbance at 340 nm resulting from the utilization of reduced pyridine nucleotide. In a total volume of 1.0 ml were included the following in micromoles: potassium phosphate at pH 6.0, 200; DPNH, 0.15; tartronic semialdehyde, 4; an appropriate amount of enzyme. All reactions were carried out at 25° in cuvettes with a 1-cm light path and absorbance changes were recorded (1) during a 3-min period. The rate of DPNH utilization was linear with respect to both time and protein concentration when absorbance changes of less than 0.2 per min were measured. A unit of activity is defined as that amount of enzyme catalyzing the formation of 1.0 pmole of DPN per min in the standard incubation mixture. Specific activity is defined in terms of activity units per mg of protein. Protein was measured colorimetrically (11) with crystalline bovine serum albumin as the standard. As determined in this manner, 1 mg of homogeneous protein in 1 ml was equivalent to 0.156 mg of nitrogen and an absorbance of 0.683 at 280 nm.

Chromatography—Paper chromatography of the product involved the following four solvent systems, the RF for glyceric acid being indicated in parentheses: Solvent 1: ethyl ether-formic acid-water 5:2:1 (0.31); Solvent 2: butanol-1 saturated with 0.3 M formic acid (0.26); Solvent 3: ethanol-ammonia-water, 7:1:1 (0.17 and 0.46); Solvent 4: ethyl ether-acetic acid-water 13:3:1 (0.13 and 0.26). Glyceric acid was visualized by staining with silver nitrate (12).

Hydrodynamic Experiments—Sedimentation velocity experiments were performed at 25° in a Spinco model E ultracentrifuge.
equipped with a temperature control unit and a phase plate as schlieren diaphragm. The rotor speed for these experiments was maintained at 60,000 rpm. Diffusion data was also obtained at 25° by utilizing a Rayleigh optical system and a double sector, artificial boundary cell rotating at 4,000 rpm for 4 hours. Protein was prepared for each of these experiments by dialysis at 2° for a total of 72 hours against three changes, over 1,000 volumes each, of 0.1 M potassium phosphate at pH 7.5, containing 3 mM mercaptoethanol.

Amino Acid Analysis—After extensive dialysis of the homogeneous protein against distilled water, 0.5-ml samples were hydrolyzed with constant boiling HCl and analyzed with a Beckman model C instrument as previously detailed (4). Samples subjected to performic acid oxidation (13) were prepared and analyzed for cysteic acid as described earlier (3).

Enzyme Purification

P. putida, strain ATCC-17642, was grown with 0.2% meso-tartrate as the sole carbon source. Methods of growth, harvest, and extraction (Step 1) of the cells have been described in detail (1). Extractable enzyme activity remained constant during storage of cells at -15° for at least 6 months. All procedures were carried out at 0-2° and all buffers contained 3 mM mercaptoethanol.

Steps 2 and 3—After extraction of 105 g of cells, the enzyme was fractionated by treatment with protamine sulfate (Step 2) and ammonium sulfate (Step 3) as described elsewhere (1). The precipitate obtained by treatment with ammonium sulfate was suspended in 0.04 M Tris-chloride at pH 7.4 supplemented to contain 20% (v/v) glycerol. The resultant protein solution was dialyzed overnight against 8 liters of the Tris-glycerol buffer.

Step 4. DEAE-cellulose—The dialyzed material was charged onto a DEAE-cellulose column (5 × 30 cm) previously equilibrated with 0.04 M Tris-chloride at pH 7.4 supplemented to contain 20% (v/v) glycerol. The resultant protein solution was dialyzed overnight against 8 liters of the Tris-glycerol buffer.

Step 5. Gel Filtration—A column of the bead form of Sephadex G-250 (5 × 65 cm) was prepared after the gel had been hydrated for 1 week in 0.04 M Tris-chloride at pH 7.4 and equilibrated for an additional day in the same buffer containing 20% glycerol. Sephadex G-25, similarly prepared, was used to overlay the column for 2 cm. The dialyzed protein solution was applied and eluted with the same Tris-glycerol buffer system; fractions of 5.8 ml were collected. Fractions 35 through 55, containing the peak of enzyme activity, were pooled and concentrated to 40 ml in an Amicon filter apparatus (model 200) with a UM-1 membrane. The concentrate was dialyzed overnight against 6 liters of 0.02 M Tris-chloride buffer, pH 7.4, which contained 20% glycerol.

Step 6. Hydroxylapatite—The concentrated fraction from Step 5 was charged onto a hydroxylapatite (Bio-Rad) column (2.5 × 20 cm) which had been washed and equilibrated with the 0.02 M Tris-chloride-glycerol buffer. Elution was carried out by a linear gradient arranged so that the reservoir flask had 500 ml of a 20% glycerol-0.06 M potassium phosphate buffer at pH 7.5 and the mixing flask contained 500 ml of the equilibrating buffer. Fractions containing enzyme were collected between 270 ml and 410 ml. The eluates were pooled and concentrated to a volume of 4 ml with a Schleicher and Schuell ultrafiltration apparatus.

Step 7. Preparative Disc Gel Electrophoresis—A preparative gel apparatus (Buchler) was prepared and cooled with circulating water at 0-2°. The gels and buffers were those described in the manufacturer's instruction manual (14) except, for the elution buffer which consisted of 0.04 M Tris-chloride, pH 7.4, containing 20% glycerol and for the separating gel which was 11.5% with respect to acrylamide. Separating gel was prepared to a height of 15 cm and the amount of stacking gel was equal to the sample volume. Mercaptoethanol was added to the upper buffer to a concentration of 3 mM prior to layering the protein solution from Step 6. A constant current of 50 ma was maintained while fractions of 5.8 ml were collected at a flow rate of 0.75 ml per min. Tartronic semialdehyde reductase was eluted in Fractions 80 through 140 (Fig. 1A); active fractions were pooled and concentrated by ultrafiltration to a volume of 2.6 ml.

Step 8. Gel Filtration—Sephadex G-150, hydrated and equilibrated with 0.1 M potassium phosphate at pH 7.0, was used to form a column (2.5 × 65 cm) to which the enzyme solution from Step 7 was applied. Protein was eluted with the same phosphate buffer while 5.8-ml fractions were collected. The distribution of tartronic semialdehyde reductase, recovered in tubes 60 through 75 (Fig. 1B), was coincident with the protein peak and had an identical specific activity in each fraction. The eluates

\[ \text{UNITS} \quad \text{PER} \quad \text{GEL} \]

Fig. 1. Elution profile of tartronic semialdehyde reductase (●) and of protein as measured at 280 nm (○) from a preparative disc electrophoresis gel column (A) and from Sephadex G-150 (B) under the conditions outlined in the text.
Table 1
Summary of purification

<table>
<thead>
<tr>
<th>Step and fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
</tr>
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<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units</td>
<td>units/mg</td>
</tr>
<tr>
<td>1. Extract</td>
<td>630</td>
<td>14,720</td>
<td>2,560</td>
<td>0.2</td>
</tr>
<tr>
<td>2. Protamine treatment</td>
<td>780</td>
<td>13,840</td>
<td>2,270</td>
<td>0.2</td>
</tr>
<tr>
<td>3. Ammonium sulfate</td>
<td>325</td>
<td>7,600</td>
<td>2,170</td>
<td>0.3</td>
</tr>
<tr>
<td>4. DEAE-cellulosea</td>
<td>35</td>
<td>125</td>
<td>965</td>
<td>7.8</td>
</tr>
<tr>
<td>5. Sephadex G-200</td>
<td>40</td>
<td>58</td>
<td>732</td>
<td>13</td>
</tr>
<tr>
<td>6. Hydroxylapatite</td>
<td>4.0</td>
<td>29</td>
<td>702</td>
<td>24</td>
</tr>
<tr>
<td>7. Preparative gel electrophoresis</td>
<td>2.6</td>
<td>10.1</td>
<td>487</td>
<td>48</td>
</tr>
<tr>
<td>8. Sephadex G-150</td>
<td>2.0</td>
<td>9.8</td>
<td>470</td>
<td>49</td>
</tr>
<tr>
<td>9. Crystallizationb</td>
<td>1.0</td>
<td>4.0</td>
<td>200</td>
<td>50</td>
</tr>
</tbody>
</table>

a Only the fraction solubilized after treatment with ammonium sulfate at 45% of saturation was used for this preparation. On other occasions, the 40% and 35% fractions were pooled with the 45% fraction and purified in the same manner, resulting in a 50% higher yield at specific activity of approximately 50 at Step 7.

b Obtained from the 40% fraction as described in the text.

FIG. 2. Crystals of the enzyme as obtained from Step 9. Photographs were taken with polarized light and a dark-field condenser at a magnification of 1000.

RESULTS

Hydrodynamic Measurements—On each occasion that the enzyme has been examined in the ultracentrifuge, it has sedimentsed or diffused as expected for a single, homogeneous entity. Sedimentation constants of 6.77 S, 6.99 S, and 7.08 S, respectively, were obtained when using concentrations of 4.8, 3.0, and 1.5 mg of enzyme per ml at 25°C in a pH 7.5, 0.1 M potassium phosphate buffer containing 3 mM mercaptoethanol. Extrapolation to infinitely small protein concentration results in a $s_{20, w}$ of 7.27 S. The diffusion coefficient, $D_{20, w}$, of $6.43 \times 10^{-6}$ was calculated from data obtained at 25°C in the same buffer at a protein concentration of 6.5 mg per ml. A partial specific volume of 0.73 was estimated for the enzyme from data derived from amino acid analysis (16). Applying these values, the molecular weight was calculated to be 104,000 with a frictional coefficient, $f/f_0$, of 1.23.

Subunit Structure—In a solution of 6 M guanidine hydrochloride and 0.1 M mercaptoethanol, the enzyme was inactivated and was converted to units smaller than the 104,000 molecular weight native protein. After dialysis for 3 days against three changes of 100 volumes each of the guanidine-mercaptopethanol solution, the enzyme sedimented as a single entity with an $s_n$ of 0.503, 0.52, 0.547, and 0.577 at protein concentrations of 10.0, 8.25, 6.7, and 4.0 mg per ml, respectively. The density of the medium, $\rho$, was determined to be 1.1446 g per ml. Extrapolation of these values to “zero protein concentration” yields an $\rho$ of 0.632. Correcting the partial specific volume of the native protein, 0.73, for the effect of guanidine hydrochloride (17) yielded an effective specific volume, $\phi^*$, of 0.72 ml per g. By use of Equa-

were pooled and concentrated by ultrafiltration to 2.0 ml. Prior to storage at $-10^\circ$, the preparation was dialysed against 0.04 M Tris-chloride at pH 7.4 containing 20% glycerol.

Step 9. Crystallization—The enzyme was precipitated by the addition of 600 mg of ammonium sulfate per ml of protein solution and the precipitate was extracted at 2°C with 1 ml of a solution 60% saturated with respect to ammonium sulfate. The suspension was centrifuged at 18,000 x $g$ for 10 min and, after the supernatant fluid had been transferred to a vessel maintained at room temperature, the residue was successively treated with ammonium sulfate solutions at 55, 45, 40, and 35% of saturation and 33% of saturation in an identical fashion. After 8 hours at room temperature all fractions were placed at 2°C. When crystallization was judged complete, the crystals were harvested by centrifugation. Crystallization could also be achieved by dialysis of the preparation against 0.05 M potassium acetate buffer at pH 5.5. The active material cited in Table I is representative of the former procedure; yields from the latter procedure are the same. Crystals obtained by ammonium sulfate extraction are similar in size and shape to those described for the $P. ovalis$ preparation (8); those obtained by dialysis in acetate buffer are presented (Fig. 2).

The purification procedure is summarized in Table I. Crystalline material and material from Step 8 demonstrated only one protein band when subjected to analytic disc gel electrophoresis at pH 9.5 (15) and pH 7.8 in a 7.5% acrylamide separating gel system with a 2.5% acrylamide stacking system. Enzyme activity was eluted (3) from the polyacrylamide gel in the area coincident with the single protein band.

The pH 7.8 system was made available prior to its publication by Dr. A. C. Crambach of the National Cancer Institute.
tion 1 which was derived by Tanford, Kawahara, and LaRue
(18) and allows approximation of the molecular weight of the
protein under these conditions, the number of amino acid resi-
dues, \( n \), per protein was calculated to be 210. Multiplying this
value by the average residue weight for the enzyme, 123, results
in an average molecular weight of 25,830 for each subunit.
The average residue weight is derived from amino acid analysis
of the enzyme (Table II).

\[
\phi_p^* / (1 - \phi_p^*) = 0.286 n^{0.478} \quad (1)
\]

The assumption implicit in these calculations is that the pro-
tein behaves as a random coil and has a chain length proportional
to \( \phi_p^* / (1 - \phi_p^*) \). The experimental data obtained with sever-
al proteins under these conditions (18, 19) is in reasonable agree-
ment with the assumption and allowed the calculation of the
constants of Equation 1.

Amino Acid Analysis—Analyses of the native enzyme were
performed at three time intervals (Table II) and the average of
the determined values was used with the exception of four amino
acids. Threonine and serine were estimated by extrapolating
their concentrations to a hydrolysis time of zero. The value
obtained for the longest period of hydrolysis, 72 hours, was used
for valine. Trypsinogen was determined spectrophotometrically
(20). From the integral numerical values assigned to each of
the amino acids, a molecular weight of 104,000 was calculated,
representing good agreement with the value derived from hydro-
dynamic experiments.

Analyses of the enzyme were also performed after treatment
with 6 M guanidine hydrochloride-0.1 M mercaptoethanol for
40 hours; there was no significant difference when compared

### TABLE II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Hydrolysis time</th>
<th>Average</th>
<th>Residues per mole</th>
<th>Integral residues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
<td>48 hours</td>
<td>72 hours</td>
<td>moles/mg protein</td>
</tr>
<tr>
<td>Lyseine</td>
<td>0.578</td>
<td>0.614</td>
<td>0.641</td>
<td>0.665</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.146</td>
<td>0.134</td>
<td>0.144</td>
<td>0.144</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.281</td>
<td>0.287</td>
<td>0.281</td>
<td>0.283</td>
</tr>
<tr>
<td>Aspartic</td>
<td>0.745</td>
<td>0.780</td>
<td>0.742</td>
<td>0.739</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.447</td>
<td>0.416</td>
<td>0.391</td>
<td>0.471*</td>
</tr>
<tr>
<td>Serine</td>
<td>0.616</td>
<td>0.480</td>
<td>0.419</td>
<td>0.684+</td>
</tr>
<tr>
<td>Glutamic</td>
<td>0.907</td>
<td>0.877</td>
<td>0.889</td>
<td>0.891</td>
</tr>
<tr>
<td>Proline</td>
<td>0.226</td>
<td>0.202</td>
<td>0.199</td>
<td>0.210</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.029</td>
<td>0.565</td>
<td>0.360</td>
<td>0.978</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.964</td>
<td>0.538</td>
<td>0.932</td>
<td>0.944</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>0.118</td>
<td>0.112</td>
<td>0.115</td>
<td>0.115</td>
</tr>
<tr>
<td>Valine</td>
<td>0.638</td>
<td>0.632</td>
<td>0.661</td>
<td>0.661*</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.013</td>
<td>0.012</td>
<td>0.012</td>
<td>0.012</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.380</td>
<td>0.410</td>
<td>0.404</td>
<td>0.404</td>
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<tr>
<td>Leucine</td>
<td>0.505</td>
<td>0.506</td>
<td>0.502</td>
<td>0.504</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.137</td>
<td>0.147</td>
<td>0.147</td>
<td>0.144</td>
</tr>
<tr>
<td>Phenylnitroine</td>
<td>0.275</td>
<td>0.292</td>
<td>0.289</td>
<td>0.286</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.071</td>
<td>0.071</td>
<td>0.071</td>
<td>0.071</td>
</tr>
</tbody>
</table>

a Extrapolation to zero time.
b Assumed to represent only a lower limit and measured as
cysteic acid in performic oxidised samples.
c Highest value obtained.
d Estimated spectrophotometrically.

![Fig. 3. Activity as a function of pH with tartronic semialde-
hyde and DPNH (——) and with TPNH and DPNX (—). Substitu-
tions of TPNH for DPNH or of solutions of hydroxy-
pyruvate for tartronic semialdehyde did not alter either the
appropriate pH optimum or the general shape of the curve. The
following buffers were used at the indicated initial concentra-
tions: 0.1 M potassium phosphate (●); 0.1 M sodium acetate (○); 0.1 M
Tris-chloride (△); 0.1 M glycine-chloride (■). Standard condi-
tions were used for assays having tartronic semialdehyde or
hydroxypyruvate as substrate. Glycerate assays contained
DL-glycerate, 10–10 mmoles, and DPN, 2–10 mmoles, per 1.0 ml final volume.

with the analysis presented in Table II. Analyses of the ox-
idized samples indicate a total of 12 half-cysteine residues per
molecule of native protein.

Specificity and Kinetics—The enzyme was active in the reduc-
tion of tartronic semialdehyde with either DPNH or TPNH.
With either reduced pyridine nucleotide, the optimum pH was
at 6.0 (Fig. 3). The \( K_m \) values for DPNH and for TPNH were
2.7 \( \times 10^{-8} \) M and 3.6 \( \times 10^{-8} \) M, respectively, in the presence of
4.6 mm semialdehyde. With 0.15 mm DPNH and
TPNH the \( K_m \) for tartronic semialdehyde was 4 \( \times 10^{-3} \) M and
1 \( \times 10^{-3} \) M, respectively. In the presence of 0.15 mm DPNH or
TPNH, the following compounds were inactive as substrates
when each was tested in the concentration range of 0.1 mm to
10 mm; malonic semialdehyde, glyoxal, glycoaldehyde, glyoxy-
late, pyruvate, DL-glyceraldehyde, dihydroxyacetone, \( \alpha \)-keto-
butyrate, \( \beta \)-ketocturate, oxalacetate, dihydroxyfumarate, \( \alpha \)-keto-
oglutarate, and \( \beta \)-ketocturate.

Although solutions of hydroxyopyruvate served as substrate,
they did so at slower rates than those observed with tartronic
semialdehyde preparations at equivalent concentration. A 10-
fold lower maximal velocity was determined. Since both struc-
tural isomers are in equilibrium with a common enol (Reaction
III), aqueous solutions of either would be expected to be con-
taminated with the other. Indeed, the enol absorption at 272
mM can be readily observed in solutions of hydroxopyruvate
and can be used to follow the extent of the reaction when tar-

* The actual concentrations of tartronic semialdehyde are
approximately 80 to 70% of that recorded. The concentration
given is that of the hydroxopyruvate used in the preparation (1)
of the aldehyde. The solution actually contains both optical
isomers of tartronic semialdehyde as well as residual hydroxy-
pyruvate. Accordingly, all such values are primarily operational
and serve mainly for the examination of mechanism.
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FIG. 4. Kinetics of the reaction with tartaronic semialdehyde and either DPNH or TPNH at pH 6.0 in 0.2 M potassium phosphate. The curves were calculated from Equation 3 or 4 and the points represent experimental values. The tartaronic semialdehyde concentrations were 0.25 mM, 0.5 mM, 0.75 mM, 1.25 mM, 1.88 mM, and 2.5 mM for Curves E through J, respectively, and 0.25 mM, 0.5 mM, 0.75 mM, 1.0 mM, 1.25 mM, 1.88 mM, and 2.5 mM for Curves G' through M', respectively. DPNH concentrations were 0.03 mM, 0.05 mM, 0.08 mM, and 0.1 mM for Curves A through D, respectively, and TPNH concentrations were 0.025 mM, 0.037 mM, 0.05 mM, 0.062 mM, 0.093 mM, and 0.124 mM for Curves A' through F', respectively.

FIG. 5. Kinetics of the reaction with D(-)-glycerate and DPN at pH 8.5 in 0.2 M Tris-chloride buffer. The curves were calculated from Equation 5 while the points are experimental values. The D(-)-glycerate concentrations are 0.021 mM, 0.042 mM, 0.064 mM, 0.096 mM, 0.159 mM, and 0.212 mM for Curves A through F, respectively, and the DPN concentrations are 0.031 mM, 0.063 mM, 0.443 mM, and 0.886 mM for Lines G through J, respectively.

The reaction was reversible with n-glycerate and either DPN or TPN in the alkaline pH range (Fig. 3). In Tris-chloride at pH 8.5 and in the presence of 0.15 mM DPN, a $K_m$ of $5 \times 10^{-5}$ M was obtained for n-glycerate. With 0.2 mM D-glycerate at the same pH, a $K_m$ of $4 \times 10^{-4}$ M was obtained for DPN.

Kinetic analysis (21) allowed the determination of the constants of the general rate equation (Equation 2) where $V_m$ designates the maximum velocity, $v$, the velocity of the reaction at a given concentration of the substrates $A$ and $B$, and where $K_a$, $K_b$, and $K_s$ are constants; tartaronic semialdehyde is abbreviated as TSA. Data were obtained for initial reaction rates when less than 10% of the substrates were utilized.

From the experimental results obtained for Equation 3 at pH 6.0 in potassium phosphate buffer and for Equation 5 at pH 8.5 in Tris-chloride buffer, the relative maximal velocity was found to be 55-fold greater in the direction of glyc erate formation under the stated conditions.

**Inhibition**—The enzyme is inhibited in a competitive manner by the following compounds, each of which was tested under otherwise standard assay conditions: $\beta$-mercaptopyruvate ($K_i = 1$ mM), glycolate ($K_i = 3$ mM), malonic semialdehyde ($K_i = 4$ mM), and succinic semialdehyde ($K_i = 10$ mM). Dihydroxyfumarate was a noncompetitive inhibitor with a $K_i$ of 0.5 mM. In the presence of dihydroxyfumarate a spectral change in tartaric semialdehyde reductase was noted which was identical with that exhibited by addition of dihydroxyfumarate to either hydroxy pyruvate reductase (5) or to glyoxylate reductase.4

In the presence of tartaric semialdehyde concentrations ranging between 0.5 and 5 mM, significant inhibition was not observed with the following compounds when each was tested over the concentration range of 0.5 to 10 mM: fluoropyruvate, $\beta$-hydroxypropionate, DL-glyceraldehyde, glycerol, glyceraldehyde, $\alpha$-ketobutyrate, $\beta$-ketobutyrate, $\alpha$-ketocaproate, $\alpha$-keto glutarate, $\beta$-keto glutarate, L-malate, oxalate, oxalacetate, phosphoenolpyruvate, 3-L-phosphoglycerate, pyruvate, DL-serine, meso-tartarate, L(+)-tartarate, and tartronate.

When tartronic semialdehyde reductase preparations were assayed after incubation with 20 mM phenylhydrazine or hydroxylamine, the system was entirely active. Under standard assay conditions, the inclusion of phenylhydrazine at concentrations equivalent to those of tartronic semialdehyde resulted in appreciable inhibition; at equivalent concentrations of this inhibitor, the reverse reaction was not affected. Sodium arsenite, 3 mM, and sodium ethylenediaminetetraacetate, 10 mM, were not inhibitory when used in the standard assay system.

Product—(−)-Glyceric acid was identified as the product of the enzyme-catalyzed reduction of tartronic semialdehyde by DPNH. Glycerate was isolated from a reaction mixture containing the following in a final volume of 50 ml: 7.5 mmoles of potassium phosphate at pH 6.0; 0.4 mg of homogeneous enzyme; 1 mmole of tartronic semialdehyde added in four equal aliquots at hourly intervals; and 1 mmole of DPNH added gradually over the course of 4 hours. A control vessel contained the same constituents except for the substitution of an equal amount of boiled enzyme for active material. The reaction was followed by measuring the decrease in absorption at 340 mp of aliquots taken periodically from the reaction vessels. After 4 hours at 25°, 0.91 mmole of DPNH had been oxidized. The reaction was terminated by the addition of sulfuric acid to a final pH of 2 and glycerate was isolated by methods previously described (5). The procedure resulted in the isolation of 0.82 mmole of glycerate as determined by assay with chromatropic acid both with (22), and without (23) prior periodate oxidation. There was no evidence for glycerate formation in the boiled enzyme control which had been subjected to the identical isolation procedure.

The isolated product was oxidized by DPN in the presence of hydroxypyruvate reductase (5) or tartronic semialdehyde reductase. Paper chromatography of the product in four solvent systems yielded identical K<sub>eq</sub> values for both product and authentic n-glyceric acid without separation of the two on chromatography. In the presence of equimolar ammonium molybdate, the K<sub>eq</sub> for two samples of product (c, 0.64 in water) was +124 and +104, respectively, when calculated for the sodium salt of glyceric acid (cf. References 4 and 5).

Salt Effects—The reduction of tartronic semialdehyde was markedly affected by the nature and concentration of anions present (Fig. 6A). Optimum activity was attained at phosphate concentrations somewhat greater than 0.15 M and the standard enzyme assay was therefore routinely performed in 0.2 M potassium phosphate buffer. Sulfates were also stimulatory whereas iodides, chlorides, bromides, and nitrates were inhibitory.

Similar effects could not be demonstrated in the direction of n-glycerate oxidation when examined at pH 8.5. Nitrates did stimulate slightly but the phosphates, sulfates, and chlorides of sodium, potassium, and ammonium did not markedly affect the reaction (Fig. 6B). However, significant stimulation of n-glycerate oxidation was obtained in the presence of Tris-chloride buffers.

**Equilibrium Constant**—The data (Table III) for the calculation of the equilibrium constant for Reaction II (Equation 6) were derived from measurements of changes in DPNH concentration. However, it is evident from the tracings depicted in Fig. 7 that a true equilibrium was not reached but rather that DPNH tended to increase slowly with time. The slow phase of the reaction is believed to be a function of both the establishment of an equilibrium with hydroxypyruvate (Reaction III) and of the gradual nonenzymatic decarboxylation of

\[
K_{eq} = \frac{[DPNH][glycerate]}{[H^+][DPNH][tartronic semialdehyde]}
\]

**Table III**

<table>
<thead>
<tr>
<th>No.</th>
<th>Buffer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Initial concentration of</th>
<th>Final concentration of</th>
<th>Calculated&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H&lt;sup&gt;+&lt;/sup&gt;</td>
<td>n-Glycerate</td>
<td>DPNH</td>
<td>DPNH</td>
</tr>
<tr>
<td>1</td>
<td>Tris</td>
<td>1.48</td>
<td>4.24</td>
<td>1.77</td>
</tr>
<tr>
<td>2</td>
<td>Tris</td>
<td>1.48</td>
<td>2.12</td>
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<tr>
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<td>Tris</td>
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<td>8.47</td>
<td>3.54</td>
</tr>
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<td>3.16</td>
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<td>1.77</td>
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<td>7.06</td>
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<tr>
<td>9</td>
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<td>4.24</td>
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<td>3.15</td>
<td>2.12</td>
<td>1.77</td>
</tr>
</tbody>
</table>

<sup>a</sup> In addition to the buffer, all reaction vessels contained 10 mM potassium phosphate and 17 units of homogeneous enzyme, except vessels No. 9 and 10 which contained 270 units of homogeneous enzyme. Tris-chloride buffers were 0.2 M; phosphate buffer was 0.15 M.

<sup>b</sup> The average calculated value was 6.4.
FIG. 7. Determination of equilibrium. Each cuvette contained, in a final volume of 1.0 ml, 4.24 pmoles of d-glycerate, 1.77 pmoles of DPN, and 200 pmoles of either Tris-chloride or potassium phosphate at pH 7.5. The pH of the solution did not vary over the course of the reaction. The enzyme concentration was 0.35 mg per ml for Curves A and C and 1 mg per ml for Curve B.

FIG. 8. Absorption spectrum of 1.0 mg of the enzyme at pH 7.0 (- - -) and in 0.1 n KOH (---).

The values for DPNH recorded in Table III were estimated by subtracting the small amount of DPNH formed as a consequence of what were considered as secondary reactions, e.g., Reactions III and IV. The "true" quantity was obtained by extrapolating the small but constant slope to zero time as shown in Fig. 7, Curve A. Within these limitations, the $K_{eq}$ at 25° was calculated to be $6 \times 10^4$, representing a $\Delta F$ of $-16.1$ kc at 25° and, at pH 7.0, a $\Delta F'$ of $-6.5$ kc.

These results must be compared with a $K_{eq}$ of $5 \times 10^4$ obtained for the same reaction using phosphate buffer and the enzyme from P. ovalis (8). Fig. 7 illustrates the approach to equilibrium with the presently described enzyme in both Tris-chloride (Fig. 7, Curve A) and inorganic phosphate (Fig. 7, Curve C); both were at the same pH and the amount of enzyme was equal to that used in the experiments with P. ovalis previously described. It is evident that these conditions result in a very slow approach to equilibrium in inorganic phosphate whereas equilibrium, as defined above, is reached within 20 min in the presence of Tris-chloride. The rate is, of course, increased by the use of greater amounts of enzyme (Fig. 7, Curve B) although 10- to 15-fold higher enzyme concentrations were necessary in phosphate to match the rate shown in Fig. 7, Curve A and to obtain the data presented for phosphate buffer in Table III.

Spectrum—Aside from absorption due to the aromatic amino acids, neutral solutions of the enzyme in 0.1 M potassium phosphate displayed a second absorption peak at 410 mp and a broad shoulder between 320 and 350 mp (Fig. 8). This observation could be made with preparations at any of the last four purification stages and, accordingly, is not considered to be a consequence of modification of the enzyme as a function of these purification steps. Exposure of the enzyme to 0.1 M KOH resulted in the loss of 410 mp absorption but in an increase of that between 320 and 350 mp. Exposure of a 2-mg sample of enzyme to 0.5 M H2SO4 for 30 min at room temperature or at 60° followed by centrifugation and solution of the precipitate in 0.1 M KOH revealed the complete loss of all but the aromatic amino acid absorption (Fig. 8). Spectral examination of the supernatant fluid obtained after acid treatment revealed no evidence of material absorbing in this spectral area nor was there spectral evidence for compounds related to pyridoxine (24, 25). Reaction with phenylhydrazine (26) did not yield a compound with major 410 mp absorption nor did chromatography of the reaction mixture in several solvent systems (24) indicate the presence of pyridoxalphenylhydrazine.

This examination was pursued since cruder enzyme preparations, prior to Step 7, were yellow and exhibited a browning reaction (27) at pH 10 in glycine buffers. Both color and browning reaction were completely eliminated from the preparation by Step 7. A protein, eluted immediately prior to tartronic semialdehyde reductase from the disc gel procedure, was found to be responsible for the browning reaction.

The possibility that the observed spectral properties were due to bound pyridine nucleotide was also considered but chromatographic methods (28) and treatment with cyanide (29) failed to elicit any evidence of its presence. The enzyme was not stimulated by the addition of DPN, FMN, or FAD.

DISCUSSION

The tartronic semialdehyde reductase from P. putida appears to differ in several properties from that obtained from P. ovalis (8) although the extent and interpretation of the dissimilarities is not clear. Comparison of the two enzymes reveals different pH optima, specific activities, and substrate specificity. The significance of the differences is obscured by the nature of the substrate. In the presently reported experiments, tartronic semialdehyde was prepared by enolization in alkaline medium of lithium hydroxyypyruvate (1). The resultant mixture con-
obtained residual hydroxypyruvate and both optical isomers of tartronic semialdehyde. The tartronic semialdehyde used in the studies with \( P. \) \( ovalis \) was the product of a glyoxylate carboligase catalyzed reaction (30) and may represent a single optical isomer of the aldehyde, contaminated only minimally by hydroxypyruvate. The solutions containing the substrates are therefore different and could easily account for the nearly %-fold decrease in \( P. \) \( ovalis \) residual hydroxypyruvate and both optical isomers of tartronic semialdehyde. The tartronic semialdehyde used in the studies with \( t \)ally generated substrate.

noted a shift in pH optimum from that obtained with enzymatic substrates prior to assay, was used, Gotto and Kornberg (8) in the studies with enzyme from \( P. \) \( ovalis, 5 \)

expected product, \( \alpha \)-hydroxypropionate, oxidized in the presence of the appropriate pyridine nucleotide. Mesoxalic semialdehyde was a potent competitive inhibitor of the tartronic semialdehyde and of mesoxalic semialdehyde. Although \( P. \) \( pufluicola, 6 \)

could result in an even greater differential between the two expected anion values. The difference is not explicable on the basis of the lability of tartronic semialdehyde in so far as the present experiments are concerned, since this argument would result in an even greater differential between the two equilibrium constants.

The highly significant difference in equilibrium constant between those reported here, \( 6 \times 10^4 \), and those observed with the enzyme from \( P. \) \( ovalis, 5 \times 10^4 \), cannot be explained to any degree of satisfactory, although the effect of different buffers on the rate of the reverse reaction (Figs. 6 and 7) may be the basis for the conflicting values. The difference is not explicable on the basis of the lability of tartronic semialdehyde in so far as the present experiments are concerned, since this argument would result in an even greater differential between the two equilibrium constants.

There are several properties common to tartronic semialdehyde and of mesoxalic semialdehyde. Although mesoxalic semialdehyde was a potent competitive inhibitor of the \( P. \) \( putida \) enzyme, the compound was not reduced nor was its expected product, \( \beta \)-hydroxypropionate, oxidized in the presence of the appropriate pyridine nucleotide. Mesoxalic semialdehyde was not tested since it was not possible to confirm that preparations of the compound (31) were not contaminated with tartronic semialdehyde.

There are several properties common to tartronic semialdehyde reductase and the three enzymes, \( \alpha \)-hydroxypropionate reductase (5), oxalolglycylate reductive dehydrogenase (4), and glyoxylate reductase \( \alpha \) (5, 32), which are able to reduce hydroxypyruvate. Each of the enzymes catalyzes the formation of the \( \delta \)-(+) isomer of glyceraldehyde, binds dihydroxyfumarate, and is significantly affected by specific anions. In contrast, \( L \)-lactate dehydrogenase, which can catalyze the reduction of hydroxypyruvate to the \( L \)-(+) isomer of glyceraldehyde, is not significantly affected by dihydroxyfumarate or by added anions. It would appear that a holistic approach to an examination of the enzymes active in glyceraldehyde formation could offer useful information on structure-function relationships as applied to the substrates discussed. Further understanding of the determinants required for oxidoreductase activity and specificity in this series might aid in evaluating other such enzyme groupings, such as malate and tartrate dehydrogenases.

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