The Dihydroxy Acid Dehydratase of Neurospora crassa*

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SUMMARY

The dihydroxy acid dehydratase of Neurospora crassa was purified 100- to 150-fold by fractionation with protamine sulfate, ammonium sulfate, Sephadex, and DEAE-Sephadex. It was found that the purified protein migrated in an electric field as a single band on acrylamide gel and in the analytical centrifuge. It had a lipid content of 44 to 50%. The enzyme activity was stabilized by the presence of magnesium ion. The Km values and pH optima were determined for both substrates, α,β-dihydroxyisovalerate and α,β-dihydroxy-β-methylvalerate.

Dihydroxy acid dehydratase EC 4.2.1.9 catalyzes the formation of the α-keto acid precursors of valine and isoleucine from their respective α,β-dihydroxy acid analogues, α,β-dihydroxyisovalerate and α,β-dihydroxy-β-methylvalerate. This enzyme has been shown to occur in a wide variety of organisms from bacteria to higher plants (1-4), and is now generally accepted as being one of the enzymes involved in the biosynthesis of both isoleucine and valine in all organisms capable of synthesizing these amino acids.

Recent findings in this laboratory indicate the existence of an enzyme system in Neurospora crassa associated with the mitochondrial fraction of homogenates prepared by sand grinding which is directly involved in the synthesis of valine from pyruvate and isoleucine from at least pyruvate and pyruvate plus α-ketoisovalerate (5-8). This system contains at least four enzymes, among them the dihydroxy acid dehydratase.

Our studies of the enzyme system indicate that it is highly labile so far as the over-all synthesis of valine from pyruvate is concerned unless sucrose or pyruvate are present (7). In an attempt to analyze the system further, we have made a partial purification of two of the enzymes, the dehydratase, as described in this communication, and the reductoisomerase, as described in the accompanying article (9).

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¶ Recipient of research Career Program Award GM-K6-18, 383 from the United States Public Health Service.

EXPERIMENTAL PROCEDURE

The strain, KJT1960a, of N. crassa used in these experiments was derived from a cross between the Emerson wild type strains Em 32568 and Em 3267a.

Mycelium was grown in long necked 2-liter flasks containing 1 liter of Vogel’s minimal medium (10). The flasks were inoculated with heavy suspensions of conidia and shaken at 28° on a rotary shaker for 21 to 24 hours. The accumulated mycelium was collected on cheesecloth.

DHI and DHV were synthesized following the modified method of Sjolander et al. (11). α-Keto-β-methylvalerate was prepared as described by Meister (12). α-Ketoisovalerate was obtained from Sigma, protamine sulfate from Nutritional Biochemicals, Sephadex G-100 and DEAE-Sephadex A-50 from Pharmacia, and the Tris buffer (Trizma Grade) from Sigma.

The buffer used in most of the experiments and referred to as Tris-Mg buffer contained 0.05 M Tris and 0.01 M MgSO₄. It was adjusted to pH 8.2 with HCl at 25°.

Sephadex G-100 was soaked in glass-distilled water for 48 hours, and then packed in a column (4.5 x 50 cm) until a gel level of 40 cm was obtained. Glass wool and glass beads were used to support the gel. The column was washed with 2 liters of Tris-Mg buffer before each use and reused repeatedly.

DEAE-Sephadex A-50 (coarse or medium grade) was soaked in glass-distilled water for 48 hours and then treated successively with 0.5 N HCl and 0.5 N NaOH. Each wash was followed by rinsing with glass-distilled water. The DEAE-Sephadex gel was then brought to pH 6.0 with 0.02 N HCl and equilibrated with several washings of the Tris-Mg buffer. Two DEAE-Sephadex columns were prepared, one with the dimensions of 2.2 x 40 cm and the other 1.0 x 30 cm. Both columns were packed with gel to a height of 15 cm, measured from the top of the glass bead bed. Since the column prepared with DEAE-Sephadex A-50, medium grade, had a very slow flow rate, the coarse grade was used in later experiments. Freshly prepared DEAE-Sephadex columns were used for each run.

Dehydratase activity was measured in a reaction mixture containing 5 mmoles of MgSO₄, 10 mmoles of one of the dihydroxy acid substrates, 0.05 ml of the enzyme preparation, and 0.05 M Tris buffer at the indicated pH, in a total volume of 2.0 ml. Controls contained everything in the reaction mixture except the substrate. The reaction mixtures were incubated at 37° for 15 min and stopped by the addition of 0.5 ml of 10% trichloroacetic acid. α-Keto acids synthesized in the mixtures

1 The abbreviations used are: DHI, α,β-dihydroxyisovalerate; DHV, α,β-dihydroxy-β-methylvalerate.
were determined by the direct method of Friedemann and Haugen (13). Enzyme activity was recorded as specific activity, i.e., as micromoles of keto acid synthesized per mg of protein per hour.

Protein concentration was determined according to the method of Lowry et al. (14).

The method of Raymond (15) was used for electrophoresis of protein in an acrylamide gel. The gel was made with 5% cyanogum in 0.1 M Tris-borate at pH 8.8 containing 0.1 mM EDTA. Electrophoresis was carried out at 400 volts, direct current, for 90 min. Under these conditions the current was 55 ma. Bromphenol blue was used to develop the sample on the acrylamide gel, and to check the front during electrophoresis.

The identification of the α-keto acids synthesized by dehydratase was carried out chromatographically following the method described by Wagner and Bergquist (16). The 2,3-dinitrophenylhydrazine derivatives of the products were applied with those of known keto acids.

The lipid content of the purified dehydratase preparation was determined by the method of Folch, Lees, and Sloane-Stanley (17), as modified by Fleischer, Klouwen, and Brierly (18). The phosphorus determinations were made by the method described by Bartlett (19).

RESULTS

Purification of α,β-Dihydroxy Acid Dehydratase—Purification procedures were carried out at pH 8.2 with the Tris-Mg buffer. Enzyme preparations were kept at 0-4°C during all steps. Table I presents the degree of purification achieved by the fractionation procedures described below.

Mycelium, 250 g, wet weight, was washed once with 1 liter of Tris-Mg buffer and resuspended in 800 ml of the same buffer. The suspension was homogenized for 10 min in a VirTis grinder at a rheostat setting of 90. The homogenate was centrifuged at 39,000 × g for 40 min in a Sorvall refrigerated centrifuge. The supernatant obtained is referred to as the crude extract. The pellet was discarded. The protein concentration of the crude extract was ordinarily found to be approximately 10 mg per ml. However, approximately 20% of the protein in the crude extract was eliminated by this procedure.

Partial purification of dehydratase was achieved by the addition of 3.4 ml of 1.5% protamine sulfate per 1,000 mg of protein in the crude extract. The suspension was stirred for 5 min and then centrifuged for 10 min at 12,000 × g in a refrigerated centrifuge. The volume of the supernatant solution was similar to that of the crude extract. However, approximately 20% of the protein in the crude extract was eliminated by this procedure.

Solid ammonium sulfate was added slowly to the supernatant until the level of 60% saturation was obtained. After 5 min of stirring, the suspension was centrifuged for 10 min at 12,000 × g. Sufficient ammonium sulfate was added to the supernatant to achieve a final saturation of 80%. The suspension was again centrifuged to obtain a precipitate. The precipitate was dissolved in 75 ml of the Tris-Mg buffer. The protein concentration found in a single fraction was 10 mg per ml.

A Sephadex G-100 column (4.5 × 40 cm) was charged with 70 ml of the 60 to 80% ammonium sulfate fraction, and then eluted with 300 ml of the Tris-Mg buffer. The flow rate was 10 ml/8 min, and 10-ml fractions were collected. The maximum protein concentration found in a single fraction was 10 mg per ml. This fraction occurred in one of the tubes numbered 15 through 18. The fraction showing the highest dehydratase activity was eluted from the column, four or five fractions before the protein peak. The specific activity of the sample possessing the highest dehydratase activity indicated that 10- to 20-fold purification had been achieved. The residual ammonium sulfate in the 60 to 80% sediment fraction was removed by the Sephadex G-100 gel.

Several fractions possessing high dehydratase activity were

<table>
<thead>
<tr>
<th>Table I</th>
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<tbody>
<tr>
<td>Purification of α,β-dihydroxy acid dehydratase from N. crassa. Enzyme activity was measured at pH 8.1 with DHV as substrate.</td>
</tr>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Crude extract</td>
</tr>
<tr>
<td>Protamine sulfate</td>
</tr>
<tr>
<td>Ammonium sulfate (60-80%)</td>
</tr>
<tr>
<td>Sephadex G-100</td>
</tr>
<tr>
<td>DEAE-Sephadex I</td>
</tr>
<tr>
<td>DEAE-Sephadex II</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Table II</th>
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</thead>
<tbody>
<tr>
<td>Effect of divalent cations on activity of α,β-dihydroxy acid dehydratase</td>
</tr>
<tr>
<td>In each assay, 10 μg of protein were used. Activity was assayed at pH 8.0 for DHI and at pH 7.9 for DHV. The final concentration of the metal ion added to each assay was 1 × 10⁻⁵ M.</td>
</tr>
<tr>
<td>Metal added</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>DHI</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>ZnSO₄</td>
</tr>
<tr>
<td>CaCl₂</td>
</tr>
<tr>
<td>FeSO₄</td>
</tr>
<tr>
<td>CaSO₄</td>
</tr>
<tr>
<td>MnSO₄</td>
</tr>
<tr>
<td>MgSO₄</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of EDTA on α,β-dihydroxy acid dehydratase</td>
</tr>
<tr>
<td>The assay mixture contained 10 μg of protein. Activity was determined at pH 8.0 for DHI and at pH 7.9 for DHV. Specific activity of the dehydratase with DHI and DHV as substrates was 72 and 65 μmoles of keto acids per mg of protein per hour, respectively.</td>
</tr>
<tr>
<td>MgSO₄</td>
</tr>
<tr>
<td>---</td>
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<tr>
<td></td>
</tr>
<tr>
<td>+</td>
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| Issue of May 10, 1966 Kiritani, Narise, and Wagner 2043 |
pooled; the resulting 200 to 250 mg of protein were then added
to the first DEAE-Sephadex A-50 (coarse grade) column (2.2 ×
15 cm), and the column was washed with 100 ml of the Tris-Mg
buffer. The protein was eluted with supplemented Tris-Mg
buffer: first with 80 ml of buffer containing 0.05 M NaCl, and
then with 150 ml of buffer containing 0.1 M NaCl. Fractions
containing 5 ml each were collected with a flow rate of 1 ml per
min. Dehydratase activity was found in the 0.1 M NaCl frac-
tions.

Two fractions possessing high dehydratase activity were pooled
and diluted 4 times with Tris-Mg buffer. The diluted sample
containing approximately 20 mg of protein was adsorbed on the
second DEAE-Sephadex A-50 (coarse or medium grade) column
(1.0 × 15 cm). The column was eluted with 40 ml of Tris-Mg
buffer containing 0.05 M NaCl and then 100 ml of buffer con-
taining 0.08 M NaCl. During elution the flow rate was 1 ml/
3 min for the coarse grade gel and 1 ml/6 min for the medium
grade gel. Most of the dehydratase activity was detected in the
fractions obtained by elution with 0.08 M NaCl. Since
dehydratase activity was routinely measured at pH 8.1 with
DHV as the substrate, the activity of purified enzyme at optimal
pH was 10 to 40% greater than that indicated in Table I. Gen-
erally 100- to 150-fold purification of dehydratase activity was
obtained after the second DEAE-Sephadex fractionation. An
indication of the purity of these preparations is given by the
finding that the protein in them migrated as a single band on
acrylamide gel. This band was not tested for enzyme activity.
Purified fractions stored at -20° were found to retain their
activity for several weeks.

The omission of Mg++ from the Tris-Mg buffer caused the
inactivation of dehydratase during the purification procedure.

Effects of Metal Ions on Purified Dehydratase—None of the
metal ions tested enhanced the enzyme activity as shown in
Table II. Of the divalent metal ions all except Fe++, Ca++, and
Mg++ inhibited dehydratase activity. Fe++ had no effect on
the activity; Ca++ had no effect on the activity for DHI, and
slightly inhibited the activity for DHV. In an occasional
assay, the addition of Mg++ slightly stimulated dehydratase
activity. It should be noted that synthesis of keto acids from
substrates as a function of time continued linearly during a 30-
min period, whether or not Mg++ was added to the assay mixture.

Since Mg++ was already present in the enzyme preparation,
Mg++ contamination as great as 2.5 × 10⁻⁴ M was unavoidable
in all assays. Dialysis of the purified enzyme preparation against
0.05 M Tris buffer, pH 8.2, resulted in a 70% loss of dehydratase
activity after 5 hours. The activity measured after dialysis
was not influenced by the presence or absence of Mg++ in the
assay system. Furthermore, incubation of the dialysate at 37°
for 30 min in the presence of 10⁻⁴ M Mg++ did not restore dehy-
dratase activity.

As shown in Table III, dehydratase activity was partially
inhibited by EDTA concentrations exceeding 10⁻⁴ M.

Effect of pH—Fig. 1 shows the effect of pH on dehydratase
activity. When a single purified fraction was tested, the optim-
um pH conditions for dehydratase activity for the two sub

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Effect of pH. Tris buffers adjusted to the desired pH
values at 25° were used. Activities at different pH values are
shown for both substrates. □—□, DHI as substrate; △—△,
DHV as substrate. The protein concentration used in the assays
was 4 μg/2 ml.

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Effect of substrate concentration on the α,β-dihydroxy
acid dehydrase activity as shown by a Lineweaver-Burk plot. Pro-
tein concentration was 3 μg in a total assay volume of 2 ml. The pH of the assay medium was 7.7 for DHV as substrate and 8.3 for
DHI. △, DHI as substrate; ○, DHV as substrate.
strates were generally different by about 0.4 unit. As shown in Fig. 1, the pH curve of dehydratase activity with DHI as the substrate possesses a shoulder at the pH where the enzyme activity for the DHV is optimal. The optimum pH for different samples was variable. As a general rule, the optimum pH was obtained within a range of 7.7 to 8.0 for DHV as substrate and 8.0 to 8.3 for DHI.

Effect of Substrate Concentration—Fig. 2, A and B, shows the effect of substrate concentration on purified dehydratase activity. $K_m$ values calculated by the method of Lineweaver and Burk were $1.2 \times 10^{-3}$ M for DHV and $5.8 \times 10^{-4}$ M for DHI.

Effect of Temperature on Activity—Samples of purified enzyme preparations were diluted 1:10 with cold 0.05 M Tris buffer to a total volume of 1 ml. One-half of each diluted sample was heated at 41°C for 30 min. The samples were then chilled immediately and assayed for remaining dehydratase activity. Table IV presents specific activities of the unheated samples and the percentage of the activity remaining in the corresponding heated samples. The dehydratase activity for both substrates was not affected when the pH was higher than 8.1. However, as the pH was lowered below 8.1, enzyme inactivation increased. Even when unheated samples were added to cold buffers, dehydratase inactivation occurred at the lower pH values. Dehydratase maintained in Tris-Mg buffer could be subjected to repeated freezing and thawing without a significant loss of activity.

Identification of Products—The products of dehydratase activity with DHV and DHI as substrates were identified by paper chromatography as α-ketoisovalerate and α-keto-β-methylvalerate, respectively. $R_f$ values for α-ketoisovalerate and α keta β methylvalerate were 0.75 and 0.78, respectively.

Lipid Content of Purified Enzyme—A number of different samples of purified dehydratase were analyzed for lipid content as indicated in Table V. The five samples ranged from 44 to 50% lipid, or chloroform-methanol-soluble material. Lipid was calculated in palmitic acid equivalents.

Phosphorus determinations were made on a number of samples of lipid extracted from the protein, but none could be detected by the method employed.

A comparison of the properties of the dehydratase of N. crassa with those of other organisms reveals a number of striking similarities. All of the dehydratases purified so far have been found to be dependent upon the presence of Mg++ or Mn++ for activity, or for the maintenance of activity or both (2–4). This includes Saccharomyces cerevisiae in addition to the organisms mentioned above. The dehydratase of S. cerevisiae has not been purified to any great extent, but in crude extracts it is clearly stimulated by the presence of Mg++ (20). Fe++ have also been found to be stimulatory for the dehydratase of E. coli (3) and P. radiatus (4), but not for spinach. Whether Fe++ will also enhance the activity of the N. crassa enzyme could not be determined by the

**TABLE IV**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Purification*</th>
<th>Protein</th>
<th>Lipidb</th>
<th>Lipid%</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0.85</td>
<td>0.705</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>105</td>
<td>0.57</td>
<td>0.455</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>0.55</td>
<td>0.396</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>0.50</td>
<td>0.435</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>95</td>
<td>0.50</td>
<td>0.396</td>
<td>44</td>
</tr>
</tbody>
</table>

* Degree of increase of dehydratase activity is presented.
* Amounts of lipid are shown as palmitic acid equivalents.

**TABLE V**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Ratio of $K_m$</th>
<th>Reference source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHV</td>
<td>12 $\times 10^{-4}$</td>
<td>Present study</td>
</tr>
<tr>
<td>DHV</td>
<td>5.8 $\times 10^{-4}$</td>
<td>Study</td>
</tr>
<tr>
<td>DHI</td>
<td>8.0 $\times 10^{-4}$</td>
<td>Study</td>
</tr>
</tbody>
</table>

**Discussion**

The data presented indicate that the procedure described to purify the dihydroxy acid dehydratase of N. crassa produces preparations which are quite homogeneous as determined by gel electrophoresis and ultracentrifugation. Furthermore, they indicate that only one dehydratase exists in N. crassa for the conversion of DHI and DHV to their respective keto acid analogues. This latter observation is in complete accord with what has been found in Escherichia coli (3), spinach Spinacea oleracea (2), and the bean Phaseolus radiatus (4).

A comparison of the properties of the dehydratase of N. crassa with those of other organisms reveals a number of striking similarities. All of the dehydratases purified so far have been found to be dependent upon the presence of Mg++ or Mn++ for activity, or for the maintenance of activity or both (2–4). This includes Saccharomyces cerevisiae in addition to the organisms mentioned above. The dehydratase of S. cerevisiae has not been purified as yet even in crude extracts it is clearly stimulated by the presence of Mg++ (20). Fe++ have also been found to be stimulatory for the dehydratase of E. coli (3) and P. radiatus (4), but not for spinach. Whether Fe++ will also enhance the activity of the N. crassa enzyme could not be determined by the
with the enzymes of the electron transport chain (20-22) and present in a fashion similar to the mitochondrial lipid associated such a complex indeed exists, then lipid might be expected to be enzyme, the reductoisomerase, and the transaminase, that are involved in the biosynthesis of isoleucine and valine (7, 8). If complex with at least three other enzymes, the condensing dehydratase is apparently associated with the mitochondria in a canc e because of the previous observation that in N. crassa the DHV as substrate, and 8.0 to 8.3 for DHI. The presence of lipid in the dehydratase is of particular significance in the investigation is the high lipid content of the purified enzyme. Perhaps the most interesting finding made as a result of this investigation is the high lipid content of the purified enzyme. The presence of lipid in the dehydratase is of particular significance because of the previous observation that in N. crassa the dehydratase is apparently associated with the mitochondria in a complex with at least three other enzymes, the condensing enzyme, the reductoisomerase, and the transaminase, that are involved in the biosynthesis of isoleucine and valine (7, 8). If such a complex indeed exists, then lipid might be expected to be present in a fashion similar to the mitochondrial lipid associated with the enzymes of the electron transport chain (20-22) and transhydrogenase system (23). However, these enzymes are generally found to be associated with phospholipids, whereas the dehydratase, at least in the partially purified state, seems to be associated with a phosphorus-free lipid of an unknown nature.

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REFERENCES

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