D-SERINE DEHYDRASE OF NEUROSPORA*

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In a previous publication from this laboratory (1), it was mentioned that cell-free extracts of Neurospora mycelium form considerable amounts of pyruvate and ammonia from D-l-serine. It was also reported that pyridoxal phosphate stimulates the activity of this system. Results similar to these have recently been obtained by Reissig (2). On further examination of the Neurospora system, it has been found that both isomers of serine are deaminated and that in each case pyridoxal phosphate stimulates keto acid production. Since D-serine dehydrase (deaminase) has not been previously studied in a cell-free system, and in view of the apparent participation of pyridoxal phosphate in the reaction, the characteristics of the Neurospora dehydrase were investigated.

There have been several reports of the deamination of D-serine by organisms other than Neurospora. The D-amino acid oxidase of mammalian tissues oxidatively deaminates D-serine (3); however, Neurospora D-amino acid oxidase does not (4). D-Serine is also rapidly deaminated by rat kidney slices, although in this case the reaction does not appear to be catalyzed by D-amino acid oxidase (5). The product of the deamination of D-serine by rat kidney slices has been identified as β-hydroxypyruvic acid (5). There have been several reports of the deamination of D-serine by strains of Escherichia coli ((6, 7); however, compare Maas and Davis (8)) and, here, pyruvate has been implicated as the probable product of the deamination (7).

Methods

A wild type strain (Em-5297a) of Neurospora crassa was employed in these studies. To obtain mycelium for enzyme extraction this strain was grown in 5 liter bottles containing 3 liters of Neurospora minimal medium (9). The bottles were incubated in a constant temperature room maintained at 30° and were continuously aerated. After 72 hours the mycelium formed was filtered through cheese-cloth and washed twice in distilled water. The washed mycelium was then either frozen and stored in this condition or lyophilized and kept in a vacuum desiccator; mycelium stored in either manner does not lose dehydrase activity. To prepare cell-free

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extracts, mycelium was homogenized in cold 0.05 M phosphate buffer at pH 7.8 with a Ten Broeck glass homogenizer. The homogenates were centrifuged in a Servall centrifuge at 12,000 r.p.m. for 30 minutes and the turbid supernatant solutions used as crude enzyme extracts. These preparations were routinely stored at \(-15^\circ\) without any loss of D-serine dehydrase activity.

Dehydrase activity was determined by the direct method for pyruvate developed by Friedemann and Haugen (10). Enzyme assays were performed in a 1 ml. volume containing \(5 \times 10^{-3} \text{ M D-serine}\), \(20 \gamma\) of barium pyridoxal phosphate, \(0.5 \text{ ml. of } 0.2 \text{ M borate buffer at pH 8.2}\), and enzyme extract. Assay tubes were generally incubated at \(37^\circ\) for 20 minutes. The reaction was stopped by the addition of 1 ml. of 10 per cent trichloroacetic acid to each tube and the precipitate removed by centrifugation. A 1 ml. portion of the supernatant solution was taken for pyruvic acid determination. A pyridoxal phosphate control and an enzyme control were run with each assay. When ammonia production was measured, a modification of the Conway microdiffusion technique (11) was employed. The protein content of the various fractions obtained during enzyme purification was determined by the method of Lowry et al. (12).

A unit of D-serine dehydrase activity is arbitrarily defined as the amount of enzyme required to form 0.1 \(\mu\text{M}\) of pyruvate in 20 minutes at \(37^\circ\) in the presence of the supplements mentioned previously.

**Results**

Preliminary experiments were performed with crude enzyme preparations to determine the effect of the period of incubation on the rate of pyruvate formation from D-serine. It was found that there is a linear production of pyruvate with time. Occasionally the rate of pyruvate production has been observed to decrease after 30 minutes incubation and for this reason a standard incubation time of 20 minutes was adopted. Pyruvate formation is proportional to enzyme concentration.

Purification of D-Serine Dehydrase—Before determining the characteristics of D-serine dehydrase of *Neurospora*, it seemed desirable to purify the enzyme partially and thus eliminate interfering factors which might be present in crude extracts. All operations were carried out in a cold room maintained at \(3^\circ\).

First Ammonium Sulfate Fractionation—To each 100 ml. of crude extract (approximately 15 mg. of protein per ml.) 24.5 gm. of solid ammonium sulfate were added with stirring. After 30 minutes the precipitate was removed by centrifugation and 10.3 gm. of ammonium sulfate were

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1 Kindly supplied by Dr. J. P. Greenstein.
2 Kindly supplied by Dr. W. W. Umbreit.
added to the supernatant solution. The mixture was centrifuged after 30 minutes and the supernatant solution discarded. The precipitate was taken up in 30 ml. of 0.05 M phosphate buffer at pH 7.8.

**Acetone Fractionation**—To this solution 19 ml. of acetone (−10°) were slowly added with stirring. After 30 minutes the precipitate was removed by centrifugation and 21 ml. of acetone were slowly added to the supernatant solution. After standing for 20 minutes, the precipitate was collected by centrifugation and suspended in 10 ml. of phosphate buffer. The mixture was stirred for several minutes and the insoluble protein removed by centrifugation and discarded.

**Second Ammonium Sulfate Fractionation**—To the supernatant solution from the acetone fractionation 2.2 gm. of ammonium sulfate were added. The mixture was allowed to stand for 30 minutes, following which the precipitate was removed by centrifugation; 1.2 gm. of ammonium sulfate were added to the supernatant fluid and the mixture allowed to stand for 30 minutes. The precipitate was collected by centrifugation. This precipitate was dissolved in 5 ml. of 0.1 M borate buffer at pH 8.2 and the resulting solution was dialyzed against the same buffer (0.01 M) for 12 to 24 hours. The dialyzed solution was centrifuged to remove a small amount of protein which had precipitated.

The relative purity and activity recovered in the various fractions in a representative run are indicated in Table I. Usually between 35- and 40-fold purification of d-serine dehydrase was obtained by this procedure. Further purification has been achieved by repeating the second and third steps in the purification.

**Characteristics of d-Serine Dehydrase**—In all subsequent determinations 35- to 40-fold purified dehydrase preparations were employed.

**Identification of Pyruvic Acid As Product of d-Serine Deamination**—5 ml. of a d-serine dehydrase preparation were incubated at 37° with 50 mg. of d-serine, 400 γ of barium pyridoxal phosphate, and 3 ml. of 0.2 M borate buffer at pH 8.2. After 4 hours, 5 ml. of 10 per cent trichloroacetic acid

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### Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Serine dehydrase</th>
<th>Protein</th>
<th>Serine dehydrase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units</td>
<td>mg.</td>
<td>units per mg. protein</td>
</tr>
<tr>
<td>Crude extract</td>
<td>2800</td>
<td>1500</td>
<td>1.9</td>
</tr>
<tr>
<td>1st ammonium sulfate fractionation</td>
<td>2200</td>
<td>350</td>
<td>6.3</td>
</tr>
<tr>
<td>Acetone fractionation</td>
<td>1850</td>
<td>115</td>
<td>16</td>
</tr>
<tr>
<td>2nd ammonium sulfate fractionation</td>
<td>1200</td>
<td>19</td>
<td>63</td>
</tr>
</tbody>
</table>
D-SERINE DEHYDRASE

were added and the protein precipitate removed by centrifugation. A solution containing 200 mg. of 2,4-dinitrophenylhydrazine in 20 ml. of 2 N HCl was added to the supernatant solution. After cooling the mixture overnight, the hydrazone was filtered and recrystallized from a 1:1 mixture (volume per volume) of ethyl acetate and ligroin. The yield was 24 mg. of hydrazone. These crystals melted at 118–119° (uncorrected) and gave no melting point depression when mixed with the 2,4-dinitrophenylhydrazone of authentic pyruvic acid.

Equivalence of Ammonia and Pyruvate Production—A comparison was made of the amount of pyruvate and ammonia formed by the deamination of D-serine. The results of two determinations are given in Table II. It is seen that at both enzyme concentrations tested there is good agreement between pyruvate and ammonia formation. Manometric experiments performed under conditions in which considerable amounts of pyruvate were being formed indicated that there is no detectable oxygen uptake associated with D-serine deamination by the Neurospora dehydrase.

pH Optimum—Typical pH-activity curves in 0.1 M phosphate buffer and 0.1 M borate buffer are shown in Fig. 1. Optimal activity is obtained at pH 8.1 to 8.2 in each case, although the dehydrase is less active in phosphate than in borate buffer. The nature of the phosphate inhibition will be considered in a later section.

Coenzyme of D-Serine Dehydrase—In experiments performed with crude dehydrase preparations pyridoxal phosphate stimulated pyruvate production from D-serine. Attempts to free the enzyme from pyridoxal phosphate by fractionation have been successful only occasionally. However, when mycelium stored at −15° for several weeks is used as starting material, purified preparations are obtained which are inactive in the absence of
pyridoxal phosphate. This method of resolving pyridoxal phosphate-requiring enzymes has been used previously (13). A pyridoxal phosphate saturation curve for a partially purified d-serine dehydrase preparation is shown in Fig. 2. From this curve the pyridoxal phosphate concentration required to restore half maximal velocity can be estimated to be approximately $3 \times 10^{-6}$ M. Additional evidence that pyridoxal phosphate is the coenzyme of d-serine dehydrase comes from the observation that the activity of crude dehydrase preparations (tested in the absence of additional pyridoxal phosphate) is completely inhibited by $10^{-3}$ M hydroxylamine.

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

Fig. 1. pH-activity curves for d-serine dehydrase. O, 0.1 M borate buffer; ●, 0.1 M phosphate buffer. Standard supplements were employed.

Fig. 2. Pyridoxal phosphate saturation curve for d-serine dehydrase. Incubation period 20 minutes in the presence of $5 \times 10^{-2}$ M d-serine, 0.5 ml. of 0.2 M borate buffer at pH 8.2, and 0.03 ml. of enzyme in a 1 ml. volume. Calcium pyridoxal phosphate of known purity (see foot-note 2) was employed and the concentrations indicated in the figure are calculated for free pyridoxal phosphate.

Adenosine-5-phosphate and glutathione, reported activators of bacterial L-serine deaminase (14), as well as yeast extract (15), pyridoxal, and pyridoxamine, were inactive in stimulating d-serine dehydrase activity when tested alone or in various combinations.

Kinetics—The results of a typical experiment designed to determine the effect of d-serine concentration on the velocity of pyruvate production by d-serine dehydrase are shown in Fig. 3. It can be seen that the velocity of the reaction varies with the initial d-serine concentration. The first order constants obtained from these curves were used to calculate the Michaelis constant. A Lineweaver-Burk plot of the data in Fig. 3 is shown in Fig. 4. The $K_m$ value for d-serine obtained from this curve is $2.6 \times 10^{-4}$ M.
Specificity—A partially purified D-serine dehydrase preparation was tested for the ability to form keto acids (with pyruvate as a standard, except in the case of D-threonine with which α-ketobutyrate was used).

![Graph showing the effect of D-serine concentration on the velocity of pyruvate formation by D-serine dehydrase.](image)

**FIG. 3.** Effect of D-serine concentration on the velocity of pyruvate formation by D-serine dehydrase. Standard supplements and conditions were employed with the initial D-serine concentrations and times indicated in the figure.

![Graph showing the Lineweaver-Burk plot for D-serine.](image)

**FIG. 4.** Lineweaver-Burk plot of kinetic data for D-serine. The $K_m$ value for D-serine obtained from this curve is $2.6 \times 10^{-4}$ M.

from the following compounds: L-serine, D-threonine, L-threonine, DL-allo-threonine, DL-glutamic acid, L-glutamic acid, DL-aspartic acid, L-aspartic acid, DL-homoserine, DL-alanine, glycine, DL-methionine, DL-tryptophan, DL-leucine, DL-valine, DL-isoleucine, DL-norleucine, DL-α-amino-n-valeric acid, DL-α-amino-n-butyric acid, and DL-homocystine. Of the compounds mentioned keto acid was produced from D-threonine, DL-glutamic acid,
and dL-aspartic acid. Since keto acid was not formed from L-glutamic acid or L-aspartic acid, it appears that only the unnatural forms of these amino acids are attacked. All n-serine dehydrase preparations examined to date, both crude and purified, produce keto acid from d-threonine, dL-glutamic acid, and dL-aspartic acid, as well as from d-serine. Of these compounds keto acid production from d-threonine proceeds at the lowest rate. The formation of keto acid from d-threonine and d-serine is enhanced in the presence of pyridoxal phosphate, while this coenzyme does not affect the production of keto acid from dL-glutamic acid or dL-aspartic acid. Furthermore, keto acid production in the presence of d-serine plus dL-glutamic acid (or d-serine plus dL-aspartic acid) is the summation of keto acid formation from each substrate tested alone. When d-serine and d-threonine are tested together, there is a marked inhibition of keto acid production (see "Inhibition studies"). The formation of keto acids from dL-glutamic acid and from dL-aspartic acid does not appear to be due to the contamination of d-serine dehydrase preparations with d-amino acid oxidase, since other d-amino acids which are readily attacked by Neurospora d-amino acid oxidase (4) were not deaminated. It seems reasonable to conclude that both d-serine and d-threonine are deaminated by the same enzyme. Whether or not a second enzyme is responsible for keto acid production from dL-glutamic acid and dL-aspartic acid cannot be stated at the present time.

Inhibition Studies—The results of an experiment designed to examine the inhibition of the deamination of d-serine by d-threonine are presented in Fig. 5. It can be seen that d-threonine is deaminated very slowly as compared with d-serine. The addition of an equivalent amount of d-
threonine ($5 \times 10^{-2}$ M) to D-serine results in a substantial reduction in the rate of keto acid production. Doubling the D-serine concentration partially reverses D-threonine inhibition. These observations are taken as further suggestive evidence that keto acid formation from D-serine and from D-threonine is catalyzed by the same enzyme. L-Threonine and L-serine are only slightly inhibitory.

The effect of various substances on the activity of partially purified D-serine dehydrase was investigated. Phosphate, citrate, cysteine, CN⁻, and 8-hydroxyquinoline inhibited enzyme activity (Table III). These findings suggest that the dehydrase may be metal-activated. Consistent with this supposition is the observation that the inhibition caused by some of these metal-binding agents could be reversed by Mg²⁺ ions. However, attempts to free the dehydrase from a metal activator by prolonged dialysis against distilled water or by dialysis against citrate buffer at pH 5.5 and 6.5 have been unsuccessful.

The inhibition of D-serine dehydrase by hydroxylamine, NH₄⁺, Co²⁺, CN⁻, or Zn²⁺, as well as the lack of inhibition by F⁻, is similar to the inhibition pattern obtained with another pyridoxal phosphate requiring enzyme, tryptophan desmolase (1, 16). The inhibition of the dehydrase

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**Table III**

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Molar concentration</th>
<th>Per cent inhibition</th>
<th>Per cent inhibition + $1 \times 10^{-4}$ M Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylamine</td>
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<td>100</td>
<td></td>
</tr>
<tr>
<td>F⁻</td>
<td>$1 \times 10^{-3}$</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>$2 \times 10^{-2}$</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>$1 \times 10^{-3}$</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>$3 \times 10^{-3}$</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.1</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>0.3</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>CN⁻</td>
<td>$5 \times 10^{-4}$</td>
<td>44</td>
<td></td>
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<td>$1 \times 10^{-3}$</td>
<td>71</td>
<td>63</td>
</tr>
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<td>Zn²⁺</td>
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<td>100</td>
<td></td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>$5 \times 10^{-4}$</td>
<td>55</td>
<td>42</td>
</tr>
<tr>
<td>&quot;</td>
<td>$1 \times 10^{-3}$</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>$1 \times 10^{-4}$</td>
<td>55</td>
<td>43</td>
</tr>
<tr>
<td>&quot;</td>
<td>$1 \times 10^{-3}$</td>
<td>98</td>
<td></td>
</tr>
</tbody>
</table>
by Co++, Cu++, or Zn++ is partially reversed by Mg++ ions. The inhibition caused by Co++, Cu++, Zn++, or 8-hydroxyquinoline is partially reversed by additional pyridoxal phosphate.

DISCUSSION

It is, of course, exceedingly difficult to account for the presence in organisms of enzymes whose specificity restricts their action to unnatural substrates. D-Serine dehydrase would fall into this category. This enzyme does not appear to serve a useful function, although it is always present in growing Neurospora mycelium and reaches a maximal level when maximal growth is attained. It is interesting that in an organism whose D-amino acid oxidase does not attack D-serine or D-threonine (4) there is a distinct enzyme which deaminates these two compounds.

Several studies (17, 18) recently reported have demonstrated the stimulatory action of pyridoxal phosphate in desulfhydrase systems. In this paper it was shown that Neurospora D-serine dehydrase requires this coenzyme for its action. It has also been found that the deamination of L-serine by extracts of Neurospora is stimulated by pyridoxal phosphate. The functioning of this coenzyme in dehydrase and desulfhydrase systems indicates another group of similar reactions involving amino acids in which pyridoxal phosphate is an essential component.

SUMMARY

The Neurospora dehydrase responsible for the production of equivalent quantities of pyruvate and ammonia from D-serine was investigated and purified 35- to 40-fold. The pH optimum of this enzyme was found to be between pH 8.1 and 8.2 in either borate or phosphate buffer. The pyridoxal phosphate concentration required to restore half maximal activity to a resolved preparation is approximately $3 \times 10^{-6}$ M. The $K_m$ value for D-serine was found to be $2.6 \times 10^{-4}$ M. Partially purified dehydrase preparations form keto acids from D-threonine, D-glutamic acid, or D-aspartic acid, as well as from D-serine. However, pyridoxal phosphate does not stimulate keto acid formation from D-glutamic acid or D-aspartic acid. D-Threonine inhibits keto acid production from D-serine and the inhibition is partially reversed by D-serine. The effect of various substances on the activity of D-serine dehydrase was investigated.

BIBLIOGRAPHY

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