PURIFICATION AND PROPERTIES OF SERINE AND THREONINE DEHYDRASES*

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The work reported herein describes the results of endeavors to isolate and characterize the enzymes involved in the dehydrative deamination of the β-hydroxyamino acids, serine and threonine. Preparations have been obtained which act on threonine and serine separately. L-Threonine dehydrase and L-serine dehydrase were separated by controlled heat denaturation and subsequent ammonium sulfate fractionation.

Methods and Materials

Assay Method—The amount of enzyme was determined by incubating an aliquot during purification with substrate and determining the quantity of keto acid formed. A unit of enzyme activity has been arbitrarily defined as that amount of enzyme which produces 1 μmole of keto acid when incubated with an excess of substrate at 37° in 1 hour. Under the above conditions the reaction was first order with respect to enzyme concentration and zero order with respect to substrate. Incubations were carried out in 20 ml. beakers in a Dubnoff metabolic shaking incubator at 37° for 30 minutes unless specified otherwise. At the end of the incubation, the enzyme reaction was stopped by the addition of 0.5 ml. of 25 per cent trichloroacetic acid, which not only stopped the reaction, but also precipitated the protein from solution. The reaction mixture was centrifuged, and suitable aliquots of the clear supernatant fluid were then assayed for keto acid, α-ketobutyric acid in the case of threonine and pyruvic acid in the case of serine.

The keto acid formed during an incubation was determined by a modification of the direct method of Friedemann and Haugen (1), which is dependent upon the measurement of the color of the 2,4-dinitrophenylhydrazone of the keto acid in an alkaline solution. The modification consisted of the addition of 2 ml. of 95 per cent ethanol to the 10 ml. reaction test-
tubes after the added dinitrophenylhydrazine reagent had been allowed to react. This prevents the development of turbidity which otherwise is a frequent occurrence. The appropriate standard curve was used to calculate the quantity of the standard curve for the keto acid being determined.

The L isomers of serine and threonine were used for all assays unless otherwise specified.

Enzyme assay values are expressed in terms of specific activity; namely, as the number of enzyme units (micromoles of keto acid formed per hour) per mg. of protein. Protein concentrations were determined by reading suitable aliquots of the sample at 277 m\(\mu\) in the Beckman quartz spectrophotometer, model DU (2). The values obtained were compared with a standard curve for the enzyme, prepared from a partially purified sample which had been analyzed in quintuplicate by the micro-Kjeldahl method.

Preparation of Homogenates—Fresh sheep liver was obtained from the abattoir, brought directly to the laboratory, and immediately washed with cold distilled water to remove the blood and to cool the liver. The liver was ground in an electric meat grinder, after removal of the large vessels and connective tissue, to facilitate homogenization. This minced liver was then homogenized in a Waring blender for \(\frac{1}{2}\) minute with 2 volumes of phosphate buffer, 0.1 M, pH 7.2. Small aliquots were centrifuged, and the clear extract was assayed for threonine dehydrase and serine dehydrase activity. The values obtained were used as the starting level, and all expressions of purification were based on this value.

Fractionation Procedure

Controlled Heat Denaturation—The crude homogenate, in batches of 500 ml., was heated to 70\(^\circ\) in a boiling water bath and cooled immediately when this temperature was reached. The heat denaturation led to the coagulation of many of the contaminating proteins but did not damage the enzyme when carried out as one of the initial steps in purification. The more purified enzyme, however, showed a 50 per cent loss of activity upon heating at 70\(^\circ\) for 5 minutes. The heat-treated material was centrifuged in 250 ml. bottles at 2500 r.p.m. (1340 \(\times\) g) for 20 minutes in an International refrigerated centrifuge. The clear supernatant liquid was retained for further fractionation, while the precipitate was discarded.

Fractionation with Ammonium Sulfate—The clear supernatant liquid from the above centrifugation was fractionated with ammonium sulfate according to the procedure outlined in Fig. 1. The purification achieved in the fractionation of the two enzymes is presented in Table I. The amount of solid ammonium sulfate to be added was calculated from the formula of Kunitz (3). All salt concentrations are expressed as per cent saturation of ammonium sulfate. The major portion of serine dehydrase came out in the
Liver homogenized in Waring blender 0.5 min. with 2 volumes 0.1 M phosphate buffer, pH 7.2

Heat homogenate to 70°; cool immediately

Filtrate I: add (NH₄)₂SO₄ (solid, in all steps) to 30% saturation; adjust pH to 7.2; allow to stand 4 hrs.; filter

Residue: discard

Filtrate II: add (NH₄)₂SO₄ to 40% saturation; adjust pH to 7.2; allow to stand 4 hrs.; filter

Ppt. I: dissolve in phosphate buffer; save for serine dehydrase preparation

Filtrate III: add (NH₄)₂SO₄ to 70% saturation; adjust pH to 7.2; allow to stand overnight; filter

Ppt. II: treat as I

Filtrate IV: discard

Ppt. III: dissolve in 0.1 M phosphate buffer (pH 7.2); dialyze against 0.02 M phosphate buffer until free of (NH₄)₂SO₄; then dialyze against 0.1 M phosphate buffer

Add (NH₄)₂SO₄ to 35% saturation; adjust pH to 7.2; allow to stand 4 hrs.; filter

Filtrate V: add (NH₄)₂SO₄ to 40% saturation; allow to stand 4 hrs.; filter

Ppt. IV: save for serine dehydrase preparation

Filtrate VI: add (NH₄)₂SO₄ to 50% saturation; adjust pH to 7.2; allow to stand 4 hrs.; filter

Ppt. V: test for serine dehydrase activity; save if active

Filtrate VII: discard

Ppt. VI (contains highest threonine dehydrase activity): redissolve; refractionate in 50% saturated (NH₄)₂SO₄ 3 or 4 times; enzyme then ready for further purification by concentration-gradient elution or crystallization with mercury

* All filtrations with Hormann pressure filter on No. D-3 asbestos pad with aid of Hyflo Super Cel.

FIG. 1. Procedure for fractionation of threonine and serine dehydrases from ground sheep liver.
30 to 35 per cent saturated ammonium sulfate solution, and the major portion of threonine dehydrase came out in the 45 to 50 per cent ammonium sulfate solution.

**Ammonium Sulfate Concentration-Gradient Elution**—Ammonium sulfate concentration-gradient elution from a Hyflo Super-Cel column represented the final step in the purification of threonine dehydrase. This technique led to a 620-fold purification over the starting material. The ammonium sulfate concentration-gradient elution technique is a modification of the method of Schwimmer (4). The protein was applied as an ammonium sulfate-precipitated suspension to the top of a column of Hyflo Super-Cel and was eluted by the gradual dilution of a 65 per cent saturated ammonium sulfate solution, buffered to pH 7.2, in the mixing chamber, in which the solution was kept thoroughly mixed by means of a magnetic stirrer. In this way the suspended protein and enzyme were eluted by a steadily decreasing concentration of ammonium sulfate. The fractions were collected in test-tubes with a Technicon time flow fraction collector, a drop counter being employed and 125 drops being collected in each tube. Each tube was subsequently analyzed for protein concentration and for enzyme activity. In Fig. 2 is a representative sample of the curves obtained. Two protein peaks were obtained, one at the higher ammonium sulfate concentration and another at a slightly lower concentration. In the first peak there was no enzymatic activity, while the activity closely followed the protein concentration in the second peak. According to subsequent ultracentrifugal analysis, the second peak is essentially a single protein, but is con-

### Table I

**Enzyme Activities on Fractionation of Serine and Threonine Dehydrases from Sheep Liver**

The values are given in micromoles of keto acid per mg. of protein per hour.

<table>
<thead>
<tr>
<th>Material</th>
<th>Serine activity</th>
<th>Threonine activity</th>
<th>Ratio, threonine:serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>0.048</td>
<td>0.056</td>
<td>1.17</td>
</tr>
<tr>
<td>Heated homogenate (centrifuged)</td>
<td>0.134</td>
<td>0.093</td>
<td>0.69</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ppt., 30% saturation</td>
<td>0.155</td>
<td>0.058</td>
<td>0.37</td>
</tr>
<tr>
<td>&quot; 35% &quot;</td>
<td>0.345</td>
<td>0.109</td>
<td>0.31</td>
</tr>
<tr>
<td>&quot; 40% &quot;</td>
<td>0.300</td>
<td>0.268</td>
<td>0.89</td>
</tr>
<tr>
<td>&quot; 45% &quot;</td>
<td>0.300</td>
<td>1.33</td>
<td>4.42</td>
</tr>
<tr>
<td>&quot; 50% &quot;</td>
<td>0.470</td>
<td>2.20</td>
<td>4.70</td>
</tr>
<tr>
<td>&quot; 60% &quot;</td>
<td>0.081</td>
<td>1.15</td>
<td>14.20</td>
</tr>
<tr>
<td>Eluate, concentration-gradient</td>
<td>0.070</td>
<td>34.6</td>
<td>35.6</td>
</tr>
</tbody>
</table>
taminated by a small amount of protein of lower molecular weight. This contamination is probably due to trailing of the first protein peak into the second. By more closely selecting the fractions retained, it was largely possible to eliminate this contamination, leading to what is apparently a single protein. The high specific activity of this protein peak and the close coincidence of the protein concentration and activity curves serve as an indicator of the purity of this preparation. Absolute purity has not been attained or established, but it is felt that a high degree of purity has been achieved. Insufficient material was available for the repeated fractionations required to remove traces of other proteins and for the many ultracentrifugal and electrophoretic analyses at different pH values required to achieve and establish the complete purity of the enzyme.

Preparation of Mercury Crystals—A crystalline material was readily obtained from preparations of threonine dehydrase when Hg\(^{++}\) (10\(^{-4}\) M) was added to a 10 per cent ethanol solution of the enzyme. The crystals appear as blunt rods. They were insoluble in water, dilute salt solutions, or phosphate buffer, but were readily soluble in glycine buffer. The crystals were inactive enzymatically, and treatment with ethylenediaminetetraac-
tate (EDTA) or cysteine produced no activation; however, upon several occasions this material was reactivated by treatment with hydrogen sulfide. The activation is difficult to reproduce and cannot be repeated consistently. Maximal activity has never been obtained from the mercury crystals. The highest specific activity that has been obtained amounts to 73.5 per cent of the activity of the enzyme preparation prior to crystallization.

Considerable work remains to be done to clarify the status of the mercury crystals. At this point the problem is not only one of getting sufficient material to conduct kinetic studies, but also to retain it in a state that can be reactivated. The crystals cannot be reactivated after storage for much longer than a week or so. Upon prolonged standing (several months) the crystals revert to an amorphous protein material.

**Other Methods of Purification**—Acetone and alcohol fractionation at low temperatures and alcohol and metal salt fractionations were tried, but were discarded as being too uneconomical of enzyme for a practical purification procedure. Calcium phosphate gel, protamine sulfate, alumina gel, charcoal, and IRA-400, XE-64, and MB-2 resins were all tried without success.

**Separation of Two Enzymes**—The indication that two enzymes might be involved came from the varying ratios of threonine to serine activity in different preparations. Initial preparations have been found to vary as much as 10-fold in the amount of threonine dehydrase present, while the amount of serine dehydrase remained roughly constant for a given animal species, suggesting not only that two enzymes might be involved, but also that dietary influences might significantly alter the level of threonine dehydrase. Indeed it has been found that the enzyme threonine dehydrase is an inducible enzyme, the formation of which is stimulated by the intraperitoneal administration of the substrate to mice and rats. By following the threonine-serine activity ratios through a typical fractionation procedure, clear evidence is indicated for the existence of two enzymes (Table I).

**Activation of Enzymes**—Striking differences have been found in the effects of activators on serine and threonine dehydrases. In threonine dehydrase there was no activation by pyridoxal phosphate, Mg++, adenylic acid, or glutathione either in freshly prepared dialyzed solutions or in the most highly purified preparations obtained. On the other hand, pyridoxal phosphate, adenylic acid, and glutathione each did produce a small activation in some lyophilized preparations (Table II).

In dialyzed serine dehydrase preparations, on the other hand, activation by pyridoxal phosphate, adenylic acid, and glutathione resulted, though not by Mg++. The combined activation by these three substances is greater than any single one alone, although the effect is not additive (Table III). Adenylic acid alone gave the highest activation.
Substrate Specificity—Serine dehydrase was found to be specific for L-serine only, and it did not attack D-serine, D- or L-threonine, DL-allothreonine, DL-homocysteine, DL-cysteine, or β-phenylserine.

The threonine dehydrase is specific for L-threonine and displays no activity against D-threonine, DL-allothreonine, D-serine, DL-homocysteine, DL-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Additions</th>
<th>Specific activity</th>
<th>Per cent increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh, 45% (NH₄)₂SO₄ ppt.</td>
<td>B-6 AMP Glut.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; 45% &quot; &quot; &quot;</td>
<td>+ - -</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>&quot; 45% &quot; &quot; &quot;</td>
<td>- + -</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>&quot; 45% &quot; &quot; &quot;</td>
<td>- - +</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>&quot; 45% &quot; &quot; &quot;</td>
<td>+ + +</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Column eluate</td>
<td>- - -</td>
<td>35.7</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>+ - -</td>
<td>35.7</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>- + -</td>
<td>36.6</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>- - +</td>
<td>35.7</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>+ + +</td>
<td>35.7</td>
<td></td>
</tr>
<tr>
<td>Year-old preparation</td>
<td>- - -</td>
<td>0.49</td>
<td>18</td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>+ - -</td>
<td>0.58</td>
<td>30</td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>- + +</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>- - +</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>+ + +</td>
<td>0.62</td>
<td>26</td>
</tr>
<tr>
<td>Lyophilized preparation</td>
<td>- - -</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>+ - -</td>
<td>0.78</td>
<td>78</td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>- + -</td>
<td>0.72</td>
<td>64</td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>- - +</td>
<td>0.74</td>
<td>68</td>
</tr>
<tr>
<td>&quot; &quot; &quot;</td>
<td>+ + +</td>
<td>0.69</td>
<td>50</td>
</tr>
</tbody>
</table>

B-6, pyridoxal phosphate; +, 10⁻⁴ M; -, absent. AMP, adenylic acid; +, 5 × 10⁻⁴ M; -, absent. Glut., glutathione; +, 10⁻³ M; -, absent. The substrate contained 50 μmoles of L-threonine; specific activity, micromoles of keto acid produced per hour per mg. of protein.

cysteine, or β-phenylserine. It decomposes L-serine slightly. The purest preparations obtained presented a threonine-serine activity ratio of 36:1.

Whether the threonine dehydrase actually acts on L-serine to a limited extent or whether the threonine dehydrase preparations are slightly contaminated with L-serine dehydrase is not now clear. The former explanation would be supported by the constant ratio of activity in the purest preparations and also by the fact that serine dehydrase appears to be much
less stable than threonine dehydrase; however, because of the similarity of properties of the two enzymes in many respects, it seems logical to assume that they would be difficult to separate. Contamination of the L-serine

**Table III**

*Activation of Serine Dehydrase*

<table>
<thead>
<tr>
<th>Substrate, 50 μmoles</th>
<th>Additions</th>
<th>Specific activity</th>
<th>Activation ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Serine</td>
<td>-</td>
<td>-</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>0.86</td>
</tr>
</tbody>
</table>

- B-6, pyridoxal phosphate; +, 10^{-8} M; -, absent. AMP, adenylic acid; +, 5 × 10^{-4} M; -, absent. Glut., glutathione; +, 10^{-2} M; -, absent. Specific activity, micromoles of pyruvic acid produced per hour per 0.2 ml. of enzyme preparation.

* Ratio of activity to activity in absence of activators.

[Fig. 3. pH-activity curve of threonine dehydrase](http://www.jbc.org/)

with L-threonine probably is not responsible, since the same rate of reaction with serine was obtained on a sample chromatographed on a Dowex 50 column (11).
Effect of pH—The pH-activity curve shows a rather shallow peak at pH 7.4 with a broad range between about pH 7.2 and 7.6 (Fig. 3). Most of the experiments reported here were carried out at pH 7.2, which is within the optimal range.
Effect of Temperature—The optimal temperature of threonine dehydrase has been determined to be 55° (Fig. 4); this is somewhat higher than is usually found with enzymes of animal origin.

The temperature-activity curves were determined at two different incubation times, so that an indication of the rate of denaturation could be obtained with increasing temperature. Duplicate samples were run at 15 and 30 minute time intervals throughout the entire temperature range studied. Average values were used in plotting each point in Fig. 4, though the agreement among the results was excellent. All the samples were preincubated for 5 minutes before the addition of substrate, and the reaction was timed from that point. At the end of the incubation period the reaction was stopped with 0.5 ml. of 25 per cent trichloroacetic acid, and the samples were assayed in the usual manner. The same enzyme preparation was used throughout so that the results would be strictly comparable.

From the rate of decrease in activity between the 15 and 30 minute incubations, the 15 minute value was corrected to zero denaturation by making the assumption that denaturation in the first 15 minute period was equal to that in the second 15 minute time period.
From these corrected values, the apparent energy of activation of the threonine dehydrase reaction was determined by a plot of the Arrhenius equation (Fig. 5) and found to be 13,600 calories as calculated from the best straight line (dash curve, Fig. 5). The calculated value for the entire temperature range studied is 13,800 calories.

The energy of inactivation of threonine dehydrase was estimated, in the absence of substrate, by determining the residual activity after exposure to heat for 5 minutes. The control value (enzyme solution with no heat exposure) was accepted as 100 per cent (maximal activity), and the subsequent results were expressed as the per cent maximal activity. Fig. 6 presents a plot of the Arrhenius equation for the thermal inactivation of threonine dehydrase. The energy of inactivation has been calculated from this plot to be 70,300 calories per mole.

**Effect of Substrate Concentration**—The initial reaction rates for the deamination of threonine with increasing substrate concentrations were measured. From these data the Michaelis-Menten constant for the reaction has been determined to be $2.9 \times 10^{-2}$ M from a Lineweaver-Burk plot (Fig. 7).

**Effect of Salt Concentration**—Threonine dehydrase becomes highly un-
stable in solutions of low ionic strength. Dialysis against a phosphate buffer of $\mu = 0.001$ caused a 73 per cent loss of activity in 24 hours. The lowest concentration of phosphate buffer required to retain enzyme activity was 0.02 M for short periods and 0.05 M for indefinite storage. In dilute protein solutions, such as those obtained with the ammonium sulfate concentration-gradient column, a fairly high salt concentration was essential to retain enzyme activity. The activity was lost rapidly on dialysis even against 0.1 M phosphate buffer, while in 30 per cent saturated ammonium sulfate ($\sim$1 M) the enzyme activity was practically all retained.

**Ultracentrifugal Analysis**—In the ultracentrifugal patterns there was only one sedimenting protein boundary, with some indication of a slight contamination by another protein. The sedimentation constant was calculated to be about 5.3 s. An accurate determination of $s$ was not obtained, as this entails a study of the sedimentation velocity as a function of the protein concentration, and sufficient material for this was not available.

**DISCUSSION**

The finding that serine and threonine dehydrases appear to be separate enzymes has been fully discussed, but it is well to reemphasize this point. Both of the enzymes appear to require pyridoxal phosphate for maximal activity, which is in accord with previous findings; however, this has been difficult to demonstrate in the case of threonine dehydrase, probably owing to the stability of its binding under most conditions.

Deoxypyridoxine, which has been found to inhibit pyridoxal phosphate-catalyzed reactions (12), does not have any inhibitory effect upon this reaction; however, this is not necessarily conclusive. Probably it would be necessary to form deoxypyridoxine phosphate ($2,4$-$d_i$-methyl-$3$-hydroxy-$5$-pyridylmethyl phosphate) and to promote an exchange reaction between this compound and pyridoxal phosphate on the enzyme before any such inhibition could be observed. It is conceivable that an energy source as well as a special enzyme system would be required for this synthesis and for this exchange to take place, and no source of high energy phosphates is provided in the system studied.

Threonine dehydrase appears not to require Mg$^{++}$ as an activator. After prolonged dialysis of the enzyme, addition of Mg$^{++}$ caused no stimulation of the reaction and in some instances produced an inhibition. In addition the reaction proceeded normally in EDTA solution, which is a good chelating agent for this cation (13).

The observed activation by adenylic acid remains unexplained, and no plausible hypothesis occurs to the authors.

The function of glutathione is probably the maintenance of enzyme sulfhydryl groups in the reduced condition, although more thorough evidence is required to prove this convincingly.
SUMMARY

1. Preparations of serine and threonine dehydrases have been obtained from sheep liver. The methods of preparation and studies of their action and properties have been reported. A purification of 620-fold over the starting material was obtained in the case of threonine dehydrase.

2. Serine and threonine dehydrases appear to depend upon pyridoxal phosphate for prosthetic groups, though this was much more difficult to demonstrate in the latter case than in the former.

3. Adenylic acid and glutathione have also been shown to be activators for both enzymes under certain conditions.

4. No evidence of a requirement for magnesium or other metal ion was obtained for either of these two enzymes.

5. Measurements of certain physicochemical properties of threonine dehydrase have been carried out.

BIBLIOGRAPHY

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