Purification and Properties of L-Threonine Dehydrase of Sheep Liver*

Jonathan S. Nishimura† and David M. Greenberg

From the Department of Biochemistry, University of California School of Medicine, San Francisco 22, California

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In an earlier publication (1), the preparation of threonine dehydrase from sheep liver with a specific activity of 34.6 units per mg of protein was reported. The present communication describes the procedure for the further purification of this enzyme to a specific activity of up to 776 units per mg of protein and the results of a study of certain of its properties.

EXPERIMENTAL PROCEDURE

Materials—L-Threonine, L-serine, and pyridoxal-P monohydrate were purchased from the California Corporation for Biochemical Research, α-ketobutyric acid from the Nutritional Biochemical Company, and DEAE-cellulose from Eastman Organic Chemicals. DL-Allithreonine and L-allithreonine were kindly furnished by Dr. Alton Meister of Tufts University School of Medicine.

Enzyme Assay Method

During purification, the enzyme was assayed as has been described previously (1). Briefly, it consisted of incubating 50 μmoles of L-threonine or L-serine, enzyme, and 0.1 M potassium phosphate buffer, pH 7.2, in a total volume of 3 ml in 10-ml vials with snap caps at 37°C for 30 minutes in a Dubnoff metabolic shaking incubator. At the end of the incubation, 0.5 ml of 25% trichloroacetic acid was added to stop the reaction, and the coagulated protein was removed by centrifugation. The keto acid was determined by adding 1 ml of 0.1% dimethylphenyl-hydrazine solution (1 mg per ml in 2 N HCl) to aliquots of the incubation mixture, and after 5 minutes, 2.0 ml of absolute ethanol were added with shaking; 5.0 ml of 2.5 N NaOH were then added rapidly from a blow-out pipet, and the solution was shaken vigorously. After the solution was allowed to stand for 10 minutes to develop the color, the optical density was read at 515 nm in a Beckman model B spectrophotometer in Pyrex glass cuvettes (2). The keto acid present was read from standard calibration curves.

Ammonia Determination—When added ingredients interfered with the keto acid estimation, the enzyme activity was assayed by the ammonia formed. This was analyzed by the Conway microdiffusion method (3). The ammonia was reacted with Nessler's reagent, and the color was read at 420 nm in the Beckman model B spectrophotometer. Comparison of the proportionality between the keto acid and ammonia formed in the enzymatic reaction showed this was in equimolar ratio. Determination of threonine or serine present in incubation mixtures was also carried out by determination of the ammonia liberated after decomposition with periodic acid (4). One mole of ammonia is obtained per mole of each of the above amino acids.

Protein Determination—During most of the enzyme purification, the protein content was determined by the biuret method (5). In the final stages of purification on a DEAE-cellulose column, the absorption in the ultraviolet at 260 and 290 nm was used (5). An absorption of 1.0 optical density unit at 280 nm of the purified enzyme in a cell with a 1-cm light path (volume, 3 ml) was equivalent to 1.0 mg of protein, as determined by the biuret reaction.

Enzyme Unit and Specific Activity—An enzyme unit is defined as the amount of enzyme producing 1 μmole of keto acid per hour. Specific activity is represented as micromoles of keto acid formed per mg of protein per hour.

Enzyme Fractionation Procedure

The levels of threonine dehydrase in individual sheep livers varied widely. Consequently, 10 livers packed in ice were brought from the abattoir for each isolation attempt. Each liver was tested rapidly for threonine dehydrase activity, by homogenizing 20 g of liver with 40 ml of 0.1 M phosphate buffer, pH 7.2, and assaying the crude homogenate. Enzyme activity varied from none to 160 units per g of liver. Only those livers (about 1 in 10) with one-half or more of the maximal activity were fractionated.

Preparation of Homogenate—The livers were washed with cold distilled water, the large blood vessels were dissected out, and the tissue was minced. The minced liver (1 kg) was then homogenized with 2 volumes of 0.1 M phosphate buffer, pH 7.2, for 1 minute in a large stainless steel Waring Blender.

First Heat Treatment—The homogenate (1-liter batches) was heated with constant stirring in a 90°C water bath to a temperature of 70°C (required heating time about 10 minutes), then quickly chilled in ice water. The heated material was centrifuged in 250-ml plastic bottles (Nalge Company) in a Lourdes refrigerated centrifuge at 0°C at 4000 × g for 15 minutes. The precipitates were extracted once with 1 volume of 0.1 M phosphate buffer, pH 7.2, equal to that of the initial supernatant.
The precipitate was redissolved and dialyzed against 0.04 M phosphate buffer at 0°C. The mixture was then centrifuged at -15°C for 5 minutes at all the acetone was added, stirring was continued for 10 minutes. It was then washed by decantation with distilled water until the pH was about 7.0. A suspension of the material was poured into a 2-cm glass column and packed and washed by gravity with 0.005 M phosphate buffer, pH 7.2, to give a bed 28 cm high.

The solution from the acetone treatment was precipitated at 60% saturation of ammonium sulfate, and the precipitate was isolated by centrifugation. The protein precipitate (approximately 400 mg) was dialyzed against 3-liter changes of 0.005 M phosphate buffer, pH 7.2, for 24 hours in the cold. The precipitate that formed during dialysis was removed by centrifugation. The amber enzyme solution was pipetted carefully onto the adsorbent bed and washed into the adsorbent with the 0.005 M phosphate buffer, pH 7.2. The eluate was collected in a Technicon fraction collector, with a drop counter. With use of the 0.005 M phosphate buffer, pH 7.2, for elution, 10 fractions of 10 ml each were collected at a flow rate of 2 ml per minute. The rest of the fractionation was by gradient elution. This was effected by the flow of 0.1 M phosphate buffer, pH 7.2, into a mixing vessel containing 200 ml of the 0.005 M phosphate buffer. Stirring in the mixing chamber was produced by a magnetic stirrer. The flow rate was maintained at about 1.8 ml per minute during elution. The gradient was not linear.

The character of the protein separation obtained in the chromatogram is shown in Fig. 1. In less pure preparations, the protein shoulder adjacent to the enzyme-containing peak was even more pronounced. The threonine dehydrase activity was associated with a sharp peak as shown in Fig. 1. A purification of 2- to 6-fold was achieved by this procedure. About 20% of the added enzyme units could usually be recovered in the best fractions. The purification obtained by the process described here is given in Table I.

The main difficulty encountered in the chromatography was the great dilution of the active protein fractions and instability of the enzyme induced thereby. The enzyme activity of fractions with less than 1 mg per ml of protein could be preserved for 2 to 3 days by keeping them at near freezing, but they lost activity on freezing or precipitation with ammonium sulfate.
TABLE I
Summary of purification of threonine dehydrase from 600 g of sheep liver

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Serine units</th>
<th>Threonine units</th>
<th>Threonine activity</th>
<th>Threonine specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1,800</td>
<td>147,600</td>
<td>8,570</td>
<td>78,120</td>
<td>9.0</td>
<td>0.53</td>
</tr>
<tr>
<td>First heat treatment</td>
<td>1,580</td>
<td>14,060</td>
<td>8,850</td>
<td>90,690</td>
<td>10.2</td>
<td>6.5</td>
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<tr>
<td>First (NH₄)₂SO₄ fraction</td>
<td>60</td>
<td>4,690</td>
<td>7,600</td>
<td>79,880</td>
<td>10.5</td>
<td>11.0</td>
</tr>
<tr>
<td>Second heat treatment</td>
<td>75</td>
<td>1,875</td>
<td>9,185</td>
<td>98,140</td>
<td>10.7</td>
<td>11.0</td>
</tr>
<tr>
<td>Second (NH₄)₂SO₄ fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third heat treatment</td>
<td>30</td>
<td>450</td>
<td>7,455</td>
<td>68,250</td>
<td>9.1</td>
<td>152</td>
</tr>
<tr>
<td>Acetone fraction</td>
<td>5.1</td>
<td>145</td>
<td>4,010</td>
<td>42,840</td>
<td>9.3</td>
<td>295</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 32</td>
<td>7.5</td>
<td>14</td>
<td>880</td>
<td>8,715</td>
<td>9.9</td>
<td>622</td>
</tr>
<tr>
<td>Fraction 33</td>
<td>7.5</td>
<td>3.2</td>
<td>220</td>
<td>1,155</td>
<td>9.8</td>
<td>675</td>
</tr>
<tr>
<td>Fraction 34</td>
<td>7.5</td>
<td>1.22</td>
<td>90</td>
<td>890</td>
<td>9.9</td>
<td>720</td>
</tr>
<tr>
<td>Fraction 35</td>
<td>7.5</td>
<td>0.98</td>
<td>81</td>
<td>760</td>
<td>9.4</td>
<td>776</td>
</tr>
</tbody>
</table>

* Units expressed as micromoles of pyruvate or α-ketobutyrate produced per hour.
† Specific activity expressed as micromoles of α-ketobutyrate produced per mg of protein per hour. All enzyme assays performed at pH 7.2.

Enzyme fractions with a protein concentration greater than 1 mg per ml were generally stable to freezing and thawing.

RESULTS

Effect of Enzyme Concentration—The influence of enzyme concentration on the rate of formation of keto acids at 10- and 30-minute incubation periods was determined with the results shown in Fig. 2. A straight line was obtained with L-threonine for the 10-minute incubation. The reaction rate tended to depart from linearity at the higher protein concentrations in the 30-minute incubation time because of the considerable depletion of the substrate. However, the keto acid production was proportional to the enzyme concentration over a 4-fold concentration range.

The proportionality between the rate of serine deamination and enzyme concentration is excellent at both incubation times even though the reaction ceases after about 6 minutes. The amounts of keto acid formed were the same on incubation for 30 minutes as for 10 minutes. The different lines shown result from multiplying the 10-minute incubation figures by 6 and those for 30 minutes by 2 to convert the results to micromoles of keto acid per hour.

Effect of pH—The effect of pH on threonine dehydrase was determined over a 5 unit pH range, with phosphate, Tris, potassium phosphate buffer, pH 7.2, and enzyme (80 μg per ml for threonine and 250 μg per ml for serine determination) in a total volume of 3 ml. Rest of procedure is the same as described under enzyme assay.

**Fig. 2.** Effect of enzyme concentration on keto acid formation. Incubation contents: L-threonine or L-serine (50 μM), 0.1 M potassium phosphate buffer, pH 7.2, and enzyme (80 μg per ml for threonine and 250 μg per ml for serine determination) in a total volume of 3 ml. Rest of procedure is the same as described under enzyme assay.

| * The authors are indebted to Dr. Alle Nagabhushanam for performing the comparisons in this experiment.

Inhibition by Sulfhydryl Reagents—Serine and homoserine dehydrases were found to be inhibited by sulfhydryl reagents (6, 7). In similar experiments on threonine dehydrase, only CMB and mercuric ions were found to be effective inhibitors. To obtain a 50% inhibition required a CMB concentration of about 7.5 × 10⁻⁴ M (Table II). Mercuric ions were about equally inhibitory. N-Ethylmaleimide caused only a 16% inhibition and iodoacetate only 10% at 10⁻⁴ M.

Attempts to reverse the inhibition by CMB with the sulfhydryl compounds, GSH, 2,3-dimercaptopropanol, and mercaptoethanol were ineffective, even after removal of excess CMB by
Sodium cyanide produced little inhibition at $10^{-3}$ M. The strong inhibitory effect of hydroxylamine was used to advantage in resolving the pyridoxal-P from the apoenzyme.

**Substrate Specificity and Inhibition by Amino Acids—**The purified enzyme preparations of L-threonine dehydrase also catalyze the deamination of L-serine and L-allothreonine. L-Cysteine, DL-homocysteine, and DL-homoserine are not deaminated but exert a strong inhibitory effect on the enzyme.

L-Threonine and DL-allothreonine are deaminated in a linear manner with time. However, the reaction of L-serine with the enzyme is unique in that the decomposition of the amino acid is essentially completed in the first 6 to 10 minutes when comparatively large amounts of serine are incubated (Fig. 4). The reaction appears to be linear during the first 3 minutes, and the time for this phase of the reaction is lengthened as the L-serine concentration is decreased.

**Effect of Carbonyl Reagents—**These reagents produce inactivation of the enzyme by reacting with the formyl group of pyridoxal-P. Of such reagents tested, only hydroxylamine was highly potent (Table III). Semicarbazide caused inactivation only at a comparatively high concentration. Contrary to its marked effect on other pyridoxal-P-mediated enzymes, potassium cyanide produced little inhibition at $10^{-3}$ M. The strong inhibitory effect of hydroxylamine was used to advantage in resolving the pyridoxal-P from the apoenzyme.

**TABLE II**

*Effect of CMB, N-ethylmaleimide, and iodoacetate on threonine dehydrase activity*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>O.D. 515 μμ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMB</td>
<td>$10^{-3}$</td>
<td>0.26</td>
</tr>
<tr>
<td>CMB</td>
<td>$10^{-4}$</td>
<td>0.48</td>
</tr>
<tr>
<td>CMB</td>
<td>$10^{-5}$</td>
<td>0.64</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>$10^{-3}$</td>
<td>0.56</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>$10^{-4}$</td>
<td>0.68</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>$10^{-5}$</td>
<td>0.68</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>$10^{-3}$</td>
<td>0.60</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>$10^{-4}$</td>
<td>0.70</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>$10^{-5}$</td>
<td>0.66</td>
</tr>
<tr>
<td>No additions</td>
<td></td>
<td>0.67</td>
</tr>
</tbody>
</table>

*Effect of Carbonyl Reagents—* These reagents produce inactivation of the enzyme by reacting with the formyl group of pyridoxal-P. Of such reagents tested, only hydroxylamine was highly potent (Table III). Semicarbazide caused inactivation only at a comparatively high concentration. Contrary to its marked effect on other pyridoxal-P-mediated enzymes, potassium cyanide produced little inhibition at $10^{-3}$ M. The strong inhibitory effect of hydroxylamine was used to advantage in resolving the pyridoxal-P from the apoenzyme.

**TABLE III**

*Semicarbazide and hydroxylamine inhibition of threonine dehydrase*

The inhibitor was incubated in a volume of 3.0 ml for 15 minutes at 37° without substrate and, after addition of substrate, incubated in a volume of 3.0 ml under standard assay conditions. In the semicarbazide experiment, the amount of threonine deaminated was measured by the ammonia produced.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylamine</td>
<td>$5 \times 10^{-4}$</td>
<td>100</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>$10^{-4}$</td>
<td>64</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>$5 \times 10^{-4}$</td>
<td>8</td>
</tr>
</tbody>
</table>

**Effect of concentration on the rate of deamination of L-serine.** Incubation contents: 1.5 mg of enzyme protein (L-allothreonine test preparation) 0.1 M potassium phosphate buffer, pH 7.2, various concentrations of L-serine, in a final volume of 1.5 ml. The enzyme and the remainder of the incubation mixture were separately raised to 37° by heating for 3 minutes. Then the enzyme was then added, and the incubation was continued at 37°. At the times indicated, 0.25 ml of 25% trichloroacetic acid was added. After removal of coagulated protein by centrifugation, 0.35 ml of each supernatant fluid was assayed for keto acid.

**FIG. 3.** pH-activity curve of threonine dehydrase. Incubation contents: 5.3 μg of protein, $3.3 \times 10^{-3}$ M KCl, 0.1 M phosphate, borate, Tris, carbonate-bicarbonate, or glycine buffers, and 0.0167 M L-threonine in a total volume of 3 ml. Incubation was for 30 minutes at 37°. Reaction stopped and protein precipitated with 0.5 ml of 25% trichloroacetic acid; 1.0 ml aliquots used for α-keto-butyrate determination. The pH was assumed equal to the pH of the particular buffer used. The pH values of the buffers were verified by measurement with a Beckman model G pH meter.

**FIG. 4.** Effect of concentration on the rate of deamination of L-serine. Incubation contents: 1.5 mg of enzyme protein (L-allothreonine test preparation) 0.1 M potassium phosphate buffer, pH 7.2, various concentrations of L-serine, in a final volume of 1.5 ml. The enzyme and the remainder of the incubation mixture were separately raised to 37° by heating for 3 minutes. Then the enzyme was then added, and the incubation was continued at 37°. At the times indicated, 0.25 ml of 25% trichloroacetic acid was added. After removal of coagulated protein by centrifugation, 0.35 ml of each supernatant fluid was assayed for keto acid. Curve 1, 0.0167 M L-serine; Curve 2, 0.0067 M L-serine; Curve 3, 0.0017 M L-serine; Curve 4, 0.0008 M L-serine.
The results of certain experiments have indicated that L-threonine and L-serine are deaminated by the same enzyme. Thus, the ratio of enzyme activity on threonine and serine remains constant during purification (Table I). The data of Fig. 2 shows that this comparison is valid even though the reaction with serine ceases in 6 to 10 minutes. When L-serine and L-threonine were incubated together with the enzyme, results very similar to those obtained with serine alone were observed (Fig. 3). Although it was not possible to distinguish between the keto acids formed, the reaction was virtually complete at 10 minutes. Even with excess pyridoxal-P, there was little additional formation of keto acid. This result indicates that L-serine must inactivate the enzyme by reacting irreversibly with the coenzyme. Added pyridoxal-P does not reactivate the enzyme, probably because the coenzyme bonding sites remain occupied. D-Serine produced virtually no inhibition. In another experiment, 2 μmoles of sodium pyruvate were incubated with threonine dehydrase before the addition of L-threonine. No inhibition occurred, ruling out inhibition by the reaction product. This property of serine was utilized to dissociate the coenzyme from the apoenzyme (see below).

Additional evidence that the same enzyme catalyzes the deamination of both L-threonine and L-serine has been obtained through two types of experiments. It will be noted in Table I that during the first and second heat denaturation steps the total units of threonine activity increased appreciably. When these enzyme fractions were assayed for serine activity, with a 3-minute incubation, the total serine activity also increased proportionally. Furthermore, when the enzyme was resolved by either L-serine or hydroxylamine treatment (see below) incubation with pyridoxal-P resulted in comparable restorations of both threonine and serine activities.

The effect of varying concentrations of D,L-homoserine on threonine deamination was determined (Fig. 6). It is apparent from these data that homoserine does not inhibit the enzyme in the same manner as does serine. The manner of inhibition by L-cysteine closely parallels that brought about by homoserine.

Surprisingly, L-allothreonine is also a substrate for this enzyme. The first experiments were performed with D,L-allothreonine, the L compound being then unavailable. On incubation of 50 μmoles of D,L-allothreonine at 37° with excess enzyme, at least 25 μmoles of keto acid were formed. This result, obtained with D,L-allothreonine samples from a number of sources, is beyond the possibility of contamination by D,L-threonine.

Subsequently, L-allothreonine was obtained through the kindness of Dr. A. Meister and a test was run with an enzyme preparation partially purified by precipitation between 40 to 50% saturation of ammonium sulfate, heat treatment at 75°C for 5 minutes, and dialysis of the supernatant fluid against 0.05 M phosphate buffer.

This material decomposed only half of a D,L-allothreonine preparation but completely decomposed the L-allothreonine. That threonine aldolase was not involved in this reaction was proved by showing the absence of glycine formation by paper chromatography. The measured rate of deamination of D,L-allothreonine was 23% of that of L-threonine.

Other amino acids that were tested and found to be unaffected by the enzyme were D-threonine, D-serine, D,L-homoserine, D,L-hydroxyaspartic acid, and D,L-β-threo-phenylserine.

In view of the dual enzymatic activities of homoserine dehydrase and serine dehydrase, the effect of threonine dehydrase was tested on a variety of sulfur amino acids. No activity was observed with any of them.

Because serine is also a substrate for the enzyme, an experiment was performed to determine if the enzyme could catalyze the condensation of homocysteine to form cystathionine. The result was negative. Both L-cysteine and L-threo-cystathionine caused inhibition of serine and threonine deamination, but no condensation of these amino acids occurred.
Resolution and Recombination of Enzyme—It is difficult to demonstrate a pyridoxal-P requirement for the sheep liver threonine dehydrase. Prolonged dialysis does not produce dissociation of the pyridoxal-P. This doubtless stems from the firm binding of the coenzyme by the apoenzyme.

Some increase in enzyme activity has been observed by the addition of pyridoxal-P and AMP after dialysis against Tris buffer (1). Dialysis against Tris buffer was used successfully in dissociating the coenzyme from serine dehydrase. This procedure with threonine dehydrase resulted in only a 40% decrease in activity after 5 days dialysis against 0.2 M Tris buffer at pH 7.8. Subsequent incubation with pyridoxal-P restored virtually all of the activity. AMP and GSH were without any activating effect.

Hydroxylamine was very effective in dissociating the coenzyme. Complete loss of activity occurred after overnight dialysis of the enzyme against 0.02 M hydroxylamine hydrochloride, neutralized and buffered to pH 7.2 with 0.1 M potassium phosphate, and subsequent dialysis against phosphate buffer to remove the hydroxylamine. Of the initial enzyme activity, 75% could be restored by incubating the inactivated enzyme with pyridoxal-P.

The great affinity of serine for the enzyme suggested that it might be utilized in dissociating the coenzyme. An experiment was performed in which 2 ml of enzyme solution were mixed with 0.5 ml of 0.25 M l-serine and allowed to stand in the refrigerator for 48 hours. The serine was then dialyzed away. This caused a loss of approximately 90% of the enzyme activity against both threonine and serine. On incubation of the inactivated enzyme with pyridoxal-P, enzyme activity was restored up to 95% of the original. Restoration of enzyme activity as a function of pyridoxal-P is shown in Fig. 7. Deoxypyridoxine-5'-P inhibited reactivation of the dissociated enzyme by pyridoxal-P (Table IV), but had no effect on the intact enzyme. In equimolar quantity with pyridoxal-P, restoration of enzyme activity was decreased 50 to 60% by deoxypyridoxine-5'-P.

An incubation time of about 1 hour at 37° was required to restore full activity with pyridoxal-P. Prior incubation of the resolved enzyme with pyruvate or 0.1 M l-threonine failed to reactivate the enzyme.

In later experiments, the enzyme was allowed to stand for 48 hours with l-threonine, d-serine, L-isoleucine, L-cysteine, and DL-homoserine. After dialysis, it was found that in no case was any appreciable activity lost. It was also observed that L-serine could effect resolution in 12 hours at 2°.

Effect of Metal Ions—A divalent metal ion requirement for the activity of threonine dehydrase seems extremely doubtful. Tests with a large series of such ions at a concentration of 10⁻⁴ M were completely negative. Only Hg⁺⁺ had an effect and, as already mentioned, this inhibited enzyme activity. Additional evidence against a divalent metal ion requirement was the negative results obtained with a variety of sequestering agents. Indeed, all of these proved to be slightly activating instead of inhibiting, probably because of the binding of inhibitory heavy metal ions. The chelating agents tested were α,α'-dipyridyl, EDTA, 2-hydroxyquinoline, 8-hydroxyquinoline, and o-phenanthroline.

Certain monovalent cations have been shown to enhance the activity of various enzymes, including some requiring pyridoxal-P (9-11). Observations in this laboratory suggested that potassium ion had a beneficial effect on the activities of serine dehydrase of rat liver and the threonine dehydrase of sheep liver. To study this more systematically, experiments were performed with the alkali elements and ammonia on the above enzyme and on crystalline homoserine dehydrase.

The experiments were carried out by dialyzing each enzyme preparation against 0.1 M sodium phosphate buffer, pH 7.5, and 0.017 M L-threonine, in a volume of 3.0 ml. Incubated 30 minutes at 37°; deproteinized with 0.5 ml of 0.25% trichloroacetic acid; 0.5 ml of supernatant assayed for keto acid. Prior incubations included 100 µg of resolved threonine dehydrase.

The incubation mixture included (besides the material added during prior incubations) 0.1 M potassium phosphate, pH 7.5, and 0.017 M L-threonine, in a volume of 3.0 ml. Incubated 30 minutes at 37°; deproteinized with 0.5 ml of 25% trichloroacetic acid; 0.5 ml of supernatant assayed for keto acid. Prior incubations included 100 µg of resolved threonine dehydrase.

![Figure 7. Effect of concentration of pyridoxal P on the reconstitution of resolved threonine dehydrase.](http://www.jbc.org/content/285/10/2089/F7.large.jpg)

**FIG. 7.** Effect of concentration of pyridoxal P on the reconstitution of resolved threonine dehydrase. Preincubation was for 1 hour at 37°, carried out with 130 µg of enzyme protein and the designated amounts of pyridoxal-P in a 0.2 ml volume buffered at pH 7.5 with 0.05 M phosphate buffer. Reconstitution determined by adjusting chilled in ice aliquots of incubation mixture to 0.1 M, pH 7.5, phosphate buffer, adding 0.017 M L-threonine and assaying for activity as described in "Experimental Procedure."

<table>
<thead>
<tr>
<th>Additions</th>
<th>First incubation</th>
<th>Second incubation</th>
<th>Enzyme activity 0.0 D. 515 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Pyridoxal-P</td>
<td>25</td>
<td>0.44</td>
</tr>
<tr>
<td>Deoxypyridoxine phosphates, 25 µg</td>
<td>Pyridoxal-P</td>
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<td>0.19</td>
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<tr>
<td></td>
<td>Pyridoxal-P</td>
<td>25</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>Deoxypyridoxine-P</td>
<td>25</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* First incubation: 0.2 ml volume, 30 minutes at 37°.  † Second incubation: 0.3 ml volume, 60 minutes at 37°.
The order of effect of the several cations was found to be \( K^+ > NH_4^+ > Rb^+ > Li^+ > Na^+ \). Serine dehydrase activity was more strongly influenced than was threonine dehydrase.

The effect of increasing concentration of monovalent cations on the reactivity of a number of enzymes has been shown to follow Michaelis kinetics (10, 11). Enzyme activity-concentration curves were run for \( K^+ \) and \( NH_4^+ \) on serine dehydrase, because of the greater monovalent cation effect on this enzyme.

From the double reciprocal plots, \( K_m \) values were estimated to be \( 4 \times 10^{-3} \) M and \( 10^{-2} \) M for \( K^+ \) and \( NH_4^+ \), respectively.

**DISCUSSION**

Threonine dehydrase was unequivocally shown to be pyridoxal-P-dependent by dissociating the coenzyme by taking advantage of the combining characteristics of hydroxylamine and of serine with the coenzyme and removing this by dialysis against the above compounds. The active enzyme can then be reconstituted by reincubating the apoenzyme with pyridoxal-P. This reactivation is a time-dependent reaction, requiring 1 hour at \( 37^\circ \) to go to completion.

Threonine dehydrase, unlike the serine and homoserine enzyme, was restricted in its catalytic activity to hydroxyamino acids, namely, threonine, serine, and allothreonine. The ability to decompose L-allothreonine is rather surprising in view of the otherwise strict stereospecificity of this enzyme. Thus, D-threonine and D-serine are inert as substrates. No explanation can be given at this time for the ability of the enzyme to dehydrate L-allothreonine.

The reaction of the enzyme with serine has interesting aspects. The early decomposition of serine to pyruvic acid can be explained in terms of the mechanism of pyridoxal-P action reviewed by Snell (12). Dehydration leads to the formation of enzyme-bound aminoaacryl acid, which spontaneously hydrolyzes to pyruvic acid on dissociation. The cause of the later inactivation of the enzyme is unclear. A tempting speculation is that it might result from the formation of an oxazolidine ring conjugate by reaction of the formyl group of pyridoxal-P with the amino and the hydroxyl group of serine. The analogous thiazolidine ring, formed by reaction of cysteine with aldehydes is well known (13, 14). However, chemically no such conjugate was observed to form between pyridoxal and the potassium salt of DL-serine in methanol (see Heyl et al. (15)).

Threonine dehydrase is an unusually stable enzyme. In the course of purification, it withstood the rigorous treatment with acetone and heating to \( 75^\circ \). The great variability observed in the enzyme content of the livers of individual animals is puzzling. Threonine dehydrase has been shown to be an inducible enzyme in the rat and the mouse (16), which may be a factor in the variability. Another possible factor that comes to mind is the inactivation of the enzyme by serine.

The progressive decrease in enzyme activity by the different alkali element cations from potassium to sodium is probably in some way related to the corresponding magnitudes of the hydrated ionic radii of these ions (9). No satisfying theory has yet been propounded in explanation. The list of enzymes exhibiting a similar monovalent cation relationship to activity is growing. Consequently, this is a general phenomenon and not restricted to the hydroxyamino acid dehydrases, or to pyridoxal-P-mediated enzymes alone.

**SUMMARY**

1. Threonine deaminase has been isolated in highly purified form from sheep liver by the use of controlled heat denaturation, ammonium sulfate fractionation, acetone precipitation, and diethylaminoethyl cellulose chromatography. A specific activity of up to 776 amoles of \( \alpha \)-keto butyric acid formed per mg of protein per hour has been attained.

2. The purified enzyme deaminates L-threonine, L-allothreonine, and L-serine. L-Serine greatly inhibits the deamination of L-threonine.

3. Pyridoxal phosphate has been shown to be the coenzyme of threonine deaminase. Both hydroxylamine and L-serine are capable of resolving the enzyme. Reactivation of the resolved enzyme can be achieved by incubation with pyridoxal phosphate. Reactivation is partially inhibited by 4-deoxy pyridoxine phosphate, an analogue of pyridoxal phosphate.
4. Both serine dehydrase of rat liver and threonine dehydrase are most active in the presence of potassium ion. The order of activation by monovalent cations seems to be: $K^+ > NH_4^+ \geq Rb^+ > Li^+ > Na^+$.

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