Purification and Properties of Inducible Hydroxyproline 2-Epimerase from Pseudomonas*

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In the pathway of hydroxyproline metabolism by an inducible strain of Pseudomonas (1), an initial reaction involves the reversible epimerization of hydroxy-L-proline to allohydroxy-d-proline by racemization at carbon 2. In subsequent steps of this sequence, allohydroxy-d-proline is oxidized to \( \Delta^1 \)-pyrroline-4-hydroxy-2-carboxylate (2) and the latter compound is oxidized to \( \alpha \)-ketoglutarate through an intermediate which has been identified as \( \alpha \)-ketoglutaric semialdehyde (9).

Preliminary studies (1) of the epimerase showed that the enzyme was inducible, was not sedimented from sonic extracts at 100,000 \( \times \) g, and appeared to catalyze rather specifically the two reactions,

\[
\begin{align*}
\text{Hydroxy-} & \text{L-proline} \rightleftharpoons \text{allohydroxy-} \text{d-proline} \quad (1) \\
\text{Hydroxy-} & \text{d-proline} \rightleftharpoons \text{allohydroxy-} \text{l-proline} \quad (2)
\end{align*}
\]

The present paper describes in detail the purification of the enzyme to a stage of homogeneity or near homogeneity, together with some properties of the enzyme and features of the reaction. Hydroxyproline 2-epimerase appears to be a small protein, and may represent the first amino acid-racemizing enzyme, of those known (10-19), to be obtained in a state of high purity. Evidence is reported here that tends to rule out pyridoxal phosphate and certain other plausible cofactors as components of the enzyme or the reaction.

**EXPERIMENTAL PROCEDURE**

**Materials**—The strain of Pseudomonas striata used had been described earlier (1). Growth on stock slants and in liquid media, as well as methods of harvesting and extracting bacteria, were as described by Yoneya and Adams (2), with the exceptions noted below under "Preparation of Enzyme.”

Hydroxy-L-proline and allohydroxy-d-proline were obtained from California Corporation for Biochemical Research or Sigma Chemical Company. The latter compound was freed of contaminating hydroxy-L-proline, if necessary, by repeated recrystallization from water-ethanol.

Freedom of hydroxy-L-proline from allohydroxyproline, or of allohydroxy-d-proline from hydroxyproline, was determined by separation of the diastereomers on a Dowex 50 column essentially as described by Piez, Irreverre, and Wolff (20). With a 150-cm column, 0.1 \( \mu \) mole of one diastereomer could be easily detected in the presence of 10 \( \mu \) moles of the other. Allohydroxy-L-proline was a sample synthesized earlier (21). Hydroxy-L-proline was a sample prepared in the laboratory of Dr. J. P. Greenstein (22) and obtained from Dr. M. Winitz. N-Acetylhydroxy-L-proline was prepared as described (23). \( \beta \)-Alanylhydroxy-L-proline, carboxybenzoylslydylhydroxy-L-proline, glycyldydroxy-L-proline, hydroxy-\( \epsilon \)-propylglycine, hydroxy-\( \beta \)-propionylglycine, methyl ester, and hydroxy-\( \beta \)-proline amide were obtained from Dr. E. I. Smith (24, 25). A sample of 5-hydroxy\( \-\)-pipecolic acid isolated from Acacia (26) was a gift from Dr. A. I. Virtanen. Samples of the two racemates of synthetic \( \Delta \)-hydroxyproline were obtained from Dr. M. A. Logan (27). Pyrrole-2-carboxylic acid was purchased from K and K Laboratories; pyridoxal phosphate from Sigma Chemical Company, polyethylene glycol (Carbowax 20,000), used for concentrating enzyme preparations, from Union Carbide Chemical Company; tritiated \( \text{H}_2\text{O} \) (specific activity, 100 \( \mu \)c per g) from Nuclear-Chicago Corporation; and \( ^3\text{P} \) as inorganic phosphate from the Oak Ridge National Laboratory.

**Methods**—Purification and assays of hydroxyproline 2-epimerase are described below. Hydroxyproline was measured by a modification of the method of Neuman and Logan (28) or by ninhydrin color (20). Pyrrole-2-carboxylate was measured by color produced directly with \( p \)-dimethylaminobenzaldehyde in acid (2). Phosphorus was measured by an ascorbic-molybdate method (29); total phosphorus was measured after digestion of protein in refluxing perchloric acid. Protein was measured either turbidimetrically (30) or colorimetrically (31). Ascending paper chromatography was performed with Whatman No. 1 paper. Hydroxyproline diastereomers were distinguished by paper chromatography as the copper salts (32) or by high voltage paper electrophoresis (33). Vertical starch gel electrophoresis followed the method of Smithies (34) with equipment obtained from Otto Miller, Madison, Wisconsin. Sucrose gradient centrifugation of enzyme was done as described by Martin and Ames (35) in linear gradients from 5 to 20% in sucrose and containing 0.005 m potassium phosphate, pH 7.5, and 0.001 m EDTA. Tubes were layered with 0.2 ml of enzyme and centrifuged at approximately 10° and 30,000 r.p.m. for periods of 18 to 24 hours. After centrifugation, the tube was punctured at the bottom and
Serial samples of approximately 0.2 ml were collected. Tritium was measured in a windowless gas flow counter with thin samples and with internal standards. Counting efficiency was about 15%.

RESULTS
Preparation and Assay of Enzyme

In initial studies, the epimerization reaction was detected by paper chromatographic recognition of allohydroxy-D-proline formed in enzyme incubations with hydroxy-L-proline, or by attempting to measure the D isomer formed from hydroxy-L-proline with the use of kidney ω-amino acid oxidase to catalyze conversion of the former to pyrrole-2-carboxylate (30). As has already been noted (2), a serious limitation of the second method is the inhibitory action by pyrrole-2-carboxylate on mammalian ω-amino acid oxidase, so that even small aliquots of allohydroxy-D-proline could not be quantitatively assayed by this method.

The two enzyme assays adopted in the present work involved either direct measurement of epimerization by optical rotation or, alternatively, a more sensitive two-stage assay based on measuring allohydroxy-D-proline (formed from hydroxy-L-proline) with the aid of the specific allohydroxy-D-proline oxidase of Pseudomonas (2).

Assay by Optical Rotation (Assay I)—The Keston polarimetric attachment2 (37, 38) for the Beckman DU spectrophotometer was used with 0.5-dm polarimeter tubes containing 3 ml of reaction mixture. Individual polarimeter tubes were calibrated with sucrose; the linear plot of the absorbance at this wavelength had a slope, with sucrose; the linear plot of calculated angle of rotation at attachment? (37, 38) for the Beckman DU spectrophotometer already been noted (2), a serious limitation of the second method.

Polarimeter Company, Hackensack, New Jersey.

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0.5 dm light path, the conversion of 1 μmole (131 pg) per ml of one diastereomer to the other would therefore produce a change in rotation of 0.0088°. The calibration factor of 4.5 converts this to an absorbance change of 0.0019.

Fig. 1. Formation of hydroxy-L-proline from allohydroxy-D-proline as a function of time and enzyme concentration. Conditions were those for Assay I. The enzyme corresponded to Fraction IV, Table II. Numbers shown on the figure represent milligrams of protein added to the 3-ml reaction volume.

For routine assays, the 3-ml incubation mixture contained 0.05 M Tris buffer, pH 8.1, 0.1 M hydroxy-L-proline or allohydroxy-D-proline, and sufficient enzyme to produce an absorbance change of about 0.001 per minute. The incubation mixture was made up in small tubes with the substrate added last, and the mixed contents were transferred to a polarimeter tube. The absorbance at room temperature was recorded at 3 and 13 minutes after substrate addition. One unit of enzyme is defined as the amount catalyzing the conversion of 1 μmole of allohydroxy-D-proline to hydroxy-L-proline per minute under the conditions stated. The reaction rate was linear with enzyme concentration and time over a useful range, although linearity was limited by the equilibrium nature of the reaction (Fig. 1).

Two-Stage Assay (Assay II)—A more sensitive assay method used to detect minute amounts of enzyme, as after elution from starch gels, was based on the enzymatic oxidation of allohydroxy-D-proline formed in the epimerizing reaction. Epimerase preparations were incubated for 20 minutes with 0.1 M hydroxy-L-proline in 0.05 M Tris, pH 8.1, in a final volume of 0.2 ml. The reaction was stopped by brief heating in a boiling water bath, and an aliquot was incubated with a partly purified fraction of bacterial allohydroxy-D-proline dehydrogenase (30) in 0.05 M glycine, pH 8.8. Phenazine methosulfate (0.004%) was present as an obligatory electron acceptor (39). After 10 minutes of incubation at room temperature, the reaction was stopped by dilution with cold water and aliquots were assayed for pyrrole-2-carboxylate. At least two concentrations of epimerase of known activity were carried through the procedure to relate pyrrole-2-carboxylate formation to units as defined by the polarimetric assay. The two-stage assay could detect less than 0.2 unit of activity and was linear with enzyme up to about 1 unit.

Purification of Enzyme—Cultures were grown in 2-liter Erlenmeyer flasks, as described earlier (2), in media containing 0.2% hydroxy-L-proline and 0.05% yeast extract (Difco) as the only organic additions. On the basis of systematic observations (Table I), cells were harvested at about 18 hours to yield enzyme at maximum yield and purity. All following procedures were carried out at 0–5°. Packed cells were washed twice in 0.25% triton-X 100.

TABLE I

<table>
<thead>
<tr>
<th>Growth time</th>
<th>Absorbance at 650 mp</th>
<th>Wet weight of cells</th>
<th>Hydroxyproline in medium</th>
<th>Enzyme</th>
<th>Protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs</td>
<td>µg/liter</td>
<td>µmole/ml</td>
<td>units/ml</td>
<td>mg/ml</td>
<td>units/mg</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.42</td>
<td>1.0</td>
<td>15.8</td>
<td>0</td>
<td>20</td>
<td>0.3</td>
</tr>
<tr>
<td>13</td>
<td>1.12</td>
<td>1.9</td>
<td>9.7</td>
<td>11</td>
<td>27</td>
<td>0.4</td>
</tr>
<tr>
<td>18</td>
<td>1.85</td>
<td>3.7</td>
<td>0.3</td>
<td>24</td>
<td>27</td>
<td>0.9</td>
</tr>
<tr>
<td>24</td>
<td>1.58</td>
<td>3.7</td>
<td>0.3</td>
<td>12</td>
<td>20</td>
<td>0.6</td>
</tr>
<tr>
<td>34</td>
<td>1.25</td>
<td>3.6</td>
<td>0.3</td>
<td>11</td>
<td>18</td>
<td>0.6</td>
</tr>
</tbody>
</table>
number of induced or derepressed enzymes have been shown to  
diameter, allowed to settle by gravity, and washed with 0.005  
Most of the enzyme units were eluted at 0.3  
and 0.001  
section and the supernatant solution was further fractionated with  
Mixture containing successively 0.1, 0.15, 0.3, and 0.4  
was sometimes  
diluted to 20 mg per ml. The suspension was centrifuged and  
the precipitate was removed by centrifugation and discarded. The supernatant solution was  
held for 10 minutes in an ice bath and the precipitate was removed by centrifugation and discarded. The supernatant solution was  
with several column volumes of the buffer mixture used for  
stirred for 30 minutes in 0.005 m potassium phosphate,  
and 0.001 m EDTA; a volume of fluid equal to the original volume of the protamine sulfate supernatant solution was used. The gel was removed by centrifugation and the supernatant solution was further fractionated with  
Starch Electrophoresis—Over a pH range from about 5 to 7.5,  
vertical starch gel electrophoresis of enzyme samples purified to  
Fraction VIIb (Table II) indicated a single prominent band. On occasion, a faint band appeared behind the main band, but it was estimated from staining intensity to represent not more than 10% of the protein present.

Attempts were unsuccessful to visualize enzyme activity directly on the starch surface; however, enzyme could be eluted from an unstained, horizontally cut half of the starch block by the technique of squeezing small blocks of starch in a syringe (after a single cycle of freezing and thawing) and assaying samples of the eluted liquid by Assay II.

Such observations indicated repeatedly that all the enzyme activity recoverable from starch (5 to 20% of that placed on the starch) was eluted from a small block that coincided closely or exactly with the major protein band.

In crude enzyme fractions (Fraction III or IV, Table II) a faint band could be detected whose position matched that of the purified enzyme (Fig. 2). When sufficiently concentrated crude samples were subjected to starch gel electrophoresis, enzyme activity was also recoverable from the region of this band and from nowhere else in the starch channel. In all cases, to elute active enzyme from starch, it was important to include dilute EDTA (0.001 M) in the starch block during electrophoresis. Enzyme activity was stable for several days at -15°C when left adsorbed to starch. After elution from starch, however, enzyme activity was lost within 24 hours on storage at -15°C. Once lost on storage, eluted enzyme activity could not be restored by incubation with DPN, TPN, FAD, heated crude enzyme, pyridoxal phosphate, or GSH.

A single major protein-staining band was also seen when purified enzyme was subjected to starch gel electrophoresis at five other pH values (5.05, 5.4, 5.9, 6.4, and 6.9). Under these conditions enzyme activity could be eluted from the prominent band at all pH values used. At pH 7.9 or 8.6, the protein-

To review the text for details of procedure. Units are based on the polarimetric assay.

### Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Total units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>units/ml</td>
<td>mg/ml</td>
<td>units/mg</td>
<td>%</td>
</tr>
<tr>
<td>I. Sonic extract</td>
<td>205</td>
<td>28</td>
<td>37</td>
<td>0.8</td>
<td>100</td>
</tr>
<tr>
<td>II. Supernatant (25,000 X g)</td>
<td>250</td>
<td>28</td>
<td>32</td>
<td>0.9</td>
<td>94</td>
</tr>
<tr>
<td>III. Supernatant (100,000 X g)</td>
<td>242</td>
<td>28</td>
<td>21</td>
<td>1.3</td>
<td>91</td>
</tr>
<tr>
<td>IV. Protamine supernatant</td>
<td>206</td>
<td>24</td>
<td>8.6</td>
<td>2.8</td>
<td>95</td>
</tr>
<tr>
<td>V. Calcium phosphate supernatant</td>
<td>327</td>
<td>18</td>
<td>4.5</td>
<td>4.0</td>
<td>79</td>
</tr>
<tr>
<td>VI. Calcium phosphate eluate</td>
<td>290</td>
<td>7</td>
<td>0.11</td>
<td>0.64</td>
<td>26</td>
</tr>
<tr>
<td>VII. DEAE-cellulose eluate</td>
<td>a. 0.15 M KCl</td>
<td>30</td>
<td>6</td>
<td>0.16</td>
<td>37</td>
</tr>
<tr>
<td>b. 0.3 M KCl</td>
<td>32</td>
<td>39</td>
<td>0.23</td>
<td>132</td>
<td>10</td>
</tr>
<tr>
<td>c. 0.4 M KCl</td>
<td>31</td>
<td>6</td>
<td>0.06</td>
<td>100</td>
<td>3</td>
</tr>
</tbody>
</table>

Purity of Enzyme

Several considerations suggested that the epimerase after the DEAE-cellulose step is homogeneous or nearly so. Since a number of induced or derepressed enzymes have been shown to represent appreciable fractions of total cell protein (41, 42), it is not surprising that 200-fold purification should yield a homogeneous protein in the present case.
FIG. 2. Starch gel electrophoresis of crude and purified enzyme fractions. The central channel represents purified enzyme (Fraction VIIb, Table II); the two outer channels represent the crude high speed supernatant solution (Fraction III, Table II), concentrated 4-fold. Approximately 4 mg of protein were applied to each slot for the crude enzyme, and about 2 mg for the purified enzyme. The starch was prepared in 0.025 M Tris, pH 7.5, containing 0.001 M EDTA; electrophoresis was performed at 10 volts per cm for 43 hours at 4°C. Enzyme activity was eluted only from the single major band of purified enzyme and only from the corresponding faint band in the crude enzyme pattern.

Sedimentation and Molecular Weight—On ultracentrifugation the purified enzyme showed a single symmetrical peak (Fig. 3); an $\xi_{20, w}$ value of 1.65 was calculated for the sedimentation constant, corresponding to a minimum molecular weight of 10,500, with an assumed partial specific volume of 0.74.

A later determination of the molecular weight was made by the method of Archibald (43). For this purpose enzyme was purified through Step VIIb (Table II), concentrated, then treated with Dowex 1-acetate (see below, under “Phosphorus Content of Enzyme”) and dialyzed for 18 hours against 1,000 volumes of 0.005 M potassium phosphate, pH 7.5, containing 0.001 M EDTA. Both the dialyzed enzyme and dialysis buffer were adjusted to 0.004 M NaCl and centrifuged at 12,590 r.p.m. at 20.1°C; no boundary correction was necessary for the buffer. From the boundary formed at the meniscus, with use of an assumed partial specific volume of 0.74, three separate frames yielded calculated values for the molecular weight of 16,500, 18,340, and 18,670, with an average of 17,930. Run in the same manner (after dialysis against 0.035 M K$_2$HPO$_4$, 0.004 M KH$_2$PO$_4$, and 0.1 M NaCl), a commercial sample (Sigma Chemical Company) of pancreatic ribonuclease gave values for the molecular weight of 15,110, 15,020, 18,500, and 17,110, with an average value of 14,450.

Sucrose Gradient Centrifugation—Samples of purified enzyme were subjected to centrifugation through a sucrose density gradient as noted in “Methods.” Assays for enzyme activity in gradient tubes regularly showed a single symmetrical peak (Figs. 4 and 8). Estimates of protein by the Lowry method (31) were surprising in that while the peak of protein always closely matched the peak of enzyme activity, there was regularly a plateau of absorbance extending beyond the enzyme peak up to the top of the sucrose gradient. The portion of this peak which did not superimpose with enzyme activity had unexpected properties; samples taken from this region showed a higher absorbance at 260 nm than at 280 nm, the $A_{260}:A_{280}$ ratio decreasing toward the top of the gradient. In contrast, tubes from the enzyme area had an $A_{280}:A_{260}$ ratio greater than 1. It was established that the plateau of material giving color in the Lowry test was not an artifact related to the addition of GSH or to the concentration of the enzyme by polyethylene glycol or with GSH, since it appeared also when samples were concentrated by lyophilisation or in the absence of GSH; further, it was not seen when buffer, against which enzyme samples had been dialyzed, was concentrated comparably and then centrifuged through sucrose gradients under similar conditions. Samples of the Lowry-positive material from the top of the gradient tube also produced turbidity on treatment with trichloroacetic acid. Unlike other protein samples, however, they failed to stain with nigrosin when dried on films of cellulose acetate.

Another observation showed that the material producing the “protein” plateau at the top of a sucrose gradient tube was derived from the enzyme during the prolonged course of a gradient centrifugation. Enzyme-containing samples from a gradient centrifugation (Fig. 4A) were pooled so as to exclude most of the stained band was smeared or fragmented and no enzyme activity was recovered.

FIG. 3. Sedimentation pattern of purified epimerase. Fraction VIIb (Table II), containing 360 units and 16.5 mg of protein per ml, was adjusted to 0.09 M NaCl and centrifuged at 59,780 r.p.m. at 3.8°C. The four frames shown were taken successively at 96, 160, 224, and 288 minutes after centrifugation was begun.
"protein" plateau. The pooled enzyme was dialyzed to remove sucrose, reconstituted by lyophilization, and recentrifuged through a second sucrose gradient. Again, a plateau of lowray positive absorbance could be measured in tubes from the top of the gradient (Fig. 4B), and represented in total an almost 2-fold increase over the units of absorbance layered on the gradient. This interpretation would account for failure to see a second, lighter boundary in the pattern obtained with the analytical ultracentrifuge.

No systematic efforts were made to define the nature of this material; a few attempts to identify possible nucleic acid components by paper chromatography after hydrolysis, and to investigate spectra before and after dialysis, did not yield interpretable results. As will be noted below (Fig. 8), lighter material separated from the enzyme peak by gradient centrifugation corresponds also with the position of phosphorus-containing material similarly separated from the enzyme peak. Treatment of purified enzyme with Dowex 1 reduced but did not entirely remove the unidentified Lowray-positive plateau at the top of sucrose gradients, while this procedure removed all but traces of phosphorus.

Properties of Enzyme

Inducibility—Table III presents data confirming the inducibility of hydroxyproline epimerase. Cells grown in media containing 0.001% hydroxy-L-proline (with glutamate as a growth substrate) yielded minimally detectable enzyme, about 3% of that found after growth on optimal concentrations of hydroxy-L-proline (Table III). Proline appeared to antagonize the inducing action of minimally effective concentrations of hydroxy-L-proline in the medium (Table III). Although growth was good on L-proline as a sole carbon and nitrogen source, no evidence could be found for the appearance of a proline racemase in conic extracts of cells grown on L-proline.

Substrate Specificity—Both hydroxy-L-proline and allohydroxy-d-proline are substrates for the enzyme. The identification of allohydroxy-d-proline as an enzymatic product of hydroxy-L-proline in crude extracts was originally made (1) with the isolated compound by optical rotation, by paper chromatographic identification (32) as an allohydroxyproline epimer, and by enzymatic identification as a d epimer (susceptibility to the action of kidney d-amino acid oxidase). These observations have been confirmed with the reaction product by the use of purified enzyme; in addition, the enzymatic product behaves as an allohydroxyproline on chromatography through Dowex 50 (39) and is a substrate for the specific allohydroxy-d-proline oxidase (2).

Allohydroxy-L-proline was similarly active as a substrate, both by direct polarimetric assay and by chromatographic demonstration (32) of its epimerization to the normal form. The limited availability of either allohydroxy-L-proline or hydroxy-d-proline prevented determination of the values of $K_m$ and $V_{max}$ for these substrates as was done for the more easily available epimers (see below). Qualitative comparison, however, indicated that allohydroxy-L-proline was an active substrate, giving an assay value at 0.1 m of about 15 units per ml of enzyme compared with 24 units per ml with 0.1 m allohydroxy-d-proline as substrate.

A number of hydroxyproline derivatives, tested at 0.1 m concentration, were inactive as substrates for the purified epimerase. Amino- or carboxyl-substituted derivatives of hydroxy-L-proline included N-acetylhydroxy-L-proline, $\beta$-alanlyhydroxy-L-proline, glycylyhydroxy-L-proline, carbobenzoxyglyclyhydroxy-L-proline, hydroxy-L-prolylglycine, and hydroxy-L-proline amide. These were tested as substrates by direct polarimetry. More critically, after incubation of each substrate at 0.1 m concentration for 1 hour with 5 units of enzyme in 1 ml, the incubation mixtures were hydrolyzed in 6 M HCl (sealed tubes, 120°, 18 hours) to release free hydroxyproline. Traces of allohydroxy-d-proline were

<table>
<thead>
<tr>
<th>Amino acid addition to growth medium</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxy-L-proline, 0.2%</td>
<td>24-33</td>
</tr>
<tr>
<td>L-Proline, 0.2%</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>L-Glutamate, 0.2%</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>L-Proline, 0.2%, + hydroxy-L-proline, 0.001%</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>L-Glutamate, 0.2%, + hydroxy-L-proline, 0.001%</td>
<td>1</td>
</tr>
</tbody>
</table>

* Growth on L-proline, 0.2%, + hydroxy-L-proline, 0.001%, also yielded extract without detectable epimerase activity.

Fig. 4. Enzyme activity and protein in samples from sucrose gradient centrifugation. A, 0.2 ml of purified enzyme which had been concentrated by lyophilization was centrifuged for 24 hours through a 5 to 20% sucrose gradient as described in the text. Enzyme activity (●) was determined by Assay Method I, and protein ($\square$, shaded area) by the method of Lowry et al. (31). Fraction 1 was the first sample from the bottom of the tube. B, recentrifugation through a sucrose gradient of enzyme-containing tubes shown in A. Contents of tubes 9 to 19 from the gradient centrifugation shown in A were pooled, dialyzed against 0.001 M potassium phosphate and 0.0005 M EDTA to remove sucrose, lyophilized, and taken up to 0.5 ml. Aliquots of 0.2 ml, containing about 15 units of enzyme and 340 mg of protein, were centrifuged through a sucrose gradient under the conditions noted for A. Other procedures were identical with those for A. Estimated recovery of enzyme units from those added (summation of assays for each sample) was about 90%; estimated similarly, recovery of "protein" was about 175%.
 searched for by chromatographic (20, 32) or enzymatic methods (2).
No epimerization of the hydroxy-\(L\)-proline residue in the substrates listed could be detected.

Free 3-hydroxyproline proved to be a substrate for the purified enzyme, although a quantitative comparison was not convenient in the absence of information concerning the specific rotation and assignment of configuration for this recently discovered compound (27, 44). Epimerization was demonstrated qualitatively by incubating with enzyme each of the two synthetic racemates (27) corresponding to the \textit{threo} and \textit{erythro} forms, and then examining incubates for the formation of the other epimers. After a 1-hour incubation of 100 \(\mu\)moles of each of the two diastereomeric racemates of 3-hydroxyproline with 15 units of enzyme, aliquots of the incubation mixture were subjected to high voltage paper electrophoresis at \(pH\) 1.9 (33). Incubation mixtures yielded two equally intense ninhydrin-stained spots corresponding to both racemates, whichever racemate had been added as the substrate. The simplest interpretation was that, as is the case with 4-hydroxyproline, each enantiomer of the two 3-hydroxyproline racemates is enzymatically converted in approximately 50\% yield to its diastereomer.

Several other related compounds were tested as possible substrates. Early experiments suggested that proline was not active as a substrate, and it has already noted that growth of cells on \(L\)-proline did not produce detectable levels of proline racemase. With large amounts of purified hydroxyproline epimerase, however, it was possible to measure low levels of proline-racemizing activity, estimated at 1 to 2\% of the activity for allohydroxy-\(L\)-proline. This low level was estimated both polarimetrically and in a two-stage incubation like that described for Assay II, in which the pyrrolone compound (\(\Delta^1\)-pyrrolone-2-carboxylate) formed from \(L\)-proline by \(\alpha\)-amino acid oxidase was detected by its reaction with \(\epsilon\)-aminobenzenesulphonyl chloride. In these experiments, \(L\)-proline chromatographically freed of hydroxyproline was used to avoid a reaction due to contaminating hydroxyproline. That the latter was not the source of either the optical rotatory change or the formation of a \(\Delta^1\)-pyrrolone with \(\alpha\)-amino acid oxidase was established by failure to obtain the sensitive test for pyrrole-2-carboxylate (36, 2) in incubation mixtures which yielded a positive color test with \(\epsilon\)-aminobenzenesulphonyl chloride. It was therefore concluded that the purified enzyme contained small quantities of a contaminating proline racemase, or that weak activity for proline racemization is a property of hydroxyproline epimerase. The latter alternative seems preferable on the basis of failure to demonstrate proline racemization in crude bacterial extracts.

5-Hydroxy-\(L\)-pipocoleic acid and \(\Delta^1\)-pyrrolone-3-hydroxy-5-carboxylate (3) were also found to be inactive as substrates for the purified epimerase. With the former compound, traces of an enzymatically formed epimer were searched for by chromatographic (20, 32) or enzymatic methods (2). No epimerization of the hydroxy-\(L\)-proline residue in the substrates listed could be detected.

When alanine racemase activity was initially recognized in crude extracts of hydroxyproline-grown \textit{Pseudomonas} (7), distinctness from hydroxyproline epimerase was based on the nondependence of the former enzyme on the growth substrate. Further observations confirmed the expected separability of alanine racemase from hydroxyproline epimerase.

The ratio of hydroxyproline epimerase to alanine racemase varied markedly at the successive purification steps shown in Table II. In one series, Fraction III gave a ratio of 15, Fraction IV, 22; Fraction VI, 47; and Fraction VIIIb, 93. Alanine racemase migrated electrophoretically on starch differently from hydroxyproline epimerase: under electrophoretic conditions described above, the former activity was eluted at 1.5 to 2 cm from the origin from an area showing no stainable protein, while hydroxyproline epimerase appeared as a deeply stained band at 3 to 4 cm. However, alanine racemase could not be distinctly separated from hydroxyproline epimerase by sucrose gradient centrifugation. Peaks of the two enzyme activities were close or coincident both in the dilute buffer described above and in gradients containing 0.1 M NaCl to reduce possible protein-protein association. This finding would suggest that alanine racemase of \textit{Pseudomonas} may also be a small protein.

A number of observations support the conclusion that alanine racemase of \textit{Pseudomonas} is a pyridoxal phosphate enzyme. In the most purified fractions of hydroxyproline epimerase, alanine racemase activity was stimulated up to 2-fold by 0.001 M pyridoxal phosphate. In trial experiments before electrophoresis, alanine racemase eluted from starch blocks was markedly stimulated by pyridoxal phosphate added to enzyme assay tubes. Hydroxylamine at \(2 \times 10^{-4}\) M inhibited alanine racemase about...
50% and inhibited completely at $10^{-3}$ M. Exposure to sodium borohydride ($2 \times 10^{-3}$ M) abolished enzyme activity. In contrast, as discussed below, no evidence could be found for the participation of pyridoxal phosphate in the action of hydroxyproline epimerase.

**Inhibitors**—The earlier observed sensitivity of the epimerase to sulfhydryl reagents (1) is shown in Table IV, together with the action of other inhibitors tested. Consistent with this type of participation of pyridoxal phosphate in the action of hydroxyproline epimerase.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Inhibition</th>
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</thead>
<tbody>
<tr>
<td>AgNO₃</td>
<td>$10^{-4}$</td>
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</tr>
<tr>
<td></td>
<td>$5 \times 10^{-6}$</td>
<td>62</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>$10^{-5}$</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-5}$</td>
<td>8</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>$10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$</td>
<td>24</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>$10^{-3}$</td>
<td>100</td>
</tr>
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<td></td>
<td>$10^{-4}$</td>
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<td>CdCl₂</td>
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<td></td>
<td>$10^{-4}$</td>
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</tr>
<tr>
<td>NaAs₂O₅</td>
<td>$10^{-2}$</td>
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</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
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<td>100</td>
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<tr>
<td>N-Ethylmaleimide</td>
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<td>100</td>
</tr>
<tr>
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<td>100</td>
</tr>
<tr>
<td>Hydrazine</td>
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<td>Hydroxylamine</td>
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<td>71</td>
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<tr>
<td></td>
<td>$10^{-2}$</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
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<tr>
<td>Semicarbazide</td>
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<td>56</td>
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<td></td>
<td>$10^{-3}$</td>
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</tr>
<tr>
<td>Isonicotinylhydrazine</td>
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<td>0</td>
</tr>
<tr>
<td>KCN</td>
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<td>20</td>
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<tr>
<td>8-Hydroxyquinoline</td>
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<td>0</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
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<td>17</td>
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<tr>
<td>$o$-Phenanthroline</td>
<td>$3.2 \times 10^{-4}$</td>
<td>33</td>
</tr>
<tr>
<td>$o$, $o'$-Dipryridyl</td>
<td>$7 \times 10^{-4}$</td>
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<tr>
<td>Atebrine</td>
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<td></td>
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<tr>
<td>Acriflavin</td>
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<td>80</td>
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<tr>
<td></td>
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<td>0</td>
</tr>
<tr>
<td>Avidin</td>
<td>0.1 unit/ml</td>
<td>0</td>
</tr>
<tr>
<td>Ultraviolet light*</td>
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<td>0</td>
</tr>
</tbody>
</table>

* Enzyme (1 ml), rotated as a thin film in an ice-chilled, round bottomed flask, was exposed for 1 hour at a distance of 3 cm to an 8-watt Gates-Raymaster B lamp with maximum output at 360 mp. The authors are indebted to Dr. M. M. Weber, Department of Microbiology, St. Louis University, for the use of this equipment.

Fig. 5. Epimerase activity as a function of pH. Open symbols and line refer to allohydroxy-n-proline; closed symbols and line refer to hydroxy-L-proline. All buffers were present at 0.05 M; the enzyme preparation was Fraction VIIb.

captopropanol at 0.01 to 0.05 M were frequently able to restore partially or wholly the activity of aged enzyme preparations.

Several carbonyl reagents, known to inhibit a number of pyridoxal enzymes, were not clearly inhibitory except at high concentration.

Atebrine, and especially acriflavin, were moderately inhibitory. Attempts to prevent acriflavin inhibition by the addition of FMN⁴⁺ or FAD gave variable and inconsistent results. With certain enzyme preparations, however, FMN or FAD ($2 \times 10^{-3}$ M) produced approximately 50% restoration of activity inhibited by $6.6 \times 10^{-4}$ M acriflavin.

Inhibition was prominent with certain cations, especially Hg²⁺ and Ag⁺, while Cu²⁺ and Zn²⁺ inhibited completely at $10^{-3}$ M. Al³⁺ and Fe³⁺ were not inhibitory at concentrations of $10^{-4}$ M or less. Mn²⁺, Ca²⁺, Ba²⁺, Mg²⁺, and Co²⁺ were noninhibitory at $10^{-2}$ M.

A group of chelating agents shown in Table IV were not inhibitory at low concentrations and gave no support to the possibility of a metal ion requirement for enzyme action.

No inhibition was observed with certain structural analogues of hydroxyproline such as L-proline, D-proline, or imidazole, all tested at 0.01 M.

It is notable that concentrations of sodium borohydride up to 1000 times the molar concentrations of enzyme present had no effect on the activity of the enzyme, when present either during assay or a preincubation period as well.

**pH Optima**—Epimerizing activity as a function of pH is shown in Fig. 5 for the two substrates, hydroxy-L-proline and allohydroxy-n-proline.

Values of $K_m$ and $V_{max}$—Fig. 6 shows direct and reciprocal plots for the relation between reaction rate and substrate concentration for hydroxy-L-proline and allohydroxy-n-proline. From the reciprocal plots, the values of $K_m$ for hydroxy-L-proline and allohydroxy-n-proline were calculated as $2.2 \times 10^{-2}$ M and $5.3 \times 10^{-2}$ M, respectively. For the specific enzyme preparations used, the values of $V_{max}$ for hydroxy-L-proline and allohydroxy-n-proline were calculated as 15 and 38 units per ml. It is apparent

⁴ The abbreviation used is: FMN, riboflavin phosphate, "flavin mononucleotide."
HYDROXY-L-PROLINE
(mM)

FIG. 6. Reaction rate as a function of substrate concentration for hydroxy-L-proline (A) and allohydroxy-D-proline (B). Enzyme (Fraction VIIb, Table II) was assayed by Assay I. The insets show reciprocal plots whose ordinates are the reciprocals of units per ml (1/v) and of millimolar substrate concentration (1/S).

from the data plotted that high concentrations of either substrate are inhibitory.

Spectrum—Solutions of enzyme at highest purity at concentrations of 10 mg of protein per ml were colorless. The absorption spectrum of the enzyme at several concentrations and at several pH values from 3.3 to 11.2 was typical of a simple protein, with no indication of components such as flavin or pyridine nucleotides or pyridoxal (Fig. 7). At neutral pH, the ratio of absorbance at 278 μm (the absorbance peak) to that at 260 μm had the value 1.57.

Flavin and Pyridine Nucleotides—Purified enzyme was examined for the presence of nucleotides by fluorometric methods. A solution containing about 30 μg of protein per ml gave a negligible fluorescence increment after treatment at 60° with 6 N NaOH, with or without added hydrogen peroxide. From the sensitivity of the fluorometric method for pyridine nucleotides

FIG. 7. Absorption spectra of purified hydroxyproline epimerase at neutral and alkaline pH. Spectra shown are for enzyme at a concentration of about 0.8 mg per ml. ●, the enzyme (5 mg per ml) was dialyzed against 0.005 M potassium phosphate, pH 7.5, containing 0.001 M EDTA, and diluted 5-fold with 0.001 M phosphate, pH 7.5; the blank cuvette contained dialysis buffer similarly diluted. ○, the contents of both cuvettes described above were adjusted to pH 11.2 by addition of 0.015 ml of 2.5 N NaOH. The spectra of the enzyme at pH 3.3 (dilute acetate buffer) were identical with those at neutral pH. At pH 7.5, spectra were identical for the enzyme in the presence or absence of 0.05 M allohydroxy-D-proline. The most concentrated solutions examined (about 4 mg per ml) showed no absorption peaks at pH 7.5 or pH 3.3 in the region from 300 to 480 μm.

Flavin and Pyridine Nucleotides—Purified enzyme was examined for the presence of nucleotides by fluorometric methods. A solution containing about 30 μg of protein per ml gave a negligible fluorescence increment after treatment at 60° with 6 N NaOH, with or without added hydrogen peroxide. From the sensitivity of the fluorometric method for pyridine nucleotides

Phosphorus Content of Enzyme—Since chemical assays of purified enzyme indicated a variable phosphorus content, the enzyme was prepared from cells grown in 32P-containing media, to permit easily monitored attempts to remove phosphorus from the enzyme. In two such experiments, purified enzyme was obtained from 121-liter cultures grown in media containing 10 or 20 μc of inorganic 32P, yielding respective initial specific activities of 6.5 × 10⁴ and 11.2 × 10⁴ c.p.m. per μatom of phosphorus. From each culture, enzyme was purified to homogeneity as indicated by starch electrophoresis. In each instance, the purified enzyme contained phosphorus; in the first preparation, 0.56 atom per mole, and in the second, 0.12 atom per mole. A variety of procedures removed most of the phosphorus from the enzyme with little or no loss of enzymatic activity or of protein. These included prolonged dialysis, sucrose gradient centrifugation (Fig. 8), and treatment with Dowex 1. After starch electropho-
resin, only 50% of the counts added were recoverable by elution, but of these, 90% moved as a single band far ahead of the enzyme. Batch treatment with Dowex 1, results of which were previously presented (8), consistently reduced the phosphorus content of the enzyme to only 0.03 to 0.05 atom per mole of enzyme, a value believed to be well below the range of error in both the protein method and the molecular weight determination. This residual phosphorus may be associated with contaminating alanine racemase or traces of other phosphorus-containing proteins present.

**Characteristics and Mechanism of Epimerase Reaction**

**Equilibrium Constant**—The equilibrium concentrations of hydroxy-\(L\)-proline and allohydroxy-\(D\)-proline in incubation mixtures were determined by the method of Piez et al. (20). A series of incubations at 25°C and pH 8.1, with enzyme at purification stage VIIb (approximately 6 units in a total volume of 3 ml), began with varying initial ratios of the two hydroxyproline epimers, both together totaling 200 to 300 \(\mu\)moles. Under these conditions, unchanging values of optical rotation were reached within 80 minutes. Incubation was allowed to continue for an additional 70 minutes, at which time the reaction was stopped by heating for 10 minutes at 100°C. The quantity of each hydroxyproline epimer in the equilibrium mixture was measured after separation by ion exchange chromatography (20). Results are summarized in Table V, the average of four determinations for the equilibrium constant of Equation 1 being 0.99. The equilibrium constant can also be calculated from the Haldane equation (48) by using the above cited values of \(K_m\) and \(V_{max}\) for each substrate. Substituting these values in the Haldane expression,

\[
K = \frac{\frac{[L]}{K_m}}{\frac{[D]}{K_m}} = \frac{V_{max}(L)}{V_{max}(D)} \cdot \frac{K_m(D)}{K_m(L)} = 0.98
\]

where the subscripts (L) and (D) refer to hydroxy-\(L\)-proline and allohydroxy-\(D\)-proline, respectively.

The equilibrium mixture of hydroxy-\(L\)-proline and allohydroxy-\(D\)-proline achieved by racemization at carbon 2 in hot barium hydroxide has also been reported to be about 1:1 (49, 22).

**Labeling of a Hydrogen**—Because enzyme-catalyzed labeling of 1 or more hydrogen atoms of hydroxyproline is relevant to the mechanism of epimerization and to the participation of possible cofactors, incorporation of tritium into hydroxyproline epimers, both together totaling 200 to 300 \(\mu\)moles. Under these conditions, unchanging values of optical rotation were reached within 80 minutes. Incubation was allowed to continue for an additional 70 minutes, at which time the reaction was stopped by heating for 10 minutes at 100°C. The quantity of each hydroxyproline epimer in the equilibrium mixture was measured after separation by ion exchange chromatography (20). Results are summarized in Table V, the average of four determinations for the equilibrium constant of Equation 1 being 0.99. The equilibrium constant can also be calculated from the Haldane equation (48) by using the above cited values of \(K_m\) and \(V_{max}\) for each substrate. Substituting these values in the Haldane expression,

\[
K = \frac{\frac{[L]}{K_m}}{\frac{[D]}{K_m}} = \frac{V_{max}(L)}{V_{max}(D)} \cdot \frac{K_m(D)}{K_m(L)} = 0.98
\]

where the subscripts (L) and (D) refer to hydroxy-\(L\)-proline and allohydroxy-\(D\)-proline, respectively.

The equilibrium mixture of hydroxy-\(L\)-proline and allohydroxy-\(D\)-proline achieved by racemization at carbon 2 in hot barium hydroxide has also been reported to be about 1:1 (49, 22).

**Incubation of tritium from water into hydroxyproline during enzymatic epimerization**

To examine the mechanism of epimerization, incorporation of tritium from water into hydroxyproline was investigated. Purified enzyme (Fraction VIIb, 18 units) was incubated under the usual conditions with 20 \(\mu\)moles of hydroxy-\(L\)-proline in a final volume of 0.57 ml containing 0.5 ml of tritium-labeled water. A control incubation was performed. Aliquots of the incubation mixture were removed at 5, 10, 20, and 40 minutes and analyzed for tritium content. Details are described in the text. All incubations were carried out at 25°C and pH 8.1 (Tris, 0.05 M).

### Table V

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial concentration*</th>
<th>Equilibrium concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxy-(L)-proline</td>
<td>Allohydroxy-(D)-proline</td>
<td>Hydroxy-(L)-proline</td>
</tr>
<tr>
<td>1</td>
<td>100 0</td>
<td>64 57</td>
</tr>
<tr>
<td>2</td>
<td>0 70</td>
<td>35 32</td>
</tr>
<tr>
<td>3</td>
<td>55 20</td>
<td>40 45</td>
</tr>
<tr>
<td>4</td>
<td>15 50</td>
<td>28 29</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These are approximate concentrations.

### Table VI

<table>
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<tr>
<th>Experiment</th>
<th>Incubation time</th>
<th>Hydroxyproline specific activity</th>
<th>(\Delta)H content*</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0.92 (\times) (10^6)</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>5(\frac{1}{2})</td>
<td>1.10 (\times) (10^6)</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>5(\frac{1}{2})</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.5</td>
<td>1.02 (\times) (10^6)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>1.07 (\times) (10^6)</td>
<td>1.02</td>
</tr>
</tbody>
</table>

* Specific activity of water in the incubation mixtures was 2.1 \(\times\) \(10^6\) d.p.m./\(\mu\)mole or 1.05 \(\times\) \(10^5\) d.p.m./atom of hydrogen.

† Hydroxyproline recovered from paper chromatography after ion exchange separation.

‡ Hydroxyproline recovered from incubation with boiled enzyme.
The studies presented describe the purification to homogeneity of an inducible epimerase for hydroxyproline and appear to eliminate pyridoxal phosphate as a participant in the racemization reaction. Negative efforts to demonstrate pyridoxal phosphate as an enzyme component or reaction participant have included tests of a number of carbonyl inhibitors, exposure to ultraviolet light and to sodium borohydride, and spectral data at enzyme concentrations (up to about 0.2 mM) and over a pH range (pH 3 to 11) which should have disclosed a pyridoxal band for a single molar equivalent, on the basis of the spectral properties of several pure pyridoxal enzymes (51-55). Since pyridoxal as a coenzyme is known to occur only as the phosphate, the demonstration that enzyme activity can be retained with much less than 1 molar equivalent of phosphorus also speaks against a pyridoxal mechanism, as well as against the participation of other phosphorus-containing components such as flavin or pyridine nucleotides.

A number of amino acid racemizing enzymes have been reported since the first description of alanine racemase in 1951 (10). None has been obtained in a state of high purity, however, and there is no direct chemical evidence for the presence of specific coenzymes. Pyridoxal phosphate has been implicated as the coenzyme of amino acid racemization, from the model experiments of Olivard, Metzler, and Snell (50) demonstrating non-enzymatic racemization catalyzed by pyridoxal, and from experiments in which racemase activity was stimulated or restored by pyridoxal phosphate (10, 18), sometimes after only very modest purification (10). In other reports, no evidence for pyridoxal phosphate participation has been obtained by the use of carbonyl reagents as possible inhibitors (15, 17). Two recent notes have presented preliminary evidence for a role of flavins in the reactions of glutamic acid racemase (17) and alanine racemase (19). In particular, the proline racemase of Clostridium sticklandii studied by Stadtman and Elliott (15), although not highly purified on a relative basis, provided no kinetic evidence for the presence or participation of pyridoxal phosphate. It would therefore seem premature to accept the generalization of pyridoxal phosphate participation in the enzymatic racemization of amino acids; the present status of this question might be summarized by the statement that there is kinetic evidence (carbonyl reagent inhibition, resolution from and activation by pyridoxal phosphate) for a pyridoxal mechanism for some racemases, but not for others, and that flavin participation has been proposed in two instances, but on the basis of rather incomplete evidence.

The model reactions proposed for pyridoxal phosphate participation in enzymatic racemization (56) include a Schiff base intermediate formed between the amino group of the substrate and the carbonyl of pyridoxal phosphate. It should be noted that in the case of the secondary amino acids, proline and hydroxyproline, such an intermediate would necessarily be a quaternary Schiff base. Quaternary Schiff bases are known, and there seems no reason a priori to dismiss them as possible enzyme-substrate intermediates. However, the empirical generalization has been made (57) that pyridoxal phosphate is not known to catalyze its typical reactions with *substituted* amino groups, and the present paper provides a specific example of an enzymatic reaction type (racemization) that does not involve pyridoxal phosphate with hydroxyproline as substrate, but does appear to involve pyridoxal phosphate in the case of other enzymes catalyzing the racemization of certain primary amino acids. It is particularly notable that in the preparations of hydroxyproline 2-epimerase examined, an alanine racemase was present which did appear, on the basis of kinetic evidence, to require pyridoxal phosphate. The absence of pyridoxal phosphate from *Pseudomonas* hydroxyproline 2-epimerase would therefore appear to be related to the nature of the substrate rather than a peculiarity of *Pseudomonas* racemases.

It is relevant to note the paucity of information regarding other pyridoxal-type enzymatic reactions involving the imino acids: transamination with these substrates has not been documented, and although the decarboxylation of proline and hydroxyproline in extracts of a pseudomonad has been reported (58), no further information is now available as to the participation of pyridoxal phosphate in these reactions.

The elimination of pyridoxal phosphate opens a consideration of other possible cofactors. The evidence excluding pyridine and flavin nucleotides has been noted, and is based on negative fluorometric observations, on the spectrum of the enzyme, and on failure to demonstrate phosphorus in the enzyme. As with the exclusion of pyridoxal phosphate, such negative evidence requires confidence in the degree of purity of the enzyme preparations examined: although this cannot be asserted as an irrevocable claim, the evidence for electrophoretic and ultracentrifugal homogeneity presented supports the belief that the enzyme is at least 75% pure as a protein.

It is of interest that despite direct evidence against flavins as an enzyme component in the present studies, some degree of inhibition of enzyme activity by flavin analogues was observed, with occasional partial reactivation by FAD and FMN. These observations are noted here to indicate that caution should be exercised in inferring flavin involvement in a reaction largely or solely from such inhibition and reactivation data (17).

The failure to identify pyridoxal phosphate, oxidation-reduction cofactors, or metals in the enzyme makes it difficult to propose a racemization mechanism. While it is clear from the tritium data that a single atom of hydrogen in the substrate is equilibrated with water, the data do not necessarily indicate that the exchange is obligatory for the enzyme reaction or quantitatively parallel to it. It seems reasonable to assume, but it has not been shown by degradation, that the atom of hydrogen labeled is the α hydrogen at carbon 2. Accepting these postulations as fact, little restriction on possible mechanisms is provided. The same observation would be anticipated if racemization proceeded via a dehydrogenated intermediate involving the carbon-nitrogen bond (viz. enzyme-bound Δ1-pyrroline-4-hydroxy-2-carboxylate) or by direct displacement of the α hydrogen.

Another hypothetical mechanism for racemization is through a decarboxylation-reacarboxylation sequence. Since a possible cofactor for such a transcarboxylation reaction with the enzyme is biotin, avidin was tested as an inhibitor but with negative results.

Finally, the evidence for a sulphydryl requirement, together with the labilization of 1 hydrogen atom, might suggest a mechan...
nism in which a monothiol or dithiol represented the source of a hydrogen involved in a direct displacement reaction, or acted as a reversible acceptor in a dehydrogenation reaction.

SUMMARY

Hydroxyproline 2-epimerase, an inducible enzyme obtained from *Pseudomonas striata*, has been purified to homogeneity or near homogeneity. The molecular weight is approximately 18,000, and no cofactors have been detected on the basis of enzyme spectrum, phosphorus content, fluorescence assays for pyridine or flavin nucleotides, and inhibitor action. One atom of hydrogen in the substrate is labilized during the enzyme reaction. Other features of the enzyme and the reaction, such as substrate specificity, equilibrium constant, and Michaelis constants, have been presented.

Acknowledgments—We wish to acknowledge the kindness of Dr. A. Holtzer and Mr. M. Yamada of Washington University in helping to obtain and interpret ultracentrifugal measurements of molecular weight.

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Purification and Properties of Inducible Hydroxyproline 2-Epimerase from *Pseudomonas*
Elijah Adams and Isabel L. Norton


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