ENZYMATIC SYNTHESIS OF L-PIPECOLIC ACID
AND L-PROLINE*

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A previous communication from this laboratory described the pyridine
nucleotide-dependent reduction of the α-keto acid analogue of lysine to
L-pipecolic acid catalyzed by preparations of mammalian liver (1). The
reaction proceeded with either DPNH or TPNH and the enzyme prep-
paration obtained from rat liver also catalyzed reduction of the α-keto acid
 analogue of ornithine and of glutamic acid-γ-semialdehyde to proline.
α-Keto-ε-aminocaproic acid, the α-keto acid analogue of lysine, exists in
solution in equilibrium with the cyclic form Δ¹-piperidine-2-carboxylic
acid (2); previous findings also indicate that α-keto-δ-aminovaleric acid
(2) and glutamic acid-γ-semialdehyde (3) exist in equilibrium with Δ¹-
pyrroline-2-carboxylic acid and Δ¹-pyrroline-5-carboxylic acid, respectively.
The present report describes further experimental work on the enzyme
system that catalyzes the formation of L-pipecolic acid (from Δ¹-piperidine-
2-carboxylic acid) and L-proline (from Δ¹-pyrroline-2-carboxylic acid).
This activity has been found in several animal and plant tissues, and a
purified preparation of the enzyme has been obtained from rat kidney;
studies with this preparation and other data indicate that Δ¹-pyrroline-5-
carboxylic acid and Δ¹-pyrroline-2-carboxylic acid are reduced to L-proline
by different enzymes. On the other hand, both Δ¹-pyrroline-2-carboxylic
acid and Δ¹-piperidine-2-carboxylic acid appear to be reduced by the same
enzyme. The available evidence suggests that Δ¹-piperidine-2-carboxylic
acid is an intermediate in the conversion of lysine to pipecolic acid. The
role of Δ¹-pyrroline-2-carboxylic acid in proline metabolism will be dis-
cussed.

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Foundation, the National Heart Institute, National Institutes of Health, United
† Traveling scholar of the J. N. Tata Endowment, India.

1 Abbreviations are as follows: Δ¹-piperidine-2-carboxylic acid, PIP-2-CA; Δ¹-pyr-
rroline-2-carboxylic acid, PYR-2-CA; Δ¹-pyrroline-5-carboxylic acid, PYR-5-CA; re-
duced diphosphopyridine nucleotide, DPNH; reduced triphosphopyridine nucleotide,
TPNH; flavin adenine dinucleotide, FAD.
EXPERIMENTAL

Materials—PIP-2-CA and PYR-2-CA were prepared as the hydrobromides from the corresponding N-carbobenzyloxy derivatives as previously described (2). The α-keto acids were dissolved in potassium bicarbonate solution immediately before being used. PYR-5-CA was prepared by boiling γ,γ-dicarbethoxy-γ-acetimidobutryaldehyde (4) under a reflux with 6 N hydrochloric acid (3).

DPNH was prepared from purified DPN as previously described (5), and enzymatically prepared DPNH was obtained from the Sigma Chemical Company; no significant differences were observed in our studies between the two DPNH preparations. TPNH was prepared from TPN (Sigma) by reduction with d-isocitrate and isocitric dehydrogenase (5). d-Isocitrate was obtained by alkaline hydrolysis of l-dimethylisocitrate lactone isolated from Bryophyllum calycinum leaves (6). When TPNH prepared by reduction with hydrosulfite (obtained from Sigma) was used, enzymatic reduction of PIP-2-CA and PYR-2-CA took place at about 20 per cent of the corresponding rates with TPNH prepared from Sigma TPN enzymatically. Studies in which Sigma TPNH was added to DPNH suggested that the TPNH prepared with hydrosulfite inhibited the reaction. Enzymatically prepared TPNH was therefore employed in the present studies. Pyrrole-2-carboxylic acid and Δ1-pyrroline-4-hydroxy-2-carboxylic acid were obtained as described (7); picolinic acid was a product of the Eastman Kodak Company.

Enzyme Preparations

Rat Kidney—10 gm. of fresh kidney were homogenized in a Potter-Elvehjem homogenizer with 30 ml. of ice-cold 0.1 M potassium phosphate buffer, pH 7.0, and the homogenate was centrifuged in a Spinco model L centrifuge at 144,000 × g at 2° for 2 hours. The clear supernatant solution was decanted and brought successively (at 5°) to 30, 40, 50, and 60 per cent of saturation with respect to ammonium sulfate by the addition of a saturated solution of ammonium sulfate adjusted to pH 7.0 with ammonium hydroxide. The precipitates obtained at 40 and 50 per cent of saturation were combined and dissolved in 10 ml. of cold 0.05 M potassium phosphate buffer, pH 7.0, and dialyzed for 12 hours at 5° against 20 volumes of the same buffer. A small inactive precipitate was removed by centrifugation and 0.45 ml. of calcium phosphate gel (100 mg. of dry weight per ml.) was added per ml. of dialyzed solution. The mixture was shaken gently at 26° for 30 minutes. After removal of the gel by centrifugation, the supernatant solution was lyophilized.

Rat Liver—The rat liver preparation was obtained by a procedure similar to that employed for rat kidney. The supernatant solution obtained by
centrifugation at 144,000 X g was fractionated with ammonium sulfate and the precipitate obtained between 43 and 55 per cent of saturation was dissolved in buffer, dialyzed, and heated at 50° for 20 minutes. The solution was cooled rapidly in ice and the precipitated protein was removed by centrifugation. The clear supernatant solution was lyophilized.

*Neurospora*—A mutant strain of *Neurospora crassa* known to be blocked in the biosynthesis of proline between PYR-5-CA and proline (strain 21863a) and the corresponding parent wild strain (strain 74a (8)) were grown on the Fries medium (9) containing 0.02 per cent L-proline. After incubation at 30° for 3 days, the mycelium was harvested, washed with distilled water, and frozen with dry ice. The frozen mycelium was treated with 5 volumes of ice-cold acetone in a Waring blender for 3 minutes and the resulting homogenate was filtered rapidly; the acetone-insoluble material was placed in a vacuum desiccator over phosphorus pentoxide at 5° for 18 hours. The acetone powders were ground in a mortar with 3 parts (by weight) of Alumina A-301 (Aluminum Company of America) and the mixture was then homogenized in a Potter-Elvehjem homogenizer with 10 volumes of 0.1 M potassium phosphate buffer, pH 7.0, at 5°. The homogenates were centrifuged in a Spinco model L ultracentrifuge at 144,000 X g for 45 minutes at 2° and the resulting clear supernatant solutions were fractionated with ammonium sulfate. Solid ammonium sulfate (17.5 gm. per 100 ml. of supernatant solution) was added rapidly at 5° and the solution was allowed to stand at this temperature for 30 minutes. The resulting precipitate was removed by centrifugation and dissolved in the minimal quantity of 0.1 M potassium phosphate buffer, pH 7.0. The preparation was dialyzed against the same buffer at 5° for 18 hours before use. The mutant and wild strain preparations were processed at the same time in identical fashion.

*Plants*—*Phaseolus radiatus* and *Pisum sativum* seeds were germinated in the dark for 48 hours. The germinated seeds were homogenized with 20 volumes of ice-cold acetone in a Waring blender and the mixture was filtered rapidly. The acetone-dried material was extracted with 5 volumes of cold 0.1 M potassium phosphate buffer, pH 7.0, in a Potter-Elvehjem homogenizer and the homogenates were centrifuged for 15 minutes at 300 X g.

*Methods*—Pipecolic acid was determined as described by Schweet (10). The determination of proline was carried out by this procedure (10) and by that of Troll and Lindsley (11). Because PYR-2-CA gives color under the conditions of these procedures, it was destroyed by treatment with hydrogen peroxide (2) as follows. Aliquots of 0.3 ml. containing 0.05 to 0.25 μmole of proline and 0.05 to 2.0 μmoles of PYR-2-CA were treated with

2 Cultures of the *Neurospora* were kindly donated by Dr. H. J. Vogel.
0.1 ml. of 0.2 M hydrogen peroxide. After 15 minutes, 0.1 ml. of a solution containing 50 units of crystalline catalase was added to destroy the peroxide. The destruction of peroxide was complete within 15 minutes and the samples were then analyzed for proline. The recovery of proline in the presence of PYR-2-CA was 98 to 100 per cent under the experimental conditions employed in these studies.

### Table I

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Homogenates*</th>
<th>Centrifuged homogenates†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PIP-2-CA</td>
<td>PVR-2-CA</td>
</tr>
<tr>
<td>Kidney</td>
<td>18.2</td>
<td>22.8</td>
</tr>
<tr>
<td>Brain</td>
<td>10.7</td>
<td>14.4</td>
</tr>
<tr>
<td>Liver</td>
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<td>6.71</td>
</tr>
<tr>
<td>Testis</td>
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<td>6.56</td>
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<tr>
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</tr>
<tr>
<td>Skeletal muscle</td>
<td>1.32</td>
<td>1.70</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.615</td>
<td>1.07</td>
</tr>
</tbody>
</table>

* The reaction mixtures consisted of 0.1 ml. of homogenate (1 gm. of fresh tissue plus 2 ml. of 0.1 m potassium phosphate buffer of pH 7.0), 0.1 ml. of DPNH (4 μmoles) in phosphate buffer, and 0.1 ml. of PIP-2-CA or PYR-2-CA (2 μmoles). The mixtures were maintained at 37° for 10 to 60 minutes and subsequently analyzed for piperolic acid or proline; the values are expressed as micromoles of imino acid formed enzymatically per hour per ml. of homogenate.

† The reaction mixtures consisted of 0.05 ml. or 0.1 ml. of centrifuged homogenate (i.e. supernatant solution after centrifugation at 144,000 X g for 2 hours in a Spinco model L centrifuge), DPNH (0.15 μmole), and PIP-2-CA (2 μmoles), PYR-2-CA (2 μmoles), or pyr-5-CA (4 μmoles) in a final volume of 1.0 ml. of 0.08 m potassium phosphate buffer of pH 6.1. The reaction was started by addition of substrate and the decrease in the absorption band at 340 μν of DPNH was followed in a Cary recording spectrophotometer at 26°; values were calculated from the ΔD for the first 60 to 180 seconds, and are expressed as micromoles of DPNH oxidized per hour per ml. of enzyme preparation.

### Results

**Formation of Proline and Piperolic Acid by Several Rat Tissues**—Homogenates of a number of rat tissues were incubated with PIP-2-CA, PYR-2-CA, or PYR-5-CA and DPNH. As indicated in Table I, all of the tissues examined were capable of reducing these substrates as determined by the appearance of proline or piperolic acid. Fractionations of liver and of kidney carried out by the procedure of Schneider and Hogeboom (12) revealed that most of the enzyme activity remained in the super-
natant fraction after high speed centrifugation. Studies with homogenates centrifuged at 144,000 \( \times g \) for 2 hours were performed by means of a spectrophotometric technique which was based on the disappearance of the band at 340 m\( \mu \) due to DPNH. In both sets of experiments the relative rates of reduction of PYR-2-CA and PIP-2-CA were approximately the same. The ratios of the values for PYR-2-CA to those for PIP-2-CA were between 1.2 and 1.7. The values for the reduction of PYR-5-CA were not according to the pattern observed with PIP-2-CA and PYR-2-CA. These studies indicate that the kidney is the most active tissue in catalyzing the reduction of PIP-2-CA and PYR-2-CA and suggest that the system responsible for the reduction of PYR-5-CA is different from that which catalyzes reduction of PIP-2-CA and PYR-2-CA.


table II

<table>
<thead>
<tr>
<th>Substrate Specificity of Liver and Kidney Preparations*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue preparation</td>
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<td>---------------------</td>
</tr>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Homogenate</td>
</tr>
<tr>
<td>Supernatant solution</td>
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<tr>
<td>Ammonium sulfate fraction</td>
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<tr>
<td>Final preparation</td>
</tr>
<tr>
<td>Kidney</td>
</tr>
<tr>
<td>Homogenate</td>
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<tr>
<td>Supernatant solution</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
</tr>
<tr>
<td>Final preparation</td>
</tr>
</tbody>
</table>

* See the text for the description of tissue preparations. The values are expressed as micromoles of DPNH oxidized in the presence of substrate per mg. of protein nitrogen per hour at 26° as described in Table I.

Studies with Purified Liver and Kidney Preparations—Table II summarizes the values obtained for the reduction of PYR-2-CA, PIP-2-CA, and PYR-5-CA by liver and kidney fractions, prepared as described above. The purification procedure brought about a 36-fold increase in the specific activities of the liver preparation toward PYR-2-CA and PIP-2-CA; the activity with respect to PYR-5-CA was increased 14-fold. A 60-fold purification was obtained with the kidney preparation for the activities responsible for the reduction of PYR-2-CA and PIP-2-CA; the activity of the kidney homogenate toward PYR-5-CA was very low, and this activity could not be detected in the final preparation.
Fig. 1. A, time-course of the reduction of PYR-2-CA and PIP-2-CA by the purified kidney enzyme preparation at 26°. The reaction mixtures consisted of 0.2 μmole of PYR-2-CA (Curve 1) or PIP-2-CA (Curve 2), 0.25 μmole of DPNH, and enzyme (26 γ of protein nitrogen) in 0.15 ml. of 0.05 M potassium phosphate buffer, pH 6.1. Samples were analyzed at intervals for proline (●) or pipecolic acid (○) and, after dilution to 1 ml., were read at 340 nm for determination of DPNH. B, pH-activity curves. The reaction mixtures consisted of reduced pyridine nucleotide (0.15 μmole), keto acid (2 μmoles) and enzyme in a final volume of 1 ml. of 0.08 M potassium phosphate buffer (●) or 0.08 M sodium acetate buffer (○); 26°. Ordinate, change in optical density at 340 nm per minute. Curve 1, DPNH, PYR-2-CA, purified liver enzyme; Curve 2, DPNH, PYR-2-CA, purified kidney enzyme; Curve 3, TPNH, PYR-2-CA, purified kidney enzyme; Curve 4, DPNH, PIP-2-CA, purified kidney enzyme. C, effect of keto acid concentration. The reaction mixtures consisted of DPNH or TPNH (0.15 μmole), keto acid as indicated, and purified kidney enzyme (13 γ of protein nitrogen) in 1 ml. of 0.08 M potassium phosphate buffer, pH 6.1; 26°. Curve 1, DPNH, PYR-2-CA; Curve 2, DPNH, PIP-2-CA; Curve 3, TPNH, PIP-2-CA. Ordinate as in B. D, effect of nucleotide concentration. The reaction mixtures consisted of keto acid (2 μmoles), nucleotide as indicated, and purified kidney enzyme (13 γ of protein nitrogen) in 1 ml. of 0.08 M potassium phosphate buffer, pH 6.1; 26°. Ordinate as in B. Curve 1, DPNH, PYR-2-CA; Curve 2, DPNH, PIP-2-CA; Curve 3, TPNH, PIP-2-CA. E, reduction of PIP-2-CA to pipecolic acid coupled with oxidation of d-isocitrate by liver preparation (ammonium sulfate fraction). The reaction mixtures consisted of TPN (0.142 μmole), potassium d-isocitrate
The time-course of the reduction of PYR-2-CA and PIP-2-CA by the purified kidney enzyme preparation is described in Fig. 1, A. Values for the disappearance of DPNH and the appearance of proline (or piperolic acid) were in close agreement, and the reaction went to completion. The reduction of PYR-2-CA and of PIP-2-CA with both liver and kidney preparations exhibited a pH optimum of approximately 6. In contrast, the pH optimum for the reduction of PYR-5-CA was about 6.8. The pH optima were the same with DPNH and TPNH (Fig. 1, B).

The effect of substrate and nucleotide concentration on the rate of reduction was studied with the purified kidney enzyme (Fig. 1, C and D). The Michaelis constants for the reduction of PYR-2-CA (with DPNH), PIP-2-CA (with DPNH), and PIP-2-CA (with TPNH) were $8.6 \times 10^{-5}$ M, $6.0 \times 10^{-5}$ M, and $12.4 \times 10^{-5}$ M, respectively (Fig. 1, C). The Michaelis constants for DPNH (with PYR-2-CA), DPNH (with PIP-2-CA), and TPNH (with PIP-2-CA) were $4.27 \times 10^{-5}$ M, $7.4 \times 10^{-5}$ M, and $3.82 \times 10^{-6}$ M, respectively (Fig. 1, D). These values are of approximately the same order of magnitude and indicate a relatively high degree of affinity for both substrate and nucleotide.

The reduction of PIP-2-CA to piperolic acid was coupled with the oxidation of d-isocitrate by using the ammonium sulfate fraction obtained from liver. This fraction exhibited significant isocitric dehydrogenase activity in the presence of manganese ions. As indicated in Fig. 1, E, appreciable formation of piperolic acid was observed in the presence of isocitrate, PIP-2-CA, and a relatively small quantity of TPN.

Both PIP-2-CA and PYR-2-CA were stoichiometrically reduced in the presence of reduced coenzyme (Fig. 1, F). Within experimental error, the decrease in the absorption at 340 nm was equivalent to the quantity of substrate added. Attempts to demonstrate DPNH and TPNH formation from the corresponding oxidized coenzymes and proline (or piperolic acid) were not successful.

(4 μmoles), PIP-2-CA (7.5 μmoles), MnCl₂ (1 μmole) and liver fraction (100 γ of protein nitrogen) in a final volume of 0.06 ml. of 0.05 M potassium phosphate buffer, pH 7.0; 37°. Ordinate, formation of piperolic acid. X, value for an experiment carried out with 2 μmoles of d-isocitrate. F, Stoichiometric reduction of PIP-2-CA and PYR-2-CA by DPNH and TPNH. DPNH, the reaction vessel contained 0.056 μmole of DPNH and 50 γ of purified kidney enzyme protein nitrogen in 0.98 ml. of 0.078 M potassium phosphate buffer, pH 6.1; 0.025 μmole of PYR-2-CA was added at Point 1; 0.025 μmole of PIP-2-CA was added at Point 2. TPN; the reaction vessel contained 0.12 μmole of TPN, 1 μmole of MnCl₂, and liver enzyme preparation (ammonium sulfate fraction; 100 γ of protein nitrogen) in 0.98 ml. of 0.078 M potassium phosphate buffer, pH 6.8; 0.06 μmole of d-isocitrate was added at point 3; 0.025 μmole of PYR-2-CA was added at point 4; 0.025 μmole of PIP-2-CA was added at point 5; 1 μmole of d-isocitrate was added at point 6. Ordinate, optical density at 340 nm (corrected for volume changes to 1.0 ml.); 26°.
The purified kidney preparation exhibited no activity with the following substrates: pyrrole-2-carboxylic acid, picolinic acid, 5-oxo-2-pyrrolidone-carboxylic acid, and \( \Delta^1 \)-pyrroline-4-hydroxy-2-carboxylic acid.

**Studies with Plant Extracts**—Preparations obtained from *P. sativum* and *P. radiatus* catalyzed conversion of PYR-2-CA and PIP-2-CA to proline and pipecolic acid, respectively, in the presence of DPNH. A detailed study of the plant tissues was not carried out; however, it was found that the activity of the plant extracts was of the same order of magnitude as that observed with rat liver homogenates.

**Studies with Microorganisms**—As indicated in Table III, enzyme preparations obtained from a mutant of *N. crassa* blocked in biosynthesis of proline between PYR-5-CA and proline (8), as well as enzyme preparations derived from the corresponding parent wild strain, catalyzed the reduction of PYR-2-CA and PIP-2-CA. On the other hand, although the enzyme preparation obtained from the wild strain catalyzed significant reduction of PYR-5-CA, no detectable conversion of PYR-5-CA to proline was observed with the mutant enzyme preparation (cf. Yura and Vogel (8)). It is of associated interest that the mutant strain exhibited a growth response to PYR-2-CA which was approximately 65 per cent of that observed with L-proline.

Studies were also carried out with two proline-requiring auxotrophs of *Escherichia coli* with use of the medium of Davis and Mingioli (13). Mutant 55-1 (3), which is known to be blocked between PYR-5-CA and proline, did not grow on basal media supplemented with either PYR-2-CA or PYR-5-CA (2). Mutant 55-25, which is blocked prior to PYR-5-CA in the biosynthetic sequence (3), exhibited a growth response to PYR-5-CA which was equivalent in magnitude to that observed with racemic proline,

* The reaction mixtures consisted of 2 \( \mu \)moles of PYR-2-CA, PIP-2-CA, or 4 \( \mu \)moles of L-PYR-5-CA, 0.15 \( \mu \)mole of DPNH, and 0.1 ml. of enzyme preparation in a final volume of 1.0 ml. of 0.067 \( \mu \) potassium phosphate buffer, pH 6.8; the values are given as micromoles of DPNH oxidized per hour at \( 26^\circ \).

† Based on dry weight of mycelia formed relative to growth response to L-proline.

**Table III**

<table>
<thead>
<tr>
<th>Source</th>
<th>Growth response</th>
<th>Enzymatic reduction*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proline</td>
<td>PYR-2-CA</td>
</tr>
<tr>
<td>Wild strain</td>
<td>100</td>
<td>65†</td>
</tr>
<tr>
<td>Mutant</td>
<td>100</td>
<td>65†</td>
</tr>
</tbody>
</table>

* The reaction mixtures consisted of 2 \( \mu \)moles of PYR-2-CA, PIP-2-CA, or 4 \( \mu \)moles of L-PYR-5-CA, 0.15 \( \mu \)mole of DPNH, and 0.1 ml. of enzyme preparation in a final volume of 1.0 ml. of 0.067 \( \mu \) potassium phosphate buffer, pH 6.8; the values are given as micromoles of DPNH oxidized per hour at \( 26^\circ \).

† Based on dry weight of mycelia formed relative to growth response to L-proline.

* The *E. coli* cultures were generously provided by Dr. B. D. Davis.
but it did not grow on PYR-2-CA. A mutant of *Aerobacter aerogenes*, isolated in this laboratory by the penicillin technique, appeared to be similar to *E. coli* mutant 55-25 in that it responded to either proline or PYR-5-CA. However, in contrast to this *E. coli* mutant, the *A. aerogenes* mutant grew equally well when equimolar quantities of PYR-2-CA or of L-proline were present in the medium.

Experiments were carried out with broken cell suspensions (prepared by sonic oscillation or by grinding with powdered glass) of the several strains of *E. coli* and *A. aerogenes* in an effort to determine whether these preparations could catalyze the reduction of PYR-2-CA, PIP-2-CA, and PYR-5-CA. Preparations obtained from the wild strains, *E. coli* mutant 55-25 and the *A. aerogenes* mutant, catalyzed formation of proline from PYR-5-CA in the presence of DPNH, although *E. coli* mutant 55-1 was not appreciably active. It is of interest that some proline was formed from PYR-5-CA in the absence of added DPNH, suggesting that reduced coenzyme was present in the preparation or possibly that another mechanism for the reduction of PYR-5-CA exists. Although proline and piperolic acid were formed from PYR-2-CA and PIP-2-CA, respectively (in the presence of DPNH), with preparations of all of the bacteria, the rates of imino acid formation were not appreciably greater than those of the corresponding non-enzymatic reactions (see below). The observation that the *A. aerogenes* mutant exhibited a growth response to PYR-2-CA suggests that it can catalyze conversion of this compound to proline; failure to demonstrate this in crude cell preparations may possibly be ascribed to the relatively low activity of this enzyme system or to competing reactions.

**Optical Configuration of Enzymatically Formed Proline and Pipersolic Acid**—That the proline and piperolic acid formed by enzymatic reduction of PYR-2-CA and PIP-2-CA, respectively, were of the L configuration was established by the failure of the enzymatically synthesized amino acids to undergo oxidation by purified D-amino acid oxidase, under conditions whereby 50 per cent of the corresponding racemic mixtures was destroyed. Reaction mixtures containing enzymatically formed piperolic acid or proline were deproteinized by addition of 3 volumes of ethanol and the ethanol extracts were evaporated to dryness. The residues were treated with purified sheep kidney D-amino acid oxidase (14), FAD, and pyrophosphate buffer, pH 8.3. The mixtures were shaken in air at 37° for 3 hours. Analyses for proline and piperolic acid were carried out before and after treatment with purified D-amino acid oxidase. There was no disappearance of imino acid under these conditions, although in control experiments with racemic proline and piperolic acid there was a 50 per cent

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*Isolated by Phyllis Fraser Downey.*
reduction in the concentration of imino acid. Although enzymatic re-
duction of DL-PYR-5-CA to proline has been demonstrated previously
(8, 15, 16), there are apparently no published data on the configuration of
the proline formed. Studies carried out with L-amino acid oxidase as
described above indicated that the product of the reduction of DL-PYR-5-
CA by preparations of liver and of E. coli is L-proline. In these experi-
ments, it was necessary to separate the proline from PYR-5-CA by chro-
matography inasmuch as the latter compound interferes with the
determination of proline by the procedures employed.

Non-Enzymatic Formation of Proline and Pipecolic Acid—When
PYR-2-CA and DPNH were incubated at 37° in the absence of an enzyme
preparation, a relatively slow formation of proline was detected. Thus,
0.187 μmole of proline was formed in a mixture consisting of 2 μmoles of
PYR-2-CA and 4 μmoles of DPNH in 0.3 ml. of 0.067 M potassium phos-
phate buffer at pH 6.2, in 2 hours at 37°. A similar experiment with
PIP-2-CA gave 0.065 μmole of pipecolic acid, whereas no proline was
formed with PYR-5-CA. The non-enzymatic reaction took place most
rapidly at pH values between 6.0 and 6.5. The proline formed in the
absence of enzyme was shown to be racemic by the procedure described
above. The non-enzymatic reaction did not take place at a measurable
rate under the conditions employed in the spectrophotometric determi-
nations.

DISCUSSION

The conversion of lysine to pipecolic acid has been demonstrated by
isotope tracer experiments in plants (17–19) and in the intact rat (20).
There is also evidence for the conversion of lysine to PIP-2-CA by enzy-
matic oxidation (2, 21). The finding that the α-keto acid analogue of lysine
exists in solution in equilibrium between cyclic and open chain forms (2)
and the observation that the α-amino group rather than the ε-amino group
of lysine is lost in the conversion of lysine to pipecolic acid in the rat (20)
suggest that PIP-2-CA is an intermediate between lysine and pipecolic
acid. The present demonstration that PIP-2-CA is enzymatically re-
duced to L-pipecolic acid by preparations of animal and plant tissues gives
support to this hypothesis. That the reduction of PIP-2-CA to L-pipecolic
acid proceeds to completion and our inability to demonstrate this reaction in
the reverse direction suggest that the equilibrium of this reaction
strongly favors pipecolic acid formation. These findings are consistent
with observations which indicate that pipecolic acid cannot substitute for
lysine in supporting the growth of rats (22) or Neurospora mutants (19).

PYR-2-CA was reduced to proline by enzyme preparations obtained
from rat tissues, higher plants, and Neurospora. Although present evi-
dence suggests that PYR-5-CA is an intermediate in the metabolism of proline in microorganisms and in mammalian tissues, it is possible that a pathway involving PYR-2-CA also exists. The finding that strains of *N. crassa* and *A. aerogenes*, genetically blocked in the synthesis of proline, can utilize PYR-2-CA for growth is compatible with the existence of such an alternative metabolic pathway.

The enzymatic data described here indicate that PYR-5-CA and PYR-2-CA are reduced by different enzymes. This conclusion is supported by the markedly different activities observed with preparations of various animal tissues (Table I) and also by studies on the purified rat kidney enzyme preparation, which exhibited no detectable activity toward PYR-5-CA (Table II). In addition, preparations of the *N. crassa* mutant exhibited no detectable activity toward PYR-5-CA, whereas both PYR-2-CA and PIP-2-CA were reduced. The present data also lead to the tentative conclusion that PIP-2-CA and PYR-2-CA are reduced by the same enzyme.

**SUMMARY**

1. Preparations from rat liver, kidney, spleen, testis, heart, skeletal muscle, and brain, in the presence of reduced diphosphopyridine nucleotide (DPNH), catalyze the reduction of Δ¹-pyrroline-2-carboxylic acid and Δ¹-pyrroline-5-carboxylic acid to proline; these preparations also catalyze the reduction of Δ¹-piperidine-2-carboxylic acid to pipecolic acid. Extracts of *Pisum sativum* and *Phaseolus radiatus* also reduce Δ¹-piperidine-2-carboxylic acid and Δ¹-pyrroline-2-carboxylic acid to pipecolic acid and proline, respectively.

2. A purified enzyme preparation was obtained from rat kidney that catalyzes the reduction of Δ¹-pyrroline-2-carboxylic acid and Δ¹-piperidine-2-carboxylic acid but not that of Δ¹-pyrroline-5-carboxylic acid, picolinic acid, pyrrole-2-carboxylic acid, 5-oxo-2-pyrrolidonecarboxylic acid, or Δ¹-pyrroline-4-hydroxy-2-carboxylic acid; the enzyme is active with both DPNH and reduced triphosphopyridine nucleotide (TPNH). The pH optima for the reactions and the Michaelis constants for the substrates and coenzymes have been determined. The reduction of Δ¹-piperidine-2-carboxylic acid has been coupled with the oxidation of isocitrate in the presence of TPN.

3. Enzyme preparations obtained from a mutant strain of *Neurospora crassa*, genetically blocked in the reduction of Δ¹-pyrroline-5-carboxylic acid to proline, and the corresponding parent wild strain catalyze the reduction of Δ¹-pyrroline-2-carboxylic acid and Δ¹-piperidine-2-carboxylic acid. The *N. crassa* mutant and a proline-requiring mutant of *Aerobacter aerogenes* exhibit a growth response to Δ¹-pyrroline-2-carboxylic acid.
4. The imino acids formed by enzymatic reduction of $\Delta^1$-pyrroline-2-carboxylic acid, $\Delta^1$-piperidine-2-carboxylic acid, and $\Delta^1$-pyrroline-5-carboxylic acid have been shown to be of the L configuration.

5. Non-enzymatic reduction of $\Delta^1$-pyrroline-2-carboxylic acid and $\Delta^1$-piperidine-2-carboxylic acid by DPNH has been observed; these reactions exhibit a pH optimum between 6.0 and 6.5. Under similar conditions, $\Delta^1$-pyrroline-5-carboxylic acid is not reduced.

BIBLIOGRAPHY

17. Lowy, P. H., Arch. Biochem. and Biophys., 47, 228 (1953).