Studies on the Metabolism of the Benzene Ring of Tryptophan in Mammalian Tissues

II. ENZYMIC FORMATION OF α-AMINOMUCONIC ACID FROM 3-HYDROXYANTHRANILIC ACID*

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In preceding reports from this laboratory, 3-hydroxyanthranilic acid was shown to be converted to glutaric acid and CO₂ by crude extracts of cat liver (1–3). Evidence was also presented that α-amino-β-carboxymuconic e-semialdehyde, the primary oxidation product of 3-hydroxyanthranilic acid by the oxygenase (4), is an obligatory intermediate in this conversion (2, 3). However, all previous experiments in vitro have indicated that picolinic acid catalyzed reaction, possibly α-aminomuconic e-semialdehyde, is far obtained from α-amino-β-carboxymuconic e-semialdehyde in mammalian liver. The enzyme responsible for the formation of picolinic acid was first reported by Mehler (6) and was referred to as picolinic carboxylase (7). Since picolinic acid was shown to be converted to glutaric acid and CO₂ by crude extracts of cat liver (1–3), evidence was also presented enzymically from α-tryptophan as described previously (3). We are indebted to Dr. L. M. Henderson for generous samples of carboxyl-1⁴C-labeled and uniformly ³H-labeled 3-hydroxyanthranilic acid. α-Tryptophan, uniformly labeled in the benzene ring with ³H, were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. 3-Hydroxyanthranilic acid, catechol, α-amino adipic acid, and other chemicals were obtained from commercial sources. Pro-tamine sulfate was a product of Sigma Chemical Company. DEAE-cellulose was a product of Serva Entwicklungs labor, Heidelberg, and was treated according to the method of Peterson and Sober (11). Calcium phosphate gel was prepared according to the method of Colowick (12) and hydroxylapatite by the method of Tiselius, Hjertén, and Levin (13).

Biological Materials—Cat liver was used as the source of enzymes. The liver was removed immediately after death, packed in ice, trimmed of connective tissues and gall bladder, washed with cold 0.85% KCl, and then used immediately or stored at −20°C.

3-Hydroxyanthranilic acid oxygenase was partially purified from the aqueous extracts of rat liver acetone powder. All subsequent manipulations were conducted at 4°C. The powder, 2 g, was extracted with 20 ml of a 10⁻⁴ M solution of FeSO₄ (4, 14, 15). To the crude extract (18 ml), 6 ml of a 0.4% solution of pronamine sulfate, pH 7, were added with continuous stirring, and inactive precipitates were removed by centrifugation. To the supernatant solution (23 ml), 19 ml of a saturated solution of ammonium sulfate, pH 7, were added with stirring. After 5 minutes the resulting precipitate was removed by centrifugation. To the supernatant solution, an additional 15.5-ml portion of a saturated ammonium sulfate solution was added. After stirring for 5 minutes, the precipitate was collected by centrifugation and dissolved in 11 ml of a 10⁻⁵ M solution of FeSO₄. To the ammonium sulfate fraction (11.5 ml), 23 ml of calcium phosphate gel suspension (30 mg, dry weight, per ml) were added with stirring. After 10 minutes the gel was collected...
by centrifugation and washed once with 20 ml of distilled water. The enzyme was then eluted twice from the gel, each time with 10 ml of 0.05 M K$_2$HPO$_4$. The combined eluates (20 ml) were made 10$^{-6}$ M with respect to FeSO$_4$. By the above procedures the enzyme was purified about 5-fold with an over-all yield of about 15%, and was essentially free from kynureninase, picolinic carboxylase, quinolinate transphosphoribosylase (16), a-hydroxymuconic e-semialdehyde dehydrogenase, a-aminomuconic acid reductase, and a-ketoglutarate dehydrogenase. The purified enzyme was unstable and prepared shortly before use.

Kynureninase was purified from P. putida-adapted cells of Pseudomonas fluorescens ATCC 11250 according to the method of Hayashi and Stanier (17), except that the dialyzed second ammonium sulfate fraction was further purified by DEAE-cellulose column chromatography. By this procedure the specific activity was increased about 2-fold and the enzyme could be separated from quinolinate transphosphoribosylase.

Crystalline metapyrocatechase (18, 19) was kindly supplied by Dr. M. Nozaki. Crystalline heart muscle lactic dehydrogenase was obtained from Sigma Chemical Company.

**Enzyme Assay**—The activity of picolinic carboxylase was conveniently assayed by measurement of the decrease of absorbance of $\alpha$-amino-$\beta$-carboxymuconic $\epsilon$-semialdehyde at 300 m$\mu$ as described by Mehler (6). However, as discussed below, this assay method may not be exactly consistent with the activity of picolinic carboxylase. Decarboxylation alone does not seem to affect the absorbance significantly, and the decrease in absorbance is probably due to the nonenzymic cyclization of the primary reaction product, $\alpha$-aminomuconic $\epsilon$-semialdehyde, to picolinic acid. Thus a parallel assay was performed by measurement of the radioactive CO$_2$ evolved from $\alpha$-amino-$\beta$-carboxymuconic $\epsilon$-semialdehyde which was generated in situ from carboxyl-$^{14}$C-labeled 3-hydroxyanthranilic acid by the oxygenase.

For the optical assay, the reaction mixture initially contained 60 mmoles of 3-hydroxyanthranilic acid, 200 mmoles of Tris-acetate buffer, pH 8.0, and an excess quantity of a purified preparation of 3-hydroxyanthranilic acid oxygenase (approximately 0.4 mg of protein) in a total volume of 2.9 ml. As shown in Fig. 1, the reaction was followed spectrophotometrically at 360 nm at 24°C. After the formation of $\alpha$-amino-$\beta$-carboxymuconic $\epsilon$-semialdehyde was complete as judged by its absorption at 360 m$\mu$, 0.1 ml of picolinic carboxylase was added. The decrease in absorbance at 360 m$\mu$ was followed at 30-second intervals against a blank incubation that contained all ingredients except the substrate. These data were corrected for the spontaneous decrease of absorbance due to the formation of quinolinic acid.

For the isotopic assay, the incubation was carried out as above except that all ingredients were placed in a Thunberg tube fitted to a quartz cell with a light path of 1 cm, and carboxyl-$^{14}$C-labeled 3-hydroxyanthranilic acid was employed as substrate. The side arm of the tube contained 0.2 ml of 0.5 N NaOH. The reaction was stopped by the addition of 0.5 ml of 2 N acetic acid. Under these conditions, $\alpha$-amino-$\beta$-carboxymuconic $\epsilon$-semialdehyde did not decompose nonenzymically to $\alpha$-hydroxymuconic $\epsilon$-semialdehyde with the elimination of the $\beta$-carboxyl and amino groups, as described below. After
is the rate-limiting step and that the expected intermediate cyclizes very rapidly to picolinic acid. The optical assay may therefore be safely used as a routine assay for picolinic carboxylase.

**Comparison of Optical and Isotopic Assays**—In order to elucidate whether the optical assay represents a reaction catalyzed by picolinic carboxylase, a comparison was made between the optical and isotopic assays. As shown in Fig. 2, both the decrease in absorption at 300 μm and the evolution of radioactive CO₂ were linear with time until about half of the substrate was removed. The two methods were in parallel with each other under any conditions tested, except that the disappearance of absorbance showed a small time lag. The rate of the reaction was directly proportional to the quantities of enzyme employed.

These results indicate that the primary decarboxylation reaction is the rate-limiting step and that the expected intermediate cyclizes very rapidly to picolinic acid. The optical assay may therefore be safely used as a routine assay for picolinic carboxylase activity.

**Purification of Picolinic Carboxylase**—Picolinic carboxylase has been demonstrated in the liver and kidney of many species of animals (6). As reported by Suhadolník et al. (26), the enzyme activity in rat liver was shown to be 30 to 50 times greater than that in rat liver. Thus the enzyme was purified from rat liver. All manipulations were carried out in the cold (0-4°C) unless otherwise noted.

Frozen rat liver, 45 g, was thawed in 180 ml of 0.14 M KCl and homogenized in a Waring Blender for 3 minutes. The homogenate was centrifuged for 15 minutes at 20,000 × g.

**Step 1:** To the supernatant solution (178 ml), 36 ml of a 0.4%
solution of protamine sulfate were added slowly. The mixture was stirred for an additional 5 minutes and the precipitate was removed by centrifugation.

Step 2: The supernatant solution (202 ml) was warmed rapidly to 58° with continuous stirring and maintained for 1 minute at this temperature. The enzyme solution was then cooled rapidly to 1-2° in an ice-salt bath, and the denatured protein was removed by centrifugation.

Step 3: To the enzyme solution (192 ml), 72 ml of peroxide-free cold acetone (-30°) were added slowly with mechanical stirring (30% final concentration of acetone). The mixture was maintained at -2 to -10° in a Dry Ice-methanol bath. The resulting precipitate was removed by centrifugation at -10°. To the supernatant solution, additional cold acetone was added in the same manner to give a 50% final concentration of acetone. After 3 minutes, the precipitate was collected by centrifugation at -10° and dissolved in 30 ml of 0.0075 M potassium phosphate buffer, pH 7.5. Insoluble material was removed by centrifugation.

Step 4: The enzyme solution (36 ml) was adsorbed on a DEAE-cellulose column (diameter, 2.5 cm; length, 5 cm) which had been equilibrated with 0.0075 M potassium phosphate buffer, pH 7.5. After the column was washed with 100 ml of the same buffer, the enzyme was eluted with 180 ml of 0.025 M potassium phosphate buffer, pH 7.5.

Step 5: To the eluate (180 ml), 220 ml of a saturated ammonium sulfate solution, pH 7, were added with stirring. After 15 minutes the precipitate was removed by centrifugation. To the supernatant solution, additional 200 ml of the ammonium sulfate solution were added. After 15 minutes the precipitate was collected by centrifugation and dissolved in 13 ml of 0.0075 M potassium phosphate buffer, pH 7.5. The solution was dialyzed overnight against 2 liters of the same buffer.

Step 6: The enzyme solution (14 ml) was adsorbed on a hydroxylapatite column (diameter, 1.6 cm; length, 2.5 cm) equilibrated with 0.0075 M potassium phosphate buffer, pH 7.5. After the column was washed with 20 ml of 0.1 M potassium phosphate buffer, pH 7.5, the enzyme was eluted with 20 ml of 0.2 M potassium phosphate buffer, pH 7.5.

By the above procedures the enzyme was purified more than 100-fold with an over-all yield of 10 to 20% (Table II). The enzyme preparation thus obtained was free from kynureninase, 3-hydroxyanthranilic acid oxygenase, quinolinate transphosphoribosylase, or hydroxymuconic semialdehyde dehydrogenase, a-aminoacrylase, and a-ketoglutarate dehydrogenase. The purified enzyme was stable and could be stored for several months at -20° without significant loss of activity.

Nonenzymic Spectral Changes of α-Amino-β-carboxymuconic α-Semialdehyde under Acidic Conditions—The absorption spectrum of α-amino-β-carboxymuconic α-semialdehyde, with maximum absorption at 315 mp, did not change within the pH range from 7.5 to 13.0. The molar extinction coefficient was calculated to be 45,000 by repeated careful comparison with the absorption of 3-hydroxyanthranilic acid and that of a-hydroxymuconic α-semialdehyde (see below). At pH 4.5, the absorption maximum shifted to 345 mp. The process was reversible, and the transient absorption spectrum was observed between pH 5.5 and 4.9 (Fig. 3b). In confirmation of the previous observations (9, 15), the absorption maximum was shown to shift irreversibly to 315 mp below pH 3. It takes about 25 minutes for this reaction as shown in Fig. 3b. In agreement with the previous reports by Mehler (27) and by Dagley, Evans, and Ribbons (28), the compound giving the absorption maximum at 315 mp below

![Diagram](https://via.placeholder.com/150)

**FIG. 1 (left).** Optical assay for picolinic carboxylase. Incubation was carried out under the standard assay conditions. The reaction was started by the addition of 3-hydroxyanthranilic acid at the first arrow. Picolinic carboxylase (Step 5, Table II) was added at the second arrow as indicated. Blanks contained all ingredients except the substrate.

**FIG. 2 (right).** Release of "CO2 and change of absorption at 390 mp as a function of time. Incubation was carried out under the standard assay conditions, except that 90 n mole of "CO2-labeled 3-hydroxyanthranilic acid (90,000 c.p.m. per µmole) were used as substrate. The reaction was started by the addition of picolinic carboxylase (Step 5, Table II, 0.15 mg). Changes in optical density at 390 mp.

**TABLE II**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ml</td>
<td>units</td>
<td>mg</td>
<td>unit/mg</td>
<td>%</td>
</tr>
<tr>
<td>Crude extract</td>
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<td>25.00</td>
<td>6686.0</td>
<td>0.0068</td>
<td>100.0</td>
</tr>
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<td>1. Protamine sulfate</td>
<td>202</td>
<td>24.45</td>
<td>5496.0</td>
<td>0.0045</td>
<td>97.8</td>
</tr>
<tr>
<td>2. Heat treatment</td>
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<td>20.16</td>
<td>2976.0</td>
<td>0.0068</td>
<td>80.6</td>
</tr>
<tr>
<td>3. Ammonium sulfate</td>
<td>36</td>
<td>15.00</td>
<td>1494.0</td>
<td>0.0100</td>
<td>60.0</td>
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<td>4. DEAE-cellulose</td>
<td>180</td>
<td>12.53</td>
<td>209.0</td>
<td>0.0800</td>
<td>50.1</td>
</tr>
<tr>
<td>5. Ammonium sulfate</td>
<td>14</td>
<td>6.50</td>
<td>52.5</td>
<td>0.1250</td>
<td>26.0</td>
</tr>
<tr>
<td>6. Hydroxylapatite</td>
<td>22</td>
<td>4.91</td>
<td>6.5</td>
<td>0.7554</td>
<td>16.6</td>
</tr>
</tbody>
</table>

**FIG. 3.** Effect of pH on the absorption spectrum of α-amino-β-carboxymuconic α-semialdehyde. A, pH was adjusted by the addition of 0.5 N HCl as indicated; B, recordings were taken at 1 minute (Curve 1), 10 minutes (Curve 2), and 25 minutes (Curve 3) after the solution was made 0.1 N with respect to HCl. After 30 minutes the solution was neutralized to pH 8.0 with 1 N NaOH. Blanks contained all ingredients except the substrate.
The activity was measured spectrophotometrically under the standard assay conditions except that 200 μmoles of potassium phosphate buffer, pH 7.0, were used and the inhibitors were added as indicated. Reaction was started by the addition of picolinic carboxylase (Step 6, Table II), and the decrease in density at 360 nm between the 90- and 150-second readings was taken as enzyme activity.

### TABLE III

**Effect of various compounds on activity of picolinic carboxylase**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (M)</th>
<th>Activity* (%)</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2 x 10⁻⁴</td>
<td>5.3</td>
<td>132</td>
</tr>
<tr>
<td>NaN₃</td>
<td>1 x 10⁻⁴</td>
<td>4.0</td>
<td>100</td>
</tr>
<tr>
<td>KCN</td>
<td>1 x 10⁻³</td>
<td>1.6</td>
<td>40</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>1 x 10⁻¹</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>1 x 10⁻⁵</td>
<td>2.6</td>
<td>64</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>5 x 10⁻³</td>
<td>3.0</td>
<td>75</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>1 x 10⁻³</td>
<td>5.6</td>
<td>140</td>
</tr>
<tr>
<td>GSH</td>
<td>1 x 10⁻⁴</td>
<td>4.0</td>
<td>100</td>
</tr>
<tr>
<td>10⁻⁴ M p-chloromercuribenzoate +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁴ M L-cysteine</td>
<td>4.5</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>10⁻⁴ M p-chloromercuribenzoate +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁴ M GSH</td>
<td>3.2</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>α-Hydroxymuconic c-semialdehyde</td>
<td>1 x 10⁻⁵</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>α-Hydroxymuconic c-semialdehyde</td>
<td>3.3 x 10⁻⁴</td>
<td>0.6</td>
<td>16</td>
</tr>
</tbody>
</table>

* The values are expressed as milliunits per 0.1 ml of picolinic carboxylase.

pH 3 was identified as α-hydroxymuconic c-semialdehyde by comparison with an authentic sample prepared from catechol by the action of metapyrocatechase. It was converted enzymically to α-hydroxymuconic acid in the presence of α-hydroxymuconic c-semialdehyde dehydrogenase and NAD, and the dicarboxylic acid thus obtained was further identified by spectrophotometric criteria, paper chromatography, and electrophoresis as described below.

**Spectral Change of α-Amino-β-carboxymuconic c-Semialdehyde by Picolinic Carboxylase**—As reported by Mehler (6), when the purified picolinic carboxylase was added to the reaction mixture containing α-amino-β-carboxymuconic c-semialdehyde, the absorption maximum at 360 nm decreased rapidly with concomitant formation of picolinic acid (Fig. 4). A small shift of the absorption maximum to 384 nm was observed during the reaction. This suggests the transient formation of an intermediate, α-amino-α-carboxymuconic c-semialdehyde. However, all attempts to isolate this intermediate have been unsuccessful and picolinic acid was shown to be the only product. Picolinic acid was isolated by Dowex 1-formate column chromatography and identified by paper chromatography and paper electrophoresis as previously described (99). The reaction proceeded stoichiometrically and was essentially irreversible.

**Effects of pH and Substrate Concentration**—As reported by Mehler (6), picolinic carboxylase exhibited a broad range of maximal activity and stability between pH 6 and 9.5. The Kₘ value for α-amino-β-carboxymuconic c-semialdehyde, as calculated from the Lineweaver-Burk plot (30), was of the order of 10⁻⁴ M.

**Inhibitor and Activator Studies**—The purified enzyme preparation (Step 6, Table II) was dialyzed for 12 hours against a large volume of 0.005 M potassium phosphate buffer, pH 7.5, and tested for activators and inhibitors. The addition of boiled extracts (5%) or yeast concentrate (0.5%) showed little effect on the activity of picolinic carboxylase. As shown in Table III, MgCl₂ (2 x 10⁻⁴ M) caused a slight acceleration of the rate of the reaction. However, the enzyme was not inhibited by either NaF or EDTA at concentrations up to 1 x 10⁻⁴ M. The activity was not affected by other metal-chelating agents, such as α,α'-dipyridyl, α-phenanthroline, and 8-hydroxyquinoline at 1 x 10⁻³ M. Among the metal tests, only CuSO₄ and HgCl₂ exhibited an inhibitory effect. 3-Hydroxyanthranilic acid metabolites, such as picoline, quinoline, nacem, neotaminide, NAD, α-ketoadipate, and glutarate, as well as α-hydroxyacetic acid, did not influence the rate of the reaction at 2 x 10⁻⁴ M. However, α-hydroxymuconic c-semialdehyde inhibited the reaction to about 15% of the rate in buffer alone, even at a low concentration of 3.3 x 10⁻⁴ M. The enzyme activity was also inhibited by p-chloromercuribenzoate. This inhibition was prevented by l-cysteine. These results suggest that a sulfhydryl group might be involved in this reaction.

**Purification and Properties of α-Hydroxymuconic c-Semialdehyde Dehydrogenase**

As described above, α-amino-β-carboxymuconic c-semialdehyde was shown to be converted nonenzymically to α-hydroxymuconic c-semialdehyde under acidic conditions. The latter compound was characterized by its absorption maximum at 375 nm at neutral and alkaline pH values (9, 27, 29, 31). In a preceding report from this laboratory, α-hydroxymuconic c-semialdehyde was shown to be oxidized to α-hydroxymuconic acid by an NAD-linked specific aldehyde dehydrogenase obtained from o-cresol-adapted cells of Pseudomonas (32). Concurrently with Henderson and Mitchell, we have observed the presence of a similar enzyme in the liver and kidney of several species of mammals. The enzyme was purified about 50- to 100-fold from cat liver (40 g) was thawed in 160 ml of 0.14 M K₂HPO₄, and 55 ml of a 0.4% solution of protamine sulfate was added with stirring. The precipitate that formed was removed by centrifugation.

**Purification**—All manipulations were carried out at 2-4° unless otherwise noted. In a representative run of purification, frozen cat liver (40 g) was thawed in 160 ml of 0.14 M KCl containing 10⁻⁴ M EDTA, and homogenized for 3 minutes in a Waring Blender. The homogenate was centrifuged for 15 minutes at 20,000 x g.

**Step 1:** The crude extract (165 ml) was brought to pH 7.0 with 1 m K₂HPO₄, and 55 ml of a 0.4% solution of protease sulfate were added with stirring. The precipitate that formed was removed by centrifugation.

**Step 2:** The supernatant solution (211 ml) was warmed rapidly to 49° with continuous stirring in a water bath. After this temperature was maintained for 3 minutes, the solution was cooled to 1-2° in an ice-salt bath, and the resulting precipitate was removed by centrifugation.

**Step 3:** To the enzyme solution (205 ml), 88 ml of peroxide-free, cold acetone (−30°) were added slowly with mechanical
stirring (30% final concentration of acetone). The temperature of the mixture was controlled at $-2$ to $-10^\circ$ in a Dry Ice-methanol mixture. The resulting precipitate was immediately removed by centrifugation at $-10^\circ$. To the supernatant solution, additional cold acetone was added in the same manner to give a 40% final concentration of acetone. After 3 minutes the precipitate was collected by centrifugation at $-10^\circ$ and dissolved in 45 ml of 0.0075 M potassium phosphate buffer, pH 7.0, containing $10^{-4}$ M EDTA. Insoluble materials were removed by centrifugation.

Step 4: The enzyme solution (31 ml) was passed through a DEAE-cellulose column (diameter, 3.5 cm; length, 5 cm), equilibrated with 0.0075 M potassium phosphate buffer, pH 7.0, containing $10^{-4}$ M EDTA. Only inactive proteins were adsorbed on the column. The column was washed with 80 ml of the buffer, and the effluent and washings were combined.

Step 5: To the combined solution (108 ml), 108 ml of a saturated ammonium sulfate solution, pH 7, were added slowly with continuous stirring. After 15 minutes, the precipitate was removed by centrifugation. To the supernatant solution, an additional 55 ml of the ammonium sulfate solution were added. After 15 minutes, the precipitate was collected by centrifugation and dissolved in 30 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing $10^{-4}$ M EDTA.

Step 6: To the enzyme solution (31 ml), 31 ml of a calcium phosphate gel suspension (30 mg, dry weight, per ml) were added with mechanical stirring. After 10 minutes, the gel was collected by centrifugation; it was washed once with 20 ml of distilled water and then twice with 0.05 M K$_2$HPO$_4$ 30 ml each time. The enzyme was then eluted twice from the gel, each time with 15 ml of 0.2 M K$_2$HPO$_4$. The eluates were combined and brought to pH 7.0 by the addition of 1 M KH$_2$PO$_4$. EDTA was added to a final concentration of $10^{-4}$ M.

By the above procedures, the enzyme was purified 90- to 100-fold with an over-all yield of 60 to 70% (Table IV). The enzyme preparation thus obtained was free from kynureninase, 5-hydroxyxanthine acid oxygenase, picolinic carboxylase, quinolinate transphosphoribosylase, a-aminomuconic acid reductase, and a-ketoglutarate dehydrogenase. The enzyme was relatively unstable. Approximately 50% of the activity was lost after storage at $-10^\circ$ for a week.

Product of Reaction—When a-hydroxymuconic e-semialdehyde was incubated with NAD and the purified enzyme, the absorption at 375 mp decreased rapidly with concomitant formation of a new compound with an absorption maximum at 295 mp, as shown in Fig. 5. No reaction occurred in the absence of either NAD or the enzyme. The maximal absorbance of the reaction product shifted to 305 mp under acidic conditions (below pH 3.5) and to 350 mp under alkaline conditions (above pH 10.5) (Fig. 5). These spectral changes of the reaction product were reversible and identical with that of a synthetic sample of o-hydroxymuconic acid (Table I).

In order to confirm that the product is o-hydroxymuconic acid, catechol-$\cdot$H, 0.2 $\mu$ mole ($i \times 10^6$ c.p.m. per $\mu$ mole), was incubated with a purified preparation of metapyrocatechase (about 10 $\mu$ g) and 200 $\mu$ moles of potassium phosphate buffer, pH 8.0, in a total volume of 20 ml. When the formation of o-hydroxymuconic e-semialdehyde was complete as judged by its absorption at 375 mp, 1 $\mu$ mole of NAD and 0.28 $\mu$ g of o-hydroxymuconic e-semialdehyde dehydrogenase (Step 6, Table IV) were added. The mixture was further incubated for an additional 20 minutes at $24^\circ$. At this point, o-hydroxymuconic e-semialdehyde disappeared completely. After the addition of 100 mg of an authentic sample of nonradioactive o-hydroxymuconic acid, the reaction was stopped by the addition of 40 ml of a saturated 2,4-dinitrophenylhydrazine solution (in 2 N HCl). After standing for 2 hours at 24°, the resulting precipitate was collected by centrifugation, washed with 20 ml of cold water, and then crystallized several times from ethanol-water and ethyl acetate-n-hexane. The specific activity of each sample remained constant (7200 c.p.m. per mg). The final product

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
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<td>1. Crude extract</td>
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<td>4. DEAE-cellulose</td>
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<td>135.5</td>
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<tr>
<td>5. Ammonium sulfate</td>
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<td>3875</td>
<td>89</td>
<td>0.0435</td>
<td>77.5</td>
</tr>
<tr>
<td>6. Calcium phosphate gel</td>
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<td>3364</td>
<td>44</td>
<td>0.0765</td>
<td>67.3</td>
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</table>
of the optical density at 340 nm. Standard assay conditions were used: 0.2 M Tris-acetate buffer, pH 8.0.

Determination of α-hydroxymuconic e-semialdehyde was followed at 375 nm. Activities for other aldehydes were assayed by the increase in absorbance at 375 nm, respectively. NADH produced was calculated from the increase in absorbance at 375 nm, respectively. NADH produced was calculated from the increase in absorbance at 340 nm upon the addition of pyruvic acid and crystalline heart muscle lactic dehydrogenase. As shown in Table V, the appearance of α-hydroxymuconic acid and NADH was exactly matched by the disappearance of α-hydroxymuconic e-semialdehyde.

**Effect of pH and Substrate Concentration**—The effects of pH on the activity and stability of the dehydrogenase are given in Fig. 6. The optimal pH for activity in 0.1 M Tris-acetate and potassium phosphate buffer, pH 7.5 and 8.0. The enzyme exhibited a relatively broad range of maximal stability between pH 7.0 and 9.0.

The Kₘ values for α-hydroxymuconic e-semialdehyde and for NAD, as calculated from the Lineweaver-Burk plot (30), were approximately 1.6 × 10⁻⁴ and 1.9 × 10⁻⁴ M, respectively.

**Substrate Specificity**—In the dehydrogenation reaction of α-hydroxymuconic e-semialdehyde, NADP could not serve as a hydrogen acceptor. The preparation of most purified enzyme also oxidized several other aldehydes, including formaldehyde, acetdehyde, n-butyraldehyde, and α-methyl-α-hydroxymuconic e-semialdehyde, but not as rapidly as α-hydroxymuconic e-semialdehyde (Table VI). Propionaldehyde and crotonaldehyde in comparable concentrations did not lead to the formation of NADH in this system.

**Inhibitor and Activator Studies**—The enzyme was inhibited by p-chloromercureibenzoate (100% inhibition at 2 × 10⁻⁴ M, 52% at 10⁻³ M). This inhibition was completely reversed by the addition of either l-cysteine or reduced glutathione at a concentration of 10⁻³ M. This suggests that the dehydrogenase might be a sulfhydryl enzyme. Among the heavy metals tested, Cu++, Ni++, Hg++, and Cu++ inhibited the enzyme almost completely at 2 × 10⁻⁴ M. Metal-chelating agents, such as EDTA, α,α′-dipyridyl, α-phenanthroline, and 8-hydroxyquinoline, at 2 × 10⁻⁴ M, showed no effect on the rate of reaction. As shown in Table IV, acetone fractionation of the enzyme resulted in a 3-fold increase of the total activity. However,
neither acetone (5%) nor boiled extract (5%) influenced the rate of the reaction. The activity was not affected by 3-hydroxyanthranilic acid metabolites, including picolinic, quinolinic, α-ketoadipate, and glutarate, at $1 \times 10^{-4}$ M.

**Formation of α-Aminomuconic Acid from α-Amino-β-carboxymuconic e-Semialdehyde by Combined Action of Picolinic Carboxylase and α-Hydroxymuconic e-Semialdehyde Dehydrogenase**

As described above, the β-carboxyl group of α-amino-β-carboxymuconic e-semialdehyde was shown to be decarboxylated to yield picolinic acid (6). The formation of the pyridinecarboxylic acid, however, appears to take place in two steps with the formation of an acyclic, unsaturated aldehyde as an intermediate. Instead of cyclization to picolinic acid, the aldehyde was shown to be oxidized to α-amino-μconic acid in the presence of NAD and α-hydroxymuconic e-semialdehyde dehydrogenase. When the purified picolinic carboxylase was added to a reaction mixture containing α-amino-β-carboxymuconic e-semialdehyde, the absorption peak at 360 μm decreased rapidly with a concomitant formation of picolinic acid as shown in Fig. 4. In addition to picolinic carboxylase, when purified α-hydroxymuconic e-semialdehyde dehydrogenase and NAD were added to the reaction mixture, a new compound was formed which showed an absorption maximum at 325 μm (Fig. 7). When NAD was omitted from the reaction mixture, or when the preparation of the dehydrogenase heated at 100° for 5 minutes was employed, no compound other than picolinic acid accumulated in the reaction mixture and the spectral change was the same as that shown in Fig. 4.

**Product of Reaction**—Because of its instability, the intermediate compound has not yet been isolated, but chemical studies were performed to characterize this compound. The absorption spectrum of the product with a maximum at 325 μm did not change within the pH range of 5.0 to 9.0. Under alkaline pH (0.1 N with respect to NaOH), the maximal absorbance shifted to 333 μm and the process was reversible. Under acidic conditions (below pH 4.5), however, the maximal absorbance shifted irreversibly to 305 μm. It took about 20 minutes for the completion of this spectral change. Thereafter, the absorption maximum shifted promptly to 297 μm under neutral conditions (pH 5.5 to 9.5) and to 350 μm at alkaline pH (above 10.5). These spectral changes of the latter product, which was obtained by acylation of the 325 μm-absorbing compound, were exactly the same as those of α-hydroxymuconic acid (Table I and Fig. 5).

In order to confirm that the acidified product of the 325 μm-absorbing compound is α-hydroxymuconic acid, 0.33 μmole of 3-hydroxykyurenine, uniformly labeled in the benzene ring with $^{14}$C (247,000 c.p.m. per μmole), was incubated with 300 μmoles of Tris-acetate buffer, pH 8.0, and a purified preparation of *Pseudomonas* kynureninase (1.76 mg of protein) in a total volume of 3 ml at 24°. The reaction was followed spectrophotometrically. When the formation of 3-hydroxyanthranilic acid was complete (about 20 minutes), the mixture was diluted to 14 ml with 0.05 M Tris-acetate buffer, pH 8.0, and 0.6 ml of 3-hydroxyanthranilic acid oxygenase (2.1 mg of protein) was added. After about 3 minutes, the optical density at 360 μm of α-amino-β-carboxymuconic e-semialdehyde reached its maximum value (theoretical, 1.017; found, 1.006). To the reaction mixture, 2 μmoles of NAD, 30 μmoles of pyruvate, 0.2 μg of crystalline heart muscle lactic dehydrogenase, 0.058 mg of the purified preparations of picolinic carboxylase, and 1.09 mg of α-hydroxymuconic e-semialdehyde dehydrogenase were added (final volume, 16 ml). The mixture was further incubated at 24°. When the formation of the 325 μm-absorbing compound was complete, the reaction was stopped by the addition of 0.8 ml of 5 N H$_2$SO$_4$ and the precipitate was removed by centrifugation. The shift of the maximal absorption from 325 μm to 305 μm was complete within 20 minutes. The supernatant solution was concentrated to about 2 ml under reduced pressure, then chromatographed on a silicic acid column (diameter, 1.2 cm; length, 50 cm) after the addition of 20 μmoles of an authentic sample of α-hydroxymuconic acid as carrier. Elution was carried out with an n-butyl alcohol-chloroform system (33). Fractions (10 g each) were titrated with 0.01 N NaOH and assayed for radioactivity. A major radioactive peak appeared which coincided with the titration peak of α-hydroxymuconic acid.

The radioactive α-hydroxymuconic acid thus obtained was treated with an equal volume of a saturated 2,4-dinitrophenylhydrazine solution (in 2 N HCl). The resulting hydrazone derivative was extracted with ethyl acetate. The organic layer was washed twice with cold distilled water and then reextracted with 0.1 N NaHCO$_3$. The aqueous layer was washed once with ethyl acetate, acidified with 6 N HCl, and then extracted again with a small quantity of ethyl acetate. The radioactive hydrazone derivative obtained was identified as that of α-hydroxymuconic acid by paper chromatography in five differ-
In another set of experiments with 2.2 μmoles of 3-hydroxy-
kyurenine-¹⁴C as substrate, the radioactive 323 μg-absorbing com-
pond was prepared in the same manner, and the reaction was
stopped finally by the addition of 15 ml of 10 N KOH. 
After the addition of 1.3 mg of an authentic samples of α-amino-
adipic acid, catalytic hydrogenation was carried out for 20 hours 
at 24° in the presence of Raney nickel. The mixture was neu-
tralized to pH 7 with 20% perchloric acid. The mixture was 
treated with activated charcoal and evaporated to dryness 
under reduced pressure. The dried residue was extracted five 
times with 80% ethanol, and an aliquot of the combined extracts 
was subjected to paper chromatography. A radioactive spot 
was detected and shown to coincide with that of α-aminoadic 
acid in two different solvent systems. The Rf value of α-amino-
adipic acid with phenol-H₂O (8:2) was 0.40, and with n-butyl 
 alcohol-pyridine-H₂O (1:1:1) it was 0.18. Radioactive α-amino-
adipic acid was further identified by high voltage paper elec-
rophoresis. The mobility of the amino acid at pH 5.0 (2000 volts, 
50 minutes) was 3.2 cm to the anode. Further evidence for the 
identity was provided by repeated crystallization from ethanol 
and water to constant specific activity (100 c.p.m. per mg). 
The final product thus obtained had the same melting point 
(212°) as an authentic sample of α-aminoacidic acid. 
The total radioactivity recovered as α-aminoacidic acid was about 
60% of that of the original substrate. As a control for this 
xperiment, α-hydroxymuconic acid was reduced in the same 
manner, but no α-aminoacidic acid was formed as judged by 
paper chromatographic and paper electrophoretic criteria. 
The results described above indicate that the product of the 
combined reaction of picolinic carboxylase and α-hydroxymu-
conic α-semialdehyde dehydrogenase is α-aminoacidic acid, 
and that the primary product of the picolinic carboxylase-
catalyzed reaction is probably α-aminoacidic acid-semialdehyde.

**DISCUSSION**

Early experiments in vivo have provided evidence that the 
benzene ring of tryptophan is rapidly degraded to CO₂ via 3-
hydroxyanthranilic and glutaric acids (34-39). However, it was 
important for a long time to demonstrate in vitro a degradative 
pathway of 3-hydroxyanthranilic acid which could account for 
the observation obtained by studies in vivo. Using cat liver 
extacts, we have recently demonstrated the conversion of 3-
hydroxyanthranilic acid to glutaric acid and have shown that 
α-amino-β-carboxymuconic α-semialdehyde is an obligatory 
intermediate in this conversion (1-3). The latter compound is 
converted to quinolinic acid as was shown first by Henderson 
and Hirsch (5), and further to niacin ribonucleotide in the pres-
ence of PP-ribose-P (16, 29, 40). The cyclization of the alicyclic 
intermediate is, however, generally regarded to be nonenzymic 
(6, 14), and picolinic carboxylase has been known as the only 
enzyme which reacts with α-amino-β-carboxymuconic α-semial-
dehyde (6). The product obtained so far has been picolinic 
acid. The latter pyridinecarboxylic acid is, however, metaboli-
cally inert and is excreted in the urine as the glycine conjugate 
when administered to mammals (7, 8).

The experimental evidence presented in this report leads us to 
the reasonable postulate that the formation of picolinic acid 
takes place in two steps: enzymic decarboxylation of the β-car-
boxyl of α-amino-β-carboxymuconic α-semialdehyde and a 
subsequent cyclization to picolinic acid. The term "picolinic 
carboxylase" was originally proposed by Mehler to designate 
the enzyme which catalyzes the decarboxylation of α-amino-β-
carboxymuconic α-semialdehyde to form picolinic acid. How-
ever, it would be more reasonable to name this enzyme α-amino-
β-carboxymuconic α-semialdehyde decarboxylase.

Although little difference was observed between the rate of 
decarboxylation and the decrease in absorption at 360 μm, a 
small shift of the absorption maximum at 360 μm of α-amino-β-
carboxymuconic α-semialdehyde to 364 μm was observed during 
the picolinic carboxylase-catalyzed reaction. This suggests the 
existence of a transient acyclic intermediate, possibly α-
ammoniac acid-semialdehyde. We also found that the un-
stable, acyclic intermediate is metabolized in the presence of an 
NAD-linked aldehyde dehydrogenase and NAD to α-amino-
ammoniac acid, rather than being cyclized nonenzymically to 
picolinic acid. α-Aminoacidic acid was identified by various 
methods described in the present paper. All attempts to isolate 
α-ammoniaconic acid from the reaction mixture, however, were 
unsuccessful since this acid is readily converted to α-hydroxy-
muconic acid under acidic conditions.

Subsequent experiments have shown that α-ammoniac acid is reductively deaminated to α-ketoadipic acid in the pres-
ence of either NADH or NADPH. The keto acid is then oxidiza-
tively decarboxylated to glutaryl-CoA. The detailed experi-
mental results will be described in a following report in this 
series.

Several experiments were performed to ascertain whether or 
not a single enzyme was responsible for the oxidation of the 
aldheyde moieties of α-ammoniac acid and α-hydroxymuconic 
α-semialdehydes. Although it is not possible to reach a con-
clusion with regard to the purity of the enzyme, there is no indication at present that the activity is separable. α-Hydroxy-
muconic α-semialdehyde dehydrogenase also acts with n-butyr-
aldheyde, formylaldheyde, and 6-methyl-α-hydroxymuconic 
α-semialdehyde to some extent, but not with propionylaldheyde. 
Acoaldheyde was a poor substrate. The nonspecific aldehyde 
dehydrogenase of the liver reported by Racker (41) oxidizes 
various aldheydes, including acetaldheyde and propionylaldheyde, 
at various rates, and acetaldheyde is the most efficient substrate. 
Steroid-sensitive liver aldheyde dehydrogenase reported by 
Maxwell and Topper (42) also oxidizes n-propionylaldheyde. 
These results, together with the difference in the optimal pH 
and heat stability, indicate that these dehydrogenases belong 
to different entities. NADP does not serve as a hydrogen as 
acceptor. The aldheyde dehydrogenases purified from yeast (33), 
Acetobacter suboxydans (44) and Pseudomonas flavescens (45, 46) 
react with NADP as well as NAD. A different type of NAD-
linked dehydrogenase found in Clostridium by Burton and 
Stadlan (47) requires CoA in addition to NAD as an essential 
cofactor.

In a preliminary report from our laboratory, α-hydroxymu-
conic α-semialdehyde and α-hydroxymuconic acid (a tautomer 
of γ-oxaloerontate) were proposed to be intermediates in the 
catabolic pathway of 3-hydroxyanthranilic acid (1). The postu-
lation was based on the results of ¹⁴C trapping experiments with 
α-hydroxymuconic acid as a co substrate. Although α-hydroxy-
muconic α-semialdehyde is metabolized rapidly to glutaryl-
CoA and CO₂ via α-hydroxymuconic acid by another liver 
enzyme system, no available evidence has been obtained to 
indicate that α-hydroxymuconic α-semialdehyde is enzymically
formed from 3-hydroxyanthranilic acid. The incorporation of the radioactivity of 3-hydroxyanthranilic acid-3C as a starting material into α-hydroxyacryloxyacetic acid is probably due to the nonenzymic conversion of α-aminomuconic acid to α-hydroxyacryloxyacetic acid under the acidic conditions employed earlier (1).

The common difficulty of the previous studies on the degradation pathway of 3-hydroxyanthranilic acid in vitro can be ascribed to the extreme instability of the primary product of the picolinic carboxylase-catalyzed reaction. This intermediate cyclizes rapidly to picolinic acid unless enzymically dehydrogenated. The relative ratios of the formation of quinolinic, picolinic, and α-aminomuconic acids depend mainly upon the relative activities of 3-hydroxyanthranilic acid oxygenase, picolinic carboxylase, and α-hydroxymuconic semialdehyde dehydrogenase. The quantitative aspects concerning the metabolism of tryptophan will be described elsewhere.

SUMMARY

In addition to picolinic acid, a new compound is produced from α-amino-β-carboxyacrylic ε-semialdehyde by the combined action of picolinic carboxylase and a nicotinamide adenine dinucleotide-linked specific aldehyde dehydrogenase. Although this compound could not be isolated in a pure form (since it is converted readily to α-hydroxymuconic acid under acidic conditions), available evidence indicates that the compound is α-aminomuconic acid. Picolinic carboxylase and the aldehyde dehydrogenase were both purified about 100-fold from cat liver extracts and were characterized. The purified preparation of the latter enzyme also reacted with several other aldehydes, including formaldehyde and n-butyrildehyde, to some extent. As judged by its substrate specificity and pH optimum, however, the enzyme appeared to be different from the nonspecific aldehyde dehydrogenases previously described by other investigators. Nicotinamide adenine dinucleotide phosphate did not serve as a hydrogen acceptor in this reaction.

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