Studies on the Metabolism of Kynurenic Acid

III. ENZYMATIC FORMATION OF 7,8-DIHYDROXYKYNURENIC ACID FROM KYNURENIC ACID

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In preceding reports from this laboratory, kynurenic acid was shown to be degraded by cell-free extracts of Pseudomonas fluorescens (ATCC 11299B) with the formation of L-glutamic acid, D- and L-alanine, and acetic acid as the major end products (2, 3). Subsequently, tracer experiments with kynurenic acid labeled with C14 at various positions demonstrated that the carbon skeleton of glutamic acid was derived directly from the benzene moiety of kynurenic acid and that the pyridine moiety also contributed to glutamic acid formation by a circuitous mechanism (4). The enzymes involved in this pathway have now been purified, and the intermediate products have been isolated and identified. Available evidence indicates the sequence of reactions shown in Fig. 1 as a pathway of kynurenic acid degradation in this microorganism (5, 6).

The present communication is concerned with the details of the initial phase of this metabolic sequence: the formation of 7,8-dihydroxykynurenic acid from kynurenic acid.

EXPERIMENTAL PROCEDURE

Materials—Kynurenic acid, 7-hydroxykynurenic acid, 8-hydroxykynurenic acid, 7,8-dihydroxykynurenic acid, and quinaldic acid were obtained from the same sources as described in the preceding report (3). trans-“Benzeneglycol” (trans-5,6-dihydroxycyclohexadiene-1,3) was kindly donated by Drs. M. Nakajima and N. Kurihara. Kynurenic yellow (2,3-dihydroxykynurenic acid), hydroxykynurenine yellow (2,3-dihydro-8-hydroxykynurenic acid) (7), and 6-hydroxykynurenic acid were provided through the courtesy of Drs. T. Sakan, S. Senoh, Y. Hirose, and T. Tokuyama. Aminopterin and folic acid were gifts from Takeda Chemicals Industries, Ltd. Tetrahydrofolic acid was prepared by the method described by Hatemi et al. (8). NAD, NADH, NADP, NADPH, L-cysteine, GSH, sodium thioglycollate, and 2-mercaptoethanol were commercial products. Crystalline lactic dehydrogenase was prepared by the method of Straub (9). Glucose dehydrogenase was purified by the procedure of Strecker (10) through the final step (specific activity, 518). Crystalline catalase was obtained from the Sigma Chemical Company. Ammonium sulfate was crystallized once from a boiling solution containing 10−3 M EDTA, and twice from boiling water. The saturated ammonium sulfate solution used for enzyme fractionation was prepared from these crystals and adjusted to pH 7.5 by the addition of ammonium hydroxide.

Analytical Methods—Enzymatic reactions were assayed spectrophotometrically with a Shimadzu recording spectrophotometer and a model DU Beckman spectrophotometer. The spectrophotometric estimation of various compounds was based on the following molar extinction coefficients in 0.1 M Tris buffer, pH 8.0, containing 10−4 M ferrous ammonium sulfate: kynurenic acid, ε293 4,940, ε320 11,100, ε350 6,860; NADH, 1 ε293 5,840, ε320 6,050, ε350 5,550; Compound I, ε293 8,750, ε320 8,480, ε340 8,000, ε350 5,150, ε360 3,270; 7,8-dihydroxykynurenic acid, ε293 20,000, ε320 9,900, ε340 4,850, ε360 4,940; 8-hydroxykynurenic acid, ε293 4,100, ε320 4,900; 7-hydroxykynurenic acid, ε293 10,100. These values were obtained by measuring absorbancess of the accurately prepared solutions containing each compound and remaining constant between pH 7.0 and 8.0, where the enzymatic reactions were carried out. Oxygen consumption was determined polarographically by a modification of the method of Longmair (19), with the use of a vibrating platinum electrode. The electrode was polarized at −0.6 volt; corrections were made for endogenous oxygen consumption and a residual current. The concentration of oxygen in the air-saturated reaction mixture was taken to be 0.24 μmole per ml (13). Protein concentration was determined spectrophotometrically (14). Paper electrophoresis was carried out on Whatman No. 1 filter paper (13.5 × 45 cm, pyridine-acetic acid-water (1:10:89), pH 3.5, 1500 V). The determination was based on the molar extinction coefficient at 340 μm of 6.22 × 104 for reduced NAD (11). The differences between molar extinction coefficients of NADH and NAD used for the assay were 1100, 3200, and 4000 at 293, 311, and 320 μm, respectively. These values were also used for the assay of NADPH.

1 The molar extinction coefficients of Compound I were determined from the stoichiometric conversion of kynurenic acid to Compound I and also from that of Compound I to 7,8-dihydroxykynurenic acid. Figures obtained by these two experiments (see below) agreed within experimental error.

2 The ultraviolet absorption spectra of 8-hydroxykynurenic acid and 7,8-dihydroxykynurenic acid, among the compounds tested, were altered by addition of ferrous ammonium sulfate. These modifications of spectra may be due to the formation of coordination compounds. It was difficult to determine the exact molar extinction coefficients, especially at a shorter wave length, of these compounds under the conditions in which enzymatic reactions were carried out. The ε293 of 7,8-dihydroxykynurenic acid ranged from 18,000 to 21,000 in several assays, but the value of 20,000 was considered most reliable for the assay of the compound in the reaction mixture. The values with 8-hydroxykynurenic acid changed in the pH range 7.0 to 8.0; the data presented were obtained at pH 8.0.
tracts of Pseudomonas (ATCC 11299B) was obtained by high speed centrifugation as described in the preceding report (3). This supernatant solution was made 0.01 M with respect to L-cysteine by addition of the solid reagent and was fractionated with saturated ammonium sulfate solution at 0-4°C. Ten minutes after ammonium sulfate was added, the precipitate was collected by centrifugation at 20,000 × g for 10 minutes and dissolved in a volume of 0.02 M Tris buffer, pH 7.5, containing 0.01 M D-cysteine, equal to the starting volume. Fractions thus obtained were designated according to the ammonium sulfate saturation at which they were precipitated, e.g. AS₄₀, AS₆₀. When AS₈₀ was precipitated again by the addition of an equal volume of saturated ammonium sulfate solution, the resulting preparation, which was referred to as AS₉₀, 14-II, contained approximately 4 mg of protein per ml.

Enzyme Assay—The enzyme catalyzing the formation of Compound I (see below) from kynurenic acid will be referred to as kynurenic acid hydroxylase. The standard reaction mixture for a routine assay contained, in a total volume of 1.0 ml, 0.08 μmole of kynurenic acid, 0.1 μmole of NADH, 0.1 μmole of ferrous ammonium sulfate, 300 units of glucose dehydrogenase, 0.20 ml of AS₄₀-50, and 100 μmoles of Tris buffer, pH 7.5. The reaction was initiated by the addition of the enzyme. Before addition of the enzyme solution, the spectrum of kynurenic acid in the reaction mixture was recorded, usually from 280 μm to 400 μm, with a reference cell containing the same components except for the omission of kynurenic acid. The activity was assayed by measuring the initial rate of the decrease in absorbance at 333 μm. All other enzymatic reactions were carried out at 23°C unless otherwise specified.

RESULTS

Formation of Compound I from Kynurenic Acid

When kynurenic acid was incubated aerobically with NADH or NADPH, glucose, glucose dehydrogenase, and kynurenic acid hydroxylase, a progressive alteration of the spectrum of the reaction mixture was observed, as shown in Fig. 2. The initial peak at 333 μm due to kynurenic acid diminished with the concomitant appearance of a new broad peak at 312 μm. The new compound, with a broad maximum around 312 μm, was designated Compound I. The formation of Compound I was strictly aerobic, as seen in Table I, and oxygen could not be replaced by other electron acceptors, such as methylene blue and 2,6-dichlorophenolindophenol. One mole of kynurenic acid was converted to approximately 1 mole of Compound I with the consumption of approximately 1 mole each of oxygen and NADPH (Table II).

Cofactor Requirements—When kynurenic acid was incubated with kynurenic acid hydroxylase alone or in the presence of NAD or NADPH, no spectral change was observed in the incubation mixture. As seen in Fig. 3, however, if the NAD or NADP present in the reaction mixture was reduced by the action of glucose and glucose dehydrogenase, a rapid decrease in absorbancy at 333 μm took place, indicating that a reduced form of these coenzymes is essential for the disappearance of kynurenic acid. NADPH was approximately 7 times as effective as NADPH. Tetrahydrofolic acid could not replace the reduced coenzyme in a final concentration of 10⁻⁴ M. Kynurenic acid disappeared at an appreciable rate in the absence of added ferrous ion when incubated with AS₉₀, 14-II, as seen in Fig. 2. However, the addition of ferrous ion or boiled extracts caused a marked increase in the rate of the reaction. With the dialyzed AS₉₀-50, ferrous ion added at a concentration of 10⁻⁴ M stimulated the reaction approximately 7-fold (Table III). EDTA and α,α'-
dipyrifyl completely inhibited the reaction at concentrations of 0.9 × 10⁻⁴ M and 0.5 × 10⁻⁴ M, respectively. The enzymatic activity could be restored by the addition of ferrous ion at the concentration indicated in Table III. Other metals tested, including Fe⁺⁺, Ni++, Zn*, Co++, Mn++, Mg++, and Ca++, did not stimulate the reaction at a concentration of 10⁻⁴ M. The addition of aminopterin (5 × 10⁻⁴ M) had no effect on the reaction rate. No significant inhibition of the reaction was observed after the addition of catalase (80 µg per ml) or hydrogen peroxide (1.4 × 10⁻⁴ M).

Isolation of Compound I—The reaction mixture contained the following (expressed in micromoles) in a total volume of 235 ml: kynurenic acid, 30; NADH, 30; ferrous ammonium sulfate, 30; Tris buffer, 25,000 (pH 7.1); d-glucose, 25,000; glucose dehydrogenase, 29,900 units; and AS₉₀, 160 mg of protein. At 27 and 74 minutes after the start of the reaction, 0.3 and 0.4 ml of 0.1 M kynurenic acid, respectively, were added. The mixture was incubated in a 5-liter Erlenmeyer flask at 23°C for 200 minutes. Aliquots were removed at intervals after the initiation of the reaction, and absorption at 270 to 400 nm was recorded in order to follow the progress of the reaction. At the end of the incubation, approximately 35% of the kynurenic acid added had disappeared as judged by the decrease in absorbance at 333 and 310 nm, respectively. The reaction mixture was then passed through a Dowex 1 (analytical grade)-formate column (3.14 cm² × 5 cm, 5% cross-linkage, 200 to 400 mesh). The column was washed with 400 ml of water, and elution was carried out successively with 200 ml of 0.5 M formic acid. All 1 N formic acid fractions were combined and lyophilized to dryness. The residue was dissolved in 2 ml of water and subjected to paper chromatography by a descending method with Whatman No. 31 extra thick paper. The solvent system used consisted of butanol-water-acetic acid (4:1:1). The band having an RF of 0.51 with faint pale blue fluorescence was cut off and eluted with water.

**Table I**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Kynurenic acid consumed</th>
<th>Compound I formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>0.235</td>
<td>0.240</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>0.020</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* The assay was performed 1 minute after the system was opened to the air. Kynurenic acid disappeared completely with the stoichiometric formation of Compound I at 20 minutes after the system was opened.

**Table II**

Stoichiometry in formation of Compound I from kynurenic acid

**Experiment 1**—The reaction mixture contained 0.3 µmole of kynurenic acid, 0.3 µmole of NADH, 0.3 µmole of ferrous ammonium sulfate, 240 µmoles of glucose, 300 µmoles of Tris buffer, pH 7.5, 350 units of glucose dehydrogenase, and 0.3 µl of AS₉₀ in a final volume of 3.1 ml. Spectral changes were recorded as described in the text. Oxygen consumption was followed polarographically. After the termination of the spectral change in both Experiments 1 and 2, kynurenic acid and Compound I were determined spectrophotometrically as described in Table I.

**Experiment 2**—The reaction mixture contained, in a total volume of 1.0 ml, 0.08 µmole of kynurenic acid, 0.2 µmole of NADPH, 0.1 µmole of ferrous ammonium sulfate, 100 µmoles of Tris buffer, pH 7.5, and 0.2 ml of AS₉₀. In a reference mixture, kynurenic acid was omitted. The spectrum from 300 to 400 nm was recorded at time intervals of 2 minutes. Decreases in absorbance at 320, 335, and 340 nm during initial 7 minutes were used for the estimation of kynurenic acid and NADPH consumed and Compound I formed. It is assumed that the decrease in absorbance at 320 nm was due to the consumption of NADPH only because an isosbestic point was found at 320 nm during the spectral change of the reaction mixture in Experiment 2, to which NADPH-generating system was added as in Experiment 1.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Kynurenic acid consumed</th>
<th>Oxygen consumed</th>
<th>NADPH consumed</th>
<th>Compound I formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.300</td>
<td>0.280</td>
<td>0.000</td>
<td>0.300</td>
</tr>
<tr>
<td>2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* The lag phase of increase in absorbancy at 310 nm almost disappeared when the reaction was carried out at pH 7.5 with AS₉₀ as enzyme (see the text and footnotes 5 and 7).

**Table III**

Effect of ferrous ion on disappearance of kynurenic acid

The standard assay system in which ferrous ion was omitted was employed, with the additions indicated.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Enzyme activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
</tr>
<tr>
<td>Boiled extracts, 0.1 ml</td>
<td>3.8</td>
</tr>
<tr>
<td>Fe²⁺, 0.9 × 10⁻⁴ M</td>
<td>6.5</td>
</tr>
<tr>
<td>EDTA, 0.9 × 10⁻⁴ M</td>
<td>0.0</td>
</tr>
<tr>
<td>α,a'-Dipyrifyl, 0.5 × 10⁻⁴ M</td>
<td>6.6</td>
</tr>
<tr>
<td>EDTA, 0.9 × 10⁻⁴ M; Fe²⁺, 2.7 × 10⁻⁴ M</td>
<td>5.1</td>
</tr>
</tbody>
</table>

* One unit is that amount which causes an absorbancy decrease of 0.01 per minute at 333 nm in the standard assay system.
† Boiled extracts were prepared by heating the supernatant fraction at 60°C for 5 minutes.
‡ Ferrous ions were added in the form of ferrous ammonium sulfate. No clear additive effects were observed with boiled extracts and Fe²⁺.
TABLE IV

Color reactions of Compound I with phenol reagents

Color reactions were performed with 0.04 to 0.1 µmole of each sample on a plate. The reaction with phosphomolybdic acid was carried out as follows. After the addition of 0.04 ml of 2% phosphomolybdic acid solution to 0.04 ml of a neutral solution of each sample, 0.04 ml of a saturated Na₂CO₃ solution was added.

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>2,6-Dichloroquinone chloride</th>
<th>Diazotised sulfanilic acid</th>
<th>Ferric chloride</th>
<th>Phosphomolybdic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound I</td>
<td>Light blue</td>
<td>Light yellow</td>
<td>Light brown</td>
<td>No color</td>
</tr>
<tr>
<td>trans-Benzeneglycol</td>
<td>Light gray</td>
<td>Light yellow</td>
<td>No color</td>
<td>No color</td>
</tr>
<tr>
<td>7,8-Dihydroxykynurenic acid</td>
<td>Brown</td>
<td>Pink</td>
<td>Green</td>
<td>Rhin</td>
</tr>
<tr>
<td>8-Hydroxykynurenic acid</td>
<td>Green</td>
<td>Red</td>
<td>Green</td>
<td>Light blue</td>
</tr>
<tr>
<td>7-Hydroxykynurenic acid</td>
<td>Greenish blue</td>
<td>Yellow</td>
<td>Light brown</td>
<td>Light blue</td>
</tr>
<tr>
<td>Kynurenic acid</td>
<td>No color</td>
<td>No color</td>
<td>Brown</td>
<td>No color</td>
</tr>
</tbody>
</table>

Color reactions

Color reactions of Compound I with various phenol reagents

5 ml of water. The eluate was taken to dryness in a flash evaporator at 30-35°C. Approximately 20 µmoles of Compound I were obtained.

Properties of Compound I—Although the instability of Compound I has prevented its crystallization, the stoichiometry of the reaction described above, as well as that of the reaction by which the compound is converted to 7,8-dihydroxykynurenic acid as described below, and the following properties of the isolated product indicate that it is probably identical with the 7,8-dihydrodiol of kynurenic acid.

1. Compound I could be distinguished clearly from either 7- or 8-hydroxykynurenic acid by comparing the ultraviolet absorption spectra (Figs. 4 and 5), the Rp values upon paper chromatography, and the mobility upon paper electrophoresis. In the butanol-water-acetic acid system, the Rp values of 7- and 8-hydroxykynurenic acid and Compound I were 0.73, 0.81, and 0.51, respectively, on Whatman No. 31 extra thick paper by the same method as that described above. Compound I ran 9.9 cm toward the anode; 7- and 8-hydroxykynurenic acid ran 3.3 and 6.7 cm, respectively, in the electrophoresis system described under "Experimental Procedure.”

2. Color reactions of Compound I with various phenol reagents were not typical of phenols, but were similar to reactions of trans-benzeneglycol, as shown in Table IV.

3. Compound I was comparatively stable in aqueous solution at room temperature even under acidic or alkaline conditions. For example, the spectra in Fig. 4 could be interconverted by the addition of acid or alkali under the conditions described. However, when the acidic solution of Compound I was taken to dryness in a vacuum at room temperature, the formation of a considerable amount of 8-hydroxykynurenic acid was detected by paper electrophoresis and paper chromatography, as well as by spectrophotometry (Table V). In an experiment designed to confirm this, the dried material obtained by acid treatment from approximately 30 µmoles of Compound I was dissolved in 0.01 N NaOH solution. Light yellow crystals, which were precipitated after the addition of 1 N HCl, were collected by filtration. After recrystallization, approximately 1 mg of pure crystals of the fluorescent material was obtained. This material was identified as 8-hydroxykynurenic acid by comparison with an authentic sample with the following criteria: (a) ultraviolet absorption spectra under acidic (0.1 N HCl), alkaline (0.1 N NaOH), and neutral (0.1 N Tris buffer, pH 8.0) conditions; (b) infrared absorption spectra (Fig. 6); (c) paper electrophoresis; (d) paper chromatography with three different solvent systems (butanol-acetic acid-water (4:1:1), Rp = 0.66; 80%
propanol, $R_f = 0.53$; ethanol-ammonia-water (18:1:1), $R_f = 0.55$. When the isolated fluorescent compound was incubated with NADH, glucose, and glucose dehydrogenase and AS$_{40}$, the progressive changes recorded in ultraviolet absorption were essentially identical with those observed in the incubation mixture with 8-hydroxykynurenic acid shown below. Compound I did not change spectrophotometrically in the same incubation system. No other fluorescent compounds were formed in appreciable amounts from Compound I under the conditions in which 8-hydroxykynurenic acid was formed, as judged by paper chromatography and paper electrophoresis, except that a very small amount of a fluorescent material giving the same $R_f$ value as 7-hydroxykynurenic acid was sometimes observed. When kynurenic acid-2-C$^4$ (4) was incubated as described under "Isolation of Compound I," labeled Compound I was isolated. The radioactivity was incorporated into the 8-hydroxykynurenic acid formed from the labeled Compound I, by the treatment described above.

Evidence for Involvement of More than One Step in Compound I Formation—Apparent isosbestic points in the changing spectrum during the course of the reaction as described in Fig. 2 altered between 315 and 325 m$\mu$, depending mainly on enzyme preparations and pH, indicating the complex nature of this reaction. Furthermore, when polarographic and spectrophotometric assays were carried out simultaneously with the same reaction mixture, the oxygen consumption and the decrease in absorbancy at 333 m$\mu$ took place immediately without any lag phase. However, an increase in absorbancy at 300 m$\mu$ was observed only after an appreciable lag period, as seen in Fig. 7, indicating that one or more intermediate steps are involved in the formation of Compound I from kynurenic acid.

Properties of Kynurenic Acid Hydroxylase—When the supernatant fraction was precipitated with ammonium sulfate in the absence of cysteine, the enzymatic activity catalyzing the disappearance of kynurenic acid was almost completely lost. Fractionation with ammonium sulfate not treated with EDTA resulted in a marked loss of the enzymatic activity. Although the supernatant fraction obtained after high speed centrifugation of crude extracts could be stored at $-10^\circ$ for several months without appreciable loss of activity, ammonium sulfate fractions frozen at $-10^\circ$ lost activity in a few days even in the presence of 0.01 M cysteine. AS$_{40-45}$ completely lost activity in 2 hours in an atmosphere of $O_2$ at $23^\circ$, although the activity was retained in an atmosphere of $N_2$, indicating a sensitivity to oxygen, as has been reported with various other oxygenases (17-19). As shown in Fig. 8, the activity was most stable at pH 9 when the enzyme was dialyzed against 0.05 M potassium phosphate buffer containing 0.01 M L-cysteine at $4^\circ$ for 20 hours. This dialyzed fraction (0.05 ml) was added to the incubation mixture as the enzyme. In the experiment to determine optimal pH (O), the reaction mixture contained 0.2 pmole of kynurenic acid, 0.3 pmole of NADH, 0.3 pmole of ferrous ammonium sulfate, 200 pmoles of glucose, 215 micromoles of Tris-malate buffer, 292 units of glucose dehydrogenase, and 0.1 ml of AS$_{40-45}$ in a total volume of 2.6 ml. The assay method in both experiments was the same as that described under "Experimental Procedure." The specific activity is expressed as units per mg of protein (see Table III).

The lag period was enhanced when AS$_{40-45}$ was used as the enzyme.
Fe+++ did not show any effect. The pH optimum was found at 7.0 (Fig. 8).7

Specificity of Substrate—Quinaldic acid and 6-hydroxykynurenic acid did not change spectrophotometrically in the standard assay system. Kynurenic yellow incubated in the standard reaction system reacted at a much slower rate than kynurenic acid, resulting in a decrease in absorbancy of the peak at 380 rnp due to the substrate and in the formation of a new absorption maximum at 357 rnp. The product was definitely distinguishable from Compound I, but its identity was not established.

When 8-hydroxykynurenic acid was incubated in those systems in which AS&-E was incubated instead of AS&-55 in the reaction mixture similar to the standard system, 7,8-dihydroxykynurenic acid disappearing, 1 mole of 7,8-dihydroxykynurenic acid was produced with the consumption of 1 mole of NADH, as additional evidence that the previous reaction product was identical with 7,8-dihydroxykynurenic acid. Spectrophotometric evidence indicates that for each mole of 8-hydroxykynurenic acid disappearing, 1 mole of 7,8-dihydroxykynurenic acid was produced with the consumption of 1 mole of NADH, as shown in Table VII. An essentially identical reaction was observed with 7-hydroxykynurenic acid incubated with the same system. These reactions took place only aerobically.

The enzyme activities for the oxidation of kynurenic acid and 8- and 7-hydroxykynurenic acid were all found exclusively in the AS&-55 fraction. The initial velocity with 8- and 7-hydroxykynurenic acid was 0.35 and 0.22, respectively, as compared with that of kynurenic acid when assayed spectrophotometrically by measuring the rate of consumption of each substrate with the standard assay system, in which kynurenic acid was replaced with each of the monohydroxykynurenic acids. NADH was 3.6 times more effective with 8- and 7-hydroxykynurenic acid than with kynurenic acid. NADPH was 7 and 3.6 times more effective with 8- and 7-hydroxykynurenic acid, respectively, than NADPH when assayed by measuring the initial rate of increase in absorbancy at 293 rnp with the same standard reaction mixture as described above.

In order to determine whether the same enzyme catalyzes the hydroxylation of kynurenic acid and 7- or 8-hydroxykynurenic acid, the effect of kynurenic acid on the hydroxylation reaction product, a new peak at 390 rnp, corresponding to the absorption maximum of 5-(γ-carboxy-γ-oxopropenyl)-4,6-dihydroxypicolinic acid, immediately appeared, thus providing additional evidence that the previous reaction product was identical with 7,8-dihydroxykynurenic acid. Details of the recording procedure were the same as those described in the legend of Fig. 2, except that the numbers indicate the time in minutes at which recordings were carried out after mixing.

**Table VI**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Addition</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMB, 0.45 X 10^-4 M</td>
<td>None</td>
<td>48</td>
</tr>
<tr>
<td>Cysteine, 1.8 X 10^-2 M</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>GSH, 1.8 X 10^-3 M</td>
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<td>21</td>
</tr>
<tr>
<td>CMB, 0.9 X 10^-4 M</td>
<td>None</td>
<td>87</td>
</tr>
<tr>
<td>Cysteine, 1.8 X 10^-3 M</td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>Iodoacetate, 10^-4 M</td>
<td>None</td>
<td>87</td>
</tr>
<tr>
<td>Cysteine, 1.8 X 10^-3 M</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>Cysteine, 4.2 X 10^-4 M</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>GSH, 4.2 X 10^-3 M</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>CuSO_4, 10^-4 M</td>
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</tr>
<tr>
<td>HgCl_2, 10^-3 M</td>
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<td>85</td>
</tr>
<tr>
<td>AgNO_3, 10^-3 M</td>
<td>None</td>
<td>25</td>
</tr>
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</table>

7 The rate of the increase in absorbancy at 310 rnp after the initial lag phase showed the maximal value when the reaction was carried out at pH 7.5.

8 Since a considerable amount of 7,8-dihydroxykynurenic acid oxygenase (see the text) was contained in AS&-55, the initial increase in absorbancy at 293 rnp was immediately followed by an increased absorbancy at 390 rnp and a decreased absorbancy at 293 rnp upon incubation of the standard assay system containing 8-hydroxykynurenic acid. A similar observation was made with the assay system containing hydroxykynurenic yellow; that is, the initial increase in absorbancy at 335 rnp was followed by an increase and a decrease in absorbancy at 380 and 335 rnp, respectively.

7,8-Dihydroxykynurenic acid oxygenase converts 7,8-dihydroxykynurenic acid to an intensely yellow compound with an absorption maximum at 390 rnp, the structure of which has been presented as 5-(γ-carboxy-γ-oxopropenyl)-4,6-dihydroxypicolinic acid (6). Details will be published elsewhere.
TABLE VII

Stoichiometry of reaction of 8-hydroxykynurenine acid in the presence of 
AS$_{40}$, NAD and NADH

The reaction mixture consisted of 290 µmoles of Tris buffer, pH 8.0, 0.3 µmole of ferrous ammonium sulfate, 0.4 µmole of NADH, 0.25 µmole of 8-hydroxykynurenine acid, 0.5 ml of AS$_{40}$, NAD, and water in a total volume of 3.1 ml. Absorbance at 203, 330, and 350 µm was recorded at 4-minute intervals after the reaction was started by the addition of the enzyme solution. Calculations are based on the assumption that the change of absorbancy at 330 µm is due only to the change of concentration of NADH, because an isosbestic point was found at 330 µm during the spectral change under the condition described in the legend for Fig. 8.

<table>
<thead>
<tr>
<th>Time after reaction started</th>
<th>8-Hydroxykynurenine acid consumed</th>
<th>7,8-Dihydroxykynurenine acid formed</th>
<th>NADH oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>µmole</td>
<td>µmole</td>
<td>µmole</td>
</tr>
<tr>
<td>4</td>
<td>0.043</td>
<td>0.050</td>
<td>0.047</td>
</tr>
<tr>
<td>8</td>
<td>0.105</td>
<td>0.102</td>
<td>0.093</td>
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</tbody>
</table>

...of 8-hydroxykynurenine acid was tested. When kynurenine acid and 8-hydroxykynurenine acid were incubated in the presence of glucose, NADH, and glucose dehydrogenase with a large amount of AS$_{40}$, 8-hydroxykynurenine acid was converted to 7,8-dihydroxykynurenine acid, which was then rapidly transformed to a yellow compound with a peak at 390 µm (see footnote 8), whereas kynurenine acid was transformed to Compound I, which could not undergo any further change in this system. Kynurenine acid at a concentration of 2 x 10^{-4} M markedly inhibited the formation of the yellow compound from 8-hydroxykynurenine acid, whereas similar compounds, such as 6-hydroxykynurenine acid and quinaldic acid, had no effect. The inhibition was reversed by increasing the concentration of 8-hydroxykynurenine acid, indicating the competitive nature of this inhibition (Fig. 11). The formation of the yellow compound from 7,8-dihydroxykynurenine acid was not inhibited by kynurenine acid at a concentration of 2 x 10^{-4} M. The KM for 8-hydroxykynurenine acid was approximately 0.3 x 10^{-4} M; the KM for kynurenine acid was about 0.6 x 10^{-4} M, which is in the same order as the KM value, 0.8 x 10^{-4} M, as described above.

Formation of 7,8-Dihydroxykynurenine Acid from Compound I

Fig. 12 shows the spectral changes that occurred during the incubation of Compound I with AS$_{40}$ in the presence of ferrous ion, NAD, and NADH. The initial spectrum with a broad peak at 312 µm due to Compound I changed progressively with the concentration of Compound I. The KM for 8-hydroxykynurenine acid was approximately 0.3 x 10^{-4} M; the KM for kynurenine acid was about 0.6 x 10^{-4} M, which is in the same order as the KM value, 0.8 x 10^{-4} M, as described above.

Formation of 7,8-Dihydroxykynurenine Acid from Compound I

Essentially similar spectral changes were observed when ferrous ion, pyruvate, and lactate dehydrogenase were added to the reaction mixture described in the legend of Fig. 2 at the end of incubation, or when kynurenine acid was incubated with AS$_{40}$, NAD, sodium pyruvate, and lactate dehydrogenase. The reaction mixture contained, in a total volume of 1.0 ml, 100 µmoles of Tris buffer, pH 7.5, 10 µmoles of sodium pyruvate, 0.1 µmole of NAD, 0.2 µmole of ferrous ammonium sulfate, 0.06 µmole of Compound I, 0.1 unit of lactate dehydrogenase, and 0.06 ml of AS$_{40}$. These recording procedures were as described in the legend of Fig. 2. A reference cell contained the same components, except for the omission of Compound I. Under the conditions described above, the oxidation of Compound I from kynurenine acid did not occur, but the formation of Compound I was observed spectrophotometrically in a reaction mixture with 8-hydroxykynurenine acid.
TABLE VIII

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Cofactor</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NAD</td>
<td>0.074</td>
</tr>
<tr>
<td>2</td>
<td>NADH</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>3</td>
<td>NADP</td>
<td>0.005</td>
</tr>
<tr>
<td>4</td>
<td>NADPH</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

**TABLE IX**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Incubation time</th>
<th>Compound I consumed</th>
<th>7,8-Dihydroxykynurenic acid formed</th>
<th>NADH formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.022</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.042</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.029</td>
<td>0.029</td>
<td>0.028</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.044</td>
<td>0.044</td>
<td>0.043</td>
</tr>
</tbody>
</table>

**Fig. 13.** Infrared absorption spectra of synthetic (B) and isolated (A) 7,8-dihydroxykynurenic acid. A KBr disk was used.

Isolation of 7,8-Dihydroxykynurenic Acid—The reaction mixture contained, in a final volume of 2150 ml, kynurenic acid, 240 μmoles; NADH, 120 μmoles; NAD, 90 μmoles; ferrous ammonium sulfate, 500 μmoles; Tris buffer, pH 8.0, 225 mmoles; and AS_{0.15} to 11, 240 ml (960 mg of protein). It was divided into equal amounts in three Erlenmeyer flasks of 5-liter volume. Aliquots were assayed for absorption at 290 to 400 μm at intervals after the initiation of the reaction. After incubation at 20° for 33 hours, the reaction was more than 80% complete as judged by the increase in absorbancy at 293 μm, and was stopped by the addition of 600 ml of 85% formic acid. The acidified reaction mixture was centrifuged at 3000 × g for 10 minutes, and the supernatant solution was evaporated to dryness at 40°. The residue was extracted with 130 ml of 22 N formic acid, and the extract, after dilution with 910 ml of water, was passed through a Dowex 1 (analytical grade)-formate column (3.14 cm² × 6 cm, 8% cross-linkage, 200 to 400 mesh). After having been washed with 170 ml of 4 N formic acid, the column was eluted successively with 570 and 100 ml of 0 and 12 N formic acid, respectively. The last two combined eluates were concentrated to dryness in a vacuum. Crystalline yellow needles weighing approximately 21 mg (85 μmoles of 7,8-dihydroxy-kynurenic acid) were obtained. This material was recrystallized several times from 47.5% HBr. The final crystallization was performed by the addition of water to the 47.5% HBr solution. The crystals were dried over silica gel and KOH in a vacuum at room temperature for 24 hours.

Identification of 7,8-Dihydroxykynurenic Acid—The compound thus isolated was identified as 7,8-dihydroxykynurenic acid by the following criteria. Both authentic and isolated materials began to darken at 275° and almost decomposed at 290° without melting. Elemental analysis was carried out with a sample dried at 110° for 3 hours in a vacuum.

C_{34}H_{37}N_{10}O_{12} (mol wt 231 17)

Calculated: C 54.30, H 3.19, N 6.33
Found: C 53.66, H 3.31, N 6.62

The isolated material gave infrared and ultraviolet absorption spectra identical with those of the authentic material (Fig. 13). The isolated material showed three absorption maxima in 3 N HCl; ε_{344} 34,400, ε_{525} 5,200, and ε_{635} 2,300. Those of an authentic sample were ε_{345} 37,200, ε_{525} 5,460, and ε_{635} 2,410. Additional evidence for this identity was obtained by paper electrophoresis and enzymatic assay (see below).

Stoichiometry—In the absence of sodium pyruvate and lactic dehydrogenase, the amount of NAD reduced in the reaction was determined spectrophotometrically. Based on the molar extinction coefficient of Compound I calculated from that of kynurenic acid and 7,8-dihydroxykynurenic acid, the disappearance of 1 m mole of Compound I was accompanied by the formation of approximately 1 m mole of NADH and 1 m mole of 7,8-dihydroxykynurenic acid (Table IX).

Specificity of Dehydrogenase—Ayungar et al. (20) have described the occurrence of a dihydrodidehydrogenase in rabbit

Three different solutions of the isolated and authentic materials used for comparison of absorption spectra were the following: 3 N HCl, 0.1 m potassium phosphate buffer, pH 5.8, and 0.1 m Tris buffer containing ferrous ammonium sulfate equimolar to the materials and 5 × 10^{-4} m cysteine.
liver that catalyzes the formation of catechol from trans-benzeneglycol with NADP as a cofactor. When trans-benzeneglycol and NAD were incubated with the supernatant fraction of this Pseudomonas extract, an increase in absorbancy at 340 \( \mu \text{m} \) was observed, suggesting the presence of a similar benzeneglycol dehydrogenase in the supernatant fraction. Therefore, the possibility that the dehydrogenation of Compound I and of benzeneglycol are catalyzed by the same enzyme in this organism was tested. As seen in Table X, the activities of Compound I dehydrogenase and benzeneglycol dehydrogenase are distributed in different proportions in AS\(_{0.42}, \) AS\(_{4.0}, \) and AS\(_{4.70} \). trans-

**Table X**

**Distribution of 7,8-dihydroxykynurenic acid-7,8-diol (Compound I) dehydrogenase and trans-benzeneglycol dehydrogenase in ammonium sulfate fraction**

The assay system for Compound I dehydrogenase contained 100 \( \mu \text{mole} \) of Tris buffer, pH 7.5, 0.2 \( \mu \text{mole} \) of ferrous ammonium sulf-
fate, 10 \( \mu \text{mole} \) of sodium pyruvate, 0.4 \( \mu \text{mole} \) of NAD, 0.085 \( \mu \text{mole} \) of Compound I, 0.1 unit of lactic dehydrogenase, and 0.1 ml of each ammonium sulfate fraction or the supernatant fraction in a total volume of 1.0 ml. A reference system contained all of the components except Compound I. The activity of Compound I dehydrogenase in each fraction was determined from the initial rate of increase in absorbancy at 300 \( \mu \text{m} \). * When Compound I was incubated with the supernatant fraction, the 7,8-dihydroxy-
kynurenic acid formed reacted partially to form the yellow compound with a peak at 390 \( \mu \text{m} \), as described in the text. Therefore, the activity in the supernatant fraction was determined from the initial rate of increase in absorbancy at both 300 and 390 \( \mu \text{m} \). For the calculation of activity, the molar extinction coefficient at 390 \( \mu \text{m} \) of the yellow compound in the reaction mixture was tentatively determined to be 22,000. † The assay of trans-benzeneglycol dehydrogenase, the following incubation mixture was used, in a total volume of 1.0 ml: 50 \( \mu \text{mole} \) of Tris buffer, pH 7.5, 0.4 \( \mu \text{mole} \) of NAD, 0.1 \( \mu \text{mole} \) of NADH, ‡ 0.5 \( \mu \text{mole} \) of trans-benzeneglycol, and 0.2 ml of each ammonium sulfate fraction or the supernatant fraction. A reference cell contained all of the same components except the substrate. The activity of the benzeneglycol dehydrogenase was assayed by measuring the initial rate of increase in absorbancy at 340 \( \mu \text{m} \).

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Activity of Compound I dehydrogenase</th>
<th>Activity of trans-benzeneglycol dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant fraction</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>AS(_{0.42})</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>AS(_{4.0})</td>
<td>61</td>
<td>19</td>
</tr>
<tr>
<td>AS(_{4.70})</td>
<td>38</td>
<td>57</td>
</tr>
<tr>
<td>AS(_{4.70})</td>
<td>7</td>
<td>33</td>
</tr>
</tbody>
</table>

* Molar extinction coefficients at 300 \( \mu \text{m} \) of Compound I (8,800) and 7,8-dihydroxykynurenic acid (17,900) were used for estimation of the activity of Compound I dehydrogenase (see Table VIII) because absorbancy at 290 \( \mu \text{m} \) could not be determined with such a reaction mixture containing the supernatant fraction. † The estimation of this value was based on the following experiment. 7,8-Dihydroxykynurenic acid (0.10 \( \mu \text{mole} \)) dissolved in a neutralized solution of 0.01 M cysteine hydrochloride was incubated with 0.05 ml of the supernatant fraction in a total vol-

ume of 2.6 ml containing 250 \( \mu \text{mole} \) of Tris buffer, pH 7.2. After 1 minute, the absorbancy at 390 \( \mu \text{m} \) reached a maximum of 0.86, which did not change for several minutes. ‡ The amount of NADH added was sufficient to saturate the NADH oxidase activity in each fraction during the estimation of the activity of benzeneglycol dehydrogenase.

Benzeneglycol added in final concentrations of 0.5 \( \times 10^{-4} \) M and 1.2 \( \times 10^{-4} \) M did not inhibit the activity of Compound I dehydrogenase. These findings indicate that Compound I is dehydrogenated by an enzyme different from trans-benzeneglycol dehydrogenase.

**Reversibility**—In spite of all efforts to demonstrate the reversibility of this reaction, the reduction of 7,8-dihydroxy-
kynurenic acid to Compound I has not been accomplished under various conditions tested.

7,8-Dihydroxykynurenic Acid as Intermediate in Kynurenic Acid Metabolism—When kynurenic acid is incubated with the supernatant fraction, an intensely yellow compound with a peak at 390 \( \mu \text{m} \) appears and then gradually disappears until no absorption is observed between 290 and 300 \( \mu \text{m} \) (21, 22). Both isolated and synthetic 7,8-dihydroxykynurenic acids in-
cubated with the supernatant fraction also rapidly formed a yellow compound with the same absorption spectrum as that of the yellow compound formed from kynurenic acid, indicating that 7,8-dihydroxykynurenic acid is not an artifact of the isola-
tion, but an intermediate in the bacterial metabolism of kynu-
renic acid.

**DISCUSSION**

The evidence presented in this paper indicates that in the presence of oxygen and reduced nicotinamide adenine dinucleo-
tides (NADH or NADPH), kynurenic acid was first converted to Compound I, which had a broad absorption maximum at 312 \( \mu \text{m} \) at pH 7.0. This Compound I was then dehydrogenated by an NAD-specific dehydrogenase to form 7,8-dihydroxykynurenic acid. The latter compound was concurrently synthesized by Behrmann and Tanaka and was proposed as a possible inter-
mediate in the enzymatic degradation of kynurenic acid (23). However, kynurenic acid, 7-hydroxykynurenic acid, 8-hydroxy-
kynurenic acid, and 7,8-dihydroxykynurenic acid were all con-
verted by the supernatant fraction to the same yellow compound with a peak at 390 \( \mu \text{m} \). Under this situation, it was difficult to determine which of these compounds are true intermediates in the metabolism of kynurenic acid. Our results indicate that kynurenic acid is converted stoichiometrically to 7,8-dihydroxy-
kynurenic acid through Compound I and that neither 7- nor 8-hydroxykynurenic acid is an intermediate, and provide defi-
nite proof that 7,8-dihydroxykynurenic acid is an intermediate in the metabolism of kynurenic acid.

The exact structure of Compound I has not been deftinately estab-
lished and must await its isolation in a crystalline form, but it could be definitely distinguished from 7- or 8 mono-
hydroxykynurenic acid by various color reactions, ultraviolet absorption spectra, and paper chromatographic and elec-
trophoretic evidence. Furthermore, both 7- and 8-hydroxykynu-
renic acid could be converted slowly to 7,8-dihydroxykynurenic acid, but these reactions required the presence of reduced nicotinamide adenine dinucleotides and oxygen, similar to the enzym-
atic conversion of kynurenic acid to Compound I, whereas the enzymatic conversion of Compound I to 7,8-dihydroxykynurenic acid required NAD and could take place under anaerobic condi-
tions. Although Compound I was rather unstable and could be converted nonenzymatically to 8-hydroxykynurenic acid under more drastic conditions, there is no evidence that such a reaction could take place enzymatically. These observa-
tions, together with the stoichiometry and the cofactor require-
ment of the enzymatic reaction, indicate that Compound I is
probably identical with or at the same oxidation level as 7,8-dihydrokynurenic acid-7,8-diol.

Available evidence strongly indicates that the formation of Compound I from kynurenic acid may involve more than one reaction step. It appears most probable that either a peroxyde (A) or an epoxide (B) is produced as a primary product. A could then undergo rearrangement, or B could be hydrated to form a dihydrodiol compound (I) (see Scheme 1). However, the absence of an isosbestic point during the conversion of kynurenic acid to Compound I makes Pathway B more likely than A, because kynurenic acid peroxyde and the dihydrodiol probably have a similar spectrum whereas the epoxide may show a spectrum different from either kynurenic acid or the dihydrodiol. Preliminary experiments with O\(^{18}\) and H\(_2\)O\(^{18}\) provided results pertinent to the mechanism of this conversion.

It has been generally assumed that the biological synthesis of catechol is carried out by the further hydroxylation of a monophenolic compound. Evidence presented in this paper indicates another possible mechanism of catechol formation, namely, an oxygenation to form a dihydrodiol compound and the subsequent dehydrogenation of this dihydrodiol compound. This type of aromatization reaction is not entirely novel, since it has been reported previously that a simple benzeneglycol, trans-5,6-dihydroxyeyclohexadiene-1,3, is dehydrogenated to form catechol by an NADP-linked dehydrogenase from rabbit liver (20). Although the biological role of this enzyme has not been clarified, various naturally occurring dihydrodiol compounds and many epoxides are well known (24-26). The mechanism of the enzymatic formation of these dihydrodiol compounds and many epoxides, however, has not been understood. Our findings suggest that kynurenic acid is oxygenated to form 7,8-dihydrokynurenic acid-7,8-diol with the consumption of 1 mole each of oxygen and reduced nicotinamide adenine dinucleotide, possibly through hydration of kynurenic acid-7,8-oxide. Concurrently Booth et al. (27) have also reported findings consistent with this hypothesis. Their experiments suggest that an epoxide of naphthalene is formed in the presence of NADPH, oxygen, and rat liver microsomes, and that the latter is further converted to a dihydro-

\[^{13}H\] Taniuchi, T. Makita, N. Itada, and O. Hayaishi, unpublished results.

were inert in the reaction system catalyzing the formation of Compound I from kynurenic acid, did not inhibit the hydroxylation. These findings suggest that the hydroxylation of 7- or 8-hydroxykynurenic acid and the formation of Compound I from kynurenic acid were catalyzed exclusively by AS\(_{0-\beta}\). The inhibition of the hydroxylation of 8-hydroxykynurenic acid by kynurenic acid appears to be competitive in nature. Quinoidal acid and 6-hydroxykynurenic acid, both of which

**SUMMARY**

1. A partially purified enzyme preparation from *Pseudomonas fluorescens* (ATCC 11299B) adapted to tryptophan catalyzes the formation of a new compound from kynurenic acid with the consumption of equimolar amounts of reduced nicotinamide adenine dinucleotide or reduced nicotinamide adenine dinucleotide phosphate and oxygen. This enzyme was referred to as kynurenic acid hydroxylase.

2. The intermediate compound was isolated in a pure form but has not been crystallized, since it decomposes to 8-hydroxykynurenic acid upon drying. Available evidences indicate that it is identical with 7,8-dihydrokynurenic acid-7,8-diol.

3. This compound was dehydrogenated by a partially purified enzyme preparation to form 7,8-dihydrokynurenic acid. NAD but not NADP was the essential cofactor. This enzyme was referred to as 7,8-dihydrokynurenic acid-7,8-diol dehydrogenase.

4. 7,8-Dihydroxykynurenic acid was isolated, identified, and established as an intermediate in the metabolism of kynurenic acid.

5. The mechanism of formation of 7,8-dihydroxykynurenic acid from kynurenic acid is discussed in connection with the
biological origin of catechol and the mechanism of enzyme hydroxylation.

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REFERENCES
Studies on the Metabolism of Kynurenic Acid: III. ENZYMATIC FORMATION OF 7,8-DIHYDROXYKYNURENIC ACID FROM KYNURENIC ACID
Hiroshi Taniuchi and Osamu Hayashi


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