In preceding reports from this laboratory, kynurenine 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 kynurenine acid labeled with C$^{14}$ at various positions demonstrated that the carbon skeleton of glutamic acid was derived directly from the benzene moiety of kynurenine 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 kynurenine 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-dihydroxykynurenine acid from kynurenine acid.

**EXPERIMENTAL PROCEDURE**

Materials—Kynurenine acid, 7-hydroxykynurenine acid, 8-hydroxykynurenine acid, 7,8-dihydroxykynurenine acid, and quinazolic 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. Kynurenine yellow (2,3-dihydroxykynurenine acid) (7) and 6-hydroxykynurenine acid were provided through the courtesy of Drs. T. Sakan, S. Senoh, Y. Hirose, and T. Tokuyama. Aminopterin and folic 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. Tetrahydrofolinic acid was prepared by the method described by Hatefi 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 Strecker (9). Glucose dehydrogenase was purified by the procedure of Strecker (10) through the final step (specific activity, 135,000). Paper electrophoresis was carried out on Whatman No. 1 filter paper (13.5 X 45 cm, pyridine-acetic acid-water (1:10:89), pH 3.5, 100 V, 3 hours). Protein concentration was determined spectrophotometrically (14). Tetrahydrofolinic acid was prepared by the method described by Hatefi 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 Strecker (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$^{-4}$ 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**—Enzymic 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: kynurenine acid, $e_{340}$ 4,940, $e_{328}$ 11,100, $e_{310}$ 8,660, NADH; $e_{340}$ 5,840, $e_{328}$ 6,050, $e_{300}$ 5,650; Compound I, $e_{340}$ 8,775, $e_{328}$ 8,480, $e_{310}$ 8,000, $e_{300}$ 5,190, $e_{290}$ 3,270; 7,8-dihydroxykynurenine acid, $e_{340}$ 20,000, $e_{328}$ 9,900, $e_{310}$ 4,850, $e_{300}$ 4,940; 6-hydroxykynurenine acid, $e_{340}$ 4,100, $e_{328}$ 4,900; 7-hydroxykynurenine acid, $e_{328}$ 10,100. These values were obtained by measuring absorancies 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 Longman (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 X 45 cm, pyridine-acetic acid-water (1:10:89), pH 3.5, 100 V, 3 hours).

The determination was based on the molar extinction coefficient at 340 μm of 6.22 X 10$^4$ for reduced NAD (11). The differences between molar extinction coefficients of NADH and NAD used for the assay were 1100, 3200, and 4000 at 285, 311, and 320 μm, respectively. These values were also used for the assay of NADPH.

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

The ultraviolet absorption spectra of 8-hydroxykynurenine acid and 7,8-dihydroxykynurenine 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 $e_{340}$ of 7,8-dihydroxykynurenine acid ranged from 18,000 to 21,000 in several assay, but the value of 20,000 was considered most reliable for the assay of the compound in the reaction mixture. The values with 8-hydroxykynurenine acid changed in the pH range 7.0 to 8.0; the data presented were obtained at pH 8.0.

**Studies on the Metabolism of Kynurenine Acid**

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

HIROSHI TANIUCHI and OSAMU HAYASHI

From the Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto, Japan

(Received for publication, June 26, 1962)
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 in 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 x g for 10 minutes and dissolved in a volume of 0.02 M Tris buffer, pH 7.5, containing 0.01 M L-cysteine, equal to the starting volume. Fractions thus obtained were designated according to the ammonium sulfate saturation at which they were precipitated, e.g. AS65, AS60. When AS60 was precipitated again by the addition of an equal volume of saturated ammonium sulfate solution, the resulting preparation, which was referred to as AS60-55, 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, 300 units of glucose dehydrogenase, 0.20 ml of AS60-55, 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 290 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 absorbancy at 333 μm. All other enzymatic reactions were carried out at 23°C unless otherwise specified.

**RESULTS**

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 NADP, 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. NADH was approximately 7 times as effective as NADPH. EDTA and α,α'-

**Enzyme Preparation—** The supernatant fraction of crude extracts 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 x g for 10 minutes and dissolved in a volume of 0.02 M Tris buffer, pH 7.5, containing 0.01 M L-cysteine, equal to the starting volume. Fractions thus obtained were designated according to the ammonium sulfate saturation at which they were precipitated, e.g. AS65, AS60. When AS60 was precipitated again by the addition of an equal volume of saturated ammonium sulfate solution, the resulting preparation, which was referred to as AS60-55, contained approximately 4 mg of protein per ml.

**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 NADP, 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. NADH was approximately 7 times as effective as NADPH. EDTA and α,α'-
dipyridyl completely inhibited the reaction at concentrations of 0.9 \times 10^{-4} \text{M} and 0.5 \times 10^{-4} \text{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} \text{M}. The addition of aminopterin (5 \times 10^{-4} \text{M}) had no effect on the reaction rate.

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 AS_{SO, \text{so}} \text{X} 160 \text{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° for 200 minutes. Aliquots were removed at intervals after the initiation of the reaction, and absorption at 290 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 absorbancy at 333 and 310 nm, respectively. The reaction mixture was then passed through a Dowex 1 (analytical grade)-formic column (3.14 cm² x 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 N formic acid. All 0.5 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

| TABLE I |

**Comparison of aerobic and anaerobic conditions for enzymatic disappearance of kynurenic acid**

The main compartment of a conventional Thunberg tube contained, in a volume of 5.0 ml, NADH, 0.5 \text{mole}; ferrous ammonium sulfate, 0.5 \text{mole}; glucose, 500 \text{mole}; and AS_{SO, \text{so}} \text{X} 100 \text{mg of protein}. The tube was incubated in a 5-liter Erlenmeyer flask at 23° for 200 minutes. Aliquots were removed at intervals after the initiation of the reaction, and absorption at 290 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 absorbancy at 333 and 310 nm, respectively. The reaction mixture was then passed through a Dowex 1 (analytical grade)-formic column (3.14 cm² x 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 N formic acid. All 0.5 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

**TABLE II**

**Stoichiometry in formation of Compound I from kynurenic acid**

**Experiment 1—**The reaction mixture contained 0.3 \text{mole} of kynurenic acid, 0.3 \text{mole} of NADH, 0.3 \text{mole} of ferrous ammonium sulfate, 240 \text{mole} of glucose, 300 \text{mole} of Tris buffer, pH 7.5, 100 \text{mole} of glucose dehydrogenase, and 0.3 \text{mole} of AS_{SO, \text{so}} \text{X} 160 \text{mg of protein}. 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 absorbancy at 320, 333, 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 absorbancy at 320 nm was due to the consumption of NADH 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.

**Experiment 2—**The reaction mixture contained, in a total volume of 1.0 ml, 0.08 \text{mole} of kynurenic acid, 0.2 \text{mole} of NADPH, 0.1 \text{mole} of ferrous ammonium sulfate, 100 \text{mole} of Tris buffer, pH 7.5, and 0.2 \text{mole of AS}_{SO, \text{so}} \text{X} 160 \text{mg of protein}. 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 absorbancy at 320, 333, 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 absorbancy at 320 nm was due to the consumption of NADH 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 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†</td>
<td>3.8</td>
</tr>
<tr>
<td>Fe^{+++}‡</td>
<td>0.9 \times 10^{-4} \text{M}</td>
</tr>
<tr>
<td>EDTA, 0.9 \times 10^{-4} \text{M}</td>
<td>0.0</td>
</tr>
<tr>
<td>α,α’-Dipyridyl, 0.5 \times 10^{-4} \text{M}</td>
<td>6.6</td>
</tr>
<tr>
<td>EDTA, 0.9 \times 10^{-4} \text{M}; Fe^{+++}‡</td>
<td>2.7 \times 10^{-4} \text{M}</td>
</tr>
<tr>
<td>α,α’-Dipyridyl, 0.5 \times 10^{-4} \text{M}; Fe^{+++}‡</td>
<td>1.4 \times 10^{-4} \text{M}</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° for 5 minutes.
‡ Ferrous ions were added in the form of ferrous ammonium sulfate. No clear additive effects were observed with boiled extracts and Fe^{+++}.

---

1. T. Horiike and O. Hayashi, 1963
2. H. Tanaka and O. Hayashi, 1963
Color reactions were performed with 0.04 to 0.1 pmole 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.

### Color reactions of Compound I with phenol reagents

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Color developed by reaction with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,6-Dichloroquinone</td>
</tr>
<tr>
<td></td>
<td>chloride</td>
</tr>
<tr>
<td></td>
<td>Diastatised sulfuric acid</td>
</tr>
<tr>
<td></td>
<td>Ferric chloride</td>
</tr>
<tr>
<td></td>
<td>Phosphomolybdic acid</td>
</tr>
<tr>
<td>Compound I</td>
<td>Light blue</td>
</tr>
<tr>
<td>trans-Benzenediglycol</td>
<td>Light yellow</td>
</tr>
<tr>
<td>7,8-Dihydroxykynurenic acid</td>
<td>Light brown</td>
</tr>
<tr>
<td>8-Hydroxykynurenic acid</td>
<td>No color</td>
</tr>
<tr>
<td>7-Hydroxykynurenic acid</td>
<td>No color</td>
</tr>
<tr>
<td>Kynurenic acid</td>
<td>No color</td>
</tr>
</tbody>
</table>

### Figures

**Figure 4 (Left).** Ultraviolet absorption spectra of Compound I at a concentration of 10⁻⁴ M. Curve 1, 0.1 M Tris buffer, pH 8.0; Curve 2, 0.1 N HCl; Curve 3, 0.1 N NaOH. The spectrum of Compound I in 1 N formic acid was the same as Curve 2.

**Figure 5 (Right).** Ultraviolet absorption spectra of 8-hydroxykynurenic acid (Curve 1) and 7-hydroxykynurenic acid (Curve 2) at a concentration of 10⁻⁴ M in 0.1 M Tris buffer, pH 8.0.

### Table IV

<table>
<thead>
<tr>
<th>Compound determined</th>
<th>Before treatment</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Hydroxykynurenic acid</td>
<td>0.30</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>&lt;0.003</td>
<td>0.10</td>
</tr>
</tbody>
</table>

### Table V

**Chemical formation of 8-hydroxykynurenic acid from Compound I**

A solution of Compound I (0.5 pmole) in 0.3 ml of 0.1 N HCl was condensed to dryness in a vacuum at room temperature and was subjected to paper chromatography and spectrophotometric analysis. The determination was based on the following molar extinction coefficients in 0.1 M Tris buffer, pH 8.0: Compound I, ε₂₅₀ 7500, ε₃₀₀ 8500; 8-hydroxykynurenic acid, ε₂₅₀ 8300, ε₃₄₄ 9000.

### Notes

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 0.81, 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 2.3 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-benzenediglycol, 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 pmoles 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 recrystallisation, 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 M 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$_{60}$, 10-11, 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$^4$ ($A$) 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 $\mu_M$, 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 $\mu_M$ took place immediately without any lag phase. However, an increase in absorbancy at 300 $\mu_M$ 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$_{60-45}$ 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. A&$\beta$-so completely lost activity in 2 hours in a nitrogen atmosphere at 23$^\circ$, whereas dialysis against 0.05 M potassium phosphate buffer containing 0.01 M L-cysteine, CMB$^6$ and 2-mercaptoethanol (0.01 M) and GSH were less effective than cysteine in stabilizing the activity. Tris buffer was much less effective than phosphate buffer. Heating the supernatant fraction at 60$^\circ$ for 5 minutes caused complete inactivation of the enzyme. The $K_m$ values were approximately 0.8 $\times$ 10$^{-4}$ M for kynurenic acid and 0.4 $\times$ 10$^{-4}$ M for NADH. Although the enzyme preparation contained 0.01 M cysteine, CMB$^6$ and iodoacetate inhibited the enzymic activity considerably; the inhibition was reversed by the addition of GSH or cysteine to the reaction mixture (Table VI). Cu$^{2+}$, Hg$^{2+}$, or Ag$^+$ also had an inhibitory effect on the disappearance of kynurenic acid, but

$^6$ The abbreviation used is: CMB, p-chloromercuribenzoate.
Fe+++ did not show any effect. The pH optimum was found at 7.0 (Fig. 8).

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 kynurenine, indicating the formation of a compound other than kynurenine. When a partially purified 7,8-dihydroxykynurenic acid was added to the incubation mixture containing the acid, resulting in a decrease in absorbancy of the peak at 380 nm upon incubation of the standard assay system, an absorption maximum at 293 nm due to the substrate and in the formation of a new absorption maximum at 390 nm. 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 ASO, 50-11 was incubated instead of ASO, 58 in the reaction mixture similar to the standard system, an absorption maximum at 293 nm appeared with a clear isosbestic point observed at 350 nm during the progressive alteration of the spectrum, indicating the formation of a compound other than Compound I (Fig. 9). Both NADH and NADPH were effective as cofactors, and NADP and NAD were totally inactive, as was the case with kynurenic acid. The final spectrum of the reaction mixture between 290 and 400 nm was identical with the spectrum of 7,8-dihydroxykynurenic acid under the same conditions (Fig. 10). When a partially purified 7,8-dihydroxykynurenic acid was added to the incubation mixture containing the reaction product, a new peak at 390 nm, corresponding to the absorption maximum of 8-(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. 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 ASO, 50-11 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 substance with the standard assay system, in which kynurenic acid was replaced with each of the monohydroxykynurenic acids. NADH 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 nm 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 of 7,8-dihydroxykynurenic acid to an intensely yellow compound with an absorption maximum at 390 nm, the structure of which has been presented as 5-(γ-carboxy-γ-oxopropenyl)-4,6-dihydroxypicolinic acid (6). Details will be published elsewhere.
and lactic dehydrogenase were present to convert NADH back, but dehydrogenase was not essential for the activity, and the reaction proceeded under anaerobic conditions if sufficient quantities of pyruvate were added. The presence of equimolar ferrous ion prevented this alteration of the product, 7,8-dihydroxykynurenic acid, which is labile above pH 7.0. Incubation at pH 7.0 resulted in an irreversible alteration of 8-hydroxykynurenic acid. Under the conditions described in the legend for Fig. 2, 8-hydroxykynurenic acid was converted to 7,8-dihydroxykynurenic acid, indicating the competitive nature of this inhibition (Fig. 11). The formation of the yellow compound from 8-hydroxykynurenic acid, as described above, was observed at 390 mp under such conditions.

Formation of 7,8-Dihydroxykynurenic Acid from Compound I

Fig. 12 shows the spectral changes that occurred during the incubation of Compound I with AS$_{66}$ in the presence of ferrous ion, NAD and NAD-generating system, pyruvate, and lactic dehydrogenase. The initial spectrum with a broad peak at 312 mp 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 absorbance at 330 mp is due only to the change of concentration of NADH, because an isosbestic point was found at 330 mp during the spectral change under the condition described in the legend for Fig. 9.

<table>
<thead>
<tr>
<th>Time after reaction started</th>
<th>8-Hydroxykynurenic acid consumed</th>
<th>7,8-Dihydroxykynurenic 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>
</tr>
</tbody>
</table>

TABLE VII

*Stoichiometry of reaction of 8-hydroxykynurenic acid incubated with AS$_{66}$, ADP, and NADH*

The reaction mixture consisted of 250 µmoles of Tris buffer, pH 8.0, 0.3 µmole of ferrous ammonium sulfate, 0.4 µmole of NADH, 0.25 µmole of 8-hydroxykynurenic acid, 0.5 µl of AS$_{66}$, ADP, and water in a total volume of 3.1 ml. Absorbance at 293, 330, and 350 mp 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 absorbance at 330 mp is due only to the change of concentration of NADH, because an isosbestic point was found at 330 mp during the spectral change under the condition described in the legend for Fig. 9.

The reaction mixture contained, in a total volume of 1.0 ml, 20 µmoles of Tris buffer, pH 7.5, 100 µmoles of glucose, 0.1 µmole of NADH, 0.1 µmole of ferrous ammonium sulfate, 0.02 to 0.1 µmole of 8-hydroxykynurenic acid, 300 units of glucose dehydrogenase, and 0.4 µl of AS$_{66}$, ADP. The reaction mixture for testing the effect of kynurenic acid (C) contained 0.1 µmole of kynurenic acid together with the same components as the reaction mixture described above. The reference cells contained the same components, except for the omission of 8-hydroxykynurenic acid, as those in the reaction cells. The increases in absorbance at 390 mp were measured at 34 to 41 minutes after the initiation of the reaction by the addition of the substrate. One unit of the velocity is expressed as an increase in absorbance at 390 mp of 0.001 per minute. The assumption is made that observed rate is proportional to the rate of the formation of 7,8-dihydroxykynurenic acid from 8-hydroxykynurenic acid, because the difference between the molar extinction coefficients of 8-hydroxykynurenic acid and 7,8-dihydroxykynurenic acid is less than 3% of the molar extinction coefficient of the yellow compound at 390 mp under such conditions (see Table X). In this system, the formation of 7,8-dihydroxykynurenic acid from kynurenic acid did not occur, but the formation of Compound I was observed spectrophotometrically in a reaction mixture without 8-hydroxykynurenic acid.

Fig. 12 shows the spectral changes that occurred during the incubation of Compound I with AS$_{66}$ in the presence of ferrous ion, NAD and NAD-generating system, pyruvate, and lactic dehydrogenase. The initial spectrum with a broad peak at 312 mp 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 absorbance at 330 mp is due only to the change of concentration of NADH, because an isosbestic point was found at 330 mp during the spectral change under the condition described in the legend for Fig. 9.

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 µl of AS$_{66}$, ADP. 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.

Fig. 12 shows the spectral changes that occurred during the incubation of Compound I with AS$_{66}$ in the presence of ferrous ion, NAD and NAD-generating system, pyruvate, and lactic dehydrogenase. The initial spectrum with a broad peak at 312 mp 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 absorbance at 330 mp is due only to the change of concentration of NADH, because an isosbestic point was found at 330 mp during the spectral change under the condition described in the legend for Fig. 9.

The reaction mixture contained, in a total volume of 1.0 ml, 20 µmoles of Tris buffer, pH 7.5, 0.1 µmole of glucose, 0.1 µmole of NADH, 0.25 µmole of 8-hydroxykynurenic acid, and water in a total volume of 3.1 ml. Absorbance at 293, 330, and 350 mp 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 absorbance at 330 mp is due only to the change of concentration of NADH, because an isosbestic point was found at 330 mp during the spectral change under the condition described in the legend for Fig. 9.

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 µl of AS$_{66}$, ADP. 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.

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 kynurenic acid was incubated with AS$_{66}$, ADP in the presence of ferrous ion and NADH. In the latter case, a catalytic amount of NADH was sufficient to permit extensive formation of the compound with a peak at 390 mp under such conditions (see Table X). In this system, the formation of 7,8-dihydroxykynurenic acid from kynurenic acid did not occur, but the formation of Compound I was observed spectrophotometrically in a reaction mixture without 8-hydroxykynurenic 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 (min)</th>
<th>Compound I consumed (µmoles)</th>
<th>7,8-Dihydroxykynurenic acid formed (µmoles)</th>
<th>NADH formed (µmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.022</td>
<td>0.021</td>
<td>0.021</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.042</td>
<td>0.039</td>
<td>0.039</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.029</td>
<td>0.029</td>
<td>0.029</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.044</td>
<td>0.044</td>
<td>0.044</td>
</tr>
</tbody>
</table>

The incubation mixture of Experiment 1 contained 250 µmoles of Tris buffer, pH 8.0, 0.3 µmole of ferrous ammonium sulfate, 40 µmoles of sodium pyruvate, 0.3 µmole of NAD, 0.2 µmole of Compound 1, 0.1 unit of lactate dehydrogenase, and 0.4 ml of ASgly-glu in a total volume of 2.8 ml. The incubation mixture of Experiment 2 was same as that of Experiment 1 in Table VIII. The changes of absorbancy at 293 and 319 mp in Experiment 1 and at 293, 311, and 340 mp in Experiment 2 were estimated with the aid of a recording spectrophotometer. It is assumed in Experiment 2 that the increase in absorbancy at 311 mp is due only to the reduction of NAD, since an isosbestic point was found at 311 mp under the conditions of Experiment 1 (see Fig. 11).

### Isoalloxazine Requirement

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

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 the NADH absorption region. The absorbance at 293 mp was estimated with the aid of a recording spectrophotometer. It is assumed in Experiment 2 that the increase in absorbancy at 311 mp is due only to the reduction of NAD, since an isosbestic point was found at 311 mp under the conditions of Experiment 1 (see Fig. 11).
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 nm 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 catalysed 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<sub>5-19</sub>, AS<sub>13-16</sub>, and AS<sub>14-70</sub>.

**Table X**

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Activity of Compound I dehydrogenase</th>
<th>Activity of benzeneglycol dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant fraction</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>AS&lt;sub&gt;5-19&lt;/sub&gt;</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>AS&lt;sub&gt;13-16&lt;/sub&gt;</td>
<td>61</td>
<td>19</td>
</tr>
<tr>
<td>AS&lt;sub&gt;14-70&lt;/sub&gt;</td>
<td>38</td>
<td>57</td>
</tr>
<tr>
<td>AS&lt;sub&gt;14-70&lt;/sub&gt;</td>
<td>7</td>
<td>33</td>
</tr>
</tbody>
</table>

* Molar extinction coefficients at 300 nm 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 380 nm 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 μ 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 volume of 2.6 ml containing 250 μ moles of Tris buffer, pH 7.2. After 1 minute, the absorbancy at 390 nm 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 × 10<sup>-4</sup> M and 1.2 × 10<sup>-4</sup> 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-dihydroxykynurenic 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 nm appears and then gradually disappears until no absorption is observed between 290 and 300 nm (21, 22). Both isolated and synthetic 7,8-dihydroxykynurenic acids incubated with the supernatant fraction also rapidly formed a yellow compound<sup>4</sup> 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 isolation, but an intermediate in the bacterial metabolism of kynurenic acid.

**DISCUSSION**

The evidence presented in this paper indicates that in the presence of oxygen and reduced nicotinamide adenine dinucleotides (NADH or NADPH), kynurenic acid was first converted to Compound I, which had a broad absorption maximum at 312 nm 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 Behrman and Tanaka and was proposed as a possible intermediate in the enzymatic degradation of kynurenic acid (23). However, kynurenic acid, 7-hydroxykynurenic acid, 8-hydroxykynurenic acid, and 7,8-dihydroxykynurenic acid were all converted by the supernatant fraction to the same yellow compound with a peak at 390 nm. 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-dihydroxykynurenic acid through Compound I and that neither 7- nor 8-hydroxykynurenic acid is an intermediate, and provide definite proof that 7,8-dihydroxykynurenic acid is an intermediate in the metabolism of kynurenic acid.

The exact structure of Compound I has not been definitely established 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 electrophoretic evidence. Furthermore, both 7- and 8-hydroxykynurenic 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 enzymatic 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 conditions. 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 observations, together with the stochiometry and the cofactor requirement 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 peroxide
(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).

In 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 peroxide and the dihydrodiol
probably have a similar spectrum whereas the epoxide may show
a spectrum different from either kynurenic acid or the dihydro-
diol. Preliminary experiments with $O^2$ and $H_2O^2$ 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 mono-
phenolic 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 cate-
chol 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 enzy-
matic 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-dihydrokynurenene
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 hy-
pothesis. Their experiments suggest that an epoxide of naphtha-
lene is formed in the presence of NADPH, oxygen, and rat liver
micosomes, and that the latter is further converted to a dihydro-

\[ \text{Scheme 1} \]

were inert in the reaction system catalyzing the formation of
Compound I from kynurenic acid, did not inhibit the hydroxyla-
tion. These findings suggest that the hydroxylation of 7- or
8-hydroxykynurenic acid and the formation of Compound I
from kynurenic acid may involve epoxidation as a common
primary step. Bloom and Shull (29) have already reported a
similar phenomenon in a microorganism capable of introducing
an epoxide grouping into a steroid.

**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 dinucleo-
tide 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-hydroxy-
kynurenic 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-dihydrokynurenene acid
NAD but not NADP was the essential cofactor. This enzyme
was referred to as 7,8-dihydrokynurenene acid-7,8-diol dehy-
drogenase.

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

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

Acknowledgments—The authors are indebted to Drs. I. P. Crawford and R. K. Ghose for their valuable aid in the preparation of this manuscript. Thanks are also due to Mr. Hiroshi Yamada for his contributions in certain phases of this work and to Drs. S. Senoh and T. Tokuyama for their generous advice and helpful discussions of this work.

REFERENCES