THE QUINOLINE PATHWAY OF TRYPTOPHAN OXIDATION BY PSEUDOMONAS: THE INITIAL STEPS IN THE OXIDATION OF KYNURENIC ACID*

E. J. Behrman** and Takehiko Tanaka***

Cancer Research Institute
New England Deaconess Hospital
Boston 15, Massachusetts

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We have reported (Behrman and Tanaka, 1959) the oxidation of kynurenic acid (KA) by cell-free preparations obtained from a Pseudomonas (RYS-1) degrading tryptophan via the quinoline pathway. At these meetings, we announced the identification of L-glutamic acid as an end product of this pathway and the stoichiometry of the over-all reaction; one mole of kynurenic acid is oxidized with the consumption of three moles of oxygen, yielding three moles of carbon dioxide together with one mole of L-glutamic acid. Hayaishi et al. (1959) have since confirmed the finding of L-glutamic acid and also reported the isolation of acetic acid.

We should now like to report some of our findings on the intermediate steps involved.

Ammonium sulfate fractionation destroyed the activity of the KA-oxidizing system; the 33-50% fraction was reactivated by the addition of cyanide, a DPNH-generating system, and ferrous ions, whereupon the preparation readily oxidized kynurenic acid, but with the uptake of only two moles of oxygen per mole of substrate and with the concomitant formation of an intensely yellow compound whose extinction coefficient was about 24,000 at 390 mμ. No carbon

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** Postdoctoral Research Fellow of the National Cancer Institute, U.S. Public Health Service.
*** Present address: Institute for Protein Research, Osaka University, Osaka, Japan.

dioxide was evolved.

The observation that both 7-hydroxykynurenic acid (7-OHKA) and xanthurenic acid (8-OHKA) could be slowly oxidized in this system led us to synthesize 7,8-dihydroxykynurenic acid (7,8-diOHKA)*. This compound was rapidly oxidized by the fractionated enzyme with the formation of the 390 mμ compound; the only necessary additions were glutathione and ferrous ions. Only one mole of O_2 was consumed. The stoichiometry of these manometric experiments is summarized in Table I.

TABLE I

The Oxidation of Kynurenic Acid and 7,8-Dihydroxykynurenic Acid by the Fractionated Enzyme

Substrate	O_2 Consumed	μmole O_2/μmole Substrate
8 μmoles KA	15.9 μmoles	1.99
8.9 μmoles 7,8-diOHKA	8.5 μmoles	0.95

For the oxidation of kynurenic acid the main compartment contained: 2.0 ml. enzyme (63.6 mg. protein); 0.2 ml. M/10 KCN; 0.2 ml. M/2 Na lactate; 0.2 ml. lactic dehydrogenase (0.1 mg. protein); 0.2 ml. M/100 DPN; 0.3 ml. M/250 $Fe(NH_4)_2(SO_4)_2$; 0.4 ml. M/10 Tris, pH 7.5. The side arm contained 0.4 ml. M/100 kynurenic acid.

For the oxidation of 7,8-dihydroxykynurenic acid the main compartment contained: 1.0 ml. Tris, M/5, pH 7.5; 1.0 ml. 0.089M 7,8-diOHKA; 0.9 ml. M/50 GSH. The side arm contained 0.3 ml. enzyme (10.6 mg. protein); 0.1 ml. M/2500 Fe^{++}.

In each case, the control flask contained buffer in place of substrate.

By following the increase in optical density at 390 mμ, the oxidation of these compounds could be followed conveniently by spectrophotometry. Table II demonstrates the necessity of various cofactors for the oxidation of KA. Under the conditions specified in Table II, the relative rates of oxidation of KA, 7-OHKA, and 8-OHKA were 1.0, 0.455, and 0.455, respectively. In the presence

* 7,8-Dihydroxykynurenic acid was obtained by a modified Conrad-Limpach synthesis (Surrey and Hammer, 1946) via condensation of diethyl oxalacetate and 2,3-dimethoxyaniline, cyclization in refluxing Dowtherm A to yield ethyl 4-hydroxy-7,8-dimethoxyquinoline-2-carboxylate, and finally hydrolysis in HBr to yield the desired compound. Yellow needles, m.p. greater than 300°.
Calcd. for $C_{10}H_7O_5N \cdot 1/2H_2O$: C: 52.2; H: 3.5; N: 6.1
Found: C: 52.5; H: 3.9; N: 6.3
Its UV spectrum showed three maxima in 2N HCl: $\epsilon_{385\ m\mu}$ 2100; $\epsilon_{325\ m\mu}$ 4600; $\epsilon_{265\ m\mu}$ 35,000.

of GSH and Fe^{++} the relative rate for 7,8-diOHKA was 19.7. Under these latter conditions, there was no oxidation of KA, 7-OHKA, or 8-OHKA. The addition of GSH was required to maintain a steady rate of oxidation of 7,8-diOHKA, but was without effect on the oxidation of KA.

TABLE II

Requirements for the Oxidation of Kynurenic Acid

System	Activity (% of complete system)
Complete	100
No enzyme	0
No DPN	0
No KCN	13
No Fe^{++}	70
No LDH (lactic dehydrogenase)	23
No lactate	25

The complete system contained: 0.2 ml. enzyme; 0.1 ml. M/10 KCN; 0.1 ml. M/50 DPN; 0.1 ml. LDH; 0.1 ml. M/2 lactate; 0.1 ml. M/2500 Fe^{++}. The reaction was started by the addition of 0.1 ml. M/50 KA.

When the oxidation of 7,8-diOHKA was followed by titration at a constant pH of 7.0, two equivalents of base per mole of substrate supplied were consumed From this observation and the manometric data, we propose that the 390 mμ compound is: β(2,6-dicarboxy-4-hydroxypyridyl-3)acrylic acid. The 390 mμ compound readily forms insoluble hydrazones from aqueous solutions. This fact may be accounted for by the reaction of the 4-hydroxy group in its keto form; the formation of hydrazones from certain 4-pyridones has been reported (Petrenko-Kritschenko and Mosseschwili, 1901; Arndt, 1930). Chromatography of the 2,4-dinitrophenylhydrazones of the 390 mμ compound prepared from KA and from 7,8-diOHKA shows identical Rf's in the solvent systems a, c, d, and e recommended by Meister and Abendschein (1956).

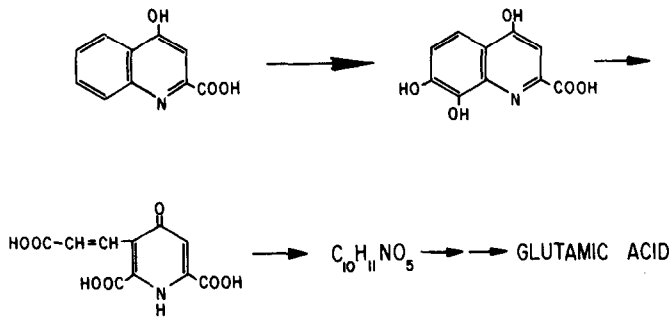
The 390 mμ compound is degraded by the 33-50% ammonium sulfate fraction upon the addition of a TPNH-generating system; the 390 mμ peak disappears rapidly under anaerobic conditions with the simultaneous appearance of a new peak at 293 mμ . We have isolated this product of the reaction, a crystalline compound, whose analysis corresponds to the empirical formula, $C_{10}H_{11}NO_5 \cdot 2H_2O$.

Samples prepared from either KA or 7,8-diOHKA as starting materials are identical according to the following criteria: X-ray powder diagrams, titration curves, UV spectra, and melting behavior.

A full report on the isolation and properties of the 293 mμ compound will appear in a forthcoming paper.

Discussion

That 7,8-diOHKA is an intermediate in this system is supported by the following lines of evidence: its rapid oxidation by extracts of tryptophan-grown cells (extracts of succinate-grown cells are inactive), the manometric stoichiometry of its oxidation, the apparent identity of the 390 mμ compounds prepared from it and from KA, and the identity of the 293 mμ compounds prepared from both substances. We therefore tentatively propose the following scheme to account for these findings:



Addendum

Kuno et al. (1959) have also observed the formation of the 390 mμ compound from KA, although the stoichiometry of their reaction is apparently different from ours. They have found as well that the radioactivity of the glutamic acid isolated from the degradation of KA labelled with C^{14} in the 2-, 3-, and 9-positions is diluted 40-50%. We have obtained similar results using 2- and 3-C^{14}KA.

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