The enzymic formation of 7,8-dihydroxykynurenic acid from kynurenic acid

The enzymic formation of L-glutamic acid from KA by a partially purified enzyme preparation from cells of *Pseudomonas* was previously reported[1,2]. In this communication, we wish to report the isolation and identification of DHKA, a presumable intermediate in this process. The incubation mixture contained, in a final volume of 3,150 ml, 240 μmoles KA, 120 μmoles DPNH, 90 μmoles DPN, 500 μmoles ferrous ammonium sulfate, 225 mmoles Tris buffer (pH 8.0) and 240 ml of enzyme* (960 mg protein). After incubation at 20° for 3.5 h, the reaction proceeded over 80% as judged by the increase in absorbancy at 293 mμ**. The reaction was stopped by the addition of 600 ml 85% formic acid. The acidified reaction mixture was centrifuged and the supernatant was evaporated to dryness at 40° *in vacuo*. The residue was extracted with 130 ml 22 *N* formic acid and the extract, after dilution with 7 vol. water, was passed through a Dowex 1 formate column (6 cm × 3.14 cm^2, 8% cross linkage). After washing with 4 *N* formic acid, the column was eluted successively with 6 *N*, 12 *N* and 22 *N* formic acid. The combined eluates were concentrated to dryness *in vacuo*. DHKA, obtained as a light yellow crystal (21 mg, 85 μmoles) was recrystallized several times from 47.5% HBr. It was identified by m.p. (both authentic*** and isolated materials started to darken at 275° and almost blacken at 290° without melting), analysis (Found: C, 40.24; H, 2.87. Calc. for $C_{10}H_7NO_5 \cdot HBr$: C, 39.76; H, 2.67), the u.v. spectrum§, the infrared spectrum and by paper electrophoresis.

Concurrently, DHKA was synthesized by BEHRMAN AND TANAKA and was found to be rapidly converted to a yellow, as yet unidentified compound by a similar bacterial extract[3]. We have previously reported[4] enzymic synthesis of DHKA starting from 3,4-dihydroxykynurenine[5] by *Pseudomonas* kynurenine transaminase in the presence of α-ketoglutarate and obtained similar results. The present communication provides definitive proof that DHKA is a metabolite of KA by the *Pseudomonas* enzyme.

When a partially purified enzyme preparation was incubated with KA in the presence of reduced pyridine nucleotides (DPNH or TPNH) and oxygen, the accumulation of a compound with λ_{max} at 314 mμ at pH 8.0, was observed. This compound was then dehydrogenated to form DHKA by a DPN-linked dehydrogenase.

Abbreviations: KA, kynurenic acid; DHKA, 7,8-dihydroxykynurenic acid; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

* The supernatant fraction, which was prepared as previously described[1], was fractionated with ammonium sulfate in the presence of 0.01 *M* cysteine. The precipitate obtained between 40 and 50% satn. was collected by centrifugation and dissolved in an amount of 0.02 *M* Tris buffer, pH 8.0, containing 0.01 *M* cysteine, equal to the volume of the original enzyme solution. The enzyme was again fractionated with ammonium sulfate and the precipitate obtained with 50% satn. was dissolved in the same buffer.

** DHKA shows a characteristic u.v. spectrum (λ_{max}, 293 mμ, ε, 18,000) in the presence of excess Fe^{++} and Tris buffer, pH 8.0.

*** An authentic sample of DHKA was synthesized by the reduction and demethylation of ethyl-β-(2-nitro-3,4-dimethoxybenzoyl-)pyruvate (T. TOKUYAMA, S. SENOH AND T. SAKAN, unpublished procedure).

§ Isolated material shows three maxima in 3 *N* HCl; ε_{266}, 34,400; ε_{325}, 5,200; ε_{386}, 2,300. Those of an authentic sample are ε_{266}, 37,200; ε_{325}, 5460; ε_{386}, 2,410.

Although the final identification must await further progress, available evidence indicates that this intermediate compound is clearly distinguishable from either 7- or 8-monohydroxy-KA and is probably identical with or at the same oxidation level as 7,8-dihydrodiol of KA. The mechanism of the conversion of this compound to DHKA, therefore, appears to be analogous to the enzymic formation of catechol from 3,5-cyclohexadiene-1,2-diol catalyzed by a TPN-linked dehydrogenase from rabbit liver[6].

This investigation was supported in part by research grants from the National Institutes of Health (C-4222), the Rockefeller Foundation, the Jane Coffin Childs Memorial Fund for Medical Research and the Scientific Research Fund of the Ministry of Education of Japan.

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Received July 28th, 1960

Biochim. Biophys. Acta, 43 (1960) 356–357

Enzymic formation of acetyl-CoA and CO_2 from glutaryl-CoA

Glutaric acid has been shown to be an intermediate metabolite of lysine degradation[1], and recently to be a product of tryptophan metabolism in the rat[2]. Although several investigators have described the conversion *in vivo* of glutaric acid to acetic acid[2,3], the exact pathway of glutaric acid metabolism is yet to be elucidated. In this communication, we wish to report that 1 mole of glutaric acid is converted to 2 moles of acetate and 1 mole of CO_2 by a partially purified enzyme preparation from *Pseudomonas*, and glutaryl-CoA is proposed to be an intermediate in this process.

Pseudomonas fluorescens (ATCC 11299) was grown as described previously[4], except that 0.1 % glutarate and 0.5 % $(NH_4)_2SO_4$ were used as major carbon and nitrogen sources, respectively. Cell-free extracts were prepared by extracting 5 g of acetone-dried cells with 50 ml 0.02 *M* potassium phosphate buffer, pH 6.8, for 20 min at 0°, followed by centrifugation at 20,000 × *g* for 30 min. The supernatant fraction thus obtained was treated with protamine sulfate.