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The Hydroxylation of Nicotinic Acid by *Pseudomonas fluorescens*

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It has been shown that cell-free extracts of *Pseudomonas fluorescens* strain KB1 convert nicotinic acid into 6-hydroxynicotinic acid and that these extracts do not further oxidize this compound (Hughes, 1955). This paper reports studies in which ^{18}O -labelled oxygen has been used as a tracer to determine the origin of the hydroxyl group. It is concluded that the oxygen appearing in the hydroxyl group of 6-hydroxynicotinic acid is derived from water. Preliminary findings of this work have already been communicated (Hunt, Hughes & Lowenstein, 1957).

EXPERIMENTAL AND RESULTS

Growth of organisms. *Pseudomonas fluorescens* strain KB1 (Kogut & Podoski, 1953) was grown in 8 l. batches of medium containing inorganic salts (Hughes, 1955), yeast extract (Oxo Ltd.) 0.05% and nicotinic acid 0.1%, aerated at the rate of 5–6 l. of air/min. After growth for 16–24 hr. at 20–25° the cells were collected by centrifuging, washed well in 0.9% NaCl and stored at –15° until needed.

Preparation and handling of cell-free extracts. The washed cells were crushed in a Hughes (1951) press at –25° to –20° without the addition of abrasive. After crushing, the very viscous material was homogenized in a stainless-steel Potter-type homogenizer in an equal volume of ice-cold 0.5M- KH_2PO_4 - Na_2HPO_4 buffer, pH 7.0, and centrifuged in 50 ml. cups for 20 min. at 1200 g. The viscous and cloudy supernatant was decanted from the more solidly packed intact cells and cell debris. It was stored at –20° until wanted and then thawed in ice-water, and added to Warburg cups containing the various ice-cooled substrates,

together with several 0.5 cm. glass beads. Oxygen uptake was followed manometrically. After completion of the reaction the liquid contents of the cups were transferred to conical centrifuge tubes.

Isolation of 6-hydroxynicotinic acid. In a previous paper (Hughes, 1955) 6-hydroxynicotinic acid was identified by paper chromatography, and isolated for chemical characterization by solvent-extraction procedures from relatively large-scale incubation mixtures. For the purposes of ^{18}O analysis it was essential to have a method yielding micro-mole quantities of 6-hydroxynicotinic acid in high purity from Warburg flasks. Preliminary experiments indicated that both nicotinic acid and 6-hydroxynicotinic acid could be absorbed and separated quantitatively by stepwise elution on Dowex-2 formate resin. The following procedure was used. After the completion of oxidation, reaction mixtures were transferred to 10 ml. conical centrifuge tubes and deproteinized by heating in a boiling-water bath for 2 min. The precipitate was removed by centrifuging. The supernatant was transferred quantitatively to a column of the formate salt of Dowex-2 ion-exchange resin (10% cross-linked, 200–400 mesh) with a resin bed 1 cm. in diameter and 5 cm. high, and a flow rate of approx. 1 ml./10 min. The column was washed with 5–6 vol. of water until the optical density of the eluate had fallen below 0.100 at 260 m μ . Small amounts of nicotinic acid, which remained after the reaction, were eluted with 0.04N-formic acid until the optical density at 260 m μ had again fallen to less than 0.100. The 6-hydroxynicotinic acid was then eluted with 0.2N-formic acid. Up to 100 μ moles of the compound could be eluted quantitatively in three or four 10 ml. fractions and the compound crystallized out from fractions containing the highest concentrations. The fractions containing 6-hydroxynicotinic acid were pooled and were evaporated slowly with gentle warming, and the 6-hydroxynicotinic acid was recrystallized once from a minimum of water and dried *in vacuo* over P_2O_5 . The ratio $E_{290\text{ m}\mu}/E_{260\text{ m}\mu}$ of the isolated material agreed with that

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found previously by Hughes (1955) for the synthetic material ($E_{280\text{ m}\mu}/E_{260\text{ m}\mu} = 0.5$).

Experiments with ^{18}O -enriched oxygen. Water enriched with ^{18}O was obtained from the Radiochemical Centre, Amersham, Bucks. Oxygen gas enriched with ^{18}O was prepared from water in a small-scale electrolysis apparatus run on a 90v battery, and transferred to an evacuated glass vessel for storage. Warburg flasks were evacuated and filled with ^{18}O -enriched oxygen from this storage vessel.

The isotopic content of samples other than oxygen gas was measured by determining the isotopic ratio 44/46 of carbon dioxide on a mass spectrometer. Oxygen in samples of 6-hydroxynicotinic acid was converted into carbon dioxide by the method of Rittenberg & Ponticorvo (1956). Oxygen in samples of water was converted into carbon dioxide by equilibration with added carbon dioxide according to the method of Cohn (1953). The isotopic content of oxygen gas was measured by determining the isotopic ratio 32/34.

The results of experiments on the enzymic oxidation of nicotinic acid in the presence of ^{18}O -labelled water or oxygen are shown in Table 1. ^{18}O was incorporated into 6-hydroxynicotinic acid when its source was water, and not when it was gaseous oxygen.

An additional experiment was performed as a control for exchange between 6-hydroxynicotinic acid and H_2^{18}O . Each flask contained 0.5 ml. of enzyme (30 mg. of nitrogen approximately), 1 ml. of ^{18}O -labelled water (containing 2.46 atoms % excess of ^{18}O), 1 ml. of 0.015M-6-hydroxynicotinic acid and 0.5 ml. of 0.5M-phosphate buffer, pH 7.0. The final volume was 3 ml., the temperature 30° and the mixture was incubated for 3 hr. 6-Hydroxynicotinic acid was isolated at the end of the incubation and analysed for ^{18}O . Duplicate experiments showed it to contain -0.013 and -0.009 atom % excess of ^{18}O . This shows that under the experimental conditions employed there

was no exchange with oxygen of 6-hydroxynicotinic acid and water.

It is concluded that the oxygen of the hydroxyl group of 6-hydroxynicotinic acid is derived from water and not from gaseous oxygen.

DISCUSSION

The oxidation of nicotinic acid by *P. fluorescens* depends on the adaptive formation of a hydroxylating system specific for nicotinic acid and some halogeno-nicotinic acids (Hughes, 1955). Other hydroxylating systems are known in this organism (Evans, Smith, Linstead & Elvidge, 1951; Hayaishi & Stanier, 1951), but the details of their reaction mechanism have not been studied. For the purpose of this Discussion, hydroxylation is defined as the replacement of a hydrogen atom by a hydroxyl group.

In hydroxylations in which the oxygen from molecular oxygen is incorporated into the hydroxyl group, the net effect is that one atom of each oxygen molecule is incorporated into the hydroxyl group while the other oxygen atom is reduced to water. A reducing agent is therefore required for the complete reaction, whether the hydroxylation proceeds via a dihydrodiol (Young, 1950) (equations 1 and 2), or via a hydroperoxide (Grant, 1956) (equations 3 and 4). The available evidence indicates that the reducing agent is supplied in the form of reduced pyridine nucleotides (Brodie *et al.* 1955; Grant, 1956; Hayano, Saito, Stone & Dorfman, 1956; Kaufman, 1957; Mitoma, 1956), but other substances have been reported which may fulfil this role (Mason, Onopryenko & Buhler, 1957).

In hydroxylations in which oxygen from water is incorporated into the hydroxyl group, the net effect is that the oxygen atom of the water

Table 1. *Enzymic oxidation of nicotinic acid*

Warburg flasks contained 2 ml. of enzyme solution (50–60 mg. of protein/ml.), 1 ml. of 0.2M-sodium nicotinate and, where indicated, 1 ml. of [^{18}O]water (2.84 atoms % excess of ^{18}O). Final volume was 4 ml.; gas phase, oxygen, or, where indicated, oxygen enriched with ^{18}O (2.46 atoms % excess of ^{18}O as determined on a sample of the flask atmosphere at the completion of the reaction). Temperature, 30°. Oxygen uptake was measured manometrically. Experiments were stopped when the rates of oxygen uptake equalled those of controls without nicotinic acid (11 μmoles of $\text{O}_2/\text{hr.}$); the values shown have this blank subtracted.

Expt.	^{18}O -enriched material	Oxygen consumption		6-Hydroxynicotinic acid isolated (mg.)	Atom % excess of ^{18}O	
		Rate ($\mu\text{moles/hr.}$)	Total O_2 consumed (μmoles)		Found	Calc.
1a	Water	30	90	10.0	0.203	0.237
1b	Water	34	100	9.3	0.206	0.237
2a	Oxygen gas	38	84	13.3	0.004	0.820
2b	Oxygen gas	38	84	10.0	0.000	0.820
3a	None	32	94	—	—	—
3b	None	38	94	—	—	—

Free or 'bound'
hydrogen peroxide

molecule is incorporated into the hydroxyl group while two hydrogen atoms are oxidized to water. An oxidizing agent is therefore required for the complete reaction (equation 5). This is true of certain aromatic hydroxylations (Young, 1950) where the reaction involves a hydration followed by a dehydrogenation (equation 5), and also of the β -hydroxylation of carboxylic acids, where the compound is first dehydrogenated and then hydrated. The oxidizing agent generally takes the form of a pyridine nucleotide or a flavoprotein.

The results presented in this paper show that the oxygen in the hydroxyl group of 6-hydroxynicotinic acid is derived from water. No dihydromonohydroxy derivative of nicotinic acid was encountered in our work, but the reaction sequence probably involves a hydration followed by dehydrogenation. Preliminary experiments indicate that the dehydrogenation reaction is linked to the cytochrome system of terminal oxidation. This is supported by the recent findings by Behrman & Stanier (1957) that cytochromes are reduced during oxidation of nicotinic acid.

SUMMARY

1. The oxidation of nicotinic acid to 6-hydroxynicotinic acid by cell-free extracts of *Pseudomonas fluorescens* has been studied with $^{18}\text{O}_2$ and H_2^{18}O as tracers. 6-Hydroxynicotinic acid was found to contain the tracer when the source of ^{18}O was water, but not when it was gaseous oxygen. It is concluded that the oxygen of the hydroxyl group is derived from water.

2. The results are discussed in relation to biological hydroxylation mechanisms.

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Oxidation of Fatty Acids by Cell-Free Extracts of a *Vibrio*

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Cell-free extracts of *Clostridium kluyveri* catalyse the oxidation by molecular oxygen of certain fatty acids with four to eight carbon atoms (Stadtman & Barker, 1949*a, b*). Work with this obligate anaerobe has shed much light on mechanisms by which fatty acids are oxidized biologically (for reviews see Barker, 1951; Stadtman & Stadtman, 1953), but little progress has been made with aerobic micro-organisms since hitherto it has not been possible to prepare from them extracts with biochemical activities comparable with those from *C. kluyveri*. Indeed, studies of enzyme induction in whole cells of *Serratia marcescens* gave results apparently opposed to β -oxidation of saturated fatty acids with 2 to 14 carbon atoms (Silliker & Rittenberg, 1951, 1952). It has been suggested (Stadtman & Stadtman, 1953), however, that in these experiments the enzymes induced may be those responsible for activation of the free fatty acids and not for their oxidation; and Ivler, Wolfe & Rittenberg (1955) have prepared an extract from *Pseudomonas fluorescens* which produced acetate from *n*-decanoate with the consumption of only 1 μ mole of oxygen/ μ mole of substrate, and which further suggested β -oxidation by its cofactor requirements and in the formation of a hydroxamate of *n*-decanoate. The evidence of Webley, Duff & Farmer (1955) strongly supported β -oxidation as the mechanism of breakdown of ω -phenyl-substituted fatty acids by whole cells of *Nocardia opaca*. Various investigations (Dagley & Rodgers, 1953; Dagley & Johnson, 1956; Dagley & Walker, 1956) indicate that the tricarboxylic acid cycle operates for the vibrio used in the present work, and Krebs, Gurin & Eggleston (1952) have suggested that for micro-organisms generally the main function of the cycle is to provide intermediates for use in synthesis. When the carbon

source for growth consists of molecules of two carbon atoms, as in acetate, or if a higher fatty acid is degraded to C_2 units by β -oxidation, this view makes it necessary to postulate an additional mechanism by which the tricarboxylic acid cycle can be replenished with intermediates drained off for synthetic reactions. In this vibrio the necessity for such a mechanism is evident, not simply from speculations concerning the function of the tricarboxylic acid cycle; for it has been demonstrated that cell suspensions synthesize and excrete 2-oxoglutarate when aerated with acetate (Dagley & Patel, 1955). Kornberg & Krebs (1957) have recently proposed the 'glyoxylate by-pass' to account for the replenishment of tricarboxylic acid cycle intermediates from acetate, in which isocitratase (Smith & Gunsalus, 1955; Saz & Hillary, 1956) is coupled with malate synthetase (Wong & Ajl, 1956).

In the present paper we describe the preparation of soluble cell-free extracts of a vibrio that catalyse the degradation of straight-chain saturated monobasic acids (C_6 to C_{10}) to acetate, or to acetate and propionate; coenzymes of the fatty acid spiral (Lynen & Ochoa, 1953; Lynen, 1954) are required for full activity; the reactions of the 'glyoxylate by-pass' are also catalysed. Parts of this work have been briefly reported elsewhere (Dagley, 1956; Callely, Dagley & Hodgson, 1957).

EXPERIMENTAL

Organism and cell-free extracts. The organism was the vibrio used in earlier work (Dagley & Patel, 1955; Dagley & Walker, 1956). For growth at 30° with forced aeration in a mineral-salt medium it utilizes as sole source of carbon certain aromatic compounds and normal saturated mono- or di-carboxylic acids; details of growth media have been given by Dagley & Walker (1956). The phosphate buffer