OBSERVATIONS ON THE OXIDATION OF HALOGENATED NICOTINIC ACIDS*

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Hughes (1) discovered that certain halogenated derivatives of nicotinic acid can be attacked by the inducible enzymes of *Pseudomonas fluorescens* which catalyze the oxidative dissimilation of nicotinic acid. He reported that the 2-fluoro and 5-fluoro derivatives were oxidized by whole cells at approximately the same rate as nicotinic acid, whereas the 5-chloro derivative was more slowly oxidized and the 5-bromo and 6-fluoro derivatives were not attacked at all. The oxidation of 5-fluoronicotinic acid was almost as extensive as that of nicotinic acid itself, whereas the other oxidizable halogenated nicotinic acids gave rise to considerably smaller total oxygen uptakes. Hughes tentatively identified 5-chloro-6-hydroxynicotinic acid as an intermediate in the oxidation of 5-chloronicotinic acid, but did not detect any intermediates in the oxidation of the fluorinated substrates. None of the halogenated nicotinic acids could support bacterial growth. The present paper reports some further observations on the metabolism of halogenated nicotinic acids.

Materials and Methods

The methods employed were those described in the preceding paper (2). Except where otherwise stated, strain N-9 of *P. fluorescens* served as biological material.

2-Fluoronicotinic acid was synthesized by the method of Minor *et al.* (3). We are indebted to Dr. D. E. Hughes for samples of 5-chloro- and 5-bromonicotinic acid, and to Dr. Arthur Roe and to Eli Lilly and Company for samples of 5-fluoronicotinic acid.

Results

Oxidation of Halogen-Substituted Nicotinic Acids—Comparative data on the oxidation of nicotinic acid and some halogenated derivatives by whole

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cells grown at the expense of nicotinic acid, and by the particulate fraction derived from them, are shown in Table I. The data on the oxidation of the 5-substituted nicotinic acids by whole cells agree broadly with those of Hughes (1). Both the 5-fluoro and 5-chloro compounds can be oxidized, the 5-fluoro more rapidly than the 5-chloro. The 5-bromo compound appears to be unoxidizable. Hughes reported that 5-fluoronicotinic acid was oxidized almost as rapidly as nicotinic acid, but in our experiments the relative rates were never greater than 1:4. The total oxygen uptake with 5-fluoronicotinic acid is almost 6 atoms per mole of substrate oxidized, some 75 per cent of the total oxygen uptake with nicotinic acid. The oxidation of 5-chloronicotinic acid was so slow that an accurate value for

TABLE I

Oxidation of Nicotinic Acid and Some Halogenated Derivatives by Whole Cells and by Particulate Fraction of P. fluorescens

Compound	Whole cells*		Particulate fraction	
	Relative rate of oxidation	Total oxygen uptake, atoms per mole substrate	Relative rate of oxidation	Total oxygen uptake, atom per mole substrate
Nicotinic acid	100	7.8	100	1.0
5-Fluoronicotinic acid	25	5.8	27	1.0
5-Chloronicotinic "	10		9	
5-Bromonicotinic "	0		2.5	
2-Fluoronicotinic "	0		0	

* Data corrected for endogenous oxygen uptake.

total oxygen uptake could not be measured. Hughes reported that 2-fluoronicotinic acid is readily oxidized by strain KB1 of *P. fluorescens*, but we found that it could not be attacked at all by strain N-9. Through the kindness of Dr. Hughes, we obtained a subculture of strain KB1, but were unable to demonstrate an oxidation of 2-fluoronicotinic acid by it. We can offer no explanation for this discrepancy.

The inducible enzyme which oxidizes nicotinic acid to 6-hydroxynicotinic acid is located exclusively in the particulate fraction of cell-free extracts (2). As shown in Table I, the particulate fraction can also oxidize the nicotinic acids substituted in the 5 position. The relative rates of oxidation of nicotinic acid, 5-fluoronicotinic acid, and 5-chloronicotinic acid by the particulate fraction correspond closely to the relative rates of oxidation of these substrates by whole cells. The total oxygen uptakes with nicotinic acid and 5-fluoronicotinic acid are identical (1 atom per mole of substrate oxidized). The particulate fraction can oxidize 5-bromo-

nicotinic acid at a very low rate (2.5 per cent of the rate of oxidation of nicotinic acid). Since this fraction consumes no oxygen in the absence of substrate, even a low rate of substrate oxidation can be accurately measured. With whole cells, on the other hand, an oxygen consumption of this magnitude might be obscured by the considerably greater endogenous respiration. It is accordingly possible that whole cells can also oxidize 5-bromonicotinic acid at a very low rate.

Enzymatic Preparation of 5-Fluoro-6-hydroxynicotinic Acid—The oxidation of nicotinic acid catalyzed by the particulate fraction results in the stoichiometric formation of 6-hydroxynicotinic acid (2). By analogy, the enzymatic oxidation of 5-fluoronicotinic acid should yield 5-fluoro-6-hydroxynicotinic acid. In order to confirm this, the reaction was conducted on a larger scale and the product was isolated.

The reaction mixture consisted of 8.0 ml. of a particulate fraction (91 mg. of protein), 8.0 ml. of 0.1 M neutralized 5-fluoronicotinic acid, and 24 ml. of 0.02 M phosphate buffer (pH 6.8). It was incubated at 30° in an Erlenmeyer flask of 250 ml. capacity, which was agitated mechanically to insure adequate aeration. The course of the oxidation was checked by determining oxygen uptake manometrically on an identical reaction mixture at one-fortieth of the scale of the main run. When oxygen uptake ceased in the pilot vessel, the mixture in the main reaction vessel was centrifuged in order to sediment the particles. The supernatant liquid was treated with Norit, heated to the boiling point, and filtered. The hot filtrate was acidified with HCl, and allowed to stand overnight at 5°. The crystalline precipitate was separated by filtration, washed with cold water, and dried. The recovery was 86.6 mg., or 68 per cent of theory for the oxidation of the quantity of substrate furnished to 5-fluoro-6-hydroxynicotinic acid.

Elementary analysis of the isolated compound gave the following results: C 45.65, H 2.65, N 8.86. Calculated values for 5-fluoro-6-hydroxynicotinic acid are C 45.87, H 2.57, N 8.92. The corrected melting point (determined in an evacuated capillary tube) was 353–355° (decomposed). The absorption spectrum is similar to that of 6-hydroxynicotinic acid (Fig. 1). Taken in conjunction with its mode of formation, these data leave little doubt that the isolated compound is 5-fluoro-6-hydroxynicotinic acid. This acid has not been previously described or synthesized, and so a comparison with the properties of synthetic material cannot at present be made.

The enzymatically prepared 5-fluoro-6-hydroxynicotinic acid is oxidized by whole cells at 32 per cent of the rate at which 6-hydroxynicotinic acid is oxidized. The total oxygen uptake is 5.2 atoms per mole of substrate furnished, or 0.6 atom less than the total oxygen uptake at the expense of

5-fluoronicotinic acid. 5-Fluoro-6-hydroxynicotinic acid can also be oxidized by the enzymes of the soluble fraction; the total oxygen uptake is 4 atoms per mole of substrate oxidized, identical with the oxygen uptake by the soluble fraction at the expense of 6-hydroxynicotinic acid.

Ultimate Metabolic Fate of 5-Fluoronicotinic Acid—As shown in Table I, whole cells of *P. fluorescens* N-9 consume approximately 8 atoms of oxygen

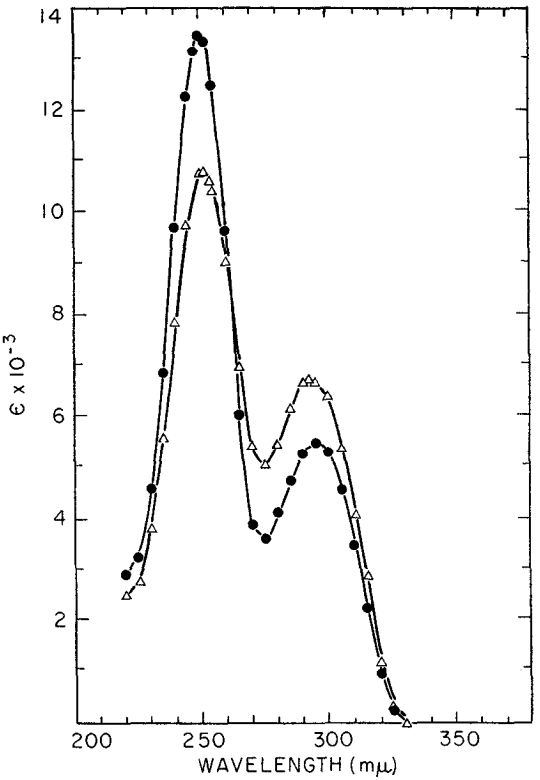

FIG. 1. The absorption spectra of 6-hydroxynicotinic acid (●) and 5-fluoro-6-hydroxynicotinic acid (△) in water at pH 6.8.

per mole of nicotinic acid oxidized. This is less than the theoretical value for complete oxidation (11 atoms per mole of substrate). The discrepancy may be reasonably attributed to the occurrence of some oxidative assimilation, which takes place at the expense of the pyruvic acid into which three of the carbon atoms of nicotinic acid are eventually converted (2). With 5-fluoronicotinic acid, whole cells consume approximately 6 atoms of oxygen per mole of substrate oxidized (Table I). As established by Hughes (1), 5-fluoronicotinic acid cannot serve as a substrate for bacterial growth despite its oxidizability. This suggests that it cannot serve as a source of

assimilable carbon. If such were the case, however, the consumption of 6 atoms of oxygen should reflect an incomplete oxidation of the substrate, with the accumulation of aliphatic end products on the average oxidation level of acetate. The fact that the soluble fraction can oxidize 5-fluoro-6-hydroxynicotinic acid and 6-hydroxynicotinic acid with the same total oxygen uptake indicated that there could be no interference with the reactions which lead in the normal oxidation to the formation of pyruvate (2), and therefore also pointed to the terminal stages of the oxidation as the site of metabolic derangement. Accordingly, an experiment was performed in order to determine whether aliphatic acids accumulate during the oxidation of 5-fluoronicotinic acid by whole cells. Cells from a culture grown at the expense of nicotinic acid were sedimented, washed, and resuspended in 0.02 M phosphate buffer (pH 6.8), at a density of 0.2 mg. of dry weight per ml. Of this suspension, 100 ml. were added to each of two 500 ml. Erlenmeyer flasks. One flask also received 1.0 ml. of 0.1 M 5-fluoronicotinic acid, and the other 1.0 ml. of 0.1 M nicotinic acid. Both flasks were incubated on a shaking machine at 30°. The course of the oxidations was followed by measurement of oxygen uptake on pilot mixtures, prepared at one-fiftieth the scale of the main reaction mixtures. When the oxidations had proceeded to completion in the pilot flasks, the contents of the main reaction flasks were analyzed. The cells were removed by centrifugation, and the supernatant liquid was evaporated to a volume of about 10 ml. Most of the phosphate was removed by precipitation as calcium phosphate, which was filtered off. The filtrate was passed through a column of Amberlite IR-120 in the hydrogen ion form, neutralized with concentrated NH_4OH, and evaporated *in vacuo* to a volume of 2 ml. The concentrate was again acidified with H_2SO_4, and extracted with ether. The ether extract was evaporated, and the residue dissolved in a small volume of water. This solution was subjected to unidirectional paper chromatography, the solvent of Buffa *et al.* (4) being used. The contents of the flask in which 5-fluoronicotinic acid had been oxidized yielded two spots, the R_F values of which were identical with those of known acetic and citric acids. No acidic end products were detected in the contents of the flask initially provided with nicotinic acid.

DISCUSSION

In conjunction with our observations on the pathway for the dissimilation of nicotinic acid (2), the data reported above permit a tentative interpretation of the metabolism of 5-fluoronicotinic acid. Since the 5 position of nicotinic acid is not involved in the initial dissimilatory reactions, fluorine substitution at this position does not prevent attack by the sequence of enzymes responsible for the initial steps in the oxidative dissimilation of nicotinic acid. 5-Fluoronicotinic acid is not as rapidly

metabolized as nicotinic acid itself, probably because the fluorine atom slightly impedes enzyme-substrate combinations. In this connection, it should be noted that substitution in the 5 position of larger halogen atoms has a much more drastic effect on oxidizability: the rate of oxidation of 5-chloronicotinic acid is only 40 per cent of the rate of oxidation of 5-fluoronicotinic acid, and 5-bromonicotinic acid is almost unoxidizable.

The data on the oxidation of 5-fluoronicotinic acid and 5-fluoro-6-hydroxynicotinic acid by both whole cells and extracts indicate that these compounds can be converted to fluorinated analogues of the aliphatic intermediary metabolites normally produced during the oxidation of nicotinic acid. The metabolic fates of the individual carbon atoms of nicotinic acid are known (2); the carbon atom in position 5 eventually becomes the β-carbon atom of pyruvate and the 2-carbon atom of acetate. The oxidation of 5-fluoronicotinic acid should, therefore, eventually yield fluoroacetate. At this point, a serious derangement of intermediary metabolism can be expected to occur. As shown by Peters and his collaborators (5), the condensation of fluoroacetate and oxalacetate to fluorocitrate blocks the operation of the tricarboxylic acid cycle, and thus prevents terminal oxidation. We have found that the oxidation of 5-fluoronicotinic acid by whole cells of *P. fluorescens* results in the accumulation of two acids chromatographically indistinguishable from acetic acid and citric acid. These two acids are presumably the fluoro acids, which cannot be differentiated by chromatography from the unsubstituted acids (4). Unfortunately, the small amount of 5-fluoronicotinic acid at out disposal precluded the accumulation of these acids in amounts sufficient for fluorine analyses. The "lethal synthesis" of fluorocitrate would also explain the failure of 5-fluoronicotinic acid to support growth, despite its ready oxidizability.

If the metabolic behavior of 5-fluoronicotinic acid provides a reliable indication, fluorinated substrates may prove of considerable value in the study of intermediary metabolism. Such compounds should be readily attacked by enzymes which act normally on the unsubstituted compounds, provided that the position of the substitution is not directly involved in the enzyme-catalyzed reaction. Consequently, dissimilation should result in the formation of a fluorine-substituted intermediate which cannot be further metabolized because the site of enzyme action is blocked by the fluorine atom. If the dissimilation results in the eventual formation of fluoroacetate, the entire process of terminal respiration will be blocked by the synthesis of fluorocitrate.

SUMMARY

The bacterial oxidation of halogenated derivatives of nicotinic acid has been reinvestigated. 5-Fluoro- and 5-chloronicotinic acids can be oxidized

by *Pseudomonas fluorescens* after induction in the cells of the enzyme system which oxidizes nicotinic acid. The particulate fraction from induced cells can convert 5-fluoronicotinic acid in good yield to a previously undescribed fluoro compound, 5-fluoro-6-hydroxynicotinic acid. This new acid is readily oxidized, both by whole cells and by the soluble fraction of cell-free extracts. The oxidation of 5-fluoronicotinic acid by cells results in the accumulation of two acids, chromatographically indistinguishable from acetic and citric acids.

These observations can be satisfactorily interpreted by the assumption that 5-fluoronicotinic acid is converted enzymatically to fluoroacetic acid, which is then condensed to fluorocitric acid, with a resulting blockage of terminal respiration.

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