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Microbial transformation of ethylpyridines

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Abstract

Pyridine and its derivatives have been found as pollutants in the environment. Although alkylpyridines constitute the largest class of pyridines contaminating the environment, little information is available concerning the fate and transformation of these compounds. In this investigation ethylpyridines have been used as model compounds for investigating the biodegradability of alkylpyridines. A mixed culture of ethylpyridine-degrading microorganisms was obtained from a soil that had been exposed to a variety of pyridine derivatives for several decades. The enrichment culture was able to degrade 2-, 3-, and 4-ethylpyridine (100 mg/L) at 28° C and pH 7 within two weeks under aerobic conditions. The degradation rate was greatest for 2-ethylpyridine and least for 3-ethylpyridine. Transformation of ethylpyridines was dependent on substrate concentration, pH, and incubation temperature. Studies on the metabolic pathway of 4-ethylpyridine revealed two products; these chemicals were identified by MS and NMR analyses as 4-ethyl-2(1H)-pyridone and 4-ethyl-2-piperidone. 6-Ethyl-2(1H)-pyridone was determined to be a product of 2-ethylpyridine degradation. These results indicate that the transformation mechanism of ethylpyridines involves hydroxylation and reduction of the aromatic ring before ring cleavage.

Introduction

Many pollutants found in the environment contain aromatic structures. Microbial transformation of homocyclic aromatic compounds has been intensively studied (Dagley 1971; Gibson 1984), but the transformation of heterocyclic aromatic compounds has not received much attention (Callely 1978; Berry et al. 1987). Pyridine is one of the most abundant aromatic heterocyclic compounds in the world (Gilchrist 1985). Although compounds containing a pyridine ring exist naturally in the environment in the form of nicotinic acid derivatives and plant alkaloids, pyridine and alkylpyridines are usually of anthropogenic origin. Large scale pollution by *N*- heterocyclic chemicals is associated with the production of synthetic liquid fuels; the use of these fuels may be the most attractive alternative to the current use of fossil fuels in the United States (Myers et al. 1977).

Pyridine and alkylpyridines are found primarily in coal liquefication products and shale oil retort water. Coal tar contains about 0.2% pyridines, mainly methyl-, dimethyl- and ethylpyridines (Gilchrist 1985). Dobson et al. (1985) analyzed the composition of oil shale retort water from Rundle, Australia, and found pyridine and alkylpyridines at concentrations of 1 to 7 mg/L. In addition, pyridine and pyridine derivatives are extensively used in industry as solvents and starting materials or as intermediates for the synthesis of pesticides, dyes, and pharmaceuticals (Sims & O'Loughlin 1989).

In contrast to the biodegradation of other pyridine derivatives, relatively little information is available on the biodegradation of alkylpyridines and even less is known about ethylpyridines. Rogers et al. (1985) reported that ethylpyridines in groundwater were degraded within 17 days under aerobic conditions. Shukla (1975) found that an *Arthrobacter* sp. adapted to 2-methylpyridine required no lag period to grow on 2-ethylpyridine and vice versa. How-

ever, *Corynebacterium* sp. and *Brevibacterium* sp., which can metabolize pyridine, are unable to use 2-ethylpyridine as a substrate (Shukla 1973).

To date the degradative pathway of ethylpyridines has not been investigated. Several reviews have described studies conducted to determine the fate of pyridine and methylated pyridines (Kost & Modyanova 1978; Sims & O'Loughlin 1989; Shukla 1984; Egorov et al. 1985). Watson & Cain (1975) investigated the pathway of pyridine degradation by means of several inhibitors and suggested that reductive ring cleavage is the primary process. *Bacillus* 4 and *Nocardia* Z1 utilized different pathways for the transformation of pyridine and produced succinate semialdehyde and glutarate semialdehyde, respectively, as intermediates. Korosteleva et al. (1981), however, reported that pyridine was degraded through an initial hydroxylation. Kaiser & Bollag (1991) showed that cultures grown on pyridine were able to transform 3-hydroxypyridine with no lag period. This result suggested that 3-hydroxypyridine might be an intermediate product in the pyridine transformation process, also indicating hydroxylation as a degradative mechanism. Shukla (1974) reported that the metabolism of 2-methylpyridine does not follow the usual pattern of stepwise hydroxylation and subsequent ring cleavage reactions that are characteristic of benzoid compounds and pyridine carboxylic acids. He proposed that ring cleavage (between the 2- and 3-position) preceding or accompanied by a ring reduction is a more likely mechanism. Conversely, Korosteleva et al. (1981) reported that the degradation of 3-methylpyridine begins with oxidation of the methyl group to an alcohol and then to a carboxyl group. The metabolism of nicotinic acid, on the other hand, has been shown to proceed through the maleamate pathway (Behrman & Stanier 1957).

Obviously, substituted pyridines may be microbially degraded by a variety of mechanisms. The purpose of this research was to investigate the biodegradability of ethylpyridines, and to elucidate the pathway of ethylpyridine transformation.

Materials and methods

Media

The following mineral salt medium was used in the experiments (g/L): NH_4Cl, 0.54; $MgCl_2 \cdot 6H_2O$, 0.41; $CaCl_2 \cdot 2H_2O$, 0.07; NaCl, 1.166; KCl, 0.18. NH_4Cl was omitted in media which did not contain nitrogen. The salt solution was sterilized by autoclaving. The medium was supplemented with the following sterile stock solutions (per liter of medium): 20 mL phosphate buffer (300 mM), 0.5 mL trace element solution, and 1 mL vitamin solution (Kaiser & Hanselmann 1982). Phosphate buffer was prepared by mixing equimolar solutions of NaH_2PO_4 and K_2HPO_4 to obtain the required pH. The pH of the medium was adjusted to 7 unless otherwise indicated.

Microorganisms and culture conditions

Enrichment techniques were used to obtain a microbial population that degrades ethylpyridines from a soil in Indianapolis, IN. The soil had been exposed to pyridines for several decades. Ethylpyridines served as the sole carbon source. Five-gram soil samples were inoculated into 50 mL of medium containing 100 mg/L 2-, 3-, or 4-ethylpyridine in 160-mL serum bottles. The bottles were incubated at 28° C in the dark. Microbial cultures were transferred into fresh media containing the respective ethylpyridine upon disappearance of the substrate. The liquid cultures were used as stock cultures for the various experiments. Cultures were grown in 50 mL mineral salt medium supplemented with 100 mg/L of 2-, 3-, or 4-ethylpyridine and incubated at 28° C. Microbial growth was monitored by measuring the optical density at 630 nm. The pH effect was tested between pH 5 and 9 at a substrate concentration of 100 mg/L and at 28° C. The influence of temperature (4°, 18°, 28°, and 37° C) was examined at pH 7 and 100 mg/L of the respective ethylpyridine. The effect of substrate concentration (100, 200, 500, and 1,000 mg/L) in the culture medium was determined at pH 7 and 28° C. All treatments were triplicated; the mineral salt medium containing the substrate but lacking inoculum served as a control.

Resting cell experiment

The microorganisms were grown at 28° C in a 1 L Erlenmeyer flask containing 500 mL of the mineral salt medium with 100 mg/L of the appropriate ethylpyridine. The cells were harvested at the exponential growth phase by centrifugation at 4,000 x g for 30 min and washed three times with 50 mM phosphate buffer (pH 7). The cells were then suspended in 100 mL phosphate buffer containing 200 mg/L of the respective ethylpyridines and were incubated for 24 h.

Analytical methods

The disappearance of 2-, 3-, and 4-ethylpyridine was monitored by high-performance liquid chromatography (HPLC); an aliquot of 0.5 mL of the culture medium was mixed with 0.5 mL of methanol, centrifuged at 3,000 x g for 20 min and filtered through a nylon membrane filter (0.45 μM pore size). All samples were stored at - 20° C prior to analysis. HPLC was performed with a Waters Associates (Milford, MA) system, consisting of a Model 6,000A pump and a Rheodyne 7125 injector. A Waters 10 cm x 5 mm Nova-Pak C_{18} column of 4 μm particle size was used. Compounds were separated using a mobile phase of methanol/water (1 : 1, v/v) with 0.068% triethylamine (v/v) buffered with 0.13% KH_2PO_4 (w/v) at a flow rate of 1.5 mL/min. Eluted peaks were monitored at 254 nm using a Lambda-Max 480 LC UV detector (Waters Associates, Milford, MA) connected to a 3392A integrator (Hewlett-Packard Co., Palo Alto, CA).

For analyses of products, the cells were removed by centrifugation at 4,000 x g for 20 min. The supernatant was extracted three times with 100 mL ethyl acetate and the extracts were combined. The solvent was then removed in a vacuum evaporator. The residues were redissolved in ethyl acetate and used for GC-MS analyses. Mass spectral analyses were carried out on a Kratos MS-25 gas chromatograph-mass spectrometer with electron impact ionization (70 eV) and isobutane chemical ionization, using 60 m x 0.32 mm ID 0.25 mm DB-5 column (J & W Scientific, Folsom, CA) programmed from 40° C to 280° C at 6° C/min, using helium as a carrier gas. High resolution mass analysis was conducted on a Kratos MS-9/50 mass spectrometer.

For nuclear magnetic resonance (NMR) analysis, residues were dissolved in methanol and purified by HPLC. The collected fractions from HPLC were extracted again with ethyl acetate which was then removed by evaporation. The residues were dissolved in deuterioacetone and proton NMR spectra were obtained on a 360-MHz FT-NMR spectrometer (Bruker MW-360, Bruker Instruments, Cambridge, MA).

The appearance of ammonium was detected using the Nessler reagent after incubation of the microorganisms and substrate in a medium which contained no ammonium. 0.2 mL Nessler reagent was mixed with 0.4 mL medium and 2 mL distilled water (Clesceri et al. 1989). Absorbance was measured at 425 nm with a spectrophotometer (Spectronic 2000, Bausch & Lomb, Rochester, New York) after 10 min. The interference of calcium, magnesium and iron was eliminated by adding one drop (0.05 mL) of EDTA reagent (50 g disodium ethylenediamine tetraacetate dihydrate dissolved in 100 mL water containing 10 g NaOH).

Chemicals

2-, 3-, and 4-Ethylpyridines (purity, 98%) were obtained from Reilly Chemicals Inc., Indianapolis, IN. Other chemicals used were of analytical grade; solvents used were of HPLC grade.

Results

A mixed microbial culture obtained from the contaminated soil in Indianapolis was incubated with 2-, 3-, and 4-ethylpyridine (100 mg/L), and the transformation of the substrate was monitored. The ethylpyridines disappeared within two weeks; transformation of 2- and 4-ethylpyridine was faster than transformation of 3-ethylpyridine (Fig. 1). Ethylpyridines (100 mg/L) were transformed most rapidly at 28° C and pH 7 (data not shown). The culture can degrade a maximum concentration of 1,000 mg/L 2-ethylpyridine, 200 mg/L 3-ethylpyridine, and 500 mg/L 4-ethylpyridine.

Morphological features of the mixed culture were determined by microscopic observation. The microorganisms were cocci, and motile and nonmotile rods. When inoculated on a nutrient agar plate, the opaque colonies formed exhibited a convex surface and an entire margin. The diameter of the colonies was 1.5 to 2 mm after a two-day incubation.

The formation of a yellow pigment was observed during the transformation of 4-ethylpyridine, but the yellow compound was not produced during the transformation of 2- en 3-ethylpyridine. On a nutrient agar plate, the pigment diffused from the colonies.

Metabolites of 4-ethylpyridine degradation

The transformation of 4-ethylpyridine proceeded with the formation of intermediate products. Characterization of the intermediates was achieved by GC-MS analysis. A peak with a GC retention time of 20.4 min gave a molecular ion $[M^+]$ of m/z 123 and fragment ions at m/z 108 ($[M-15]^+$, 1.1%), m/z 94 $[M-29]^+$, m/z 80 $[M-43]^+$ (Fig. 2A). The molecular ion corresponds to the molecular formula C_7H_9NO (Calculated mass 123.0684, measured mass 123.0691). The frag-

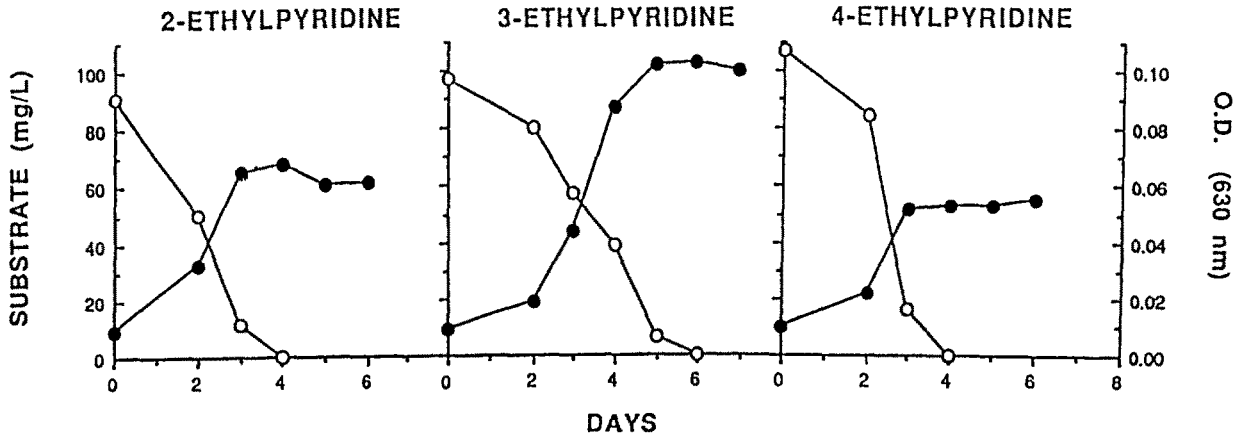
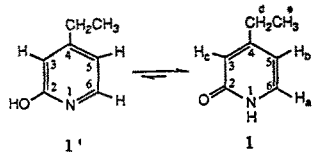
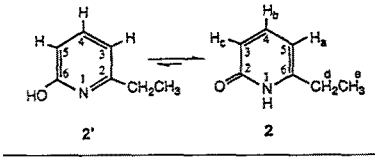


Fig. 1. Degradation of 2-, 3-, and 4-ethylpyridine vs. microbial growth: –o– substrate, –•– optical density.

Table 1. Proton NMR data for the degradation products of ethylpyridines.*

Compound	Chemical shift, ppm	Splitting and integral*	Proton assigments
 1' 1	7.45	d, J = 6.7, 1H	H_a
	6.25	dd, J_1 = 6.7, J_2 = 1.6, 1H	H_b
	6.36	d, J = 1.6, 1H	H_c
	2.51	q, J = 7.6, 2H	H_d
	1.18	t, J = 7.6, 3H	H_e
 2' 2	6.05	d, J = 6.9, 1H	H_a
	7.38	dd, J_1 = 6.9, J_2 = 9.2, 1H	H_b
	6.21	d, J = 9.2, 1H	H_c
	2.58	q, J = 7.6, 2H	H_d
	1.24	t, J = 7.6, 3H	H_e

* d = doublet; dd = doublet of doublets; t = triplet; q = quartet.

ments are due to the losses of CH_3, CH_3CH_2, and HOCN, respectively. Chemical ionization confirmed the molecular weight by providing an MH^+ at m/z 124. Additional characterization of the structure was obtained by proton nuclear magnetic resonance (NMR) analysis (Table 1). The NMR spectrum clearly showed the presence of an ethyl group and the three aromatic hydrogens. The coupling pattern of hydrogens on the pyridine ring indicated that the proton at the 2-position was missing. This intermediate was determined as 4-ethyl-2-hydroxypyridine, 1′, which is known to exist predominantly as its tautomer 4-ethyl-2(1H)-pyridone, 1 (Schofield 1967; Joule & Smith 1978; Gilchrist 1985). The NMR data match well with the homologue, 2-hydroxy-4-methyl-pyridine (4-methyl-2(1H)-pyridone) which shows signals at 6.06 (d), 6.17 (s), and 7.29 (d) (Pouchert & Behnke 1993).

The peak at a retention time of 17.0 min by GC analysis gave a molecular ion $[M^+]$ of m/z 127 and fragments at m/z 112 ($[M\text{-}15]^+$, 1.7%) m/z 99 $[M\text{-}28]^+$, and m/z 84 ($[M\text{-}43]^+$, 1.8%) (Fig. 2C). This molecular ion is consistent with the molecular formula $C_7H_{13}NO$ and the fragments are the result of losing CH_3, CO, and O = C-NH groups. The low intensity (8%) molecular ion supports the fact that compound 3 is not aromatic, because aromatic compounds (see compounds 1′ and 2′) show more intense molecular ions. Chemical ionization provided an MH^+ at m/z 128, confirming the molecular weight of 127 Da. On the basis of these spectral data and the isolation of com-

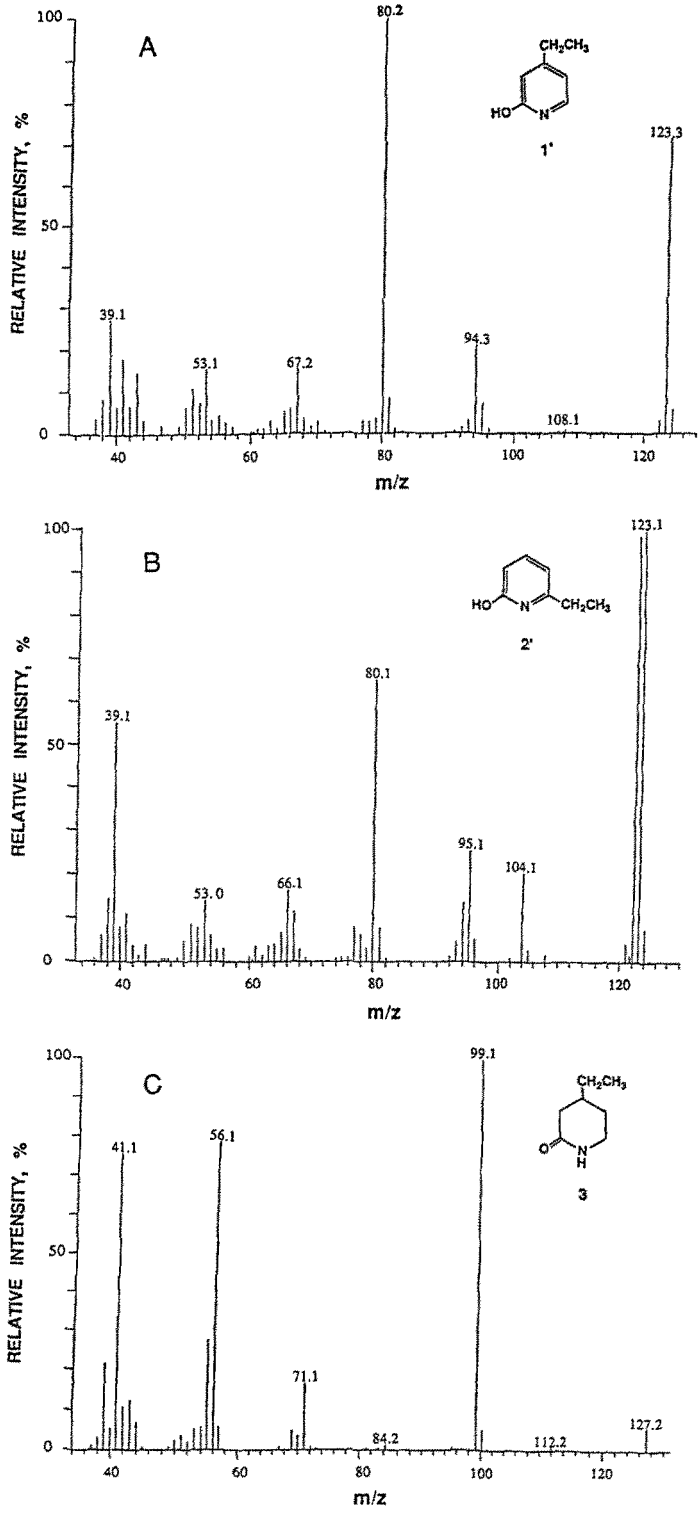


Fig. 2. Mass spectrum of (A) 4-ethyl-2-hydroxypyridine (M.W. 123), (B) 2-ethyl-6-hydroxypyridine (M.W. 123), and (C) 4-ethyl-2-piperidone (M.W. 127).

pound 1 from the same reaction, this product structure is proposed as 4-ethyl-2-piperidone, 3. Lack of sufficient material prevented NMR analysis of compound 3. Experiments were performed in which the mixed culture was inoculated into a medium which did not contain nitrogen. Since it was possible to detect the formation of ammonium in this medium, it may be concluded that the nitrogen originated from the pyridine ring and that ring-fission of the pyridine ring took place.

Metabolites of 2-ethylpyridine degradation

A new product was found in resting cell experiments when 2-ethylpyridine was used as the substrate. This product gave a peak in GC analysis with a retention time of 20.3 min and a molecular ion of 123 (confirmed by chemical ionization), consistent with the molecular formula C_7H_9NO (Fig. 2B). The fragments at m/z 122, m/z 104, m/z 95, and m/z 80 result from the loss of H, H_2O and H, $CH_2 = CH_2$, and HOCN groups, respectively. The loss of the HOCN group indicated that the oxygen atom was at the 6-position. Further confirmation of the structure was obtained by NMR analysis (Table 1) which showed the presence of the intact ethyl group indicating that the initial transformation did not involve the side chain. The presence of three aromatic hydrogens was determined by integration, the chemical shifts and coupling patterns allowed assignment of these to ring positions 3, 4, and 5. Thus the product was identified as 2-ethyl-6-hydroxypyridine, 2′, which exists primarily as its tautomer, 6-ethyl-2(1H)-pyridone, 2. The methyl analogue of 2 has aromatic proton signals at 6.07 (d), 6.41 (d), and 7.37 (m) (Pouchert & Behnke 1993).

Discussion

The presence of an alkyl substituent on the pyridine ring provides two possible pathways of degradation: oxidation of either the aromatic ring or the alkyl substituent. Results of this research indicate that the initial step of ethylpyridine degradation involves the oxidation of the aromatic ring. The transformation of both 2- and 4-ethylpyridine was initiated by hydroxylation on the pyridine ring. An additional ring reduction product, 4-ethyl-2-piperidone, was also found as an intermediate of the transformation of 4-ethylpyridine. Ring cleavage and degradation also occurred since ammonium could be detected in the culture medium. Based on these results, a scheme of the degradation of these two ethylpyridines by a mixed culture is proposed (Fig. 3).

Hunt et al. (1958) have reported that nicotinic acid is transformed via hydroxylation to 6-hydroxynicotinic acid and that the oxygen of the hydroxyl group is derived from water. Similar observations have been reported for quinoline which is converted to 2-hydroxyquinoline by *Pseudomonas putida* (Bauder et al. 1990). In this case as well, water is the source of oxygen used for the hydroxylation reaction under aerobic conditions. It is well possible that the oxygen appearing in the hydroxyl group of the transformed ethylpyridines also derived from water.

Transformation of homocyclic aromatic compounds involves a second hydroxylation on the aromatic ring before ring cleavage. For instance, Gibson et al. (1973) reported that ethylbenzene is mostly oxidized to a *cis*-dihydrodiol by *Pseudomonas putida*, and only 2% of the oxidation products are the product of oxidation on the side chain. Transformation of some substituted pyridines, such as picolinic acids and hydroxypyridines (pyridones), are reported to form dihydroxylated intermediates before ring cleavage (Shukla 1984; Sims & O'Loughlin 1989). However, formation of dihydroxylated intermediates during alkylpyridine degradation has not been reported.

The idea that reduction steps are involved in aerobic degradation of pyridines has been proposed by several researchers (Shukla 1974; Watson & Cain 1975) although the intermediates were not isolated. The ability of nicotinamide nucleotides to serve as universal redox coenzymes in biological systems may underlie the reduction of the pyridine ring. Microorganisms may use nicotinamides for the degradation of pyridine derivatives as follows: $XH + H_2O + NAD^+ \rightarrow XOH + NADH + H^+$ (X = pyridine ring), $NADH + H^+ + 1/2O_2 \rightarrow NAD^+ + H_2O$. The net result is oxidation of the pyridine with water as the source of the oxygen atom.

It should be noted that hydroxypyridines are subject to tautomerism which occurs via proton transfer between the molecule's oxygen and nitrogen atoms (Schofield 1967; Joule & Smith 1978; Gilchrist 1985). While 3-hydroxypyridine cannot exist in a pyridone form, in almost all solvents, 2- and 4-hydroxypyridines exist predominantly as the pyridones because of polar solvation effects (Joule & Smith 1978; Newkome & Paudler 1982). Thus, products exist predominately in the pyridone forms, **1** and **2**, not in the hydroxypyridine forms, **1′** and **2′**. However, the hydrox-

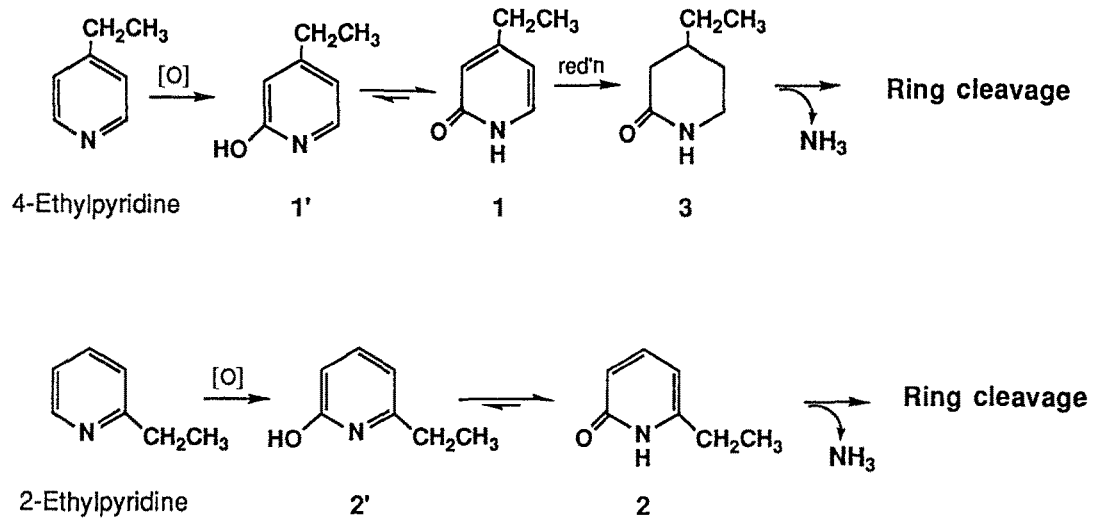


Fig. 3. Proposed pathway of 2- and 4-ethylpyridine degradation by a mixed culture under aerobic conditions.

ypyridine tautomer appears to be more stable in the gas phase (Gilchrist 1985), and consequently hydroxyethylpyridines, **1′** and **2′** , would be the form that is present in the mass spectrometer.

2-Ethyl-6-hydroxypyridine, **2′**, and 4-ethyl-2-hydroxypyridine, **1′**, showed different mass spectral fragmentation patterns. 2-Ethyl-6-hydroxypyridine exhibited a strong peak at $[M\text{-}1]^+$, whereas the loss of hydrogen is not significant in the spectrum of 4-ethyl-2-hydroxypyridine. Biemann (1962) has shown the same trend in mass spectral analysis of isomeric ethylpyridines with 2-ethylpyridine showing a much more intense $[M\text{-}H]^+$ than 4-ethylpyridine. The elimination of a CH_3CH_2 group was favored for 4-ethyl-2-hydroxypyridine, but 2-ethyl-6-hydroxypyridine lost $CH_2 = CH_2$, presumably via a McLafferty rearrangement involving nitrogen. The loss of HOCN to give m/z 80 was a major fragmentation pathway for both **1′** and **2′** and proves that the hydroxy group is *ortho* to the nitrogen of the heterocycle.

The slow transformation of 3-ethylpyridine is consistent with the observation that substituents in the *meta* position make compounds more resistant to microbial attack. Sims & Sommers (1986) observed that both 2- and 4-methylpyridine (100 mg/L) were degraded within 24 days by indigenous soil microorganisms, whereas 3-methylpyridine (100 mg/L) persisted in soil suspensions.

A yellow pigment was produced during the transformation of 4-ethylpyridine, but not during the transformation of 2- and 3-ethylpyridine. The formation of the yellow pigment during metabolism of pyridine and 2-methylpyridine has been observed by several investigators (Shukla 1974; Sims & O'Loughlin 1992), and the yellow pigment has been identified as riboflavin (vitamin B_2) and its derivatives. The role of the yellow pigment is unknown.

To date alkylpyridine metabolic pathways have all been investigated using methylated pyridines as discussed in the introduction. This study represents the first investigation of the metabolic pathway of ethylpyridines and identification of intermediate products. The data indicate that 2- and 4-ethylpyridine were rapidly transformed by adapted microorganisms via a mechanism involving an initial ring hydroxylation followed by ring cleavage.

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