Metabolism of pyridine and 3-hydroxypyridine under aerobic, denitrifying and sulfate-reducing conditions

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Summary. The transformation of pyridine and several hydroxypyridines was investigated under different physiological conditions. Pyridine and 2-, 3- and 4-hydroxypyridine were metabolized aerobically within 9 days in a mineral salt medium inoculated with 10% sewage sludge. Under anaerobic conditions in the presence of nitrate or sulfate, pyridine and 3-hydroxypyridine were metabolized after a lag period of 2 months, while no transformation of 2- or 4-hydroxypyridine was detected. Experiments with ^{14}C-labeled-pyridine showed that under denitrifying as well as under sulfate-reducing conditions pyridine was oxidized to carbon dioxide. Disappearance of nitrate and sulfate under anaerobic conditions was 79% and 85%, respectively, of the amount predicted from the stoichiometric equation for the complete transformation of pyridine.
Key words. Pyridine metabolism; hydroxypyridines; anaerobiosis; denitrifying conditions; sulfate-reducing conditions.

Increasing amounts of aromatic compounds are produced by various industries every year[1]. Many of these substances are xenobiotic compounds and some are difficult to degrade. Most of them are hazardous for humans even at low concentrations. Two-thirds of the known organic compounds belong to the group of heterocyclic chemicals[2]. Although it has been recognized in recent years that recalcitrant and toxic groundwater contaminants often have heterocyclic structures[3], the knowledge about the fate of these compounds in a certain environment is still unknown[4]. A better knowledge of the microbial, physical and chemical degradation potential would allow predictions of the fate of xenobiotic compounds in a natural ecosystem[5]. Aerobic metabolism of aromatic substances has already been studied intensively and has been reviewed by Bayly and Barbour[6] and Gibson and Subramanian[7]. However, the metabolism of natural and xenobiotic aromatic chemicals, especially the transformation of heterocyclic compounds such as pyridine derivatives, under anaerobic conditions has not been investigated until recently[4].

Pyridine and its derivatives are found in nature in the form of coenzymes, such as nicotinamides, pyridoxyl derivatives and plant alkaloids. Also, natural derivatives such as nicotine, anabasin and cavadin have been used as insecticides for many years[8]. Pesticides such as paraquat, diquat and picloram, as well as certain industrial solvents are also pyridine derivatives[9]. Furthermore, pyridine is found in effluents derived from the mining and processing of coal and shale[10,11].

Contamination of the environment by pyridine and its hydroxylated and alkylated derivatives is a serious problem, since these compounds have been observed to persist in surface and subsurface soils and may be hazardous to human health[12,13]. Pyridine and its alkyl isomers have also been found in groundwater, reducing the potability of water due to their undesirable taste and odor[11,14].

Much is known about the physical and chemical characteristics of pyridine, and its aerobic degradation is well characterized. For instance, several pure cultures of aerobic microorganisms, such as *Brevibacterium* sp., *Corynebacterium* sp.[15,16] and *Micrococcus* sp.[17], have been demonstrated to utilize pyridine as a growth substrate. In contrast, almost nothing is known about the microbial metabolism of pyridine and its derivatives under anaerobic conditions[18,19]. Since they are important representative heterocyclic compounds, pyridine and its hydroxylated derivatives were selected as model compounds in our study of the biotransformation of heterocyclic compounds under anaerobic conditions.

Materials and methods

Media and culture conditions. The following salt medium was used for growth of microorganisms under aerobic conditions (in g/l): NH_4Cl, 0.54; $MgCl_2 \cdot 6H_2O$, 0.41; $CaCl_2 \cdot 2H_2O$, 0.07; NaCl, 1.17; KCl, 0.18; and resazurin 0.001. Resazurin was also added aerobically as a control to ascertain that it had no adverse effect. The salt solution was acidified with 0.6 ml 2 M HCl per liter and subsequently autoclaved. After cooling the medium was supplemented with the following sterile stock solutions (per liter of medium): 20 ml 300 mM potassium-phosphate; 0.5 ml trace elements and 1 ml vitamins[20], and 2.5 ml 100 mM sulfide and 30–50 mg carbon substrates. The substrates (pyridine and 2-, 3-, and 4-hydroxypyridine) were analytical grade and purchased from Sigma Chemical Company, St. Louis, MO.

For growth of microorganisms under sulfate-reducing conditions the medium was modified as follows: NaCl was replaced by Na_2SO_4 (2.84 g/l). After autoclaving, the medium was cooled under an oxygen-free gas phase to avoid redissolving of oxygen in the medium. Then the medium was supplemented with the following sterile stock solutions (per liter of medium): 20 ml 300 mM potassium phosphate; 0.5 ml trace elements and 1 ml vitamins[20]. N_2 was passed through the solution to completely replace oxygen and the medium was amended

with 2.5 ml 100 mM sulfide, 10 mg sodium dithionite and 30–50 mg carbon substrates.

For growth of microorganisms under denitrifying conditions, the following salt medium was used (in g/l): K_2HPO_4, 0.87; KH_2PO_4, 0.54; KNO_3, 0.3. After autoclaving and cooling under an oxygen-free gas phase, the medium was supplemented with the following sterile stock solutions (per liter of medium): 2 ml 400 mM $MgSO_4 \cdot 7H_2O$; 0.5 ml trace elements and 1 ml vitamins[20] and 2.5 ml 100 mM sulfide, 10 mg sodium dithionite and 30–50 mg carbon substrates.

Mixed culture isolation. For enrichment with pyridine or hydroxypyridines as sole carbon source, media for sulfate or nitrate reducers were inoculated with sewage sludge (10%) and incubated anaerobically at 28 °C. Upon transformation of the substrates, the cultures were transferred to fresh medium. After 6 such transfers, all solid particles from the sewage sludge were removed. The cultures contained at least 3–4 different microorganisms (as determined by microscopic observation).

Analytical methods. Substrates were analyzed on a high-performance liquid chromatograph (HPLC) equipped with a 6000 A pump and U6K injector (Waters Associates, Inc., Milford, MA) using a reverse phase column (C_{18}, particle size 5 µm, 15 cm × 4.6 mm, Supelco Inc., Bellefonte, PA). The mobile phase was a methanol/water mixture (2/8, v/v) with 0.068% triethylamine (v/v) buffered with 0.13% KH_2PO_4 (w/v) (pH 6.8). The flow rate was 1.5 ml/min. Compounds were detected by ultraviolet absorption at 254 nm using a Lambda-Max 480 LC spectrometer (Waters Associates, Inc., Milford, MA) connected to a 3392 A integrator (Hewlett-Packard Co., Palo Alto, CA).

All substrates were measured and determined by HPLC and quantified using the external standard method. Samples (0.5 ml) were removed periodically from serum bottles, mixed with methanol (1/1), v/v) and stored at −20 °C. Prior to injection onto HPLC, samples were centrifuged at 10,000 × g for 10 min at 4 °C and the supernatants were filtered through a nylon filter (0.45 µm pore size, MSI, Fisher Scientific Co., Fair Lawn, NJ).

Mineralization of ^{14}C-labeled pyridine was measured by trapping the $^{14}CO_2$ which was released during pyridine degradation. Experiments were performed in anaerobic culture tubes (18 × 150 mm) sealed with butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ). The culture medium (10 ml) was amended with 0.45 mM pyridine and 1.7×10^3 Bq [2,6-^{14}C]-pyridine (specific activity of 6.7×10^8 Bq/mM and of 98% purity, Amersham Corp., Arlington, IL). Samples were then incubated under anaerobic conditions at 28 °C and evolved $^{14}CO_2$ was trapped on 2-phenylethylamine-soaked chromatographic paper (Whatman No. 1) after adding 1 ml 6 M HCl. Radioactivity was measured after 24 h using Scintiverse scintillation cocktail (Fisher Scientific Co., Fair Lawn, NJ) with a Beta Trac 6895 Liquid Scintillation Counter (Tracor Analytic Corporation, Elk Grove Village, IL). The efficiency of the assay was 46% and all data reported were corrected accordingly.

Sulfide, nitrate and ammonium were measured colorimetrically. Sulfide was determined by the methylene blue method according to Gilboa-Garber[21] nitrate by the phenoldisulfonic acid method[22], ammonium according to Nessler[23], and carboxyl intermediates according to Miwa et al.[24].

Results

A) *Transformation of pyridine and hydroxypyridines under aerobic conditions.* Under aerobic conditions pyridine, 2-, 3- and 4-hydroxypyridine (0.45 mM), each individually present as the only carbon source, were transformed by a 10% sewage sludge inoculum within 9 days. 2-Hydroxypyridine was transformed after a lag period of 2 days, whereas the metabolism of 3- and 4-hydroxypyridine required a lag period of 3–4 days. Pyridine transformation occurred after a lag period of 4 days, and the initial concentration of 0.42 mM disappeared within 5 days (fig.).

Experiments with ^{14}C-labeled pyridine under aerobic conditions showed that 71% of the added radioactivity was converted to $^{14}CO_2$, whereas under anaerobic conditions the amount of labeled CO_2 produced was higher. The amount of radioactivity remaining in the medium was higher when pyridine metabolism occurred under aerobic conditions than under anaerobic conditions (table 1).

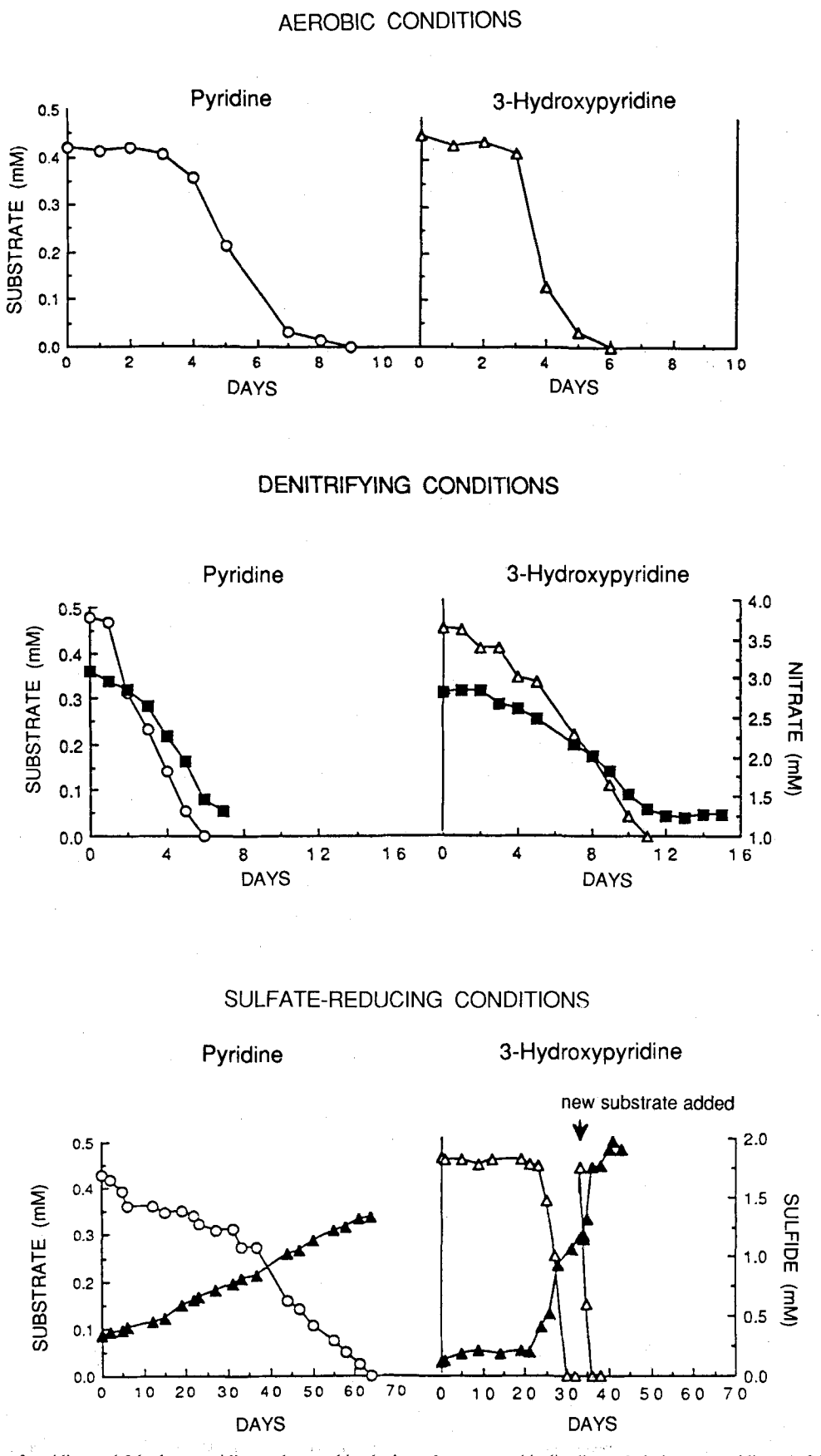
B) *Transformation of pyridine and 3-hydroxypyridine under denitrifying conditions.* Enrichment cultures were obtained under denitrifying conditions after 6 transfers of the pyridine- or 3-hydroxypyridine-metabolizing cultures initially inoculated with 10% sewage sludge. The mixed cultures contained at least three microorganisms, two cocci and a rod. The isolated cells transformed 0.36 mM pyridine within 6 days (fig.). Growth occurred at temperatures between 20 °C and 40 °C, and a pH range between 6.0 and 8.5.

Experiments showed that 79% of the nitrate necessary for complete stoichiometric degradation of pyridine was reduced (fig.). During the transformation of 0.36 mM pyridine, 0.32 mM ammonium were produced. Pyridine metabolism under denitrifying conditions seems to follow equation I, listed in table 2.

This pyridine-transforming culture was also able to transform 3-hydroxypyridine. 88% of the expected amount of nitrate required for complete stoichiometric

Table 1. Recovery of $^{14}CO_2$ on mineralization of ^{14}C-pyridine by a mixed culture obtained from anaerobically digested sludge

	Radioactivity recovered (%)		
	$^{14}CO_2$	In medium	Total
Aerobic conditions	71	19	90
Anaerobic conditions			
Denitrifying conditions	95	10	105
Sulfate-reducing conditions	88	6	94



Transformation of pyridine and 3-hydroxypyridine under aerobic, denitrifying and sulfate-reducing conditions by a mixed culture enriched from anaerobically digested sludge. ○ pyridine, △ 3-hydroxypyridine, ■ nitrate, ▲ sulfide.

Table 2

Denitrifying conditions	
A) Pyridine transformation	
$5\ C_5NH_5 + 22\ NO_3^- + 9\ H_2O + 2\ H^+ \rightarrow 25\ HCO_3^- + 5\ NH_4^+ + 11\ N_2$	I
B) 3-Hydroxypyridine transformation	
$C_5NOH_5 + 4\ NO_3^- + 2\ H_2O \rightarrow 5\ HCO_3^- + NH_4^+ + 2\ N_2$	II
Sulfate-reducing conditions	
A) Pyridine transformation	
$4\ C_5NH_5 + 11\ SO_4^{-2} + 16\ H_2O \rightarrow 20\ HCO_3^- + 4\ NH_4^+ + 11\ HS^- + 5\ H^+$	III
B) 3-Hydroxypyridine transformation	
$2\ C_5NOH_5 + 5\ SO_4^{-2} + 8\ H_2O \rightarrow 10\ HCO_3^- + 2\ NH_4^+ + 5\ HS^- + 3\ H^+$	IV

transformation of 3-hydroxypyridine to carbon dioxide was reduced (fig.). During the transformation of 0.44 mM 3-hydroxypyridine, 0.36 mM ammonium were produced. The experimentally determined amounts are thus in agreement with the stoichiometry for a complete transformation of 3-hydroxypyridine under denitrifying conditions (fig.; stoichiometric equation II in table 2).

C) *Transformation of pyridine and 3-hydroxypyridine under sulfate-reducing conditions.* Sulfate-reducing pyridine and 3-hydroxypyridine cultures, obtained in a manner similar to that used to isolate denitrifying pyridine-degraders, consisted of at least four microorganisms. Microscopic observations identified two types of coccoidal cells, non-motile curved rods and motile spirals which were present in large numbers. The isolated mixed culture transformed 0.43 mM pyridine within 64 days (fig.). Growth occurred at temperatures ranging from 20 °C to 40 °C and at pH values between 6.0 and 8.0.

Analysis showed that 85% of the sulfate necessary for a complete stoichiometric transformation of pyridine was reduced (fig.). The transformation of pyridine under sulfate-reducing conditions follows stoichiometric equation III (table 2).

Similar to the pyridine-metabolizing culture under denitrifying conditions this mixed culture was also able to convert 3-hydroxypyridine. 91% of the expected sulfate amount necessary for complete stoichiometric transformation of 3-hydroxypyridine to carbon dioxide was reduced (fig.).

The transformation of 3-hydroxypyridine under sulfate-reducing conditions follows stoichiometric equation IV (table 2).

Discussion

The fast transformation of pyridine and monohydroxylated pyridines under aerobic conditions are in accord with the observations of Sims and Sommers[25] who showed that pyridine and monohydroxylated pyridines were rapidly converted by soil microorganisms in the presence of oxygen. Not all carbon in the substrate was dissimilated to carbon dioxide; part was assimilated into the biomass. This appears to be the main reason why the disappearance of electron acceptors was always lower than the expected theoretical amount. Our experiments show that under aerobic conditions a greater amount of the carbon substrate is incorporated into biomass than under anaerobic conditions. In the absence of oxygen usually less than 10% of the carbon from the substrate is assimilated into the cell biomass[26]; the same phenomenon was observed under denitrifying and sulfate-reducing conditions.

Cultures grown on pyridine were also able to transform 3-hydroxypyridine with no lag period. 3-Hydroxypyridine might therefore be an intermediate product in the pyridine transformation pathway, as hydroxylation is often involved in the degradation of aromatic compounds[27,28]. In contrast to our observations, Shukla[16] reports that a *Corynebacterium* sp. and a *Brevibacterium* sp. capable of growing on pyridine are not able to transform 3-hydroxypyridine. Houghton and Cain[8] report that *Nocardia* Z1 grown on pyridine is able to transform 3-hydroxypyridine at a much lower rate and therefore 3-hydroxypyridine is not suspected as an intermediate product during the transformation of pyridine by *Nocardia* Z1.

Sims et al.[17] note that under aerobic conditions 79% of the nitrogen in the pyridine ring is released into the medium as ammonium. Bak and Widdel[29] also suggest that indole, another heterocyclic compound, is completely converted to carbon dioxide and ammonium under sulfate-reducing conditions. The latter authors mention that these pyridine enrichment cultures grow very slowly. The same was true for our enrichment cultures with complete degradation of 0.43 mM pyridine requiring approximately 64 days. Thus, the sulfate-reducing bacteria required 10 times longer to degrade pyridine than did cultures under denitrifying conditions. Even cultures that had successfully degraded pyridine showed no significant increase in the transformation of pyridine when new substrate was added (data not shown).

Experiments with ^{14}C-labeled pyridine showed that pyridine was also completely degraded to carbon dioxide under sulfate-reducing conditions: 88% of the radioactivity was converted to $^{14}CO_2$ (table 1). Similar to the denitrifying cultures, cultures capable of degrading pyridine under sulfate-reducing conditions were also able to metabolize 3-hydroxypyridine.

Degradation of 3-hydroxypyridine under sulfate-reducing conditions was more rapid than the transformation of pyridine; 0.45 mM 3-hydroxypyridine was metabolized by a mixed culture within 30 days (fig.). A polar functional group on a heterocyclic ring facilitates the anaerobic degradation of the respective compound[3]; this also seems to be true for the transformation of 3-hydroxypyridine under sulfate-reducing conditions. With sulfate as well as with nitrate as electron acceptor no intermediate products could be detected during the trans-

formation of pyridine or 3-hydroxypyridine. It is likely that during the transformation of these substrates compounds are produced that contain carboxyl groups. The failure to detect carboxylated intermediate products may have been due to concentrations below the detection limit even though the method used would allow detection of carboxylated compounds in concentrations as low as 1 µmol/l[24]. The fact that no carboxylated end products (e.g. acetate) accumulated agrees with our radiolabeled experiments where it has been shown that the pyridine-carbon is efficiently transformed to $^{14}CO_2$.

In conclusion, microorganisms obtained from digested sewage sludge were able to oxidize pyridine and 3-hydroxypyridine to carbon dioxide under anaerobic conditions when an electron acceptor like nitrate or sulfate was available.

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Sexual dimorphism in the defensive secretion of a carabid beetle*

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Summary. The defensive secretions of male and female *Oodes americanus* display striking qualitative differences. Altogether 13 carboxylic acids were identified in the secretions of the two sexes. Methacrylic, crotonic, and tiglic acids are produced exclusively by the female; the male lacks these unsaturated components, but produces their saturated analogs. 2-Methylbutyric acid is a major component produced by both sexes. Shared components also include hexanoic, (*E*)-2-hexenoic, benzoic, and (*E*)-2-octenoic acid, of which the latter two had not previously been reported from carabid beetles.
Key words. Carabidae; defensive secretion; sexual dimorphism; carboxylic acids.

The chemistry and morphology of the defensive glands of carabid beetles have been intensely investigated. Over 300 species have been studied[1], and a wide range of chemicals has been characterized, including carboxylic acids, esters, carbonyl compounds, and aromatic components[2–5]. In no species had evidence been presented indicating that the secretion might differ in males and females. We here report the demonstration of sexual dimorphism in the defensive secretion of *Oodes americanus*, a member of the carabid tribe Oodini of the supertribe Callistitae[6], a lineage of relatively recent evolutionary origin within the family Carabidae.