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Oxidation of Nitrapyrin to 6-Chloropicolinic Acid by the Ammonia-Oxidizing Bacterium *Nitrosomonas europaea*

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Suspensions of *Nitrosomonas europaea* catalyzed the oxidation of the commercial nitrification inhibitor nitrapyrin [2-chloro-6-(trichloromethyl)-pyridine]. Rapid oxidation of nitrapyrin (at a concentration of 10 μM) required the concomitant oxidation of ammonia, hydroxylamine, or hydrazine. The turnover rate was highest in the presence of 10 mM ammonia (0.8 nmol of nitrapyrin per min/mg of protein). The product of the reaction was 6-chloropicolinic acid. By the use of $^{18}O_2$, it was shown that one of the oxygens in 6-chloropicolinic acid came from diatomic oxygen and that the other came from water. Approximately 13% of the radioactivity of [2,6-^{14}C]nitrapyrin was shown to bind to cells. Most (94%) of the latter was bound indiscriminately to membrane proteins. The nitrapyrin bound to membrane proteins may account for the observed inactivation of ammonia oxidation.

Overly rapid nitrification of ammonia applied to agricultural fields can lead to loss of nitrogen products through leaching and denitrification. In attempts to retard such losses, inhibitors of nitrification such as nitrapyrin [2-chloro-6-(trichloromethyl)-pyridine] are employed. Nitrapyrin inhibits ammonia oxidation by the nitrifying bacterium *Nitrosomonas europaea* (3) and methane oxidation by the methylotropic bacteria *Methylosinus trichosporium* and *Methylococcus capsulatus* (12). Though nitrapyrin specifically inhibits the aerobic oxidation of ammonia (in contrast to the oxidation of the intermediate hydroxylamine), the mechanism of inhibition remains unknown (7). We show here that nitrapyrin was converted to 6-chloropicolinic acid in an oxygenating reaction which may involve ammonia monooxygenase and that an intermediate of this reaction derivatizes membrane proteins indiscriminately. A preliminary report of this observation has appeared previously (13).

MATERIALS AND METHODS

Chemicals. Nitrapyrin (90%), [2,6-^{14}C]nitrapyrin (5.51 mCi/mmol), and 6-chloropicolinic acid were gifts from Dow Chemical Company, Midland, Mich., and $^{18}O_2$ gas (95%) was purchased from Monsanto Research Corp., Miamisburg, Ohio. All other chemicals were obtained from Aldrich Chemical, Milwaukee, Wis.

Bacterial strains. *N. europaea* was grown and harvested as previously described (1, 6).

Reaction conditions. Incubations were in 125-ml serum vials sealed with Pierce (Rockford, Ill.) "Tuf-Bond" Teflon-laminated silicone discs and hypovial aluminum clasps. The headspace contained air. Either ammonium sulfate, hydroxylamine, or hydrazine was added to a 50-ml solution of 50 mM phosphate buffer (pH 7.5) along with 10, 30, 50, or 100 μM nitrapyrin from a 100 mM stock solution in dimethyl sulfoxide. The reaction was initiated by the addition of 100 or 200 μl of a suspension of cells (200 mg [wet weight] per ml) to a final protein concentration of 15 or 30 mg of protein per ml. The suspension was stirred at 23°C for 90 min.

Gas chromatography. For the determination of nitrapyrin, a 0.1-ml sample of the cell suspension, taken each 10 min with a gas-tight syringe, was injected into 0.5 ml of pentane in a 1.5-ml Wheaton (Millville, N.J.) autosampler vial sealed with a Teflon-coated rubber septum. Pentachlorobenzene (1 μM) was present in the pentane solution as an internal standard. Chromatography of the pentane phase was carried out on a 0.25-mm-inside-diameter DB-5 capillary column (J & W Scientific, Folson, Calif.) in a Hewlett-Packard 5890 gas chromatograph equipped with an electron capture detector and a 7673A auto sampler. The conditions were as follows: 1-μl sample volume, 1.5 ml min^{-1} He carrier gas flow, and 150°C oven, 160°C injector, and 280°C detector temperatures.

HPLC. For the identification and determination of 6-chloropicolinic acid, 1.5 ml of the cell suspension was centrifuged for 2 min at 13,800 × *g* to remove the cells. A 0.5-ml sample was injected into a Hewlett-Packard 1090 high-performance liquid chromatograph (HPLC). Separation was achieved on a Polypore H column (250 by 4.6 mm) (Applied Biosystems, Foster City, Calif.) equipped with a Polypore H guard cartridge (30 by 4.6 mm). The solvent used was 50 mM acetic acid; the flow rate was 1.0 ml/min. Identification of the elution peak due to 6-chloropicolinic acid was based on comparison of its retention time and UV spectrum with those of authentic 6-chloropicolinic acid. Peaks were observed by scanning at 274 nm, and appropriate fractions were collected manually for analysis by scintillation counting.

GC-MS. For identification of their mass spectra, 6-chloropicolinic acid and ^{18}O-containing 6-chloropicolinic acid were extracted from experimental solutions at pH 3.5 into ether. The ether was then evaporated, and the concentrated solutions were reacted with diazomethane (4) to methylate the acids. By this procedure, both carboxylate oxygens were retained and subsequently observed in the analysis of the molecular ion peaks. The methyl ester derivatives of both the experimental fractions and an authentic standard of 6-chloropicolinic acid were assayed by use of a Kratos MS25 70-eV electron impact gas chromatography-mass spectrometer (GC-MS) with a DB-1 capillary column (30 m by 1 μm) (J & W Scientific) with a 60 to 180°C temperature ramp.

Experiments with $^{18}O_2$. The 125-ml serum reaction vials

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TABLE 1. Rates of oxidation of nitrapyrin[a]

Substrate	Concn of:		Rate of oxidation of nitrapyrin[b]
	Substrate (mM)	Nitrapyrin (μM)	
Ammonia (NH_3)	1	10	0.61
	10	10	0.75
		30	0.21
		50	0.25
		100[c]	0
	40	10	0.26
Hydrazine (NH_2NH_2)	1	10	0.40
	10	10	0.44
Hydroxylamine (NH_2OH)	1	10	0.55
	0	10	0
None		10	0.043

[a] Disappearance of nitrapyrin was measured as described in Materials and Methods. Rates were taken during the linear phase of the reaction and were reproducible within plus/minus 10%.
[b] In nanomoles per minute per milligram of protein.
[c] At concentrations of 100 μM, nitrapyrin was not oxidized under any of the conditions listed here.

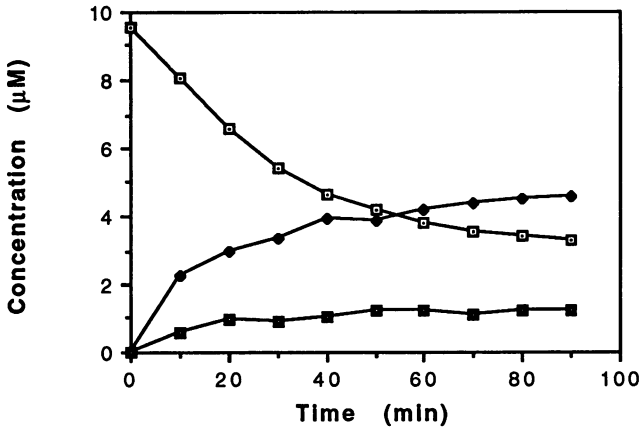

FIG. 1. Time course of the oxidation of nitrapyrin to 6-chloropicolinic acid by *N. europaea*. The reaction was initiated by the addition of cells to a reaction mixture containing 10 μM nitrapyrin and 10 mM ammonia. ⊡, concentration of nitrapyrin; ◆, concentration of 6-chloropicolinic acid; ■, amount of carbon-14-labeled nitrapyrin irreversibly bound to whole cells.

containing the reaction solution without cells were repeatedly evacuated and flushed with nitrogen to remove oxygen gas. A positive pressure of $^{18}O_2$ gas was introduced after the final evacuation. The positive pressure was subsequently released. Analysis by GC-MS of the headspace both before and after the incubation indicated that 95% of the total headspace gas was $^{18}O_2$. Cells were then added, and the incubation was carried out. Values of peak heights from GC-MS were compared and normalized against calculated abundances of the isotopes of oxygen and chlorine that are naturally present. Contributions to peaks from the presence of chlorine-37 were thus eliminated so that oxygen-18 incorporation could be determined.

Scintillation counting. Scintillation counting of ^{14}C was carried out with a Beckman LS3801 device with ECOLUME (ICN Biomedicals, Inc., Irvine, Calif.) scintillation fluid.

Electrophoresis and fluorography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by using the method of Laemmli (9) on 10 and 12% gels. Cells were broken by repeated freezing and thawing of a 20% cell suspension. Soluble and membrane proteins were separated by centrifugation of cell extracts at 13,800 × *g* for 6 min. The protein samples were heat treated at 100°C for 1 min in the presence of 1.5% β-mercaptoethanol. The gels were stained with Coomassie blue and treated for fluorography by using the method of Bonner (2).

Other assays. Production of nitrite, rates of usage of oxygen, and inactivation by acetylene were carried out as described previously (1, 6). Protein concentration was determined by using the bicinchoninic acid method (11).

RESULTS

Incubations of nitrapyrin with ammonia, hydroxylamine, or hydrazine in the absence of cells resulted in no detectable loss of nitrapyrin during 90 min. However, nitrapyrin was rapidly transformed by cells in the presence of ammonia, hydroxylamine, or hydrazine (Table 1 and Fig. 1). The greatest rate of disappearance was with 10 mM ammonia (Table 1). The time course of the disappearance of nitrapyrin was approximately first order and corresponded to the time course of the appearance of nitrite (Fig. 2). The disappearance of nitrapyrin was inhibited by hydroxylamine at a concentration of 10 mM but not at that of 1 mM. Concentrations of nitrapyrin greater than 10 μM inhibited the oxidation of ammonia and nitrapyrin by the cells. At 100 μM nitrapyrin, no transformation of either nitrapyrin or ammonia took place. In the absence of added ammonia, hydroxylamine, or hydrazine, cells oxidized nitrapyrin at low but detectable rates. Cells preincubated with acetylene were completely inhibited with respect to the oxidation of both ammonia and nitrapyrin (data not shown). Rates of transformation of nitrapyrin were highly reproducible in a given

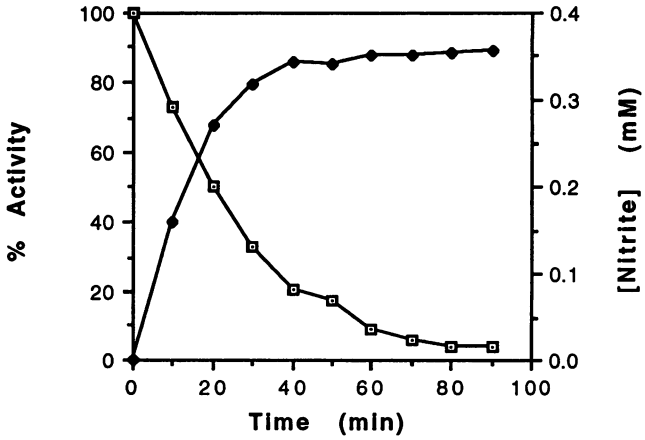

FIG. 2. Time course of production of nitrite and inactivation of *N. europaea* during degradation of nitrapyrin. The reaction was initiated by the addition of cells to a reaction mixture containing 10 μM nitrapyrin and 10 mM ammonia. Ammonia-oxidizing activity of *N. europaea* (⊡) was measured as the rate of oxygen utilization of cells which had been removed from the reaction mixture by sedimentation and resuspended in reaction solution containing ammonia but lacking nitrapyrin. The concentration of nitrite (◆) was measured in aliquots taken from the original reaction mixture at the indicated times.

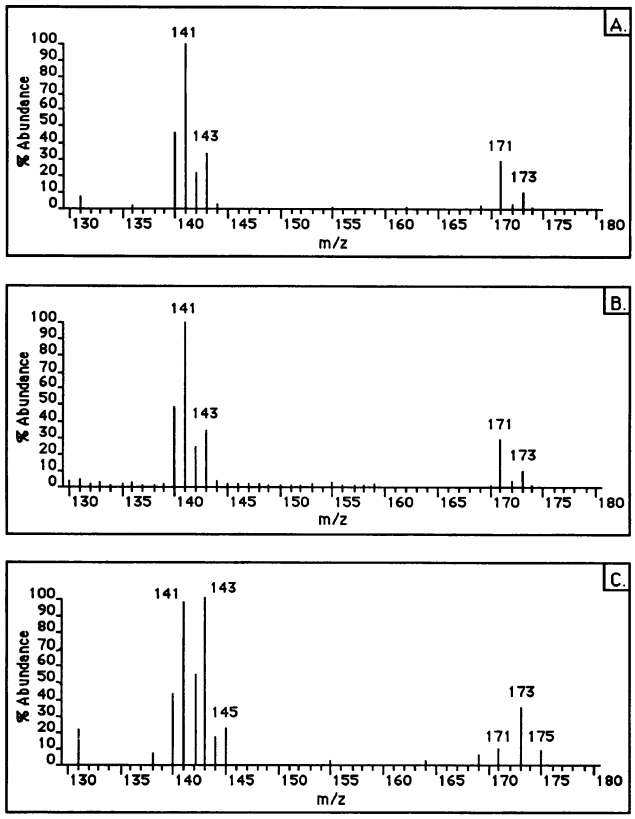

FIG. 3. Mass spectra of the methyl ester of 6-chloropicolinic acid. (A) Authentic standard, (B) product of oxidation of nitrapyrin, (C) product of oxidation of nitrapyrin in the presence $^{18}O_2$ gas.

batch of cells but might vary twofold between batches of cells.

Identification of 6-chloropicolinic acid as the product was carried out by using GC-MS (Fig. 3B). The product was converted to its methyl ester and then identified as 6-chloropicolinic acid by comparison of mass spectra and retention times with those of authentic standards (Fig. 3A and B). The dual molecular ion peaks (at 171 and 173) are characteristic of a tetra-chloro compound and arise from the natural abundances of the isotopes ^{35}Cl and ^{37}Cl. Production of 6-chloropicolinic acid accompanied nitrapyrin disappearance with a nearly one-to-one stoichiometry (Fig. 1). Most ring ^{14}C-labeled nitrapyrin was converted to labeled 6-chloropicolinic acid. Some of the label (13%) was found to be bound to cellular material and was retained after repeated washing (Fig. 1). This labeling also followed the time course of the disappearance of nitrapyrin. Essentially all of the ^{14}C of transformed nitrapyrin was found either bound to the cell or as 6-chloropicolinic acid. Most (94%) of the bound ^{14}C-labeled nitrapyrin was found in the membrane fraction. Analysis by fluorography of the membrane proteins separated by SDS-PAGE indicated that all or most of the membrane proteins had been derivatized (data not shown).

During the oxidation of nitrapyrin, the rate of ammonia oxidation progressively diminished, though the substrate, ammonia, was still present in excess. Analysis of the ammonia-oxidizing activity of washed cells indicated that the ammonia monooxygenase system was being inactivated by nitrapyrin. Ammonia-oxidizing ability and the rate of disappearance of nitrapyrin diminished at similar times in the incubation (Fig. 1 and 2).

During the transformation of nitrapyrin in the presence of $^{18}O_2$ gas, ^{18}O was incorporated into the 6-chloropicolinic acid product (Fig. 3). By comparison of the corrected heights of the molecular ion peaks 171 and 173 in the mass spectrum of 6-chloropicolinic acid (Fig. 3C), the level of incorporation of ^{18}O was determined to be 39% of the total oxygen. Since the headspace gas employed in the experiments contained 95% ^{18}O, the maximum possible level of incorporation of ^{18}O in 6-chloropicolinic acid was 95%. The observed value of 39% is close enough to a theoretical value of 47.5% to indicate that only one atom from $^{18}O_2$ was incorporated into 6-chloropicolinic acid. The deviation from the theoretical may be due to exchange between the solvent water and 6-chloropicolinic acid.

According to analysis by Dow Chemical, the sample-grade nitrapyrin used in this work is 90% 2-chloro-6-(trichloromethyl)-pyridine and 10% 2,4-dichloro-6-(trichloromethyl)-pyridine and other related compounds. The dichloropyridine compound was identified here by GC. It was found to have a retention time several minutes longer than that of nitrapyrin. Mass spectra showed that the dichloropyridine compound was converted to 2,4-dichloropicolinic acid and that $^{18}O_2$ was incorporated (data not shown).

DISCUSSION

The present work shows that nitrapyrin [2-chloro-6-(trichloromethyl)-pyridine] can be oxidized to 6-chloropicolinic acid by whole cells of *N. europaea*. Rapid oxidation requires the concomitant oxidation of ammonia, hydroxylamine, or hydrazine; i.e., it is a "cooxidation." Although we cannot preclude the involvement of other enzymes, the possible involvement of the ammonia monooxygenase system in all or part of this reaction is suggested by the following. (i) Ammonia- and nitrapyrin-oxidizing activities are both inactivated by preincubation with acetylene, a compound thought to be a suicide substrate for ammonia monooxygenase (8). (ii) The inactivation of ammonia- and nitrapyrin-oxidizing activities follows the same time course during nitrapyrin degradation. (iii) Incorporation of $^{18}O_2$ into the product suggests the action of a monooxygenase. We emphasize that, because the present results are from studies with intact cells, conclusions that can be made regarding the mechanism(s) of an enzyme(s) are minimal.

From data presented thus far with cells of *N. europaea* and cells or enzymes of the methylotrophs, dehalogenation of substrates appears to occur only as a secondary, and probably nonenzymic, reaction of an oxygenated intermediate compound such as an alcohol or an epoxide. The formation of an oxygenated compound has thus far been seen to be limited to reactions with a C-H carbon bond or available π-electrons. Thus, methyl carbons with free hydrogens such as methylene chloride or chloroform are degraded by *N. europaea*, whereas a methyl carbon lacking a free hydrogen, such as in carbon tetrachloride, resists degradation (14). The latter point is also illustrated by the oxidation by *N. europaea* of 1,1,1-trichloroethane, which occurs only at a free hydrogen of the 2 position to form 2,2,2-trichloroethanol (10). Systems with π-electrons are oxidized by monooxygenases to the epoxide (5). Compounds such as 1,1-dichloroethene and trichloroethene are degraded by *N. europaea* (14). The probable importance of accessible π-electrons is illustrated by the conversion of another fully halogenated compound, chlorotrifluoroethene, to its epoxide

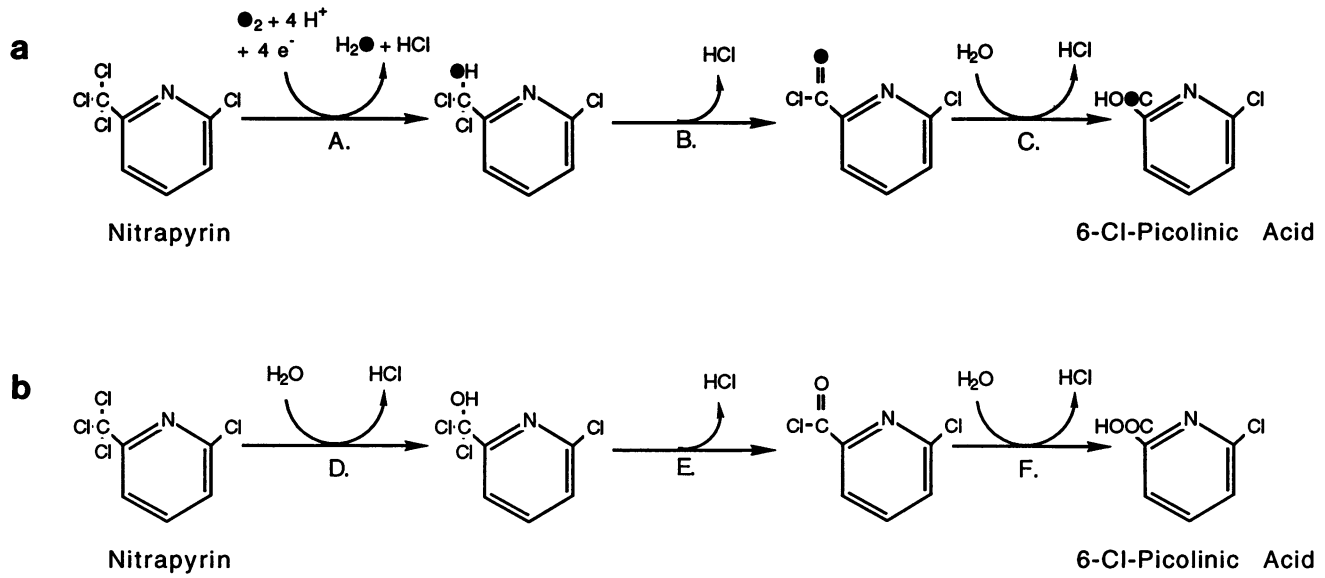

FIG. 4. (a) Possible mechanisms for the production of 6-chloropicolinic acid-^{18}O,^{16}O. Steps: A, four-electron oxidation of nitrapyrin to an oxygen-18 alcohol intermediate; B, spontaneous release of hydrochloric acid to form an oxygen-18 acid chloride; C, chemical hydrolysis of the acid chloride to form 6-chloropicolinic acid-^{18}O,^{16}O. Filled or open oxygens represent ^{18}O or ^{16}O, respectively. (b) Hydrolytic breakdown of nitrapyrin. Steps: D, hydrolysis of nitrapyrin to an oxygen-16 alcohol intermediate; E, spontaneous release of hydrochloric acid to form an acid chloride; F, chemical hydrolysis of the acid chloride to form 6-chloropicolinic acid-^{16}O,^{16}O.

by methane monooxygenase (5). With this background, the conversion of nitrapyrin to 6-chloropicolinic acid by *N. europaea* is unusual in that a chlorinated carbon lacking either a free hydrogen or π-electrons is oxidized. The reaction is also unusual in that it appears to involve four rather than two electrons in what is, overall, a monooxygenase-type reaction. As is suggested in Fig. 4, future work may demonstrate a reductive dechlorination of the trichloromethyl group either prior to or simultaneous with a two-electron monooxygenase reaction.

In Fig. 4, proposed reaction mechanisms are shown for the incorporation of $^{18}O_2$ during the formation of 6-chloropicolinic acid from nitrapyrin. The biological reaction may involve a concerted four-electron oxygenation reaction (Fig. 4a, step A) in which chloride is displaced and a halogenated alcohol is formed. The alcohol would then spontaneously release hydrochloric acid (Fig. 4a, step B) to form an acid chloride which would undergo nonbiological hydrolysis to form 6-chloropicolinic acid (Fig. 4a, step C). The oxidation mechanism (Fig. 4a) is consistent with the results of the ^{18}O experiments in which ^{18}O was found in only one of the two oxygens of 6-chloropicolinic acid; the other presumably came from water. Hydrolysis of the acid chloride would account for this. If 6-chloropicolinic acid had been produced by the hydrolysis of nitrapyrin (Fig. 4b), incorporation of $^{18}O_2$ into 6-chloropicolinic acid would not have occurred and all incorporated oxygens would have come from $H_2{}^{16}O$.

Carbon-14-labeled nitrapyrin was found to bind irreversibly to cellular protein. As suggested in Fig. 4, an acid chloride intermediate would be expected to react with nucleophilic side chains of proteins, thus accounting for the labelling of proteins and inactivation of the cells. In notable contrast to labelling by acetylene (8), all or most membrane proteins are labelled by nitrapyrin.

It is possible that the oxidation reaction (Fig. 4a, step A) occurs in two separate steps. First, a two-electron, reductive dehalogenation of nitrapyrin may occur to form a dichloromethyl compound. This compound could then be oxygenated in the usual two-electron manner to form an alcohol which could then follow steps B and C in Fig. 4a to form 6-chloropicolinic acid.

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