JOURNAL OF BACTERIOLOGY, Aug. 1973, p. 480-485
Vol. 115, No. 2

Printed in U.S.A.

Specific Inhibitors of Ammonia Oxidation in *Nitrosomonas*

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Received for publication 25 October 1972

The following compounds or treatments have been shown to inhibit the oxidation of ammonia, but not the oxidation of hydroxylamine in cells of *Nitrosomonas*: (i) metal-binding agents such as allylthiourea or potassium cyanide; (ii) compounds such as SKF 525 which interact with cytochrome P-450 of mammalian microsomes; (iii) carbon monoxide; (iv) inhibitors of catalase, peroxidase, and amine oxidases such as thiosemicarbazide, ethylxanthate, and iproniazid, respectively; (v) uncouplers of oxidative phosphorylation such as *m*-chlorocarbonylcyanidephenylhydrazone; (vi) electron acceptors such as phenazine methosulfate; (vii) compounds such as methanol or N_2O which react with free radicals; and (viii) illumination with 420 lux (5,000 foot candles) of light.

Although the preparation of cell-free extracts capable of oxidizing ammonia to nitrite has been reported from the ammonia-oxidizing autotrophic bacteria (18, 20), the existing systems have not readily yielded information regarding the mechanism of ammonia oxidation. Studies with suspensions of intact cells on the effect of inhibitors (A. B. Hooper and K. R. Terry, Bacteriol. Proc., p. 139, 1971) on and the kinetics (10) of ammonia oxidation have suggested the involvement of a metal ion and a preincubation-induced state for ammonia oxidation, respectively. The present work extends the categories of inhibitors of ammonia oxidation, but not hydroxylamine oxidation, to include carbon monoxide, uncouplers of oxidative phosphorylation, certain electron acceptors such as phenazine methosulfate (PMS), illumination with light, compounds such as N_2O or methanol which can act as free-radical trapping agents, and inhibitors of peroxide-metabolizing enzymes. Although the data presented here cannot establish any component of the mechanism of ammonia oxidation, they point the way toward analysis in cell-free systems. A report of part of this work has appeared previously (A. B. Hooper and K. R. Terry, Bacteriol. Proc., p. 139, 1971).

MATERIALS AND METHODS

The chemicals used were analytical-grade reagents. SKF 525 was a gift from the Smith, Kline & French Laboratories, Philadelphia; N-Serve (2-chloro-6-trichloromethyl-pyridine) was from Dow Chemical Co., Midland, Mich.; 1799 ($\alpha\alpha'$-bis(hexafluoroacetonyl)-cyclohexanone) as from E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.; and Lilly 18947 and 53325 were from the Lilly Research Laboratories, Indianapolis. Washed cell suspensions of *Nitrosomonas europaea* (Schmidt strain) were prepared as described previously (11).

Nitrite formation was assayed by the diazotization method (12) in a reaction mixture containing 0.2 mg (wet weight) of *Nitrosomonas* cells per ml, 5×10^{-4} M $(NH_4)_2SO_4$, or 10^{-3} M NH_2OH in 0.05 M phosphate, pH 7.5. Unless otherwise indicated, the rate of nitrite production was linear during the first 20 min and proportional to the amount of cells present. The test tubes were rotated at 20 to 30 rpm in a nearly horizontal position to facilitate aeration. Inhibitors were added at the specified concentrations. The effect of CO or N_2O was tested by injecting the cell suspension into a Thunberg tube containing the reaction mixture equilibrated with the appropriate gas mixture. To test reversibility of inhibition, cells (4 mg/ml) were equilibrated for 15 min at room temperature in the presence of levels of inhibitors previously found to have caused 100% inhibition of ammonia oxidation. Cells were sedimented and resuspended in fresh buffer four times. The rate of nitrite production from ammonia was assayed and compared with a control which had been treated in an identical manner, except that the inhibitor was absent.

PMS-dependent nitrite synthetase was assayed as described previously (9) as the rate of nitrite production in a reaction mixture containing cell-free extract, 10^{-4} M NH_2OH, 5 μM phenazine methosulfate, and 0.05 M tris(hydroxymethyl)aminomethane (pH 8.0).

RESULTS AND DISCUSSION

A great many compounds inhibited: (i) ammonia oxidation specifically, (ii) both ammonia

and hydroxylamine oxidation, or (iii) neither ammonia nor hydroxylamine oxidation (Tables 1 to 5). For each inhibitor, data were selected from a plot of percent inhibition versus the concentration at which the inhibitory effect on ammonia was maximal and the effect on hydroxylamine oxidation was minimal. Inhibitors are grouped in tables according to possibly related modes of action.

Metal-binding compounds. Lees (14) first showed that allylthiourea and diethyldithiocarbamate (DTC) were potent and specific inhibitors of ammonia oxidation, and we show here (Table 1) that KCN and $\alpha\alpha'$-dipyridyl are as well. These effects suggest the involvement of a metal ion, possibly copper, in ammonia oxidation. Inhibition by DTC or KCN was not reversed by the usual washing or by washing with $FeCl_3$, $MgSO_4$, $SnCl_2$, bovine serum albumin, chelated copper, or adenosine 5'-triphosphate. However, washing with $CuCl_2$ increased the rate of nitrite synthesis from ammonia in DTC-treated cells from 6 (DTC treated and buffer washed) to 41% (DTC treated and $CuCl_2$ washed), and in KCN-treated cells from 14 (KCN treated and buffer washed) to 30% (KCN treated and $CuCl_2$ washed). Full recovery was not expected, for we have observed that Cu^{2+} inhibits cell-free, PMS-dependent nitrite synthetase.

Sodium sulfide (10^{-4} M) and dithiol (toluene-3,4-dithiol, 1 mM) were potent inhibitors of both ammonia and hydroxylamine oxidation.

The presence of ethylenediaminetetraacetate (10^{-2} M), 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline ("Bathocuproine," 1 mM), disodium 1,2-dihydroxybenzene-3,5-disulfonate ("Tiron," 10^{-2} M), or desferrioxamine mesylate ("Desferal," 1 mM) caused essentially no inhibition of either ammonia or hydroxylamine oxidation by cells of *Nitrosomonas*.

Enzyme and heme protein-binding compounds. The possible dependence of ammonia oxidation on a factor similar to cytochrome P-450 of mammalian microsomes was suggested by the specific sensitivity to SKF 525, Lilly 18947, Lilly 53325, and CO (Table 2).

P-460, a novel cytochrome from *Nitrosomonas*, has a high affinity for CO and also binds the ammonia oxidation inhibitor NH_2NH_2 (5). In addition, we have observed a cytochrome *b*-like spectrum in dithionite-reduced plus CO (95% O_2:5% CO) minus dithionite-reduced difference spectra of extracts of *Nitrosmonas*. This pigment may be an *o*-type cytochrome (17) or possibly a peroxidase similar to the CO-binding cytochrome *c* peroxidases observed by Yamanaka in *Thiobacillus* (21) and *Nitrosomonas* (T. Yamanaka, personal communication). In the partially purified state, cytochrome a_1 of *Nitrosomonas* appears to bind CO only when oxygen is essentially absent (6).

Ammonia oxidation in *Nitrosomonas* was inhibited by several inhibitors of catalase (15): thiosemicarbazide, diphenylthiocarbazone, 3-aminotriazole, or aminoguanidine (as well as

TABLE 1. *Metal-binding compounds*

Inhibitor	Concn (M)	Rate of HNO_2 synthesis by intact cells (% control)		
		NH_3 substrate	Reversibility	NH_2OH substrate
Specific inhibitors of NH_3 oxidation				
Allylthiourea	10^{-6}	18	+	100
KCN	5×10^{-6}	22	$+(Cu^{2+})^a$	83
Diethyldithiocarbamate	10^{-5}	0	$+(Cu^{2+})^a$	69
8-Quinolinol[b]	10^{-5}	0		70
o-Phenanthroline	5×10^{-5}	0		76
$\alpha\alpha'$-Dipyridyl[b]	10^{-4}	0		96
Nonspecific inhibitors				
NaN_2	10^{-3}	10		45
Na_2S	10^{-4}	0	+	9
Dithiol	10^{-3}	0		0

[a] For reversal of inhibition, cells incubated with 10^{-3} M DTC or KCN in phosphate buffer were washed four times with (250 ml/g of cells) 0.05 M Tris-hydrochloride, pH 7.5, in the presence of: no additives; 1 mM $CuCl_2$, $FeCl_3$, $MgSO_4$, or $SnCl_2$; 0.05% bovine serum albumin; 15 μg of chelated copper per ml (Geigy Sequestrene); or 6.4 mM ATP. The cells were then washed once again before assay in phosphate buffer with no additives. Abbreviations: DTC, diethyldithiocarbamate; Tris-hydrochloride, tris(hydroxymethyl)aminomethane-hydrochloride; ATP, adenosine 5'-triphosphate; DMSO, dimethylsulfoxide.

[b] Dissolved in 0.4% (final concentration) DMSO.

CO and NH_2NH_2). Significantly, allythiourea or diethyldithiocarbamate may be similar enough in structure to 3-aminotriazole to inhibit a catalase or catalase-like enzyme. This suggests that a catalase-like enzyme is involved in ammonia oxidation, either directly (to generate [OH] or as a hydroxylase) or indirectly (to remove inhibitory levels of H_2O_2, for example). A number of compounds including thiosemicarbazide have been shown to inhibit biological ammonia oxidation in preference to hydroxylamine oxidation in sludge (19), and the authors point out that such compounds can occur in rubber. We have found that ammonia oxidation by cells of *Nitrosomonas* is very sensitive to certain kinds of rubber stopper or tubing.

Ethyl xanthate inhibits peroxidase of *Nitrosomonas* (3), and we have observed that it inhibits a soluble oxidase from *Nitrosomonas* (R. H. Erickson and A. B. Hooper, Bacteriol. Proc., p. 171, 1968) and is a specific inhibitor of ammonia oxidation (Table 2) as shown previously (14).

We confirm the previously observed (4) sensitivity of ammonia, but not hydroxylamine, oxidation to Dow N-Serve. Ammonia oxidation was also more sensitive than hydroxylamine oxidation to high concentrations of iproniazid, which is an inhibitor of diamine oxidases (22) as are others of the ammonia oxidation inhibitors employed here (diethyldithiocarbamate, aminoguanidine, hydroxylamine, cyanide, hydrazine, and thiosemicarbazide). The fact that several compounds which are generally considered to be potent, but nonpermeable, enzyme inhibitors, including *p*-hydroxymercuribenzoate (5×10^{-4} M) and 7-diazonium-1,3-naphthalene disulfonic acid (1 mM), were not inhibitory to ammonia or hydroxylamine oxidation may indicate that the ammonia oxidation system is not on the extreme outer surface of *Nitrosomonas*. Both compounds inhibited PMS-dependent nitrite synthesis in cell-free extracts.

Uncouplers of oxidative phosphorylation and inhibitors of electron transport. 2,4-Dinitrophenol (DNP), *m*-chlorocarbonylcyanidephenylhydrazone (*m*CCP), 1799, and tetrachlorosalicylanilide are excellent specific inhibitors of ammonia oxidation (Table 3). In bacteria they appear to inhibit oxidative phosphorylation, ATP-dependent nicotinamide adenine dinucleotide phosphate reduction, proton pumping, and ion transport (8). *N*,*N*'dicyclohexylcarbodiimide is an inhibitor of membrane-bound electron transport and an uncoupler in

TABLE 2. *Enzyme and heme protein-binding compounds*

Inhibitor	Concn (M)	Rate of HNO_2 synthesis by intact cells (% control)		
		NH_3 substrate	Reversibility	NH_2OH substrate
β-Diethylaminoethyldiphenylpropyl-acetate (SKF 525)[a]	5×10^{-5}	35	–[b]	75
2,4-Dichloro-6-phenylphenoxyethyl-diethylamine (Lilly 18947)[c]	10^{-4}	4		100
2,4-Dichloro(6-phenylphenoxy)ethyl-amine hydrobromide (Lilly 53325)	10^{-4}	4		75
NH_2NH_2	2×10^{-3}	16		86
CO (95% O_2:5% CO)	0.05	8	+[d]	100
Thiosemicarbazide	10^{-5}	5		79
diphenylthiocarbazone[c]	3×10^{-5}	50		100
3-Aminotriazole	10^{-3}	0	+	150
Aminoguanidine	10^{-3}	26		80
Ethyl xanthate	10^{-4}	0		94
2-Chloro-6-trichloromethyl pyridine (Dow N-Serve)[c]	5×10^{-5}	14	–	96
1-Isonicotinyl-2-isopropyl-hydrazine (iproniazid)	0.01	12		80

[a] Caused bleaching of cells.
[b] Cells were lysed during wash procedure.
[c] Dissolved in 0.4% (final concentration) dimethylsulfoxide.
[d] Inhibition was reversed after exposure to air.

TABLE 3. *Uncouplers and inhibitors of electron transport*

Inhibitor	Concn (M)	Rate of HNO_2 synthesis by intact cells (% control)		
		NH_3 substrate	Reversibility	NH_2OH substrate
m-Chlorocarbonylcyanidephenylhydrazone	10^{-5}	17	+	128
Tetra chlorosalicylanilide[a]	10^{-5}	41		100
$\alpha\alpha'$-Bis(hexafluoroacetonyl)cyclohexanone (duPont 1799)[a]	2×10^{-5}	27		100
N,*N'*-Dicyclohexylcarbodiimide	5×10^{-5}	40	-	82
2,4-Dinitrophenol	2×10^{-4}	27	+	100
Phenazine methosulfate	5×10^{-5}	0	+	375
Methylene blue	10^{-4}	0		100
2,6 Dichlorophenolindephenol	10^{-3}	0		160

[a] Dissolved in 0.4% (final concentration) dimethylsulfoxide.

bacteria (13). DNP and *m*-CCCP are reported to inhibit ATP-dependent pyridine nucleotide reduction in extracts of *Nitrosomonas* (1). Thus, one or more of these processes may be directly or indirectly involved in ammonia oxidation. The effect of uncouplers at least suggests the dependence of ammonia oxidation on a functionally intact membrane as has been indicated by work with cell-free systems (18, 20).

Ammonia oxidation was specifically inhibited by PMS, 2,6-dichlorophenolindophenol, and methylene blue at concentrations at which, as rapidly oxidized electron acceptors, they stimulate NH_2OH oxidation. Specific sensitivity to methylene blue has been shown previously (2). We suggest that PMS inhibits ammonia oxidation by oxidizing an intermediate compound or a reduced enzyme or by bypassing reactions coupled to hydroxylamine oxidation. In this regard, it is significant that the intermediate product of PMS reduction is a free radical which is presumably reactive with other free radicals, that the oxidation of PMS by O_2 yields H_2O_2, and that PMS is an inhibitor of catalase (16).

Short-chain alcohols and amines. A number of alcohols were specific inhibitors of ammonia, but not of hydroxylamine oxidation (Table 4). Short-chain primary alcohols were the most effective as was indicated by comparing the effects of methanol, ethanol, *n*-butanol, and *t*-butanol. Formate, acetaldehyde, and glyoxylate were not especially inhibitory to ammonia oxidation, and their effect on hydroxylamine oxidation could not be determined. Possibly compounds such as acetone or ethyl acetate act as organic solvents and disrupt membrane or enzyme function. The alcohols may react with a peroxide-metabolizing enzyme, as has been shown for catalase (15), or act as free-radical trapping agents.

Methylamine, but not ethanolamine or tris-(hydroxymethyl)aminomethane, specifically inhibited ammonia oxidation. In fungi, methylamine competes with ammonia for a permease (7).

Miscellaneous factors. N_2O, which can act as a free-radical trapping agent, specifically inhibited ammonia but not hydroxylamine oxidation (Table 5).

Illumination during the reaction with 420 lux (5,000 foot-candles) of light resulted in complete and irreversible inactivation of ammonia oxidation with no effect on hydroxylamine oxidation. Preliminary analysis indicated that the photoinactivation followed first-order kinetics and did not occur under anaerobic conditions. The presence of high concentrations of ammonia in the reaction mixture decreased the rate of photoinactivation; whereas illumination for 10 min in the absence of added ammonia or hydroxylamine caused complete inactivation of subsequent ammonia oxidation and 70% inactivation of hydroxylamine oxidation. These observations suggested the destruction of factors necessary for ammonia and hydroxylamine oxidation by a process of photooxidation which could be prevented by keeping the cells in a substrate-reduced or anaerobic state.

A decrease in the incubation temperature caused greater decrease in the rate of ammonia than in hydroxylamine oxidation. Arrhenius plots were linear between 4 and 30° and indicated apparent energies of activation of 4.63 and 3.74 kJ/mol for ammonia and hydroxylamine oxidation, respectively.

The present results suggest the following about ammonia oxidation: the process depends on (i) a metal ion such as copper, (ii) a CO-binding factor, (iii) a P-450-like protein,

TABLE 4. *Short-chain alcohols and amines*

Inhibitor	Concn (M)[a]	Rate of HNO_2 synthesis by intact cells (% control)		
		NH_3 substrate	Reversibility	NH_2OH substrate
Specific inhibitors of NH_3 oxidation				
Methanol	5×10^{-3}	0	+	100
Methylamine	0.01	18		87
Ethanol	0.09	0	+	100
n-Propanol	0.33	0		95
i-Propanol	0.13	9		100
n-Butanol	0.11	0		73
t-Butanol	0.11	65		120
Ethyl acetate	0.23	0		85
Aminoethanol	0.2	57		80
Nonspecific inhibitors				
Acetone	0.14	0	+	[b]
Polymyxin B[c]	10^{-4} U/ml	0	−[c]	0
Noninhibitory compounds				
Acetaldehyde	10^{-2}	89		[b]
Formate	0.1	70		[b]
Glyoxylate	0.1	77		[b]
Cholate	1%	94		68
Glycerol	1.6	100		100
Dimethylsulfoxide	0.13	100		100
Acetate	0.1	91		100
Tris(hydroxymethyl)amino-methane	0.25	100		100

[a] Except as otherwise noted.
[b] Inhibitor reacted with NH_2OH.
[c] Caused cell lysis.

TABLE 5. *Miscellaneous factors*

Treatment	Conditions	Rate of HNO_2 synthesis by intact cells (% control)		
		NH_3 substrate	Reversibility	NH_2OH substrate
N_2O	10% N_2O:90% O_2	0	+[a]	100
Light[b]	420 lux	0	−	100
Temperature (as compared with 25 C)	15 C	23		50

[a] Inhibition was reversed after exposure to air.
[b] Cells were illuminated during the reaction by a Kodak model 800 Carousel slide projector with a 200-W bulb at a measured light intensity of 420 lux. The temperature of the mixture was maintained at 25 C. For reversal, cells inhibited by light were allowed to remain in the dark for up to 1 h.

and (iv) a functionally intact membrane. In addition, (v) a role for an H_2O_2 or OH radical producing, utilizing, or detoxifying enzyme system is strongly indicated by the specific sensitivity to many inhibitors of catalase, peroxidase, or amine oxidases, and (vi) the involvement of a free radical is suggested by sensitivity to N_2O and methanol.

ACKNOWLEDGMENTS

This investigation was supported by research grant GB 15101 from the National Science Foundation.

We are grateful to Rita Young for help in growing batch cultures of *Nitrosomonas*.

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