Applied and Environmental Microbiology, Nov. 1993, p. 3728–3735 Vol. 59, No. 11
0099-2240/93/113728-08$02.00/0
Copyright © 1993, American Society for Microbiology

# Mechanism-Based Inactivation of Ammonia Monooxygenase in *Nitrosomonas europaea* by Allylsulfide

LISA Y. JULIETTE, MICHAEL R. HYMAN, and DANIEL J. ARP*

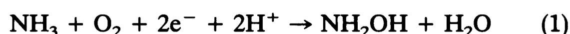
*Laboratory for Nitrogen Fixation Research, Oregon State University, Corvallis, Oregon 97331-2902*

Received 21 June 1993/Accepted 27 August 1993

**Allylsulfide caused an irreversible inactivation of ammonia monooxygenase (AMO) activity (ammonia-dependent $O_2$ uptake) in *Nitrosomonas europaea*. The hydroxylamine oxidoreductase activity (hydrazine-dependent $O_2$ uptake) of cells was unaffected by allylsulfide. Anaerobic conditions or the presence of allylthiourea, a reversible noncompetitive AMO inhibitor, protected AMO from inactivation by allylsulfide. Ammonia did not protect AMO from inactivation by allylsulfide but instead increased the rate of inactivation. The inactivation of AMO followed pseudo-first-order kinetics, but the observed rates did not saturate with increasing allylsulfide concentrations. The time course of recovery of AMO-dependent nitrite production after complete inactivation by allylsulfide required de novo protein synthesis. Incubation of cells with allylsulfide prevented the $^{14}C$ label from $^{14}C_2H_2$ (a suicide mechanism-based inactivator of AMO) from being incorporated into the 27-kDa polypeptide of AMO. Some compounds structurally related to allylsulfide were unable to inactivate AMO. We conclude that allylsulfide is a specific, mechanism-based inactivator of AMO in *N. europaea*.**

The ammonia-oxidizing nitrifying bacteria, characterized by such species as *Nitrosomonas europaea*, obtain all their energy for growth solely from the oxidation of ammonia to nitrite. Ammonia oxidation by nitrifying bacteria can initiate environmentally and economically deleterious effects by rapidly oxidizing ammonia-based fertilizers applied to croplands. On the other hand, nitrifying bacteria also play a crucial role in the beneficial process of removal of ammonia from sewage. Therefore, it is not surprising that there has been considerable interest in the metabolism and inhibition of ammonia oxidation by nitrifying bacteria.

The oxidation of ammonia by *N. europaea* is initiated by the enzyme known as ammonia monooxygenase (AMO) (equation 1):



$$NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O \quad (1)$$

Despite the interest in the catalytic activity of AMO, purification of this enzyme has not been achieved. Much of what is known about the catalytic properties of AMO have been deduced from inhibitor studies using whole cells of *N. europaea* (8, 13, 17, 26). Many compounds inhibit AMO solely by interacting as substrates at the active site(s) of the enzyme (15, 16). Other substrates oxidized by AMO can lead to the generation of reactive intermediates (24). These reactive intermediates can disrupt ammonia oxidation by permanently damaging intracellular protein(s). In some cases, these effects are nonspecific. For example, the oxidation of trichloroethylene by cells of *N. europaea* leads to inactivation of AMO (24). The inactivation of AMO by trichloroethylene requires $O_2$ and is prevented by allylthiourea, a reversible inhibitor of AMO. This indicates that the inactivating effect of trichloroethylene requires catalytic turnover by AMO to generate a reactive species (12, 24). However, the effects of trichloroethylene oxidation are not limited to AMO because incubation of cells with $^{14}C$-labeled trichloroethylene resulted in nonspecific and covalent binding of the reactive species to many intracellular proteins. This indicates that the reactive species generated by AMO is able to diffuse away from the active site.

In contrast to the nonspecific effects caused by oxidation of trichloroethylene by AMO, the effects of acetylene ($C_2H_2$), a mechanism-based inactivator, are specific for AMO (12, 17). Incubation of cells with $^{14}C_2H_2$ gives rise to the covalent attachment of $^{14}C$ label almost exclusively to a polypeptide with an apparent $M_r$ of 27,000. The inactivation of AMO by $C_2H_2$ is diminished in the presence of high ammonia concentrations and is prevented under anaerobic conditions when AMO is inactive (17). It is proposed that $C_2H_2$ inactivates AMO as a result of the attempted oxidation of the acetylenic triple bond resulting in the generation of a reactive intermediate which binds to and inactivates the enzyme. The covalent modification of this polypeptide by $^{14}C$ label from $^{14}C_2H_2$ is a saturable process and corresponds with the amount of ammonia-oxidizing activity present in the cells (12). The ammonia-oxidizing activity of cells inactivated by either trichloroethylene or $C_2H_2$ was shown to recover by a process requiring de novo protein synthesis (12, 24). Acetylene-inactivated cells recovered their nitrite-producing activity faster than cells inactivated by trichloroethylene. The difference in the rate of recoveries was suggested to be due to the limited protein synthesis required when cells were inactivated by a specific inactivator like acetylene in contrast to the extensive protein synthesis required following nonspecific effects produced when cells are incubated with trichloroethylene (12).

During our characterizations of the properties of inhibition by some sulfur-containing compounds of ammonia oxidation by *N. europaea*, we found that allylsulfide was a potent inactivator of ammonia oxidation (17a). In contrast to a variety of other organic sulfides that were shown to be oxidized to sulfoxides by the enzyme AMO, allylsulfide both specifically and potently inactivated AMO. In this article, we have further characterized the inactivation of the ammonia-oxidizing activity of cells of *N. europaea* by this compound. Some of the methods previously used to unequivocally demonstrate that $C_2H_2$ acts as a mechanism-based inactiva-

* Corresponding author.

tor of AMO were applied to our characterization of the inactivation of AMO by allylsulfide. The inactivation of the ammonia-oxidizing activity of cells by allylsulfide was time dependent and followed pseudo-first-order kinetics. The inactivation of AMO by allylsulfide required catalytic turnover of AMO. The inhibitory effects of other compounds structurally related to allylsulfide were also examined, and the results emphasize the unique nature of allylsulfide.

## MATERIALS AND METHODS

**Materials.** Allylthiourea was obtained from Eastman Kodak Co. (Rochester, N.Y.). All other allylic compounds and $CS_2$ (spectrophotometric grade, 99+%) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Chemicals were >97% pure by manufacturer analysis, except allylmercaptan (70+%) and diallyldisulfide (80%), which were supplied as technical-grade reagents and used without further purification. Reagents for electrophoresis and fluorography were obtained from ICN Biochemicals (Costa Mesa, Calif.). $^{14}C_2H_2$ was generated from $Ba^{14}CO_3$ (specific activity, 45.8 mCi/mmol) (Sigma Chemical Co., St. Louis, Mo.) as described previously (11). Unlabeled $C_2H_2$ was obtained from a cylinder (AIRCO, Oregon) or generated in a gas-generating bottle from calcium carbide (technical grade; Aldrich Chemical Co., Inc.) as described previously (9).

**Growth and preparation of cells.** *N. europaea* (ATCC 19178) was grown in batch cultures (1.5 liters) in Erlenmeyer flasks (2 liters) in an unlit room at constant temperature (30°C) on rotary shakers in previously described growth medium (12). The bacteria were harvested by centrifugation (20,000 × *g*, 15 min), resuspended in assay buffer (1.5 ml, 50 mM sodium phosphate buffer [pH 7.8] containing 2 mM $MgCl_2$), and stored on ice for use within 12 h of harvesting.

**$O_2$ electrode measurements.** The ammonia- and hydrazine-dependent $O_2$ uptake rates were determined with a Clark-style $O_2$ electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) mounted in a glass water-jacketed reaction vessel containing assay buffer (1.8 ml). Ammonia-dependent $O_2$ uptake rates of the cells were measured in the presence of ammonium sulfate (2.75 mM). Once a steady-state rate of $O_2$ uptake was recorded, allylthiourea (100 μM) was then added to the $O_2$ electrode chamber to inhibit ammonia oxidation. Hydrazine hydrochloride (750 μM) was then added to the electrode chamber. The hydrazine-dependent $O_2$ uptake rate was then recorded and used as an estimate of the hydroxylamine oxidoreductase (HAO) activity. All substrates and inhibitors were added from aqueous stock solutions and delivered by syringe. The solubility of $O_2$ in air-saturated buffer at 30°C was taken to be 230 μM, and that of $C_2H_2$, in water equilibrated at 25°C with 1 atm of $C_2H_2$, was taken to be 42 mM (13).

**Requirements of $O_2$ and AMO turnover conditions for inactivation by allylsulfide.** The requirement of $O_2$ for inactivation of AMO by allylsulfide was examined under the following conditions. The cell suspensions, assay buffer, and assay vials (10 ml) containing assay buffer (0.7 to 0.9 ml) and ammonium sulfate (2.5 mM) were stoppered with Teflon-lined silicone stoppers (Alltech Associates, Inc., Deerfield, Ill.) and then repeatedly evacuated and flushed with $N_2$ to remove $O_2$. An $O_2$-free stock solution of allylsulfide was made by the addition of pure allylsulfide (5 μl) to a vial filled with $O_2$-free assay buffer, and an aliquot of this solution (0.3 μmol, 0.2 ml) was added to the appropriate vials. The vials were incubated (5 min) in a shaker bath (30°C; 160 rpm). Cell suspensions (0.1 ml, 1.3 mg of protein) were added to initiate the reaction. After 10 min, the cells were recovered from reaction mixtures by sedimentation (14,000 × *g*, 4 min) and washed by three cycles of sedimentation and resuspension in assay buffer (3× 1.5 ml). The cells were then resuspended in assay buffer (1.0 ml) and their ammonia- and hydrazine-oxidizing activities were examined with an $O_2$ electrode, as described above. The rates of ammonia- and hydrazine-dependent $O_2$ uptake were compared for cells incubated (i) anaerobically with or without allylsulfide and (ii) aerobically with or without allylsulfide.

The ability of allylthiourea, a reversible AMO inhibitor, to protect AMO from inactivation by allylsulfide was determined by $O_2$ uptake measurements after incubating cells (30 min) with or without allylsulfide in the presence or absence of ATU (100 μM). The assay vials were prepared as described above except that (i) the amount of allylsulfide was 0.5 μmol and (ii) all conditions were aerobic.

**Recovery of ammonia-dependent nitrite-producing activity.** The ammonia-oxidizing activity of the cells was inactivated by treatment in the presence of ammonium sulfate (2 mM) with either $C_2H_2$ (1.3 μmol) or allylsulfide (2.5 μmol). Butyl rubber-stoppered glass serum vials (40 ml) containing assay buffer (4.7 or 3.7 ml, respectively) were prepared. Allylsulfide (1 ml) was added to the vials from a stock solution (2.5 mM), and $C_2H_2$ was added as a gas (2 ml) as an overpressure. The vials were placed on a rotary shaker (200 rpm), and the reactions were initiated (5 min after the addition of either allylsulfide or $C_2H_2$) by the addition of the cell suspensions (0.3 ml, 4 mg of protein). Uninhibited cells were preincubated in assay buffer (4.7 ml) containing ammonium sulfate (2 mM) and were treated in the same manner as the inhibited cells. After 20 min, the reaction mixtures were diluted (approximately eightfold with assay buffer) and then sedimented (20,000 × *g*, 10 min). The cells were resuspended with assay buffer (0.3 ml) and transferred into cotton-stoppered Erlenmeyer flasks (50 ml) containing growth medium (20 ml). The flasks were placed on a rotary shaker (200 rpm) in an unlit room at constant temperature (30°C). The recovery of AMO-dependent nitrite-producing activity was monitored by removing a sample (5 μl) of the growth medium by syringe and determining nitrite accumulation colorimetrically as described previously (7).

**Pretreatment of cells with various amounts of allylsulfide prior to $^{14}C_2H_2$ labeling.** Cells of *N. europaea* were inactivated by allylsulfide under aerobic conditions in the presence of ammonium sulfate (5 mM). Allylsulfide (25 to 600 nmol, 10 to 240 μl) was added to sealed vials. The vials were preincubated (5 min) in a shaking water bath (30°C, 160 rpm). Cell suspensions (0.1 ml, 1.9 mg of protein) were then added to the vials to initiate the reaction. After incubation with allylsulfide (10 min), the cells were washed (as described above) and the residual AMO and HAO activities of a portion of this suspension (50 μl, 0.2 mg of protein) were examined by $O_2$ uptake measurements. The remaining portion of the suspension was used to determine the residual AMO activity by using a $^{14}C_2H_2$ labeling reaction (see below).

**$^{14}C_2H_2$ labeling incubations.** Cells were exposed to $^{14}C_2H_2$ in glass serum vials (6 ml) stoppered with butyl rubber septa and capped with aluminum crimp seals (Wheaton Scientific, Millville, N.J.). Assay buffer (1.5 to 1.7 ml) was injected into the stoppered vial to create an overpressure. Five minutes before use, an aliquot (50 μCi) of $^{14}C_2H_2$ was added to the vials. After the vials were allowed to preincubate for 5 min in a shaking water bath (30°C, 300 rpm), the labeling reactions were initiated by the addition of the cell suspension (0.4 ml,

1.5 mg of protein). The reactions were terminated by the addition of allylthiourea (to 100 μM), and the cells were harvested from the reaction medium by sedimentation in a microcentrifuge (14,000 × *g*, 4 min). The supernatant was removed, and the sedimented cells were immediately solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and stored at −20°C until further use.

**SDS-PAGE and fluorography.** Protein solubilization, electrophoresis, and fluorography were conducted as previously described (11, 12). Protein samples (200 μg per lane) for analysis by SDS-PAGE and fluorography were obtained from a constant volume of cell suspension. Radiolabel incorporation into the 27-kDa polypeptide component of AMO was determined by densitometric scanning of the fluorograms with a GS300 densitometer (Hoefer Scientific Instruments, San Francisco, Calif.) interfaced to a chart recorder. The apparent molecular weight of the $^{14}C$-labeled polypeptide was determined from fluorograms by comparison with $R_f$ values for molecular weight standards of bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), and cytochrome *c* (12,300).

**Effects of the various allylic compounds on ammonia oxidation by *N. europaea*.** Stock solutions of the allylic compounds were prepared daily by the addition of the compound to sealed glass serum vials filled with assay buffer. Incubations were conducted in glass serum vials containing ammonium sulfate (5 mM) and assay buffer. The allylic compound (0.5 μmol, 0.25 to 0.5 ml) was added to assay vials and allowed to equilibrate for approximately 2 min at 30°C in a shaking water bath. Cell suspensions (50 μl, 1.2 mg of protein) were added to initiate the reactions. After 10 min, a liquid sample was withdrawn from the assay vial for quantification of nitrite. The rate of nitrite production by cells in the presence of only ammonium sulfate (5 mM) and assay buffer (0.95 ml) remained constant during the time course of the assay.

The reversibility of the effects of the compounds on the activities of AMO and HAO was examined as described above. The cells were resuspended in assay buffer (0.5 ml), and a portion of the washed cells (50 μl, 0.12 mg of protein) was added to assay buffer (1.8 ml) in the chamber of an $O_2$ electrode. The ammonia- and hydrazine-dependent $O_2$ uptake rates were recorded for the cells as described above. These rates were compared with the rates of cells treated in the same manner except incubated only in the presence of ammonium sulfate (5 mM) and assay buffer (0.95 ml).

**Protein determinations.** Protein concentrations were determined with the biuret assay (6) after solubilization of cell protein in aqueous 3 N NaOH (30 min at 60°C) and sedimentation of insoluble material by centrifugation (14,000 × *g*, 5 min).

## RESULTS

**Inactivation of AMO by allylsulfide: requirements of $O_2$ and AMO turnover conditions.** Allylsulfide was demonstrated to be a potent inactivator of ammonia oxidation in *N. europaea* (17a). However, the mechanism of inactivation was not determined. Two properties of AMO-specific inactivators which have been previously used to distinguish the mode of inactivation were investigated. These properties were (i) the requirements of $O_2$ for inactivation, which would indicate a requirement of AMO catalysis for the inactivation, and (ii) the use of allylthiourea, a reversible inhibitor of AMO, to prevent inactivation by inhibiting AMO turnover. If the inactivation of AMO by allylsulfide was prevented by either or both of these conditions, the catalytic involvement of AMO in the inactivation could be concluded.

Cells incubated with allylsulfide (0.5 μmol) and ammonium sulfate (5 mM) under anaerobic conditions retained 90% of their ammonia-dependent $O_2$ uptake rate (38 μmol of $O_2$ consumed $h^{-1}$ mg of protein$^{-1}$) compared with cells incubated either aerobically or anaerobically solely in the presence of ammonium sulfate. In contrast, the ammonia-oxidizing activity of the cells incubated aerobically with allylsulfide was completely inactivated. These results indicated that inactivation of AMO by allylsulfide required $O_2$ and therefore conditions under which AMO would be catalytically active. The activity of HAO (hydrazine dependent $O_2$ uptake) remained the same (7.5 μmol of $O_2$ consumed $h^{-1}$ mg of protein$^{-1}$) regardless of the presence of allylsulfide.

To further confirm the requirement for the catalytic activity of AMO in inactivation by allylsulfide, the ability of a reversible AMO inhibitor to prevent inactivation was examined. Cells were pretreated with allylthiourea (100 μM) in either the presence or absence of allylsulfide (0.5 μmol). The cells were washed to remove the inhibitor(s). Cells incubated with only allylthiourea recovered 60% of the ammonia-dependent $O_2$ uptake activity relative to cells incubated in the absence of any inhibitor. Cells incubated with allylthiourea and allylsulfide recovered 55% of their ammonia-dependent $O_2$ uptake activity relative to cells incubated in the absence of any inhibitor. In contrast, cells incubated with only allylsulfide retained none of their ammonia-dependent $O_2$ uptake activity. The hydrazine-dependent $O_2$ uptake activity was unchanged by the presence of either allylthiourea or allylsulfide. Both the requirements of $O_2$ and the substantial protection from inactivation afforded by allylthiourea suggest that allylsulfide may be a mechanism-based inactivator of AMO.

**Inactivation of ammonia-dependent $O_2$ uptake by allylsulfide followed first-order kinetics.** The inactivation of enzyme activity in the presence of an excess of a mechanism-based inactivator should follow pseudo-first-order kinetics (27). To determine whether the loss of AMO activity which occurred in the presence of allylsulfide was an apparent first-order process, the time-dependent inhibitory effect of allylsulfide on the rate of ammonia-dependent $O_2$ uptake was monitored with an $O_2$ electrode. Cells were added to the $O_2$ electrode chamber, followed by ammonium sulfate. Once a constant rate of $O_2$ uptake was observed, various amounts of allylsulfide were then added to the $O_2$ electrode chamber from an aqueous stock solution. Figure 1 shows a semilogarithmic plot of the rate of $O_2$ uptake as a function of the time following the addition of allylsulfide to the $O_2$ electrode chamber. The straight lines for each concentration of allylsulfide imply a first-order rate of inactivation of the AMO activity (Fig. 1).

**Dependence of the rate of inactivation of ammonia-dependent $O_2$ uptake by allylsulfide on the concentration of ammonia.** To characterize the dependence of the rate of inactivation of AMO by allylsulfide on the ammonia concentration, a series of $O_2$ electrode experiments were conducted. The range of ammonium ion concentrations used covered the concentrations both above and below the apparent $K_m$ for ammonium sulfate, as determined from initial rates of $O_2$ uptake (Fig. 2A). The effect of the ammonium sulfate concentration on the rate of inactivation was determined by estimating the time required for allylsulfide to inactivate the ammonia-dependent $O_2$ uptake activity by 50%. The results of this experiment and of comparable experiments with allylthiourea and acetylene are presented in Fig. 2B. Over

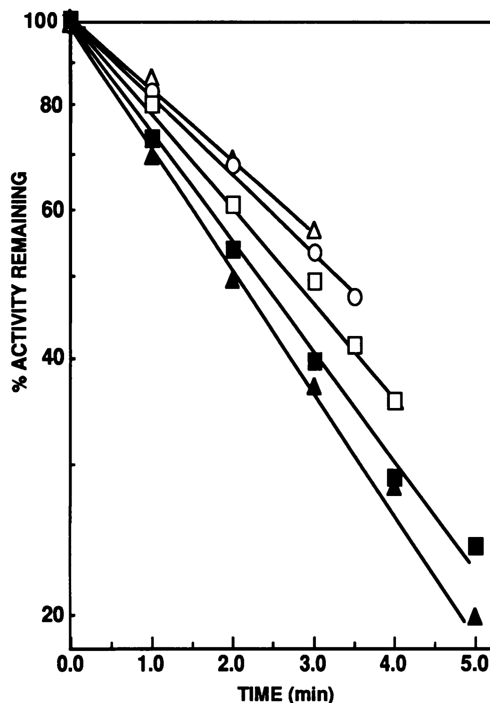



FIG. 1. First-order inactivation of ammonia-dependent $O_2$ uptake by allylsulfide. A series of $O_2$ electrode experiments were conducted with cells of *N. europaea* (0.06 mg of protein) in the presence of ammonium sulfate (2.7 mM). Once a steady-state rate of ammonia-dependent $O_2$ uptake (1.6 μmol of $O_2$ consumed $min^{-1}$ mg of $protein^{-1}$) was established, allylsulfide at 22 (△), 26 (○), 31 (□), 35 (■), or 40 (▲) μM was added to the $O_2$ electrode chamber. The results are presented as a semilogarithmic plot of the percent $O_2$ uptake rate remaining (in relationship to the preinhibited rate) versus the time after the addition of allylsulfide to the $O_2$ electrode chamber.

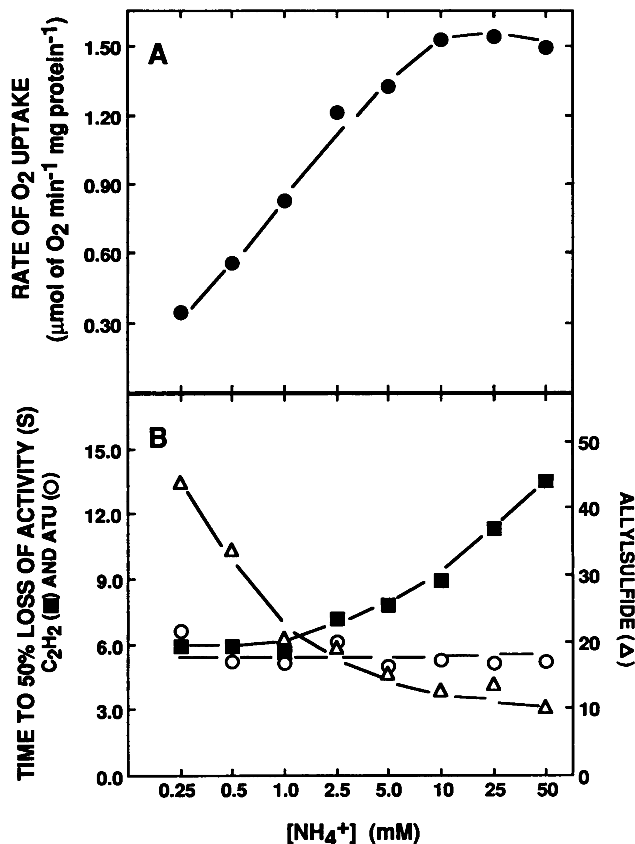



FIG. 2. Effect of the ammonium sulfate concentration on the $t_{1/2}$ of inhibition of ammonia oxidation by acetylene, allylthiourea (ATU), and allylsulfide. A series of $O_2$ electrode incubations was performed in which cells (0.14 mg of protein per ml) were assayed for rates of ammonia-dependent $O_2$ uptake in the presence of a range of ammonium ion concentrations (0.25 to 50 mM). When a steady-state rate of $O_2$ uptake had been established for each concentration of ammonium sulfate, the inhibitor (to 200 μM) was added. The time required for the rate of $O_2$ uptake to decline to 50% of the preinhibited rate was then determined. (A) Effect of initial ammonium ion concentration on the preinhibited rate of $O_2$ uptake; (B) effect of ammonium ion concentration of the $t_{1/2}$ of ammonia-dependent $O_2$ uptake activity of cells inhibited with 200 μM $C_2H_2$ (■), 200 μM allylthiourea (○), or 200 μM allylsulfide (△).

the range of ammonium sulfate concentrations tested, there was essentially no change in the half-life ($t_{1/2}$) for the inhibition caused by allylthiourea, implying that the inhibition by allylthiourea was independent of ammonium ion concentration. The $t_{1/2}$ for inactivation by acetylene progressively increased with successive increases in ammonium ion concentrations, as previously observed (13). In contrast to acetylene, a progressive decrease in $t_{1/2}$ for allylsulfide inactivation of AMO occurred with increasing ammonium sulfate concentrations. Thus, the inactivation rate increased with increasing ammonia concentrations. The addition of sodium chloride (44 mM) to the $O_2$ electrode chamber containing ammonium sulfate (2.7 mM) did not increase the rate of inactivation by allylsulfide (44 nmol), suggesting that the increased rate of inactivation with higher concentrations of ammonium sulfate was probably not due to an increase in ionic strength. Furthermore, the increased rate of inactivation by allylsulfide approached a maximum as the rate of ammonia-dependent $O_2$ uptake approached its maximum (Fig. 2). This confirms that the increasing rate of inactivation was likely to be a result of increasing ammonium sulfate concentration and hence AMO turnover rate rather than a consequence of increasing ionic strength.

**Comparison of the rate of recovery of nitrite production by allylsulfide- and acetylene-treated cells.** Cells of *N. europaea* can recover their ammonia-dependent nitrite-producing activity after exposure to either acetylene or trichloroethylene if the cells are subsequently placed in growth medium in the absence of the inhibitor (12, 24). The recovery of ammonia-oxidizing activity after inactivation of AMO by $C_2H_2$ or inactivation by trichloroethylene is known to require de novo protein synthesis (12, 24). It is also known that $^{14}C_2H_2$ binds to and inactivates predominantly one polypeptide, which is presumed to be an active-site-containing component of AMO. In contrast, the covalent modifications caused by the activated $^{14}C$-labeled trichloroethylene product are nonspecific and result in $^{14}C$ incorporation into numerous polypeptides of *N. europaea* (24). Cells inactivated by trichloroethylene recover their nitrite production more slowly than cells inactivated by $C_2H_2$ (12). It was suggested that the slower rate of recovery for trichloroethylene-treated cells is a consequence of the nonspecific protein inactivations resulting from trichloroethylene oxidation (i.e., several proteins were modified), which is in contrast to the faster rate of recovery of cells inactivated by the specific inactiva-

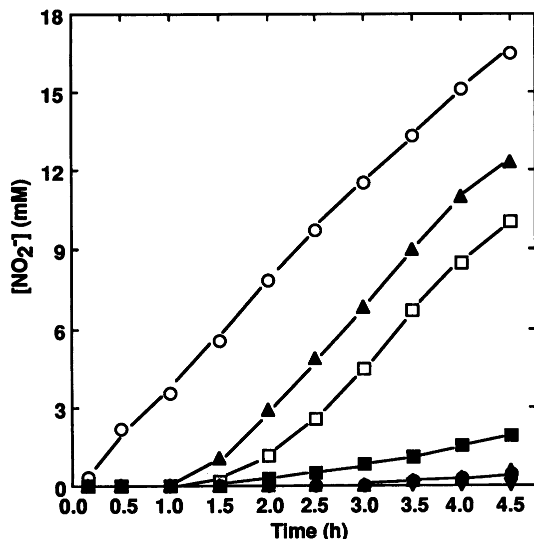



FIG. 3. Time course of recovery of ammonia-dependent nitrite-producing activity of cells after inactivation by either $C_2H_2$ or allylsulfide. Cells were pretreated with $C_2H_2$ or allylsulfide as described in Materials and Methods. The time course of recovery of their nitrite-producing activity was monitored as nitrite accumulation produced by uninhibited cells (○), cells which had been preincubated with $C_2H_2$ (□, ●, ▼), or cells which had been preincubated with allylsulfide (▲, ■, △). The washed cells (0.3 ml, 14 mg of protein per ml) were then added to Erlenmeyer flasks (50 ml) containing 20 ml of the following media: complete growth medium (○, □, ▲), complete growth medium plus rifampin (100 μg/ml) (●, ■), and complete growth medium plus chloramphenicol (400 μg/ml) (▼, △). The time course of the recovery of uninhibited cells was unaffected by incubation in the presence of either rifampin or chloramphenicol (data not shown).

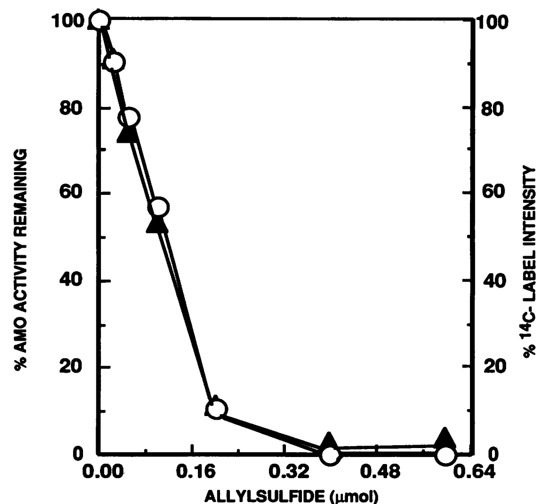



FIG. 4. Comparison of the ammonia-dependent $O_2$ uptake rate of cells of *N. europaea* with their ability to incorporate $^{14}C$ label from $^{14}C_2H_2$ after prior inactivation by allylsulfide. Cells were sequentially exposed to allylsulfide and $^{14}C_2H_2$ as described in Materials and Methods. Shown is a plot of the residual AMO activity and the level of $^{14}C$ label incorporation versus the amount of allylsulfide. The intensity of the $^{14}C$-labeled 27-kDa polypeptide, as determined by densitometry, of cells inactivated by allylsulfide is expressed as a percentage relative to the intensity of the $^{14}C$ label which was incorporated by the uninhibited cells (○). The remaining AMO activity of the cells, as determined by ammonia-dependent $O_2$ uptake, is expressed as a percentage relative to the AMO activity of the uninhibited cells (▲).

tion resulting from acetylene oxidation (i.e., only a single polypeptide was modified) (12).

As an additional technique to assess whether the inactivation of AMO by allylsulfide was specific, we compared the recovery rates of cells whose ammonia-dependent $O_2$ uptake activities had been completely inactivated with either acetylene or allylsulfide. The rates of nitrite production by uninhibited cells and by cells treated with either allylsulfide or acetylene are shown in Fig. 3. After a 2- to 3-h lag, the rate of nitrite production by acetylene-treated cells gradually increased to the rate of uninhibited cells, demonstrating the recovery of their ammonia-oxidizing activity. Cells treated with allylsulfide also regained their ammonia-oxidizing activity but required slightly less time to recover than cells treated with acetylene. The fact that the rate of recovery after allylsulfide inactivation did not have a lag time longer than that shown for acetylene suggests that the inactivation of AMO by allylsulfide did not involve the inactivation of many protein components. If, as in the case of trichloroethylene, several proteins had been damaged by allylsulfide, the cells would be expected to have a recovery rate slower than that of cells inactivated by acetylene.

A second characteristic of the recovery of ammonia-dependent nitrite-producing activity by cells of *N. europaea* after inactivation of AMO by acetylene is that de novo protein synthesis of the 27-kDa polypeptide is required. The recovery of nitrite production by allylsulfide-inactivated cells was also prevented with either the transcriptional inhibitor rifampin or the translational inhibitor chloramphenicol (Fig. 3). This result demonstrates that de novo protein synthesis is also required for the recovery of allylsulfide-inactivated cells and further suggests that a protein component was covalently modified as a result of incubation of cells with allylsulfide.

**Effect of allylsulfide treatment on the incorporation of $^{14}C$ label from $^{14}C_2H_2$ into the 27-kDa polypeptide.** It is proposed the $C_2H_2$ inactivates AMO as a result of the attempted oxidation of the acetylenic triple bond resulting in the generation of a reactive intermediate which binds to and inactivates the enzyme (17). This interpretation is supported by the observation that incubation of cells with $^{14}C_2H_2$ gives rise to the covalent attachment of $^{14}C$ label to a membrane-bound polypeptide with an apparent $M_r$ of 27,000, as determined by SDS-PAGE and fluorography. The incorporation of $^{14}C$ label from $^{14}C_2H_2$ into the polypeptide with an apparent $M_r$ of 27,000 is well characterized (12, 17). The level of covalent modification of this polypeptide by $^{14}C$ label from $^{14}C_2H_2$ and the extent of the inhibition of ammonia-oxidizing activity are proportional. The incorporation of $^{14}C$ label was saturable and could be used to estimate the levels of active AMO present in cells of *N. europaea* (12). The specific, time-dependent inactivation of AMO by allylsulfide resulted in a concomitant and proportional decrease in the ability of cells to incorporate $^{14}C$ label from $^{14}C_2H_2$ into the polypeptide with an apparent $M_r$ of 27,000 (Fig. 4). Thus, the levels of active AMO as measured by label incorporation decreased proportionally with the levels of active AMO measured as ammonia-dependent $O_2$ uptake. This result indicates that inactivation of ammonia-oxidizing activity by allylsulfide decreases the amount of catalytically competent AMO present in cells.

TABLE 1. Comparison of the effects of various allylic compounds on ammonia oxidation by *N. europaea*

| Chemical structure of compound | Amt added (μmol) | % Nitrite produced relative to uninhibited cells from 10 mM $NH_4^+$ [a] | % Reversibility relative to uninhibited cells for[b]: | |
|---|---|---|---|---|
| | | | AMO | HAO |
| $(H_2C{=}CHCH_2)_2C$ | 0.5 | 97 | 96 | 104 |
| $(H_2C{=}CHCH_2)_2O$ | 0.5 | 92 | 101 | 108 |
| $(H_2C{=}CHCH_2)_2NH$ | 0.5 | 101 | 98 | 100 |
| $(H_2C{=}CHCH_2)_2S_2$ | 0.5 | 9 | 25 | 108 |
| $H_2C{=}CHCH_2SH$ | 0.5 | 4 | 20 | 104 |
| $(H_2C{=}CHCH_2)_2S$ | 0.5 | 10 | 4 | 110 |

[a] The percentages are expressed relative the amount of nitrite produced by cells incubated only in the presence of ammonium sulfate. The relative errors among the percentages ranged from ±2% to ±10% for the triplicate samples.

[b] To examine the reversibility of the effect, the cells were sedimented and resuspended three times with buffer to remove the compound. The activities of AMO and HAO were then measured as the rates of ammonia- or hydrazine-dependent $O_2$ uptake. The activities are expressed as a percentage relative to a rate of 54 μmol of $O_2$ consumed $h^{-1}$ mg of protein$^{-1}$ for the activity of AMO or 11 μmol of $O_2$ consumed $h^{-1}$ mg of protein$^{-1}$ for the activity of HAO. The errors ranged between ±1% and ±11% among triplicate samples.

**Effects of compounds with structures similar to that of allylsulfide on ammonia oxidation by *N. europaea*.** To determine whether allylsulfide was atypical in its ability to inactivate the ammonia-oxidizing activity of *N. europaea*, we examined other compounds with structures related to that of allylsulfide for their effects on the activity of AMO. The effects of these compounds on ammonia-dependent nitrite production and the reversibility of their effects on the ammonia- and hydrazine-oxidizing activities are presented in Table 1. Compounds analogous to allylsulfide but which did not contain sulfur, i.e., 1,6-heptadiene, diallylamine, and diallylether, did not have any irreversible effects on the AMO activity of cells of *N. europaea* (Table 1). Allylmercaptan and allylsulfide did have irreversible effects on AMO; however, both these reagents contained sulfides and/or allylic sulfides as impurities and thus their effects could not be attributed exclusively to these compounds.

## DISCUSSION

Two essential determinants of a mechanism-based inactivator are that (i) the target enzyme must catalyze its own destruction and (ii) the inactivation must result from the reaction of the enzyme with a species produced within its own active site (23). This article provides evidence that the inactivation of AMO by allylsulfide occurred as a result of or during the process of oxidation of allylsulfide by AMO and that the inactivation was specific for AMO. As characteristic of a mechanism-based inactivator, the inactivation required the same conditions required for catalytic turnover of the target enzyme. Incubation of cells with allylsulfide under anaerobic conditions or when the turnover of AMO was inhibited with allylthiourea substantially prevented the inactivation of the ammonia-oxidizing activity of cells. Therefore, AMO seems the most likely target for the effects of allylsulfide. However, because the experiments were conducted with whole cells, our data cannot rigorously exclude the possibility that allylsulfide may inactivate an accessory component required for ammonia oxidation.

Inactivation of AMO activity by allylsulfide followed pseudo-first-order kinetics (Fig. 1). Increasing ammonia concentrations increased the rate of inactivation of AMO by allylsulfide (Fig. 2). This suggests that stimulating the rate of turnover of AMO increases the rate of inactivation. The recovery of nitrite production by allylsulfide-inactivated cells required de novo protein synthesis, indicating that an irreversible modification of a protein component of AMO had occurred, as would be expected of a mechanism-based inactivation. Furthermore, the recovery rate of cells exposed to allylsulfide closely followed the recovery rate of cells exposed to acetylene and was faster than the rate previously characterized for the recovery of cells exposed to the nonspecific inactivations produced by trichloroethylene (12). These results are consistent with the idea that the inactivation of ammonia-oxidizing activity by allylsulfide is specific for a single essential protein component involved in ammonia oxidation. Cells treated with allylsulfide were incapable of incorporating $^{14}C$ label from $^{14}C_2H_2$ into the 27-kDa polypeptide of AMO. This result demonstrates at the molecular level the catalytic inactivation of AMO. Furthermore, the amount of ammonia-dependent $O_2$ uptake which remained after treatment with progressively larger amounts of allylsulfide corresponded to the amount of $^{14}C$ label from $^{14}C_2H_2$ which was incorporated into the 27-kDa polypeptide (Fig. 4). This result verified that the level of inactivation of AMO by allylsulfide, as measured by ammonia-dependent $O_2$ uptake, correlated with the AMO activity remaining the cells. Other criteria for establishing that an inactivator is mechanism based, such as protection with either exogenous nucleophiles or an alternate substrate for AMO (27), were not applicable for use in our whole-cell system. The most satisfying test of a mechanism-based inactivator is the demonstration of a covalent modification of an active site component, such as a polypeptide or prosthetic group, as has been shown for the inactivation of AMO by $^{14}C_2H_2$. However, radiolabeled allylsulfide is not commercially available, and therefore this remains to be determined. Nonetheless, our results suggest that the attempted oxidation of allylsulfide by AMO produces a reactive intermediate which, like $C_2H_2$, can specifically inactive AMO.

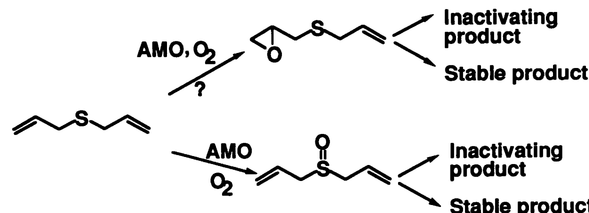



FIG. 5. Two possible pathways for the oxidation of allylsulfide by AMO which may lead to the inactivation of AMO.

Two possible oxidative sites which may be relevant to the production of a reactive species which could inactivate AMO are present in allylsulfide. Oxidation of allylsulfide by AMO could occur at either the terminal alkene or the central sulfenyl moiety (Fig. 5). While oxidation at either of these sites is possible, oxidation at only one site is likely to result in the reactive species which leads to the inactivation of AMO (Fig. 5). The oxidation of terminal alkenes to stable epoxides by cells of *N. europaea* is well documented (14, 16). However, the inactivation of cytochrome P-450-associated monooxygenases by terminal alkenes is also well established (23). Given the similar catalytic natures of these two broad-substrate monooxygenases and the known reactivity of epoxides, the inactivation of AMO by allylsulfide may

result during the oxidation of the alkene moiety. However, previous results for the oxidation of organic sulfides by AMO suggest that the oxidation of the sulfur atom is a more frequent occurrence. Enzymatic preference for the oxidation of the sulfenyl moiety over the alkene moiety might be expected in view of the ease of oxidation of the sulfenyl moiety (20). Additionally, none of the structurally related compounds listed in Table 1, which did not possess a sulfur atom and for which an epoxide might be an expected product, had any inactivating effect on AMO. If activation of the alkene moiety of allylsulfide is the initiating step which results in the inactivation of AMO, then one might expect some epoxide to be formed and therefore some inactivation to occur during the oxidation of allylmethylsulfide by *N. europaea*. However, cells were not substantially inactivated by allylmethylsulfide, but rather a substantial amount of allylmethylsulfoxide was produced by AMO (17a). While oxidation of the alkene moiety of allylsulfide is theoretically possible, results for allylmethylsulfide suggest that this is an infrequent occurrence and therefore the inactivation of AMO by allylsulfide is mostly likely to involve the attempted oxidation at the sulfenyl moiety of allylsulfide.

The inactivation of AMO by allylsulfide raises the controversial issue of allelopathic inhibition of nitrification. The ability of a plant to inhibit nutrient consumption by soil microorganisms would undoubtedly be an advantage, and the effects of volatile compounds from plant residues on microorganisms are well established (28). However, despite the facts that allelopathic inhibition of nitrification has been suggested (30) and theoretically debated (29) and two natural products have been investigated as potential nitrification inhibitors (25), the inhibition of a pure culture of ammonia-oxidizing nitrifier by a distinctive plant product has never been conclusively demonstrated (19). Interestingly, numerous allylic sulfide compounds are known to be present in plants of the *Allium* spp., especially the garlic and chive varieties, and these compounds are suspected to function to either attract or retard insects and/or pests (1). Allylsulfide and possibly allyldisulfide may be two compounds which can inhibit microorganisms, including ammonia-oxidizing nitrifiers, and which are present in the immediate environment surrounding bulbs of the *Allium* spp. Allylsulfide could, therefore, constitute a nitrification inhibitor which is both a natural product and an example of an allelopathic agent.

The biological activity of allylic sulfides in the soil environment is not unknown. Germination of sclerotia of the white rot fungus *Sclerotium cepivorum* Berk. is specifically triggered only by allylic sulfur compounds produced by members of the genus *Allium*. It is suggested that the exudates containing alkyl- and alkenyl-*l*-cysteine sulfoxides from the roots of *Allium* spp. are metabolized by the soil microflora to yield a range of volatile allylic thiols and sulfides which specifically activate the germination of sclerotia (5, 18). However, despite our suggestion that exudates from plants of the *Allium* spp. may inhibit ammonia consumption by nitrifiers, the requirement of *Allium* spp. for nitrogen fertilizer does not appear to differ from the requirement of fertilizer by other crop species (4). Although field studies suggest that nitrification may not in general be inhibited in soils containing garlic, it may be of interest in light of our findings with regard to the inactivation by AMO by allylsulfide to determine whether the rate of nitrification in crops of *Allium* spp. differs from that in crops of other species which do not produce allylsulfides.

The inhibition of ammonia oxidation by allylsulfide may also have a direct practical use as a tool in ecological studies of nitrification. One important inhibitor used in the study of the nitrogen cycle is acetylene. Through various mechanisms acetylene inhibits nitrogenase, AMO, nitrous oxide reductase, and nitrate reductase (10). The acetylene blockage assay for nitrous oxide reductase activity is the most widely used assay for denitrifying activity (21). However, this assay is complicated in the field by the inhibitory effects of acetylene on the process of nitrification. Nitrification supplies the electron acceptor, nitrate, for the process of denitrification. What is needed to estimate natural nitrogen-transforming activities is a series of inhibitors specific for each process. Toward this end, methyl fluoride and dimethyl ether have recently been shown to be effective inhibitors of methane oxidation and nitrification with little effect on methanogenesis and denitrification (22). Allylsulfide is another potential specific inhibitor of nitrification which has several attractive features for field studies. From a practical point, allylsulfide is highly volatile and can be applied as effectively as acetylene and other gaseous inhibitors. Perhaps more important, allylsulfide acts as a mechanism-based inactivator of AMO. Its effects are therefore likely to be more similar to those of acetylene than are those of methyl fluoride and dimethyl ether, which both appear to inhibit nitrification by acting as alternate substrates for AMO.

The unique properties of allylsulfide have also been recognized in another area of research. Both allylsulfide and its biologically more oxidized products, i.e., allylsulfoxide and allylsulfone, were shown to inhibit microsomal P-450 2E1 monooxygenase. Allylsulfide has been suggested to inhibit carcinogenesis as a result of its inhibition of microsomal monooxygenase P-450 2E1 (2). However, allylsulfide was concluded not to be a general inhibitor of monooxygenases because other microsomal monooxygenase activities were unaffected (3). In light of the similar oxidations catalyzed by AMO and P-450-associated monooxygenases, it is also interesting that the bulk of the sulfur compounds which inactivate cytochrome P-450 monooxygenases have been concluded not to be mechanism-based suicidal agents but instead to partition out of the active site (23). However, our data for the inactivation of AMO by allylsulfide in cells of *N. europaea* suggest that allylsulfide is a mechanism-based inactivator and that the inactivating species is unlikely to diffuse far from the catalytic site.

## ACKNOWLEDGMENTS

This research was supported by U.S. Department of Agriculture grant 88-37120-4200, U.S. Environmental Protection Agency grant R816531, and the Oregon Agricultural Experiment Station.

## REFERENCES


1. **Block, E.** 1992. The organosulfur chemistry of the genus *Allium*—implications for the organic chemistry of sulfur. Angew. Chem. Int. Ed. Engl. **31**:1135–1178.
2. **Brady, J. F., H. Ishizake, J. M. Fukuto, M. C. Lin, A. Fadel, J. M. Gapac, and C. S. Yang.** 1991. Inhibition of cytochrome P-450 by diallyl sulfide and its metabolites. Chem. Res. Toxicol. **4**:642–647.
3. **Brady, J. F., D. Li, I. Hiroyuki, and C. S. Yang.** 1988. Effect of diallyl sulfide on rat liver microsomal nitrosamine metabolism and other monooxygenase activities. Cancer Res. **48**:5937–5940.
4. **Buwalda, J. G.** 1986. Nitrogen nutrition of garlic (*Allium Sativum L.*) under irrigation. Components of yield and indices of crop nitrogen status. Scientia Hortic. **29**:69–76.
5. **Coley-Smith, J. R., and D. Parfitt.** 1986. Some effects of diallyl disulphide on sclerotia of *Sclerotium cepivorum*: possible novel control method for white rot disease of onions. Pestic. Sci. **37**:587–594.

6. **Gornall, A. G., C. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177:**751–766.
7. **Hageman, R. H., and D. P. Hucklesby.** 1971. Nitrate reductase from higher plants. Methods Enzymol. **23:**491–503.
8. **Hofman, T., and H. Lees.** 1953. The biochemistry of the nitrifying organisms. 4. The respiration and intermediary metabolism of *Nitrosomonas*. Biochem. J. **54:**579–583.
9. **Hyman, M. R., and D. J. Arp.** 1987. Quantification and removal of some contaminating gases from acetylene used to study gas-utilizing enzymes and microorganisms. Appl. Environ. Microbiol. **53:**298–303.
10. **Hyman, M. R., and D. J. Arp.** 1988. Acetylene inhibition of metalloenzymes. Anal. Biochem. **173:**207–220.
11. **Hyman, M. R., and D. J. Arp.** 1990. The small-scale production of [U-$^{14}$C]acetylene from $Ba^{14}CO_3$: application to labeling of ammonia monooxygenase in autotrophic nitrifying bacteria. Anal. Biochem. **190:**348–353.
12. **Hyman, M. R., and D. J. Arp.** 1992. $^{14}C_2H_2$- and $^{14}CO_2$-labelling studies of the *de novo* synthesis of polypeptides by *Nitrosomonas europaea* during recovery from acetylene and light inactivation of ammonia monooxygenase. J. Biol. Chem. **267:**1534–1545.
13. **Hyman, M. R., C. Y. Kim, and D. J. Arp.** 1990. Inhibition of ammonia monooxygenase in *Nitrosomonas europaea* by carbon disulfide. J. Bacteriol. **172:**4775–4782.
14. **Hyman, M. R., I. B. Murton, and D. J. Arp.** 1988. Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes, and alkynes. Appl. Environ. Microbiol. **54:** 3187–3190.
15. **Hyman, M. R., and P. M. Wood.** 1983. Methane oxidation by *Nitrosomonas europaea*. Biochem. J. **212:**31–37.
16. **Hyman, M. R., and P. M. Wood.** 1984. Ethylene oxidation by *Nitrosomonas europaea*. Arch. Microbiol. **137:**155–158.
17. **Hyman, M. R., and P. M. Wood.** 1985. Suicidal inactivation and labelling of ammonia mono-oxygenase by acetylene. Biochem. J. **227:**719–725.

17a. **Juliette, L. Y., M. R. Hyman, and D. J. Arp.** 1993. Inhibition of ammonia oxidation in *Nitrosomonas europaea* by sulfur compounds: thioethers are oxidized to sulfoxides by ammonia monooxygenase. Appl. Environ. Microbiol. **59:**3718–3727.

18. **King, J. E., and J. R. Coley-Smith.** 1969. Production of volatile alkyl sulphides by microbial degradation of synthetic alliin and alliin-like compounds, in relation to germination of sclerotia of *Sclerotium cepivorum* Berk. Ann. Appl. Biol. **64:**303–314.
19. **McCarty, G. W., J. M. Bremner, and E. L. Schmidt.** 1991. Effects of phenolic acids on ammonia oxidation by terrestrial autotrophic nitrifying microorganisms. FEMS Microbiol. Ecol. **85:**345–349.
20. **Oae, S.** 1991. Organic sulfur chemistry: structure and mechanism, p. 253–281. CRC Press, Boca Raton, Fla.
21. **Oremland, R. S., and D. G. Capone.** 1988. Use of "specific" inhibitors in biogeochemistry and microbial ecology. Adv. Microb. Ecol. **10:**285–383.
22. **Oremland, R. S., and C. W. Culbertson.** 1992. Evaluation of methyl fluoride and dimethyl ether as inhibitors of aerobic methane oxidation. Appl. Environ. Microbiol. **58:**2983–2992.
23. **Ortiz de Montellano, P. R.** 1988. Suicide substrates for drug metabolizing enzymes: mechanisms and biological consequences, p. 99–149. *In* G. G. Gibson (ed.), Progress in drug metabolism. Taylor and Francis, New York.
24. **Rasche, M. E., M. R. Hyman, and D. J. Arp.** 1991. Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*: cometabolic inactivation of ammonia monooxygenase and substrate specificity. Appl. Environ. Microbiol. **57:** 2986–2994.
25. **Sahrawat, K. L., and D. R. Keeney.** 1985. Perspectives for research on development of nitrification inhibitors. Commun. Soil Sci. Plant Anal. **16:**517–524.
26. **Shears, J. H., and P. M. Wood.** 1985. Spectroscopic evidence for a photosensitive oxygenated state of ammonia monooxygenase. Biochem. J. **226:**499–507.
27. **Silverman, R. B.** 1988. Mechanism-based enzyme inactivation: chemistry and enzymology, p. 3–30. CRC Press, Inc., Boca Raton, Fla.
28. **Stotzky, G., and S. Schenck.** 1976. Volatile organic compounds and microorganisms. Crit. Rev. Microbiol. **4:**333–382.
29. **White, C. S.** 1986. Nitrification inhibition by monoterpenoids: theoretical mode of action based on molecular structures. Ecology **69:**1631–1633.
30. **White, C. S.** 1986. Volatile and water-soluble inhibitors of nitrogen mineralization and nitrification in a ponderosa pine ecosystem. Biol. Fertil. Soils **2:**97–104.