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Factors Limiting Aliphatic Chlorocarbon Degradation by *Nitrosomonas europaea*: Cometabolic Inactivation of Ammonia Monooxygenase and Substrate Specificity

MADELINE E. RASCHE, MICHAEL R. HYMAN, AND DANIEL J. ARP*

Laboratory for Nitrogen Fixation Research, Department of Botany and Plant Pathology, 2082 Cordley Hall, Oregon State University, Corvallis, Oregon 97331-2902

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The soil nitrifying bacterium *Nitrosomonas europaea* is capable of degrading trichloroethylene (TCE) and other halogenated hydrocarbons. TCE cometabolism by *N. europaea* resulted in an irreversible loss of TCE biodegradative capacity, ammonia-oxidizing activity, and ammonia-dependent O_2 uptake by the cells. Inactivation was not observed in the presence of allylthiourea, a specific inhibitor of the enzyme ammonia monooxygenase, or under anaerobic conditions, indicating that the TCE-mediated inactivation required ammonia monooxygenase activity. When *N. europaea* cells were incubated with [^{14}C]TCE under conditions which allowed turnover of ammonia monooxygenase, a number of cellular proteins were covalently labeled with ^{14}C. Treatment of cells with allylthiourea or acetylene prior to incubation with [^{14}C]TCE prevented incorporation of ^{14}C into proteins. The ammonia-oxidizing activity of cells inactivated in the presence of TCE could be recovered through a process requiring de novo protein synthesis. In addition to TCE, a series of chlorinated methanes, ethanes, and other ethylenes were screened as substrates for ammonia monooxygenase and for their ability to inactivate the ammonia-oxidizing system of *N. europaea*. The chlorocarbons could be divided into three classes depending on their biodegradability and inactivating potential: (i) compounds which were not biodegradable by *N. europaea* and which had no toxic effect on the cells; (ii) compounds which were cooxidized by *N. europaea* and had little or no toxic effect on the cells; and (iii) compounds which were cooxidized and produced a turnover-dependent inactivation of ammonia oxidation by *N. europaea*.

Nitrosomonas europaea is an obligate chemolithotrophic nitrifying bacterium which derives its energy for growth exclusively from the oxidation of ammonia to nitrite. Nitrification in *N. europaea* is initiated by the reductant-dependent oxidation of ammonia to hydroxylamine (NH_2OH) through the action of ammonia monooxygenase (AMO). Reductant for AMO-catalyzed reactions is provided by the further oxidation of hydroxylamine to nitrite by hydroxylamine oxidoreductase (27).

Recent evidence indicates that AMO in cells of *N. europaea* is also capable of cooxidizing hydrocarbons (14–16, 18) and aliphatic halogenated hydrocarbons (2, 17, 23–25), including the agricultural nematicidal fumigant 1,2-dibromo-3-chloropropane (24) and industrial pollutants such as trichloroethylene (TCE) (2, 25). Because many halogenated hydrocarbons are suspected human carcinogens, increasing concern about the presence of these chemicals in soil and groundwater supplies (5, 20) has stimulated interest in characterizing the activity and physiology of bacteria which exhibit biodegradative potential. Nitrifying bacteria are excellent candidates for study because it may be possible to enhance the biodegradative capacity of these ubiquitous soil bacteria with the simple addition of ammonia and O_2 to support halocarbon cometabolism.

However, if bacteria are to be used effectively in bioremediation schemes, it is important to understand the limitations as well as the potential of the biodegradative systems. One factor which potentially limits biodegradative capacity is the effect of halocarbon cometabolism on the physiology of the microorganism. For example, Wackett and Householder (26) demonstrated that TCE cometabolism produces a toxic effect in *Pseudomonas putida* strains containing toluene dioxygenase activity. Other investigators have described TCE toxicity associated with methane monooxygenase activity in methylotrophic bacteria (1, 9, 21, 22). However, Arciero et al. (2) have reported that ammonia oxidation in the nitrifying bacterium *N. europaea* is not inactivated during short-term (15-min) exposure to TCE. The absence of a toxic effect on the cells as a result of TCE oxidation would make nitrifiers unique among bacteria known to cooxidize TCE and as such would represent a considerable advantage of nitrifiers in bioremediation schemes.

However, in contrast to the results of Arciero et al. (2), we have observed a turnover-dependent inactivation of AMO in *N. europaea* cells incubated with TCE and several other halogenated hydrocarbons. To investigate the physiological consequences of halocarbon cometabolism on *N. europaea*, we first studied the inactivation of ammonia oxidation by cells during TCE biodegradation. Our results showed that inactivation by TCE was accompanied by covalent modification of cellular proteins and loss of O_2 uptake activity associated with both ammonia oxidation and hydrazine oxidation. We then examined a series of chlorinated methanes, ethanes, and additional ethylenes for AMO turnover-dependent inactivation of ammonia oxidation. This approach revealed that a strong correlation exists between halocarbon biodegradability and the effect of cometabolism on ammonia-oxidizing activity. The chlorinated hydrocarbons could be divided into the following three classes on the basis of their biodegradability and inactivating potential: (i) compounds which were not biodegradable by *N. europaea* and which had no toxic effect on the cells; (ii) compounds which were cooxidized by *N. europaea* and had little or no toxic

* Corresponding author.

effect on the cells; and (iii) compounds which were cooxidized and produced a turnover-dependent inactivation of ammonia oxidation by *N. europaea*.

MATERIALS AND METHODS

Growth and preparation of cells. Cells of *N. europaea* ATCC 19718 were grown in batch cultures (1 to 2 liters) and harvested by centrifugation as described previously (15). The growth medium consisted of 10 mM $(NH_4)_2SO_4$, 3 mM KH_2PO_4, 750 μM $MgSO_4$, 200 μM $CaCl_2$, 10 μM $FeSO_4$, 16 μM EDTA, 1 μM $CuSO_4$, and 0.04% (wt/vol) Na_2CO_3. The medium was buffered with the addition of a phosphate solution (pH 8.0) to final concentrations of 43 mM potassium phosphate and 4 mM sodium phosphate. For experiments involving the measurement of chloride, cells were grown in the same medium with $CaSO_4$ used in place of $CaCl_2$. Cells were harvested by centrifugation and suspended in chloride-free phosphate buffer (50 mM potassium phosphate [pH 7.8], 2 mM $MgSO_4$). In all cases, cell suspensions (0.2 g [wet weight] per ml) were stored on ice and used within 24 h of harvesting. The AMO activities of different bacterial preparations were compared by measuring the AMO-dependent conversion of ethylene to ethylene oxide (18) over 1 h in the presence of 5 mM $(NH_4)_2SO_4$ and 10% (vol/vol) ethylene gas (Airco, Vancouver, Wash.), as described previously (23). The AMO activities measured for various bacterial preparations in this work ranged from 1.3 to 2.4 μmol of ethylene formed in 1 h per mg of protein.

TCE degradation assays. Assays for TCE degradation by *N. europaea* were conducted in serum vials (37 ml) sealed with Teflon-lined silicone septa (Alltech Associates, Deerfield, Ill.). The incubation medium (5 ml) consisted of phosphate buffer (50 mM potassium phosphate [pH 7.8], 2 mM $MgSO_4$) and 5 mM $(NH_4)_2SO_4$. TCE was added as a saturated aqueous solution in phosphate buffer (as described above) at room temperature (approximately 23°C). The solubility of TCE in water was assumed to be 1.1 g of TCE per liter as reported previously (12). After equilibration of the gas and liquid phases at 30°C (160 shaking cycles per min), the reactions were initiated by the addition of bacteria (approximately 5 mg of protein). For time course assays, a sample (100 μl) of the gas phase was removed for analysis of TCE by gas chromatography by using a flame ionization detector with a stainless steel column (0.125 by 16 in. [ca. 0.317 by 41 cm]) packed with Porapak Q (80/100 mesh; Waters Associates, Inc., Framingham, Mass.) and run at a column temperature of 150°C. A sample (400 μl) of the assay mixture was removed immediately (20 s) after the start of the assay and at subsequent time points. The liquid was mixed with 400 μl of ice-cold phosphate buffer (as described above), and cells were removed by microcentrifugation. The concentration of nitrite in the supernatant was determined colorimetrically as described by Hageman and Hucklesby (8), and chloride release was quantified as outlined below.

The rates of nitrite production in the presence of either ammonia or hydroxylamine were measured for cells that had been inactivated with various amounts of TCE. Aliquots (0.45 ml each) of the assay mixture were removed and combined with ice-cold phosphate buffer (0.45 ml; as described above). Cells were washed three times with phosphate buffer (1 ml) and resuspended in 0.45 ml of buffer. The rates of ammonia-dependent and hydroxylamine-dependent nitrite production were determined over a period of 30 min by incubating a portion of washed cells (100 μl) in phosphate buffer (1 ml) containing either 5 mM $(NH_4)_2SO_4$ or 2.5 mM hydroxylamine.

The effects of TCE cometabolism on the rates of ammonia-dependent and hydrazine-dependent O_2 uptake were determined after the cells were washed and resuspended to the original volume. O_2 uptake rates were measured in the chamber of an O_2 electrode after the sequential addition of $(NH_4)_2SO_4$ (to 5 mM), allylthiourea (to 50 μM), and hydrazine (to 600 μM). For anaerobic incubations, cells were pretreated with TCE in serum vials (10 ml) sealed with butyl rubber stoppers. O_2 was removed from liquid solutions by three cycles of evacuation on a vacuum manifold and purging with N_2 (Industrial Welding Supply, Albany, Ore.). Vials containing the initial cell suspension were made anaerobic by purging with N_2 for 3 min prior to initiating the assays. TCE (80 nmol) was added as a saturated solution in anaerobic buffer (as described above). After the cells were incubated for 1 h with TCE, the gas phase was removed from each vial and replaced with N_2. The vials were then purged with N_2 for 5 min. The cells were washed and O_2 uptake rates were determined as described above.

^{14}C labeling of proteins. Proteins from *N. europaea* cells incubated under AMO turnover conditions in the presence of either $^{14}C_2H_2$ or [^{14}C]TCE were covalently labeled with ^{14}C. The relative distribution of the label among the proteins was determined by fluorography after separating proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13). The time course of labeling in the presence of [^{14}C]TCE (4.1 mCi/mmol) (Sigma Chemical Co., St. Louis, Mo.) was initiated with the addition of cells into a 37-ml vial containing phosphate buffer (final volume of 5 ml) 5 mM $(NH_4)_2SO_4$ and [^{14}C]TCE (7.2 μCi; 1.8 μmol of TCE). A portion (600 μl) of the assay mixture was removed immediately, and the reaction was terminated in an Eppendorf tube with allylthiourea (final concentration, 50 μM). The nitrite content of the medium was determined, and cells were sedimented, suspended in SDS-PAGE sample buffer (200 μl), and stored on ice. Additional samples were removed at 5, 20, 45, 90, and 135 min. Cells were labeled in the presence of $^{14}C_2H_2$ synthesized from $Ba^{14}CO_3$ as described previously (13). Before electrophoresis, cells labeled in the presence of $^{14}C_2H_2$ were diluted 12-fold with unlabeled cells. Protein samples (300 μg per lane) were separated on a 13.5% polyacrylamide gel at a constant voltage of 7 mA. After the gel was stained with Coomassie blue, it was dehydrated with dimethyl sulfoxide, impregnated with the fluor 2,5-diphenyloxazole, dried, and exposed to X-ray film with an intensifying screen at −80°C for 3 weeks (13). The effect of AMO-specific inhibitors on the incorporation of ^{14}C from [^{14}C]TCE into proteins was investigated by pretreating cells for 20 min with either 8 μmol of acetylene (a suicide substrate for AMO) (19) or 500 μM allylthiourea (a specific noncompetitive inhibitor of AMO) (11) and then incubating cells with [^{14}C]TCE for 1 h (as described above) in the presence of the appropriate AMO-specific inhibitor.

Recovery of cells from inactivation by TCE. Cells (8.8 mg of protein per ml of assay medium) were preincubated in 10-ml serum vials containing phosphate buffer (50 mM potassium phosphate [pH 7.8], 2 mM $MgSO_4$), 5 mM $(NH_4)_2SO_4$, and either 1.1 μmol of TCE or 8 μmol of acetylene. Control cells were preincubated with 5 mM $(NH_4)_2SO_4$ alone for 45 min. After preincubation, cells were washed and resuspended to the original volume. A portion of the cells (0.9 mg of protein) was transferred to a 37-ml serum vial containing 5 ml of nutrient growth medium (described above) which contained 10 mM $(NH_4)_2SO_4$. Ammonia-oxidizing activity was moni-

tored as nitrite production. Nitrite-producing activity was also monitored for cells pretreated with TCE for 10 min and then placed in nutrient growth medium in the presence of the protein synthesis inhibitors chloramphenicol (400 μg/ml) or rifampin (100 μg/ml).

Degradation of chlorinated hydrocarbons. Chlorocarbon degradation assays were conducted in serum vials (10 ml) sealed with Teflon-lined silicone septa. The incubation medium (1 ml) consisted of 50 mM potassium phosphate (pH 7.8), 2 mM $MgSO_4$, 5 mM $(NH_4)_2SO_4$, and the indicated amounts of chlorocarbon substrates. Chloromethane (Liquid Carbonic, Inc., Chicago, Ill.), chloroethane (Eastman Kodak Co., Rochester, N.Y.), and vinyl chloride (Alltech Associates) were delivered as gases to sealed vials. The molar quantity added was estimated assuming ideal gas volume. The remaining substrates were added from saturated aqueous solutions in chloride-free phosphate buffer (as described above) at room temperature (approximately 23°C). The amount of substrate added from saturated solutions was estimated from solubility tables (12). The liquid chlorocarbon substrates and products were obtained from Aldrich Chemical Co. (Milwaukee, Wis.), except for 1,1-dichloroethane which was purchased from American Tokyo Kasei (Portland, Ore.).

The reactions were initiated by the addition of bacteria (1 to 2 mg of protein). Samples of the gas phase (100 to 200 μl) or liquid phase (5 μl) were removed immediately after the start of the assay and after 1 h of shaking at 30°C (160 cycles per min). The chlorocarbon content was analyzed by using a gas chromatograph equipped with a flame ionization detector and a stainless steel column packed with Porapak Q as described above. The column was operated at temperatures between 50 and 170°C. Peak areas were quantified by using a Shimadzu C-R3A integrator. Substrates and some organic products were identified by coelution with authentic standards. To test the specificity of AMO for chlorocarbon degradation, 8 μmol of acetylene or 50 μM allylthiourea was used in additional assays.

Formaldehyde produced from the oxidation of chloromethane was measured by using a coupled assay to formaldehyde dehydrogenase from *P. putida* (Sigma Chemical Co.) as described previously (23). CO released into the gas phase during assays with dichloromethane was detected with a gas chromatograph equipped with a 0.125 by 84 in. (ca. 0.317 by 213 cm) molecular sieve 13X column (Alltech Associates) and a thermal conductivity detector operated at a current of 60 mA. The column was run at 23°C with helium as the carrier gas. The standards consisted of various amounts of CO added to 10-ml Teflon-sealed vials containing 1 ml of phosphate buffer. CO was also identified by using a heme-binding assay (4).

Detection of chloride. Chloride released by cells into the assay medium was measured by using a colorimetric assay (3). Briefly, cells were removed by microcentrifugation and a portion (750 μl) of the supernatant was combined with 125 μl of 0.25 M ferric ammonium sulfate (Sigma Chemical Co.) in 9 N HNO_3 and 125 μl of ethanol saturated with mercuric thiocyanate (EM Science, Gibbstown, N.J.). After 10 min, the A_{460} was measured and the chloride content was determined by comparison to a NaCl standard curve. Nitrite in the assay medium also produced a weak positive response to the colorimetric chloride assay. Therefore, the nitrite concentration for each sample was determined, and the chloride values were corrected for absorbance due to nitrite.

Toxic effects of chlorocarbon cooxidation in *N. europaea*. To determine whether the chlorocarbon substrates or their cometabolism produced toxic effects in *N. europaea*, the O_2 uptake rates associated with ammonia oxidation by AMO and hydrazine oxidation by hydroxylamine oxidoreductase were measured for cells after the assays for substrate degradation. Cells were incubated for 1 h with ammonia and the indicated chlorocarbon substrate, washed, and then resuspended. A portion (100 to 200 μl) of the cell suspension was added to phosphate buffer (2 ml) in the chamber of an O_2 electrode, and the steady-state rates of ammonia-dependent and hydrazine-dependent O_2 uptake were determined after the sequential addition of $(NH_4)_2SO_4$ (to 5 mM), allylthiourea (50 μM), and hydrazine (600 μM). The rates of O_2 uptake for cells preincubated with ammonia and the chlorocarbon substrate plus allylthiourea to inhibit AMO activity (nonturnover conditions) were also measured after the cells were washed. Between 30 and 100% of the AMO activity was recovered in washed allylthiourea-treated cells. The O_2 uptake rates were expressed as percentages of the rates obtained with control cells which were preincubated with ammonia alone for 1 h.

Protein determinations. Protein content was determined by using the Biuret assay (7) after the cells were solubilized in 3 N NaOH for 60 min at 60°C. Bovine serum albumin was used as the standard.

RESULTS

Time course of TCE biodegradation and toxicity. The biodegradation of TCE by cells of *N. europaea* was accompanied by the loss of ammonia-oxidizing activity during a 1-h exposure of cells to ammonium and TCE (2.8 μmol added to the vial; approximately 130 μM TCE in the liquid phase) (Fig. 1). During incubation with TCE, nitrite production by the cells decreased with time, and only 1.9 mM total nitrite was accumulated over 1 h despite the presence of 8.1 mM ammonium remaining in the incubation medium. In the absence of added chlorocarbon, cells typically produced nearly 10 mM nitrite over the same time period (Fig. 2).

We tested whether the loss of ammonia-oxidizing activity was reversible by washing cells to remove excess TCE and water-soluble oxidation products and then measuring the rates of ammonia-dependent and hydroxylamine-dependent nitrite production by the cells. Progressive irreversible loss of ammonia-oxidizing activity was observed in cells exposed to TCE (Fig. 2). With the lowest concentration of TCE tested (0.38 μmol of TCE in the vial; approximately 18 μM in the liquid phase), 7.6 mM nitrite was formed after 1 h. In contrast, cells incubated with ammonium in the absence of TCE converted all of the ammonium to nitrite (10 mM) within 45 min (Fig. 2A). After 1 h of incubation with TCE, the rate of ammonia-dependent nitrite formation of washed TCE-treated cells declined to 20% of the initial rate of control cells preincubated with ammonium alone (Fig. 2B). The rate of hydroxylamine-dependent nitrite formation was reduced to 80% of the control rate during the first 20 s of the assay (Fig. 2C). However, in contrast to the effect on ammonia-dependent nitrite production, hydroxylamine oxidation by TCE-pretreated cells was not further inactivated with time and appeared to increase slightly over the time course of the assay. When cells were preincubated with higher concentrations of TCE, less nitrite was accumulated during the 1-h preincubation, and ammonia oxidation by the cells was more rapidly inactivated. After 1 h, only 3% of the ammonia-oxidizing activity remained in cells treated with the higher TCE concentrations. Thus, the effect of TCE cometabolism on ammonia oxidation by *N. europaea* varied,

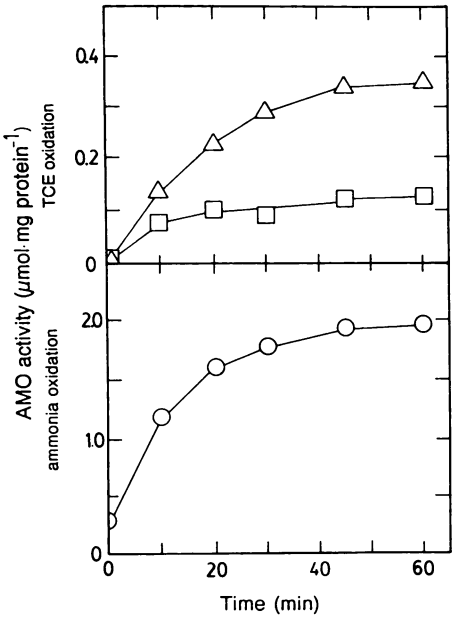


FIG. 1. Time course of TCE cometabolism by *N. europaea*. Cells (5.5 mg of protein) were incubated in serum vials (37 ml) containing phosphate buffer (5 ml) (50 mM potassium phosphate [pH 7.8], 2 mM $MgSO_4$), 5 mM $(NH_4)_2SO_4$, and TCE (2.8 μmol). Aliquots (0.4 ml) of the assay mixture were removed at the indicated time points, and the reaction was terminated with allylthiourea (to 100 μM). The levels of substrates and products were determined as described in the text. Values were corrected for substrate lost and a low background level of chloride and nitrite detected in the presence of allylthiourea-treated cells. Cells pretreated with allylthiourea and incubated with chlorocarbon, ammonia, and 50 μM allylthiourea showed no production of chloride or nitrite. Symbols: □, TCE degraded; △, chloride released; ○, nitrite released.

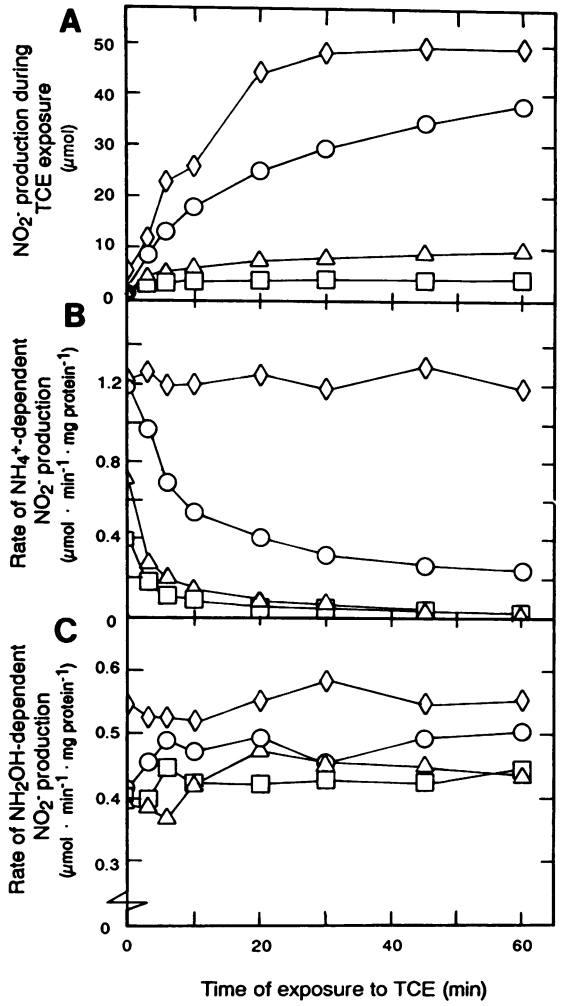


FIG. 2. Effect of TCE concentration on nitrite production and inactivation of ammonia oxidation by *N. europaea*. Cells (5 mg of protein) were incubated as described in Materials and Methods in the presence of 50 μmol of ammonium and the following amounts (in micromoles) of TCE: 0 (◇), 0.38 (○), 2.8 (△), and 5.7 (□). These quantities of TCE corresponded with approximate liquid-phase concentrations of 0, 18, 130, and 270 μM TCE, respectively. Twenty seconds after initiating the assay and at the indicated time points, 0.45-ml aliquots of the assay mixture were removed, and the concentration of nitrite was determined (A). The cells were washed, and the rates of ammonia-dependent nitrite production (B) and hydroxylamine-dependent nitrite production (C) were determined as outlined in Materials and Methods.

depending on the time of exposure to TCE and the concentration of TCE present during biodegradation.

Incorporation of ^{14}C label from [^{14}C]TCE into cellular proteins. *N. europaea* cells treated with [^{14}C]TCE incorporated ^{14}C label into a number of cellular proteins (Fig. 3A). At least 15 separate radioactively labeled protein bands could be identified, including a 27-kDa polypeptide which was also labeled when cells were inactivated with the AMO-specific mechanism-based inactivator [^{14}C]acetylene (Fig. 3A, lane 7). In addition to proteins, small molecules which ran with the dye front were also labeled with ^{14}C. During the first 45 min of treatment with [^{14}C]TCE, increasing amounts of label were incorporated into the proteins. By 90 and 135 min, nitrite production was decreased to 2% of the initial activity, and little additional label appeared to be incorporated into the proteins. The saturating effect of ^{14}C labeling suggests that the ability of the cells to covalently modify cellular proteins correlated with the amount of ammonia-oxidizing activity remaining.

When cells were incubated with [^{14}C]TCE in the presence of the AMO inhibitor allylthiourea or acetylene, ^{14}C label was not incorporated into proteins (Fig. 3B), indicating that covalent modification by TCE required catalytic activity by AMO. This result is consistent with the proposal that TCE is activated by AMO to form a reactive product which is involved in the covalent modification of proteins. In either the presence or absence of AMO inhibitors, a substantial amount of label was detected at the dye front of the gel. The reason for this label is not known.

Turnover dependence of TCE inactivation of O_2 uptake by *N. europaea*. Two of the major O_2-consuming reactions of *N. europaea* can provide insight into the physiological state of the cells. O_2 consumption in the presence of ammonium reflects AMO activity in addition to terminal oxidase activity. Both of these O_2-consuming reactions utilize electrons derived from the oxidation of hydroxylamine, which itself is generated by ammonia oxidation. When ammonia oxidation is inhibited by the addition of the AMO-specific inhibitor allylthiourea, the capacity for hydroxylamine oxidation can be monitored separately by adding hydrazine, a competitive,

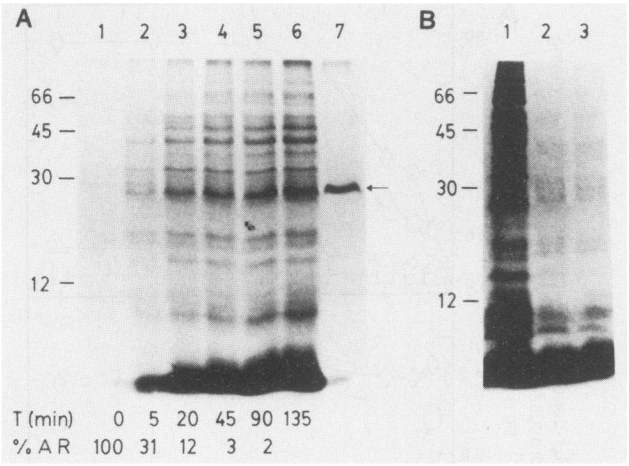


FIG. 3. Time course of incorporation of ^{14}C from [^{14}C]TCE into proteins and effect of AMO inhibitors on protein labeling. Proteins in cells of *N. europaea* were labeled with ^{14}C from [^{14}C]TCE, separated by gel electrophoresis, and detected by fluorography as described in Materials and Methods. (A) Fluorogram of the ^{14}C-labeled protein gel. Lanes contain cells incubated with [^{14}C]TCE for 0 (lane 1), 5 (lane 2), 20 (lane 3), 45 (lane 4), 90 (lane 5), and 135 (lane 6) min. Lane 7 shows separation of proteins from cells labeled in the presence of $^{14}C_2H_2$. Protein standards used were bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and cytochrome *c* (12 kDa). The arrow indicates the position of the major polypeptide labeled when cells were incubated with $^{14}C_2H_2$. The values for the percentage of nitrite-producing activity remaining in cells (%AR), determined by measuring the difference in the amount of nitrite produced between time points, are shown at the bottom of the gel. (B) Effect of allylthiourea and acetylene pretreatment on incorporation of ^{14}C from [^{14}C]TCE into proteins of *N. europaea*. Cells were incubated with [^{14}C]TCE and prepared for fluorography as described in Materials and Methods. Lanes: 1, cells incubated with [^{14}C]TCE; 2, cells pretreated with 500 μM allylthiourea and incubated with [^{14}C]TCE and allylthiourea; 3, cells pretreated with 8 μmol of acetylene and then incubated with [^{14}C]TCE and acetylene.

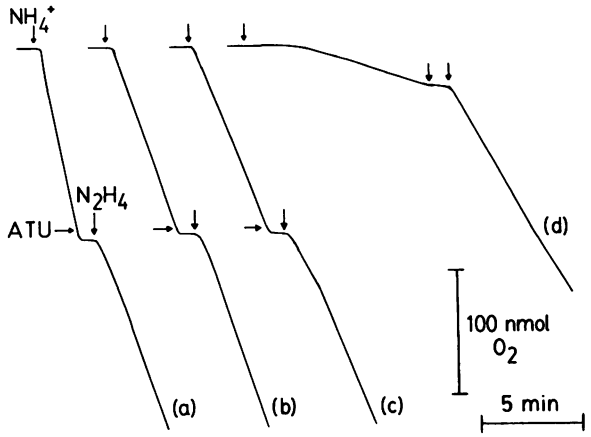


FIG. 4. O_2 uptake activity remaining in cells preincubated in the presence or absence of TCE. Cells (1.2 mg of protein) were pretreated for 1 h in sealed serum vials (10 ml) containing phosphate buffer (1 ml) under various conditions. Traces: a, active cells incubated with 5 mM $(NH_4)_2SO_4$; b, cells treated with 500 μM allylthiourea (ATU) for 15 min prior to incubation with 5 mM $(NH_4)_2SO_4$ and 100 μM ATU; c, ATU-treated cells incubated with 5 mM $(NH_4)_2SO_4$, 100 μM ATU, and 570 nmol of TCE; d, active cells incubated with 5 mM $(NH_4)_2SO_4$ and 570 nmol of TCE. After preincubation, the cells were washed, and a portion of the washed cells (0.12 mg of protein) was added to phosphate buffer (1.6 ml) in the chamber of an O_2 electrode at time zero for each tracing. After 40 s, $(NH_4)_2SO_4$ was added to a final concentration of 5 mM (first arrow), and then ATU (to 50 μM) and N_2H_4 (to 600 μM) were added at the indicated positions in each tracing.

alternate substrate for hydroxylamine oxidoreductase (10). The effects of TCE cooxidation on the rates of ammonia-dependent and hydrazine-dependent O_2 uptake were determined by preincubating cells with TCE and ammonium for 1 h, washing the cells, and then measuring the rates of O_2 consumption. Compared with control cells that had been pretreated with ammonium alone for 1 h, TCE-pretreated cells retained only 7% of the ammonia-dependent O_2 uptake activity and 60% of the hydrazine-dependent activity (Fig. 4, traces a and d). When cells were preincubated with the AMO-specific inhibitor allylthiourea and then washed, some of the ammonia-dependent and hydrazine-dependent O_2 uptake activity was lost (Fig. 4, trace b). However, the O_2 uptake rates were similar for cells pretreated with allylthiourea in either the presence or absence of TCE (Fig. 4, traces b and c), suggesting that the presence of allylthiourea during preincubation with TCE protected against the inactivating effect of TCE.

Further evidence that the toxic effect of TCE involved an AMO turnover-dependent process was illustrated by the O_2 requirement for inactivation. Cells preincubated with TCE and ammonium under anaerobic conditions retained 100% of both the ammonia-dependent and hydrazine-dependent O_2 uptake rates compared with control cells preincubated either anaerobically or aerobically in the presence of ammonium alone (data not shown). Under the conditions used in this experiment (80 nmol of TCE initially present; approximately 12 μM in the liquid phase), the ammonia-dependent and hydrazine-dependent O_2 uptake rates of cells preincubated aerobically with TCE were reduced to 20 and 70%, respectively, of the rates obtained with aerobically pretreated control cells.

Recovery of cells from inactivation by TCE. Ammonia-dependent nitrite-producing activity was inactivated in cells pretreated with either TCE or the mechanism-based inactivator acetylene. Nitrite-producing activity could be recovered if cells were washed and placed in a growth-supporting nutrient medium (Fig. 5). After a 2- to 3-h lag, the rate of nitrite production by acetylene-treated cells increased to the rate of control cells, demonstrating recovery of ammonia-oxidizing activity. Cells pretreated with TCE also recovered ammonia-oxidizing activity but required several hours longer to recover than acetylene-treated cells. If cells were inactivated with 8 μmol of acetylene prior to incubation with TCE, nitrite-producing activity recovered and exhibited the same kinetics as that of cells inactivated with acetylene alone, suggesting that AMO activity was required for TCE-mediated inactivation. Recovery of TCE-treated cells was inhibited by the transcriptional inhibitor rifampin and the translational inhibitor chloramphenicol.

Correlation between chlorocarbon biodegradability and inactivation of ammonia oxidation by *N. europaea*. To investigate the possibility that the cooxidation of other chlorocarbons in addition to TCE could inactivate ammonia oxidation by *N. europaea*, we determined the effect of chlorocarbon cometabolism on ammonia-dependent and hydrazine-dependent O_2 uptake by cells. In addition, to provide insight into the biochemical mechanism of inactivation by chlorocar-

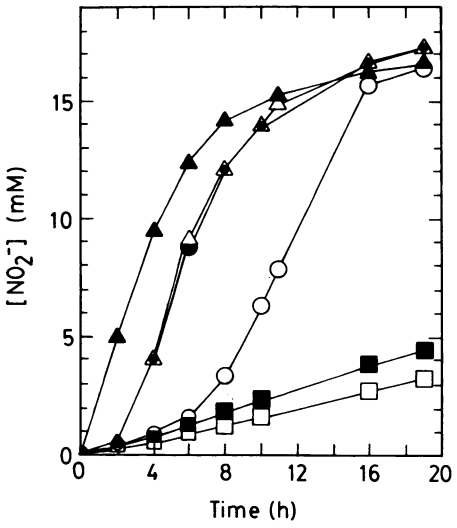


FIG. 5. Recovery of ammonia-oxidizing activity by *N. europaea* cells inactivated with TCE or acetylene. Cells (8.8 mg of protein per ml of assay medium) were preincubated with either 1.1 μmol of TCE or 8 μmol of acetylene as described in Materials and Methods. After preincubation, cells were washed and a portion of the cells (0.9 mg of protein) was transferred to a 37-ml serum vial containing 5 ml of nutrient growth medium with 10 mM $(NH_4)_2SO_4$. Nitrite released into the medium was monitored colorimetrically (8). Preincubation treatments included cells incubated with ammonia alone for 45 min (▲) or with ammonia and 8 μmol of acetylene for 45 min (△), cells inactivated with 8 μmol of acetylene for 15 min prior to preincubation with ammonia plus 1.1 μmol of TCE for 10 min (●), and active cells treated with ammonia and 1.1 μmol of TCE for 10 min (○). TCE-pretreated cells were also incubated in nutrient growth medium in the presence of 100 μg of rifampin per ml (■) or 400 μg of chloramphenicol per ml (□).

bons, we identified cooxidation products whenever possible and correlated inactivation of the ammonia-oxidizing system with the ability of *N. europaea* to cooxidize and dechlorinate individual chlorocarbons. Because dehalogenation appears to be a rate-limiting step in the degradation of halocarbons in the environment, the measurement of chloride release was of particular importance as an indicator of the ability of *N. europaea* to initiate biodegradation through dechlorination reactions. Cells were preincubated for 1 h with ammonium and the amount of individual chlorocarbons that produced the greatest amount of substrate depletion in preliminary trials. After the cells were washed, the ammonia-dependent and hydrazine-dependent O_2 uptake rates were measured. The O_2 uptake activities for washed, chlorocarbon-pretreated cells were expressed as percentages of the O_2 uptake rate remaining compared with the rate obtained for control cells preincubated for 1 h with ammonium alone (Table 1).

Three classes of chlorocarbons were identified on the basis of the ability of *N. europaea* to cooxidize the compound and the tendency of chlorocarbon cometabolism to inactivate O_2 uptake by cells. The first class of chlorocarbons included carbon tetrachloride and tetrachloroethylene, which were not cooxidized by *N. europaea* and had no toxic effect on the cells as measured by O_2 uptake activity (Table 1). No chlorocarbon depletion or chloride release was detected, even over an 80-fold concentration range of carbon tetrachloride (0.05 to 4 μmol added) or tetrachloroethylene (10 to 800 nmol added) (data not shown). The ability of *N. europaea* to produce a high level of nitrite (9.5 mM) during incubation with carbon tetrachloride or tetrachloroethylene indicated that the chlorocarbon itself was not toxic to the cells.

The second class consisted of compounds which did not inactivate O_2 uptake by cells to a great extent but which were cosubstrates for AMO, as determined by substrate depletion, chloride release, and product formation. Included in this group were chloromethane, chloroethane, 1,2-dichloroethane, and 1,1,1,2-tetrachloroethane. Only partial inhibition of ammonia-dependent O_2 uptake (up to 20%) and hydrazine-dependent O_2 uptake (up to 25%) was observed after preincubating cells with these compounds for 1 h (Table 1). In a previous study (23), no inactivation of ammonia-dependent or hydrazine-dependent O_2 uptake was observed for cells preincubated for 10 min with either a high concentration of chloroethane or 1 mM acetaldehyde. The partial inactivation exhibited in the current experiment may reflect damage of the cells due to prolonged (1-h) exposure to the aldehyde product. The previous work (23) also showed that the cooxidation of chloromethane and chloroethane by *N. europaea* results in the production of formaldehyde and acetaldehyde, respectively, through an oxidative dechlorination mechanism. We report here the identification of chloroacetaldehyde and trichloroacetaldehyde (chloral) as products of 1,2-dichloroethane and 1,1,1,2-tetrachloroethane cometabolism, consistent with an oxidative dechlorination mechanism at the monochlorinated carbon.

The final class of chlorocarbons consisted of compounds which were cosubstrates for AMO, as indicated by substrate depletion and chloride production, and which produced a turnover-dependent inactivation of one or both of the O_2 uptake activities measured. In this group, an effect on both the ammonia-dependent and hydrazine-dependent O_2 uptake activities accompanied the cooxidation of chloroform, 1,1-dichloroethane, 1,1-dichloroethylene, and trichloroethylene (Table 1). Moderate or severe inhibition of ammonia-oxidizing activity with little or no effect on hydrazine oxidation occurred when cells were preincubated with 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane, 1,1,1-trichloroethane, vinyl chloride, *cis*-1,2-dichloroethylene, and *trans*-1,2-dichloroethylene at the indicated concentrations. Interestingly, dichloromethane was the only halocarbon which strongly inhibited hydrazine-dependent O_2 uptake while only moderately affecting the rate of ammonia-dependent uptake. For all cosubstrates which had a deleterious effect on O_2 uptake activities, the addition of allylthiourea during the 1-h preincubations with the chlorocarbon protected the cells against inactivation, suggesting that turnover conditions (i.e., oxidation of the cosubstrate) were necessary for inactivation (data not shown).

DISCUSSION

Turnover dependence of TCE-mediated inactivation of ammonia oxidation by *N. europaea* and incorporation of ^{14}C from [^{14}C]TCE into proteins. The results presented here demonstrate that inactivation of ammonia oxidation by *N. europaea* occurred during biodegradation of trichloroethylene and a number of other chlorinated hydrocarbons (Fig. 1 and 2 and Table 1). Incubation of cells with TCE under conditions which supported AMO turnover resulted in the progressive, irreversible loss of ammonia-oxidizing activity, as measured by the ability of cells to convert ammonia to nitrite (Fig. 2) and couple ammonia oxidation to O_2 uptake (Fig. 4). The extent of inactivation depended on the time of exposure to TCE and the initial TCE concentration, factors which

TABLE 1. Effect of chlorocarbon cometabolism on O_2 uptake by *N. europaea*

Chlorocarbon	Amt[a] (μmol) added	% of O_2 uptake activity remaining after incubation with chlorocarbon and ammonia[b]		Chlorocarbon degraded[c]	Ratio of Cl^- released to substrate degraded	Product detected
		NH_3-dependent	N_2H_4-dependent			
Class I (not biodegradable; no inactivation)						
Carbon tetrachloride	1	88	98	−	ND[d]	
Tetrachloroethylene	0.05	96	102	−	ND	
Class II (biodegradable; minimal inactivation)						
Chloromethane	4	80	84	+	0.7	Formaldehyde
Chloroethane	9	97	79	+	1.1	Acetaldehyde
1,2-Dichloroethane	5	89	74	+	0.9	Chloroacetaldehyde
Class III (biodegradable; substantial inactivation)						
Dichloromethane	4	60	21	+	2.5[e]	CO
Chloroform	2	1	3	+	2.2	
1,1-Dichloroethane	5	11	20	+	2.1	Acetic acid[f]
1,1,1-Trichloroethane	5	53	91	+	ND	2,2,2-Trichloroethanol
1,1,2-Trichloroethane	1.6	8	80	+	2.0	
1,1,2,2-Tetrachloroethane	1.4	4	79	+	1.0	
1-Chloroethylene	0.20	30	86	+	0.4	
1,1-Dichloroethylene	0.10	3	37	+	2.5	
cis-1,2-Dichloroethylene	0.36	4	120	+	1.0	
trans-1,2-Dichloroethylene	0.65	50	99	+	ND	
Trichloroethylene	0.57	3	44	+	3.2	

[a] Amount of chlorocarbon which supported the greatest amount of chlorocarbon depletion in preliminary trials.
[b] Cells were preincubated for 1 h with 5 mM $(NH_4)_2SO_4$ and the indicated amount of chlorocarbon. After the cells were washed, the rates of ammonia-dependent and hydrazine-dependent O_2 uptake were determined as described in Materials and Methods. Values indicate the percentages of O_2 uptake activity remaining in chlorocarbon-pretreated cells compared with that of control cells pretreated for 1 h with ammonium alone. Each value is the average of duplicate determinations.
[c] +, greater substrate depletion by active cells (0.8 to 1.7 mg of protein) than by acetylene-treated or allylthiourea-treated cells; −, no substrate depletion detected.
[d] ND, no chloride detected.
[e] Ratio of Cl^- released to CO produced.
[f] A peak coeluting with acetate was detected with active cells, but not with acetylene-treated cells.

would also affect the rate of TCE cooxidation by the cells. These results are in contrast with a previous report (2) which indicated that loss of ammonia-oxidizing activity did not accompany TCE biodegradation by *N. europaea* during a 15-min incubation of cells with 1 mM ammonium and a nominal TCE concentration of 11 μM. The discrepancy between our results and those of Arciero et al. (2) may be accounted for by differences in the experimental system, such as reductant concentration, cell densities, and TCE concentrations.

The TCE-mediated inactivation of ammonia oxidation by *N. europaea* required AMO turnover conditions, as indicated by the O_2 requirement for biodegradation of and inactivation by TCE and by the ability of the AMO-specific inhibitor allylthiourea to protect against inactivation (Fig. 4 and Table 1). Incorporation of ^{14}C label from [^{14}C]TCE into proteins occurred only under conditions which supported AMO turnover (Fig. 3) and indicated that cometabolic inactivation by TCE was accompanied by covalent modification of cellular proteins. Notably, one of the major labeled bands comigrated with the 27-kDa polypeptide labeled in the presence of $^{14}C_2H_2$ and presumed to carry the active site of AMO (19). Similar examples of a turnover-dependent, cometabolic inactivation of TCE-oxidizing bacteria have been reported for methanotrophic bacteria (1, 9, 21) and for *P. putida* cells expressing toluene dioxygenase activity (26).

Recovery of cells from inactivation by TCE. *N. europaea* cells inactivated with TCE or acetylene and then placed in a growth-supporting medium were able to recover ammonia-dependent nitrite-producing activity after removal of the inactivators. However, TCE-inactivated cells required a much longer time period to recover activity than did acetylene-treated cells (Fig. 5). For both inactivators, inhibition of recovery by rifampin and chloramphenicol demonstrated that recovery involved de novo protein synthesis. With TCE, the higher number of covalently modified proteins and longer recovery time relative to those of acetylene-treated cells may be interpreted as inactivation of other proteins in addition to AMO that are required for energy generation or protein synthesis during recovery.

The ability of cells to recover from the deleterious effects of TCE cooxidation indicates that it may be possible to establish biodegradation conditions, such as specific cosubstrate concentrations, ammonia-to-cosubstrate ratios, or microbial densities, that would protect against complete inactivation and allow sufficient ammonia oxidation to support cell growth or continued metabolism. Preliminary evidence in our laboratory demonstrates that cells incubated in growth-supporting medium in the continuous presence of low concentrations of TCE were able to maintain nitrite-producing activity for up to 5 days (data not shown). One strategy that could be utilized in a bioreactor system to

reduce the TCE concentration to nontoxic levels for bacteria would be to absorb TCE onto activated charcoal. This procedure would allow a gradual release of TCE during the period of exposure of cells to the halocarbon. Success with this approach, as measured by increased biodegradation and longevity of cells, has been demonstrated for TCE biodegradation by methanotrophic bacteria incubated in the presence versus the absence of activated charcoal (21). An alternative strategy would involve reinoculation with active bacteria to maintain continued biodegradation over a period of time.

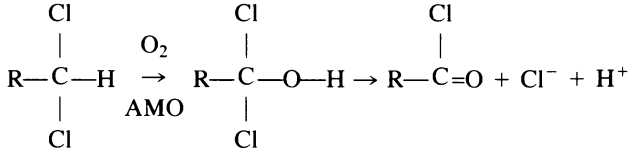
Correlation between chlorocarbon biodegradability and metabolic inactivation of *N. europaea*. The inactivating potential of additional C_1 and C_2 chlorocarbons was correlated with the ability of *N. europaea* to cooxidize and dehalogenate the chlorinated compounds. This systematic characterization of the substrate specificity, oxidation products, and inactivating potential of halogenated hydrocarbons would contribute to elucidating the mechanism of halocarbon cooxidation by AMO and the chemical basis of the cometabolic inactivation of AMO. The three types of interactions observed between *N. europaea* and the chlorinated methanes, ethanes, and ethylenes can be summarized as follows: Class I, $X \not\rightarrow X^*$ (not biodegradable and no inactivating effect); Class II, $X \rightarrow X^* \rightarrow$ innocuous product (biodegradable and low inactivating potential); and Class III, $X \rightarrow X^* \rightarrow$ toxic product or alkylating agent (biodegradable and high inactivating potential).

Except for carbon tetrachloride and tetrachloroethylene, all of the chlorinated methanes, ethanes, and ethylenes containing up to four chlorine substituents were cometabolized by *N. europaea*, as indicated by substrate depletion under AMO turnover conditions (Table 1). The biodegradable compounds had in common the presence of either a carbon-hydrogen or a carbon-carbon double bond available for oxidation by AMO. Cooxidation of the degradable chlorocarbons was accompanied by the release of chloride ions into the medium in all cases except for 1,1,1-trichloroethane and *trans*-1,2-dichloroethylene. The organic products identified were all consistent with the simple hydroxylation of a carbon-hydrogen bond or an oxidative dehalogenation mechanism.

(i) Class I. The first class of compounds was not biodegradable by *N. europaea* and did not inactivate ammonia oxidation by the cells. Incubation of *N. europaea* cells with carbon tetrachloride and tetrachloroethylene at the concentrations tested did not prevent AMO from oxidizing ammonia during the time course of the assay and did not affect the rates of ammonia-dependent and hydrazine-dependent O_2 uptake, indicating that the inability to degrade the compounds was not due to inactivation of AMO or a general toxic effect on the cell. Class I compounds would include halocarbons which do not bind to AMO as well as compounds which bind to AMO but are not cooxidized.

(ii) Class II. When a single chloride ion was removed from a monochlorinated carbon, the corresponding aldehyde (formaldehyde, acetaldehyde, chloroacetaldehyde, trichloroacetaldehyde) was produced. Cooxidation of the substrates chloromethane, chloroethane, 1,2-dichloroethane, and 1,1,1,2-tetrachloroethane did not inactivate AMO or strongly inhibit the rates of hydrazine-dependent O_2 uptake, indicating that the oxidation products were not toxic to the cell during short-term exposures.

(iii) Class III. Cometabolism of the third class of substrates resulted in an AMO turnover-dependent inactivation of ammonia oxidation by *N. europaea*. This group included chlorinated ethylenes and those compounds containing a dichlorinated carbon. Hydrolysis of chlorinated ethylene epoxides, the proposed products of chlorinated ethylene oxidation by methane monooxygenase from *Methylosinus trichosporium* OB3b (6), may produce acyl chloride alkylating agents and would account for the turnover-dependent inactivation of AMO in nitrifying bacteria. The production of alkylating agents may also provide the basis for inactivation of ammonia-dependent O_2 uptake by 1,1-dichloroethane, 1,1,2-trichloroethane, and 1,1,2,2-tetrachloroethane oxidation. Acyl chlorides could result from the hydroxylation of the dichlorinated carbon followed by the elimination of one of the chlorines, as illustrated below:



These compounds could act as protein-modifying agents which inactivate enzymes or proteins such as electron carriers through covalent modification.

Potentially, a fourth class of halocarbon compounds exists which, although not transformed by *N. europaea*, could produce a toxic effect in the cells. Interestingly, no compounds of this type were identified in the range of chlorocarbons studied in this work.

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