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The toxic effects of degrading the chlorinated hydrocarbons trichloroethylene (TCE), chloroform (CF) and cis-1,2-dichloroethylene (cis-1,2-DCE) were studied in the bacterium Nitrosomonas europaea. N. europaea is an ammonia-oxidizing bacterium that obtains all of its energy from the oxidation of ammonia to nitrite. This metabolic process involves two enzymes, ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). AMO has a broad substrate range and is also capable of oxidizing TCE, CF, and cis-1,2-DCE. Effects of degrading these chlorinated compounds on both AMO and HAO were studied. Cells were inactivated with known inhibitors of both AMO (light) and HAO (hydrogen peroxide) to provide comparison studies. Oxidation of the three chlorinated hydrocarbons did not always result in similar toxic effects to the cells. Whole cell studies indicated that oxidation of TCE and CF resulted in a loss of both NH4+- and N2H4- dependent O2 uptake rates, while in vitro studies indicated that at lower concentrations of both TCE (≤ 0.05 mM) and CF (≤ 0.10 mM) neither AMO or HAO appear to be the primary sites of inactivation. The oxidation of cis-1,2-DCE appeared to specifically inactivate AMO both in in vivo
and *in vitro* assays. *N. europaea* cells were also pretreated with the AMO inhibitor acetylene and incubated with the chlorinated hydrocarbons. Results of both whole cell O$_2$ uptake rates and the *in vitro* HAO assay confirms the hypothesis that the chlorinated hydrocarbons must be turned over in order to produce a toxic effect in *N. europaea* cells.
Effects of Chlorinated Aliphatic Hydrocarbon Degradation on the Metabolic Enzymes in Nitrosomonas europaea

by

Kimberly A. Fawcett

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CONTRIBUTION OF AUTHORS

Dr. Michael Hyman was involved in the design, analysis, and writing of the manuscript. Dr. Daniel Arp was involved in the design and analysis of the manuscript as well as provided the necessary lab space and equipment. Dr. Kenneth Williamson was involved in the design and writing of the manuscript.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Chlorinated Hydrocarbon Background</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Trichloroethylene</td>
<td>2</td>
</tr>
<tr>
<td>1.1.2 Chloroform</td>
<td>3</td>
</tr>
<tr>
<td>1.1.3 cis-1,2-Dichloroethylene</td>
<td>4</td>
</tr>
<tr>
<td>1.2 Bioremediation</td>
<td>4</td>
</tr>
<tr>
<td>1.3 Cometabolism</td>
<td>6</td>
</tr>
<tr>
<td>1.4 Monoxygenases</td>
<td>8</td>
</tr>
<tr>
<td>1.5 <em>Nitrosomonas europaea</em></td>
<td>9</td>
</tr>
<tr>
<td>1.6 Toxicity Associated with <em>N. europaea</em></td>
<td>10</td>
</tr>
<tr>
<td>1.7 Methane-oxidizers</td>
<td>12</td>
</tr>
<tr>
<td>1.8 Toxicity in Methanotrophs</td>
<td>12</td>
</tr>
<tr>
<td>1.9 Toluene-oxidizers</td>
<td>18</td>
</tr>
<tr>
<td>1.10 Toxicity in Toluene-oxidizers</td>
<td>19</td>
</tr>
<tr>
<td>2. Investigations into the Mechanism of Inactivation with Trichloroethylene, Chloroform, and cis-1,2-Dichloroethylene Cometabolism by <em>Nitrosomonas europaea</em></td>
<td>21</td>
</tr>
<tr>
<td>2.1 Abstract</td>
<td>22</td>
</tr>
<tr>
<td>2.2 Introduction</td>
<td>22</td>
</tr>
<tr>
<td>2.3 Materials &amp; Methods</td>
<td>25</td>
</tr>
<tr>
<td>2.4 Results</td>
<td>29</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.5 Discussion</td>
<td>43</td>
</tr>
<tr>
<td>3. Summary and Conclusions</td>
<td>47</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>52</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>1.</td>
<td>The effect of trichloroethylene on NH$_4^+$ and N$_2$H$_4$-dependent O$_2$ uptake activity, incorporation of $^{14}$C from $^{14}$C$_2$H$_2$ into the 27 kDa polypeptide and <em>in vitro</em> HAO activity</td>
</tr>
<tr>
<td>2.</td>
<td>The effect of light and H$_2$O$_2$ on NH$_4^+$ and N$_2$H$_4$-dependent O$_2$ uptake activity, incorporation of $^{14}$C from $^{14}$C$_2$H$_2$ into the 27 kDa polypeptide and <em>in vitro</em> HAO activity</td>
</tr>
<tr>
<td>3.</td>
<td>The effect of chloroform on NH$_4^+$ and N$_2$H$_4$-dependent O$_2$ uptake activity, incorporation of $^{14}$C from $^{14}$C$_2$H$_2$ into the 27 kDa polypeptide and <em>in vitro</em> HAO activity</td>
</tr>
<tr>
<td>4.</td>
<td>The effect of <em>cis</em>-1,2-dichloroethylene on NH$_4^+$ and N$_2$H$_4$-dependent O$_2$ uptake activity, incorporation of $^{14}$C from $^{14}$C$_2$H$_2$ into the 27 kDa polypeptide and <em>in vitro</em> HAO activity</td>
</tr>
<tr>
<td>5.</td>
<td>The effect of light, H$_2$O$_2$, TCE, CF and <em>cis</em>-1,2-DCE on active and acetylene pretreated cells</td>
</tr>
<tr>
<td>6.</td>
<td>The effects of light, TCE and TCE &amp; acetylene treated cells on the incorporation of $^{14}$C from $^{14}$CO$_2$</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

This research investigated the effects of degrading the chlorinated hydrocarbons trichloroethylene, chloroform and cis-1,2-dichloroethylene on the metabolic enzymes of the bacterium *Nitrosomonas europaea*. The effects to both ammonia monooxygenase and hydroxylamine oxidoreductase were examined by both *in vivo* and *in vitro* assays. *N. europaea* was utilized in this study due to its relatively simple two enzyme metabolic system and our ability to examine the effects to these enzymes individually.

1.1. Chlorinated Hydrocarbon Background

Environmental contamination of chlorinated hydrocarbons often results from accidental spills, leaking storage tanks, improper disposal, and from landfill leachates (73). Contamination of ground water often results in concern for human exposure. Several of the most common groundwater pollutants are trichloroethylene (TCE), chloroform (CF) and cis-1,2-dichloroethylene (cis-1,2-DCE).
1.1.1. **Trichloroethylene**

TCE has been used primarily as an industrial solvent and degreasing agent. Its extensive use has resulted in it being one of the most widely distributed organic ground water pollutants in the United States. TCE is one of the top ten most commonly found chemicals at hazardous waste sites and was found at 28 percent of waste sites examined (44). According to the Agency for Toxic Substances and Disease Registry (ATSDR), trichloroethylene has been found in at least 852 of the 1,430 National Priorities List sites identified by the Environmental Protection Agency (EPA) (http://atsdr1.atsdr.cdc.gov:8080/ tfacts19.html, 9/19/98 at 12:30 pm).

Although acute doses in mammals can result in cardiac arrhythmias (66) and central nervous system depression (67), health concerns about TCE stem primarily from its carcinogenic potential. Chronic exposures of TCE to mice by gavage appear to induce hepatocellular carcinomas (45, 60). Chronic exposures by gavage in rats appear to cause nephrotoxicity and a low incidence of renal tubular adenocarcinomas (60). As a result of these and other studies, TCE has been classified as a possible human carcinogen (2, 19, 53).

Further concern in TCE contaminated groundwater results from the degradation products produced, such as vinyl chloride and dichloroethylenes. Under anaerobic conditions, TCE can be dechlorinated to dichloroethylenes and vinyl chloride (59, 74). The presence of vinyl chloride is less desirable than TCE because vinyl chloride is a known carcinogen. However, aerobic degradation of TCE and its products can provide a reasonable method of removing the
contaminant from groundwater and it is this possibility that has lead to an increased interest in microorganisms with the capability of degrading halogenated compounds. To date, there is no known microorganism with the capability of using TCE as their sole carbon and energy source. However, TCE can be degraded cometabolically by a number of organisms that grow on the following substrates: toluene (76), phenol (20), methane (57), propane (50, 75), propylene (15), cumene (13), isoprene (18), and ammonia (4, 64).

1.1.2. Chloroform

Chloroform (CF) was originally used as an anesthetic and as a sweetener (http://www.iet.msu.edu/journal/choro.html) and is currently used as a solvent in sewage treatment, fire extinguishers, refrigerants and in the manufacturing of dyes, drugs, plastics and pesticides (33). CF is mass-produced at a rate of $2.5 \times 10^5$ tons per year and it has been estimated that 10 percent of this is released into the environment each year (61). CF may be released into the environment through accidental spills, leaking storage tanks, improper disposal, and landfill leaching. According to ATSDR, CF has been found in at least 717 of the 1,430 EPA National Priority List sites (http://atsdr1.atsdr.cdc.gov:8080/tfacts6.html, 9/19/98 at 12:30pm). CF has been classified as a health hazard by the U. S. National Institute for Occupational Safety, and exposure may result in liver and kidney tumors as well as damage of the central nervous system (61).

Microbial degradation of chloroform can also serve as an effective method for remediating contaminated sites. Biodegradation of CF under aerobic conditions
has been observed in methane-oxidizing (3, 10, 70), ammonia-oxidizing (64), and
toluene-oxidizing (51) bacteria.

1.1.3. *cis-1,2-Dichloroethylene*

* cis-1,2-Dichloroethylene (*cis*-1,2-DCE) is used as a solvent in the
manufacturing of pharmaceuticals, in the extraction of fats and oils from fish and
meat, as a chemical intermediate for perfumes, dyes, lacquers, thermoplastics, fats,
phenols, camphor, and rubber. (http://Intp-server.niehs.nih.gov/htdocs/
Results_status/ResstatD/c51581.html, 8/13/98 2:30 pm). Under anaerobic
conditions *cis*-1,2-DCE may exist because it is a breakdown product in reductive
dehalogenation of TCE and tetrachloroethylene (http://www.speclab.com/
compound/c156592.htm, 8/13/98 2:30 pm). According to ATSDR, *cis*-1,2-
dichloroethene has been found in at least 146 of the 1,430 National Priorities List
sites identified by the EPA. ATSDR also suggests that exposure to low doses of
*cis*-1,2-DCE may cause effects on the blood, such as a decrease in red blood cell
counts, and effects on the liver (http://atsdr1.atsdr.cdc.gov:8080/tfacts87.html,
9/19/98 at 12:30 pm).

1.2. **Bioremediation**

Bioremediation is defined by Atlas and Bartha as “the use of biological
agents to reclaim soils and waters polluted by substances hazardous to human
health and/or the environment” (7). The use of bioremediation technologies
provides several advantages over physical remediation methods. Bioremediation
is relatively inexpensive in comparison to physical methods. The potential to mineralize the compound is another advantage of bioremediation. Finally, bioremediation strategies generally require no transport of the contaminated media, unlike in physical remediation strategies where the contaminated media may have to be moved to another site.

In general, there are three approaches to bioremediation. They include bioaugmentation, biostimulation and intrinsic methods. Bioaugmentation entails adding the appropriate microorganism to the contaminated area. Biostimulation requires modifying the contaminated environment in order to enrich for the appropriate microbial populations. Intrinsic remediation requires monitoring of the natural microbial populations but no manipulations are done (7).

Bioremediation is not always a viable option for degrading environmental pollutants. Several conditions must be met in order for the necessary processes to occur. First, microorganisms with the necessary catabolic enzymes must exist in the contaminated environment unless using the bioaugmentation approach. These microorganisms must not only exist but the pollutant must be accessible to the organism. Secondly, other conditions in the environment must allow for the population degrading the pollutant to proliferate. If these conditions are not met, then the environmental conditions must be modified such as in the biostimulation approach. Finally, if the initial enzymes that attack the pollutant are extracellular then the products of this initial reaction must be accessible to intracellular enzymes in order for complete degradation to occur (1). Other organisms may also be
present in the same local environment that may be capable of further degrading these products.

1.3. Cometabolism

The term cometabolism was defined by Dalton and Stirling as "the fortuitous biotransformation of a non-growth supporting substrate in the obligate presence of a growth supporting substrate or some other transformable compound" (14). Cometabolism can occur under both aerobic and anaerobic conditions. Anaerobic organisms can use TCE as a non-growth-supporting electron acceptor and this results in a less chlorinated compound (74). Aerobic cometabolic transformations are catalyzed by nonspecific monooxygenase enzymes that are typically used to initiate metabolism of the growth substrate (39). Aerobic cometabolism has an advantage over anaerobic transformations in that aerobic cometabolism can result in complete mineralization of the pollutant.

Three factors that influence the rate of degradation of compounds in an aerobic cometabolic process are inhibition, inactivation and recovery. Inhibition results from the presence of both a growth and nongrowth substrate. Though several types of inhibition are known (competitive, noncompetitive and uncompetitive), TCE is a competitive inhibitor of ammonia oxidation in *N. europaea* (39). Inhibition results in a decrease in the consumption of the growth substrate, but it also has other consequences. First, the decrease in growth substrate consumption can result in a decrease in the generation of reductant (39). For example, in the case of TCE degradation in *N. europaea*, the presence of TCE
results in less ammonia being oxidized by the reductant-dependent AMO to hydroxylamine. The second enzyme hydroxylamine oxidoreductase produces the reductant necessary to fuel the degradation of both TCE and ammonia by AMO. Since we now have less of the substrate hydroxylamine, less reductant will be provided to the cell and AMO. A second complication can result if the growth substrate is needed as an inducing compound to maintain the necessary cometabolic enzymes (17). A final important characteristic of inhibition is that it is a reversible process.

A second factor which can affect the rate of degradation is the toxicity that can be associated with degradation of chlorinated compounds. Toxicity is usually observed as the irreversible inactivation of the monooxygenase activity. This toxicity may result from the binding, to the monooxygenase, of short-lived reactive intermediates produced during oxidation of these chlorinated compounds. This mechanism of inactivation has been suggested in the case of TCE degradation (17, 21, 57, 64).

A third factor which affects this rate of degradation is the cells ability to recover from the observed toxic effects. This recovery process requires de novo protein synthesis (39). If the cells are able to recover quickly they may be better at maintaining a higher degradation rate of the chlorinated compounds.

A variety of physiologically distinct bacteria are capable of cometabolizing TCE. These include Methylosinus trichosporium OB3b (58), Xanthobacter (15), Burkholderia cepacia G4 (20), Rhodococcus species (50) and Nitrosomonas europaea (4, 64).
1.4. Monooxygenases

Oxygenases are a class of enzymes that catalyze the incorporation of oxygen from molecular oxygen into substrates. There are two kinds of oxygenases, dioxygenases and monooxygenases. Dioxygenases incorporate both atoms of O\textsubscript{2} into the molecule. Dioxygenases need not require a reductant source because both atoms of O\textsubscript{2} are reduced when incorporated into the substrate.

Monooxygenases incorporate one atom of oxygen into the substrate, usually as a hydroxyl group, and the other atom of oxygen is reduced to water (49). Monooxygenases require a reductant source to reduce the second atom of oxygen to H\textsubscript{2}O. An important component of these enzymes is the prosthetic groups they contain. Although the type of group varies between organisms, they all serve a similar function, the activation of O\textsubscript{2}. The possible prosthetic groups include flavin, heme, binuclear iron clusters, mononuclear iron centers, and copper.

Monooxygenases can catalyze several types of reactions including epoxidations and O-dealkylations. These monooxygenases appear to be relatively nonspecific in their substrate ranges and can oxidize a variety of chemical compounds. For example, the soluble methane monooxygenase of *Methylococcus capsulatus* (Bath) has a broad substrate specificity catalyzing the oxidation of a wide range of alkanes, alkenes, ethers, and alicyclic, aromatic, and heterocyclic compounds (12). Two other examples of monooxygenases are ammonia monooxygenases and toluene-4-monooxygenase. This study looks at ammonia monooxygenase in *N. europaea*. 
1.5 *Nitrosomonas europaea*

*N. europaea* is an obligate chemolithoautotrophic nitrifying bacterium. All energy utilized by the cells is provided exclusively from the oxidation of ammonia to nitrite. This oxidation is done by a two enzyme pathway. First, ammonia monooxygenase (AMO) catalyzes the oxidation of ammonia to hydroxylamine. Molecular oxygen is required and one atom is incorporated into the substrate ammonia, while the second atom is reduced to water. The oxidation of hydroxylamine to nitrite is catalyzed by hydroxylamine oxidoreductase (HAO). This reaction provides the electrons necessary to maintain AMO activity. The remaining two electrons are used in an electron transport chain to synthesize ATP. AMO is a membrane bound enzyme which, to date, has not been purified with activity. Though unpurified, information exists about this enzyme. It has been theorized that a 27 kDa polypeptide contains the active site of AMO; this is supported by the observation that this polypeptide becomes covalently modified upon inactivation of ammonia oxidation in the presence of acetylene (43). A 40 kDa polypeptide also copurifies with the 27 kDa polypeptide, though this polypeptide is not labeled by acetylene (52). It has also been suggested that the prosthetic groups in AMO may contain both Cu (16) and Fe (79). HAO has been purified and is a periplasmic enzyme (78). HAO contains 7 c-type hemes and a P-460 heme in each subunit of the homotrimer consisting of an α3 arrangement (5).

*N. europaea* is known to oxidize a wide variety of alternative substrates via the non-specific AMO. These substrates include alkanes, alkenes and aromatic hydrocarbons, ethers, thioethers and primary amines (6, 37, 40-42).
Inhibitors of both AMO and HAO can provide us with ways of examining each enzyme individually. There are many known inhibitors of AMO (39). These include metal-chelating compounds, such as allylthiourea (ATU) which binds to copper and other C=S containing compounds (9). Other substrates that compete for the active site of the enzyme such as methane, ethylene, and halogenated hydrocarbons also serve as inhibitors (35, 38, 62, 72). Finally, suicide substrates such as acetylene also inhibit the enzyme. Suicide substrates can irreversibly and specifically bind to an enzyme. It has been proposed that acetylene inactivates AMO after attempting to oxidize the triple bond, resulting in the production of a reactive intermediate which binds to and inactivates the enzyme (43). Finally, light specifically inactivates AMO (32, 68). An important note is that both light and acetylene appear to act specifically on AMO with no effects on HAO.

Inactivation of HAO occurs in the presence of hydrogen peroxide. A study done by Hooper and Terry (31) showed that incubation with hydrogen peroxide resulted in the rapid and irreversible loss of the ability to catalyze the conversion of hydroxylamine.

1.6. Toxicity Associated with *N. europaea*

Arciero, *et al.*, (4) were the first to report that TCE was degraded by *N. europaea* and since then others have shown that various other chlorinated hydrocarbons, including chloroform and cis-1,2-dichloroethylene, are also oxidized (62, 63, 72).
Arceiro, et al. (4) reported that ammonia-oxidation in *N. europaea* was not inactivated during short-term (15 min) incubations with 1.1 mM TCE. However, a study done by Rasche, et al. (64) saw inactivation of AMO activity in cells incubated with TCE and other halogenated compounds. Rasche noted that the differences seen in these two studies may be due to experimental designs. The study done by Rasche went on to separate a number of chlorinated compounds into three classes based on both the cells ability to degrade the compound as well as the inactivating potential of the compound. Class 1 consists of compounds such as carbon tetrachloride and tetrachloroethylene that were neither biodegraded nor toxic to the cells. Class 2 consists of compounds that were degraded by the cells but had little or no toxic effect on the cells. Examples of Class 2 compounds are chloromethane, chloroethane, and 1,2-dichloroethane. Class 3 compounds, such as TCE, CF, and cis-1,2-DCE, are cooxidized but also produce a turnover-dependent inactivation of ammonia-oxidation. Examining the inactivation of ammonia oxidation by TCE also revealed that the extent of inactivation depended on both the time of exposure as well as the concentration of TCE that cells were exposed to during incubations. The study also noted that inactivation of ammonia oxidation by TCE required AMO turnover. Inactivation was prevented in the presence of allylthiourea, an AMO specific inhibitor. In the same study, *N. europaea* cells were incubated in the presence of $^{14}$C-TCE resulting in the labeling of the 27 kDa polypeptide of AMO as well as 14 other polypeptides.
1.7. Methane-oxidizers

A group of bacteria, collectively referred to as the methanotrophs are capable of utilizing methane as their sole source of both carbon and energy. Methanotrophs contain the enzyme methane monooxygenase (MMO) which enables the bacteria to introduce an oxygen atom into methane. Two forms of MMO have been found in methanotrophs: soluble (sMMO) and particulate (pMMO). sMMO has been purified from several methanotrophs and contains three components: a hydroxylase component (245 kDa), a B component (15.8 kDa) and a reductase component (38.4 kDa). The hydroxylase component contains the active site and the prosthetic group that activates the O₂, a hydroxy-bridged diiron center. Component B serves a regulatory role. The reductase component serves to transfer electrons from NADH to the hydroxylase (47). pMMO has not been purified to homogeneity, but it is known that all methanotrophs can form this membrane-bound form of MMO in the presence of copper. On the other hand, sMMO appears to be formed only under copper limited conditions and is not found in all methanotrophs. sMMO has a broad substrate specificity and is capable of cooxidizing compounds such as TCE and CF (26).

1.8. Toxicity in Methanotrophs

The toxicity to the bacteria during cometabolism of TCE and other chlorinated hydrocarbons has been best studied in methanotrophs. Many studies have been done examining the effects of cometabolizing TCE and CF on
methanotrophs. The general thought is that toxicity or loss of methane-oxidizing capabilities may occur as a result of the inactivation of MMO. It has been suggested that during TCE cometabolism, one of the cometabolic products may cause this inactivation. Scheme 1 is a graphical representation of possible TCE degradation pathways and can be referred to for structures of the degradation products for both methane- and toluene-oxidizers.

Little et al. (48) were the first to show TCE degradation by a pure culture of methanotrophs. Methane-oxidizing bacterium strain 46-1 converted TCE to both water soluble products (15.1 percent of the initial TCE concentration) and CO₂ (11.4 percent of the initial TCE concentration). Further analysis of the water soluble products revealed that both glyoxylic acid and dichloroacetic acid were present. Both of these products have been seen when TCE epoxides decompose in mammalian systems (30, 54). These results suggest that an epoxide may be an intermediate in TCE oxidation.

A study done by Oldenhuis et al. (58), tested the effects of incubating Methylosinus trichosporium OB3b cells with increasing concentrations of TCE. They found that TCE concentrations above 0.2 mM in the liquid phase resulted in inhibition of TCE oxidation. They also noted that during TCE oxidation greater than 90 percent of the organic chlorine in TCE was released as inorganic chlorine, suggesting that only small amounts of chlorinated intermediates/products should be expected to remain. Two of these products were determined to be 2,2,2-trichloroethanol and trichloroacetaldehyde. The authors suggested that a TCE epoxide was a possible intermediate in TCE oxidation by M. trichosporium OB3b.
Scheme 1. Theorized TCE degradation pathway for methane- and oxidizers Adapted from TCE Graphical Pathway Map from The University of Minnesota Biocatalysis/Biodegradation Database (http://www.labmed.umn.edu/umbbd/tce/tce_image_map.html, 12/27/98 12:30pm)
This study also looked at the degradation of both CF and cis-1,2-DCE and found that both were degraded to below detection levels within 24 hours.

Fox et al. (21) did another study on TCE degradation in *M. trichosporium* OB3b. In this study they used 4-(p-nitrobenzyl)-pyridine as a trapping reagent to look for epoxide formation. They found that a semistable epoxide was produced during TCE oxidation. Formate and carbon monoxide accounted for 88% of the total products formed. Glyoxylate, dichloroacetate and chloral were also produced in small quantities. The authors went on to compare these products with those produced from the hydrolysis of TCE epoxide. The hydrolysis of the epoxide produced a similar distribution of products with the exception of chloral. These results suggest that all the products observed in this study came from the non-enzymatic breakdown of the TCE epoxide with the exception of chloral.

The authors then went on to show that the oxidation of TCE resulted in the time-dependent inactivation of the MMO. The addition of the competitive inhibitor ethylene to the TCE reactions slowed the rate of inactivation of the enzyme. Experiments were also done in the presence of cysteine and again resulted in a reduced rate of inactivation. This finding suggests that the inactivating product may be diffusible. The incubation of MMO components with TCE resulted in the modification of all the enzyme components roughly in accordance with the estimated exposed surface area of each component. The incorporation of reaction products into the enzyme lent further evidence to the
idea of a diffusible inactivating agent. This study suggests that inactivation is probably not occurring exclusively at the active site.

The addition of cysteine also resulted in an increase in epoxide formation. This increase in epoxide formation was, as stated above, associated with a decrease in the inactivation rate suggesting that the TCE epoxide is not the inactivating agent. Components of MMO were also incubated with chloral and this incubation did not result in inactivation of the enzyme. This suggests that chloral is not causing the loss in activity. The authors suggest that the reactive species may be an acyl chloride. Acyl chlorides are known to be highly reactive and can be hydrolyzed to nonreactive acidic products in solution. They are also known to react with nucleophiles, which would explain the protective effect of cysteine in this study.

Another study done on *M. trichosporium* OB3b by Oldenhuis *et al.* (57) showed that in the presence of TCE these cells exhibited a decrease in both methane- and methanol-dependent oxygen consumption. This suggests that both the first and second enzymes (methanol dehydrogenase) in the pathway are affected by TCE conversion. However, in experiments done in the presence of $^{14}$C-TCE, the hydroxylase component of sMMO was labeled but the methanol dehydrogenase was not labeled. This suggests that there may be a general metabolic inactivation in the cell in addition to the specific inactivation of sMMO.

Further studies have been done by Alvarez-Cohen *et al.* (2) to look at the transformation products of TCE metabolism in a mixed methanotrophic culture. Using $^{14}$C-TCE they found that 93% of the $^{14}$C was found as transformation
products after 21 hours. Though they did not identify the transformation products they found that 58% of the $^{14}C$ was as nonvolatile compounds and the remaining 35% was detected as CO$_2$. The study then went on to look at the limited capacity of the cells to transform TCE. Repeated additions of TCE in the absence of methane results in a limited capacity to transform TCE. They found that the addition of formate resulted in an increase in the capacity to transform TCE. This is likely due to the subsequent increase in NADH which serves as an electron donor for sMMO and may also have helped in recovery of damaged cellular components. However, this addition did not completely restore the cells ability to degrade TCE. This suggested to the authors that the loss seen in transformation capacity was due to the depletion of reductant stores and/or the toxicity to cells of TCE or the metabolites of TCE oxidation.

A recent study looked at the viability of cells after exposure to various chlorinated hydrocarbons and compared it to inhibition of transformation rates. The viability of cells decreased exponentially with the amount of TCE converted. The loss of viability was probably not due to the inactivation of the monooxygenase activity because cell counts were similar on both methanol and methane amended plates. Exposure to cis-1,2-DCE resulted in the rapid decrease of both cell viability and transformation rates. Experiments done with acetylene pretreated cells provided evidence to suggest that loss in cell viability was due to transformation products rather than the chlorinated compounds themselves. The authors suggest that acyl chlorides may be the reactive species due to their highly reactive nature and the possibility that they can be produced through
rearrangement of the epoxides hypothesized to be intermediates of the oxidation of these compounds (71).

1.9. Toluene-Oxidizers

Toluene is also used as a growth substrate by a number of bacteria. Five different enzymes can be utilized to initially attack toluene. *Burkholderia cepacia* G4 uses the enzyme toluene-2-monooxygenase (T2MO). This enzyme inserts the oxygen atom as a hydroxyl group onto the ortho position of the molecule. It is interesting to note that the same enzyme (T2MO) also converts the *o*-cresol produced from toluene oxidation to the subsequent catechol. *Pseudomonas pickettii* PK01 contains a toluene-3-monooxygenase which converts the toluene into *m*-cresol. A toluene-4-monooxygenase is used by *Pseudomonas mendocina* KR1 to oxidize toluene to *p*-cresol. *Pseudomonas putida* MT2 contains a xylene monooxygenase. This enzyme attacks the methyl group on toluene which results in the production of benzyl alcohol. Finally, a toluene dioxygenase exists in *Pseudomonas putida* F1. This enzyme reduces both atoms of O$_2$ during incorporation into the substrate, resulting in the production of a catechol. Several of these toluene-oxidizers have shown promise for use in the field of bioremediation due to their broad substrate ranges, but *B. cepacia* G4 shows considerable promise due to its apparent ability to deal with the toxicity exhibited by TCE or TCE metabolites in comparison to other types of bacteria that are also capable of cometabolizing TCE.
1.10. Toxicity in Toluene-Oxidizers

Several studies have also been done looking at toxicity during cometabolism of TCE in toluene-oxidizers. It is again believed that the degradation of TCE via the oxygenases leads to the formation of toxic products that ultimately limit the potential for bioremediation (28). The hypothesis that the oxygenase plays an integral role in the toxicity observed during TCE degradation is supported by a study done by Heald and Jenkins in P. putida F1 (28). They observed a loss in cell viability when toluene-grown cells were incubated with TCE, while the noninduced cells maintained viability in the presence of TCE. A previous study showed that metabolism of TCE by P. putida F1 resulted in the inhibition of growth and the covalent modification of cellular molecules (77). This study used mutants that lacked toluene dioxygenase activity. The authors used these mutants to confirm that TCE was oxidized via the oxygenase and to determine that growth of the mutant cells was not inhibited in the presence of TCE. In the same study cells were incubated in the presence of radiolabeled TCE. It was determined that 16.6% of the radioactivity was accounted for in the total cell fractions. The majority of this label was associated with the protein fraction but small amounts were also associated with RNA, DNA and lipid fractions. In an attempt to determine possible TCE oxidation products, the protein fraction was hydrolyzed and HPLC analysis suggested that both glyoxylate and formate were associated with the protein. Studies done by Li and Wackett (46) also suggested that the major TCE oxidation products in this organism were formate and glyoxylate, with intermediates of formyl chloride and
glyoxyl chloride. These oxidation products were both diffusible and capable of modifying proteins and reduced nucleotides in solution.

A study done in 1997 by Newman and Wackett (56) supports much of what was found in the previous studies. Incubation of radioactive TCE with B. cepacia G4 resulted in the production of glyoxylic acid, which accounted for 45% of the total $^{14}$C-TCE added. Utilizing the purified toluene-2-monoxygenase, the authors went on to study the possible products produced during TCE oxidation by this enzyme. Incubation of TCE with the purified T2MO resulted in the production of both glyoxylic acid and formate as well as the volatile product carbon monoxide. This study also confirmed the presence of a TCE epoxide intermediate by trapping the epoxide with 4-($p$-nitrobenzyl)pyridine.
Chapter 2

Investigations into the Mechanism of Inactivation Associated with Trichloroethylene, Chloroform and cis-1,2-Dichloroethylene Cometabolism by

*Nitrosomonas europaea*

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2.1 Abstract

The toxicity associated with the cometabolism of trichloroethylene, chloroform and cis-1,2-dichloroethylene in the ammonia-oxidizing bacterium *Nitrosomonas europaea* was investigated. *N. europaea* obtains all of its energy from the oxidation of ammonia to nitrite by the enzymes ammonia monooxygenase and hydroxylamine oxidoreductase. Ammonium and hydrazine-dependent O₂ uptake activities, [¹⁴C] acetylene labeling of ammonia monooxygenase and an *in vitro* hydroxylamine oxidoreductase assay were utilized to further elucidate the mechanism of inactivation associated with cometabolism of these chlorinated solvents. The three chlorinated solvents did not inactivate the cells by the same mechanism. *cis*-1,2-Dichloroethylene appeared to be a specific inactivator of ammonia monooxygenase. At lower trichloroethylene (≤ 0.05 mM) and chloroform (≤ 0.10 mM) concentrations, neither ammonia monooxygenase nor hydroxylamine oxidoreductase appeared to be the primary site of attack. The inactivation that resulted from the cometabolism of these two compounds differed from results obtained by inactivating the cells with known inhibitors of either ammonia monooxygenase (light) or hydroxylamine oxidoreductase (hydrogen peroxide).

2.2 Introduction

Trichloroethylene (TCE) has been used extensively as an industrial solvent and degreasing agent and its widespread use has resulted in groundwater contamination. Biological processes have been used to clean up soil and groundwater contamination. Several physiologically different bacteria known to cometabolically degrade TCE have been identified. These including methane- (57), propane- (75), propylene- (15), isoprene-
(18), cumene- (13), toluene- (69), phenol- (55), 2,4-dichlorophenoxyacetate- (27) and ammonia- (4) oxidizing bacteria. Several common features exist between bacteria that aerobically degrade these compounds. First, these organisms contain broad substrate range oxygenases that are utilized in the aerobic degradation of these chlorinated hydrocarbons. A second common feature is that the majority of these bacteria suffer a toxic effect as a result of cometabolizing these compounds.

The mechanisms and cellular targets of these chlorinated compounds remains unclear. In mammalian systems, except at acute doses, TCE does not appear to be the toxic compound. Instead, bioactivation of TCE by cytochrome P-450 enzymes results in the production of reactive metabolites such as epoxides and chloral (11, 22, 54). However, the molecular mechanisms behind the resulting cytotoxicity are still unclear. Studies have shown that these reactive intermediates can covalently bind to protein, RNA, DNA and inactivate the catalytic enzyme (11, 22, 54).

In bacterial systems, TCE and other chlorinated solvents such as chloroform (CF) and cis-1,2-dichloroethylene (cis-1,2-DCE) can be degraded aerobically by cometabolism. This fortuitous oxidation is catalyzed by nonspecific oxygenases and requires the concurrent metabolism of a growth supporting substrate (14). Toxic effects associated with TCE oxidation can be a major factor limiting the effectiveness of bioremediation approaches based on aerobic cometabolism.

It has been suggested that the toxicity associated with cometabolism of TCE is caused by reactions of short-lived reactive intermediates, possibly TCE epoxide, generated during the oxidation of TCE (17, 21, 57, 64). Though the mechanisms of inactivation still remain unclear, toxic intermediates may covalently bind the catalytic
enzyme or diffuse from the enzyme and inactivate other cellular components. In studies with the methane-oxidizer Methylosinus trichosporium OB3b expressing the soluble form of methane monooxygenase (sMMO), it has been observed that TCE and methane oxidation is limited by toxicity (57). Studies with [1,2-\(^{14}\)C] TCE demonstrated that TCE oxidation resulted in the non-specific covalent modification of numerous proteins in whole cells, including the components of sMMO (57). Similar [1,2-\(^{14}\)C] TCE radiolabeling studies, conducted with the purified sMMO, have shown that all three components of the enzyme become covalently radiolabeled during [1,2-\(^{14}\)C] TCE oxidation (21). Similar observations have been seen in toluene-oxidizers. Pseudomonas putida F1 contains a dioxygenase enzyme which catalyzes the oxidation of both toluene and TCE. Studies with whole cells (77) and a purified enzyme system (46) have shown that this enzyme is susceptible to inactivation as a consequence of TCE oxidation. Radiolabeling of the purified protein components (46) as well as cellular macromolecules (77) after the oxidation of [1,2-\(^{14}\)C] TCE was observed. The toluene-2-monooxygenase present in Burkholderia cepacia G4 has recently been purified and all three enzyme components became covalently radiolabeled after the oxidation of [1,2-\(^{14}\)C] TCE (56). In ammonia-oxidizers, Rasche \textit{et al.} (64) showed in \textit{Nitrosomonas europaea} that [\(^{14}\)C] TCE oxidation resulted in covalent attachment of \(^{14}\)C label to the 27 kDa active-site containing polypeptide of AMO, in addition to other proteins.

\textit{N. europaea}, an ammonia-oxidizing bacterium, obtains all its energy from the oxidation of ammonia to nitrite. The initial oxidation of ammonia is catalyzed by ammonia monooxygenase (AMO) resulting in the production of hydroxylamine. Hydroxylamine is then oxidized to nitrite by a second enzyme, hydroxylamine
oxidoreductase (HAO). The electrons generated from this second reaction are used to reduce AMO for further ammonia oxidation, for CO₂ fixation and for cell growth (78). N. europaea provides an ideal system in which to study the toxicity involved with the metabolism of TCE and other chlorinated solvents, due to its relatively simple two enzyme metabolic system. Since we have both the in vivo and in vitro assays to examine the effects of TCE oxidation on both AMO and HAO individually, we can examine the effect on each enzyme.

This study is built on a previous observation that the level of ¹⁴C-radiolabel incorporation from [1,2-¹⁴C] TCE into cellular proteins in N. europaea does not correlate well with the observed level of inactivation of ammonia-oxidizing activity (64). This suggests that covalent modification of AMO components by TCE metabolites and inactivation of ammonia-oxidizing activity are largely unrelated events. In this study we have concentrated on separating the effects of chlorinated hydrocarbons on AMO and HAO-activities. Results indicate that toxicity exhibited in ammonia- and hydrazine-activities during cometabolism of TCE and CF is not caused by a loss in either AMO or HAO activities. However, toxicity exhibited by cis-1,2-DCE does appear to result from the inactivation of AMO.

2.3 Materials & Methods

Materials. Cells of N. europaea (ATCC 19178) were grown in 1.5-liter batches and harvested by centrifugation as previously described (34). Concentrated cell suspensions were stored in the dark at 4°C and used within 24 h. TCE was obtained from Aldrich Chemical Co. (Milwaukee, WI). CF was obtained from Mallinckrodt (Paris, KY). Cis-
1,2-DCE was obtained from Aldrich Chemical Co. $^{14}$Na$_2$CO$_3$ and $^{14}$BaCO$_3$ were obtained from Sigma Chemical Co. (St. Louis, MO).

**Inactivation of cells with chlorinated hydrocarbons.** Cells were exposed to TCE, CF or cis-1,2-DCE in small scale incubations conducted in glass serum vials (10 ml) as described previously (39). In summary, the vials were prepared by adding 50 mM sodium phosphate buffer, pH 7.8 (up to 850 μl) and (NH$_4$)$_2$SO$_4$ (10 mM NH$_4^+$). The vials were crimp sealed with Teflon Faced Butyl septa (Supelco, Bellefonte, PA) and the appropriate volume of buffer (up to 500 μl), saturated with the chlorinated hydrocarbon, was added to the vial. In acetylene (C$_2$H$_2$) pre-treated reactions, 1.5% C$_2$H$_2$ was added to the headspace of the reaction vial. The vials were placed in a shaking water bath (30°C, 150 rpm) for 10 min to allow an equilibrium distribution between gas and liquid phases to be established. The reactions were initiated by the addition of cells (50 μl; approximately 0.9 mg of protein) to yield a final reaction volume of 1 ml. The vials were then returned to the shaking water bath. After 10 min, the vials were removed and a sample of the reaction medium (800 μl) was added to a microcentrifuge tube (1.5 ml) and centrifuged (1 min, 14,000 x g). The resulting supernatant was discarded and the remaining pellet was immediately resuspended in fresh buffer without added hydrocarbon (800 μl). The cells were sedimented again by centrifugation and were then resuspended in fresh buffer (200 μl). The washed cells were stored on ice until analysis for residual ammonia and hydrazine-dependent O$_2$ uptake activity, *in vitro* HAO activity, and labeling of the cells with either $^{14}$C$_2$H$_2$ or $^{14}$CO$_2$. 
Inactivation of hydrazine-oxidizing activity by hydrogen peroxide. Cells were treated with hydrogen peroxide (H$_2$O$_2$) to inactivate HAO activity under similar conditions as those used in incubations with chlorinated solvents. Glass serum vials were prepared by adding 50 mM sodium phosphate buffer, pH 7.8 (up to 950 μl) and H$_2$O$_2$ (up to 5 mM). The vials were then crimp sealed with Teflon Faced Butyl septa and incubated in a shaking water bath (30°C, 150 rpm) for 10 min. The reactions were initiated by the addition of cells (50 μl, 0.9 mg of protein) to yield a final reaction volume of 1 ml. Vials were returned to the shaking water bath and incubated for 10 min. Cells were harvested and stored on ice as described for chlorinated solvent exposures.

Inactivation of ammonia-oxidizing activity by light. The AMO activity in cells was inactivated by light, as previously described (34). In summary, 160 ml glass serum vials were prepared by adding 19.5 ml sodium phosphate buffer, pH 7.8. Vials were crimp sealed with Teflon Faced Butyl septa and reactions were initiated by the addition of cells (500 μl, 9 mg of protein) to yield a final reaction volume of 20 ml. Vials were placed on an orbital shaker (200 rpm) and illuminated by a 500-W tungsten halogen projector bulb at a distance of 25 cm. At various time points, 1 ml samples were removed and added to a microcentrifuge tube (1.5 ml) and centrifuged for 1 min. The supernate was decanted and the pellet was resuspended in 200 μl of fresh buffer. The washed cells were stored on ice until analysis for residual ammonia and hydrazine-dependent O$_2$ uptake activity, in vitro HAO activity, and labeling of the cells with either $^{14}$C$_2$H$_2$ or $^{14}$CO$_2$.

O$_2$ uptake measurements. The effects of exposure to chlorinated hydrocarbons, H$_2$O$_2$, and light on AMO and HAO activities were determined, as described previously (34), by measurements of NH$_4^+$- and hydrazine (N$_2$H$_4$) -dependent O$_2$ uptake activities.
at 30°C, respectively. In summary, samples of washed cells (25 μl, 0.1 mg of protein) obtained from chlorinated hydrocarbon, H₂O₂, and light treatments were added to the reaction chamber of a Clark-style O₂ electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) containing sodium phosphate buffer (1.8 ml) and (NH₄)₂SO₄ (5 mM). Once a stable rate of O₂ uptake had been established and measured, the NH₄⁺-dependent O₂ uptake activity was inhibited by the addition of allylthiourea (100 μM). After 2 min, N₂H₄•H₂SO₄ (750 μM) was added to the reaction mixture. The N₂H₄-dependent O₂ uptake rate was then measured once a steady rate of O₂ uptake had been re-established.

**Radiolabeling of cells.** Cells were labeled with ¹⁴C₂H₂ or Na₂¹⁴CO₃ immediately following exposure to chlorinated hydrocarbon, H₂O₂, or light treatments (see above). ¹⁴C₂H₂ was generated from Ba¹⁴CO₃ as described previously (36). Incubations with ¹⁴C₂H₂ (36) or Na₂¹⁴CO₃ and subsequent separation of radiolabeled polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were done as described previously (34). Cells were exposed to ¹⁴C₂H₂ in serum vials (6 ml) stoppered with butyl rubber stoppers and aluminum crimp seals. Each vial contained phosphate buffer (875 μl) and 120 mM hydrazine sulfate. Vials were preincubated in a 30°C shaking water bath (30°C, 200 rpm) for 30 min. Two milliliters of ¹⁴C₂H₂ gas were added to each vial as an overpressure. The reactions were initiated by the addition of cells (125 μl, 0.45 mg protein) and vials were immediately returned to the shaking water bath. After 1 hr (sufficient time to saturate the labeling) vials were removed and 900 μl of the reaction medium were placed in a microcentrifuge tube and centrifuged (14,000 x g, 4 min). The supernate was decanted and the cells were resuspended in SDS-PAGE sample buffer (100
Samples were fractionated by SDS-PAGE and incorporation of $^{14}$C was analyzed with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) by using Imagequant software (Molecular Dynamics).

**In vitro HAO activity.** *In vitro* HAO enzyme activity was assayed as described previously (31). Cell extracts were prepared in microcentrifuge tubes from chlorinated hydrocarbon, H$_2$O$_2$, and light treated cells (25 µl, 0.1 mg protein) by 4 cycles of freezing and thawing. Five microliters of deoxyribonuclease I (1 mg/ml) was added to the microcentrifuge tube and suspensions were incubated for 10 min. The samples were centrifuged (2 min, 14,000 x g), and the resulting pellet was discarded.

Spectrophotometric assays were performed in glass cuvettes and contained Tris-HCl, pH 8 (2 ml, 50 mM), NH$_2$OH (100 µM), dichlorophenylindolephenol (100 µM) and phenazine methosulfate (5 µM). Assays were initiated by adding 20 µl of cell extract. HAO activity was measured as the rate of decrease in absorbance of dichlorophenylindolephenol at 600 nm.

**Protein assay.** Protein concentrations were determined by the biuret assay (23) after solubilization of cells in aqueous 3 N NaOH (30 min at 60°C) and sedimentation of insoluble material by centrifugation (14,000 x g, 5 min).

**Statistical Analysis.** Standard deviations for all O$_2$ uptake activities were determined by Microsoft Excel Version 5.0a.

2.4 **Results**

**Effects of TCE cometabolism on AMO and HAO in N. europaea** - NH$_4^+$- and N$_2$H$_4$-dependent O$_2$ uptake activities were used to assess the *in vivo* affects of TCE on cells.
Fig. 1. The effect of trichloroethylene on NH₄⁺ and N₂H₂-dependent O₂ uptake activity, incorporation of ¹⁴C from ¹⁴C₂H₂ into 27 kDa polypeptide and in vitro HAO activity. N. europaea cells were exposed to TCE (0-0.76 mM in aqueous phase) in serum stoppered vials for 10 min and then washed as described in Experimental Procedures. Residual NH₄⁺ (●) and N₂H₂-dependent (■) O₂ uptake measurements were determined at each TCE concentration (n=5). The ability of TCE treated cells to incorporate ¹⁴C₂H₂ was demonstrated by incubating treated cells with ¹⁴C₂H₂, separating polypeptides by SDS-PAGE and analyzing radioactivity by phosphoimaging. Concentration-dependent changes in incorporation of ¹⁴C into the 27 kDa polypeptide (○) are shown (n=2). In vitro HAO activity (□) was monitored by utilizing cell extracts obtained from TCE treated cells (n=2). Extracts were obtained as described in Experimental Procedures. Results of all assays are expressed as a percentage of the untreated sample.
NH₄⁺-dependent O₂ activity showed a concentration-dependent loss of activity throughout the concentration range tested (0-0.76 mM; Fig. 1). Acetylene is known to be a mechanism-based inactivator of AMO (43). ¹⁴C₂H₂ can therefore be used to determine the active AMO population in each treatment. The results of the acetylene labeling indicate that the AMO population is not affected at TCE concentrations below 0.05 mM although 70% of the NH₄⁺-dependent O₂ uptake activity had been lost. This result implies that AMO does not contribute to the loss of in vivo activity seen in NH₄⁺-dependent O₂ uptake data at this concentration. At concentrations above 0.05 mM, the acetylene labeling and thus the active AMO populations decrease and possibly contribute to the loss of in vivo NH₄⁺-dependent activity.

Hydrazine-dependent O₂ uptake activity was used to monitor the in vivo effect of TCE on HAO (Fig. 1). A loss in activity was observed at all concentrations tested. However, activity appears to stabilize at concentrations greater than 0.05 mM at about 45% of the untreated cells. The reason for this leveling off is not known. In vitro HAO activity was determined spectrophotometrically by assaying cell extracts. Results indicated that the HAO enzyme population was active over the entire concentration range tested. This again indicates that HAO is not responsible for the loss seen in in vivo activity.

The in vivo data indicates that metabolism of TCE causes a loss in activity that affects the cells’ ability to utilize both ammonia and hydrazine. The ¹⁴C₂H₂ labeling indicates that at concentrations below 0.05 mM inactivation of AMO is not the reason for this loss in in vivo activity. The in vitro HAO assay indicates that at all
concentrations tested, inactivation of HAO is also not the reason for the loss of \textit{in vivo} activity.

\textbf{Inactivation of Cells by light and H}_2\text{O}_2 - In order to have a standard for both the loss of AMO and HAO, cells were exposed to two known inactivators of either AMO or HAO. These specific inactivators allowed for comparisons with results seen in chlorinated solvent exposures. Light is known to specifically inactivate ammonia oxidation with no effect on hydroxylamine oxidation (32), and results of light inactivation with both the \textit{in vivo} and \textit{in vitro} assays were used as a comparison for loss of AMO activity. Hydrogen peroxide has been shown to inactivate HAO (31) and was again used to look at the effects of the loss of HAO.

Inactivation of cells by light (Fig 2a) shows the time-dependent loss of NH$_4^+$-dependent O$_2$ uptake. Incorporation of $^{14}$C$_2$H$_2$ into cells exposed to light also shows a decrease in the active AMO population. Since light is a specific inactivator of AMO, the N$_2$H$_4$-dependent O$_2$ uptake activity is not lost when exposed to light over the 60 minute time course. \textit{In vitro} HAO assays also indicated that HAO is still active throughout the exposure time. Light therefore provided us with a standard for loss of AMO function with which to compare inactivation by chlorinated solvents.

Inactivation of \textit{N. europaea} with hydrogen peroxide provided us with an example of HAO inactivation. Exposure to a range of H$_2$O$_2$ concentrations (0-5 mM) resulted in the loss of both the N$_2$H$_4$-dependent O$_2$ uptake and \textit{in vitro} HAO activity (Fig. 2b). The reason for the discrepancy between the N$_2$H$_4$-dependent O$_2$ uptake and the \textit{in vitro} HAO assay is unknown. Since the oxidation of NH$_2$OH to NO$_2^-$ generates the necessary reductant for AMO catalyzed reactions, the inactivation of HAO would
Fig. 2. The effects of light and H$_2$O$_2$ on NH$_4^+$ and N$_2$H$_4$-dependent O$_2$ uptake activity, incorporation of $^{14}$C from $^{14}$C$_2$H$_2$ into 27 kDa polypeptide and in vitro HAO activity.  

a, $N$. europaea cells were illuminated by a projector bulb for 60 min and samples were removed at the indicated time points for analysis. 
b, $N$. europaea cells were exposed to H$_2$O$_2$ (0-5 mM in aqueous phase) in serum stoppered vials for 10 min and then washed as described in Experimental Procedures. Residual NH$_4^+$ (●) and N$_2$H$_2$-dependent (■) O$_2$ uptake measurements were determined (n=3). The ability of TCE treated cells to incorporate $^{14}$C was demonstrated by incubating treated cells with $^{14}$C$_2$H$_2$, separating polypeptides by SDS-PAGE and analyzing radioactivity by phosphoimaging. Concentration (H$_2$O$_2$) or time (light) -dependent changes in incorporation of $^{14}$C into the 27 kDa polypeptide (○) are shown (n=2). In vitro HAO activity (□) was monitored by utilizing cell extracts obtained from TCE treated cells (n=2). Extracts were obtained as described in Experimental Procedures. Results of all assays are expressed as a percentage of the untreated sample.
Figure 2. The effects of light and H$_2$O$_2$ on NH$_4^+$ and N$_2$H$_4$-dependent O$_2$ uptake activity, incorporation of $^{14}$C from $^{14}$C$_2$H$_2$ into 27 kDa polypeptide and in vitro HAO activity.
result in a loss of NH$_4^+$-dependent O$_2$ activity (Fig. 2b). However, $^{14}$C$_2$H$_2$ label incorporation indicates that the AMO population is still active.

**Inactivation of cells by Chloroform and cis-1,2-Dichloroethylene** - In order to evaluate if all chlorinated solvents had similar mechanisms of inactivation, cells were exposed to both chloroform and cis-1,2-dichloroethylene. The same set of assays used for TCE, light and H$_2$O$_2$ were used to study these compounds. Exposure of cells to CF (0-1.28 mM) resulted in concentration-dependent losses of both NH$_4^+$- and N$_2$H$_4$-dependent O$_2$ uptake (Fig. 3). $^{14}$C$_2$H$_2$ label incorporation indicated that the cells' ability to incorporate $^{14}$C$_2$H$_2$ into AMO was not lost at concentrations below 0.10 mM CF. Therefore, inactivation of AMO is not the reason for the loss in NH$_4^+$-dependent O$_2$ uptake at low CF concentrations. Then, as in TCE inactivation (Fig. 1), acetylene incorporation appears to decrease with an increase in CF concentration, thus inactivation of AMO may contribute to the loss in *in vivo* activity. After exposure to CF, the *in vitro* HAO assays indicated that a slight loss in HAO occurred, approximately 10% below CF concentrations of 0.21 mM and 40% at the highest concentration tested, 1.28 mM (Fig. 3). However, this loss in *in vitro* activity does not completely account for the loss seen in the O$_2$ uptake assays. At lower concentrations (below 0.10 mM), neither loss in AMO or HAO accounts for the loss of *in vivo* activity. The pattern produced by CF inactivation is similar to what is seen in TCE inactivation and the possibility exists that the two mechanisms of inactivation are similar.

Inactivation of cells by cis-1,2-dichloroethylene produced a different pattern than the two previous compounds. *cis*-1,2-DCE appears to specifically target AMO (Fig. 4) and results in a pattern similar to what is seen in light inactivation (Fig 2a).
Fig. 3. The effect of chloroform on NH$_4^+$ and N$_2$H$_4$-dependent O$_2$ uptake activity, incorporation of $^{14}$C from $^{14}$C$_2$H$_2$ into 27 kDa polypeptide and in vitro HAO activity. N. europaea cells were exposed to CF (0-1.28 mM in aqueous phase) in serum stoppered vials for 10 min and then washed as described in Experimental Procedures. Residual NH$_4^+$ (●) and N$_2$H$_2$-dependent (■) O$_2$ uptake measurements were determined at each TCE concentration (n=3). The ability of TCE treated cells to incorporate $^{14}$C$_2$H$_2$ was demonstrated by incubating treated cells with $^{14}$C$_2$H$_2$, separating polypeptides by SDS-PAGE and analyzing radioactivity by phosphoimaging. Concentration-dependent changes in incorporation of $^{14}$C into the 27 kDa polypeptide (○) are shown (n=2). In vitro HAO activity (□) was monitored by utilizing cell extracts obtained from TCE treated cells (n=2). Extracts were obtained as described in Experimental Procedures. Results of all assays are expressed as a percentage of the untreated sample.
Fig. 4. The effect of cis-1,2-dichloroethylene on NH$_4^+$ and N$_2$H$_4$-dependent O$_2$ uptake activity, incorporation of $^{14}$C from $^{14}$C$_2$H$_2$ into 27 kDa polypeptide and *in vitro* HAO activity. *N. europaea* cells were exposed to cis-1,2-DCE (0-199 μM in aqueous phase) in serum stoppered vials for 10 min and then washed as described in Experimental Procedures. Residual NH$_4^+$ (●) and N$_2$H$_2$-dependent (■) O$_2$ uptake measurements were determined at each TCE concentration (n=3). The ability of TCE treated cells to incorporate $^{14}$C$_2$H$_2$ was demonstrated by incubating treated cells with $^{14}$C$_2$H$_2$, separating polypeptides by SDS-PAGE and analyzing radioactivity by phosphoimaging. Concentration-dependent changes in incorporation of $^{14}$C into the 27 kDa polypeptide (○) are shown (n=2). *In vitro* HAO activity (□) was monitored by utilizing cell extracts obtained from TCE treated cells (n=2). Extracts were obtained as described in Experimental Procedures. Results of all assays are expressed as a percentage of the untreated sample.
Over the concentrations tested (0-199 μM), cometabolism of cis-1,2-DCE resulted in the loss of NH$_4^+$-dependent O$_2$ uptake as well as the incorporation of $^{14}$C$_2$H$_2$ label. However, both in vivo and in vitro HAO assays indicate that neither N$_2$H$_4$-dependent O$_2$ uptake nor the in vitro HAO activity is lost after exposure to cis-1,2-DCE (Fig. 4). The results of these assays indicate that cis-1,2-DCE may be a mechanism-based inactivator of AMO. Since cometabolism of cis-1,2-DCE resulted in a different mechanism of inactivation than either TCE or chloroform, it can not be assumed that all chlorinated solvents have similar mechanisms of inactivation.

**Exposure of Acetylene Pretreated Cells to Inactivating Treatments** - In order to ensure that toxicity resulted from the turnover of each chlorinated solvent, rather than the actual solvent, cells were exposed to treatments in the presence of C$_2$H$_2$, a suicide substrate, to prevent turnover of the inactivating compound. All treatments were tested at the highest concentrations that we investigated and N$_2$H$_4$-dependent O$_2$ uptake and in vitro assays were run on each sample in the presence and absence of acetylene. NH$_4^+$-dependent O$_2$ uptakes and $^{14}$C$_2$H$_2$ labeling were not obtained since pretreatment of acetylene would negate them. Untreated cells were used as controls for the experiment and showed no loss of activity in either in vivo or in vitro assays (Fig. 5). Acetylene pretreatment of both light and H$_2$O$_2$ samples served as controls for loss of either AMO or HAO respectively. These samples gave expected results and exposure to acetylene had no effect. Light inactivation resulted in a minimal loss in both assays. Hydrogen peroxide resulted in the expected loss of activity in both assays. Exposure to TCE in the presence of C$_2$H$_2$ resulted in no significant loss in either O$_2$ uptake activity or the in vitro assay. Results in the absence of acetylene resulted in approximately a 50% loss of O$_2$ uptake.
activity but no loss in the *in vitro* assay. These results are consistent with what we saw previously (Fig. 1) and further support the idea that HAO is also not a target of TCE metabolites. This also supports previous observations that TCE must be turned over in order to exhibit toxicity (3, 21, 46, 56, 57, 64, 77). Treatment with chloroform resulted in a slight loss of *in vitro* activity in acetylene treated cells and no loss in O₂ uptake. In the absence of acetylene, both the O₂ uptake and the *in vitro* assays were significantly reduced, 70% and 40% respectively. However, there is a significant difference between the two assays and loss of *in vitro* HAO activity does not account for all the toxicity exhibited in *in vivo* studies. cis-1,2-DCE treatments, in the presence and absence of acetylene, further substantiate that HAO is not a target of cis-1,2-DCE. An increase in the O₂ uptake activity is seen when cells are exposed to cis-1,2-DCE. The reason for this increase in *in vivo* activity is unclear but has been seen previously (64).

**1⁴CO₂ Labeling of Light and TCE Treated Cells** - *N. europaea* are autotrophs and therefore have a strict requirement for CO₂ as a carbon source. We examined the cells’ ability to incorporate ¹⁴C from ¹⁴CO₂ into polypeptides during the *de novo* protein synthesis required for recovery from the inactivating treatments. Samples of cells were inactivated by a range of light (0-60 min), TCE (0-0.76 mM) or TCE and C₂H₂ (0-0.76 mM TCE & 1.5% C₂H₂ in the headspace) treatments and then incubated in the presence of ¹⁴CO₂; hydroxylamine was also added as an energy source for the cell. Nitrite measurements were taken at the conclusion of the reaction to ensure that all samples utilized the same amount of energy. Approximately 1.5 mM NO₂⁻ was produced in each reaction (data not shown).
Fig. 5. The effect of light, H$_2$O$_2$, TCE, CF and cis-1,2-DCE on active and acetylene pretreated cells. *N. europaea* cells were incubated in either the presence (+) or absence (-) of acetylene (1.5% (vol/vol)). Cells were then exposed (10 min) to the highest treatment utilized in previous experiments: light (60 min), H$_2$O$_2$ (5mM), TCE (0.79 mM), CF (1.28 mM) and cis-1,2-DCE (199 μM) (n=3). N$_2$H$_4$ dependent O$_2$ uptake activity (solid bars) and *in vitro* HAO assays (striped bars) were performed and are shown as percentages of the untreated control cells.
Fig. 6. The effects of light, TCE and TCE & acetylene treated cells on the incorporation of $^{14}$C from $^{14}$CO$_2$. N. europaea cells were treated with either TCE (0-0.76 mM), TCE & acetylene (0-0.76 mM and 1.5% acetylene) or light (0-60 min) as described in materials and methods. Treated cells were then incubated in the presence of Na$_2^{14}$CO$_3$ and subsequently separated by SDS-PAGE. Incorporation of $^{14}$C was analyzed by a Phosphoimager. The relative density was calculated for the 27-kDa polypeptide.
Figure 6. The effects of light, TCE and TCE & C₂H₂ on the incorporation of H\(^{14}C\) from \(^{14}C\)CO\(_2\) into polypeptides.
Incorporation of $^{14}$C into polypeptides was analyzed by SDS-PAGE and phosphorimaging. As cells were inactivated with increasing amounts of TCE incorporation of $^{14}$C into polypeptides decreased (Fig. 6). Light exposed cells, in comparison, resulted in a relatively constant incorporation of $^{14}$C into polypeptides. Since light specifically inactivates AMO, we can hypothesize that the toxicity exhibited in TCE incubations does not result entirely from inactivation of AMO, and that some other cellular component(s) are possible targets. Some component of the electron transport system may be a possible target resulting in problems with energy flow in the cell. Treatment with TCE and C$_2$H$_2$ further supports the theory that it is the metabolic products of TCE that are inactivating the cell rather than TCE itself dissolving the cell.

2.5 Discussion

*N. europaea* was used to study the metabolism of TCE and the subsequent consequences of its oxidation on both AMO and HAO. The ability to look at each of these enzymes individually allowed us to assess the effects of TCE metabolism in *N. europaea* as well as aid in elucidation of molecular and cellular mechanisms of toxicity in both mammalian and bacterial systems. Evidence from this study leads to several conclusions. First, at lower concentrations of TCE and CF the metabolic enzymes do not appear to be primary targets of inactivation by either the TCE or CF oxidation products. Second, comparing the three chlorinated solvents TCE, CF, and cis-1,2-DCE, we can conclude that not all chlorinated solvents act by similar mechanisms in the inactivation in *N. europaea*. 
Previous studies have shown that toxicity of TCE generally results from bioactivation of the compound. In mammalian systems, TCE is oxidized by cytochrome P-450 enzymes (24, 29, 65), most likely P-450 2E1 (24, 25) to reactive metabolites. Evidence for this bioactivation also exists in bacterial systems (3, 21, 56, 77) and has been shown in *N. europaea*. Rasche *et al* (64) showed that pretreatment with acetylene, a specific inactivator of AMO (43), prevented incorporation of [14C]TCE into proteins. In this study, pretreatment of *N. europaea* cells with acetylene and subsequent exposure to TCE, CF and cis-1,2-DCE (fig. 5) further supported evidence suggesting that TCE does need to be bioactivated in order to exhibit toxic effects.

Evidence for target sites of these diffusible intermediate products has varied widely. Early studies in mammalian systems have shown that covalent binding of [14C]TCE intermediates can occur in both microsomal proteins and exogenous DNA in *vitro* (8). In a study by Miller and Guengerich (53) covalent binding to DNA, RNA and protein was observed in hepatocytes, suggesting that the reactive metabolite was stable enough to migrate through the plasma membrane. This study also suggests that heme destruction resulted in the inactivation of the catalytic enzyme. This suicide inactivation of the catalytic enzyme is a common theory in TCE mediated toxicity. Studies done by Fox *et al* (21) in *M. trichosporium* OB3b with the purified MMO have shown that covalent binding to the catalytic enzyme does occur. *In vivo* studies have also shown that nonspecific binding of these reactive metabolites to protein occurs. Nonspecific binding to proteins was observed in *M. trichosporium*, indicating that this nonspecific covalent binding caused the inactivation observed in cells degrading TCE. [14C]TCE incubations with *N. europaea* also showed that numerous proteins were covalently
modified after incubation with TCE (64). Therefore, in the case of TCE, our study does not support the idea that it is the inactivation of the catalytic enzyme that is initially causing the toxicity observed in cells. Utilizing assays to monitor both AMO and HAO, results did not support the theory that the inactivation of AMO, the catalytic enzyme, is a factor in toxicity at concentrations below 0.05 mM. Inactivation of HAO also does not appear to be part of the TCE mediated toxicity at these concentrations. Similar results are also seen with CF cometabolism in *N. europaea*.

In examining the recovery of these cells to treatment with TCE (fig. 6) in the presence of $^{14}$CO$_2$ we can see that it is likely that more than just AMO is involved in the exhibited toxicity. When looking at the proteins synthesized after inactivation by light a consist pattern of proteins are synthesized. In comparison, recovery from TCE inactivation results in significantly less new protein production with increasing concentrations of TCE. This probably indicates that some other component of the cell involved in energy generation is being modified. It will therefore be beneficial to look closer at other possible sites of inactivation such as DNA, RNA and other cellular components to further elucidate possible binding sites of TCE and CF metabolites.

Interestingly, cometabolism of *cis*-1,2-DCE presented a different picture. Inactivation by this compound appears to be specific to AMO (fig. 4). The inactivation pattern exhibited by this compound is similar to what is seen when cells are inactivated by light (this study; (34). Evidence presented in this study lends support to the possibility that *cis*-1,2-DCE is a mechanism-based inactivator of AMO. This difference in the mechanisms of inactivation between TCE and CF and *cis*-1,2-DCE suggests we can not assume that all chlorinated solvents will inactivate bacterial systems by the same
mechanisms. This idea may be important to aspects such as modeling in bioremediation.

In an attempt to predict the degradation of these compounds in the environment many models may make the incorrect assumption that inactivation occurs by the same mechanism and this may affect model predictions.
Chapter 3

Summary and Conclusions

In this study, effects on the metabolic enzymes in the bacterium *N. europaea*, during the degradation of the chlorinated hydrocarbons TCE, CF and *cis*-1,2-DCE, were examined. Assays were utilized to look at consequences to AMO and HAO during degradation of these chlorinated hydrocarbons.

As seen in both this study and in previous studies, all three chlorinated hydrocarbons were not toxic to *N. europaea*, but instead they needed to be bioactivated by AMO to reactive products. Though these compounds were not identified in this study, studies in other organisms have suggested that an epoxide may be an intermediate in the cases of TCE and *cis*-1,2-DCE. Either the epoxide or one of the degradation products of the epoxide, may actually be the compounds causing the effects seen in this study.

This study utilized both an O$_2$ uptake assay to monitor the *in vivo* effects to both AMO and HAO, as well as two additional assays to look at both AMO and HAO *in vitro*. The effect on AMO was quantified by utilizing the fact that acetylene is a suicide substrate. Though this is not technically an *in vitro* assay, this allowed us to use radiolabeled acetylene to quantify comparative amounts of AMO in cells after exposure to the chlorinated hydrocarbons. HAO was quantified utilizing an *in vitro* spectrophotometric assay. Utilizing these assays the effects to both AMO and HAO were examined.
TCE degradation in *N. europaea* resulted in a concentration-dependent inactivation of the \( \text{NH}_4^+ \)-dependent \( \text{O}_2 \) uptake rates in whole cells during 10 minute incubations. The \( \text{N}_2\text{H}_4^- \)-dependent \( \text{O}_2 \) uptake rate was also inhibited in a concentration-dependent manner up to approximately 0.05 mM TCE. At higher concentrations of TCE the \( \text{O}_2 \) uptake leveled off at approximately 50 percent of the control without TCE. The reason(s) for this leveling off effect are unknown. However, when we look at the *in vitro* assays we get a different picture as to the effect of TCE degradation on these enzymes. Both AMO and HAO appear to be unaffected after 10 minute incubations at concentrations below 0.05 mM TCE. This leads us to believe that the loss seen in \( \text{O}_2 \) uptake rates in whole cell assays is not due to the inactivation of either of these enzymes. At concentrations above 0.05 mM TCE, results of the \( ^{14}\text{C}_2\text{H}_2 \) assay show that there is a 30-70 percent loss of AMO. This suggests that a partial loss of active AMO may play a role in the loss of \( \text{NH}_4^+ \)-dependent \( \text{O}_2 \) uptake activity above 0.05 mM TCE. The *in vitro* HAO assay, however, indicates that there is not a significant loss in the *in vivo* activity of this enzyme over the entire TCE concentration range tested, indicating that a loss in HAO is not contributing to the loss seen in either \( \text{NH}_4^+ \)- or \( \text{N}_2\text{H}_4^- \)-dependent \( \text{O}_2 \) uptake rates in whole cells.

Degradation of CF by *N. europaea* during 10 minute incubations resulted in a similar pattern as seen in TCE degradation. CF degradation resulted in a concentration dependent loss of \( \text{NH}_4^+ \)-dependent \( \text{O}_2 \) uptake activity at tested CF concentrations in whole cells. Loss of \( \text{N}_2\text{H}_4^- \)-dependent \( \text{O}_2 \) uptake was also concentration dependent up to 0.2 mM CF. At concentrations above 0.2 mM, \( \text{N}_2\text{H}_4^- \)-dependent \( \text{O}_2 \) uptake levels off at approximately 30 percent of the no CF control vial. Again, the reason(s) for this leveling off effect is unknown.
When we look at the results of the \textit{in vitro} assays we again see a different picture than the results of the whole cell assays. The $^{14}\text{C}_2\text{H}_2$ assay indicates that there is no loss in the amount of active AMO at concentrations at or below 0.1 mM CF. This is in stark contrast to the drastic decrease in \textit{in vivo} activity seen at those concentrations. For example, at 0.1 mM CF the NH$_4^+$-dependent O$_2$ uptake activity is at approximately 30 percent of the no CF control sample, while the $^{14}\text{C}_2\text{H}_2$ assay shows the AMO population is still at approximately 100 percent of the no CF control sample. This suggests that loss of AMO is not the reason for the loss seen in the \textit{in vivo} activity. At concentrations above 0.1 mM CF the $^{14}\text{C}_2\text{H}_2$ assay results show that a significant decrease in the AMO population occurs at these concentrations and the loss of AMO, therefore may contribute to the loss of NH$_4^+$- dependent O$_2$ uptake.

The \textit{in vitro} HAO assay indicates that at concentrations of CF up to 0.2 mM, no significant loss in HAO is seen in comparison to the no CF control sample. Again this is in contrast to the drastic losses seen in the N$_2$H$_4$-dependent O$_2$ uptake assay where a significant loss in activity was seen at these concentrations. At concentrations above 0.2 mM a small loss in the \textit{in vitro} HAO assay rate is seen and may contribute to the decrease seen in the whole cell NH$_4^+$- and N$_2$H$_4$-dependent O$_2$ uptake assay.

Results of treating \textit{N. europaea} cells with increasing concentrations of \textit{cis}-1,2-DCE resulted in a different pattern than seen in TCE and CF experiments. \textit{cis}-1,2-DCE appears to inactivate AMO specifically. This inactivation is similar to what is seen with the specific inactivation of AMO by light. A concentration-
dependent loss is seen throughout the tested concentration range (0-200 μM cis-1,2-DCE) in the NH$_4^+$-dependent O$_2$ uptake assay. The $^{14}$C$_2$H$_2$ assay also shows a concentration dependent loss of the AMO population. Both of these assays indicate that AMO has been inactivated by the product(s) of cis-1,2-DCE degradation. In contrast to AMO, both the N$_2$H$_4$-dependent O$_2$ uptake assay and the in vitro HAO assay indicate that HAO is unaffected by the degradation of cis-1,2-DCE.

These differences in toxicities exhibited towards chlorinated hydrocarbons in *N. europaea* is interesting. Models describing the degradation of chlorinated hydrocarbons often assume that the mechanisms of inactivation are the same for all chlorinated hydrocarbons. This study clearly indicates that this is not true. When modeling degradation of these compounds, it may be important to take into account the differences in toxicities between various chlorinated hydrocarbons. This may alter the assumption of a common mechanism of inactivation that many cometabolic models are based on.

Finally, experiments done to look at protein synthesis after incubation with increasing TCE concentrations gives further evidence that more than just the cometabolic enzyme, AMO, is being inactivated. *N. europaea* cells were treated with increasing amounts of either TCE, TCE and acetylene, or light. Cells were then incubated in the presence of both an energy source and $^{14}$CO$_2$. Protein gels were used to examine proteins synthesized upon recovery from the above treatments. Both the TCE & acetylene and light treatments produced relatively constant patterns of proteins across the tested ranges, while the TCE treated cells
show a decrease in new protein synthesis with increasing concentrations of TCE. Since both acetylene and light inactivate AMO specifically and a relatively constant pattern of new protein synthesis occurs after light inactivation, this lends evidence to the idea that more than just AMO is affected during TCE degradation. Possibly some portion of the electron transport chain is affected, resulting in a decrease in energy production. This experiment also further substantiates the idea that TCE must be bioactivated in order to exhibit toxic effects to the cell.

This study demonstrated that toxicity exhibited towards cells during cometabolism of chlorinated hydrocarbons may be more complex than originally thought. Evidence was presented to disprove the idea that the cometabolic enzyme is the only site of inactivation during degradation of chlorinated hydrocarbons, though this is possible as we saw with cis-1,2-DCE. Both TCE and CF appear to have other factors involved in the observed loss of NH$_4^+$- and N$_2$H$_4$-dependent O$_2$ uptake rates in whole cells. This study hopefully begins to indicate the complexity involved in cometabolism of chlorinated hydrocarbons.
Bibliography


