Physiology, Biochemistry, and Specific Inhibitors of CH$_4$, NH$_4^+$, and CO Oxidation by Methanotrophs and Nitrifiers

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INTRODUCTION

An objective of this review is to summarize aspects of the biochemistry and physiology of methanotrophs and ammonia oxidizers which are relevant to the oxidizing activities of these organisms in their natural habitats. Ecological aspects have been extensively reviewed elsewhere (51, 128, 130), as have general aspects of methanotrophs (4, 5, 25, 55) and ammonia oxidizers (61, 174). Particular attention is given to the ammonia monoxygenase of ammonia oxidizers and the methane monoxygenase of methanotrophs since these are both key enzymes in the metabolism of CH$_4$, NH$_4^+$, and CO.

Many methanotrophs (family Methylcoccaceae) and chemolithotrophic ammonia oxidizers (family Nitrobiacteriaceae) have been found to carry out the following reactions (25, 39, 61, 70, 110, 157):

\[
\text{CH}_4 + \text{O}_2 + \text{AH}_2 \rightarrow \text{CH}_3\text{OH} + \text{H}_2\text{O} + \text{A} \quad (1)
\]

\[
\text{CO} + \text{O}_2 + \text{AH}_2 \rightarrow \text{CO}_2 + \text{H}_2\text{O} + \text{A} \quad (2)
\]

\[
\text{NH}_4^+ + \text{O}_2 + \text{AH}_2 \rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O} + \text{A} \quad (3)
\]

The immediate source of reductant (AH$_2$) can be reduced nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] in methanotrophs, but in ammonia oxidizers it may be a cytochrome c (25, 157). All of the above oxidations are apparently catalyzed by monoxygenase enzymes: methane monoxygenase in methanotrophs and ammonia monoxygenase in ammonia oxidizers (25, 61). Only CH$_4$ can support growth in the former organisms, and only NH$_4^+$ can support growth in the latter (39, 79, 80, 139). In both methanotrophs and ammonia oxidizers, there are reports that CH$_3$OH is metabolized to CO$_2$ and cell C (25, 79, 164). While the pathway of CH$_3$OH oxidation is well characterized in methanotrophs, in ammonia oxidizers it remains incompletely understood (148, 149). In addition, during the production of NO$_3^-$ from NH$_4^+$ by both methanotrophs and ammonia oxidizers, small amounts of NO$_2^-$ are evolved (174, 177, 178). Although there is evidence that, in certain environments, methanotrophs and ammonia oxidizers may carry out oxidation of the growth substrate of the other as well as CO, few, if any, attempts have been made to measure directly the
relative contributions of these two kinds of organisms to in situ CO, CH₄, and NH₄⁺ metabolism.

NO₃⁻ peaks located at the boundary between anoxic and oxygenated waters are a common feature of moderately productive lakes (67). Several different processes, including nitrification and denitrification, could lead to the formation of such peaks (91). In the metalimnion of Lake Mendota (Wisconsin), an NO₃⁻ maximum was found to occur at the depths where CH₄ oxidation was most pronounced, prompting the suggestion that methanotrophs might be responsible for NO₃⁻ accumulation in this environment (51, 52). An alternative explanation (79) is that chemolithotrophic ammonia oxidizers were oxidizing CH₄ in addition to NH₄⁺. Recently, it has been suggested that methanotrophs could be involved in the process of nitrification in lakes under winter ice (90). Methanotrophs may also be responsible for some of the nitrification observed in soils (130, 159). S. R. Megraw and R. Knowles (unpublished data) have obtained strong evidence that, under certain conditions, essentially all of the NH₄⁺ oxidation measured in a particular humisol is carried out by methanotrophs.

Biological oxidation is an important sink in the worldwide cycle of CO (104). Carboxydobacteria are aerobic organisms that can use this compound as sole source of carbon and energy (105). Most of the carboxydobacteria known at the present time exhibit half-saturation constants for CO oxidation which are considerably higher in value than naturally occurring CO concentrations. For this reason, it has been suggested that these organisms may not play a significant role in CO cycling. On the other hand, both methanotrophs and ammonia oxidizers exhibit high affinities for the compound, which has led some investigators to speculate about the importance of these organisms in the environmental metabolism of CO (27, 80).

The participation of methanotrophs and nitrifiers in the atmospheric cycles of CH₄, N₂O, and CO can have increasingly important implications for global warming, due to the infrared absorption of CH₄ and N₂O (31), and for stratospheric chemistry (including ozone) since all three gases participate in vital reactions (78). An increased understanding of the relative roles of methanotrophs and nitrifiers in such processes is therefore desirable.

To determine the nature of the organism(s) responsible for an oxidation in an environment, one might use an inhibitor which selectively blocks only methanotrophs or only ammonia oxidizers. Several inhibitors which act upon ammonia oxidizers are used to delay nitrification in agricultural soils (135). Nitrification blockers have also been used to quantify nitrification in aquatic environments (e.g., see reference 137). However, it has been found that almost all agents that inhibit ammonia oxidizers also inhibit methanotrophs (129, 155, 156). This raises the possibility that in certain environmental studies activity attributed to ammonia oxidizers was in fact due to methanotrophs. We conclude by reviewing our present knowledge of inhibitors of these organisms and examine the limitations associated with the use of nitrification blockers in ecological studies.

**OXIDATION OF CH₄, NH₄⁺, AND CO BY METHANOTROPHS AND NITRIFIERS**

Similarities between Methanotrophs and Ammonia Oxidizers

Methanotrophs (family *Methylococcaceae*) can obtain all of their carbon and energy from CH₄ under aerobic conditions (170). The pathway of methane oxidation is outlined in Fig. 1. Obligate methanotrophs grow only on CH₄ and CH₃OH. In addition to these substrates, facultative methanotrophs can also grow on multicarbon compounds. Methane oxidizers are grouped into two types based in part on their pathways of carbon assimilation (22). Type I methanotrophs fix formaldehyde by the ribulose monophosphate pathway, while type II organisms use the serine pathway to assimilate carbon dioxide and formaldehyde (4).

Ammonia oxidizers are chemolithotrophs, members of the family *Nitrobacteraceae* (165). They oxidize NH₄⁺, which provides energy for the fixation of CO₂ via the Calvin cycle (167). The pathway of NH₄⁺ oxidation is summarized in Fig. 1.

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**FIG. 1.** Pathway of methane oxidation in methanotrophs and ammonia oxidation in ammonia oxidizers (after references 25 and 34). * PQQ, Pyroquinoline quinone. **X and XH₂ are the oxidized and reduced forms of an unknown electron donor.
In addition to their usual source of energy, both methanotrophs and ammonia oxidizers can oxidize a variety of nongrowth substrates. Table 1 lists compounds which are oxidized by both groups of organisms. Methanotrophs can oxidize NH$_4^+$ and ammonia oxidizers can oxidize CH$_4$, although neither type of organism can use the energy from the reaction for growth (79, 127). Methane oxidizers can modify a great number of organic compounds in addition to those mentioned in Table 1 (47). The multiplicity of oxidations carried out by methanotrophs and ammonia oxidizers reflects the wide substrate specificities of the methane monooxygenase of the former organisms and the ammonia monooxygenase of the latter (34, 56, 70, 71).

Other similarities between the two groups of organisms can be summarized as follows. (i) An NH$_4$OH oxidase resembling that in ammonia oxidizers, and distinct from methanol dehydrogenase, may be present in at least some methanotrophs (136). (ii) A complex array of intracytoplasmic membranes is present in both types of organisms (28, 107). (iii) Ammonia oxidizers and type I methanotrophs have predominantly 16:0 and 16:1 fatty acids (111, 100, 109). (iv) The type I methanotrophs possess a tricarboxylic acid cycle which lacks α-ketoglutarate dehydrogenase (169). (v) Both groups of organisms tend to favor the same kinds of habitats: aerobic-anaerobic interfaces where CH$_4$, NH$_4^+$, and O$_2$ are readily available (70, 127).

The possibility of an evolutionary link between methanotrophs and ammonia oxidizers has often been mentioned (86, 123, 169), and recent SS and 16S ribosomal ribonucleic acid studies show the ammonia oxidizers in groups β-3 and γ-1 and a type I methanotroph in the γ-1 group of the purple bacteria (172, 173). However, the elucidation of the relationship between methanotrophs and ammonia oxidizers may be rendered more difficult by recent discoveries which suggest that cross-species gene transfer contributes in a significant way to species evolution (150).

The present review concentrates on CH$_4$ oxidation by ammonia oxidizers. NH$_4^+$ oxidation by methanotrophs, and CO oxidation by both groups of organisms; activities which, as indicated earlier, may be of importance in the natural environment. First, the characteristics of the respective monooxygenases will be discussed.

**Ammonia Monooxygenase**

Although the enzyme involved has yet to be purified, it appears likely that the first step of NH$_4^+$ oxidation in *Nitrosomonas europaea* is a monooxygenase-catalyzed oxidation of NH$_4^+$ to NH$_2$OH (35, 59). Evidence that NH$_3$ rather than NH$_2$OH is the substrate for the monooxygenase comes from an examination of the effect of pH on NH$_3^+$ oxidation in cell-free extracts of this organism (144). $K_m$ values for the oxidation decreased markedly with increasing pH. However, the values remained essentially constant when the reaction was carried out in terms of unprotonated NH$_3$ instead of total ammonia (NH$_3$ plus NH$_2$OH). NH$_2$OH is a probable immediate in NH$_3^+$ oxidation (58, 59, 96, 108). It is oxidized to NO$_2^-$ by whole cells and cell extracts of *N. europaea* and accumulates during NH$_3^+$ oxidation in the presence of hydrazine, a powerful inhibitor of NH$_2$OH oxidation (58, 59, 96, 108). The inhibitor sensitivities of NH$_3^+$ and NH$_2$OH oxidation are very different (58, 62) see Table 3. Furthermore, NH$_2$OH is the substrate of an oxidoreductase which has been isolated from *N. europaea* and extensively purified and characterized (for reviews, see references 32 and 61).

$^{15}$O from $^{16}$O was incorporated into NO$_2^-$ during NH$_3^+$ oxidation by *N. europaea* (126). Cell suspensions of this organism containing added hydrazine were used in isotopic studies of the NH$_3$-to-NH$_2$OH reaction step (35, 59): the oxygenase nature of the reaction mechanism was demonstrated with $^{16}$O$_2$, H$_2$,$^{18}$O, and $^{15}$NH$_4$Cl. The oxygenase is likely to be of the mixed-function (monooxygenase) type (59); i.e., it inserts one O atom from dioxygen into ammonia while reducing the other to H$_2$O: NH$_3$ + AH$_2$ + O$_2$ → NH$_2$OH + A + H$_2$O.

The immediate source of reductant “A” has not been identified with any certainty (61; see discussion below). The ability of ammonia oxidizers to oxidize small organic molecules provides further evidence for a monooxygenase mechanism (see section on CH$_4$ oxidation by ammonia oxidizers).

Cell extracts which oxidize NH$_4^+$ have been prepared from *N. europaea* (146, 149) and *Nitrososphaera oceanus* (166). In the former species, bovine serum albumin, Mg$^{2+}$, or spermine in the presence of adequate concentrations of phosphate or other anions was required for activity (146, 149). In *Nitrososphaera oceanus*, tris (hydroxymethyl)aminomethane, adenosine triphosphate, phosphate, and Mg$^{2+}$ were requirements (166). In a cell-free extract of *N. europaea*, ammonia monooxygenase activity was associated with the membrane fraction (63).

**Source of reducing power.** Reducing power for the monooxygenase is apparently regenerated via NH$_2$OH oxidation (145). Close coupling of NH$_2$OH and NH$_3^+$ oxidation has been found in resting cells (60), spheroplasts (145), and cell-free extracts of *N. europaea* (63). The ammonia monooxygenase of *N. europaea* appears not to utilize NADH as a direct electron donor (148, 149). However, NADH can initiate NH$_3^+$ oxidation in cell-free extracts (148, 149), and it may do this by reducing cytochromes which are required for the reaction (149). Ascorbate is also ineffective as a source of reductant (148). A likelier candidate for the role may be cytochrome c$_{553}$ (157). When added to a membrane fraction obtained from a cell-free extract of *N. europaea* containing NH$_4^+$ and NH$_2$OH oxidizing activities, as well as cytochrome oxidase, cytochrome c$_{553}$ reconstituted NH$_4^+$-linked O$_2$ uptake (147). In this system, Tsang and Suzuki (157) found that NH$_3^+$, NH$_2$OH, and NH$_3$NH$_2$ (a substrate for NH$_2$OH oxidoreductase) all reduce the cytochrome. Reduction by NH$_3^+$ occurs at a slower rate than reduction by NH$_2$OH and NH$_3$NH$_2$, and there is a lag between addition of NH$_3^+$ and reduction of the cytochrome. Reduced cytochrome c$_{553}$ in the presence of membrane fraction is immediately oxidized upon addition of NH$_4^+$ or CO. The extent of the oxidation is dependent on the concentration of NH$_4^+$ or CO. These observations are consistent with a role as electron donor for cytochrome c$_{553}$ (157). The cytochrome...
c.54 obtained from *N. europaea* has a midpoint potential \( E_{m,0} \) of +20 mV (106). It is a protein of \( M_r \), 25,000, which contains four type \( e \) hemes and is located in the periplasmic space (5, 32). Although it may bind substrate (e.g., \( O_2 \)), its properties as determined by optical, Mössbauer, and electron paramagnetic resonance spectroscopies are more suggestive of an electron transfer role (3).

Tri- and tetramethylhydroquinone, two artificial electron donors, can support the activity of ammonia monooxygenase in whole cells of *N. europaea* (133). These compounds may be useful in the eventual purification of the enzyme.

The structure and workings of the monooxygenase itself are not known in any detail. One component appears to be a protein of \( M_r \) 28,000. This was identified by gel electrophoresis and autoradiography of proteins obtained from cells of *N. europaea* which had been incubated in the presence of \(^{14}\text{C} \text{C}_2\text{H}_4\) (73). \(^{14}\text{C} \text{C}_2\text{H}_4\) appears to be a suicide substrate for the ammonia monooxygenase (73), and it is suggested that the attempted oxidation of \(^{14}\text{C} \text{C}_2\text{H}_4\) by the monooxygenase produces a reactive compound which can bind to the enzyme (73).

**Possible role of copper**. That copper plays an important role in \(^{14}\text{NH}_2\) oxidation was first proposed more than 40 years ago by Lees (95). The presence of copper in ammonia monooxygenase is suggested by the ability of agents which bind the metal to block \(^{14}\text{NH}_2\) oxidation in *N. europaea* (62, 97). Furthermore, \(^{14}\text{NH}_2\) oxidation is stimulated by low levels of copper (99, 153), although an absolute requirement for the metal has never been demonstrated. Finally, it has been suggested that ammonia monooxygenase possesses a photosensitive oxygenated state similar to that of other copper-containing enzymes (132).

Expanding on work done by Hooper and Terry (63), Shears and Wood (132) studied the light sensitivity of *N. europaea*. They found that illumination of cells with ultraviolet light leads to inactivation of \(^{14}\text{NH}_2\) but not \(^{14}\text{NH}_3\)OH oxidation. Anaerobiosis, copper-chelating agents, and organic substrates of the monooxygenase tend to mitigate the effect. Difference absorption spectrophotometry of cell suspensions of *Nitrosomonas* spp. shows that, upon exposure to ultraviolet light, there is a bleaching in the 350- to 400-nm region. Two copper proteins, tyrosinase and hemocyanin, have similar spectroscopic characteristics. They are also sensitive to a number of chelating agents which also inhibit ammonia monooxygenase. Based on these findings, Shears and Wood have proposed a catalytic cycle for the monooxygenase (Fig. 2) modeled on the cycle known from tyrosinase (98). Two copper atoms are hypothesized to be at the active site as in tyrosinase. The ammonia monooxygenase could be present in one of three states: reduced (deoxygenated), oxygenated (oxy), and oxidized (met). \( \text{O}_2 \) reacts reversibly with the deoxy form: the Cu(I) atoms are oxidized to Cu(II), while oxygen is bound as peroxide ion (\( \text{O}_2^{2-} \)). The enzyme, now in the activated oxy state, can be inactivated by ultraviolet light at a wavelength of 345 nm. This type of illumination causes the bond between copper and \( \text{O}_2^{2-} \) to break. A release of \( \text{O}_2 \) can radially occur. Organic substrates protect the enzyme from inactivation by rapidly shifting it from the oxy to the met state. Oxymonooxygenase inserts one atom of oxygen into the substrate while the other is reduced to water. The enzyme, now in the met state, can be reduced by a two-electron transfer to the deoxy state. Chelating agents, such as thioourea, protect the monooxygenase by keeping it in this ultraviolet-insensitive form. Thioourea has a high affinity for Cu(I).

![FIG. 2. Proposed catalytic cycle for ammonia monooxygenase, involving oxidation and reduction of a binuclear copper site. (Modified from reference 172 with permission.) U.V., Ultraviolet.](image)

**MMO**

The first step of CH\(_4\) oxidation is: CH\(_4\) + O\(_2\) + NADH \( \rightarrow \) CH\(_3\)OH + H\(_2\)O + NAD\(^+\). The catalyst for the reaction is methane monooxygenase (MMO) (25). In cells of *Methylococcus capsulatus* and *Methylococcus methylaut oxidants*, \(^{18}\text{O}\) from added \(^{16}\text{O}\), but not \(^{18}\text{H} \text{H}_2\text{O}\) was inserted into CH\(_4\), yielding CH\(_3\)\(^{18}\text{OH}\), demonstrating the monooxygenase nature of the reactions (57).

This initial step of CH\(_4\) oxidation has been most investigated in two species of methanotrophs: *Methylococcus capsulatus* Bath and *Methylosinus trichosporum* OB3b. Both of these organisms produce a membrane-bound ("particulate") MMO and a cytoplasmic ("soluble") MMO. Because only the soluble enzymes of the two organisms have been extensively purified and characterized, it is not entirely clear whether soluble and particulate forms are distinct or whether they share components (26, 41). In the case of *Methylococcus capsulatus* Bath, the former possibility appears likeliest (45). At least one other species, *Methylbacterium* sp. strain CRL-26, can apparently express both a soluble and a particulate enzyme (115, 116). *Methylococcus albus* B8 and *Methylocystis parvus* may possess only a particulate form (25). Dalton and Leak (25) suggested that soluble MMOs are restricted to only a few species of methanotrophs. There are no reports of organisms possessing this type of enzyme alone.

**Role of copper**. Copper may exert considerable influence on the nature of the monooxygenase expressed and on cell morphology (14, 26, 122). In both *Methylococcus capsulatus* Bath and *Methylosinus trichosporum* OB3b when Cu is present in limiting amounts, methane oxidation is carried out mostly by the soluble MMO. When Cu is present in nonlimiting quantities, it is the particulate MMO which dominates (14, 26). Cu(I) and Cu(II) inactivate the soluble enzyme of *Methylococcus capsulatus* Bath (44). Copper appears to be a requirement for particulate MMO activity in both this species (122) and *Methylbacterium* sp. strain CRL-26 (115). Increasing Cu(II) availability appears to lead to increased content of intracytoplasmic membranes in cells of *Methylosinus trichosporum* and *Methylococcus capsulatus* (14, 122, 131).

Gel electrophoresis of cell extracts of *Methylococcus capsulatus* Bath grown in continuous culture shows that, as
copper supply increases, polypeptide bands corresponding to soluble MMO disappear while other bands possibly related to particulate MMO increase in intensity (122). A similar pattern was noted in *Methylosinus trichosporium OB3b* (14).

**Source of reductant.** In an extensive review of the evidence, Anthony (5) concluded that, at least in cell-free systems, all forms of MMO can utilize NAD(P)H as electron donor. It has been suggested that, in a variety of methanotrophs, electrons from the oxidation of CH$_3$OH by CH$_3$OH dehydrogenase can be recycled directly to be particulate MMO via one or more electron transport proteins (93, 101, 154; Fig. 1). The existence of such a pathway is not certain. It could explain the greater carbon conversion efficiency of cells expressing the particulate MMO when compared with that of cells expressing the soluble enzyme (94).

**Substrates.** In *Methylococcus capsulatus* and *Methylosinus trichosporium*, the particulate MMO appears to have a narrower range of substrates than the soluble MMO. Both enzymes can oxidize alkenes and alkanes up to pentane (134, 26, 140). But, unlike soluble MMO, particulate MMO cannot oxidize aromatic and alicyclic compounds or higher (> 5) alkanes (14, 140). These differences could be due to dissimilar active sites or to disruption of the activity of the particulate MMO (26). It is interesting that ammonia monoxygenase differs from particulate MMO in that it can oxidize certain aromatic and alicyclic compounds (34, 69).

**Inhibitors.** The MMOs of *Methylococcus capsulatus* and *Methylosinus trichosporium* share similar inhibitor profiles (26, 131, 141, 142). The particulate MMOs are inhibited by a wide variety of compounds including thiol and metal chelators and electron transport inhibitors (26, 131). The soluble MMOs are sensitive only to acetylenic compounds and 8-hydroxyquinoline (141, 142). CH$_3$H$_2$ inhibits both forms of the enzyme and apparently acts as a suicide substrate (121), thus resembling ammonia monoxygenase as mentioned earlier. Because of its inhibitor profile and membrane location, ammonia monoxygenase is thought to more closely resemble the particulate MMO (70).

**Regulation.** The mechanisms of regulation of synthesis of the enzymes are not yet clear. The soluble MMO of *Methylococcus capsulatus* may be induced only in the presence of CH$_4$, since its activity could not be detected in cells grown on CH$_3$OH (122). When the organism was grown on CH$_3$OH, particulate MMO activity was always present, whether or not copper had been added to the growth medium (122). Increasing copper concentrations led to increased particulate enzyme activity (122).

**Soluble MMO.** The soluble MMO of *Methylococcus capsulatus* has been purified and extensively characterized (19, 20, 44, 45, 175). The picture that emerges from these studies is briefly outlined below.

Soluble MMO consists of three components, labeled A, B, and C. Component A is a nonheme iron protein of Mr 210,000 which binds CH$_4$. Recent spectroscopic evidence indicates the presence of a binuclear Fe center in *Methylococcus capsulatus* (40). Evidence regarding the presence of a Cu-Fe-S cluster associated with the Fe cluster in this and other organisms is conflicting (38, 42, 87, 119). Protein A apparently uses reducing equivalents obtained from component C to activate O$_2$, allowing insertion of an oxygen atom into CH$_4$.

Component B is a low-molecular-weight (Mr 15, 700) protein which lacks any prosthetic groups. It converts the MMO from an oxidase to an oxygenase. In its absence, proteins A and C catalyze the 4-e$^-$ reduction of O$_2$ to H$_2$O.

Component B may play a role in controlling cellular levels of NAD. In the presence of protein B, NADH oxidation becomes tightly coupled to CH$_4$ oxidation. The oxidase activity is switched off.

Component C is an iron-sulfur flavoprotein of Mr 42,000. It possesses a single polypeptide chain, one flavin adenine dinucleotide molecule, and an Fe$_2$S$_2$ center. It is the reductase component of the MMO, funnelling electrons from NADH to component A.

Based on steady-state kinetic analyses, Green and Dalton (45) have proposed a mechanism of action for the soluble MMO of *Methylococcus capsulatus*. CH$_3$OH binds to the monoxygenase, followed by NADH which reduces the enzyme. NAD$^+$ is released, and the reduced enzyme combines with O$_2$ to form a ternary complex which breaks down with the release of CH$_3$OH and H$_2$O (a "Bi Uni Uni Bi Ping Pong" mechanism). The $K_m$ values for CH$_3$OH, NADH, and O$_2$ were 3, 55.8, and 16.8 $\mu$M, respectively.

Early efforts to purify the soluble MMO from *Methylosinus trichosporium* failed due to the instability of enzyme preparations (142). However, the ability of purified components B and C from the soluble MMO of *Methylococcus capsulatus* to restore MMO activity to a fraction obtained from a soluble extract of *Methylosinus trichosporium* emphasizes the similarity between the enzymes of the two organisms. A binuclear Fe cluster is also reported for protein A from *M. trichosporium* (42).

A soluble MMO obtained from *Methyllobacterium* sp strain CRL-26 has been partially purified and characterized (113, 115, 120). It consists of three components named A, B, and C by analogy to the soluble MMO of *Methylococcus capsulatus*. Component B of *Methyllobacterium* sp. was not required for MMO activity as measured by the oxidation of propylene to propylene oxide (113, 115). Component A of this organism is an iron protein similar in molecular weight to component A of *Methyllobacterium capsulatus* (113), and it also contains a binuclear Fe-Fe cluster (119). Component C of *Methyllobacterium* sp. is a flavoprotein which is reduced by NADH and the redox properties of which, as determined by optical and electron spin resonance spectra, are very similar to those of the corresponding protein in *Methylococcus capsulatus* (120). Components A and C of *Methyllobacterium* sp. catalyze the epoxidation of propylene (113). An immunoglobulin fraction of antisera raised against these two purified components cross-reacted with certain proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis from crude extracts of *Methyllobacterium organophilum* XX, a facultative type II organism; *Methylococcus* sp. strain CRL-25, a type I organism; and *Methylomomas* sp. strain 761 M, an obligate type I organism (115). All immunological studies cited point to a similarity between the soluble MMOs of what are, in some cases, seemingly very different organisms.

**Particulate MMO.** A particulate MMO was purified from cell extracts of *Methylosinus trichosporium OB3b* (154). Three components were present: a soluble CO-binding cytochrome c (Mr 13,000), a copper protein (Mr, 47,000), and a small protein (Mr 9,400). The function of the different components and the manner in which they interact to
catalyze CH₄ oxidation are not clearly understood. Ascorbate and the methanol-methanol dehydrogenase couple could both donate electrons to the system. However, a later effort to duplicate these results was unsuccessful (131). In fact, on this occasion, only NAD(P)H was found to be an effective electron donor. A recent attempt to purify particulate MMO from Methylosinus trichosporium OB3b failed due to loss of activity of the enzyme upon solubilization (14).

The mechanism of action of the particulate MMOs may be different from that of the soluble MMOs. Joergensen (75) studied the kinetics of O₂ and CH₄ utilization by whole cells of an unidentified type II methanotroph. Growth conditions were such as to ensure that the only MMO expressed would be particulate. Intracellular NADH concentrations were apparently always above saturation. In this system, the kinetics are consistent with a random bireactant mechanism. Either CH₄ or O₂ binds to the enzyme, and this results in a decrease of affinity for the substrate which remains to be bound. The Kₘ,S for O₂ and CH₄ were 0.14 and 1 μM, respectively.

Methane Oxidation by Ammonia Oxidizers

Products of CH₄ oxidation. Cell suspensions of a variety of ammonia oxidizers incubated in the presence of nanomolar concentrations of ¹⁴C CH₄ produced ¹⁴C CO₂ and incorporated ¹⁴C into cellular material with or without NH₄⁺ (79). Species and strains tested included Nitrosomonas oceanica, N. europaea, and NitrosoVectoma marina as well as a number of marine and freshwater isolates, suggesting that the ability to oxidize CH₄ is widespread among the ammonia oxidizers. At the concentration (approximately 10 nM) used to screen their ability to oxidize CH₄, none of the ammonia oxidizers tested incorporated more than 17% of the CH₄ carbon utilized (79). The effects of CH₄ and NH₄⁺ levels on incorporation were examined in N. europaea and NitrosoVectoma oceanica (79). In general, the presence of NH₄⁺ appeared to decrease the proportion of CH₄ carbon entering cell material. This proportion tended to increase with rising CH₄ concentrations. The maximum observed value, 63%, occurred in N. europaea in the absence of NH₄⁺ and in the presence of approximately 120 μM CH₄.

In the above study, the suspending medium was not examined for the presence of small organic intermediates of CH₄ oxidation. However, Hyman and Wood (70) used gas-liquid chromatography to monitor the accumulation of CH₄OH in the supernatant of cell suspensions of N. europaea incubated with CH₄ in concentrations of 200 μM and more. Levels of CH₄OH as high as 500 μM were measured (70). Voysey and Wood (163) used ¹³C nuclear magnetic resonance spectroscopy to follow the fate of ¹³C CH₄OH added to cell suspensions of N. europaea. In the supernatant, the concentration of CH₄OH decreased continuously over the period of incubation, while initially, both HCHO and HCOOH increased, the latter at a slower rate than the former. After several hours, HCHO levels began to decrease while HCOOH continued to increase and did so over the duration of the experiment (30 h). Experimental conditions were such that CO₂ release could not be adequately assessed (163). The distribution of intracellular ¹³C was not monitored.

HCHO accumulation during CH₄OH oxidation may have important consequences since exogenously supplied HCHO strongly inhibits NO₃⁻ production in N. europaea (163). HCHO can react with NH₂OH, an intermediate in NH₄⁺ oxidation, to form formaldoxime: HCHO + NH₂OH → H₃C=N=OH + H₂O. Formaldoxime is a powerful inhibitor of NH₂OH oxidation in N. europaea and may be a substrate for NH₂OH oxidoreductase (163). Cell suspensions of this organism incubated in the presence of ¹³C-formaldoxime produced ¹³C-formate, albeit at slow rates (163). The production of formaldoxime within cells has, however, never been demonstrated (163).

Ward (164) confirmed the production of ¹⁴CO₂ and ¹⁴C-labeled particulate matter from ¹⁴CH₄ in N. oceanica. These products also appeared on incubation of the organism with ¹³CH₄OH (164). Under the single set of incubation conditions used by Ward, approximately equal amounts of CO₂ and particulate carbon were formed with either CH₄ or CH₃OH as substrate.

The findings of Jones and Morita (79), Hyman and Wood (70), Voysey and Wood (163), and Ward (164) appear to contradict previous reports that ammonia oxidizers cannot metabolize CH₄ or CH₃OH. In their study of N. europaea, Hyman and Wood (70) measured an apparent half-saturation constant (Kₘ) for CH₄ oxidation of 2 mM, leading them to suggest that the concentration of CH₄ used by Drozd (33) and Hynes and Knowles (74), 0.1 mM, was too low to allow the detection of oxidation activity. Jones and Morita (79) pointed out that the measurement of O₂ uptake as used by Suzuki et al. (148) and Drozd (33) is relatively insensitive when compared with methods in which radioactively labeled substrates are used.

The pathway of incorporation of CH₄ carbon into cellular material is unknown. HCHO reacts reversibly with proteins and nucleic acids to product additional compounds (117). At the present time, the possibility that at least part of the observed incorporation of CH₄ carbon occurs via this abiotic route cannot be dismissed. In N. europaea, NH₄⁺ oxidation provides energy for CO₂ fixation via the Calvin cycle (124). NH₄⁺ stimulates H¹⁴CO₃⁻ uptake in this organism (124). In N. europaea and NitrosoVectoma oceanica, CH₄ alone did not stimulate H¹⁴CO₃⁻ uptake (79). It should be noted, however, that the maximum concentration of CH₄ utilized in these experiments did not exceed 150 μM.

Attempts to grow ammonia oxidizers on either CH₄ or CH₃OH have failed (79, 163), and 50 mM CH₄ did not enhance the survival of NH₄⁺-starved cells of a marine strain of NitrosoVectoma sp. over a 25-week period (83). It is not clear whether ammonia oxidizers derive any net benefit from CH₄ oxidation.

Involvement of ammonia monooxygenase. Based on their study of N. europaea, Hyman and Wood proposed that ammonia monooxygenase catalyzes CH₄ oxidation to CH₃OH (70). They found that CH₄ inhibited NH₄⁺ oxidation to NO₂⁻ in cell suspensions with a Kₘ of 50 μM. Furthermore, CH₄ alone or with hydrazine, a source of reductant for ammonia monooxygenase (62), stimulated O₂ consumption. The stimulation was abolished in the presence of allylthiourea, an inhibitor of the monooxygenase (62). Finally, the stoichiometry of O₂ consumed to CH₃OH produced was consistent with the following reaction: CH₄ + 2[H] + O₂ → CH₃OH + H₂O. This is analogous to the first step of NH₄⁺ oxidation in ammonia oxidizers (35, 59). Similar evidence has been used to implicate the ammonia monooxygenase of N. europaea in the oxidation of several other organic substrates including bromomethane (71), ethylene (72), benzene (69), and, notably, methanol (163). Other ammonia oxidizers may also utilize their ammonia monooxygenase to catalyze the first step of CH₄ oxidation. Ward (164) estimated a Kₘ of 6.6 μM.
for the inhibition of NH₄⁺ oxidation to NO₂⁻ by CH₄ in cell suspensions of *Nitrosoccus oceanicus*. Consistent with this finding, Jones and Morita (79), working with the same organism, found that NH₄⁺ oxidation to NO₂⁻ was inhibited by >80% in the presence of 119 μM CH₄.

In cell suspensions of *N. europaea*, the rate of CH₃OH production from CH₄ is stimulated by low concentrations of NH₄⁺ and inhibited by higher concentrations (70). A similar effect was noted on the rate of production of CO₂ and cell-C from CH₄ in suspensions of *N. europaea* and *Nitrosooccus oceanicus* (79). The same general pattern also occurred in the oxidation of ethylene and methanol by *N. europaea* cells (72, 163). The effect of NH₄⁺ on the various oxidations listed above can only be understood if it is remembered that, during the oxidation of NH₄⁺ to NO₂⁻, reducing power required by ammonia monoxygenase comes from the further oxidation of NH₂OH to NO₂⁻ (61) and that NH₄⁺ and CH₄ are apparently competing for the same active site on the monoxygenase. It was proposed (70, 72, 163) that addition of small amounts of NH₄⁺ results in the production of reducing power which can be used by ammonia monoxygenase in the oxidation of CH₄ and other organic substrates. Higher NH₄⁺ levels competitively inhibit CH₄ oxidation.

As indicated above, it appears that, in *N. europaea*, CH₃OH can be oxidized to HCHO by ammonia monoxygenase. It has been known for some time that CH₃OH inhibits NH₄⁺ oxidation in *N. europaea* (62, 148, 163). The methanol dehydrogenase of conventional methanotrophs is characterized by a pyruvovinolinequinoine prosthetic group (5). Efforts to detect the presence of this group in cells of *N. europaea* were unsuccessful (163). Reaction steps beyond formaldehyde which lead to the formation of formate, cell-C, and CO₂ remain unknown (163, 164).

CH₄ oxidation rates and affinities. There are few available estimates of CH₄ oxidation rates and affinities for ammonia oxidizers (70, 79). What limited data exist are summarized in Table 2. The maximum methane oxidation rate for an ammonia oxidizer was still five times lower than the lowest value for a methanotroph. The apparent Km (6.6 μM) for CH₄ oxidation by *Nitrosococcus oceanicus* is comparable to that of methanotrophs (Table 2). Although in their work on cell-free extracts of *N. europaea* Suzuki et al. (148) measured a Km of 50 μM for CH₄ inhibition of NH₄⁺ oxidation, in whole cells Hyman and Wood (70) noted a much higher value (2 mM). There may thus be considerable interspecies and interstrain variation in affinity for CH₄ among ammonia oxidizers.

### Ammonia Oxidation by Methanotrophs

There is extensive evidence that many methanotrophs can oxidize NH₄⁺ to NO₂⁻. This activity was exhibited by 21 of 67 enrichment cultures of marine methaner oxidizers (68), and in another study all of the 104 isolates of methanotrophs obtained from a variety of environments produced NO₂⁻ from NH₄⁺ (171). Three species of obligate methanotrophs oxidized NH₄⁺ to NO₂⁻ in the presence of CH₄ (127).

Furthermore, during NH₄⁺ oxidation by some methane oxidizers, including *Methylisotus trichosporium* OB3b, small amounts of N₂O can be evolved (91a, 110, 171, 178). However, attempts to grow methanotrophs on NH₄⁺ in the absence of CH₄ have failed (68). NH₄⁺, although it is oxidized by whole cells of *Methylisotus capsulatus* Bath (24), does not support CO₂ fixation in this organism (139).

### Involvement of MMO. Cell-free extracts of *Methylisotus capsulatus* Bath containing soluble MMO activity oxidized NH₄⁺ to NO₂⁻ (24). During the oxidation, NH₂OH accumulated transiently and was the first detectable product of the reaction. O₂ was a requirement for the reaction. The kinetics of NH₄⁺ oxidation to NH₂OH were complex but conformed to the Michaelis-Menten model when NH₄⁺ concentrations fell between 20 and 200 μM. In this range, the calculated Km was 87 μM at pH 7. CH₄ in all concentrations tested exhibited an inhibitory effect on NH₄⁺ oxidation. NH₂OH, hydroxyquinoline and acetylenc, well-established inhibitors of the soluble MMO of *Methylisotus capsulatus* (141), strongly inhibited NH₄⁺ oxidation to NH₂OH but not NH₂OH oxidation to NO₂⁻. Inhibitors specific to the particulate MMO of the organism (26) had little effect on CH₄ or NH₄⁺ oxidation in the extracts. Based on the above results, it was hypothesized that the soluble MMO of *Methylisotus capsulatus* catalyzes the oxidation of NH₄⁺ to NH₂OH (24).

A cell-free extract of *Methylisotus methanica* containing particulate MMO activity catalyzed NH₄⁺-stimulated NADH oxidation (21). The activity was blocked by a variety of inhibitors, including metal-chelating agents, which were later shown to be specific to particulate MMOs (26, 131). In addition, NH₄⁺ in high concentrations inhibited the oxidation by the extracts of bromomethane (an analog of methane). Regrettably, this study and the one on *Methylisotus capsulatus* extracts cited above are the only investigations in which it can be determined, with some degree of certainty, whether a soluble or a particulate MMO was present in the system carrying out NH₄⁺ oxidation. Further efforts should be made to characterize each type of enzyme with respect to this activity.

Studies on whole cells also appear to implicate MMO in NH₄⁺ oxidation. NH₄⁺ stimulated O₂ uptake by *Methylisotus trichosporium* OB3b (110). The stoichiometry suggested a reaction identical to that catalyzed by ammonia oxidizers: NH₄⁺ + 1.5O₂ → HNO₂ + H₂O. Formate and formaldehyde enhanced NH₄⁺ oxidation, presumably via NADH production. Except for a stimulatory effect at very low concentra-
tions. CH₄ generally inhibited NO₂⁻ production. The stimulation, confirmed in a later study (91a), may have been due to the release of NADH during the oxidation of intermediates in the pathway of CH₄ metabolism. Presumably, CH₄ in higher concentrations excludes NH₄⁺ from the active site of the MMO. In this context, it is interesting to note that, in some methanotrophs, nitrification occurs only in the presence of CH₄ (68, 127).

NH₄⁺ can reduce the growth rate of many methanotrophs by inhibiting CH₄ oxidation (171). The degree of inhibition varies between species and strains. Ferenci et al. (40) noted the competitive nature of the inhibition in cell suspensions of Methylococcus methanica and measured a Ki of 10 mM NH₄⁺ at pH 7. For Methylosinus trichosporium OB3b, Ki values varied between 17.5 mM at pH 6 and 0.2 mM at pH 8 (110). The dependence on pH suggested that, similarly to the ammonia monooxygenase of N. europaea (144), NH₄⁺, not NH₃, might be the substrate for the MMO present in this culture of Methylosinus trichosporium (110). However, in cell suspensions of Methyllococcus capsulatus Bath, the Km for NH₄⁺ oxidation increased from pH 7 to 8 and no activity could be observed at pH 9 (24).

NH₂OH oxidation. In crude extracts of Methyllococcus capsulatus Bath, added NH₂OH was readily oxidized to NO₂⁻ and the rate of reaction was strongly enhanced by the addition of phenazine methosulfate (PMS) (24). The oxidation was not significantly inhibited by the addition of 8-hydroxyquinoline or acetylene, agents which blocked the activity of the MMO present in the extracts (see above). Neither did methanol dehydrogenase appear to be responsible for NH₂OH oxidation. Cyanide, a powerful inhibitor of methanol dehydrogenase in Methyllococcus capsulatus (24) actually stimulated NH₂OH oxidation. The activity was not affected by the addition of CH₃OH. The presence of a hydroxylamine oxidase was therefore postulated (24).

The CH₃OH dehydrogenase and NH₂OH oxidase activities present in soluble extracts of Methyllococcus thermophilus, an obligate methane utilizer, were separated by ion-exchange chromatography (136). The NH₂OH oxidase which was isolated shares a number of characteristics with the corresponding enzyme in ammonia oxidizers: a PMS-stimulated ability to oxidize NH₂OH to NO₂⁻, NH₂OH-cytochrome c oxidoreductase activity, a pH optimum of 9, and similar absorption spectra in the visible region (136). Upon addition of NH₂OH, membrane preparations of Methyllococcus thermophilus consumed O₂, reduced NAD⁺ and cytochrome c, and produced adenosine triphosphate (102). It is not known whether NH₂OH oxidation can contribute significantly to the energy budget of methanotrophs which possess this activity. In fact, it remains unclear whether the ability to oxidize NH₂OH is widespread among methanotrophs.

NH₄⁺ oxidation rates and affinities. The methanotroph’s ability to nitrify is clearly not as developed as that of the ammonia oxidizers. The maximum rate of NO₂⁻ production determined for a methanotroph is still more than 20 times lower than one of the lowest rates reported for an ammonia oxidizer (Table 2). Although there is overlap in the Km values obtained in the two groups of organisms, the lowest Km (NH₄⁺) for ammonia oxidizers is 300 times lower than the lowest Km for methanotrophs (Table 2). Considerable interspecies variation in ability to nitrify appears to occur among the methanotrophs (110, 171).

CO Oxidation by Methanotrophs and Ammonia Oxidizers

A number of methanotrophs (39, 40, 65, 143) and ammonia oxidizers (80, 81, 157) have been found to oxidize CO to CO₂. Methanomonas which exhibit this activity include Methylococcus albus BGR (65), Methylosinus trichosporium OB3b (65), Methylosinus methanica (39), and Methyllococcus capsulatus Bath (143). Nitrifiers which oxidize CO include all of the species tested by Jones and Morita (80), i.e., Nitrosococcus oceanus and various species and strains of Nitrosomonas, including N. europaea.

CO does not appear to support the growth of either methanotrophs or ammonia oxidizers (39, 80). No increase in survival rate was noted for cells of a marine Nitrosomonas starved of NH₄⁺ for 25 weeks in the presence of 50 nM CO (83). In ammonia oxidizers, carbon from CO is incorporated into cell material only in the presence of NH₄⁺ (80). Apparently, NH₄⁺ oxidation provides energy for the Calvin-cycle-mediated fixation of CO₂ produced from the oxidation of CO.

Involvement of methane and ammonia monooxygenases. In the ammonia oxidizers, it appears that CO oxidation is catalyzed by ammonia monooxygenase. Because the studies on CO metabolism by methanotrophs were carried out at a time when the fundamental distinction between particulate and soluble MMOs had not yet been made, there are, in some cases, no clues as to which type of enzyme was responsible for an observed activity. However, the soluble MMO of Methyllococcus capsulatus Bath was shown to carry out CO oxidation (128). Furthermore, the extreme sensitivity to cyanide exhibited by the MMO extracts of Methylococcus methanica used by Ferenci et al. (40) suggested that the enzyme present was of the particulate type (70). Further evidence is required to clarify the issue.

In both methanotrophs and ammonia oxidizers, CO stimulates O₂ uptake and oxidation of sources of reducing power for the monooxygenases (65, 157). Stoichiometries are as expected for reactions catalyzed by mixed-function oxygenases: CO + AH + O₂ → CO₂ + A + H₂O. In some cases (39, 40, 143, 157), CO is oxidized only in the presence of a substrate which can directly, or via its own oxidation, provide reducing power to the monooxygenase. CO oxidation was inhibited by cyanide (39). The action of CO is apparently not at the level of cytochrome oxidase since the affinity of this enzyme for CO is apparently much less (6, 176).

NH₄⁺ at 3.6 mM inhibits CO oxidation in two species of Nitrosomonas, and Nitrosococcus oceanus was inhibited by >50% (81). A significant inhibitory effect was seen at NH₄⁺ levels as low as 70 μM (81). NH₄⁺ inhibited CO oxidation by Methylosinus methanica with a Ki of 12 mM (40). CO is a powerful inhibitor of MMO with a Ki of 0.2 mM (91a). CO oxidation in ammonia oxidizers is blocked by numerous inhibitors of nitrification (82). When cyanide is added in concentrations low enough not to affect the terminal oxidases, it blocks CO oxidation in Methylococcus methanica (40).

CO oxidation rates and affinities. Maximum rates of CO oxidation by methanotrophs show considerable overlap with those of ammonia oxidizers (Table 2). Both groups of organisms can oxidize CO when it is present in concentra-
Inhibitors of Methanotrophs and Ammonia Oxidizers

Inhibitors of NH₄⁺ Oxidation in the Study of Nitrification

The activity of chemoautotrophic ammonia oxidizers is specifically blocked by numerous compounds (62), and a number of these have received considerable attention for their ability to inhibit nitrification in agricultural soils (135). Inhibitors have also been used to estimate nitrifier activity in the environment (see references below). The method can be summarized as follows. A sample of soil, sediment, or water is divided equally between two sets of replicates. One set receives inhibitor dissolved in a carrier; the other receives an equal amount of carrier alone. The disappearance of endogenous NH₄⁺ (49, 50, 54, 90) and O₂ (90) or the appearance of NO₃ (16, 48, 49, 50, 54, 90, 168, 179, 180) can then be followed. Alternatively, a substrate which can be metabolized by chemoautotrophic ammonia oxidizers is added and its utilization is monitored. Substrates used have included NH₄⁺ (50, 180), ¹⁴CO₂ (10, 17, 37, 48, 90, 137, 160, 161, 162), ¹⁴CO and ¹⁴CH₄ (84). Compared with nonradioactive compounds, radiolabeled ones provide greater sensitivity and allow shorter (<24-h) incubation times (84, 152).

When ¹⁴CO₂ is used, a ratio of nitrogen oxidized to carbon fixed is required to estimate rates of nitrification (10). An average ratio obtained from pure-culture experiments is usually applied. Molar ratios fall between 3 and 42 in ammonia oxidizers and 25 and 132 in nitrifiers (43, 85). Because of these wide variations, the validity of the ¹⁴CO₂ uptake method as a direct measure of nitrification has been questioned (48, 85, 152).

The derivation of estimates of nitrification from ¹⁴CO₂ uptake measurements is even more problematic. NH₄⁺ depresses CO oxidation in nitrifiers (80, 81). As noted above, this is because both NH₄⁺ and CO are substrates for ammonia monoxygenase (157). Nitrification (2-chloro-6-trichloromethyl-pyridine) has been the inhibitor most often favored by investigators (152), although allylthiourea (49, 50) and thiourea (180) have also been used. These compounds block the NH₄⁺-to-NH₂OH step of ammonia oxidation (15). The mode of action of nitrification inhibitors is further discussed in the next section. Various problems associated with the use of these compounds in field studies are outlined in the following paragraphs.

Inhibitors may not act immediately upon addition. Powell and Prosser (118) noted a 10-h lag between amendment with 6-chloropicolinic acid (2 μM) and slowing of growth in a culture of N. europaea. Although eventually effective, low nitrification concentrations (<0.9 μM) did not immediately block NH₄⁺ oxidation by various strains of nitrifiers (7). These problems may be alleviated by using higher levels of inhibitor (7) or by preincubating samples with inhibitor before substrate addition (160–162, 168).

Not all species and strains of nitrifiers are similarly affected by a given compound. For seven strains belonging to three separate genera of ammonia oxidizers, nitrification concentrations required for complete inhibition ranged from 0.9 to 43 μM (7). In a study of nine inhibitors, Jones and Morita (82) noted several differences in susceptibility among three species of nitrifiers. For example, 3-aminotriazole in a concentration of 1.19 mM completely inhibited NH₄⁺ oxidation by two species of Nitrosomonas but left Nitrosococcus oceanus unaffected. At this concentration most of the other inhibitors tested decreased activity by >90%. The level of nitrapyrin originally recommended for use in the ¹⁴CO₂ uptake assay was 22 μM (10). It was suggested that higher amounts would affect other microorganisms.

The extent of inhibition may depend on the substrate added. With many inhibitors, it appears that NH₄⁺ and CH₄ oxidation are more affected than is the oxidation of CO (82). This could be related to the high affinity of ammonia monoxygenase for CO.

Nitrification can be lost through volatilization or degradation (125). Both of these processes are probably of little importance in short-term (<24-h) incubations or in assays on sealed water samples. No loss in effectiveness was seen in sealed lakewater samples incubated for more than 40 days (90). In sediments, the situation appears to be different. Hall (49) noted relief of inhibition in freshwater sediment after 24 h of incubation. To prevent nitrification from occurring in samples of marine sediment, Henriksen (54) found it necessary to add fresh nitrapyrin at intervals of 1 or 2 days. In the environment, nitrapyrin is hydrolyzed to 6-chloropicolinic acid (12, 125). Although this compound appears to inhibit nitrification (118), at equal concentration it is less effective than nitrapyrin (12, 92, 118). Little is known of the fate of the thioureas in the environment.

The influence of various physicochemical characteristics of soil on the effectiveness of nitrapyrin has been extensively studied (135). The effects of these parameters on the thioureas are poorly documented. It appears that, at least in some environments, these compounds may be less effective than nitrapyrin. In pure cultures of N. europaea, allylthiourea and nitrapyrin completely block NH₄⁺ oxidation at approximately the same concentration: 5 μM (15). But, when added to soils in a concentration of 10 mg kg⁻¹, thiourea and allylthiourea had no effect on nitrification while, at an equivalent level, nitrapyrin significantly inhibited this activity (13).

Nitrification inhibitors can influence other biological processes. The biological effects of nitrapyrin are summarized by Blangen and Kerkhoff (135). The possible effects of the thioureas have received little attention. Allylthiourea and allylthiourea inhibit CH₄ oxidation by methanotrophs (66), as does nitrapyrin (155, 156).

Because nitrapyrin is only sparingly soluble in water, it is usually dissolved in an organic solvent before addition to samples. The carrier itself may have an inhibitory effect (12). Ethanol has been used in a number of studies (10, 17, 48, 137, 160). In a concentration of 90 mM, it blocks NH₄⁺ oxidation by N. europaea (62). Hall (48) found that 1.2 mM ethanol had no effect on nitrifiers. In most of the studies cited above, it appears that ethanol was near or below this level. Dimethyl sulfoxide has also been used as a carrier (84, 90). When present at 1 ml liter⁻¹, it had no effect on NH₄⁺, CH₄, and CO oxidation by various nitrifiers (82). Potential problems with the carrier are avoided by using allylthiourea and thiourea, which are soluble in water.

Studies designed to compare estimates of nitrification obtained with different inhibitors are almost nonexistent. Hall (49) found that measurements of nitrification in freshwater sediments, using allylthiourea, were higher than those made with nitrapyrin. The author proposed that volatilization of the latter compound may have been an important
factor. Billen (10) found nitrapyrin to be more satisfactory for determining $^{14}$CO$_2$ uptake by nitrifiers than either thio- 
urea or hydrazine, although no specific data in support of 
this were provided.

Despite all of the drawbacks listed above, inhibitor studies 
have yielded estimates of nitrification which agree within an 
order of magnitude with measurements made by using other 
approaches (10, 17, 37, 48, 90, 137). This was the level of 
precision originally claimed for the method (10).

Since many of the inhibitors used in studies of nitrifica-
tion also inhibit the oxidation of NH$_4^+$ and CH$_4$ by methan-
otrophs, as noted above, it is often not possible to conclude 
onequivocally that observed nitrification is due to the am-
monia oxidizers alone. A differential inhibitor would there-
fore be useful in ecological studies.

Differential Inhibition of Methanotrophs and 
Ammonia Oxidizers

An inhibitor specific to either methanotrophs or ammonia 
oxidizers would be useful for distinguishing which of these 
organisms is responsible for NH$_4^+$, CH$_4$, or CO oxidation in 
environments in which such activities occur (129). As noted 
above, nitrapyrin, thiourea, and alIyIthiourea all inhibit 
methanotrophs. Topp and Knowles (155) suggested that 
measurements of nitrapyrin-sensitive $^{14}$CO$_2$ uptake in sam-
iples in which methanotrophs were present might yield over-
estimates of nitrifier activity. A similar problem exists with 
nitrapyrin-sensitive $^{14}$CO uptake (84).

Table 3 lists 35 agents which have been tested on metha-
notrophs and nitrifiers. The compounds inhibit one or the 
other or both types of organisms. Where pure culture data 
were not available, results from sewage sludge or soil studies 
are included. With very few exceptions, the inhibitors in 
Table 3 act on both groups of organisms.

In most of the studies of methanotrophs cited in Table 3, 
the nature of the monooxygenase, either particulate or 
soluble, was not monitored. Given what is now known about 
the inhibitor profiles of the two forms of monooxygenase 
(26), it appears that in a majority of the organisms investi-
gated it was a particular enzyme which was responsible for 
most of the observed methane oxidation. The results ob-
tained on Methyllobacter capsulatus Bath (24, 141) clearly 
do not fit this pattern. At the time of these studies, the 
monooxygenase under investigation was known to be solu-
ble. With the possible exception of KCN, the inhibitor profile of 
Methyllobacter capsulatus Bath obtained by the 
investigators appears to be that which has now been recog-
nized to be typical of the soluble MMOs (26).

Some of the inhibitors in Table 3 have more than one 
effect. For example, KCN blocks both cytochrome oxidase 
and particulate MMO (75). But most of the compounds 
listed, when added in low enough concentration, affect 
mainly the CH$_4$-to-CH$_3$OH or NH$_4^+$-to-NH$_3$OH steps in 
the sequence of oxidations carried out by methanotrophs and 
ammonia oxidizers.

A number of agents in Table 3 are thought to act by 
binding a metal (in all likelihood, Cu) which is essential for 
the activity of the particulate MMOs and ammonia monoox-
genase. Such inhibitors would include those listed as "che-
lateI agents" and "monodentate ligands" as well as ni-
trapyrin and histidine (15, 62, 97, 141). Lees (97) exam-
ined the effect of eight different chelating agents on the 
oxidation of NH$_4^+$ by Nitrosomonas spp. That the slope of 
the curve relating inhibitor concentration to the percent 
inhibition was the same for all the compound tested sug-
gested that a common mechanism was involved. Inhibitor 
strength was closely related to the ability to chelate. Finally, 
in the cases of allylthiourea, guanidine, and L-histidine, 
the inhibition was noncompetitive.

Involvement of copper in CH$_4$ and NH$_4^+$ oxidation has 
been deduced in part from the high affinities of inhibiting 
chelators for this metal (66, 97). Hooper and Terry (62) found 
that, in N. europaea, the inhibition of NH$_4^+$ oxidation 
caused by KCN or diethylthiocarbamate could be re-
versed, at least in part, if cells were treated with a solution 
containing Cu$^{2+}$. This same ion relieved the inhibition of 
CH$_4$ oxidation by 8-hydroxyquinoline, α-dipryridyl, and 
thiosemicarbazide in cells of Methylosinus trichosporum 
OB3b (66). Cu$^{2+}$ did not restore NH$_4^+$ oxidation in N. 
europaea or CH$_4$ oxidation in Methylosinus trichosporum 
OB3b when these organisms were exposed to allylthiourea 
(62, 66). This may be because allylthiourea has a higher 
affinity for the Cu$^{2+}$ ion (66). Addition of Cu$^{2+}$ to cell 
suspensions of N. europaea treated with nitrapyrin restored 
the ammonia-oxidizing capability (15). Similar treatment did 
not relieve the inhibition of CH$_4$ oxidation by nitrapyrin in 
Methylosinus trichosporum OB3b (156). This suggests that 
nitrapyrin might function differently in methanotrophs and 
ammonia oxidizers.

It is possible that pyridine and its derivatives (Table 3) 
could act by binding key copper atoms, but a more direct 
interaction with the active site of the monooxygenases is 
suggested by the following findings. Benzene, an analog of 
pyridine, is actively oxidized by cell suspensions of N. 
europaea (69). Pyridine is a substrate for soluble MMO (23) 
and inhibits particulate MMO (66). However, particulate 
MMO does not oxidize benzene or other aromatic com-
pounds (14). Because of its resemblance to benzene and 
phenol, aniline, which inhibits NH$_4^+$ oxidation in N. eu-
ropaea (153), may also be a substrate for ammonia mono-
oxegenase.

The target of some of the inhibitors may not be the 
ammonia monooxygenase or MMO. It has been suggested 
that, in methanotrophs, chelating agents may interact with 
an electron transport protein closely associated with the 
particulate MMO (26). Bhandari and Nicholas (9) proposed 
that NaN$_3$, diethyldithiocarbamate, thiourea, nitrapyrin, and 
2-trichloromethyl pyridine inhibit a copper-dependent trans-
locase in ammonia oxidizers. However, the evidence ad-
vanced by these authors is also consistent with direct 
inhibition of NH$_4^+$ oxidation (88). Furthermore, CH$_4$ and 
CO oxidation in ammonia oxidizers is also inhibited by 
nitrapyrin (82). Since CO and CH$_4$ must enter the cells by 
passive diffusion, it seems likely that the inhibitors listed 
above function on the oxidative machinery of ammonia 
oxidizers, not NH$_4^+$ transport.

C$_2$H$_2$ is a suicide substrate for both ammonia monooxy-
genase (73) and the MMO of Methyllobacter capsulatus 
(121). This may be the mechanism underlying the action of 
alI acetylenic compounds which block CH$_4$ and NH$_4^+$ ox-
idation. The cytochrome P-450 monooxygenase of hepatic 
microsomes is subject to inhibition by a wide variety of 
acetylenic compounds (111). Here, too, a suicide mechanism 
is thought to be involved. Certain P-450 inhibitors have been 
shown to inhibit CH$_4$ and NH$_4^+$ oxidation, although high 
concentrations are required (Table 3).

Inhibition of the MMO of Methyllobacter capsulatus 
(strain unspecified) by threonine and phenylalanine is be-
lieved to be due to end product inhibition of enzymes 
essential for the synthesis of certain key amino acids (36).
### TABLE 3. Inhibitors of methanotrophs and ammonia oxidizers

| Name of Inhibitor | Formula | Mol wt (10^3) | State at 25°C | Minimum inhibitory concn (μM) | Effect on methanol oxidation | Minimum inhibitory concn (μM) | Effect on ammonia oxidation | References*’ |
|-------------------|---------|---------------|---------------|-------------------------------|-----------------------------|-------------------------------|-------------------------------|----------------|
| Chelating agent   |         |               |               |                               |                             |                               |                               |               |
| Thiosemicarbazide (hydrazinecarbothiamide) | NH₂CSNHN₂ | 91.14 S | 100’ | No | 10 | No | 62, 66, 114, 141 |
| Diethyl dithiocarbamate (diethylcarbamodithioic acid sodium salt) | (C₂H₅₂NCS₂Na | 171.27 S | 100 | No | 10 | No | 9, 62, 97, 141, 151 |
| Thiourea | H₂NCSN₂ | 76.12 S | 1,000 | No | 37.4 | No | 62, 66, 97, 158 |
| Potassium ethyl xanthate (potassium xanthogenate) | C₂H₅OCS₂K | 160.30 S | 100’ | No | 9 | ND | 9, 64, 97, 151 |
| Allylthiourea [12-propenylthiourea] | CH₂=CHCH₂NHCSN₂ | 116.19 S | 10 | No | 1 | No | 15, 58, 62, 66, 70, 82, 97 |
| Thioacetamide (1,10-phenanthroline, 1,10-phenanthroline) | CH₂CSN₂ | 75.00 S | 100’ | No | 10 | No | 153 |
| 8-Hydroxyquinoline (8-quinolone) | NH₂CNH₂ | 145.16 S | 100 | No | 10 | No | 62, 66, 114, 141 |
| α,β-Dipyridyl (2,2’-bipyridine) | | 158.18 S | 100’ | No | 100 | No | 62, 64, 114, 141 |
| Aminoguanidine (hydrazinecarboximidamide) | NH₂CNHNH₂ | 74.09 S | 10,000 | No | 1,000 | No | 62, 66 |
| Monodentate ligand |         |               |               |                               |                             |                               |                               |               |
| Sodium azide | NaN₃ | 65.02 S | 1,000 | No | 1,000 | Yes (inhibition) | 9, 62, 66 |
| Potassium cyanide | KCN | 65.11 S | Kᵣ = 0.3 | No | 5 | No | 62, 64, 66, 75, 76, 114, 141 |
| 3-Aminotriazole | | 84.08 S | 10,000 | No | 1,000’ | Yes (stimulation) | 62, 66, 82 |
| Pyridine compound |         |               |               |                               |                             |                               |                               |               |
| Pyridine | | 79.10 L | 1,000 | No | 1,264’ | Yes | 66, 129, 138, 141 |
| 6-Chloro-2-picoline (2-chloro-6-ethyl-pyridine) | | 127.57 S | 430’ | ND | >10 | ND | 129, 156 |
| Nitrapyrin (2-chloro-6-trichloromethyl pyridine) | | 230.88 S | 9 | No | 1 | No | 9, 15, 82, 129, 155 |
| Picolinic acid (2-pyridine carboxylic acid) | | 123.11 S | 10 | ND | >10 | ND | 129 |
| Chloropicolinic acid | | 158.56 S | >10 | ND | 2.2” | ND | 118, 129 |
| 1-Isonicotinyl-2-isopropyl hydrazide [4-pyridine carboxylic acid 2-(1-methyl-ethyl) hydrazine] | | 179.22 S | >1,000” | ND | 10,000 | No | 21, 62 |
| Acetylenic compound |         |               |               |                               |                             |                               |                               |               |
| Acetylene (ethyne) | HC=CH | 26.02 G | 1,000 | No | 0.25 (Kᵣ) | No | 30, 74, 82, 141 |
| Propyne | HC=CH₂ | 42.08 G | 1,000 | No | 10 Pa” | ND | 103, 141 |
| 2-Butyne | CH₃C=CH₃ | 54.09 G | 1,000 | No | >10 Pa” | ND | 103, 141 |

Continued on following page
TABLE 3—Continued

| Inhibitor | Name<sup>a</sup> | Formula | Mol wt (10<sup>3</sup>) | State at 25°C | Minimum inhibitory concn (μM)<sup>b</sup> | Effect on methanotrophs | Minimum inhibitory concn (μM)<sup>c</sup> | Effect on hydroxylamine oxidation | Reference(s)<sup>d</sup> |
|-----------|------------------|---------|-------------------------|--------------|---------------------------------|------------------------|---------------------------------|---------------------------------|------------------|
| 1-Butyne  | Propargylamine (2-propyn-1-amine) | HC=C=CH<sub>2</sub> | 54.09 L 10.000 No | 4.000 | No | 10 Pa | 1.000 μmol kg<sup>-1</sup> | ND | 103, 141 |
| 2-Propyn-1-ol<sup>e</sup> | HC=C=CH<sub>2</sub>OH | 56.06 S >1.000 No | 4.000 | No | 1.000 μmol kg<sup>-1</sup> | ND | 103, 141 |
| Amino acid | 1-Histidine | NH<sub>2</sub>-CH-COOH | 155.16 S 10.000 No | 5.000 | ND | 18, 36, 66, 97 |
| | L-Threonine | CH<sub>3</sub>CHNH<sub>2</sub> | 119.12 S 1.000<sup>f</sup> ND | >5.000<sup>g</sup> | ND | 18, 36, 97 |
| | L-Phenylalanine | (CH<sub>3</sub>CH)CH<sub>2</sub>NH<sub>2</sub> | 165.19 S 1.000<sup>f</sup> ND | >5.000<sup>g</sup> | ND | 36, 97 |
| Cytochrome P-450 inhibitor | SKF 525-A (β-diethylamino ethylidiphenylpropiol acetate) | C<sub>41</sub>H<sub>42</sub>Cl<sub>2</sub>O<sub>3</sub>N<sub>4</sub> | 353.5 S 5.000 No | 50<sup>h</sup> | No | 62, 66 |
| Lilly 18947 (2,4-dichloro-6-phenyl phenoxethyl-dietfylamine) | C<sub>18</sub>H<sub>16</sub>Cl<sub>2</sub>NO<sub>2</sub> | 338.28 S 10.000 No | 100 | No | 62, 66 |
| Lilly 53325 [2,4-dichloro-6-phenyl phenoxethylamine hydrobromide] | C<sub>18</sub>H<sub>16</sub>BrO<sub>3</sub>N<sub>2</sub> | 363.08 S 5.000 No | 100 | No | 62, 66 |

- Miscellaneous compounds

| Inhibitor | Name | Formula | Mol wt (10<sup>3</sup>) | State at 25°C | Minimum inhibitory concn (μM)<sup>b</sup> | Effect on methanotrophs | Minimum inhibitory concn (μM)<sup>c</sup> | Effect on hydroxylamine oxidation | Reference(s)<sup>d</sup> |
|-----------|------|---------|-------------------------|--------------|---------------------------------|------------------------|---------------------------------|---------------------------------|------------------|
| Hydrazine | H<sub>2</sub>NNH<sub>2</sub> | 32.05 L >2.000 Yes | 2.000 | Yes | 58, 62, 66 |
| Aniline | C<sub>6</sub>H<sub>5</sub>NH<sub>2</sub> | 93.12 L >100 No | 100 | ND | 141, 153 |
| Hydroxylamine | NH<sub>2</sub>OH | 33.03 S 100 No | 2.000 | No | 2, 66 |

<sup>a</sup> All inhibitor studies on pure cultures are listed. When such data were not available, results from soil experiments are included.

<sup>b</sup> The commonly used appellation is given. When applicable, the correct terminology is given in parentheses.

<sup>c</sup> S. Solid; L, liquid. G, gas.

<sup>d</sup> The minimum tested concentration found to inhibit methane/ammonium oxidation by >70% in at least one species of methanotroph or ammonia oxidizer.

<sup>e</sup> An inhibitor is judged to show an effect if the rate of oxidation in its presence differed from that in the control without inhibitor by more than 30%. ND, Not determined.

<sup>f</sup> Does not inhibit Methylococcus capsulatus Bath. See text for interpretation.

<sup>g</sup> Stirling and Dalton (141) reported a 40% reduction in the rate of CH<sub>3</sub>OH oxidation by whole cells of Methylococcus capsulatus Bath in the presence of 100 μM diethylthiocarbamate. In cell-free extracts of this species (124) and whole cells of other species (see references 66 and 141), CH<sub>3</sub>OH oxidation was not affected by the inhibitor.

<sup>h</sup> Inhibition of NH<sub>4</sub><sup>+</sup> oxidation was 65%.

<sup>i</sup> Of three ammonia oxidizer species tested, only one, Nitrosoccus oceanus, showed no inhibition of NH<sub>4</sub><sup>+</sup>, CO<sub>2</sub>, or CH<sub>4</sub> oxidation in the presence of 1,190 μM 3-aminoantrolide (82).

<sup>j</sup> CH<sub>4</sub> oxidation was inhibited by 64%. See the discussion concerning pyridine in the text.

<sup>k</sup> Inhibition of NH<sub>4</sub><sup>+</sup> oxidation in sludge was approximately 50% (138).

<sup<l> No inhibition of NH<sub>4</sub><sup>+</sup> oxidation at 10 μM (129).

<sup>m</sup> Inhibition of NH<sub>4</sub><sup>+</sup> oxidation was 60% (118).

<sup>n</sup> CH<sub>4</sub> oxidation in cell-free extracts (23).

<sup>o</sup> Inhibition of nitrification in soils.

<sup>p</sup> 2-Propyn-1-ol is a substrate for methanol dehydrogenase in Methylococcus capsulatus Bath (141). At 100 μM, the compound inhibits methane oxidation by 40%.

<sup>q</sup> Inhibition of exponential growth over a 6- to 12-h period (136).

<sup>r</sup> No inhibition of short-term (3-h) NO<sub>3</sub><sup>-</sup> production in concentrated cell suspensions of Nitrosomonas spp. (97). Accumulation of NO<sub>3</sub><sup>-</sup> over a 3-day period was inhibited by >70% in cultures of N. europae ammended with amino acid in a concentration of 4 mg liter<sup>-1</sup> (118).
This leads to an imbalance in amino acid metabolism. Threonine and lysine inhibit NO$_3^-$ production in growing cultures of *N. europaea* (18) but the inhibition is, apparently, not immediate (97). Again, end product inhibition is suggested.

Hydrazine blocks NO$_3^-$ production by *Nitrosomonas* spp. by acting as an alternative substrate for hydroxylamine oxidoreductase (58). The oxidation of hydrazine can actually stimulate the action of ammonia monoxygenase by providing it with reducing power (70, 174).

In high enough concentration, hydroxylamine inhibits its own production in cells of *N. europaea* (2). Hyman and Wood (70) proposed that this inhibition serves a regulatory purpose by helping to prevent accumulation of NH$_2$OH in cells.

Salvas and Taylor (129) showed that picolinic acid in a concentration of 10 μM blocks NH$_3^+$ oxidation by *Methylotus trichosporium* OB3b but not in two species of marine ammonia oxidizers. Table 3 suggests that other compounds may selectively inhibit one or the other of the groups of organisms. More specifically, the possible use of certain acetylenic compounds and pyridine derivatives should be examined.

### CONCLUDING REMARKS

The question of the ecological roles of the methanotrophs and nitrifiers in the oxidation of CH$_4$, NH$_3^+$, and CO$_2$ in nature remains perplexing. It will be resolved only by detailed physiological and biochemical studies of the mechanisms involved, coupled with the use of the more sophisticated ecological approaches. It is hoped that this review will provide some incentive for such studies.

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