Methane oxidation by *Nitrosomonas europaea*

Michael R. HYMAN and Paul M. WOOD*
Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.

(Received 19 August 1982/Accepted 20 December 1982)

1. Methane inhibited NH$_4^+$ utilization by *Nitrosomonas europaea* with a $K_i$ of 2 mM. O$_2$ consumption was not inhibited. 2. In the absence of NH$_4^+$, or with hydrazine as reductant, methane caused nearly a doubling in the rate of O$_2$ uptake. The stimulation was abolished by allylthiourea, a sensitive inhibitor of the oxidation of NH$_4^+$. 3. Analysis revealed that methanol was being formed in these experiments, with yields approaching 1 mol of methanol per mol of O$_2$ consumed under certain conditions. 4. When cells were incubated with NH$_4^+$ under an atmosphere of 50% methane, 500µM-methanol was generated in 1 h. 5. It is concluded that methane is an alternative substrate for the NH$_3$-oxidizing enzyme (ammonia mono-oxygenase), albeit with a much lower affinity than for methane mono-oxygenase of methanotrophs.

The oxidation of NH$_4^+$ to NO$_3^-$ forms one stage in the biological nitrogen cycle. It is brought about for the most part by autotrophic bacteria, typified by *Nitrosomonas europaea*. In chemical terms the conversion is quite a complex process; the NH$_4^+$ ion must lose 6 reducing equivalents, and two N–O bonds must be formed. Hofman & Lees (1953) demonstrated that hydroxylamine is an intermediate, a conclusion confirmed by later studies (Hollocher et al., 1981). The work of Hollocher et al. (1981) and Suzuki et al. (1974) provides good evidence that the first step involves incorporation of one oxygen atom from molecular O$_2$ and has uncharged NH$_3$ as substrate:

$$\text{NH}_3 + 2[\text{H}] + \text{O}_2 \rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O} \quad (1)$$

The enzyme catalysing reaction (1) has been given many names, the most appropriate being ammonia mono-oxygenase. Ammonia mono-oxygenase is fractionated with cell membranes; it is likely to contain copper, but nothing more is known of its constitution (Hooper, 1978). The immediate H-atom donor is unknown. Up to now, NH$_3$ has been regarded as the unique substrate for this hydroxylating system. The present paper provides evidence that methane is also a substrate, in what we believe to be an analogous reaction:

$$\text{CH}_4 + 2[\text{H}] + \text{O}_2 \rightarrow \text{CH}_3\text{OH} + \text{H}_2\text{O} \quad (2)$$

Methane was chosen for study because it is the parent substrate of a closely related enzyme, is biochemically very inert, and can occur naturally along with NH$_4^+$ and O$_2$.

During normal growth on ammonium salts the reducing equivalents required by reaction (1) will be derived from oxidation of NH$_2$OH, with exactly 2e$^-$ returned per molecule of NH$_2$OH when the NH$_2$OH concentration is in steady state. By contrast, reaction (2) is expected to result in a net drain of reducing equivalents from the cell; methanol dehydrogenase has not been reported for *N. europaea*, and we have found no evidence for its presence. Thus in experiments with methane the question of a source of reducing equivalents is an important one, and various alternatives are described below.

The experimental results in the literature for *N. europaea* that come closest to those reported in the present paper are from Suzuki et al. (1976). They found that the rate of NADH oxidation by a membrane fraction was stimulated about 4-fold when NH$_3$, methane, CO or methanol was added. Nevertheless they reported 'neither CH$_4$, CO nor CH$_3$OH was oxidized by *Nitrosomonas* cells or extracts'. No analytical details were given in support of this statement, and no explanation was put forward as to how these molecules could stimulate an oxidation without being chemically altered themselves. Drozd (1976) and Hynes & Knowles (1982) tested for methane oxidation, with negative results. Both used what was probably too low a concentration, 100µM. Drozd (1976) used as reductant 1 mM-NH$_2$OH, which in our experience inhibits the mono-oxygenase, and the high NH$_4^+$ concentration used by Hynes & Knowles (1982) would likewise be counter-productive. These points are explained below.

**Experimental**

*Nitrosomonas europaea* (A.T.C.C. 19178) was kindly supplied by Dr. N. Walker (Rothamsted
Experimental Station, Herts., U.K.). It was grown at 28°C in semi-batch culture in a 10-litre fermenter fitted with pH-stat control (LH Engineering, Stoke Poges, Bucks., U.K.). The growth medium was based on that described by Skinner & Walker (1961) and contained, per litre, 3.3g of \((NH_4)_2SO_4\), 0.53g of \(KH_2PO_4\), 67mg of \(MgSO_4\cdot7H_2O\), 67mg of \(CaCl_2\cdot2H_2O\), plus 0.67mg of Fe added as an equimolar mixture of \(FeSO_4\) and EDTA. The pH was adjusted to 7.8 before inoculation and maintained at this value by addition of autoclaved 5% (w/v) \(Na_2CO_3\). Cells were harvested at centrifugation at 4°C (28,000g for 40 min), by resuspension in medium containing 50mm-sodium phosphate buffer, pH 7.5, 2mm-\(MgCl_2\) and 0.15mm-\((NH_4)_2SO_4\) and re-centrifugation (38,000g for 20 min). The pellet was resuspended in 50mm-sodium phosphate buffer, pH 7.7, containing 2mm-\(MgCl_2\) at 0.2g wet wt./ml, stored at 0°C and used within 24 h.

O\(_2\) measurements made use of a Clark-type oxygen electrode (Hansatech, King’s Lynn, Norfolk, U.K.). In experiments with simultaneous monitoring of NH\(_4\)\(^+\) a wider model was used, with an internal diameter of 16mm (Rank, Bottisham, Cambridge, U.K.). Measurements of NH\(_4\)\(^+\) were made with a Philips ion-selective electrode (Pye-Unicam, Cambridge, U.K.). This was mounted in the oxygen-electrode chamber with a nylon sleeve to minimize contact with atmospheric O\(_2\). The sleeve had a vertical slit to allow additions with a micro-syringe, and through this slit passed a length of cannula tubing filled with 1% agarose gel plus 0.1m-NaNO\(_3\). This tubing also made contact with a standard calomel reference electrode (Philips RH 44/2-SD/1) via a small reservoir of 1m-KNO\(_3\). The voltage between the NH\(_4\)\(^+\) and reference electrodes was measured with a Pye-Unicam pH/mV-meter and displayed along with the oxygen-electrode reading on a two-pen chart recorder. A prior titration showed that for \([NH_4]^+\) > 100µM the response fitted closely to \(E = \text{constant} + 570 \log [NH_4^+]\), for \(E\) in mV.

Methanol was detected by g.l.c. with a Perkin-Elmer F-11 chromatograph (Perkin-Elmer, Beaconsfield, Bucks., U.K.) fitted with a flame ionization detector and a 1m column of Tenax GC (60-80 mesh). A sample volume of 5µl was used and an N\(_2\) flow of 20ml/min. The injection port was maintained at 200°C and the column at 70°C.

All chemicals were research-grade products of BDH Chemicals, Poole, Dorset, U.K., except for methane (CP grade; British Oxygen Co., London, U.K.) and allylthiourea (Sigma Chemical Co., Poole, Dorset, U.K.). A Heps [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer was used for experiments with the NH\(_4\)\(^+\) electrode and a sodium phosphate buffer for all other purposes. The reason for this difference was that Hepes buffers gave a distinct methanol peak when injected into the g.l.c. system, whereas for work with the NH\(_4\)\(^+\) electrode it was desirable to use a relatively low [Na\(^+\)] (selectivity coefficients states as NH\(_4\)\(^+\) = 1, Na\(^+\) = 0.002; K\(^+\), with a coefficient of 0.2, was avoided).

For oxygen-electrode experiments with methane present the gas was bubbled into the reaction mixture from a fine syringe needle, with the assumption that the fractional saturation with methane equalled the fractional amount of O\(_2\) displaced. For experiments with an atmosphere of 50% methane in air, the gas flows were regulated with ball-type flowmeters, the viscosity of methane being taken as 0.60 of that for air (Washburn, 1929). The solubility of methane was taken as 1.24mM for 0.1MPa (1atm) pressure at 30°C (Washburn, 1928), and the solubility of O\(_2\) in air-saturated medium at 30°C as 230µM (Truesdale & Downing, 1954).

### Results

Suzuki et al. (1976) reported that methane had an inhibitory effect on O\(_2\) uptake by cells of \(N. europaea\) incubated with NH\(_4\)\(^+\). Even with 0.5mm-methane, inhibition was only partial; its extent decreased as the NH\(_4\)\(^+\) concentration was raised, but did not fit a simple competitive behaviour. Our cells, prepared slightly differently, showed similar behaviour if NH\(_4\)\(^+\) was monitored directly with an ion-selective electrode. Table 1 shows data for the range of NH\(_4\)\(^+\) concentrations over which the electrode was most responsive and least subject to error, with bubbling of the controls with N\(_2\) to make them strictly comparable. Higher extents of inhibition could be attained with very low NH\(_4\)\(^+\) concentrations, say 20-50µM, but the assumption that the electrode is responding to NH\(_4\)\(^+\) alone then becomes more questionable. It is useful to have a rough quantitative value for the effectiveness of methane as inhibitor, and for that purpose Fig. 1 shows reciprocal plots similar to those given by Suzuki et al. (1974, 1976). The \(K_m\) value for NH\(_4\)\(^+\) works out at 1.2mM, implying \(K_m = 50µM\) for NH\(_4\)\(^+\) (p\(K_a = 9.1 \text{ at } 30^\circC\); Sillén & Martell, 1964). This can be compared with a \(K_m\) for NH\(_3\) of 29µM found by Suzuki et al. (1974), for 25°C and pH 7.5. If the rates with 0.6mm-methane are treated as for competitive inhibition, \(K_m' = 1.6µM\), implying a \(K_i\) for methane of 2mm.

Although methane inhibited NH\(_3\) oxidation, it had little effect on O\(_2\) consumption (see Table 1). That methane can actually stimulate O\(_2\) uptake is shown more clearly by experiments without added NH\(_4\)\(^+\) (see Table 2). This presents rates with and without one of the most effective inhibitors of ammonia.
Table 1. *Effect of methane on the rates of utilization of NH₄⁺ and O₂ by N. europaea*

NH₄⁺ and O₂ were monitored simultaneously. The rates given are for when the reaction had reached full speed. The initial NH₄⁺ concentration was corrected for that consumed by this stage assuming \( E = \text{constant} + 57\log[\text{NH}_4^+] \), in mV, and the same formula was used to calculate the rate of NH₄⁺ utilization from the chart-recorder trace. The medium consisted of 15mM-Hepes/NaOH buffer, pH 7.75 at 30°C, containing 2mM-MgCl₂. It was gassed with N₂ or methane until 50% of the O₂ had been displaced. The cells were present at 3mg wet wt./ml. The temperature was 30°C.

<table>
<thead>
<tr>
<th>Concen. of NH₄⁺ (µM)</th>
<th>Rate of NH₄⁺ utilization (µM/min)</th>
<th>Inhibition (%)</th>
<th>Rate of O₂ utilization (µM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No CH₄</td>
<td>0.6mM-CH₄</td>
<td></td>
</tr>
<tr>
<td>530</td>
<td>31.4</td>
<td>26.1</td>
<td>17</td>
</tr>
<tr>
<td>340</td>
<td>23.0</td>
<td>18.2</td>
<td>21</td>
</tr>
<tr>
<td>260</td>
<td>19.0</td>
<td>15.0</td>
<td>21</td>
</tr>
<tr>
<td>180</td>
<td>13.1</td>
<td>9.5</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 2. *Stimulated O₂ uptake by N. europaea in the absence of NH₄⁺*

Measurements were made with an oxygen electrode. The medium consisted of 50mM-sodium phosphate buffer, pH 7.7, containing 2mM-MgCl₂. For experiments with allylthiourea the cells were mixed with the inhibitor 1min before addition. For experiments with methane the medium was gassed until 50% of the O₂ had been displaced. The cells were present at 5.5mg wet wt./ml. The temperature was 30°C.

<table>
<thead>
<tr>
<th>Addition(s)</th>
<th>Rate of O₂ utilization (µM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without allylthiourea</td>
<td>5.3</td>
</tr>
<tr>
<td>With 10µM allylthiourea</td>
<td>9.4</td>
</tr>
<tr>
<td>0.6mM-CH₄</td>
<td>23</td>
</tr>
<tr>
<td>0.6mM-N₂H₄</td>
<td>37</td>
</tr>
<tr>
<td>+ 0.6mM-CH₄</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. *Kinetic parameters for NH₄⁺ and methane utilization by N. europaea*

The Figure shows a Lineweaver-Burk plot for the NH₄⁺-utilization data in Table 1, with additional points for high NH₄⁺ concentrations and no methane, derived from oxygen-electrode traces assuming a 2:3 stoichiometry for NH₄⁺/O₂. ○, No methane; ●, 0.6mM-methane. The line drawn through the points for methane present is for simple competitive inhibition.

Methane oxidation by *Nitrosomonas europaea*

Mono-oxygenase, allylthiourea (Hooper & Terry, 1973). The normal endogenous rate of O₂ uptake was little affected by allylthiourea, implying that it was largely independent of NH₄⁺ oxidation (Bömecke, 1939; Hollocher et al., 1982). If methane was bubbled to give 50% saturation, the rate was nearly doubled, and significantly this increase was prevented by allylthiourea. Table 2 also lists rates with hydrazine, a substrate analogue for the hydroxylamine-oxidizing enzyme with free N₂ as product (Nicholas & Jones, 1960; Wallace & Nicholas, 1969). The much higher rate of O₂ uptake was again stimulated by methane, provided that allylthiourea was not present.

How can the increased O₂ uptake be explained? Fig. 2 demonstrates that methanol was being formed in such experiments, and that allowing an oxygen-electrode reaction mixture to run until anaerobic resulted in an easily assayable yield of methanol. Methanol formation, like the increase in respiration, was inhibited by allylthiourea. A series of experiments without added NH₄⁺ was conducted in the oxygen electrode, differing only in the fraction of O₂ displaced by methane before the cells were added. In each case the methanol produced before anaerobicosis was determined as for the experiment shown.
Fig. 2. *Analysis of methanol formed by N. europaea during oxygen-electrode experiments*

Oxygen-electrode experiments similar to those described for Table 2 were allowed to run until the medium became anaerobic. Allylthiourea (10μM) was then added. Then 1 min later 1.5 ml was transferred to an Eppendorf-type tube and centrifuged (12,000g for 10 min) in a mini-centrifuge (Micro-Centaur; MSE, Crawley, Sussex, U.K.). The supernatant was poured into a similar tube and stored stoppered at 0°C pending analysis. The traces show typical results. (a) Not gassed, no additions; (b) gassed with methane until 16% of O₂ displaced (implying [CH₄] = 190μM, [O₂] = 200μM); (c) gassed with methane until 55% of O₂ displaced ([CH₄] = 700μM; [O₂] = 105μM); (d) as (c) but with allylthiourea at 10μM final concentration mixed with the cells 1 min before their addition. The medium was as described in Table 2, the cells were present at 4 mg wet wt./ml, and the temperature was 30°C. The peaks are identified as follows: 1, dissolved methane still present; 2, an artifact associated with H₂O injection; 3, methanol (confirmed with the pure reagent).

Fig. 3. *Methanol production by N. europaea as a function of methane concentration*

A 2 ml volume of medium as described for Fig. 2 was placed in the oxygen-electrode chamber at 30°C, part of the dissolved O₂ was displaced with methane, and the stopper was inserted. Cells were then added at 7 mg wet wt./ml. The mixture was left until the O₂ was exhausted, after which it was treated exactly as described in Fig. 2. The graph shows a plot of (methanol produced)/(O₂ consumed) as a function of initial methane concentration.

In Fig. 2, and Fig. 3 shows a graph of (methanol yield)/(O₂ consumed) versus methane concentration. With about 60% of the O₂ displaced by methane, methanol/O₂ stoichiometries approaching 1:1 were achieved. This much greater efficiency than might be expected from the Kᵣ value reflects the low rate of endogenous electron transport, and can be compared with the greater effectiveness of methane in a cell-free system found by Suzuki et al. (1976). A 1:1 relationship corresponds to reaction (2).

The data in Fig. 3 and Table 2 taken together imply a rate of methanol production of about 1.5 μM/min for cells at 1 mg wet wt./ml. Higher rates could be achieved with NH₄⁺ present, though the stoichiometry relative to O₂ uptake was much lower. Fig. 4 shows time profiles for 1 mm- and 10 mm-NH₄⁺, with 5 mg wet wt. of cells/ml under an atmosphere of 50% methane. The 10 mm-NH₄⁺ profile started more slowly, consistent with competition between NH₃ and methane, but the rate with 1 mm-NH₄⁺ declined with time as the NH₄⁺ became depleted. By 45 min both yielded methanol concentrations of 500μM. Adjustment of the various parameters with a view to optimizing methanol
production would require considerable further work, but it should be pointed out that methane as a subject for detailed study suffers from two major disadvantages: its low solubility relative to its active concentration, and the likelihood that as the methanol concentration rises it too will act as a substrate in its own right (see the Discussion section).

Discussion

If these results are considered together, the only simple explanation is that methane is an alternative substrate for ammonia mono-oxygenase. As a hypothesis this is made much more plausible by a consideration of the properties of methane mono-oxygenase, and this is done below. First, however, Fig. 5 shows the reactions that we believe are taking place. For simplicity a single pool of hydrogen atoms is shown, whereas in reality the terminal oxidase at least is separated from the initial reductases by a proton-pumping electron-transport chain. Figs. 5(b) and 5(c) show why methane stimulates O₂ uptake in experiments with endogenous substrates or hydrazine. Fig. 5(a) shows the reactions taking place with NH₄⁺, for NH₂OH concentration in the steady state. This poses the question why we have not used added NH₂OH as reductant, at say 100 μM to 1 mM. The answer is that even at the lower end of this concentration range NH₂OH depresses the activity of the mono-oxygenase, with both NH₄⁺ and organic substrates (P. M. Wood, unpublished work). This important fact is scarcely mentioned in the literature. Without feedback inhibition of this sort, the NH₂OH concentration would tend to rise indefinitely during normal growth on ammonium salts.

Methane mono-oxygenase of methane-oxidizing
bacteria (methanotrophs) is an extraordinarily unspecific enzyme, capable of inserting oxygen atoms into C–H bonds in a wide range of uncharged carbon compounds and adding oxygen across C=C double bonds to yield epoxides (Higgins et al., 1980; Dalton, 1981). CO is another substrate. It can also add oxygen across certain N–H bonds, and substrates in this category include NH₃ (Dalton, 1977; O’Neill & Wilkinson, 1977). Several studies have shown that NH₄⁺ in the growth medium of methanotrophs becomes oxidized to NO₂⁻ (Whittenbury et al., 1970; O’Neill & Wilkinson, 1977). Moreover, Sokolov et al. (1981) have found the NH₂OH-oxidizing machinery in Methyllococcus methylophilus to be very similar to that in N. europaea.

Inhibition sensitivity distinguishes two very different forms of methane mono-oxygenase. Most recent work has been done with a soluble form present in Methyllococcus capsulatus Bath and Methylosinus trichosporium (Colby & Dalton, 1978; Stirling & Dalton, 1979; Dalton, 1981). This has a very limited range of inhibitors, virtually restricted to acetylenic compounds and 8-hydroxyquinoline. It also has NADH as donor, which for thermodynamic reasons would be unsuitable for Nitrosomonas: 

\[ E_{m,\text{N}} (\text{NH}_2\text{OH}/\text{NO}_2^-) = +60 \text{mV}, \text{far less favourable for} \]

for coupling to NADH formation than \[ E_{m,\text{N}} (\text{HCHO}/\text{HCO}_3^-) = -530 \text{mV} \] and \[ E_{m,\text{N}} (\text{HCO}_3^-/\text{HCO}_2^-) = -410 \text{mV} \] (Thauer et al., 1977). Much more relevant is the bound form, which appears to be more widely distributed but has not been purified (Patel et al., 1980; Dalton, 1981; Higgins et al., 1981). This is sensitive to cyanide, thioureas, aadipyridyl and N-Serve (2-chloro-6-trichloromethylpyridine) at almost exactly the same concentrations as affect NH₃ oxidation in N. europaea (Hooper & Terry, 1973; Colby et al., 1975; Hubley et al., 1975; Topp & Knowles, 1982).

The inhibitors just mentioned are all well-known metal-complexing agents. Oxidation of NH₃ by N. europaea is also sensitive to a range of small organic molecules, many of which have little or no complexing ability. Examples are methane, methanol, low concentrations of CO, bromomethane and ethanol (Thiagalingam & Kanehiro, 1971; Hooper & Terry, 1973; Suzuki et al., 1976; Wood et al., 1981). In the past this has been mysterious, although it has been suggested that short-chain alcohols might act as radical traps (Hooper & Terry, 1973). The present results point to a rationalization: all are substrates for methane mono-oxidase.

Indeed, our preliminary experiments with all these compounds point to their oxidation, as with methane (M. R. Hyman, D. J. Miller & P. M. Wood, unpublished work).

It is instructive to compare the \( K_m \) for NH₄⁺ (1.2 mm) and \( K_f \) for methane (2 mm) estimated above with values for methanotrophs. In this respect it is important to note that the \( K_m \) for a competing substrate is the same as its \( K_f \) as a competitive inhibitor (see, e.g., Cornish-Bowden, 1979). For NH₄⁺, O’Neill & Wilkinson (1977), working with Methylosinus trichosporium, found similar values for \( K_m \) and \( K_f \) with the strong pH-dependence expected if NH₃ is the active form: \( K_f = 17.5 \text{mm} \) at pH 6.0, 0.2 mm at pH 8.0; \( K_m = 7.1 \text{mm} \) at pH 6.5, 0.4 mm at pH 7.5. For Methylobomas methanica, Ferenci et al. (1975) reported \( K_f = 10 \text{mm} \) at pH 7.0. In terms of free NH₃, these values and the \( K_m \) for N. europaea all lie in the range 10–50 μM. The soluble enzyme in Methyllococcus capsulatus Bath had a much lower affinity and different pH-dependence: \( K_f = 31 \text{mm} \) at pH 7.0, 66 mm at pH 8.0 (Dalton, 1977). Published \( K_m \) values for methane are all far lower than the \( K_f \) found here: 15 μM for Mm. methanica (Ferenci et al., 1975), 45 μM for Ms. trichosporium (O’Neill & Wilkinson, 1977), and 160 μM for the soluble enzyme from Mc. capsulatus Bath (Colby et al., 1977).

Despite its high \( K_m \), there will be situations where methane affects N. europaea in the wild. In some environments methane is present at higher concentrations than is NH₄⁺ (Jones & Simon, 1981). Besides, even a small drain in reducing equivalents will be detrimental to growth. We have worked at pH 7.7 and 30°C; for every fall in pH by 0.3 unit or in temperature by 10°C the proportion of free NH₃ will be halved.

Nitrification and methane oxidation frequently occur together, for instance in the aerobic zone above anaerobic decomposition of organic matter. The present results make their interaction yet more complex: not only can the methanotrophs oxidize NH₃, but the nitrifiers may also oxidize methane. One classical distinction has been that methanotrophs cannot live on NH₃ and CO₂ alone, and autotrophic nitrifiers cannot touch organic carbon.

[A methanotroph with ribulose bisphosphate carboxylase has been described, but autotrophic growth was not achieved (Taylor et al., 1981).] Can N. europaea benefit from its weak ability to oxidize methane? Or is it merely adapted away from methane as far as the poor selectivity of its mono-oxygenase will allow? The answer will have to await further research.

Note added in proof (received 1 February 1983)

Since this work was submitted, a paper has been published reporting CO oxidation by ammonia mono-oxygenase (Tsang & Suzuki, 1982). It has also been pointed out to us that Drozd (1980) has demonstrated the oxidation of propylene, benzene and cyclohexane by this system, although he states that methane is not a substrate.
We are grateful to Professor A. E. Walsby, Dr. D. H. Brown and Dr. D. J. Hill for use of a gas chromatograph in the Department of Botany, University of Bristol. We thank the Science and Engineering Research Council for a research grant and a research studentship to M. R. H., and Miss S. O. Small for valuable technical assistance.

References