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Methane oxidation by *Nitrosomonas europaea*

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1. Methane inhibited NH_4^+ utilization by *Nitrosomonas europaea* with a K_i of 2mM. 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 1mol 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 1h. 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_2^- 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:

$$NH_3 + 2[H] + O_2 \rightarrow NH_2OH + H_2O \qquad (1)$$

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:

$$CH_4 + 2[H] + O_2 \rightarrow CH_3OH + H_2O \qquad (2)$$

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_2OH, with exactly 2e$^-$ returned per molecule of NH_2OH when the NH_2OH 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_3OH 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 1mM-NH_2OH, which in our experience inhibits the mono-oxygenase, and the high NH_4^+ concentration used by Hynes & Knowles (1982) would likewise be counterproductive. These points are explained below.

Experimental

Nitrosomonas europaea (A.T.C.C. 19178) was kindly supplied by Dr. N. Walker (Rothamsted

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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, 3.3 g of $(NH_4)_2SO_4$, 0.53 g of KH_2PO_4, 67 mg of $MgSO_4,7H_2O$, 67 mg of $CaCl_2,2H_2O$, plus 0.67 mg 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 by centrifugation at 4°C (28000 ***g*** for 40 min), followed by resuspension in medium containing 50 mM-sodium phosphate buffer, pH 7.5, 2 mM-$MgCl_2$ and 0.15 mM-$(NH_4)_2SO_4$ and re-centrifugation (38000 ***g*** for 20 min). The pellet was resuspended in 50 mM-sodium phosphate buffer, pH 7.7, containing 2 mM-$MgCl_2$ at 0.2 g 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 16 mm (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 0.1 M-$NaNO_3$. This tubing also made contact with a standard calomel reference electrode (Philips RH 44/2-SD/1) via a small reservoir of 1 M-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 $[NH_4^+] > 100\,\mu M$ the response fitted closely to $E = \text{constant} + 57 \log [NH_4^+]$, 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 1 m column of Tenax GC (60–80 mesh). A sample volume of 5 μl was used and an N_2 flow of 20 ml/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 Hepes [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 coefficinets 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 1.24 mM for 0.1 MPa (1 atm) pressure at 30°C (Washburn, 1928), and the solubility of O_2 in air-saturated medium at 30°C as 230 μM (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 0.5 mM-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 20–50 μM, 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 1.2 mM, implying $K_m = 50\,\mu M$ for NH_3 ($pK_a = 9.1$ at 30°C; Sillén & Martell, 1964). This can be compared with a K_m for NH_3 of 29 μM found by Suzuki *et al.* (1974), for 25°C and pH 7.5. If the rates with 0.6 mM-methane are treated as for competitive inhibition, $K'_m = 1.6$ mM, implying a K_i for methane of 2 mM.

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 NH_4^+ and O_2 by N. europaea*

NH_4^+ and O_2 were monitored simultaneously. The rates given are for when the reaction had reached full speed. The initial NH_4^+ concentration was corrected for that consumed by this stage assuming $E = \text{constant} + 57\log[NH_4^+]$, in mV, and the same formula was used to calculate the rate of NH_4^+ utilization from the chart-recorder trace. The medium consisted of 15mM-Hepes/NaOH buffer, pH 7.75 (at 30°C), containing 2mM-$MgCl_2$. It was gassed with N_2 or methane until 50% of the O_2 had been displaced. The cells were present at 3mg wet wt./ml. The temperature was 30°C.

Concn. of NH_4^+ (μM)	Rate of NH_4^+ utilization (μM/min)		Inhibition (%)	Rate of O_2 utilization (μM/min)	
	No CH_4	0.6mM-CH_4		No CH_4	0.6mM-CH_4
530	31.4	26.1	17	46.1	47.1
340	23.0	18.2	21	34.3	35.8
260	19.0	15.0	21	30.5	32.0
180	13.1	9.5	28	25.2	26.5

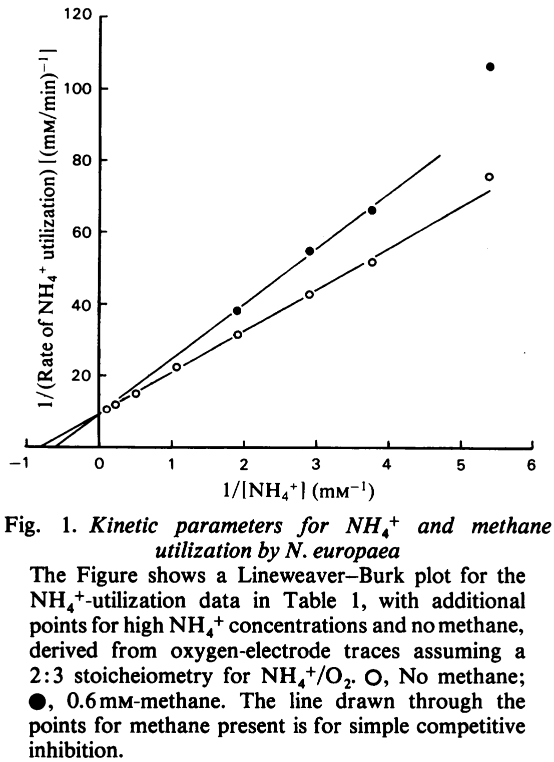

Fig. 1. *Kinetic parameters for NH_4^+ and methane utilization by N. europaea*

The Figure shows a Lineweaver–Burk plot for the NH_4^+-utilization data in Table 1, with additional points for high NH_4^+ concentrations and no methane, derived from oxygen-electrode traces assuming a 2:3 stoicheiometry for NH_4^+/O_2. ○, No methane; ●, 0.6mM-methane. The line drawn through the points for methane present is for simple competitive inhibition.

mono-oxygenase, allylthiourea (Hooper & Terry, 1973). The normal endogenous rate of O_2 uptake was little affected by allylthiourea, implying that it was largely independent of NH_4^+ 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

Table 2. *Stimulated O_2 uptake by N. europaea in the absence of NH_4^+*

Measurements were made with an oxygen electrode. The medium consisted of 50mM-sodium phosphate buffer, pH 7.7, containing 2mM-$MgCl_2$. 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 O_2 had been displaced. The cells were present at 5.5mg wet wt./ml. The temperature was 30°C.

Addition(s)	Rate of O_2 utilization (μM/min)	
	Without allylthiourea	With 10μM-allylthiourea
None	5.3	4.8
0.6mM-CH_4	9.4	5.1
0.6mM-N_2H_4	23	23
0.6mM-N_2H_4 + 0.6mM-CH_4	37	23

with hydrazine, a substrate analogue for the hydroxylamine-oxidizing enzyme with free N_2 as product (Nicholas & Jones, 1960; Wallace & Nicholas, 1969). The much higher rate of O_2 uptake was again stimulated by methane, provided that allylthiourea was not present.

How can the increased O_2 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 NH_4^+ was conducted in the oxygen electrode, differing only in the fraction of O_2 displaced by methane before the cells were added. In each case the methanol produced before anaerobiosis was determined as for the experiment shown

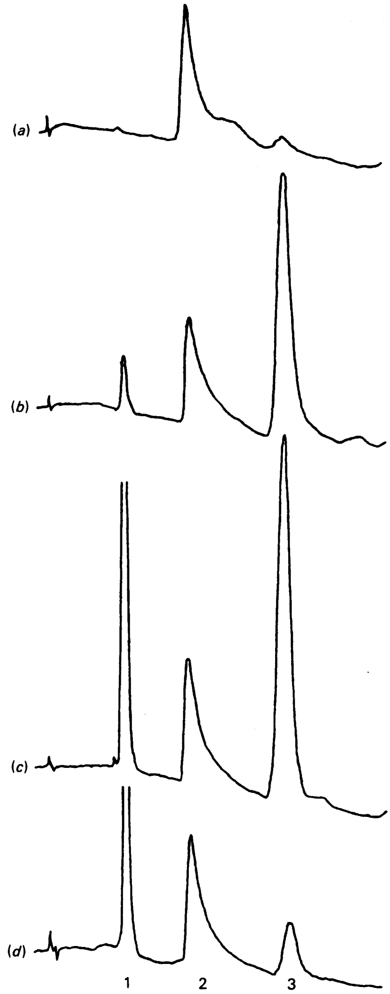

Fig. 2. *Analysis of methanol formed by N. europaea during oxygen-electrode experiments*

Oxygen-electrode experiments similar to those described for Table 2 were allowed to run until the medium became anaerobic. Allylthiourea ($10\,\mu$M) was then added. Then 1 min later 1.5 ml was transferred to an Eppendorf-type tube and centrifuged (12000 *g* 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 O_2 displaced (implying $[CH_4] = 190\,\mu$M, $[O_2] = 200\,\mu$M); (*c*) gassed with methane until 55% of O_2 displaced ($[CH_4] = 700\,\mu$M; $[O_2] = 105\,\mu$M); (*d*) as (*c*) but with allylthiourea at $10\,\mu$M 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 H_2O injection; 3, methanol (confirmed with the pure reagent).

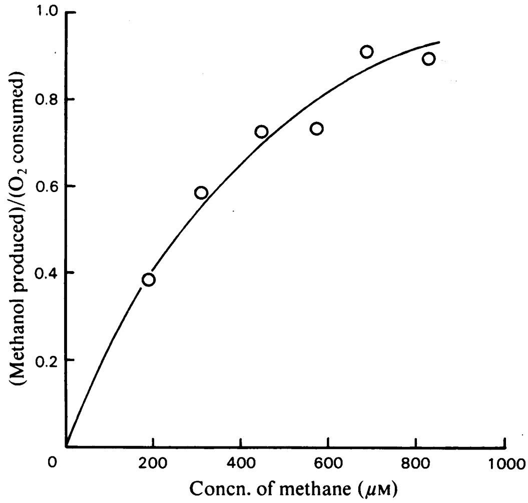

Fig. 3. *Methanol production by N. europaea as a function of methane concentration*

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 O_2 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 O_2 was exhausted, after which it was treated exactly as described in Fig. 2. The graph shows a plot of (methanol produced)/(O_2 consumed) as a function of initial methane concentration.

in Fig. 2, and Fig. 3 shows a graph of (methanol yield)/(O_2 consumed) versus methane concentration. With about 60% of the O_2 displaced by methane, methanol/O_2 stoicheiometries approaching 1:1 were achieved. This much greater efficiency than might be expected from the K_i 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 $1.5\,\mu$M/min for cells at 1 mg wet wt./ml. Higher rates could be achieved with NH_4^+ present, though the stoicheiometry relative to O_2 uptake was much lower. Fig. 4 shows time profiles for 1 mM- and 10 mM-NH_4^+, with 5 mg wet wt. of cells/ml under an atmosphere of 50% methane. The 10 mM-NH_4^+ profile started more slowly, consistent with competition between NH_3 and methane, but the rate with 1 mM-NH_4^+ declined with time as the NH_4^+ became depleted. By 45 min both yielded methanol concentrations of $500\,\mu$M. Adjustment of the various parameters with a view to optimizing methanol

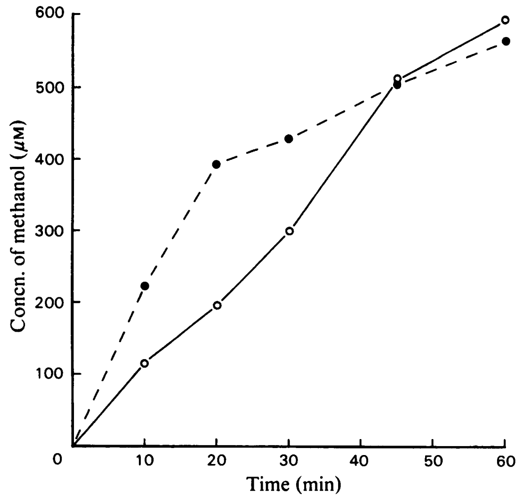

Fig. 4. *Time courses for methanol production by N. europaea with NH_4^+ present*

The side arm of a 500ml Buchner flask was sealed with a Suba-Seal rubber stopper (Gallenkamp, London, U.K.). Then 20ml of medium as described for Fig. 2 was placed in the flask, plus 1mM-NH_4Cl (●) or 10mM-NH_4Cl (○). The flask was flushed with a gas stream of 50% methane in air. Cells were added at 5mg wet wt./ml of medium, and the flask was immediately stoppered. It was then transferred to a shaking water bath at 30°C. Samples (0.5ml) were withdrawn at intervals, by piercing the rubber seal with a syringe. Each was immediately mixed with allylthiourea at 10μM final concentration, and centrifuged as described for Fig. 2. The supernatants were analysed for methanol.

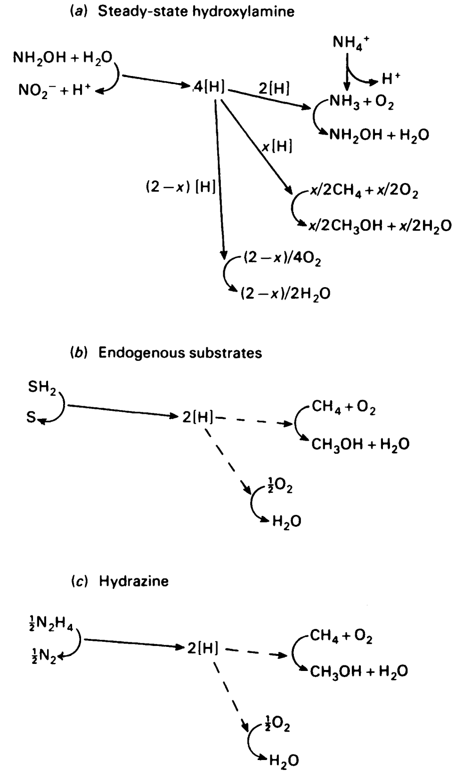

Fig. 5. *Reductants for methane oxidation*

In (*a*) for simplicity the 4 reducing equivalents from NH_2OH are shown as equivalent, implying $x \leqslant 2$. If the one-electron step

$$[NO] + H_2O \rightarrow NO_2^- + 2H^+ + e^-$$

cannot be coupled in the same way as the others, $x \leqslant 1$. A small part of the reducing equivalents shown as proceeding to the terminal oxidase will be used to reduce $NAD(P)^+$ for CO_2 fixation and other biosynthetic purposes.

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 O_2 uptake in experiments with endogenous substrates or hydrazine. Fig. 5(*a*) shows the reactions taking place with NH_4^+, for NH_2OH concentration in the steady state. This poses the question why we have not used added NH_2OH as reductant, at say 100μM to 1mM. The answer is that even at the lower end of this concentration range NH_2OH depresses the activity of the mono-oxygenase, with both NH_4^+ and organic substrates (P. M. Wood, unpublished work). This important fact is scarcely mentioned in the literature. Without feedback inhibition of this sort, the NH_2OH 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 NH_3 (Dalton, 1977; O'Neill & Wilkinson, 1977). Several studies have shown that NH_4^+ in the growth medium of methanotrophs becomes oxidized to NO_2^- (Whittenbury *et al.*, 1970; O'Neill & Wilkinson, 1977). Moreover, Sokolov *et al.* (1981) have found the NH_2OH-oxidizing machinery in *Methylococcus 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 *Methylococcus 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*: $E_{m,7}$ (NH_2OH/NO_2^-) = +60 mV, far less favourable for coupling to NADH formation than $E_{m,7}$($HCHO/HCO_2^-$) = −530 mV and $E_{m,7}$(HCO_2^-/HCO_3^-) = −410 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, αα′-dipyridyl and N-Serve (2-chloro-6-trichloromethylpyridine) at almost exactly the same concentrations as affect NH_3 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 NH_3 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-oxygenase. 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 NH_4^+ (1.2 mM) and K_i 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_i as a competitive inhibitor (see, e.g., Cornish-Bowden, 1979). For NH_4^+, O'Neill & Wilkinson (1977), working with *Methylosinus trichosporium*, found similar values for K_m and K_i, with the strong pH-dependence expected if NH_3 is the active form: K_i = 17.5 mM at pH 6.0, 0.2 mM at pH 8.0; K_m = 7.1 mM at pH 6.5, 0.4 mM at pH 7.5. For *Methylomonas methanica*, Ferenci *et al.* (1975) reported K_i = 10 mM at pH 7.0. In terms of free NH_3, these values and the K_m for *N. europaea* all lie in the range 10–50 μM. The soluble enzyme in *Methylococcus capsulatus* Bath had a much lower affinity and different pH-dependence: K_i = 31 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_i 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 NH_4^+ (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 NH_3 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 NH_3, but the nitrifiers may also oxidize methane. One classical distinction has been that methanotrophs cannot live on NH_3 and CO_2 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.

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