Oxidation of Dimethyl Ether, Methyl Formate and Bromomethane by *Methylococcus capsulatus* (Bath)

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Suspensions of *Methylococcus capsulatus* strain Bath oxidized dimethyl ether, methyl formate and bromomethane. The rate of disappearance of dimethyl ether was enhanced up to 11-fold in the presence of co-substrates such as formaldehyde. Dimethyl ether was oxidized by the purified methane mono-oxygenase from *M. capsulatus* (Bath) to give various amounts of methanol and formaldehyde. As *M. capsulatus* (Bath) cannot grow on dimethyl ether it was concluded that this ether is a non-growth substrate which can be fortuitously oxidized. Possible reasons for the lack of growth on dimethyl ether are discussed. Methyl formate and bromomethane were oxidized by the purified methane mono-oxygenase to yield equal stoicheiometric amounts of formaldehyde and formic acid, and formaldehyde alone, respectively. *Methylococcus capsulatus* (Bath) grew on methyl formate but not on bromomethane and it was concluded that the latter is a non-growth substrate which can be fortuitously oxidized. Preliminary evidence is presented for carbon assimilation during the oxidation of bromomethane.

INTRODUCTION

The ability of methane-oxidizing bacteria to oxidize and utilize dimethyl ether as a sole source of carbon and energy was first reported in a review by Wilkinson (1971). Since then, the assimilation of dimethyl ether by such bacteria has been described only on a few occasions (Davey, 1971; Hazeu, 1975; Ribbons, 1975; Patel *et al.*, 1976) although a number of reviews and papers refer to the remarks of Wilkinson (1971, 1975) as evidence for dimethyl ether oxidation. On the basis of the unpublished observations of Bryan-Jones & Wilkinson (Wilkinson, 1971) and further unpublished results of Mitton involving $^{18}O_2$ incorporation, Davey (1971) proposed a pathway for methane oxidation involving dimethyl ether as an intermediate. He suggested that methane was oxidized to dimethyl ether which in turn was oxidized by a CO-sensitive oxygenase to methyl formate. Methyl formate was then hydrolysed by means of an esterase to methanol and formate. Such a mechanism would not be consistent with the ^{18}O studies of Higgins & Quayle (1970) and, in contrast with the earlier proposals, Thomson (1974) showed that dimethyl ether was oxidized by suspensions of methane-utilizing bacteria via 2-ethoxyethanol and 2-ethoxyacetaldehyde to 2-ethoxyacetate. Furthermore, Wilkinson (1975) reported that no ^{14}C incorporation into dimethyl ether or methyl formate was detected when suspensions of various methane-utilizing bacteria were incubated with [^{14}C]methane, although [^{14}C]methanol was readily detectable (Wilkinson, 1975). To incorporate these findings and still retain a role for dimethyl ether as an intermediate in methane oxidation, a new scheme was suggested in which the initial product of dimethyl ether oxidation was the unstable hemi-acetal, methoxymethanol. This could either spontaneously decompose to give formaldehyde and methanol or be oxidized by the primary alcohol dehydrogenase to methyl formate (Thomson, 1974). Even with this revised hypothetical pathway there was no direct evidence for the involvement of dimethyl ether as an intermediate in methane oxidation. This, combined with the fact that studies with cell-

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free methane mono-oxygenase systems from *Methylococcus capsulatus* (Bath) (Colby *et al.*, 1977) and *Methylosinus trichosporium* OB3b (Tonge *et al.*, 1977; Stirling *et al.*, 1979) had indicated that methanol was the initial product of methane oxidation, means that the possibility of dimethyl ether being a normal intermediate in methane oxidation is very remote. Our present study is concerned with the nature of this apparently fortuitous oxidation (see Stirling & Dalton, 1979).

Crude extracts of two organisms, *Methylomonas methanica* and *Methylococcus capsulatus* (Bath), have been reported to be capable of catalysing the disappearance of bromomethane (Colby *et al.*, 1975; Colby & Dalton, 1976). In both instances bromomethane oxidation was thought to be catalysed by the methane mono-oxygenase. The present paper is also concerned with the oxidation of bromomethane by suspensions of *Methylococcus capsulatus* (Bath) and by purified methane mono-oxygenase and attempts to determine the nature of the oxidation and its effect on the organism.

METHODS

Growth of bacteria and preparation of washed suspensions. Methylococcus capsulatus (Bath) was grown in continuous culture and cell suspensions were prepared as described previously (Stirling & Dalton, 1979). The ability of *M. capsulatus* (Bath) to grow on dimethyl ether plus formate or methyl formate was studied using 250 ml conical flasks containing 25 ml sterile ammonium/mineral salts medium (Dalton & Whittenbury, 1976) and various amounts of the substrates. Appropriate controls either with methane (50 ml) as sole source of carbon or without carbon source were included. All flasks were inoculated with 0·5 ml of a chemostat culture as described previously (Stirling & Dalton, 1979) and incubated at 45 °C for up to 7 d.

Respiration studies. Respiration studies were done at 45 °C in a Clark-type oxygen electrode (Rank Bros, Bottisham, Cambridge) with a reaction chamber of 3 ml capacity. The reaction vessel contained 160 μM-sodium phosphate buffer (pH 6·8), cell suspension to give a final A_{540} of 2 (0·815 mg dry wt cells) prepared as described above, and substrates and inhibitors at the final concentrations indicated.

Whole-cell oxidation studies. The assays were done in 7 ml conical flasks essentially as described previously (Colby *et al.*, 1977) except that extract was replaced by cell suspension (0·53 mg dry wt cells). Dimethyl ether gas and the co-substrates were present at the concentrations indicated. Dimethyl ether disappearance was followed by flame ionization/gas chromatography as described previously (Colby *et al.*, 1977).

Identification of the contaminant in dimethyl ether. The contaminant in saturated dimethyl ether solutions was identified using a Pye Series 104 flame ionization/gas chromatograph fitted with a 2·1 m glass column (internal diam. 4 mm) packed with Porapak N (Waters Associates, Milford, Mass., U.S.A.). The column was operated at 160 °C with a N_2 gas flow-rate of 30 ml min^{-1}. The contaminant was identified by comparison of its retention time with those of authentic standards and its concentration was estimated by comparison of peak areas with the standards.

Purified methane mono-oxygenase assays. The three components of the methane mono-oxygenase from *M. capsulatus* (Bath) (Colby & Dalton, 1978) were purified as follows: component C was purified to homogeneity as previously described (Colby & Dalton, 1978, 1979) and components A and B were purified to about 95% homogeneity as judged by gel electrophoresis (unpublished results). The assays were done in 7 ml conical flasks essentially as described previously (Colby *et al.*,1977). Each flask contained 1 ml potassium phosphate buffer (20 mM, pH 6·8), 3·4 mg component A, 1·6 mg component B, 0·15 mg component C, 5 μmol NADH and substrate at the concentration indicated. Methanol was identified and estimated as described by Colby *et al.* (1977), formaldehyde by the colorimetric method of Nash (1953), and formate by the colorimetric method of Lang & Lang (1972).

Materials. Chemicals and biochemicals were generally of the best grade available and were obtained from sources described previously (Colby *et al.*, 1977). Methyl formate was obtained from BDH.

RESULTS AND DISCUSSION

Respiration studies with dimethyl ether

The respiration rates observed in an oxygen electrode containing suspensions of *M. capsulatus* (Bath) with dimethyl ether (99%) as substrate are shown in Table 1. Very rapid oxygen consumption occurred with high concentrations of dimethyl ether (100 mM) even in the presence of ethyne or 8-hydroxyquinoline, both of which are potent inhibitors of methane

Table 1. *Substrate respiration studies using suspensions of M. capsulatus (Bath)*

Substrate (mM)	Oxidation rate [nmol O_2 consumed min^{-1} (mg dry wt cells)$^{-1}$]
CH_4 (0·12)	463
CH_4 (0·12)+ethyne (1·2)	0
CH_3OCH_3 (100)	473
CH_3OCH_3 (0·1, 3)	0
CH_3OCH_3 (100)+ethyne (1·2)	468
CH_3OCH_3 (100)+ethyne (5)	334
CH_3OCH_3 (100)+8-hydroxyquinoline (1)	463
CH_3OCH_3* (100)	8
CH_3OCH_3* (100)+ethyne (1·2)	0
CH_3OCHO (2·5)	290
CH_3OCHO (2·5)+ethyne (1·2)	0
CH_3Br (2)	48
CH_3Br (2)+ethyne (1·2)	0

* Scrubbed dimethyl ether (see text).

mono-oxygenase (Stirling & Dalton, 1977). Only 30% inhibition was caused by ethyne at a final concentration of 5 mM which was approximately five times the concentration required to inhibit methane oxidation completely.

When a solution of dimethyl ether was prepared by first scrubbing the gas through water, the oxidation rate was only 8 nmol O_2 consumed min^{-1} (mg dry wt cells)$^{-1}$ (Table 1). Gas chromatographic analysis (see Methods) of the dimethyl ether solutions indicated that methanol was present at a concentration in excess of 5 mM in the unscrubbed solution but was completely absent from the scrubbed solution. This was confirmed when the unscrubbed solution was tested as a substrate for methanol dehydrogenase in soluble extracts of *M. capsulatus* (Bath) under optimum conditions for the enzyme in *Pseudomonas* M27 (Anthony & Zatman, 1964). A rapid reduction of 2,6-dichlorophenolindophenol was observed which was not inhibited by ethyne. The scrubbed dimethyl ether solution gave no similar reduction.

Dimethyl ether disappearance with whole-cell suspensions

The rate of disappearance of dimethyl ether was proportional to its concentration up to 10 mM; higher concentrations had little effect on the rate. The low rate of dimethyl ether disappearance (Table 2) was similar to the oxidation rate observed when monitoring oxygen consumption using the scrubbed dimethyl ether solution (Table 1). In the presence of a co-substrate which could generate reducing power, the rate of dimethyl ether disappearance increased markedly (Table 2). These results effectively eliminate the possibility that the low rate of oxidation of dimethyl ether with whole organisms was due to the toxicity of substrate or product. A more probable explanation would be either inability to take up the substrate or inability to generate reducing power for the mono-oxygenase from the subsequent oxidation of the substrate.

Oxidation of dimethyl ether by purified methane mono-oxygenase

No products from dimethyl ether oxidation by whole-cell suspensions or cell-free extracts (Colby *et al.*, 1977) of *M. capsulatus* (Bath) were detected by the gas chromatographic techniques described in Methods. To resolve this situation, dimethyl ether oxidation was studied using purified methane mono-oxygenase (Table 3). Methanol and formaldehyde were produced from dimethyl ether, but not in equal amounts as would be expected for these two products. This was thought to be due to the oxidation of the resultant methanol to

Table 2. *Rates of dimethyl ether disappearance with suspensions of M. capsulatus (Bath)*

Substrate(s) (μmol)	Rate of dimethyl ether disappearance [nmol CH_3OCH_3 min^{-1} (mg dry wt cells)$^{-1}$]
CH_3OCH_3 (10)	19
CH_3OCH_3 (10) + CH_3OH (4)	175
CH_3OCH_3 (10) + HCHO (4)	125
CH_3OCH_3 (10) + HCOOK (4)	223

Table 3. *Oxidation studies using purified methane mono-oxygenase from M. capsulatus (Bath)*

Assays were done as described in Methods.

Substrate (μmol per reaction flask)	Substrate oxidized (μmol oxidized after 5 min)	Product (μmol formed after 5 min)	Activity (nmol substrate utilized min^{-1})
CH_4 (134)	ND	CH_3OH (2·02) HCHO (1·05)	614*
CH_3OCH_3 (3)	1·05	CH_3OH (0·38) HCHO (1·75)	208
CH_3OCHO (2)	1·1	HCHO (1·1) HCOOH (1·05)	220
CH_3Br (2·5)	0·97	HCHO (1·0)	243

ND, Not determined.
* Rate of methane oxidation was determined from the amount of product formed.

formaldehyde by the methane mono-oxygenase, as methanol has been shown to be a substrate for the enzyme (Colby *et al.*, 1977). The results of the assay containing methane and the purified enzyme (Table 3) confirm this explanation as formaldehyde was produced from methanol, the initial product from methane oxidation. Similar assays continued for longer than 5 min still showed a linear increase in the total amount of products but the proportion of formaldehyde increased.

The oxidation of dimethyl ether to methanol and formaldehyde probably proceeds via the unstable hemiacetal methoxymethanol which dismutates to give equal amounts of methanol and formaldehyde. Interestingly, no methyl formate was detected from dimethyl ether oxidation, thus contradicting the first revised methane oxidation pathway envisaged by Davey (1971), at least in this species.

Dimethyl ether can be oxidized, albeit very poorly, by suspensions of *M. capsulatus* (Bath) and readily oxidized by extracts and purified methane mono-oxygenase preparations, although it is a non-growth substrate (Stirling & Dalton, 1979). Meyers & Ribbons (1978) recently reported that *M. capsulatus* strain Texas could not grow on dimethyl ether although it was oxidized by cell suspensions. Dimethyl ether is clearly a non-growth substrate which can be fortuitously oxidized [as defined by Stirling & Dalton (1979)]. To determine whether any carbon assimilation can occur as a result of dimethyl ether oxidation will require ^{14}C incorporation studies. If methanol and formaldehyde are the products of dimethyl ether oxidation *in vivo*, as would seem likely, it is possible that some carbon assimilation might occur. This poses the problem, why does *M. capsulatus* (Bath) not grow on dimethyl ether? If methanol and formaldehyde are the products of dimethyl ether oxidation *in vivo*, then the explanation, given above, that the low rate of oxidation by intact bacteria was due to the lack of reductant generated from the complete oxidation of dimethyl ether, cannot be correct. Both methanol and formaldehyde could generate NADH from their subsequent

oxidation by the NAD^+-linked formaldehyde and formate dehydrogenases (Stirling & Dalton, 1978). This suggests that the low rate of oxidation of dimethyl ether by suspensions of *M. capsulatus* (Bath), and consequently the inability to grow on dimethyl ether, is the result of either the organisms being relatively impermeable to dimethyl ether or that the products of the oxidation *in vivo* are not methanol and formaldehyde. The latter explanation must remain a possibility since no products from dimethyl ether oxidation *in vivo* have been detected. The fact that up to 11-fold increases in the rates of dimethyl ether disappearance with *M. capsulatus* (Bath) suspensions were observed in the presence of a co-substrate (Table 2) could suggest the involvement of an active transport mechanism for substrate uptake. If this was the case then it should be possible to grow *M. capsulatus* (Bath) on dimethyl ether in the presence of an exogenous energy source. Attempts to grow *M. capsulatus* (Bath) on dimethyl ether in the presence of various concentrations of potassium formate gave inconsistent results. Growth, albeit sparse, was observed in a number of flasks containing formate at low concentrations. The inconsistencies were probably the result of the toxic nature of formate at comparatively low concentrations (e.g. 5 mM). Dimethyl ether is fairly lipid-soluble and one would not expect *a priori* that an active transport mechanism would be necessary for the substrate to enter the cell.

Validity of reports of dimethyl ether oxidation

The results obtained for dimethyl ether oxidation when assayed by oxygen consumption raise possible doubts about the validity of four other reports of dimethyl ether oxidation by methane-utilizing bacteria (Davey, 1971; Hazeu, 1975; Ribbons, 1975; Patel *et al.*, 1976) in which dimethyl ether oxidation was monitored by oxygen consumption. None of these workers referred to the purity of gas used; although its purity in the present study was supposed to be 99%, it contained a contaminant which could accumulate to significant concentrations during the preparation of gas-saturated buffer solutions. Methanol contamination of dimethyl ether solutions was recently reported by Meyers & Ribbons (1978) who obtained similar misleading results during respiration studies with *M. capsulatus* (Texas). It is therefore suggested that all oxidation studies involving dimethyl ether should, where possible, measure either dimethyl ether disappearance or the formation of products.

Oxidation of methyl formate

As methyl formate had been proposed as an intermediate in methane oxidation (Davey, 1971), the oxidation of this compound by *M. capsulatus* (Bath) was investigated. Methyl formate was oxidized readily by whole-cell suspensions (Table 1) and, as with the other mono-oxygenase substrates tested, the oxidation was potently inhibited by ethyne. When assayed with purified methane mono-oxygenase, methyl formate was again readily oxidized (Table 3), yielding formaldehyde and formic acid and not methanol and formic acid as predicted or reported by others (Davey, 1971; Wilkinson, 1975; Meyers & Ribbons, 1978).

If formaldehyde was produced from methyl formate oxidation *in vivo* it is possible that *M. capsulatus* (Bath) could grow on methyl formate as sole source of carbon and energy. We found that *M. capsulatus* (Bath) could indeed consistently grow on methyl formate, although only when the substrate concentration was between 5 and 10 mM. With lower or higher concentrations no growth was ever observed. This was a surprising result in view of the inability of *M. capsulatus* to grow on dimethyl ether, but nevertheless provides a potentially useful tool for the further study of C_1 metabolism in *M. capsulatus* (Bath), for instance by selecting for *M. capsulatus* (Bath) mutants which lack methanol dehydrogenase.

Methylococcus capsulatus (Texas) has recently been reported to grow on, and oxidize, methyl formate (Meyers & Ribbons, 1978); however, on the evidence of studies with cell-free extracts, these authors proposed that methyl formate was oxidized by the primary alcohol dehydrogenase as opposed to the methane mono-oxygenase.

Oxidation of bromomethane

Suspensions of *M. capsulatus* (Bath) can catalyse bromomethane disappearance, the rate of which is not increased by the presence of formaldehyde (Stirling & Dalton, 1979). The results in Table 1 show that the oxidation of bromomethane by suspensions of *M. capsulatus* (Bath) was completely inhibited by ethyne, suggesting that this oxidation was catalysed by the methane mono-oxygenase. To confirm this, bromomethane oxidation was monitored using a purified methane mono-oxygenase preparation. Bromomethane was stoicheiometrically oxidized to formaldehyde (Table 3), presumably via the unstable intermediate bromomethanol which would dismutate to give formaldehyde.

Attempts to grow *M. capsulatus* (Bath) on bromomethane were unsuccessful (Stirling & Dalton, 1979), probably due to the toxicity of bromide ions produced from the dismutation of bromomethanol to formaldehyde. This view was supported by results obtained from attempts to grow *M. capsulatus* (Bath) on methane in the presence of various amounts of bromine or bromomethane. Several flasks were set up as for the growth studies (see Methods) with methane as the carbon source. Bromine or bromomethane (5, 10 or 50 μmol) was added to flasks and these, together with appropriate control flasks, were inoculated and incubated as before. Growth was observed only in flasks containing methane alone. Nevertheless, as formaldehyde is the first stable intermediate of bromomethane oxidation it is possible that carbon assimilation could result from the oxidation pathway. Preliminary experiments using bromo[^{14}C]methane showed that carbon from the bromomethane was incorporated into cell material (Stirling, 1978). Therefore, for *M. capsulatus* (Bath) bromomethane is a non-growth substrate which can be fortuitously oxidized to provide some assimilable carbon (see Stirling & Dalton, 1979).

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