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In vivo ^{13}C NMR Investigations of Methanol Oxidation by the Obligate Methanotroph *Methylosinus trichosporium* OB3b

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In vivo ^{13}C NMR has been used to observe metabolism of exogenously supplied methanol by suspensions of *Methylosinus trichosporium* OB3b grown under a variety of conditions. Formaldehyde, formate and bicarbonate ions were the only metabolites of methanol to be detected. Accumulation of formaldehyde was observed only with suspensions grown under conditions which yield particulate, membrane-bound, methane mono-oxygenase (MMO). Ethyne abolished MMO activity, partially inhibited methanol oxidation in whole organisms, and prevented growth of the organism on methanol (1%, v/v) in batch culture. Oxidation of ethanol, a substrate of methanol dehydrogenase, was not affected by ethyne. Ethyne caused accumulation of formaldehyde in all suspensions of the organism incubated with methanol, although oxidation of exogenously added formaldehyde was not affected. These observations are consistent with the proposal that in *M. trichosporium* OB3b both MMO and methanol dehydrogenase oxidize exogenously supplied methanol and suggest that the further oxidation of formaldehyde is stimulated by the consumption of reducing equivalents by MMO.

INTRODUCTION

The oxidation by methanotrophs of methane to methanol is catalysed by MMO (Fig. 1) (Higgins *et al.*, 1981). Scott *et al.* (1981*a*, *b*) found that *Methylosinus trichosporium* OB3b possessed either soluble or particulate cell-free MMO depending on the growth conditions used. Particulate activity was found only when the organism was grown under oxygen limitation in shake flasks or in continuous culture under oxygen-limited, nitrate-excess conditions, whereas

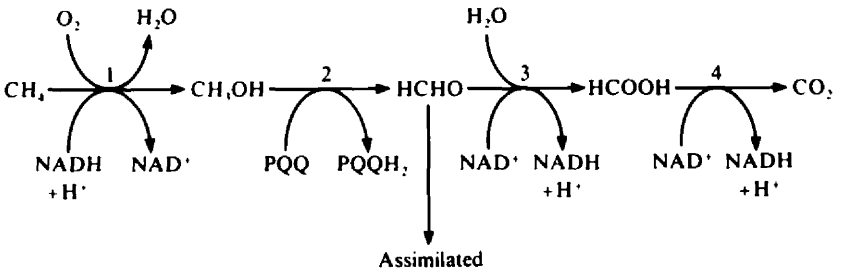

Fig. 1. A generalized scheme for methane oxidation in methanotrophs. 1, methane mono-oxygenase (MMO); 2, methanol dehydrogenase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; PQQ, pyrrolo quinoline quinone.

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Abbreviation: MMO, methane mono-oxygenase.

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soluble MMO was present in nitrate-depleted continuous cultures whether growth was oxygen- or methane-limited (Scott *et al.*, 1981*a*, *b*). Both MMOs required NADH for cell-free activity but had distinct inhibitor profiles (Scott *et al.*, 1981*a*). Stanley *et al.* (1983) have shown that for both *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath), the type of MMO is determined by the availability of cupric ions – organisms grown at a high Cu^{2+} : biomass ratio contain particulate MMO, whilst soluble MMO is present in organisms subjected to 'copper-stress' (Stanley *et al.*, 1983). The conditions used by Scott *et al.* (1981*a*, *b*) masked this underlying effect of copper.

Many methanotrophs are also able to use methanol as sole source of carbon and energy (Whittenbury *et al.*, 1970; Linton & Vokes, 1978; Hou *et al.*, 1979; Best & Higgins, 1981), although some tolerate only low concentrations of this alcohol (Leadbetter & Foster, 1958; Stocks & McCleskey, 1964; Whittenbury *et al.*, 1970). *Methylococcus* NCIB 11083 has been reported to accumulate formaldehyde during growth on methanol in batch culture (Linton & Vokes, 1978).

Methanol is a substrate for the soluble MMO of *Methylococcus capsulatus* (Bath) *in vitro* (Colby *et al.*, 1977), and *Methylosinus trischosporium* OB3b retains soluble MMO during growth on methanol (Best & Higgins, 1981). It is possible, therefore, that MMO contributes to methanol oxidation during growth on this 'unnatural' substrate, using reducing equivalents which might otherwise be available for biosynthesis (Fig. 1) (Anthony, 1982). This would be reflected in lower growth yields on methanol than would have been predicted.

Clearly, the manner in which methanotrophs metabolize exogenously supplied methanol requires further investigation. The technique of *in vivo* ^{13}C NMR is well suited to this purpose since it can be used to follow metabolism of methanol, formaldehyde and formate simultaneously using intact organisms (Hunter *et al.*, 1984).

In the present study we have investigated the metabolism of 98% enriched [^{13}C]methanol by *Methylosinus trichosporium* OB3b. By using enriched ^{13}C-labelled substrates the background spectrum due to the low natural abundance of ^{13}C (1·1%) is reduced to insignificant levels and consequently only ^{13}C-labelled metabolites are monitored. In this way it has been possible to study the metabolism by *Escherichia coli* of glucose (Ugurbil *et al.*, 1978) and formaldehyde (Hunter *et al.*, 1984), and methanol metabolism by acetogens (Kerby *et al.*, 1983). Our results reveal that the pattern of metabolite accumulation by suspensions oxidizing methanol is profoundly dependent on the conditions under which the organism is grown.

METHODS

Organisms. Methylosinus trichosporium OB3b was originally isolated by Professor R. Whittenbury, Department of Biological Sciences, University of Warwick, UK. The facultative methanol-utilizers *Pseudomonas* AM1 and *Pseudomonas extorquens* were obtained from Professor J. R. Quayle, Department of Biochemistry, University of Sheffield, UK, and Professor D. E. F. Harrison, Biological Laboratory, University of Kent, UK, respectively.

Medium. A nitrate salts medium (NSM) was used throughout these studies. This had the following composition (g per litre of distilled water): $NaNO_3$, 0·85; KH_2PO_4, 0·53; Na_2HPO_4, 0·86; K_2SO_4, 0·17; $MgSO_4.7H_2O$, 0·037; $CaCl_2.2H_2O$, 0·007. Trace elements (2 ml per litre of medium) were added as a solution prior to autoclaving. The solution contained in 1 litre distilled water (g): $ZnSO_4.7H_2O$, 0·287; $MnSO_4.4H_2O$, 0·223; H_3BO_3, $6{\cdot}2 \times 10^{-2}$; $CuSO_4.5H_2O$, 0·125; $NaMoO_4.2H_2O$, $4{\cdot}8 \times 10^{-2}$; $CoCl_2.6H_2O$, $4{\cdot}8 \times 10^{-2}$; KI, $8{\cdot}3 \times 10^{-2}$. H_2SO_4 (1 mM), 1 ml l^{-1}. $FeSO_4.7H_2O$ ($1{\cdot}12 \times 10^{-2}$ g per litre of medium) in 1 M-HCl was added after sterilization by autoclaving.

Growth on methane. Methylosinus trichosporium OB3b was grown on methane in continuous culture at 30 °C under conditions yielding organisms containing either entirely soluble or entirely particulate cell-free MMO. Organisms containing soluble MMO were grown under oxygen limitation in a 4 litre LKB Ultroferm fermenter using the following conditions: methane, 100 ml min^{-1}; air, 400 ml min^{-1}; impeller speed, 700 r.p.m.; dilution rate, 0·04 h^{-1}; biomass density, 1·6–1·8 g dry wt l^{-1}. Organisms containing particulate cell-free MMO were grown using the conditions described above but with the concentration of $CuSO_4$ in the medium increased from 1 to 5 μM. Alternatively, organisms with particulate MMO were grown under oxygen-limitation in a 10 litre LKB Ultroferm fermenter under the following conditions: methane, 250 ml min^{-1}; air, 60 ml min^{-1}; impeller speed, 500 r.p.m.; dilution rate, 0·02 h^{-1}; biomass density, 0·15–0·2 g dry wt l^{-1}.

Growth on methanol. Methylosinus trichosporium OB3b was grown on methanol (1%, v/v) using a modification of the method described by Best & Higgins (1981). A methane-grown plate culture (7 d) was resuspended in 5 ml NSM. Samples (0·01–0·1 ml) of the suspension were used to inoculate 250 ml conical flasks containing NSM

(50 ml) and methanol (0·5 ml) was added to a centre-well. The flasks were plugged with cotton wool, sealed with aluminium foil and shaken at 140 r.p.m. at 30 °C. Growing cultures were used to inoculate 2 litre flasks containing NSM (500 ml) and methanol (1 %, v/v) was added directly to the medium. Growth was followed by measuring the optical density of the cultures at 600 nm.

Preparation of organisms for assays. Organisms were harvested by centrifugation (4500 ***g***, 20 min, 4 °C). The pellets were washed once with buffer (20 mM-sodium phosphate, 5 mM-$MgCl_2$, pH 7·0) and resuspended in the same buffer to give the required density (see below). Suspensions were stored on ice and used in assays within 8 h of harvesting.

Effect of ethyne on the growth of organisms on methanol. Methanol-grown *M. trichosporium* OB3b was inoculated into 250 ml Buchner flasks containing NSM (50 ml) and methanol (1 %, v/v) and stoppered with rubber bungs. Ethyne (20 ml) was injected through a bacteriological filter (0·2 μm) attached to the side-arm, which was then clamped. Control cultures were not injected with ethyne. As a further control, *Pseudomonas* AM1 and *P. extorquens* were grown under identical conditions in either the presence or absence of ethyne. Flasks were shaken at 140 r.p.m. at 30 °C.

Measurement of oxygen uptake. Substrate-stimulated oxygen consumption was measured at 30 °C using a jacketed Clarke oxygen electrode (Rank Bros, Bottisham, Cambridge, UK) containing: 20 mM-sodium phosphate buffer, pH 7·0, 5 mM $MgCl_2$, 2·8 ml; cell suspension (10–12 mg dry wt ml^{-1}), 0·1 ml; and methane-saturated buffer, 0·1 ml or methanol (300 mM), 0·1 ml. At 30 °C the solubility of methane in water is 1·23 mM (International Critical Tables of Numerical Data). Rates measured in the presence of substrate were corrected for endogenous oxygen consumption.

MMO activity in whole organisms. MMO activity was measured using 1 ml of cell suspension (10–12 mg dry wt) by following the epoxidation of propene (Stirling & Dalton, 1979), over a time course (15 min) in the presence of sodium formate (20 mM) as a source of reducing equivalents. Suspensions were shaken (130 oscillations min^{-1}) at 30 °C in 10 ml conical flasks. Propene gas (2 ml) was injected through Suba-seals. Propene oxide was measured by gas chromatography using a glass column (2·1 m × 4 mm i.d.) of Porapak Q in a Pye Unicam 204 instrument fitted with a flame ionization detector (nitrogen flow rate, 40 ml min^{-1}; oven temperature, 140 °C).

MMO activity in cell-free extracts. Soluble and particulate fractions of *M. trichosporium* OB3b were prepared and stored as described previously (Scott *et al.*, 1981*a*). Cell-free MMO activity was measured by following the epoxidation of propene in the presence of 5 mM-NADH (Scott *et al.*, 1981*a*).

Inhibition of MMO activity by ethyne. Suspensions (1 ml) of *M. trichosporium* OB3b were shaken at 30 °C in 10 ml conical flasks in the presence of ethyne (2 ml gas, injected through Suba-seals) for 10 min. These organisms were then used immediately for oxygen electrode, MMO or substrate-disappearance assays. MMO activity could not be restored by sparging ethyne-treated organisms with air (25 ml min^{-1}, 60 min).

Methanol and ethanol oxidation. This was measured by following substrate disappearance using gas chromatography. Reactions were carried out in 10 ml conical flasks containing: cell suspension (2·5–10 mg dry wt ml^{-1}), 1 ml; and methanol (20 mM) or ethanol (20 mM). The flasks were plugged with cotton wool and shaken (130 oscillations min^{-1}) at 30 °C. Samples were analysed at 4 min intervals over 20–40 min. Substrates were measured in a Pye-Unicam 204 gas chromatograph fitted with a flame ionization detector and compared to standards using a Pye-Unicam CDP4 computing integrator. Methanol was analysed using a glass column (2·1 m × 4 mm i.d.) of 20 % (w/w) Carbowax 20M at 110 °C. Ethanol was analysed using a glass column (2·1 m × 4 mm i.d.) of Porapak Q at 140 °C. Nitrogen was used as a carrier gas at 40 ml min^{-1} in both cases.

Assay of pure methanol dehydrogenase. Methanol dehydrogenase from *M. trichosporium* OB3b was a gift from Dr D. J. Best, Biotechnology Centre, Cranfield Institute of Technology. The enzyme preparation was greater than 95 % pure as judged from analysis by SDS-PAGE. Activity was assayed polarographically as described by Dunstan *et al.* (1972).

In vivo ^{13}C *NMR experiments.* 100·6 MHz ^{13}C NMR spectra were obtained with a Bruker WH400 spectrometer. Generally, 125 or 250 transients were acquired, using a 45° pulse, and 8096 data points over 25000 Hz spectral width. Low-level (0·5 W) proton irradiation was used for 1·8 s before each pulse to build up nuclear Overhauser enhancement, and high-level (3 W) irradiation was used during the pulse and acquisition to ensure good proton decoupling. Measurements were made on suspensions of *M. trichosporium* OB3b (2 ml, 20–36 mg dry wt in 10 mm diameter tubes) containing 20 % (v/v) D_2O to provide a lock signal. The temperature was maintained at 30 °C and aeration (10 ml min^{-1}) *in situ* was achieved with a drawn-out pipette which was fixed in place with glass wool. Before Fourier transformation, 15–30 Hz line-broadening was applied to the signal to improve the signal-to-noise ratio. Chemical shifts, δ (p.p.m.), were measured using formaldehyde, 83·2 δ, as secondary standard (Hunter *et al.*, 1984).

In these experiments, the viable count of the suspensions was $1·2 \times 10^{10}$ ml^{-1}. No significant loss of viability was observed when samples were incubated with 10 mM-methanol and aerated in the spectrometer for 1 h.

Materials. Chemicals were obtained from BDH and Sigma. Methanol (98 % ^{13}C) was obtained from Prochem (London SW19, UK). [^{13}C]Formaldehyde was prepared from paraformaldehyde (91 % ^{13}C) as described previously (Hunter *et al.*, 1984).

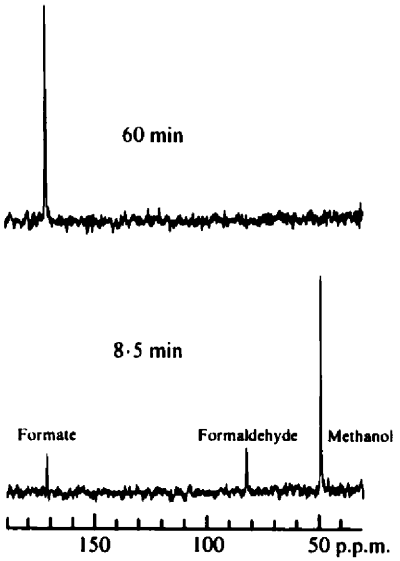

Fig. 2. 100·6 MHz ^{13}C NMR spectra of an aerated suspension of *M. trichosporium* OB3b incubated with 10 mM-[^{13}C]methanol. The organism was grown on methane under oxygen limitation in continuous culture (dilution rate, 0·02 h^{-1}; cell density, 0·15 g dry wt l^{-1}; Cu^{2+}, 1 μM) and contained only particulate MMO. For each spectrum, 250 transients were collected up to the times indicated. The density of the suspension was 13·3 mg dry wt ml^{-1}.

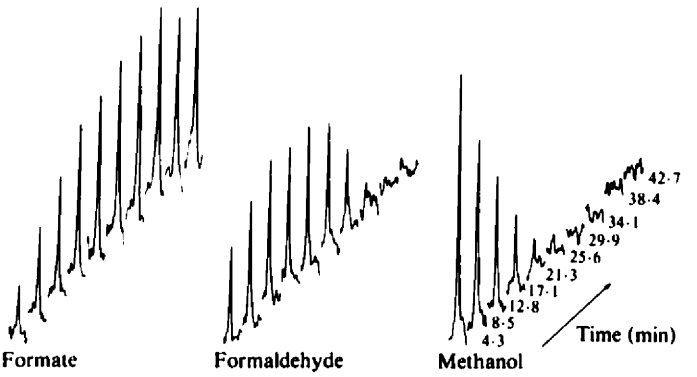

Fig. 3. Time course showing metabolism of [^{13}C]methanol (10 mM) by a suspension of *M. trichosporium* OB3b. The organism was grown under the conditions given in Fig. 2, and contained only particulate MMO. The figure shows the intensities of the ^{13}C NMR signals of methanol, formaldehyde and formate. For each spectrum 125 transients were collected between the times indicated.

RESULTS

Oxidation of [^{13}C]*methanol*

The proton decoupled ^{13}C NMR spectrum of 10 mM aqueous solutions of [^{13}C]methanol contained only a singlet at 50·1 δ. No metabolism was observed when suspensions of *M. trichosporium* OB3b were incubated with 10 mM-[^{13}C]methanol in the spectrometer without aeration. This was true irrespective of the conditions under which the organism had been grown. Metabolism of [^{13}C]methanol was observed when spectra were recorded during aeration of suspensions within the spectrometer, although the pattern of synthesis of metabolites was dependent on the conditions used to grow the organism.

Fig. 2 shows the metabolism of [^{13}C]methanol (10 mM) by aerated suspensions of *M. trichosporium* OB3b grown on methane in continuous culture (cell density, 0·15 g dry wt l^{-1};

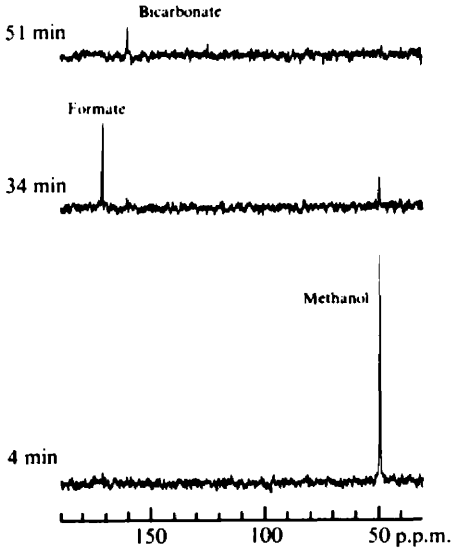

Fig. 4. 100·6 MHz ^{13}C NMR spectra of an aerated suspension of *M. trichosporium* OB3b incubated with 10 mM-[^{13}C]methanol. The organism was grown on methane under oxygen limitation in continuous culture (dilution rate, 0·04 h^{-1}; cell density, 1·7 g dry wt l^{-1}; Cu^{2+}, 1 μM) and contained only soluble MMO. For each spectrum, 125 transients were collected between the times indicated. The density of the suspension was 15·6 mg dry wt ml^{-1}.

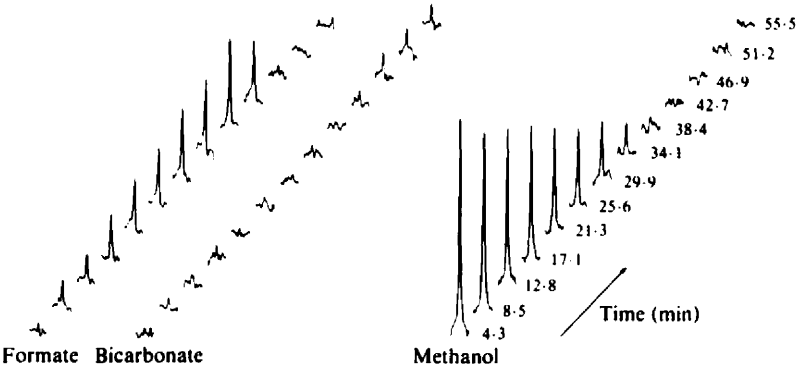

Fig. 5. Time course of the experiment of Fig. 4, showing the intensities of the ^{13}C NMR signals of methanol, formate and bicarbonate. Formaldehyde was not detected at any time. For each spectrum 125 transients were collected.

Cu^{2+}, 1 μM) under conditions which yield organisms with particulate MMO. On aeration, two signals, in addition to methanol, were observed which were identified by their chemical shifts as formaldehyde hydrate (83·2 δ) and formate (172·2 δ). Fig. 3 shows successive ^{13}C NMR spectra recorded at 4 min intervals. All the methanol was consumed within 25 min of starting aeration. The concentration of formaldehyde rose then fell, while formate accumulated and was in turn consumed with continued aeration. Formaldehyde and formate accumulated in a linear fashion when similar suspensions were incubated with 50 mM-[^{13}C]methanol (not shown).

Suspensions of *M. trichosporium* OB3b grown on methane in continuous culture under conditions yielding soluble MMO (cell density, 1·7 g dry wt l^{-1}; Cu^{2+}, 1 μM) never produced detectable amounts of formaldehyde when incubated with [^{13}C]methanol. This was true even when the initial concentration of methanol was as high as 250 mM (1%, v/v). These suspensions oxidized methanol (10 mM) through to formate (Fig. 4) which rapidly disappeared once all the methanol had been consumed (Fig. 5). A low-intensity signal at 161·2 δ due to bicarbonate ions

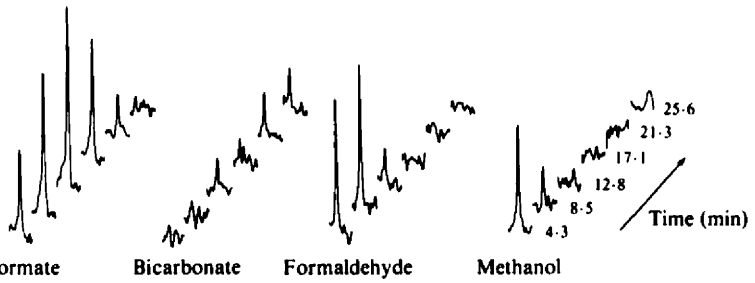

Fig. 6. 100·6 MHz ^{13}C NMR spectra showing metabolism of methanol (10 mM) by an aerated suspension of *M. trichosporium* OB3b grown on methane at high cell density on copper-supplemented medium. The organism was grown under oxygen limitation in continuous culture (dilution rate, 0·04 h^{-1}; cell density, 2 g dry wt l^{-1}; Cu^{2+}, 5 μM) and contained only particulate MMO. After starting aeration, 125 transients were collected for each spectrum. The density of the suspension was 14 mg dry wt ml^{-1}.

was observed (Figs 4 and 5). Formate continued to accumulate and reach a steady state when [^{13}C]methanol was added at an initial concentration of 250 mM (see Fig. 9).

Methylosinus trichosporium OB3b was also grown on methane in continuous culture under identical conditions to those used to produce organisms containing soluble MMO, except that the concentration of Cu^{2+} in the NSM feedstock was raised from 1 to 5 μM (cell density, 2·0 g dry wt l^{-1}). On increasing the Cu^{2+}:biomass ratio MMO activity was found to be located in the particulate membrane fraction of extracts as originally discovered by Stanley *et al.* (1983). Organisms grown under these high-copper conditions produced both formaldehyde and formate when aerated with 10 mM-[^{13}C]methanol (Fig. 6). The pattern of methanol metabolism observed was essentially the same as that found with suspensions grown at low density (0·15 g dry wt l^{-1}; Cu^{2+}, 1 μM) which also contained particulate MMO (Fig. 3). For each set of growth conditions the pattern of metabolism was reproducible.

Suspensions of methanol-grown *M. trichosporium* OB3b never produced detectable amounts of formaldehyde when incubated with 10 mM-[^{13}C]methanol, and the observed rates of methanol disappearance were generally lower than those obtained with methane-grown suspensions. The most active methanol-grown suspensions tested were prepared from a batch culture harvested in mid-exponential phase (cell density, 0·08 g dry wt l^{-1}). This suspension removed all the [^{13}C]methanol (10 mM) after 33 min but accumulation of formaldehyde or formate was not observed. Suspensions prepared from late-exponential phase cultures (cell density, 0·4 g dry wt l^{-1}) took 2–3 h to oxidize the same initial concentration of [^{13}C]methanol and produced small amounts of formate.

Formaldehyde, formate and occasionally bicarbonate were the only metabolites of methanol observed in any of the experiments described above. The signals were present in the supernatant following centrifugation of the suspensions, indicating that these labelled metabolites were outside the cells.

Metabolism of [^{13}C] *formaldehyde*

The pattern of metabolism of 10 mM-[^{13}C]formaldehyde by suspensions of *M. trichosporium* OB3b was the same irrespective of the conditions under which the organism had been grown or the carbon source used. Aerated suspensions removed all the formaldehyde, producing formate which subsequently disappeared. No metabolism was observed in the absence of aeration, and formaldehyde and formate were the only ^{13}C-labelled species detected in these experiments.

Effects of ethyne on the metabolism of methanol

The results of the *in vivo* ^{13}C NMR studies described above indicate that the production of formaldehyde by suspensions of *M. trichosporium* OB3b incubated with [^{13}C]methanol correlates with the presence of particulate MMO. These findings prompted us to investigate the possibility that either or both MMOs participate in the oxidation of exogenously supplied

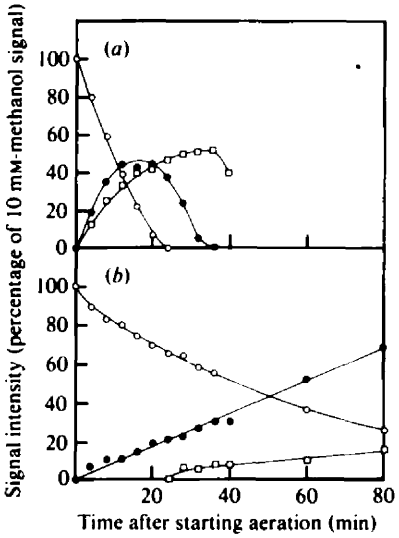

Fig. 7

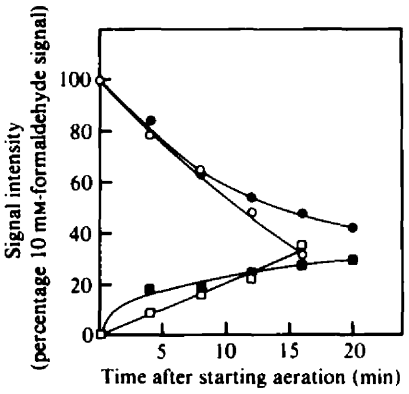

Fig. 8

Fig. 7. Effect of ethyne on the metabolism of [^{13}C]methanol (10 mM) by a suspension of *M. trichosporium* OB3b grown on methane at low cell density. The organism was grown under oxygen limitation in continuous culture (cell density, 0·2 g dry wt l^{-1}; dilution rate, 0·02 h^{-1}; Cu^{2+}, 1 μM) and contained only particulate MMO. The signal intensities of methanol (○), formaldehyde (●) and formate (□) are shown in successive ^{13}C NMR spectra recorded at intervals after starting aeration; 125 transients were collected for each spectrum. The experiment was carried out on untreated (*a*) and ethyne-treated suspensions (*b*). The density of the suspension was 17·3 mg dry wt ml^{-1}.

Fig. 8. Effect of ethyne on the metabolism of [^{13}C]formaldehyde (10 mM) by suspensions of *M. trichosporium* OB3b grown at low cell density. The suspensions were from the same batch of organisms as used in the experiments of Fig. 7. The intensities of ^{13}C NMR signals of formaldehyde (○) and formate (□) in suspensions treated with ethyne, and of formaldehyde (●) and formate (■) in suspensions of untreated organisms are shown; 125 transients were collected for each spectrum.

methanol. We therefore examined the effect of ethyne, a potent inhibitor of both types of MMO (Scott *et al.*, 1981*a*, Stanley *et al.*, 1983), on methanol metabolism.

In vivo ^{13}C NMR investigations. Fig. 7 illustrates the effect of ethyne on the metabolism of [^{13}C]methanol by suspensions of *M. trichosporium* OB3b which contained only particulate MMO (Cell density, 0·2 g dry wt l^{-1}; Cu^{2+}, 1 μM). It is clear that ethyne treatment reduced the rate of oxidation of methanol and caused accumulation of formaldehyde (Fig. 7). The latter effect cannot be attributed to inhibition of formaldehyde oxidation by ethyne, since the rate of removal of exogenous formaldehyde was similar whether or not the organisms were treated with ethyne (Fig. 8). Metabolism of methanol by suspensions containing soluble MMO (cell density, 1·8 g dry wt l^{-1}; Cu^{2+}, 1 μM) was also modified by exposure to ethyne. Whereas suspensions of organisms from these growth conditions never produced formaldehyde, even when incubated with 250 mM ^{13}C-methanol, it accumulated in suspensions pre-treated with ethyne (Fig. 9).

Similarly, ethyne reduced the rate of methanol oxidation by suspensions which had been grown at a high biomass density and contained particulate MMO (cell density, 1·8 g dry wt l^{-1}; dilution rate, 0·04 h^{-1}; NSM supplemented with Cu^{2+}, 5 μM). The rates of formation of formaldehyde and formate were also lowered, although both these metabolites accumulated to a higher level before they were removed. Ethyne had no effect on the removal of exogenous [^{13}C]formaldehyde (10 mM) by these organisms.

Growth experiments. Methylosinus trichosporium OB3b would not grow on methanol (1%, v/v) in batch culture under an atmosphere of ethyne/air (1:10, v/v). Growth of the facultative methanol-utilizers, *Pseudomonas* AM1 and *Pseudomonas extorquens*, was not affected under the same conditions.

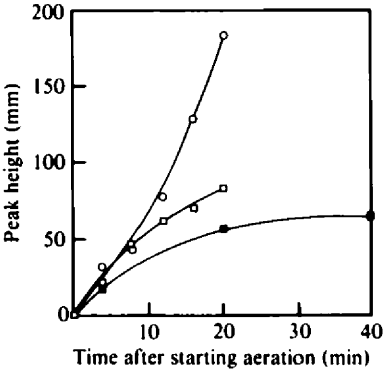

Fig. 9. Effect of ethyne on the production of formaldehyde and formate by aerated suspensions of *M. trichosporium* OB3b incubated with 250 mM-[^{13}C]methanol. These organisms were grown on methane under oxygen limitation in continuous culture (cell density, 1·8 g dry wt l^{-1}; dilution rate, 0·04 h^{-1}; Cu^{2+}, 1 μM) and contained only soluble MMO. Ethyne-treated organisms produced formaldehyde (○) and formate (□) while in suspensions of untreated organisms only formate accumulated (■). For each spectrum 125 transients were collected. The density of the suspension was 10 mg dry wt ml^{-1}.

Table 1 *Effect of ethyne on the oxidation of propene, methane, methanol and ethanol by M. trichosporium OB3b*

Washed suspensions of methane-grown *M. trichosporium* OB3b were untreated (−) or exposed to ethyne (20%, v/v; 10 min) (+). Bacterial concentrations are given in Methods. Methane, methanol and ethanol oxidation were measured polarographically. Rates are given as nmol O_2 consumed min^{-1} (mg dry wt)$^{-1}$ and are corrected for endogenous activity which was 0–5 nmol O_2 consumed min^{-1} (mg dry wt)$^{-1}$. Propene oxidation was measured by gas chromatography in the presence of sodium formate (20 mM) as a reducing source. The rate is given as nmol propene oxide produced min^{-1} (mg dry wt)$^{-1}$.

Growth condition	MMO location	Propene		Methane		Methanol		Ethanol	
		−	+	−	+	−	+	−	+
High biomass, 1 μM-copper	Soluble	96·2	ND	46·5	ND	49·4	20·8	15·8	15·4
High biomass, 5 μM-copper	Particulate	113·5	ND	66·6	ND	77·0	27·6	28·1	26·1
Low biomass, 1 μM-copper	Particulate	105·0	ND	64·2	ND	77·4	25·6	NT	NT

ND, Not detectable; NT, not tested.

Effect of ethyne on propene, methane, methanol and ethanol oxidation. Table 1 shows the effect of ethyne on the oxidation of propene, methane, methanol and ethanol by suspensions of *M. trichosporium* OB3b grown under a variety of conditions. In all cases, preincubation of organisms with ethyne (20%, v/v; 10 min) abolished both propene oxidation and methane-dependent oxygen consumption (Table 1). Methanol-dependent oxygen consumption was partially inhibited, suggesting that, at a concentration of 10 mM, a significant proportion of the methanol was oxidized by an ethyne-sensitive enzyme system. This was confirmed by comparing the initial rates of disappearance of methanol and ethanol measured using gas chromatography. The rate of methanol disappearance was partially inhibited (45%) by ethyne whereas ethanol oxidation was not affected, suggesting that ethanol is oxidized only by the non-specific methanol (alcohol) dehydrogenase. The rate of ethanol disappearance was similar to the ethyne-inhibited rate of methanol disappearance. This suggests that methanol dehydrogenase was responsible for the ethanol and ethyne-insensitive methanol oxidation.

Effect of ethyne on pure methanol dehydrogenase from M. trichosporium OB3b. Pure methanol dehydrogenase was not affected by preincubation with ethyne. The rate of methanol oxidation

was 3·2 ± 0·2 μmol O_2 consumed min^{-1} (mg protein)$^{-1}$ (mean of five measurements on the same preparation, ± SEM), whether or not the enzyme was treated with ethyne.

DISCUSSION

The results presented here show that using *in vivo* ^{13}C NMR it is possible to observe simultaneously metabolism of methanol and its oxidation products by suspensions of *M. trichosporium* OB3b. This method allows metabolites to be readily identified from their chemical shifts and offers excellent time resolution. Previous *in vivo* studies on metabolism of one-carbon compounds (Kerby *et al.*, 1983; Hunter *et al.*, 1984) have looked at slow metabolic processes which could only be monitored satisfactorily with a time resolution of about 20 min per spectrum. In the present work our measuring time per spectrum was only 4 min, allowing us to follow easily the accumulation and consumption of metabolites in less than 30 min. Using the present accumulation time, the lower limit of detection for both formaldehyde and formate is approximately 1 mM. The use of larger sample tubes and higher fields may enable sensitivity to be increased by an order of magnitude whilst polarization transfer techniques should result in even further improvement.

The acquisition parameters necessary to observe one-carbon compounds against the macromolecular background do not yield signal intensities which correspond exactly to relative concentrations (Hunter *et al.*, 1984). Nevertheless, the method does allow metabolic patterns to be compared and the results show that the mode of methanol metabolism observed varies according to the conditions used to grow the organism. Calibration of signal intensities using standards of known proportion could, in principle, give more quantitative results. No intermediates of the serine pathway of formaldehyde assimilation were detected in any of these experiments although they have been readily measured in methanotrophs using ^{14}C-labelling methods (Lawrence *et al.*, 1970). The failure to observe such assimilation products results from their low concentration.

Ethyne has been reported to be an inhibitor of MMO in a number of methanotrophs (Dalton & Whittenbury, 1976; Stirling & Dalton, 1977; Scott *et al.*, 1981*a*; Stanley *et al.*, 1983), yet it has been reported not to affect methanol oxidation (Dalton & Whittenbury, 1976; Stirling & Dalton, 1977). The results given here indicate that ethyne is a specific inhibitor of both soluble and particulate MMOs of *M. trichosporium* OB3b and that these enzymes play a significant role in the oxidation of exogenously supplied methanol. Most of our experiments were carried out using 10 mM (0·04%, v/v) methanol, which is well below the toxic threshold for the organism and below the concentration routinely used to support growth in batch culture (Best & Higgins, 1981). Our observations are therefore likely to provide an accurate reflection of the events that occur when methane-grown suspensions of *M. trichosporium* OB3b are inoculated into batch cultures containing methanol, but they may not be applicable to methanol-limited continuous cultures in which the extracellular methanol concentration is very low.

In vivo ^{13}C NMR has allowed us to observe unexpected features of methanol metabolism in *M. trichosporium* OB3b which support the conclusion that MMO oxidizes exogenous methanol. One observation, which appears anomalous at first sight, is that while ethyne inhibits the rate of methanol oxidation it also causes formaldehyde and formate to accumulate to higher levels. If it is accepted that MMO is involved in the oxidation of exogenously added methanol then these findings may be readily explained in terms of Fig. 1. During growth on methane, MMO requires reducing equivalents which are supplied by the further oxidation of methanol. By oxidizing exogenous methanol, MMO would act as a sink for the reducing equivalents which are generated by further oxidation of formaldehyde and/or formate. After inhibition of MMO with ethyne, formaldehyde would continue to be produced by way of methanol dehydrogenase, although at a lower rate. Under these circumstances MMO would no longer contribute to the regeneration of NAD^+, and the activities of NAD^+-linked formaldehyde and formate dehydrogenases would be largely dependent on the activity of NADH dehydrogenase. This activity is probably low in methanotrophs (Anthony, 1982), hence formaldehyde accumulates.

A further unexpected, and as yet unexplained finding is that untreated suspensions (i.e. not exposed to ethyne) which are oxidizing methanol produce significant amounts of formaldehyde only if they contain particulate MMO. Whereas the soluble MMO has an absolute requirement for NADH, recent evidence suggests that particulate MMO may also obtain reducing equivalents from non-NAD^+-linked alcohol or aldehyde dehydrogenases (Leak & Dalton, 1983). If this is the case, then the lower demand for NADH during methanol oxidation by the latter enzyme might result in depletion of NAD^+ and accumulation of formaldehyde. In order to fully explain these findings it will be necessary to compare the carbon flux through each segment of the oxidation and/or assimilation pathway(s) *in vivo* using organisms grown under the conditions defined here. This is currently under investigation.

It is clear from the present work that the capacity to produce significant concentrations of formaldehyde depends on the physiological status of the organism at the time of the methanol challenge. This may explain previous difficulties encountered in adapting methanotrophs to grow on methanol (Linton & Vokes, 1978; Hou *et al.*, 1979; Best & Higgins, 1981).

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