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Oxygenation of Methane by Methane-Grown *Pseudomonas methanica* and *Methanomonas methanooxidans*

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1. Experimental conditions have been found in which small amounts of methanol (approximately 2.5mM) accumulated when washed cell suspensions of methane-grown *Pseudomonas methanica* and *Methanomonas methanooxidans* were incubated with methane+oxygen mixtures in Warburg flasks. 2. The methanol formed could be separated completely from water by fractional distillation through glass helices followed by gas chromatography using 20% polyethylene glycol 400 on a Celite 545 support. 3. By using ^{18}O-enriched oxygen gas the abundance of ^{18}O in the methanol formed from oxidation of methane was measured with a Perkin–Elmer 270 combined gas chromatograph/mass spectrometer. The results showed that the oxygen in methanol was derived exclusively from gaseous oxygen in both micro-organisms. 4. Control experiments using [^{18}O]water in incubation mixtures confirmed that there was negligible incorporation of the oxygen atom from water into methanol.

Evidence from studies with whole cells (Brown, Strawinski & McCleskey, 1964) and with cell-free extracts (Johnson & Quayle, 1964) indicates that the route of methane oxidation by methane-utilizing bacteria is as follows:

$$CH_4 \rightarrow CH_3 \cdot OH \rightarrow H \cdot CHO \rightarrow H \cdot CO_2H \rightarrow CO_2$$

Johnson & Quayle (1964) reported enzymes in such bacteria that can catalyse the stepwise oxidation of methanol to carbon dioxide, but virtually nothing is known about the mechanism of the first step, the oxidation of methane to methanol. By analogy with oxidation of higher alkanes (Peterson, Kusunose, Kusunose & Coon, 1967), a possible reaction might be one which is mediated by a mixed-function oxidase (mono-oxygenase):

$$CH_4 + O_2 + XH_2 \rightarrow CH_3 \cdot OH + H_2O + X \quad (1)$$

where XH_2 is a reducing agent. Another possibility might be an oxygenase system in which two molecules of methane are oxygenated in the overall reaction:

$$2CH_4 + O_2 \rightarrow 2CH_3 \cdot OH \quad (2)$$

The evidence in favour of the operation of an oxygenase is, however, only indirect: Leadbetter & Foster (1959) grew *Pseudomonas methanica* in the presence of $^{18}O_2$ and found that if the carbon source was methane, the incorporation of ^{18}O into cell material was 16 times that found when the micro-organism was grown on methanol.

On the other hand, there is indirect experimental evidence which argues against the major involvement of a mono-oxygenase. Brown *et al.* (1964), using suspensions of *Methanomonas methanooxidans*, found that the presence of 3mM-iodoacetate during oxidation of methane caused at least 75% of the methane consumed to accumulate as methanol, and that this concentration of iodoacetate inhibited methanol oxidation without inhibiting methane oxidation. Whittenbury (1969) has pointed out that since the further oxidation of methanol would be the only source of reducing power for a mono-oxygenase reaction, the results of Brown *et al.* (1964) could be inconsistent with the involvement of such an enzyme. Further, the consumption of reducing power by a mono-oxygenase might result in the cell yield of a methane-grown organism being less than that of the same organism grown on methanol. Whittenbury (1969) stated that cell yields measured in his laboratory on methane as carbon source were approximately twice those obtained on equimolar amounts of methanol, and hence he suggested that the transformation of methane into methanol is energy-yielding and might be a hydroxylation reaction, yielding methanol and two electrons for energy transduction:

$$CH_4 + H_2O \rightarrow CH_3 \cdot OH + 2[H] \quad (3)$$

It is thus evident that the fundamental nature of the bacterial transformation of methane into methanol is not at all clear and calls for direct

experimentation using ^{18}O as tracer. The present paper records the results of such a study.

MATERIALS AND METHODS

Maintenance and growth of micro-organisms. *Pseudomonas methanica* and *Methanomonas methanooxidans* were grown in mineral salts media as described by Leadbetter & Foster (1958) and Jayasuriya (1955) respectively. *P. methanica* was maintained on agar slopes, *M. methanooxidans* in 10ml liquid cultures. Agar slopes and 10ml starter cultures were incubated at 30°C in large desiccators gassed with methane+air (50:50). The gas mixture was sterilized by passage through a sterile cotton-wool filter attached to the desiccator inlet tap. After 2–3 days growth the starter cultures were used to inoculate 50ml volumes of sterile medium in 250ml conical flasks. The latter cultures were used to inoculate 500ml volumes of sterile medium in 2-litre conical flasks after 2 days growth. Both sets of flasks were gassed with methane+air (50:50) and shaken on a rotary shaker at 30°C.

Preparation of cell suspensions. After 2–3 days growth in 2-litre conical flasks, cells were harvested by centrifugation (5000*g* for 20min at 5°C), washed once with 20mM-potassium phosphate buffer, pH7.0, and resuspended in the same buffer.

Special chemicals. Methane, used as a routine growth substrate, was obtained from Middlesex County Council Main Drainage Department, Isleworth, Middx., U.K. Typical analysis of the gas gave the following values: methane, 95–97%; CO_2, 3–4%; O_2, 0.1–0.2%; N_2, 0.3–0.4%. Pure methane for experiments with washed cells was obtained as 99.99% methane from Air Products Ltd., New Malden, Surrey, U.K. [^{18}O]Methanol (77.0 atom % excess of ^{18}O), $^{18}O_2$ (55.93 atom % excess of ^{18}O) and [^{18}O]water (23.06 atom % excess of ^{18}O) were purchased from Miles–Yeda Ltd., Rehovoth, Israel.

Measurement of gas uptake. Uptake of methane+O_2 mixtures by washed-cell suspensions was measured by conventional Warburg manometry with 10% (w/v) NaOH in the centre well to absorb CO_2. The gas mixture was usually methane+O_2 (70:30), and an absorption coefficient of 0.03 was assumed for both methane and O_2. Unless stated otherwise, incubation mixtures contained 200μmol of potassium phosphate buffer, pH7.3, and approx. 9 and 4mg dry wt. of cells for *M. methanooxidans* and *P. methanica* respectively, in a total volume of 2.8ml. After incubation at 30°C for 3h the reactions were stopped by cooling the incubation mixtures to 0°C. These were the optimum experimental conditions for accumulation of methanol.

Methanol determination. Methanol was assayed colorimetrically by oxidation to formaldehyde followed by reaction of the latter compound with chromotropic acid (Feldstein & Klendshoj, 1954). A standard curve was prepared by using standard solutions of pure methanol.

Preparation of methane+[^{18}O]oxygen mixtures. $^{18}O_2$ was transferred from sealed ampoules to storage bottles by using a Toepler pump (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). The displacement fluid in the Toepler pump and storage bottles was aq. 5% (w/v) $CuSO_4,5H_2O$ solution, previously freed of O_2. The $CuSO_4$ was present as a biocide. After transfer of the $^{18}O_2$ into the storage bottle, a measured amount of methane was admitted into the same storage bottle to give the required gas mixture. The latter was then introduced into Warburg manometers containing the bacterial suspensions, after the manometers had been first flushed with pure methane and evacuated to a pressure of 75mmHg, by allowing the gas mixture to enter the manometers until atmospheric pressure was regained.

Preparation of samples for g.l.c.–mass spectrometry. After manometry the suspension mixtures in the Warburg cups were immediately placed on ice before removal of the cells by centrifugation (30000*g* for 20min at 0°C). The methanol contained in the supernatant after centrifugation was concentrated 10–15-fold by fractional distillation through a column (10cm×1cm) one-third filled with glass helices, the first few drops of the low-boiling fraction being collected.

Measurement of the abundance of ^{18}O in methanol in dilute aqueous solution. The abundance of ^{18}O in methanol was determined in 1μl samples injected by syringe into a Perkin–Elmer 270 combined gas chromatograph–mass spectrometer (Perkin–Elmer Ltd., Beaconsfield, Bucks., U.K.). The separation of methanol from water was effected by g.l.c. by using a coiled glass column (1.83m×2.0mm internal diam.) packed with 20% (w/w) polyethylene glycol 400 (Phase Separations Ltd., Queensferry, Flints., U.K.) on 100–120-mesh acid-washed Celite 545 (Diatomite C; Pye Unicam Ltd., Cambridge, U.K.). Analyses were made under the following conditions: column temperature, 80°C; injector temperature, 120°C; helium inlet pressure, 81b/in^2; glass frit molecular separator temperature, 265°C; ion-source temperature, 220°C. Mass spectra were recorded at 20eV with an accelerating voltage of 2kV and a filament emission current of 90μA. The methanol peaks, which were displayed on a pen recorder monitoring total ion current, were scanned at the apex by using an ultraviolet recording scan speed of 10s/decade.

Fig. 1 shows line diagrams of part of the methanol mass spectra for [^{16}O]methanol and for ^{18}O-enriched methanol obtained by injecting 1μl of the appropriate aqueous methanol solution (30mM) into the Perkin–Elmer 270 instrument. [^{16}O]Methanol gave ions at mass 29, 31, 32 and [^{18}O]methanol gave ions at mass 31, 33 and 34. The mass 31 and 33 peaks were chosen to calculate the abundance of [^{18}O]methanol as the background was always relatively lowest for these peaks; this background was recorded immediately before injection into the column and was subtracted from the corresponding methanol peaks. The mass 33 peak is due entirely to [^{18}O]methanol in the mixture but both methanol species contribute to the mass 31 peak. The following expression was used to calculate the abundance of [^{18}O]methanol.

$$\text{Atom \% excess of } ^{18}O \text{ in methanol} = \frac{I_{33}\times 100}{I_{33}+(I_{31}-x\cdot I_{33})}$$

where I is (peak height − background) and x is a factor that corrects for the proportion of the peak height at mass 31 that is due to [^{18}O]methanol. The value of x was assumed to be the same as the ratio of the peak heights at mass 29 and 31, measured at the time with pure [^{16}O]-methanol. The precise value was determined for each set of measurements and was always approx. 0.3.

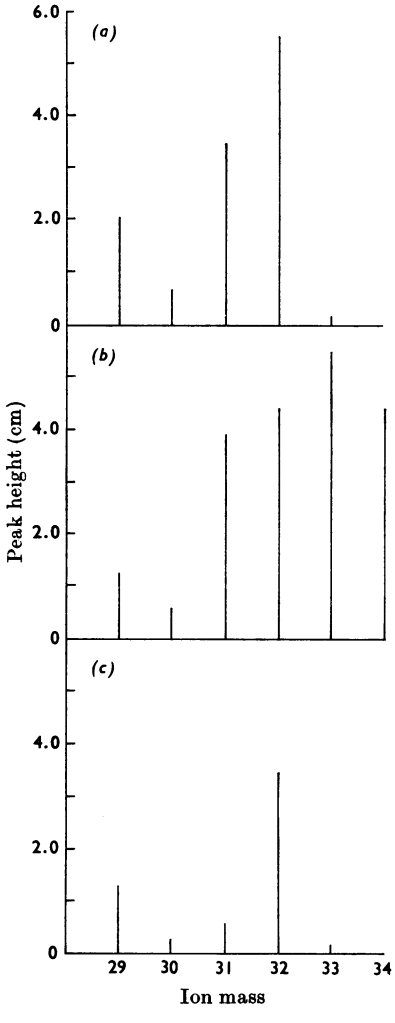

Fig. 1. Line diagrams of part of the methanol mass spectra obtained from aqueous solutions of (*a*) [^{16}O]methanol and (*b*) ^{18}O-enriched methanol (77 atom % excess of ^{18}O). These spectra have not been corrected for background. The background was individually determined for each spectrum and a typical background spectrum is shown in (*c*).

RESULTS AND DISCUSSION

Factors affecting accumulation of methanol during oxidation of methane

The success of any attempt to determine the nature of the transformation of methane into methanol depends on obtaining sufficient methanol from the bacterial oxidation of methane to permit the necessary isotopic analyses. No detectable quantities of methanol (when assayed chemically as described in the Materials and Methods section) accumulated when washed suspensions of *Methanomonas methanooxidans* and *Pseudomonas methanica* were incubated for 1–2h with methane+oxygen mixtures in Warburg cups at cell densities of approx. 1mg dry wt./ml in 20–40mM-potassium phosphate buffer, pH7.0. Therefore, following the work of Brown *et al.* (1964), preliminary attempts were made to cause methanol accumulation from methane by washed cell suspensions of *M. methanooxidans* by using iodoacetate as an inhibitor. However, no methanol could be detected under a wide range of conditions, including those used by Brown *et al.* (1964), either with *M. methanooxidans* or with *P. methanica*. In all experiments, uptake of a methane+oxygen mixture by the bacteria was actually more sensitive to iodoacetate than was methanol-dependent oxygen uptake. Typical results are shown in Table 1. Attempts to cause methanol accumulation with *p*-nitrophenylhydrazine, a known inhibitor of methanol dehydrogenase (Anthony & Zatman, 1964), were also unsuccessful. However, in one experiment with *M. methanooxidans*, methanol was found to have accumulated in a control flask that did not contain inhibitor. The incubation mixture in this case differed only in having a higher concentration of phosphate buffer than had previously been used. This effect was further investigated.

Table 1. *Effect of iodoacetate on gas uptake rates when washed cell suspensions of* M. methanooxidans *are incubated with methane or methanol*

Cell suspensions were either used immediately or stored for 43h at 4°C. Experimental conditions were as described in the Materials and Methods section except that reaction mixtures contained 100μmol of potassium phosphate buffer, pH7.0, 4.7mg dry wt. of cells and the gas uptakes were measured for 2h. The gas phase was air in the case of methanol as substrate. The concentration of sodium iodoacetate was 18mM and 6mM for the fresh and stored-cell experiments respectively.

	% Inhibition of gas uptake in the presence of iodoacetate	
Substrate	Fresh cells	Cells stored for 43h
CH_4	50.7	42.0
$CH_3.OH$ (36mM)	29.0	35.1

Factors affecting methanol accumulation in phosphate buffer. Fig. 2 shows the effect of the pH of 72mM-potassium phosphate buffer on gas uptake and methanol accumulation by *M. methanooxidans* cells. The optimum pH for methanol accumulation was 7.3 and for gas uptake 6.0. Other experiments showed that gas uptake was decreased at pH values lower than 5.5. Methanol accumulated only within

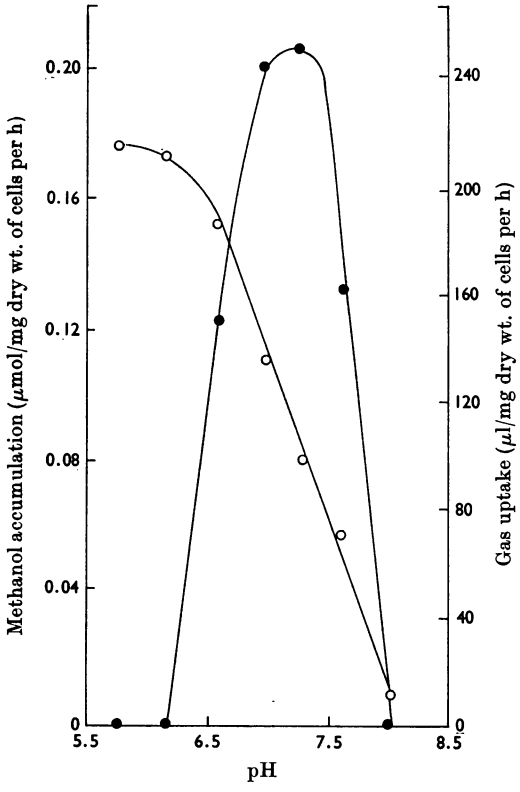

Fig. 2. Effect of pH on gas uptake (○) and methanol accumulation (●) by washed suspensions of *M. methanooxidans*. The experimental procedure was as described in the Materials and Methods section except for the pH of the buffer. Reaction mixtures contained 8.2mg dry wt. of cells.

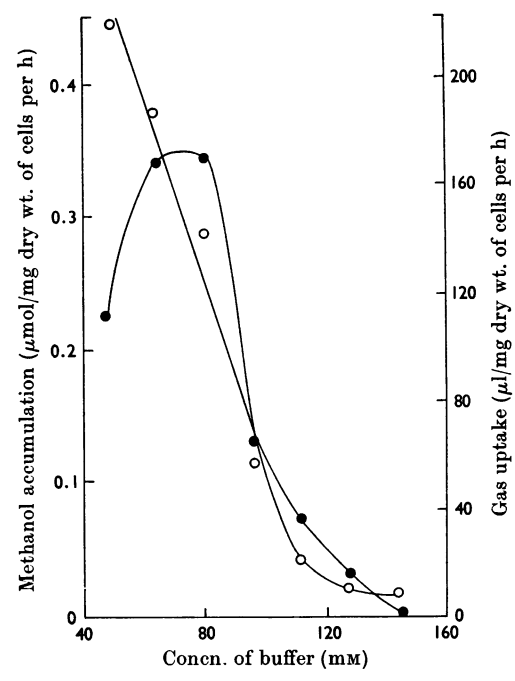

Fig. 3. Effect of concentration of potassium phosphate buffer, pH7.3, on gas uptake (○) and methanol accumulation (●) by washed suspensions of *M. methanooxidans*. Experimental conditions other than potassium phosphate concentration were as described in the Materials and Methods section. Each reaction mixture contained 7.3mg dry wt. of cells.

the pH range 6.3–7.9. Fig. 3 shows the effect of the concentration of potassium phosphate buffer at pH7.3 on gas uptake and methanol accumulation. The optimum buffer concentration for methanol accumulation was 70–80mM, and the accumulation of methanol decreased sharply on either side of this optimum buffer strength. Similar results were obtained using sodium phosphate buffer in place of potassium phosphate, but no accumulation took place with tris–HCl buffer at a wide variety of pH values and concentrations. It seems therefore that phosphate is concerned in methanol accumulation, although the addition of 80mM-potassium phosphate buffer, pH7.3, to reaction mixtures buffered with 60mM-tris–HCl, pH7.3, did not cause methanol to accumulate.

A time-course experiment with suspensions of *M. methanooxidans* in 72mM-potassium phosphate buffer, pH7.3, showed that the concentration of methanol reached a maximum after about 3h, and that thereafter the concentration slowly decreased. Maximum gas uptake rates were observed in mixtures of methane+oxygen that contained 50–70% methane and maximum methanol accumulation occurred in mixtures containing 70% methane. *P. methanica* also accumulated methanol under the conditions that were optimum for *M. methanooxidans*. For a given set of experimental conditions there was an optimum cell density for methanol accumulation in both organisms. Fig. 4 shows the effect of cell density on gas uptake and methanol accumulation for *M. methanooxidans* and *P. methanica*. In both cases the relationship between cell density and rate of gas uptake was linear. The optimum cell density per flask for methanol accumulation was approx. 9 and 4mg dry wt. of cells for *M. methanooxidans* and *P. methanica* respectively.

Effect of iodoacetate on methanol accumulation. Since Brown *et al.* (1964) have reported that iodoacetate caused methanol to accumulate when *M. methanooxidans* was incubated under methane+

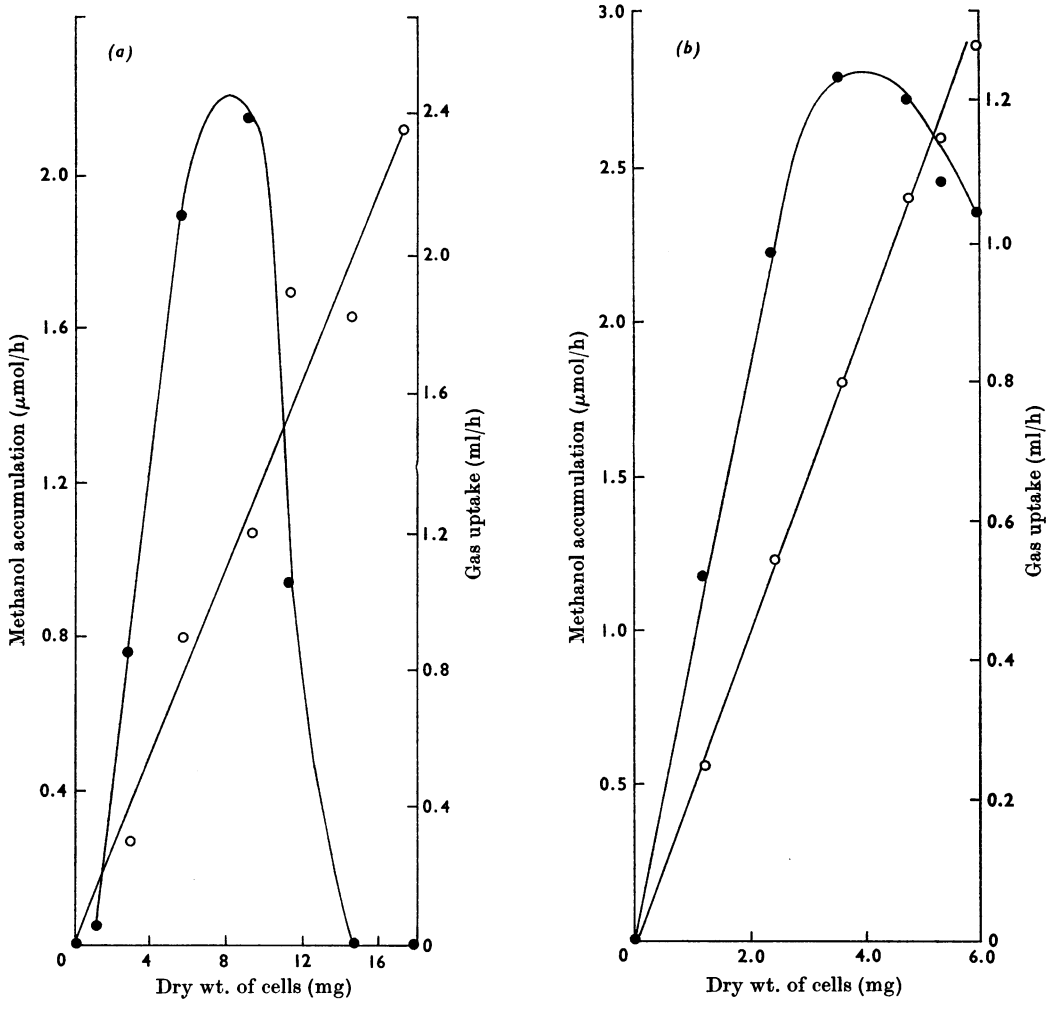

Fig. 4. Effect of cell density on gas uptake (○) and methanol accumulation (●) by (*a*) *M. methanooxidans* and (*b*) *P. methanica*. Experimental conditions other than cell density were as described in the Materials and Methods section.

oxygen mixtures, it was important to test if iodoacetate affected methanol accumulation under conditions otherwise optimum for such accumulation. Fig. 5 shows that iodoacetate under such conditions inhibits methanol accumulation. This behaviour is thus consistent with our earlier finding that, in our hands, in contrast with the report of Brown *et al.* (1964), iodoacetate inhibits methane oxidation more than methanol oxidation. The results of Brown *et al.* (1964) were interpreted by Whittenbury (1969) as indicating that a mixed-function oxidase is not involved in the oxidation of methane. This deduction is clearly not valid unless the results are reproducible.

Oxygenation of methane with [^{18}O]*oxygen*

Our studies of the specific conditions leading to methanol accumulation during oxidation of methane by *M. methanooxidans* and *P. methanica* permitted concentrations of up to 2.5mM-methanol to be obtained in cell suspensions. It was then possible to perform mass spectral analysis of the methanol as described in the Materials and Methods section. To test the accuracy of the analytical methods, analyses were made by using standards of methanol containing known amounts of ^{18}O. These were prepared by mixing an aqueous solution (1:1000, v/v) of [^{18}O]methanol of known enrichment with

various volumes of a similar solution of [^{16}O]-methanol (A.R. grade). These standard solutions were then analysed for their oxygen isotope abundance ratios and the results are shown in Table 2.

These experimental methods were now applied to analysis of methanol obtained from oxidation of methane by *M. methanooxidans* and *P. methanica* in the presence of labelled oxygen or labelled water. Flasks were prepared in triplicate with *M. methanooxidans* and also with *P. methanica*, and, in the case of *M. methanooxidans*, both sets of experiments were duplicated with independently grown batches of the micro-organism. With *P. methanica* the experiments involving [^{18}O]water, but not those involving $^{18}O_2$, were duplicated with two indendently grown batches of the organism. The results in Table 3 show that within the limits of experimental error, the $^{18}O/^{16}O$ abundance ratio in the methanol is the same as that in the molecular oxygen in the gas phase in both micro-organisms. The results of control experiments in which $^{16}O_2$ was used in place of $^{18}O_2$ in the gas phase but the suspension mixtures contained [^{18}O]water in place of [^{16}O]water are shown in Table 4. There was negligible incorporation into methanol of oxygen derived from water, thus corroborating the results obtained with labelled molecular oxygen.

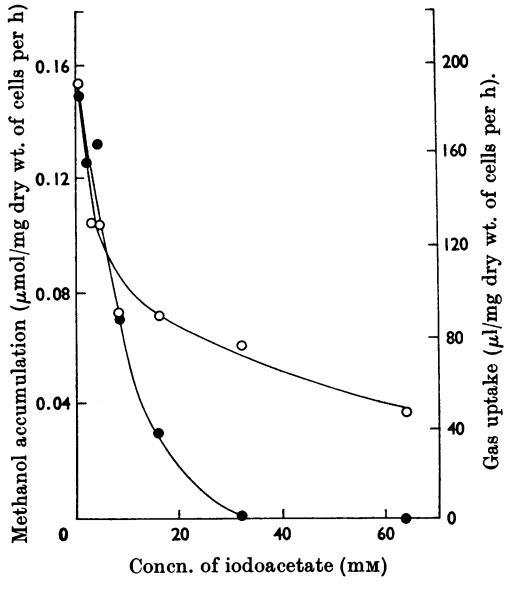

Fig. 5. Effect of iodoacetate on gas uptake (○) and methanol accumulation (●) by washed cells of *M. methanooxidans*. Conditions other than iodoacetate addition were as described in the Materials and Methods section.

Nature of the oxygenase reaction

The data presented here show unequivocally that the oxygen atom in methanol that is formed by oxidation of methane by *M. methanooxidans* and *P. methanica* is derived from molecular oxygen and not from water. The enzyme system involved must therefore be an oxygenase catalysing either reaction (1) or (2). If, by analogy with higher alkane oxidation, the reducing agent for the mono-oxygenase were a reduced nicotinamide nucleotide, the standard free-energy changes for the two reactions at pH 7 would be −82.1 and −59.5 kcal/mol respectively. If the cell yield/mol of methane is higher than the cell

Table 2. *Measurement of* ^{18}O*-enrichments in standard methanol solutions*

The solutions were prepared by dissolving [^{18}O]methanol (77.0 atom % excess of ^{18}O) in 1000 vol. of water and mixing with various volumes of a similar solution of [^{16}O]methanol. The experimental procedure is described in the Materials and Methods section.

Atom % excess of ^{18}O in methanol (calculated from weighings and enrichment data supplied by Miles–Yeda Ltd.)	Atom % excess of ^{18}O in methanol (calculated from mass spectrum)	Mean of triplicates	Standard error of the mean
77.0	77.0		
	77.1	76.7	±0.39
	75.9		
62.2	64.8		
	63.8	62.5	±1.83
	58.9		
47.4	47.5		
	47.4	47.8	±0.39
	48.6		
4.1	4.1		
	4.4	4.0	±0.24
	3.6		

Table 3. *Incorporation of label from $^{18}O_2$ into methanol formed by oxidation of methane by* M. methanooxidans *and* P. methanica

The experimental procedure was as described in the Materials and Methods section, conditions being optimum for methanol accumulation. Total gas uptake for *M. methanooxidans* was 5.6 ml and for *P. methanica* 1.24 ml.

			Atom % excess of ^{18}O in derived methanol				
Micro-organism	Batch no.	Flask no.	Atom % excess of ^{18}O (duplicate measurements)	Mean of duplicates	Overall mean	Standard error of the mean	Atom % excess of ^{18}O in enriched oxygen used
M. methanooxidans	1	1	57.7 58.1	57.9	57.5	±0.84	55.93
		2	57.2 60.0	58.6			
		3	55.2 60.9	58.1			
	2	1	55.7 57.2	56.5			
		2	54.8 53.0	53.9			
		3	60.8 58.8	59.8			
P. methanica	1	1	54.0 54.8	54.4	55.4	±0.89	
		2	56.6 58.8	57.7			
		3	53.3 54.8	54.1			

Table 4. *Determination of ^{18}O in methanol formed by oxidation of methane by* M. methanooxidans *and* P. methanica *in the presence of* [^{18}O]*water*

The experimental procedure was as described in the Materials and Methods section, conditions being optimum for methanol accumulation. The potassium phosphate buffer was prepared with [^{18}O]water (23.06 atom % excess of ^{18}O). Incubation mixtures contained 2.3 ml of this buffer in a total volume of 2.8 ml. Total gas uptakes were 6 ml for *M. methanooxidans* and 2.7 ml for *P. methanica*.

			Atom % excess of ^{18}O in derived methanol		Atom % excess of ^{18}O in enriched water used
Micro-organism	Batch no.	Flask no.	Atom % excess of ^{18}O	Mean	
M. methanooxidans	1	1	1.0	0.2	19.0
		2	0		
		3	0		
	2	1	0		
		2	0		
		3	0		
P. methanica	1	1	0.1	0.1	
		2	0.2		
		3	0		
	2	1	0.3		
		2	0		
		3	0		

yield/mol of methanol, then this would imply that the exergonic oxygenation of methane is indeed coupled to ATP synthesis. This might take place in a novel coupled electron-transport system for reaction (1) in which the electrons from the reduced nicotinamide nucleotide, during the process of methane oxygenation, are passed to one oxygen atom as terminal electron acceptor. The apparent sensitivity of methane oxygenation to phosphate concentration may be relevant in this respect.

The isotope incorporation studies described here were made possible by the discovery of the narrow range of conditions under which methanol accumulated. It should be noted that, even so, the proportion of methane utilized which accumulated as methanol was small (about 5–10% under optimum conditions for accumulation). A biochemical explanation of the interplay of the effects of the buffer concentration, its pH, the cell concentration and the composition of the gas mixture on methanol accumulation during oxidation of methane may become apparent when the oxygenase system is further characterized.

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