Oxidation of Carbon Monoxide and Methane by *Pseudomonas methanica*

By T. FERENCI, T. STRØM* AND J. R. QUAYLE

Department of Microbiology, University of Sheffield,
Sheffield, S10 2TN, U.K.

(Received 21 March 1975; revised 30 May 1975)

SUMMARY

The oxidation of carbon monoxide and methane by suspensions and ultrasonic extracts of *Pseudomonas methanica* was studied. A continuous assay for the oxidation of CO to CO₂ was devised, using O₂ and CO₂ electrodes in combination. Stoichiometries of CO-dependent CO₂ formation, O₂ consumption and NADH oxidation, and the partial stoichiometries of methane-dependent NADH oxidation, suggest the involvement of a mono-oxygenase in these oxidations. Evidence is presented suggesting methane and CO oxidation are catalysed by a single enzyme system, distinct, at least in part, from the NADH oxidase present in extracts. Ethanol was able to provide the reductant necessary for CO oxidation by cell suspensions, though the metabolism of ethanol by *P. methanica* was found unlikely to result in substrate-level formation of NADH; the means whereby alcohol oxidation could supply reductant for the mono-oxygenase are discussed.

INTRODUCTION

Ribbons & Michalover (1970) briefly reported a methane-stimulated NADH oxidase in cell-free extracts of *Methylococcus capsulatus*. Although many speculative reviews deal with this topic (Foster, 1962; Whittenbury, 1969; Ribbons, Harrison & Wadzinski, 1970; Wilkinson, 1971; Quayle, 1972; Van Dijken & Harder, 1975), no other study of methane oxidation by cell-free extracts has been published. This may be because of the apparent inability to obtain cell-free extracts which reproducibly show methane-oxidizing activity (e.g. Quayle, 1972). This paper describes some properties of a methane-dependent NADH oxidase system in extracts of methane-grown *Pseudomonas methanica*, prepared as previously described (Ferenci, 1974). The term NADH oxidase does not necessarily refer to a single, defined activity but to any activity, under the conditions specified, resulting in oxidation of NADH with oxygen as the terminal electron acceptor.

Washed suspensions of methane-utilizing bacteria catalyse the slow oxidation of carbon monoxide (CO) to CO₂ (Hubley, Mitton & Wilkinson, 1974), and CO stimulates the consumption of oxygen by these organisms (Hubley *et al.* 1974; Ferenci, 1974). This paper confirms the suggestions of Ferenci (1974) that the oxidation of CO in *P. methanica* is catalysed by an NADH-requiring mono-oxygenase. The relationship between the enzymes of CO oxidation and those of methane oxidation, and possible sources of reductant for these oxidations, are considered.

* Present address: Institute of Fishery Technology Research, Box 1159, Tromsø, Norway.

METHODS

Materials. Carbon monoxide and methane (research grade) were obtained from The British Oxygen Co., Special Gases, London, ethane (99 %) from BDH, and methane (approximately 95 %, used for the growth of organisms only) from the Greater London Council, Department of Public Health Engineering, Isleworth, Middlesex. Gases were added to all assays as saturated solutions in water at 25 °C and atmospheric pressure; the concentrations of gases in solution were taken from solubility tables (Seidell, 1920). Coenzymes and isocitrate dehydrogenase were from Boehringer Corp., London, and carbonic anhydrase was from Sigma.

Growth of the organism and preparation of extracts. *Pseudomonas methanica* was grown and harvested as previously specified (Ferenci, 1974). Suspensions were washed once in buffer containing 20 mM-potassium phosphate–5 mM-MgCl₂, pH 7.0, before preparation of extracts and the particulate fraction of extracts as previously described (Ferenci, 1974).

Simultaneous measurement of CO₂ formation and O₂ uptake. A water-jacketed Rank oxygen electrode chamber (Rank Bros., Bottisham, Cambridge), in which the O₂ electrode is housed at the base, was used as the reaction vessel. The CO₂ electrode was incorporated into the lid of the vessel; the lid consisted of a disc of stiff plastic (of a diameter to give a tight fit into the reaction chamber) through which a small hole had been drilled for the addition of reagents and removal of air bubbles. The CO₂ electrode (Radiometer type E5036, Radiometer A/S, Copenhagen) was coupled to a Radiometer PHM 71 mark II pH meter with a PHA 935 pCO₂ module. The signal from the pH meter was displayed on a Rikadenki B 241 two-channel recorder (TEM Sales Ltd., Crawley, Sussex); the second channel was used via the O₂ electrode to monitor O₂ disappearance from the reaction chamber. The signal from the pH meter was offset by 150 mV, which was registered at zero CO₂ concentration, to enable the signal to be displayed on the recorder. References to CO₂ throughout are to the total CO₂ in solution, including the hydrated forms of CO₂.

The CO₂ electrode was used essentially as described by Nicholls, Shepherd & Garland (1967). It was calibrated with CO₂ generated from isocitrate in the presence of isocitrate dehydrogenase; NaHCO₃ solutions were used as secondary standards (Nicholls *et al.* 1967). A silicone rubber membrane (Radiometer type D606) was used with the CO₂ electrode and the pH electrode compartment contained 50 mM-NaCl₂, 1 mM-NaHCO₃ and 100 µg carbonic anhydrase/ml. The time for a 90 % response to the addition of 50 µM-NaHCO₃ to the reaction chamber of the electrode containing 50 mM-sodium–potassium phosphate buffer pH 7.0, and 100 µg carbonic anhydrase/ml, was variable from membrane to membrane but was usually under 30 s. The sensitivity and stability of the CO₂ electrode was such that it could be routinely used with a full-scale deflection on the recorder equivalent to less than 20 µM-CO₂. The suitability of the CO₂ electrode for measuring enzyme-dependent rates of CO₂ formation was shown by the linear dependence of the rate of CO₂ formation, as measured by the slope of the recorder tracing, on the concentration of isocitrate dehydrogenase in the presence of isocitrate in the assay described below. The specific activity of isocitrate dehydrogenase, as calculated from CO₂ output, was 0.793 µmol CO₂ formed/min/mg protein; this agreed with that of the same enzyme preparation assayed spectrophotometrically under identical conditions, namely 0.778 µmol NADPH formed/min/mg protein.

Oxidations of substrates by cell suspensions were assayed as previously described (Ferenci, 1974).

Carbon monoxide mono-oxygenase and methane mono-oxygenase activities. Each assay mixture is described in the legend to the relevant Figure; all assays were performed at 25 °C.

The mono-oxygenase activities were also followed spectrophotometrically at 25 °C, by measurement of CO- or methane-dependent rates of NADH oxidation as previously described (Ferenci, 1974). Specific activities of enzyme preparations are expressed in terms of the CO- or methane-dependent rates of NADH oxidation/min/mg protein, i.e. corrected for substrate-independent NADH oxidation.

Inhibition of carbon monoxide mono-oxygenase activity. Assays were performed in the CO₂ electrode; the assay mixture, in a total volume of 2 ml at 25 °C, consisted of 50 mM-sodium-potassium phosphate buffer pH 7.0, 100 µg carbonic anhydrase/ml, 0.2 mM-NADH and 0.1 ml of particulate membrane fraction plus inhibitors. The specific activity of the particulate membrane fraction was 23.6 nmol CO₂ formed/min/mg protein. The reaction was initiated by adding CO to a concentration of 24 µM.

Alcohol and aldehyde oxidase and dehydrogenase activities were assayed in the Rank O₂ electrode at 25 °C with a reaction volume of 2 ml. The assay mixtures contained 50 mM-sodium phosphate buffer pH 8.0, cell-free extract containing 0.5 to 1.0 mg protein and either methanol, ethanol, formaldehyde or acetaldehyde (1 mM). To demonstrate the dehydrogenase activities, 10 mM-NH₄Cl plus 1 mM-phenazine methosulphate (PMS) were also added to the assay mixture in the O₂ electrode. The glutathione-dependent, NAD-linked formaldehyde dehydrogenase was assayed as described by Johnson & Quayle (1964).

Formate dehydrogenase. This enzyme was assayed spectrophotometrically, at 340 nm and 25 °C in a 1 ml reaction mixture containing 50 mM-sodium-potassium phosphate buffer pH 7.0, 0.2 mM-NAD, 2.5 mM-sodium formate and cell-free extract containing up to 80 µg protein. The order of addition of reagents suggested by Johnson & Quayle (1964) was followed.

Isocitrate dehydrogenase. The assay mixture in the CO₂ electrode, total volume 2 ml at 25 °C, contained: 50 mM-sodium-potassium phosphate buffer pH 7.0, 2 mM-MgCl₂, 0.8 mM-NADP, 0.5 mM-isocitrate and 200 µg carbonic anhydrase. The reaction was started by adding known amounts of isocitrate dehydrogenase.

Protein was estimated by the method of Lowry *et al.* (1951).

RESULTS

The carbon monoxide mono-oxygenase activity

In the presence of a particulate membrane preparation, simultaneous stimulations of NADH oxidation and O₂ uptake were accompanied by the oxidation of CO to CO₂ (Fig. 1). Carbon monoxide alone (Fig. 1*a*) or NADH alone (Fig. 1*b*) gave little stimulation of CO₂ formation but the latter stimulated O₂ uptake. If the rates of CO₂ formation and O₂ uptake observed without CO (Fig. 1*b*) were maintained during CO-dependent respiration, and are corrected for, the addition of 93 nmol CO resulted in the formation of 110 nmol CO₂ and the consumption of 101 nmol O₂. The equivalence of CO added, CO₂ formed, O₂ consumed and NADH oxidized, confirmed by further experiments (Table 1), is consistent with the following reaction for CO oxidation:

If the rate of CO-independent respiration was not (as assumed) maintained during CO oxidation, the amounts of O₂ and NADH consumed would be even higher, making less likely the alternative mechanism:

Fig. 1. Carbon monoxide oxidation by a particulate fraction of cell-free extracts. The assay mixtures in a final volume of 2 ml in the dual CO_2 - O_2 electrode vessel, contained 50 mM-sodium-potassium phosphate buffer pH 7.0 and 200 μg carbonic anhydrase, together with: (a) 0.75 mg protein, obtained by centrifuging a cell-free extract (Ferenci, 1974) for 60 min at 38 000 g and resuspending the pellet formed in 20 mM-sodium-potassium phosphate buffer pH 7.0 containing 5 mM- MgCl_2 ; (b) 0.6 mg protein, prepared as for (a). The additions, at the points arrowed, were: A, 0.186 μmol CO ; B, 0.4 μmol NADH; C, 0.093 μmol CO . The figures in parentheses give the rates of O_2 consumption or CO_2 formation, in nmol/min.

Table 1. *Stoichiometries of NADH-dependent oxidations of methane and carbon monoxide by cell-free extracts*

CO_2 formation and oxygen uptake were measured simultaneously as described in Methods. The assay mixture (2.4 ml) contained 50 mM-sodium-potassium phosphate buffer pH 7.0, 0.28 mM-NADH and 50 μg carbonic anhydrase/ml; the gaseous substrates were added as known amounts of saturated solutions in water at 25 °C. With CO as substrate, the 38000 g pellet fraction was used to catalyse the reaction, with 0.26 mg protein/ml in the assay. With methane as substrate, cell-free extract was used, at 0.68 mg protein/ml. NADH oxidation was followed separately, spectrophotometrically, as described in Methods. The concentrations of the reactants, in a volume of 1.2 ml, were identical to those above except that carbonic anhydrase was omitted. The values quoted have been corrected for substrate-independent CO_2 formation, O_2 uptake and NADH oxidation. All results are given in $\mu\text{mol}/\text{ml}$ assay mixture.

Substrate added	CO_2 produced	O_2 removed	NADH oxidized
Carbon monoxide			
15.5	17.4	13.8	16.3
31.0	32.4	34.3	36.5
Methane			
21.3	—	17.4	21.1
42.0	—	36.2	39.0

Fig. 2. Effect of ammonium chloride on the kinetics of methane and CO oxidation by cell suspensions. (a) The reaction mixture in the oxygen electrode vessel (total volume 2 ml) contained 50 mM-sodium-potassium phosphate pH 7.0, 0.25 mg dry wt *P. methanica*, and methane at the concentrations shown. $\square$, No further additions; $\circ$, with 10 mM- NH_4Cl ; $\bullet$, with 20 mM- NH_4Cl . Rates of oxygen uptake, v , are expressed as nmol O_2 consumed/min. (b) The reaction mixture in the dual CO_2 - O_2 electrode vessel (total volume 2 ml) contained 50 mM-sodium-potassium phosphate pH 7.0, 200 μg carbonic anhydrase, 1 mM-ethanol, 0.0475 mg dry wt *P. methanica*, CO at the concentrations shown, and other additions as for (a). Rates of CO_2 formation, v , are expressed as nmol CO_2 formed/min.

The methane mono-oxygenase activity

Cell-free extracts of *P. methanica* exhibited, in addition, a methane-dependent NADH oxidase activity (Ferenci, 1974) similar to that described for *M. capsulatus* (Ribbons & Michalover, 1970). The partial stoichiometry of methane-dependent NADH oxidation and O_2 consumption, assuming endogenous rates continued unchanged during methane oxidation (Table 1), is consistent with methane oxygenation being catalysed by a mono-oxygenase:

The stoichiometry indicates that methanol is the product; this was not directly demonstrated. If the rate of methane-independent NADH oxidation and O_2 uptake was not (as assumed) constant during methane-dependent NADH oxidation, more O_2 and NADH would be consumed for each amount of methane added thus making less likely the alternative reactions:

Reaction (5) was proposed by Wilkinson (1971).

Comparison of methane and carbon monoxide oxidation

Effect of ammonium chloride. Ammonium ion inhibited methane oxidation by *Methylosinus trichosporium* OB3B, and high NH_4^+ concentrations inhibited growth of some methane-utilizing bacteria to varied extents (Whittenbury, Phillips & Wilkinson, 1970). Wilkinson (1971) stated that NH_4^+ was a competitive inhibitor of methane oxidation. Double reciprocal plots (Lineweaver & Burk, 1934) of the kinetics of methane-dependent O_2 consumption show that NH_4Cl competitively inhibited the oxidation of methane by *P. methanica* (Fig. 2a). Half-maximal rates of methane oxidation were reached at about 15 μM -methane concentration; this compares with an $\text{S}_{0.5}$ for methane of 20 μM , found for a methane-oxidizing pseudomonad (Harrison, 1973). The K_i for NH_4Cl was about 10 mM. As Whittenbury *et al.* (1970) stated that methane-utilizing bacteria could oxidize ammonia, it is relevant that, with

20 mM- NH_4Cl , the observed rate of NH_4Cl -dependent O_2 consumption in these studies was below 1 nmol O_2 consumed/min/mg dry wt cells. The effect of NH_4Cl on the kinetics of CO oxidation by cell suspensions was followed by measuring rates of CO-dependent CO_2 formation in the presence of 1 mM-ethanol. The ethanol acted as the reductant for CO oxidation by intact cells, but its oxidation did not give rise to CO_2 (Fig. 5*b*). Under these conditions (Fig. 2*b*) the $S_{0.5}$ for CO was 2.7 μM and NH_4Cl was a competitive inhibitor of CO oxidation, with a K_i of about 12 mM. Ammonium chloride also inhibited NADH-dependent CO oxidation by particulate membrane preparations: 23 % at 20 mM and 35 % at 40 mM.

Effect of ethane. It was difficult to measure the competitive interaction of CO oxidation and methane oxidation because either oxidation resulted in the uptake of O_2 and the formation of CO_2 . Ferenci (1974) showed that high concentrations of CO completely inhibited the oxidation of both substrates. *Pseudomonas methanica* oxidizes ethane to acetaldehyde and acetate, with only a small production of CO_2 (Leadbetter & Foster, 1960). In the present short-term studies, the oxidation of ethane by *P. methanica* gave negligible rates of CO_2 formation. Therefore the CO_2 electrode was used to study the effect of 30 μM - and 90 μM -ethane on the kinetics of oxidation of CO, using experimental conditions similar to those described in Fig. 2*b*). Ethane competitively inhibited CO oxidation with a K_i of about 25 μM ; the $S_{0.5}$ for CO in this experiment was 2.3 μM .

Ethane also inhibited NADH-dependent CO oxidation by particulate membrane preparations: 42 % at 90 μM and 63 % at 180 μM . Both compounds (ethane and NH_4Cl) that inhibited CO oxidation by intact cells also inhibited the NADH-dependent oxidation of CO, suggesting that the system responsible for the NADH-dependent activity is the same as that responsible for the oxidation of CO by intact cells. Cell-free extracts catalysed both ethane-dependent NADH oxidation and, to a much lesser extent, an NH_4Cl -stimulated disappearance of NADH. Specific activities (nmol NADH oxidized/min/mg protein), using the spectrophotometric assay, were: methane, 27.8; CO, 27.1; 0.3 mM-ethane, 16.2; 100 mM- NH_4Cl , 7.7.

Stability on storage of extracts at 25 °C. Methane mono-oxygenase and CO mono-oxygenase activities were lost simultaneously, and separately from the substrate-independent NADH oxidase activity also present in extracts (Fig. 3).

pH-Dependence. The optimal pH for both the mono-oxygenase activities in phosphate buffer was pH 7.0, whereas optimal rates of substrate-independent NADH oxidase activity were reached at about pH 8 (Fig. 4).

Enzyme inhibitors. The effect of enzyme inhibitors was measured spectrophotometrically using extracts. Dithiothreitol (5 mM) and reduced glutathione (5 mM) inhibited the methane mono-oxygenase by 84 and 63 % respectively and the CO mono-oxygenase by 92 and 72 % respectively; the substrate-independent NADH oxidase was not significantly affected by either compound. The inhibitions by sulphydryl reagents may not be due simply to a chelating effect, as 10 mM-EDTA did not significantly inhibit any of the activities. Iodoacetic acid (2 mM) and *N*-ethyl maleimide (1 mM) caused only minor inhibitions. Cyanide was a potent inhibitor of both mono-oxygenase activities and of the substrate-independent NADH oxidase, though the concentration of cyanide (2 μM) that gave near-total inhibition of the mono-oxygenase activities only inhibited the activity of NADH oxidase by 50 %. The cyanide sensitivity of the NADH oxidase contrasts with the cyanide-insensitive NADH oxidase found by Davey, Whittenbury & Wilkinson (1972) in extracts of various methane-utilizers.

Fig. 3

Fig. 4

Fig. 3. Stabilities of methane mono-oxygenase, CO mono-oxygenase and substrate-independent NADH oxidase activities at 25 °C. Samples from cell-free extract (Ferenci, 1974) stored at 25 °C and sampled at the times indicated, were assayed spectrophotometrically for NADH disappearance. The specific activities at the beginning of the experiment were (nmol NADH oxidized/min/mg protein): methane mono-oxygenase ($\square$), 20.6; CO mono-oxygenase ($\triangle$), 19.4; substrate-independent NADH oxidase ($\circ$), 10.2.

Fig. 4. pH-dependence of methane mono-oxygenase, CO mono-oxygenase and substrate-independent NADH oxidase activities. Methane mono-oxygenase ($\square$), CO mono-oxygenase ($\triangle$) and substrate-independent NADH oxidase ($\circ$) activities were assayed spectrophotometrically in 50 mM-sodium phosphate buffer at the pH values indicated.

Generation of reductant for the mono-oxygenase in intact organisms

The mechanism whereby ethanol could act as the source of reductant for the mono-oxygenase was investigated (Fig. 5). The oxidation of CO to CO₂ and the concomitant faster O₂ uptake were dependent on ethanol; the rate of CO₂ formation from CO (Fig. 5a) was slow and that from ethanol (Fig. 5b) negligible, when these substrates were present separately. When small amounts of CO were added to organisms oxidizing ethanol (Fig. 5b) the stimulated rates of O₂ consumption and CO₂ formation only continued until the added CO had been exhausted. During CO-stimulated respiration, virtually the complete rate of O₂ consumption could be accounted for by the rate of CO oxidation to CO₂; the mono-oxygenase may therefore have been acting as the terminal oxidase for the reducing power generated from ethanol oxidation. The nearly twofold stimulation of the rate of O₂ consumption by the organism oxidizing ethanol after addition of CO was consistent with a switch from a normal terminal oxidase catalysing:

to the mono-oxygenase catalysing:

(6)

(7)

No NAD-linked enzyme was found to be involved in the oxidation of ethanol by *P. methanica*, which is limited to partial oxidation to acetate (Leadbetter & Foster, 1958) and

Fig. 5

Fig. 5. Ethanol-dependent CO oxidation by cell suspensions. The reaction mixtures (total volume 2 ml in the dual CO_2 - O_2 electrode vessel) initially contained 50 mM-sodium-potassium phosphate buffer pH 7.0 and 200 μg carbonic anhydrase. The following were added at the times indicated: A, 0.26 mg dry wt *P. methanica*; B, 93 nmol CO; C, 2 μmol ethanol. Two experiments, (a) and (b), were performed, with the additions made in a different order. The figures in parentheses give the rates of O_2 consumption or CO_2 formation, in nmol/min.

Fig. 6

Fig. 6. Effect of length of sonic disruption on *P. methanica*. Suspensions (6 ml) cooled in ice, were treated with an MSE 150 W ultrasonic disintegrator using the 3/8 inch-diameter titanium probe. The disintegrator was used at the 'low' power setting, with a transducer amplitude of about 7.5 μm peak to peak. Treatment was for 45 s periods, between which both the suspensions and the probe were cooled in ice-water for 1 min. The resultant preparations were centrifuged at 6000g for 10 min. The supernatants were assayed for: ●, protein released; △, formate dehydrogenase activity; □, methane mono-oxygenase activity; ■, CO mono-oxygenase activity; ○, NADH oxidase activity. The spectrophotometric assay of NADH oxidation was used for □, ■ and ○.

not to CO_2 (Fig. 5b). Acetaldehyde can be oxidized by *P. methanica*, but acetate cannot. A dehydrogenase activity dependent on ammonium ions and coupled to PMS could be demonstrated (Table 2); methanol, ethanol, formaldehyde and acetaldehyde were all substrates in the PMS-linked assay. It is likely all these activities are due, at least in part, to the presence of the same enzyme, namely the primary alcohol dehydrogenase of broad substrate specificity purified from *M. capsulatus* (Patel *et al.* 1972) and methanol-utilizing bacteria (Sperl, Forrest & Gibson, 1974). An alcohol (and aldehyde) oxidase activity independent of added electron acceptor was also observed (Table 2). A similar oxidase activity has been found in *M. capsulatus* by Wadzinski & Ribbons (1975). The glutathione-dependent NAD-linked formaldehyde dehydrogenase activity described by Johnson & Quayle (1964) was not detected in extracts of *P. methanica*, even when bacteria were grown and extracts prepared under identical conditions; nor was an NAD-linked acetaldehyde dehydrogenase activity observed.

From these results, it appeared that no substrate-level reduction of NAD was involved in the metabolism of ethanol by *P. methanica*. Ethanol was tested directly as a reductant with extracts. No ethanol-dependent CO_2 formation from CO could be detected with particulate membrane preparations, even though these preparations were capable of NADH-dependent

Table 2. *The alcohol and aldehyde oxidase and dehydrogenase activities in cell-free extracts and the effect of sonication*

The procedure for the assays and the concentrations of substrates used are described in Methods. The endogenous rate of O_2 uptake, 0.8 nmol O_2 /min/mg protein, was subtracted to obtain the values shown below. Activities are expressed in units of nmol O_2 consumed/min/mg protein.

	Time of sonication					
	1.5 min			3.0 min		
	Without additions	+ NH_4Cl	+ NH_4Cl + PMS	Without additions	+ NH_4Cl	+ NH_4Cl + PMS
Methanol	14.3	19.9	52.8	4.8	4.8	37.1
Ethanol	14.6	17.9	57.5	4.8	5.0	39.0
Formaldehyde	13.7	16.5	51.1	4.5	4.5	37.9
Acetyldehyde	10.9	14.0	39.2	4.5	4.5	36.0

CO oxidation and also possessed alcohol oxidase activity. Methane-dependent respiration by intact organisms was not stimulated by ethanol; this was not unexpected, as the complete oxidation of methane to CO_2 should give rise to more than enough reductant to ensure the initial oxygenation of methane. In contrast, the oxidation of ethane (which was originally at a rate of 16.3 nmol O_2 consumed/min/mg dry wt cells, i.e. one-fifth the rate of methane) was stimulated by exogenously supplied ethanol or formate to 31.5 or 55.0, respectively. A similar stimulation by formate and ethanol of the rates of CO oxidation has been observed previously (Ferenci, 1974).

Release by ultrasonic disruption of the enzymes involved in the oxidation of one-carbon compounds

Not all the factors governing the initial specific activity and stability of the methane mono-oxygenase system in cell-free extracts are understood. Although the procedure of Ferenci (1974) for the growth of cells and preparation of extracts was adhered to, the specific activities of the extracts used in these studies varied between 15 and 30 nmol NADH oxidized/min/mg protein for both CO mono-oxygenase and methane mono-oxygenase activities, in 85 to 90% of extracts prepared.

The treatment time used in breaking the organisms by ultrasonication strongly affected the final enzyme activities (Fig. 6). As *P. methanica* is relatively fragile, 45 s treatment under the conditions outlined in the legend to Fig. 6 released over 80% of the protein, together with the other activities illustrated, into a supernatant obtained by centrifuging at 6000g for 10 min. Treatment for a further 45 s resulted in a slight increase in all the activities tested, but longer periods resulted in a sharp decrease of all activities except the formate dehydrogenase.

As the membrane-bound enzymes (the methane/CO mono-oxygenase, NADH oxidase, alcohol oxidase; Table 2) lost more activity than the soluble enzymes (formate dehydrogenase, primary alcohol dehydrogenase), the destructive effect of ultrasonic treatment may have been due to the disruption of the complex membrane structure characteristic of methane-utilizing bacteria (Davies & Whittenbury, 1970).

Fig. 7. Scheme for the oxidative metabolism of *P. methanica*. X represents the unknown physiological electron acceptor for the reactions catalysed by the primary alcohol dehydrogenase.

Fig. 8. Scheme for two possible routes, (a) and (b), for the channelling of reductant from alcohol oxidation to the CO mono-oxygenase. X represents the unknown physiological electron acceptor for the reactions catalysed by the primary alcohol dehydrogenase, and Y represents a postulated carrier or set of electron carriers between NADH and the CO mono-oxygenase.

DISCUSSION

The evidence presented in this paper and elsewhere (Ribbons *et al.* 1970; Ribbons & Michalover, 1970; Quayle, 1972) is consistent with the pattern of oxidative metabolism shown in Fig. 7. Proof that the CO and ethane mono-oxygenase activities are secondary activities of the methane mono-oxygenase will have to await purification of the enzyme(s) responsible for these activities, but the evidence so far strongly suggests that these activities are catalysed by the same enzyme system. Indeed, in no property tested in cell-free extracts have the three activities differed significantly; the only major difference between CO, ethane and methane oxidation by whole cells is that a co-oxidizable substrate is required for CO oxidation and stimulates ethane oxidation. This is entirely consistent with the involvement of a mono-oxygenase in these oxidations. By several criteria, the mono-oxygenase activities in extracts behaved differently from the substrate-independent NADH oxidase activity. Therefore it is likely that NADH oxidase involves at least one protein distinct from those required for mono-oxygenase activity, which makes it unlikely that the observed methane-, ethane- and CO-dependent stimulations of NADH oxidation resulted from stimulation of the substrate-independent NADH oxidase.

The physiological significance, if any, of the ability of *P. methanica* to oxidize CO and ethane is probably limited to that of co-metabolism. *Pseudomonas methanica* is unable to

grow on CO or ethane as a source of carbon and energy, and CO inhibits the growth of *P. methanica* on either methane or methanol (Ferenci, 1974). Ethane is also inhibitory to the growth on methane of some methane-utilizing bacteria (Whittenbury *et al.* 1970).

The interaction of the mono-oxygenase with CO offers advantages over methane in studies of the methane mono-oxygenase system. The oxidation of CO is a one-step oxidation with CO_2 as product, which can be sensitively and continuously assayed by the procedure described in this paper. The oxidation of methane by the relatively crude membrane systems now available results in the formation of a number of products as methanol is further metabolized, in some circumstances as far as CO_2 (unpublished observations). If, as the whole-cell oxidation studies indicate, the reductant for the mono-oxygenase can be generated from further oxidation of methanol, there are advantages in using for the mono-oxygenase a substrate such as CO which cannot generate any reductant through further oxidation.

The involvement of a mono-oxygenase in methane oxidation demands that 1 mol of reducing agent be utilized for each mole of methane utilized. During growth of *P. methanica* on methane, most of the carbon is channelled to cellular material at the level of formaldehyde (Kemp & Quayle, 1967) and a smaller proportion of methane is oxidized to CO_2 , as can be judged from the growth stoichiometry data collected by Quayle (1972). Therefore during growth on methane, the relatively low amounts of NADH produced from further oxidation of formaldehyde (Fig. 7) might be insufficient to allow an NADH-linked mono-oxygenase to catalyse methane oxidation. It has been postulated that NADH might be generated from the reductant formed from alcohol and aldehyde oxidation (designated XH_2 in Fig. 7), by energy-dependent reversed electron transport as in Fig. 8(a) (Van Dijken & Harder, 1975). However, as the half-reduction potential of the X-XH_2 couple is unknown, it is premature to postulate energy-dependence for the reduction of NAD by XH_2 . Nor need reversal of electron transport to generate NADH be invoked if XH_2 itself is the reducing agent for the mono-oxygenase or can reduce the true agent Y as in Fig. 8(b). Such intermediate electron carriers have been recognized between NADH and several other mono-oxygenases (Peterson *et al.* 1967; Gunsalus *et al.* 1974; Kaufman & Fisher, 1974). The present results indicate that alcohol oxidation can supply reducing power to the mono-oxygenase *in vivo*; its failure to do so in cell-free extracts could be due to the absence of conditions for reversed electron transport to take place, or to disruption, solubilization or dilution of essential electron transfer components on preparation of extracts.

Ribbons *et al.* (1970), Kelly (1971) and Wilkinson (1971) have pointed out the similarities between methane-utilizing bacteria and lithotrophic bacteria. There is a striking similarity between the methane-CO mono-oxygenase system in *P. methanica* and the ammonia-oxidizing system recently described by Suzuki (1974) in *Nitrosomonas*. The analogy between hydroxylamine (or NADH) stimulating ammonia oxidation by cell-free extracts or hydroxylamine stimulating ammonia oxidation by whole cells or sphaeroplasts (Hooper, 1969; Suzuki & Kwok, 1969), and the effect of alcohols on the methane-CO mono-oxygenase system in whole cells of *P. methanica*, is clear. Ammonia oxidation is strongly inhibited by CO (Hooper & Terry, 1973) though whether CO is oxidized by *Nitrosomonas* was not tested. Carbon monoxide can be oxidized by a variety of bacteria other than methane-utilizers, as reviewed by Schlegel (1974), though by mechanisms different from that found in *P. methanica*.

The non-reproducibility and instability of methane-oxidizing activity in extracts of methane oxidizing bacteria have hitherto hindered study of the oxidative enzymes involved. The reproducibility of the mono-oxygenase activity in extracts of *P. methanica*, the recognition of its sensitivity to sonication, and awareness of the advantages of using CO as a substrate analogue of methane may aid future studies in this field.

We thank the Science Research Council for financial support under grant No. B/RG/27118, and Norges Teknisk-Naturvitenskapelige Forskningsråd for additional support to T.S.

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