Studies on the physiology of methane-utilising bacteria

by

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Degree of Doctor of Philosophy

University of Edinburgh

1975

I declare that this thesis has been composed entirely by myself and the experiments and results described herein are a product of my own work.

John H. Hubley.

The Microbe is so very small
You cannot make him out at all,
But many sanguine people hope
To see him through a microscope.
His jointed tongue that lies beneath
A hundred curious rows of teeth;
His seven tufted tails with lots
Of lovely pink and purple spots,
On each of which a pattern stands,
Composed of forty separate bands;
His eyebrows of a tender green;
All these have never yet been seen -
But Scientists, who ought to know,
Assure us that they must be so ...

Oh! let us never, never doubt

What nobody is sure about!

HILAIRE BELLOC

SUMMARY

1. The oxidation of methane by washed cell suspensions of Methylosinus trichosporium OB3B was inhibited by 22 compounds including metal-binding compounds such as KCN (10^{-6}M), αα'dipyridyl (10^{-4}M), 8-quinolinol (10^{-4}M), thiosemicarbazide (10^{-5}M), thiourea (10^{-4}M), hydroxylamine (10^{-3}M), histidine (10^{-2}M), British Anti-Lewisite (5×10^{-3}M) and miscellaneous known inhibitors of other oxygenases. The lack of any significant inhibition of methanol and formate oxidation by the same concentrations of these compounds and the ability of Methylomonas albus BG8 to grow with methanol but not methane as carbon source in their presence indicates that these compounds are specific inhibitors of the methane oxygenase. A role for copper in the methane oxygenase system was suggested by the pattern of inhibition and relief of inhibition by added metal ions.

2. The nitrogen-stabiliser 2-chloro,6(trichloromethyl) pyridine ('N-SERVE') was also found to selectively inhibit methane oxidation at a concentration of 0.67×10^{-4}M in both M. albus BG8 and M. trichosporium OB3B.

3. Hydrazine sulphate at a concentration of 2×10^{-3}M selectively inhibited methanol oxidation in suspensions of M. albus BG8 and M. trichosporium OB3B.

4. The ability of M. trichosporium OB3B to oxidise ammonia was confirmed by the demonstration in washed cell suspensions of both ammonia and hydroxylamine-stimulated nitrite production and the former was found to be stimulated by the addition of formate. Ammonia-stimulated nitrite production in the presence of formate was inhibited to a greater extent

than the hydroxylamine-stimulated nitrite production by 8-quinolinol (10^{-4}M), aa'dipyridyl (10^{-4}M), allyl thiourea (10^{-4}M), thiosemicarbazide (10^{-4}M) dithyldithiocarbamate (7.5×10^{-4}M) and N-SERVE (0.67×10^{-4}M).

5. Ethylene was shown to disappear from incubations of suspensions of M. trichosporium OB3B and the addition of formate stimulated this disappearance. Both the formate-stimulated ethylene and ethane oxidation were inhibited by the inhibitors of methane oxidation 8-quinolinol (10^{-4}M), aa'dipyridyl (10^{-4}M), thiosemicarbazide (10^{-4}M), thiourea (10^{-4}M), hydroxylamine (10^{-3}M) and N-SERVE (0.67×10^{-4}M).

6. The specific inhibition of methane oxidation by carbon monoxide was confirmed and the sensitivity of ethylene oxidation and ammonia-stimulated nitrite production in M. trichosporium OB3B to inhibition by CO also demonstrated. Carbon monoxide was found to stimulate respiration and CO_2 production by washed cell suspensions of M. albus BG8 and M. trichosporium OB3B and ^{14}C tracer techniques were used to confirm the oxidation of carbon monoxide by these two methylobacteria. Carbon monoxide-stimulated respiration by M. trichosporium OB3B was inhibited by nine of the specific inhibitors of methane oxidation described in (1) and an oxygenase mechanism for the oxidation of carbon monoxide by methylobacteria was indicated by the demonstration by mass spectrometry of the ^{18}O-enrichment of the CO_2 evolved by incubations of M. trichosporium OB3B with CO and ^{18}O-enriched molecular oxygen. Formate was found to stimulate CO respiration by M. albus BG8 and M. trichosporium OB3B and a role for endogenous metabolism in the oxidation of CO by the latter organism was suggested by the ability of CO to

stimulate endogenous $^{14}CO_2$ production from ^{14}C-labelled *M. trichosporium* OB3B.

7. Suspensions of methylobacteria were examined by Electron Paramagnetic Resonance Spectroscopy (E.P.R.) and an E.P.R.-detectable Cu (II)-like species was found in *Methylomonas albus* BG8, *Pseudomonas methanica*, *Methylosinus trichosporium* OB3B, and *Methylosinus trichosporium* OB4. Examination of the proposed facultative methylotroph XX revealed the presence of an E.P.R.-detectable species resembling Cu (II) in suspensions of the methane-grown but not the glucose-grown cells. An E.P.R.-detectable Cu (II) species was not observed in suspensions of *Escherichia coli* B and the hydrocarbon utiliser *Brevibacterium* JOB5, indicating some significance for the proposed copper species, however the lack of reproducible reduction of the E.P.R.-detectable Cu(II)-like species on addition of methane prevented the conclusive assignment of a role for this species in methane oxidation.

CONTENTS

ABBREVIATIONS

ATP	:	adenosine triphosphate
NAD(H)	:	nicotinamide adenine dinucleotide (reduced)
NADP(H)	:	nicotinamide adenine trinucleotide (reduced)
FAD(H)	:	flavin adenine dinucleotide (reduced)
PMS	:	phenazine methosulphate
DCPIP	:	2,6-dichlorophenolindolphenol
P.H.B.	:	poly- β -hydroxybutyrate
N.M.S.	:	nitrate mineral salts medium
A.M.S.	:	ammonium mineral salts medium

INTRODUCTION

general introduction

The state of knowledge of the properties of methane-oxidising bacteria has advanced considerably since the initial isolation by Söhngen (1906) of Bacillus methanica from aquatic plant material which was followed up by further isolations leading to the characterisation of Pseudomonas methanica (Dworkin and Foster 1956, Leadbetter and Foster 1958), Methanomonas methano-oxidans (Strawinski and Brown 1957, Brown and Strawinski 1958 and Brown et al. 1964) and Methylococcus capsulatus (Foster and Davis 1965). Growing interest in the potential of methane as a substrate for the production of single-cell protein led to a break-through in isolation techniques by Whittenbury et al. (1970) who, by isolating over a hundred strains of methane-oxidising bacteria forming five distinct morphological groups from samples of mud, water and soil from a number of countries, firmly established the ubiquity of these organisms in nature.

The widespread occurrence of methane-oxidising bacteria is not surprising considering the large quantities of methane produced in nature from biological sources, estimated to be 1,600 million tons per year on a global level (Robinson and Robbins 1967) or 3000 pounds per acre each year from swamps or rice paddies (Stevens, 1972). Other authors quote figures of methane production from less exotic sources such as an eructating cow (Quayle, 1972)! The fate of biologically produced methane in the atmosphere is generally considered to be the conversion by photochemically-produced hydroxyl radical to formaldehyde, carbon monoxide and carbon dioxide

(McConnel et al., 1971) and a significant role for methane-oxidising bacteria in the removal of methane has yet to be established. The ecology of methane oxidising bacteria has received little attention but reports of the ability of these organisms to fix atmospheric nitrogen (Coty, 1967, Whittenbury et al. 1970, de Bont and Mulder 1974), oxidise ammonia (Whittenbury et al. 1970), carbon monoxide (Ferenci 1974) and ethylene (de Bont and Mulder, 1974) reveals the versatility of this group.

The prefix 'methylo' was introduced by Foster and Davis (1965) to describe bacteria with an obligate growth requirement for methane and this convention was followed by Whittenbury et al. (1970) in naming their isolates. However the isolation of bacteria with an obligate growth requirement for C_1 substrates other than methane such as the methylated amines (Colby and Zatman 1972) and methanol (Dahl et al. 1972, Sperl et al., 1974) has led to the expansion of the original concept of methylobacteria to include bacteria with an obligate growth requirement for any C_1 substrate except CO_2 and the term 'methylotroph' has been introduced by Colby and Zatman (1972) to describe this group. The term methylobacteria will, however, be used for methane-oxidising bacteria in this study unless it is stated otherwise. All methylobacteria which have been studied in any detail have shown an obligate growth requirement for methane, methanol or dimethyl ether, are strict aerobes, Gram-negative, oxidase-and catalase-positive and may contain resting stages or be motile. In view of the notorious contamination

problems encountered in handling methylobacteria the earlier reports of heterotrophic methane oxidisers must be viewed with suspicion. The bacterium JOB 5 has been reported to grow on methane in addition to higher hydrocarbons (Ooyama and Foster, 1965; Perry, 1968) but no further reports of the methane-oxidising ability of this bacterium have appeared. This generalisation may have to be amended in the light of recent reports (Patt, et al., 1974; Naguib and Overbeek, 1970; Cappenberg 1972) of the isolation of methylobacteria which are capable of heterotrophic growth on carbon sources such as sugars but the validity of these controversial claims has yet to be fully established.

Electron micrographs of thin sections of methylobacteria have revealed the presence of two contrasting types of highly developed internal membrane systems (Whittenbury 1969, Davies and Whittenbury 1970, Smith and Ribbons 1970, De Boer and Hazeu 1972) with the membranes organised in stacks at the centre of the cell (type I) and along the periphery (type II) and it has been speculated that the metabolism of methane takes place in these membrane structures (Ribbons and Michalover 1970). The presence of triterpenoids such as squalene and sterols has been demonstrated in Methylococcus capsulatus (Bird et al. 1971a, Bird et al. 1971b) but the role of these compounds, considered until quite recently to be absent from prokaryotes (Stanier 1970), in the membranes of methane-oxidising bacteria remains to be established. The extension of the differences between membrane-type I and type II methylobacteria to include not only the membrane systems but also the pathway of carbon assimilation (Lawrence

and Quayle 1970), the presence or absence of a complete Krebs cycle (Davey et al. 1972), the presence of polyhydroxybutyrate as a storage polymer (Thomson 1974) and the cytochrome content (Davey and Mitton 1973) strongly supports the existence of a fundamental evolutionary division between these two groups. The difference in properties of the two membrane-types are summarised in table 1.

Table 1: Difference between membrane-type I and II methylobacteria

	Type I	Type II
Internal membrane	stacked	peripheral
Carbon assimilation	hexulose phosphate	serine
Tricarboxylic acid cycle	incomplete	complete
Polyhydroxybutyrate	absent	present
Glucose-6-phosphate and Gluconate-6-phosphate dehydrogenases	present	absent

The present study has been directed at understanding the nature of the oxidation of methane, and accordingly, the following review is confined to the existing state of knowledge of the biochemical pathway of methane oxidation and will not discuss the isolation, morphology and taxonomy of methylobacteria which has been well reviewed by Quayle (1972) or the applied aspects of the production of single-cell protein from methane which is discussed by Wilkinson (1971). A full discussion of the limited state of knowledge of the genetics of Methylobacteria can be found in Williams and Bainbridge (1974) and Shimmin (1974).

PATHWAY OF METHANE AND ETHANE METABOLISM

The pathway of methane oxidation

The oxidation of methane is generally assumed to take place by the following series of steps:

$$CH_4 \longrightarrow CH_3OH \longrightarrow HCHO \longrightarrow HCOOH \longrightarrow CO_2 \qquad (1)$$

Quayle (1972) has provided an excellent review of the evidence for the above pathway which will only be summarised below. The main evidence comes from the following observations:

1) The ability of washed cell suspensions of methylobacteria to oxidise the above compounds and of methanol to act as a growth substrate.

2) The demonstration of methanol, formaldehyde and formate oxidation in cell-free extracts of methylobacteria.

3) The accumulation and detection of the above compounds in suspensions of methylobacteria during methane oxidation. Quayle (1972) discusses the inconsistencies in the literature in the observations of different groups on the effect of trapping compounds. The accumulation of compounds in the presence of trapping agents such as semicarbazide or bisulphite (Brown and Strawinski, 1958) should be interpreted with caution as possible interactions between the added compounds and the cells may provide alternative explanations for the accumulation of a proposed intermediate.

4) The demonstration that the incorporation of carbon from methane into cellular material takes place at the level of formaldehyde in membrane-type I

methylobacteria and formaldehyde or formate in type II (Lawrence and Quayle, 1970).

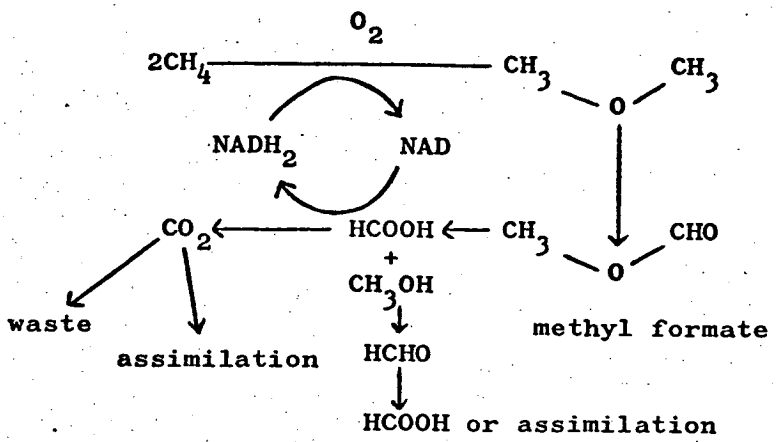
The proposal that methane was metabolised by an oxygenase mechanism was initially made by Leadbetter and Foster (1959) who showed that cells of Pseudomonas methanica, grown on methane in the presence of ^{18}O-enriched molecular oxygen, incorporated sixteen times more ^{18}O than cells grown on methanol. Although a non-oxygenase mechanism for methane oxidation was proposed by Whittenbury et al. (1970), all doubt on the matter was dispelled by Higgins and Quayle (1970) who, by preferentially inhibiting with high phosphate concentrations methanol oxidation in suspensions of Pseudomonas methanica and Methanomonas methanooxidans incubated with methane and $^{18}O_2$, were able to demonstrate a close correlation between the ^{18}O-content of the molecular oxygen and the accumulated methanol. Consistent with an oxygenase mechanism for methane oxidation they were able to demonstrate the absence of ^{18}O-incorporation into methanol by cell suspensions incubated with methane in the presence of ^{18}O-enriched water. Davey and Mitton (1973) and Ferenci (1974) subsequently showed that the methane oxygenase reaction was inhibited by carbon monoxide. The state of knowledge of the methane oxygenase system will be discussed in a later section of this introduction together with the mechanism of hydrocarbon oxidation in general.

the role of dimethyl ether

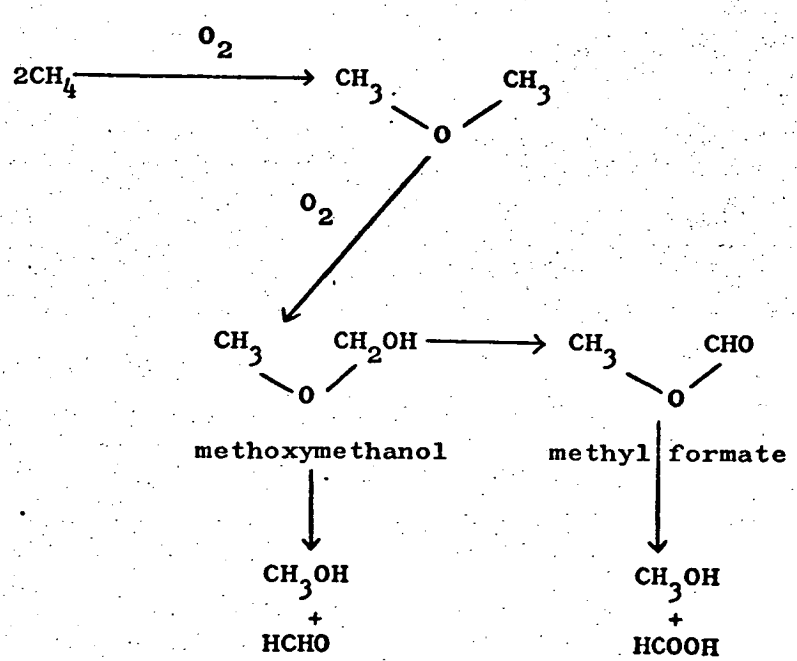
The evidence for the above pathway for methane oxidation is good for the subsequent metabolism of methanol, all the

Fig. 1: Pathways of methane oxidation with dimethyl ether as intermediate

A) After Davey (1971)



B) After Mitton (personal communication)



steps of which can be demonstrated in cell-free extracts, but does not rule out an intermediate between methane and methanol. The unpublished observations of Bryan-jones and Wilkinson (Wilkinson, 1971) that dimethyl ether can act as a growth substrate for methylobacteria and be oxidised in cell suspensions has led to the suggestion that instead of the oxygenation of methane proceeding directly to methanol, the initial product formed is dimethyl ether according to the following reaction:

$$2CH_4 + O_2 \longrightarrow CH_3OCH_3 + H_2O \qquad (2)$$

Wilkinson (1971) discussed the advantages such a reaction would have in explaining the cellular growth yields of methylobacteria. Using ^{18}O studies, dimethyl ether was subsequently shown by Dr Mitton (personal communication) to be metabolised by a carbon monoxide-sensitive oxygenase reaction to form methyl formate which was hydrolysed to formate and methanol by means of an esterase. Davey (1971) incorporated these observations into a proposed pathway of methane oxidation shown in figure 1A.

Mitton et al. (in preparation) however were unable to detect ^{14}C-incorporation into methyl formate by cell suspensions of Methylomonas albus BG8 and Methylosinus trichosporium OB3B incubated with $^{14}CH_4$ under conditions where $^{14}CH_3OH$ was readily detectable. In order to preserve a role for dimethyl ether, these authors suggested the scheme in fig. 1B in which the initial product of dimethyl ether might be the unstable hemi-acetal methoxymethanol, which could either decompose spontaneously to form formaldehyde and methanol or form

methyl formate. The former route is more attractive since the methyl formate route results in the conversion of one molecule of methane to formate which is at an oxidation level too high for direct incorporation into the serine and hexulose phosphate one-carbon assimilation pathways and in order to obtain cellular yields above 50% (see Wilkinson 1971 and Quayle 1972) fixation of most of the CO_2 arising from formate oxidation is required.

Dimethyl ether has been shown to accumulate in small amounts when cell suspensions of methylobacteria are incubated with methane but the inability to define reproducible conditions for this accumulation makes the evidence extremely unsatisfactory (Thomson 1974; Mitton et al. in preparation). Hence there is no direct evidence for the involvement of dimethyl ether as an intermediate although its participation under certain ill-defined conditions cannot be ruled out. It is unfortunate that in neither of the two studies where the oxidation of methane has been demonstrated in cell-free extracts have any product of methane oxidation been reported (Ribbons and Michalover, 1970; Ferenci 1974).

pathway of ethane oxidation

Ethane and higher alkanes can be oxidised by all methylobacteria although they cannot act as growth substrates (Leadbetter and Foster 1960; Whittenbury et al. 1970; Davey 1971) and it has been suggested that the methane oxygenase system is responsible for this reaction.

This led Thomson (1974) to carry out a detailed study of the metabolism of ethane by Methylomonas albus BG8 and Methylosinus trichosporium OB3B in the hope that such a study would shed light on the pathway of methane oxidation. He was able to confirm the observations of Leadbetter and Foster (1959) that suspensions of methylobacteria were able to oxidise ethane, ethanol, acetaldehyde and the accumulation of acetaldehyde and its subsequent metabolism to acetate was confirmed by gas chromatography. These results suggested a pathway of ethane oxidation analogous to (1):

$$C_2H_6 \longrightarrow C_2H_5OH \longrightarrow CH_3CHO \longrightarrow CH_3COOH \qquad (3)$$

The ability of suspensions of the two methylobacteria to carry out the carbon monoxide-sensitive oxidation of diethyl ether at low rates introduced the possibility of an ether intermediate analogous to that proposed for methane oxidation. However the metabolism of diethyl ether was shown to proceed, not to acetaldehyde and acetate, but by terminal hydroxylation and subsequent metabolism to 2-ethoxyacetic acid. This ruled out as unlikely an initial step in ethane oxidation involving formation of an ether intermediate and assuming the analogy between methane and ethane oxidation in methylobacteria. Thomson (1974) suggested that these results provided evidence for the non-involvement of dimethyl ether as an intermediate in methane oxidation.

In order to test this assumption strenuous efforts were made by Thomson to prove that methane, ethane, dimethyl ether and diethyl ether were all oxidised by the same enzyme and, in spite of the sensitivity of all these reactions to inhibition by carbon monoxide, the contradiction between the

insensitivity of ethane and diethyl ether oxidation to inhibition by dimethyl ether and the extreme sensitivity of methane oxidation to the same compound led Thomson, reluctantly, to the conclusion that methane and ethane were metabolised by different oxygenase enzymes.

The Assimilation of Carbon by Methylobacteria

Incorporation experiments with $^{14}CO_2$ have shown that CO_2 can be assimilated into cellular materials, however the amounts incorporated were insufficient to account for all the cell carbon (Leadbetter and Foster 1958, Johnson and Quayle 1965, Eccleston and Kelly 1973). This evidence, together with the absence of carboxydismutase from Pseudomonas methanica (Johnson and Quayle 1965) emphasises the distinction between methylotrophs and autotrophs which derive their carbon from the fixation of CO_2 (Rittenberg, 1969).

Subsequent studies, reviewed in detail by Quayle (1972), showed the existence of two distinct pathways of incorporation of C_1 units and that the distribution of the two different pathways among methylobacteria corresponded to the division between the two membrane-types.

Membrane-type I methylotrophs contain a 3-hexulose phosphate cycle in which a molecule of formaldehyde condenses with ribulose-5-phosphate to form what was initially identified as allulose phosphate (Kemp and Quayle, 1966, 1967) but which has now been shown to be D-erythro-L-glycero-3-hexulose phosphate (Kemp, 1974; Strøm et al.,1974; Ferenci et al., 1974).

Membrane-type II methylotrophs, as well as some methanol-utilisers, contain a 'serine pathway' of incorporation in

which the principal reaction is the condensation of a tetrahydrofolate with an unknown C_1 product of methane or methanol oxidation and the condensation of the resulting 5.10-CH_2-tetrahydrofolate derivative with glycine to form serine which subsequently reacts with glyoxylate to form hydroxypyruvate. A major advancement since the review by Quayle (1972) has been the identification of the role of the enzyme malyl-coenzyme A lyase in the generation of glyoxylate units (Salem et al. 1973, Hersh and Bellion 1972, Bellion and Hersh 1972, Hacking and Quayle 1974).

That the two pathways may not be mutually exclusive is suggested by the recent demonstrations of the presence of two of the major enzymes of the serine pathway hydroxypyruvate reductase (Strøm et al., 1974) and malyl Coenzyme lyase (Salem et al., 1974) in the membrane-type I methylobacteria Pseudomonas methanica and Methylococcus capsulatus and the incorporation of ^{14}C-formaldehyde into serine and glycine by whole cells of the latter organism (Eccleston and Kelly, 1973).

THE OXIDATION OF METHANOL AND ETHANOL

distribution and properties of methanol dehydrogenase

Methanol dehydrogenase activity has now been demonstrated in cell-free extracts of methane-utilisers, obligate and facultative methanol-utilisers (see table 2) and the property of the extracts of oxidising only primary alcohols with rates of oxidation decreasing with chain length has led to the term 'primary alcohol dehydrogenase' (P.A.D.) supplanting 'methanol dehydrogenase'. With one exception such cell-free systems

Table 2. Methanol Dehydrogenase Activity in extracts of Facultative and Obligate Methylotrophs

Group	Organism	References
Methane-Oxidisers type II	Methylosinus trichosporium OB3B	Davey (1971)
	" 5	Quayle (1972)
	Methylosinus sporium 12	Davey (1971)
	Methylocystis parvus OBBP	"
type I	Methylomonas albus BG8	"
	Methylomonas methanica	"
	Methylococcus minimus TMC	"
	Methylococcus capsulatus (Texas strain)	Patel and Hoare (1971)
	Pseudomonas methanica	Johnson and Quayle (1964)
Obligate Methanol-Utilisers	Pseudomonas W1	Dahl et al. (1972)
	Hyphomicrobium WC	Sperl et al. (1974)
	Pseudomonas TP1	
Facultative Methanol-utilisers	Pseudomonas AM1	Johnson and Quayle (1964)
	Protoaminobacter	"
	Vibrio extorquens	"
	Pseudomonas PP	Ladner and Zatman (1969)
	Pseudomonas M27	Anthony and Zatman (1964a 1964b)

could not use Nicotinamide Adenine Dinucleotides as electron acceptors but showed a strict requirement for phenazine methosulphate as primary acceptor, a requirement for activation by ammonia or methylamine and a pH optima of 9. The exception is the demonstration of NAD-linked dehydrogenation in a methanol-, but not methane-, utilising organism identified as Pseudomonas methanica (Harrington and Kallio, 1960). The reaction was considered to proceed by a peroxidative attack because of the requirement for an H_2O_2 generating system. Johnson and Quayle (1964) point out this reaction may be an in vitro artefact having nothing to do with in vivo methanol oxidation and the absence of such activity in Pseudomonas M27 (Anthony and Zatman, 1964b) and Pseudomonas AM1 (Johnson and Quayle, 1964) would support this view. Harrington and Kallio (1960) did not examine their extracts for the presence of P.M.S.-dependent methanol dehydrogenase activity.

The ability of certain yeasts to utilise methanol as sole carbon source is now well established (Ogata 1974) and studies on the methanol-oxidising enzyme from Kloeckera spp show major differences from the P.M.S.-linked enzyme in bacteria. No P.M.S.-linked primary alcohol dehydrogenase was found, instead there was a FAD-containing alcohol oxidase enzyme catalysing the oxidation of methanol to formaldehyde with the formation of hydrogen peroxide.

purification and properties of primary alcohol dehydrogenase

The enzyme has now been purified in Pseudomonas M27 (Anthony and Zatman, 1967a; Anthony and Zatman, 1967b), Methylococcus capsulatus texas strain (Patel et al. 1972) and

Hyphomicrobium strain WC, Pseudomonas W1 and Pseudomonas TP1 (Sperl et al. 1974). Similar methods of purification were used in each case and involved ammonium sulphate fractionation followed by chromatography on DEAE cellulose and Sephadex. The P.A.D. from Pseudomonas M27 was purified to more than 95% homogeneity as judged by ultra-centrifugal and electrophoretic analysis and similar criteria were used to establish the purity of the other P.A.D.s. The enzyme from Pseudomonas M27 constituted 10% of the cell protein (Anthony and Zatman 1967a).

The properties of the purified enzymes show similarities in their molecular weight of 120,000, in having equal sub-units, an absorption spectrum with a peak at wavelength 350 nm, a pH optimum of 9 and a requirement for activation by ammonia or methylamine.

Patel et al. (1972) have carried out a detailed comparison of the purified enzymes from Pseudomonas M27 and Methylococcus capsulatus and showed that they each consist of two sub-units of molecular weight 62,000 which were non-covalently joined. The enzymes differed in their electrophoretic mobilities although the amino acid contents showed strong similarities, differing only in the amounts of the charged amino acids, which could account for the faster mobilities of the enzyme from Methylococcus capsulatus.

Anti-sera against these two enzymes have been prepared (Patel et al., 1973) and showed that each of the two enzymes possess distinct as well as shared antigenic determinants which would indicate differences in conformational, and hence

primary structure. Their speculation that shared antigenic regions represent active sites of the enzymes was reinforced by the inhibition of enzyme activity found when each enzyme was treated with anti-sera prepared from either enzyme. Examination of antibody precipitation and gel diffusion reactions between these two anti-sera and crude cell extracts from a number of methylotrophs revealed that membrane type I methylobacterial strains Methylococcus capsulatus Bath and Texas strains, Methylococcus minimus, Culture CF (Methylobacter sp.) and Pseudomonas methanica form a natural group as do the type II methylotrophs Methylosinus sporium and Methylosinus trichosporium strain 5 with the methanol-oxidisers Hyphomicrobium B 522, Pseudomonas M27 and "pink organism". Extracts from one methanol-utiliser Pseudomonas W showed no antibody reactions with the two anti-sera.

Their limited survey thus suggests that, despite the sharing of common antigenic determinants, the difference between the membrane-type I and II methylobacteria extends to the level of the methanol dehydrogenase enzyme and that some methanol-utilisers can be considered related to type II methylobacteria which would be expected on the grounds of the common possession of the serine pathway of carbon assimilation (Lawrence and Quayle, 1970). Although these studies appear promising, cross-reaction data from a wider range of methylotrophs will be required before taxonomic conclusions of any real significance can be drawn.

Dual specificity of alcohol dehydrogenase

There is now good evidence that the primary alcohol dehydrogenase can bring about the P.M.S.-dependent oxidation

of formaldehyde to formate in the presence of ammonium ions. This was originally claimed by Ladner and Zatman (1969), but the validity of their report is questionable because of their discovery at a late stage of the presence of methanol as an impurity in their formaldehyde. Further circumstantial evidence for the dual specificity of the enzyme comes from the demonstration by Heptinstall and Quayle (1970) that extracts of Pseudomonas AM1, under conditions of assay for methanol dehydrogenase, oxidised formaldehyde at 26% of the rate of methanol and that their mutant strain M-15A, which lacked methanol dehydrogenase, was unable to oxidise formaldehyde. More satisfactory evidence comes from the demonstration that the purified P.A.D. from Methylococcus capsulatus oxidised both formaldehyde and methanol and that the ratios of specific activity of methanol and formaldehyde-oxidising activity were constant under widely-differing conditions of pH and ammonium ion concentration (Patel and Hoare, 1972). Studies of the properties of the purified P.A.D.s from three methanol-utilisers Hyphomicrobium WC, Pseudomonas strain TP1 and Pseudomonas W (Sperl et al. 1974) further support the dual specificity of the alcohol dehydrogenase and also revealed that, when oxygen uptake was followed manometrically over a fifteen minute period acetaldehyde, but not aldehydes of longer carbon chain length, could be oxidised at a low rate. The inability of other laboratories to detect acetaldehyde oxidation was ascribed to the short time interval over which the spectrophotometric dye reduction assays were usually carried out. The property of formaldehyde, and acetaldehyde, but not higher aldehydes,

of undergoing hydration to the gem-diols was suggested as the reason why these two aldehydes alone act as substrates for the primary alcohol dehydrogenase.

Mechanism of action of primary alcohol dehydrogenase enzyme

The mechanism of action of the P.A.D. is unknown: the enzyme does not contain a metal or a flavin (Anthony and Zatman, 1967a; Anthony and Zatman, 1967b) but it is now well-established that the enzyme contains a pteridine derivative whose role in the oxidation of methanol has yet to be understood. Anthony and Zatman (1967b) showed that acid and alkali treatment as well as boiling released a green fluorescent material which was considered to be a pteridine derivative on the basis of the fluorescence and absorption spectrum of the partially-purified compound. There appeared to be a direct relationship between inactivation of the enzyme and release of the fluorescent material although as Quayle (1972) points out this does not necessarily imply a causal relationship.

Evidence for the presence of pteridine derivatives in methylobacteria, but not necessarily their involvement in the methanol dehydrogenase reaction, comes from the isolation and chemical characterisation of four pteridine derivatives from Methylosinus trichosporium PG and Methylosinus sporium strain S (Urushibara and Forrest, 1970). This group has recently reported, without presenting their evidence in detail, the isolation of the same fluorescent compound from the purified primary alcohol dehydrogenases from Pseudomonas TP1, Hyphomicrobium sp W.C., Methylococcus capsulatus Texas

strain, Pseudomonas M27 and Pseudomonas methanica, and from the effects of chemical treatment on the fluorescence and absorption spectra, the compound was considered to be a derivative of lumazine, a pteridine (Sperl et al., 1974).

Two mechanisms have been proposed by which pteridines might play a part in methanol oxidation (Quayle 1972; Anthony and Zatman 1967b). The first includes a novel reaction involving the binding of the methyl group of methanol to the N-5 of the pyrazine ring derivative and the subsequent formation of a 5, 10 methylene tetrahydro derivative.

A second mechanism is one in which the pteridine derivative might function as the natural electron acceptor for methanol dehydrogenase and support for this hypothesis comes from the similarity of the standard electrode potential for the quinonoid dihydropterin couple (E_o^1 = + 0.5v.) and the dehydrogenation of methanol to formaldehyde. The known involvement of pteridine derivatives as electron donors in mono-oxygenase reactions raises the interesting possibility that methanol dehydrogenation might provide electrons for methane oxidation through such a cofactor.

A role for cytochrome c in the oxidation of methanol by Pseudomonas AM1 is suggested by the inability of cells from a cytochrome c-deficient mutant containing normal levels of methanol dehydrogenase to oxidise methanol (Anthony, 1975) and the higher levels of cytochrome c in methanol- than succinate-grown cells of Pseudomonas AM1 (Tonge et al., 1974). The evidence from cell-free studies of a coupling between the methanol dehydrogenase enzyme and cytochrome c is still inconclusive (Anthony, 1975). The lack of reduction

of Nicotinamide Adenine Dinucleotides and FAD by the methanol dehydrogenase also raises the problem of the identity of the natural electron acceptor.

Any speculation on the mechanism of methanol oxidation and the subsequent fate of the electrons generated must be made with caution. The first mechanism is attractive because it suggests how one-carbon units can be assimilated in membrane-type II methylotrophs (containing the serine pathway) through the formation of co-enzyme bound C_1 units. The advantage of the second mechanism is the possible role such a reduced co-factor might play in methane oxidation or, in the absence of any other acceptor, in electron transfer to the cytochrome chain.

THE METABOLISM OF FORMALDEHYDE AND FORMATE

Although a NAD-linked formaldehyde dehydrogenase activity has been observed in some methanol-utilisers (for review see Quayle, 1972) and a facultative methylamine-utiliser Bacterium 4B6 (Colby and Zatman, 1972), the absence of a NAD-linked and the presence of a PMS-linked ammonia-requiring formaldehyde dehydrogenase activity in a wide range of both membrane-types I and II of methylobacteria (Johnson and Quayle, 1964; Davey, 1971; Patel and Hoare, 1971) together with the ability of the purified methanol dehydrogenase enzyme to carry out the oxidation of formaldehyde and acetaldehyde (Patel et al., 1972; Sperl et al., 1974) supports a role for the methanol dehydrogenase enzyme in the in vivo metabolism of formaldehyde and acetaldehyde.

Formate dehydrogenase activity linked to NAD reduction is widespread in micro-organisms and the occurrence of this enzyme in methylotrophs has been reviewed (Ribbons *et al.*, 1970; Quayle, 1972). Since these reviews the presence of this enzyme in ten methylobacteria, including representatives of both membrane types, has been demonstrated by Davey (1971) and in *Methylococcus capsulatus* Texas strain (Patel and Hoare, 1971).

Studies on the biochemistry of this enzyme in methylobacteria have not been extensive. The partially-purified enzyme from *Pseudomonas* AM1 (Johnson and Quayle, 1964) was specific for formate and was inhibited by CN^- (95% inhibition by 5×10^{-4}M), ferrous ions (50% inhibition by 10^{-6}M) and copper (92% inhibition by 2×10^{-5}M Cu^{2+}) and an even greater sensitivity to CN^- (85% inhibition by 2×10^{-6}M) was shown by the formate dehydrogenase in extracts of *Methylococcus capsulatus* (Patel and Hoare, 1971).

Formate oxidation is the only step in the proposed pathway of methane oxidation (equation 1) which is known to generate NADH and the possible significance of this will be discussed in a later section.

THE METHANE OXYGENASE

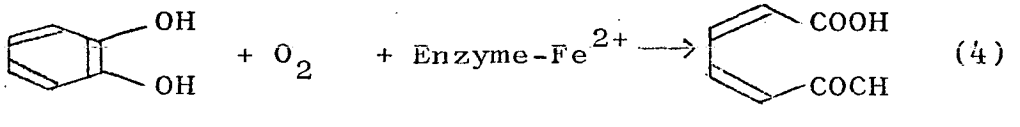
Introduction to oxygenases

The first demonstration that molecular oxygen could be directly incorporated into a substrate was by Mason *et al.* (1955) who showed the incorporation of ^{18}O into 5, 4 dimethyl catechol by a Pseudomonad species in the presence of ^{18}O-enriched molecular oxygen. The term has now come into use to describe all reactions where oxygen is introduced into a

molecule from molecular oxygen instead of water.

Much of the early interest in this class of reactions lay in the commercial potential of hydrocarbon-oxidising micro-organisms for the production of single-cell protein (see Wilkinson 1971) but more recently interest has been stimulated by the increasing awareness of the important role oxygenase reactions play in the metabolism of hormones, drugs and carcinogens in mammalian organisms, especially those reactions involving cytochrome P-450 (Boyd, 1972). The application of physical techniques such as visible and mössbauer spectroscopy, electron paramagnetic resonance spectroscopy and circular dichroism to the study of oxygenase reactions both in cell-free extracts and the purified enzymes has led to a rapid expansion of the now-sophisticated field of oxygenase biochemistry which has been the subject of a considerable number of reviews and symposia (Hayaishi, 1966; Hayaishi, 1969; Hamilton, 1969, Boyd 1972, Mason, 1965; Jurtshuk and Cardini, 1971; King et al., 1973; Hayaishi, 1974). Only a brief summary of this well-documented field will be presented below with examples derived mainly from hydrocarbon-oxidising systems rather than those of drug metabolism.

Dioxygenase is the term given to the class of reaction where both of the oxygen atoms in molecular oxygen are incorporated into the substrate and an example of this class of reactions is the m-pyrocatechol oxygenase of Pseudomonas arvilla which brings about the ring cleavage reactions shown below (Hayaishi, 1955):



(4)

When only one of the oxygen atoms is introduced into the substrate the reaction is termed a mono-oxygenase. The oxygen atom that is not incorporated into the substrate is reduced to water and because of the dual nature of the reaction the term 'mixed function oxidase' has been introduced (Mason, 1957). Where RH is the substrate and XH_2 a reducing agent, a mono-oxygenase hydroxylation reaction can be represented as follows:

$$RH + O_2 + XH_2 \longrightarrow ROH + X + H_2O \qquad (5)$$

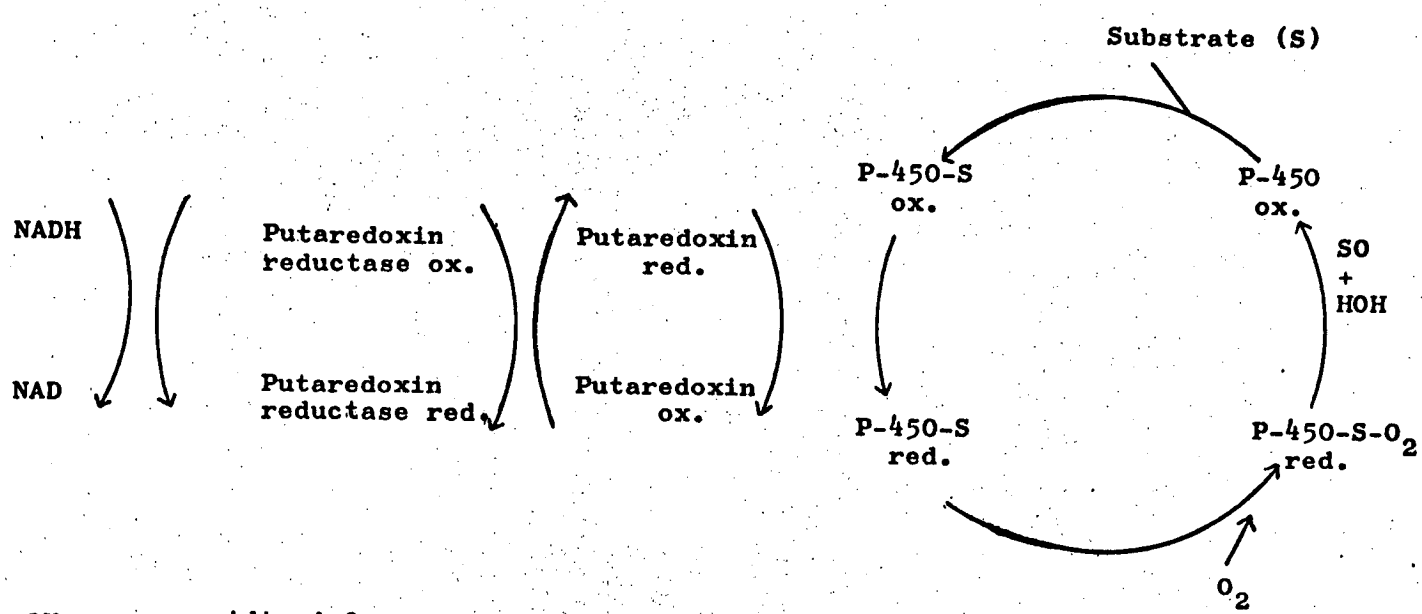
The reducing agent can be part of the substrate ('internal mono-oxygenase') or, as is usually the case, an exogenous reductant such as NADH, NADPH or ascorbic acid ('external mono-oxygenase'). The ability of a compound to serve as an electron donor in cell-free systems does not, however, imply that the compound is involved in the reaction in vivo.

The transfer of electrons from the exogenous reductant to the oxygenase enzyme has been shown to involve co-factors such as the ferredoxin and rubredoxin-type non-haeme iron proteins (reviews Yoch and Valentine, 1972; Orme-Johnson, 1973;Lovenberg, 1973), pteridines and flavins (Hayaishi, 1969).

mechanism of hydrocarbon oxidation

Comprehensive reviews of the mechanism of biological hydrocarbon oxidation have been made by Klug and Markowetz (1971), Jurtshuk and Cardini (1971), Van der Linden and Thijsse(1965) and McKenna and Kallio (1963) and accordingly only a brief discussion follows.

Fig. 2 Proposed Mechanism for the Hydroxylation of Camphor by Pseudomonas Putida[1]



Key ox. = oxidised form
red. = reduced form

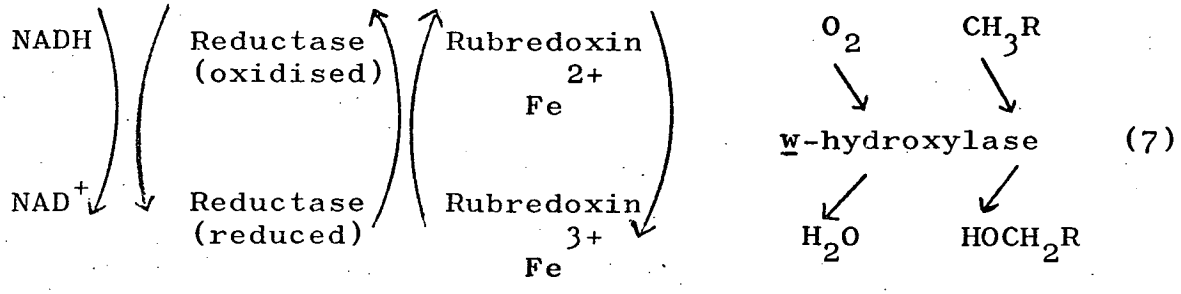
1 taken from Katagiri et al. (1968)

The Cytochrome P-450 mediated hydroxylation of camphor by Pseudomonas putida has been the subject of an intensive study by Gunsalus and his group (for reviews see Gunsalus and Marshall, 1971, Gunsalus et al., 1974) and takes place by the following mono-oxygenase reaction:

$$\text{D-camphor} + \text{NADH}_2 + \text{O}_2 \longrightarrow \text{exo-5-hydroxycamphor} + \text{H}_2\text{O} + \text{NAD} \quad (6)$$

The individual components responsible for the mono-oxygenase reaction have now been isolated and purified and shown to consist of Cytochrome $\text{P-450}_{\text{cam}}$, an iron-sulphur redox protein 'putaredoxin', a flavoprotein FAD reductase and NAD dehydrogenase and electron transport to the oxygenase enzyme is thought to take place according to the left hand portion of sequence in figure 2 (Katagiri et al., 1968).

The n-octane hydroxylase system from Pseudomonas oleovorans, which also w-oxidises a series of fatty acids, has been studied in less detail (Baptist et al., 1963; Gholson et al., 1963; Peterson et al., 1966; Kusunose et al., 1967; Peterson, 1970) but three components have been identified from studies with cell-free systems: an iron-sulphur protein rubredoxin, NADH-rubredoxin reductase and the alkane-1-hydroxylase enzyme. The electron transfer sequence can be represented as follows:



(7)

In addition to the above two studies should be mentioned the demonstration by Cardini and Jurtshuk (1968) and reviewed in detail by Jurtshuk and Cardini (1971), of the oxidation of n-octane by cell-free extracts of Corynebacterium spp strain 71EC which required molecular oxygen, NADH, cytochrome P-450 and a flavoprotein. This reaction was sensitive to inhibition by carbon monoxide as was the Cytochrome P-450 mediated camphor hydroxylase of Pseudomonas putida (Gunsalus et al. 1974). However n-alkane hydroxylation by Pseudomonas oleovorans was insensitive to inhibition by CO (Peterson et al. 1966) raising the question of the identity of the functional group of the mono-oxygenase system. Pseudomonas oleovorans does not contain Cytochrome P-450 (Peterson, 1970) and the observation that n-alkane-grown cells contained higher levels of cytochrome o than cells grown on glucose led Peterson (1970) to suggest that cytochrome o, which has been implicated as a terminal oxidase in other bacteria (Castor and Chance, 1959) may be fulfilling the same function as Cytochrome P-450 in other hydrocarbon-oxidising bacteria. This is highly indirect evidence and conclusive proof for a role for cytochrome o awaits the demonstration of the requirement of a cell-free hydrocarbon oxidising system for added cytochrome o.

The anaerobic oxidation of hydrocarbons by a non-oxygenase mediated reaction has been proposed for Pseudomonas aeruginosa (Chouteau et al., 1962), Candida rugosa and Candida tropicalis (Lebeault et al., 1969)

and a discussion of the evidence against this unlikely mechanism can be found in Klug and Markowetz (1971).

The chemical form of the oxygen atom that is inserted is a subject of considerable controversy and speculation and possible candidates are the hydroxyl radical $^{\cdot}OH$, the oxygen atom O or 'oxene' group, the protonated oxygen atom ^{+}OH and the superoxide anion O_2^- (see Coon et al., 1973 for a discussion). A mono-oxygenase can be envisaged as consisting of a number of distinct steps:

1. Reduction of oxygenase enzyme
2. Combination of oxygen molecule with the enzyme
3. Reduction of one oxygen molecule to water
4. Insertion of one oxygen atom into the substrate

Considerable effort is being directed towards understanding the sequence in which the above steps take place or whether they take place simultaneously through a concerted mechanism. A proposed sequence for camphor hydroxylation (Katagiri et al., 1968) is presented in figure 2. There is also continuing debate as to whether the initial product formed is a hydroxyl or hydroperoxide compound. These two mechanisms are shown in equations 8 and 9 and it can be seen that in the case of hydroperoxide formation the second oxygen would be reduced after insertion of the oxygen into the substrate.

$$RH + (O) \longrightarrow ROH \qquad (8)$$

$$RH + O_2 \longrightarrow ROOH \xrightarrow[2H]{} ROH + H_2O \qquad (9)$$

The involvement of a hydroperoxide free radical intermediate and a free radical equilibrium between C_1 and C_2 position was proposed by Leadbetter and Foster (1960) to

account for the formation of methyl ketones during hydrocarbon oxidation by Pseudomonas methanica. The demonstration by Thomson (1974) that acetone is produced from the breakdown of polyhydroxybutyrate during the metabolism of ethane by Methylosinus trichosporium might account for the production of acetone found by Leadbetter and Foster (1960) but not for the production of higher ketones. Thomson (personal communication, 1974) was unable to detect polyhydroxybutyrate in Pseudomonas methanica, suggesting that Leadbetter and Foster's (1960) observations were not artefacts. The formation of ketones during hydrocarbon oxidation and the possible involvement of hydroperoxide free radical intermediates has been reviewed by Markowetz (1971).

Support for the involvement of hydroperoxide intermediates comes from the demonstration that alkyl hydroperoxides are substrates for the n-alkane oxidising cell-free extracts from Pseudomonas oleovorans but attempts to detect hydroperoxide intermediates have proved negative and n-alkyl hydroperoxides did not act as substrates for the n-alkane hydroxylating system of rat liver (Coon et al. 1973). This does not, however, rule out the participation of hydroperoxides as enzyme-bound intermediates. The participation of epoxide intermediates in mono-oxygenase reactions has also been proposed (Hayaishi, 1969; Gibson, 1971).

the methane oxygenase

Studies of the methane oxygenase have been limited by the lack of reproducible cell-free systems capable of

oxidising methane (Ribbons et al., 1970; Quayle, 1972), however both NADH-stimulated methane respiration as well as methane-stimulated NADH oxidation have now been demonstrated in cell-free extracts of Methylococcus capsulatus texas strain (Ribbons and Michalover, 1970) and Pseudomonas methanica (Ferenci, 1974) and their experimental procedures and results are summarised in table 3. Similar levels of activities were obtained and in both cases the bulk of the activity resided in the particulate fraction. The ability of the extract from Methylococcus capsulatus to carry out an ethane-stimulated NADH dependent respiration and NADH oxidase activity was shown by Ribbons and Michalover (1970) and Ferenci (1974) was able to show carbon monoxide-stimulated NADH dependent respiration and NADH oxidase in cell-free extracts from Pseudomonas methanica.

Neither of these studies showed the uptake of methane by cell-free extracts or confirmed the nature of the product of methane oxidation. A stoichiometry of methane and oxygen uptake for the cell-free reaction was not calculated by Ribbons and Michalover (1970) because of the difficulty in assessing the levels of methane-independent NADH oxidation in the presence of methane and presumably a similar problem was encountered by Ferenci (1974) who also did not give a stoichiometry. In both cell-free systems the ratio of methane-stimulated oxygen uptake and NADH disappearance was 1:1 which is consistent with a mono-oxygenase mechanism for methane oxidation but not proof.

Table 3. Methane-oxidising Cell-free Extracts from Methylobacteria

	Ribbons and Michalover (1970)	Ferenci (1974)
Organism	Methylococcus capsulatus (Texas)	Pseudomonas methanica
Breakage	French Press	Sonication @ 90 secs.
	$MgSO_4$ 10mM.	$MgCl_2$ 5mM
	20 mM. Phosphate buffer pH 7.2	20mM Phosphate buffer pH7
	14.4 mg protein/ml	5 - 10 mg dry wt cells/ml
Methane-stimulated activities[1]		
(1) extract	22.6nm[3] O_2/min/mg protein (48%[2])	32.9nm O_2/min/mg protein (84%)
	28.4nm NADH/min/mg Protein (73%)	32nm NADH/min/mg protein (320%)
(2) Pellet	40,000g, 16 min ('P2')	38,000g, 60 min
	46.3 nm O_2/min/mg prot. (262%)	47.9 nm O_2/min/mg prot.
	38.8 nm NADH/min/mg prot. (246%)	52. nm NADH/min/mg prot.
Stoichiometry O_2:NADH	1:1.3 and 1:0.8	1:1 and 1:1.1
Other Substrates	Ethane	Carbon Monoxide

1 All activities refer to rates corrected for the methane independent rates

2 Figures in brackets refer to the % stimulation over the rate in absence of methane

3 nm = nmoles

No further reports of the characterisation of the constituent components of the cell-free extracts have appeared and in the case of the earlier report of Ribbons and Michalover (1970) this is undoubtedly due to the low level of reproducibility (Ribbons, personal communication 1973) and the lack of success of other workers in reproducing their results using the same method (Thomson, 1974).

energetics of the methane oxygenase reaction

Although both oxygenase enzymes and cytochrome oxidase in mammals are linked to specialised electron transport chains which pass electrons ultimately to molecular oxygen, oxidative phosphorylation has not been shown to occur with the former (Hayaishi, 1969). Thus mono-oxygenase reactions in mammals are used for anabolic processes only and are dependent on cellular metabolism for the generation of reducing power for the mono-oxygenase reaction. In the case of hydrocarbon-utilising bacteria the substrate for the mono-oxygenase reaction is also the energy source for the cell and it is generally assumed that subsequent metabolism of the oxygenated product provides both energy for cellular metabolism and growth and reducing power for the initial mono-oxygenase reaction which by analogy with oxygenase reactions in mammals is assumed not to be coupled to phosphorylation. There is no experimental evidence to support this assumption and even if the absence of phosphorylation were to be demonstrated, the extreme difficulty encountered in obtaining cell-free oxidative phosphorylation in bacteria (Gel'man et al., 1967) would render the significance of such negative results questionable.

Whereas the loss in available energy through an initial attack by a mono-oxygenase is small in comparison to the total available energy in the case of a long chain hydrocarbon substrate, a mono-oxygenation of methane according to reaction (10) is likely to be energetically an extremely costly process:

$$CH_4 + O_2 + NADH_2 \longrightarrow CH_3OH + H_2O + NAD \quad (10)$$

$$2CH_4 + O_2 \longrightarrow 2CH_3OH \quad (11)$$

Higgins and Quayle (1970) have calculated that the mono-oxygenase reaction (10) involves a standard free energy change of -82.1 kCal $mole^{-1}$ at pH 7 whereas reaction (11) involves -59.8 kCal $mole^{-1}$ and that these free energy changes represent 40% and 16% respectively of the total energy available from the complete oxidation of substrate. If the metabolism of methane took place by the non-energy yielding mono-oxygenase mechanism in reaction 10, as both Ribbons and Michalover (1970) and Ferenci (1974) propose, a substantial proportion of the total energy available from methane would be unavailable and indeed reducing power would be required from the subsequent metabolism of methanol to activate the mono-oxygenase.

The reported molar growth yields (moles cell carbon per mole substrate utilised) of methylobacteria on methane are summarised in table 4 and it can be seen that there are numerous reports of yields in excess of 60% incorporation of substrate carbon which compare favourably with yield data for heterotrophic bacteria which are reviewed by Payne (1970). Whittenbury et al. (1970) reported that cell yields

Table 4 Growth Yields and Stoichiometries of Methylobacteria

Organism	Reference	Stoichiometry of $CH_4/CH_3OH + O_2 \rightarrow CO_2 + Cells$ moles: CH_4	CH_3OH	O_2	CO_2	Cell Carbon (YM)	remarks
Batch Studies							
Pseudomonas methanica	Dworkin and Foster (1956)	1		0.5	0.23	0.71	
" "	Leadbetter and Foster (1958)	1		0.4	0.21	-	
Methanomonas methanooxidans	Brown *et al.* (1964)	1		1.1	0.19	-	
Methylococcus capsulatus	Foster and Davis (1966)	1		0.58	0.18	0.95	
Methylomonas albus BG8) *Methylomonas methanica* 25)	Whittenbury *et al.* (1970)	1		1.0-1.2	0.2-0.3	0.63-0.68	
			1	1.0-1.1	0.5-0.6	0.37-0.5	
Methylosinus trichosporium	Dugan and Weaver (1974)	1		1.5-1.7	0.45-0.5	-	
Continuous Culture Studies							
Methylomonas albus BG8	Phillips (1970)	1		1.1	0.3	0.66-0.71	30-32°C
Methylococcus capsulatus	Harwood and Pirt 1972	1		1.8	0.34	0.66	Methane-limited chemostat 37°C
		1		0.3	0.05	0.2	Oxygen-limited chemostat
Gram-negative mixed culture	Sheehan and Johnson (1971)	1		1.4		0.2	CH_4 and O_2 - limited chemostat cultures $^{2}{}_{45}$°C
Mixed culture	Wilkinson *et al.* (1974)	1		1.7	0.47	0.53	Methane-limited chemostat 32°C
		1		1.24	0.33	0.67	Oxygen-limited chemostat 32°C

in gm gells/gm substrate of two non-capsular, non slime-forming methylobacteria were half the value of cells grown on methane and interpreted this to indicate an energy-yielding step between methane and methanol and Higgins and Quayle (1970) speculated that such an exergonic oxygenation of methane might proceed by a mechanism in which electrons from the reduced nicotinamide nucleotide during the process of methane oxygenation are passed to one oxygen atom as terminal electron acceptor. Quayle (1972) later points out that the yield data could also be explained by an energy-dependent uptake of methanol. There is however no evidence to support this latter suggestion.

The lowering of the molar yields of Methylococcus capsulatus from 0.66 to 0.2 and the excretion of methanol into the growth medium on changing from a methane-limited to an oxygen-limited chemostat (Harwood and Pirt, 1972) emphasises the problems in the interpretation of yield data from batch culture studies where the growth conditions are not controlled. In apparent contrast the mixed-culture continuous culture studies of Sheehan and Johnson (1970) and Wilkinson et al. (1974) showed higher yields in the oxygen-limited continuous culture and this effect has been ascribed by the latter authors to the scavenging effect of the methanol-utilising symbionts thus emphasising the difficulty in interpreting yield data from mixed cultures.

Methanol is toxic to most methylobacteria which can only use it as a growth substrate when it is present at low substrate concentrations (Phillips, 1970). Although the two organisms used by Whittenbury et al. (1970) were

tolerant to methanol at higher concentrations than their other methylobacterial isolates, the low yields on methanol may be a result of growth under sub-optimal conditions. The problem of methanol toxicity can be overcome by studying yields in a methanol-limited chemostat but, although Phillips (1970) has confirmed the high yield of Methylomonas albus BG8 on methane substrate in continuous culture, there is no published information on the growth yields in continuous culture of methanol-grown methylobacteria. Continuous culture studies of the methanol-utilising bacteria Methylomonas methanica (Dostalek et al., 1972), Pseudomonad C (Mateles, 1974), Protoaminobacter ruber and Pseudomonas extorquens (Harrison et al., 1972) have shown molar growth yields of 0.47, 0.53, 0.33 and 0.53 respectively demonstrating that molar growth yields of methanol-utilisers can approach those of methylobacteria grown on methane.

It is thus by no means conclusively established whether molar growth yields for methane substrate are higher than methanol. A rigorous continuous culture study of the growth of a methane-utilising bacterium on both carbon substrates under a variety of substrate-limiting conditions will be required to establish whether the initial oxidation of methane is energy-yielding or energy dependent.

the stoichiometry of the methane oxygenase reaction

The two cell-free studies of methane oxidation (Ribbons and Michalover, 1970; Ferenci, 1974) were not able to obtain stoichiometries for the initial oxygenase

reaction which would have distinguished between the mechanisms shown in equations 10 and 11. Although the dimethyl ether pathway envisaged by Bryan-Jones and Wilkinson (see Wilkinson, 1970) requires only one mole of oxygen to oxidise two moles of methane, the subsequent metabolism of dimethyl ether involves an oxygenase reaction (Mitton and Wilkinson, in preparation) and thus the resulting stoichiometry of the initial oxidation of methane would be greater than 0.5:1 for O_2:CH_4 consumption.

The oxidation of methane to methanol by the mono-oxygenase reaction in equation 10 would require the O_2:CH_4 stoichiometry of growing cells to be above 1:1 and the existing data on the stoichiometry, discussed in detail by Quayle (1972), are summarised in table 4 and reveal a confused picture. Whittenbury *et al*. (1970) and Brown *et al*., (1964) in batch studies and Phillips (1970) in continuous culture studies found a CH_4:O_2:CO_2 stoichiometry of 1:1.0-1.1:0.19-0.3. It is difficult to accommodate these results into a scheme which requires one mole of oxygen to oxidise methane to methanol and impossible to fit the stoichiometries for *Pseudomonas methanica* (Dworkin and Foster, 1956, Leadbetter and Foster, 1958) of 1:0.5 and 1:0.4 respectively. An explanation for the wide variation in stoichiometries is provided by the investigation by Harwood & Pirt (1972) of the growth of pure cultures of *Methylococcus capsulatus* which showed the O_2:CH_4 stoichiometry under oxygen limitation to be 0.3:1 and under methane-limiting conditions to be 1.8:1. The presence of contaminants makes the stoichiometries derived from

continuous culture studies with mixed cultures difficult to interpret.

The data on the stoichiometry of methane and oxygen uptake during growth of pure cultures of methylobacteria do not support the mono-oxygenase reaction and instead support a mechanism of methane oxidation in which one mole of oxygen or even less combines with two moles of methane.

source of reducing power for the methane oxygenase

Ribbons and Michalover (1970) and Ferenci (1974) both showed a requirement for NADH for cell-free methane-stimulated respiration consistent with a mono-oxygenase mechanism and the inability of NADPH to substitute as electron donor was established by the former group for Methylococcus capsulatus. The only known reaction in the metabolism of methane which yields NADH is formate dehydrogenase, however a mechanism which requires all the carbon derived from methane to be oxidised to CO_2 in order to provide NADH for the first step is clearly impossible since fixation of carbon by methylobacteria occurs at the level of formaldehyde and only to a slight extent CO_2 (Quayle, 1972). In support of a role for formate metabolism, Thomson (1974) found that the oxidation of ethane by methylobacteria was stimulated by formate and this stimulation can be interpreted as the provision of reducing power for the mono-oxygenase mediated metabolism of a substrate whose further metabolism, unlike that of methane, does not produce reducing power.

An alternative to the mono-oxygenase mechanism (equation 10) is the dimethyl ether pathway (equation 2) and the unsatisfactory state of the evidence for this pathway has

already been reviewed. The requirement for reducing power by the methane oxygenase, and the possible sources, will be discussed later in the light of results obtained in this study.

cytochromes of methylotrophs

Preliminary studies on representatives of each membrane type of methylobacteria Methylomonas albus BG8 and Methylosinus trichosporium OB3B (Davey 1971, Davey and Mitton 1973) revealed quantitative, but not qualitative differences between the two types. Accurate absorption wavelengths could not be determined by Davey (1971) who used a SP800 spectrophotometer and the later study by Davey and Mitton (1973) using an Aminco-Chance Dual Wavelength Split beam recording spectrophotometer must be considered the more reliable. Difference spectra revealed that both strains possessed a c type cytochrome with α and soret bands of 553 and 423 nm respectively and a slight shoulder on the α band was the only indication of the presence of a b type. A shoulder at 443 nm in the difference spectrum suggested the presence of an a type cytochrome which was confirmed by the presence in the CO difference spectrum of a shoulder at 425 nm on a peak at 416 which was identified as cytochrome o. Although both CO binding species were present in each organism the level of the a type cytochrome was higher in Methylosinus trichosporium OB3B.

The role of the CO-binding cytochromes was discussed in the light of their observation that the oxidation by whole cells of methane was inhibited by carbon monoxide

whereas the oxidation of other presumed intermediates methanol, formaldehyde and formate was unaffected. This observation has subsequently been confirmed by Thomson (1974) and Ferenci (1974). The involvement of cytochrome o in methane oxidation was initially proposed by Davey (1971) by analogy with its suggested involvement in n-octane metabolism by Pseudomonas oleovorans (Peterson 1970). The inconclusive nature of the evidence for this suggestion together with the contrast between the carbon monoxide insensitivity of n-hexane oxidation by Pseudomonas oleovorans (Peterson et al., 1966) and the carbon monoxide sensitivity of methane oxidation led Davey and Mitton (1973) to propose the involvement of the a-type cytochrome in methane oxidation, relegating cytochrome o to the role of terminal electron acceptor in the metabolism of the products of methane oxidation. The insensitivity of the metabolism of these products to inhibition by carbon monoxide was explained by postulating a terminal acceptor which combined very slowly with carbon monoxide and there is evidence that cytochrome o from Bacillus megaterium combines with CO more slowly than with cytochrome a (Broberg and Smith, 1967).

The involvement of cytochrome o in the methane oxygenase system becomes unlikely in the light of findings of Tonge et al. (1974), that the CO-binding cytochrome corresponding to the cytochrome o of Davey and Mitton (1973) is present in the soluble fraction of the cell and not in the membrane fraction where cell-free methane-stimulated respiration and methane-stimulated NADH oxidase activity have been shown to occur (Ribbons and Michalover, 1970; Ferenci, 1974).

Tonge et al. (1974) examined the cytochromes of the facultative methanol-utilisers Pseudomonas extorquens, Hyphomicrobium sp., Pseudomonas AM1 and the methylobacteria Pseudomonas methanica and Methylosinus trichosporium OB3B. They found in all of these organisms a soluble CO-binding pigment with a CO difference spectrum absorption maximum at 415 nm but from the absence of pyridine haemochromogen corresponding to protohaeme concluded that this cytochrome, together with the 'cytochrome o' of Davey and Mitton (1973) was a CO-binding cytochrome c and that the cytochrome c that did not bind with CO did not represent a second cytochrome c but merely incomplete binding.

The presence of a CO difference spectrum corresponding to cytochrome o in whole cells of a cytochrome c deficient mutant of Pseudomonas AM1 has led Anthony (1975) to disagree with the findings of Tonge et al. (1974) and propose that Pseudomonas AM1 contains an a-type cytochrome and both cytochrome o and a CO-binding c-type cytochrome. Anthony (personal communication, 1975) suggests that the principle reason for the confusion lies in the difficulty of extraction of haeme from cells with a low concentration of haeme a and haeme b and proposes that the organisms studied by Davey and Mitton (1973) contain both cytochrome o and the CO-binding c-type cytochrome. The extrapolation of results from cytochrome studies of methanol-utilisers to the cytochromes of obligate methylobacteria is highly speculative and a further study is needed to establish the identity of the CO-binding cytochromes of methylobacteria.

The observation that CO stimulates the respiration of suspensions of methylobacteria led Ferenci (1974) to propose

an alternative mechanism of inhibition by CO of methane oxidation in which CO competes with methane for the methane oxygenase enzyme and the evidence for this mechanism will be discussed in detail in the light of results presented in this study.

Thus it can be seen that studies of the cytochromes of methylobacteria have not proceeded beyond the initial characterisation stage and their role in both the methane oxygenase reaction and in the energetics of methylobacterial growth is still obscure and will remain so until such studies are carried out with cell-free extracts capable of methane oxidation.

CONCLUSION

The demands of the initial oxygenase reaction on cell organisation, metabolism and energy production are likely to dictate many of the unusual properties of methylobacteria and the obligate growth requirement for methane (Ribbons et al., 1970). Although progress has been made in the understanding of the mechanism of methanol oxidation and carbon assimilation in methylotrophs, little is known about this initial oxidation which forms the subject of the present study.

MATERIALS AND METHODS

organisms

Most of the strains of methylobacteria used in this study were isolates of Whittenbury *et al.* (1970) and in addition *Pseudomonas methanica* was a gift of Professor Quayle (Department of Microbiology, University of Sheffield), the proposed facultative methylotroph XX, was a gift of Professor Hanson (Department of Bacteriology, University of Wisconsin, U.S.A.), *Escherichia coli* B wild type was a gift of Dr J. Dawes (Department of Microbiology, University of Edinburgh) and *Brevibacterium* JOB 5 was a gift of Dr J.J. Perry (Department of Microbiology, North Carolina State University, U.S.A.). Following the approach of previous work in this laboratory (Davey, 1971; Thomson, 1974) *Methylomonas albus* BG8 and *Methylosinus trichosporium* OB3B were used as representatives of the two membrane types of methylobacteria and will be referred to by the shortened names *M. albus* BG8 and *M. trichosporium* OB3B.

maintainance and growth of cultures

The nitrate mineral salts (N.M.S.) and ammonia mineral salts (A.M.S.) of Whittenbury *et al.* (1970) were used for all flask-grown cultures. Where appropriate the N.M.S. was supplemented with methanol (0.3 or 0.5% v/v.) or glucose (0.1% W/V).

Stock cultures of methylobacteria were maintained on N.M.S. agar (1.2% agar) in a methane-air atmosphere at 30°C as described in detail by Davey (1971). *Brevibacterium* JOB5, *Escherichia coli* B and XX were maintained on nutrient agar slopes at 30°C.

Washed cell suspensions of methylobacteria and XX in the exponential phase of growth were prepared from shake cultures of 21 flasks containing 400 mls of N.M.S. incubated at 30°C in the presence of methane and the method used in both the growth of the cultures and the preparation of the cell suspensions was identical to that of Thomson (1974) except that the cells were resuspended in phosphate buffer pH 6.8 (1g /l Na_2HPO_4; 0.8g/l$K_2H_2PO_4$) and not the growth medium.

The preparation of washed cell suspensions of Brevibacterium JOB5, glucose-grown XX and Escherichia coli B was identical to the above procedure except that with Brevibacterium JOB5, propane replaced methane as carbon source, with XX 0.25% W/V glucose replaced methane and with Escherichia coli B nutrient broth in N.M.S. salts medium replaced methane.

Unless otherwise stated all experiments with cell suspensions were carried out within 24 hours of harvesting and before being used cell suspensions were maintained at 4°C.

Prior to preparation of washed cell suspensions all flasks of methylobacteria were tested for contamination by sub-culturing onto nutrient agar and if any growth appeared after 48 hours the results of the experiment were discounted.

respiration studies

The respiration of the organisms at 30°C wes determined with a Clark-type oxygen electrode with a reaction chamber of capacity 2.7 ml (Estabrook 1967). An amount of air-saturated pH 6.8 phosphate buffer at 30°C was initially

added to the chamber such that the final volume of buffer, cells and reagents was 2.7 ml. Substrate was added one minute after a volume of cells equivalent to 5 mg dry weight had been added to the chamber. The substrates sodium formate, ethanol, methanol, formaldehyde and acetaldehyde were added as 100 µl of 100m Molar solutions and gases, such as CH_4, C_2H_6, C_3H_8, C_4H_{10} and CO were added as 0.6 ml of gas-saturated buffer at 30°C unless otherwise stated. Where required 100 µl of inhibitor solution was added immediately after the addition of cells to give the desired concentration.

For the determinations of the Km of methane-stimulated respiration the gas-saturated buffer was added before the cells as the changes in oxygen concentration on the addition of the oxygen-depleted methane-saturated buffer obscured the initial rate of substrate respiration.

Air-saturated buffer at 30°C was used to calibrate the 100% saturated oxygen level and a few crystals of sodium dithionite were added to calibrate the zero oxygen level. Unless otherwise stated all respiration rates given have been corrected for the endogenous respiration in absence of substrate.

the determination of methane, ethane, ethylene and methanol disappearance rates

The rates of methane, ethane, ethylene and methanol disappearance from incubations of washed cell suspensions in sealed flasks were determined by periodic sampling and analysis by gas chromatography of the liquid phase of the cell suspensions in the case of the determination of methanol disappearance and the gaseous phase in the case of methane, ethylene and ethane. Sampling was carried out over a

twenty-minute period in the case of methane and methanol and thirty minutes in the case of ethane and ethylene. The incubations were initiated by the injection of a volume of cell suspension corresponding to 47 mg dry weight of cells (1 ml of optical density 100 at 610 nm) into sealed 25 ml round-bottomed 'Quickfit' flasks containing the required reagents dissolved in the pH 6.8 phosphate buffer described earlier to give a final volume of 5.4 ml in the case of the experiments with inhibitors described in tables 5 and 6 in the Results and Discussion section of this report and 5.0 ml in all other cases. Where appropriate the amount of methane ethylene or ethane present was 1 ml and methanol 10 mM. In experiments where the effect of dimethyl ether on ethane disappearance was being determined an amount of gaseous dimethyl ether was injected into the flasks such that after shaking for 10 minutes to allow the dimethyl ether to dissolve the concentration of dimethyl ether in solution 10 mM.

After the reaction had been initiated the flasks were shaken on a Griffin and Tatlock 'Microid' flask shaker in a constant temperature room at 30°C.

In experiments investigating the effects of inhibitors on the rates of methane and methanol disappearance 0.2 ml of a solution of inhibitor in pH 6.8 phosphate buffer was added to the incubation to give the final desired concentration of inhibitor and the resulting rates of methane and methanol disappearance were determined as described above. The low solubility of αα'dipyridyl and 8-quinolinol necessitated the shaking of suspensions for at least one hour at 30°C to dissolve the inhibitors completely. N-SERVE

proved particularly insoluble in buffer and it was necessary to work with a saturated solution. 4, 2, 1, 0.4 mls of a saturated solution of N-SERVE were added to the incubation mixture and the corresponding concentrations of N-SERVE in a total volume of 5 ml were calculated from the solubility of N-SERVE in water (40 mg $liter^{-1}$; Dow Chemical Company, 1973) to be 13, 6.7, 3.25 and 1.3×10^{-5}M respectively.

determination of reversibility of inhibition

One ml aliquots of suspensions of M. trichosporium OB3B containing 47 mg dry weight bacteria were held for 30 minutes with inhibitor present at a concentration known to cause 100% inhibition of methane oxidation. Cells were sedimented and resuspended in 20 volumes phosphate buffer, sedimented again and finally resuspended in the original volume. Methane-stimulated respiration rates were measured and compared with a control which had been treated similarly in absence of inhibitor.

A different procedure was adopted in the case of the inhibition of methane oxidation by N-SERVE. After establishing for M. trichosporium OB3B the complete inhibition of methane disappearance by 1.3×10^{-4}M N-SERVE as described in the previous section 15 ml of phosphate buffer was added to the contents of the flask and the diluted suspension was sedimented and resuspended in 20 ml buffer twice and then sedimented and the pellet taken up in 5 mls buffer and the rate of methane disappearance from an incubation of the washed cells determined in the usual manner.

restoration of activity on addition of metal ions

Relief by metal ions of inhibition of methane-stimulated respiration was determined in the oxygen electrode. Metal ion solution (100 µl) was added after full inhibition had been reached and the resultant rate was compared with uninhibited control in the presence of the appropriate metal ion.

gas chromatograph systems

The gas chromatograph systems routinely used to assay ethane, methane, oxygen, carbon dioxide, helium and methanol have been described in considerable detail by Thomson (1974) and accordingly only a brief description follows.

Methane, ethane, oxygen, helium and carbon dioxide were assayed by injection of gas samples (0.2 ml) into a gas chromatograph (PYE 104, 2.8 m silica gel column, carrier flow rate 30 ml N_2/min^{-1}, 140°C) with a katharometer detector. Helium, oxygen and methane gave line peaks of retention times 0.5, 0.7 and 1.3 minutes respectively and hence the peak heights could be taken as proportional to the amounts present and the rates of methane oxidation calculated. However carbon dioxide gave a broad peak of retention time 4.3 min, and the peak heights were standardised against known samples.

Sharper peaks with shorter retention times were obtained in the studies of ethane and ethylene disappearance by using a 1.6 m column with the above conditions which gave peaks with retention times 0.5, 0.5, 0.7, 0.9, 1.9, 2.3 and 2.6 minutes for hydrogen, helium, oxygen, methane, ethane, carbon dioxide and ethylene respectively.

Methanol and acetone were measured in solution by injection of 2.5 µl of the cell suspension into a gas chromatograph (PYE 104 series, 1.6 m Chromosorb 101 column, carrier flow rate 30 ml N_2 min^{-1}, 150°C) with a flame ionisation detector. Retention times were 0.8 and 1.6 minutes respectively for methanol and acetone.

^{14}CO studies

(a) Radioactive counting

Scintillation counting was carried out in either a Beckmann (Beckmann Instruments Limited, Glenrothes, Scotland) ambient temperature liquid scintillation counter or in a Packard Tricarb liquid scintillation spectro~~(photo)~~meter, model 3300 (Packard Instruments Limited, Caversham, Berkshire).

The scintillant routinely used was a solution of 2,5-diphenyloxazole (PPO, 0.5% W/V) and 1, 4-di-2 (5-phenyl-oxazolyl) benzene (POPOP, 0.01% W/V) in toluene. PPO, POPOP and hyamine hydroxide (1M solution in methanol) were obtained from Nuclear Enterprises Limited, Sighthill, Scotland.

(b) standardisation of ^{14}CO source

Standardisation of ^{14}CO source: an ampoule of ^{14}CO (500 µCi, Radio-chemical Centre, Amersham) was broken in unlabelled CO (70 ml) and the specific activity of the ^{14}CO was determined by the following method. A known amount of the ^{14}CO was introduced into a 25 ml roundbottom flask fitted with a gas-tight 'suba-seal' whose volume had been accurately determined by weighing empty and filled with water; 0.2 ml samples from the flasks were then injected into a gas chromatograph (PYE 104 Series, carrier gas flow rate 15 ml N_2 min^{-1}, 50°C) fitted with a flame ionisation

detector (F.I.D.). The $^{14}CO_2$ produced by the burning of the CO in the F.I.D. flame was trapped by sparging the exhausted gases through 10 ml hyamine hydroxide (1M solution in methanol) which is a good solvent trap for CO_2 (Hash, 1972). The resulting trapped radioactivity was measured by making a 1:9 dilution of the $^{14}CO_2$-hyamine hydroxide with scintillator and counting in a scintillation counter. No quenching due to the presence of hyamine hydroxide was detected.

(c) determination of specific activity of evolved $^{14}CO_2$

Three round-bottomed flasks whose exact volumes had been measured as described in the previous section were set up with 4 mls N.M.S. and, as a control against injection errors, 1 ml helium. Flask A was a control to measure the endogenous CO_2 production. The diluted ^{14}CO (1 ml) was injected into the sealed flask B. An equal amount of washed cell suspension containing 47 mg dry weight cells was added to each flask and they were mounted on a wrist action shaker in a room thermostatically-maintained at 30°C. The gas phase in each of the flasks was analysed by injecting 0.2 ml samples at timed intervals over a 3 hr or longer period into a gas chromatograph with katharometer detector and the CO_2 production rate determined as described under gas chromatographic methods. At different times during the incubations (from one minute before the appearance of the CO_2 peak until one minute after) the gas stream of the gas chromatograph was sparged through 10 ml of hyamine hydroxide and counted as described above.

The expression $x/(y-z)$ was used to calculate the "specific activity of the excess CO_2" where x was the total

counts min^{-1} $^{14}CO_2$ in the flask with ^{14}CO, y the amount of CO_2 in μmoles in the flask with ^{14}CO and z the amount of endogenously-produced CO_2 in the control flask.

(d) establishing linear rates of $^{14}CO_2$ production from ^{14}CO

The linear rate of production of $^{14}CO_2$ in the presence of ^{14}CO was established by the following method: incubations of cell suspensions containing 47 mg cells in sealed 25 ml round-bottomed flasks of known volumes were set up in the usual manner with 3 ml of ^{14}CO and incubated at 30°. Gas samples (0.2 ml) were taken at 20 minute intervals and injected directly into scintillation vials containing 0.5 ml hyamine hydroxide and sealed with suba-seals. After ten minutes the suba-seals were removed and any ^{14}CO present was removed by leaving the vials for 1 hour in a fume cupboard. Scintillant was then added (4 mls) and the ^{14}C content of each vial counted as described above. From the total ^{14}C dissolved and the volume of the flask the total $^{14}CO_2$ content of the original flask at the time of sampling was calculated and the corresponding amount of ^{14}CO oxidised could be determined from the specific activity of the ^{14}CO source.

(e) Effect of CO on endogenous $^{14}CO_2$ production from ^{14}C-labelled cells

^{14}C-labelled cell suspensions of *M. albus* BG8 and *M. trichosporium* OB3B were prepared by inoculating ten 250 Erlenmeyer flasks containing 50 ml N.M.S. medium with 4 ml of a methane-grown culture in the exponential phase of growth. After sealing the flasks with sterile 'Suba-seals' 50 ml of unlabelled methane were added by syringe followed by two ml

of $^{14}CH_4$ of approximate specific activity 5μCi ml^{-1}. After 24 hours incubation on a reciprocal shaker at 30°C the cultures had grown and a sample from the gas phase from each flask was injected into the gas chromatograph to confirm that the CH_4 had not been totally consumed and that the cells were in the exponential phase. Washed cell suspensions were prepared in the usual manner and 47 mg dry weight of cells were incubated in sealed 25 ml round-bottomed 'Quickfit' flasks with 1 ml of helium marker and in the presence and absence of 3 mls unlabelled CO. Gas samples (0.5 ml) were taken at 20 minute intervals during the course of the incubation and injected into sealed scintillation vials containing 0.5 ml hyamine hydroxide and the trapped $^{14}CO_2$ counted as described above. During the course of the incubation gas samples (0.2 ml) from the flasks were chromatographed and the rates of CO_2 production determined.

Determination of the effect of inhibitors on growth

(a) The following procedure was adopted for all inhibitors listed in table 9: solutions of inhibitors that had been sterilised by passage through a 0.45μ millipore filter were added to 250 ml erlenmeyer flasks containing 20 ml NMS supplemented with 0.3% v/v methanol or 20 ml methane and the flasks were then inoculated with 2 ml of a liquid culture of M. albus BG8 taken from the exponential phase of growth. The growth after five days incubation with shaking at 30°C was determined by nephelometry (Evans Electroselenium Limited, Halstead, Essex).

(b) Effect of CO on growth (table 20): twelve flasks with NMS and 2 ml inoculum were set up as above except with 0.5%

methanol and 20 ml of CO were added to six of the flasks. After five days incubation with shaking at 30° the complete uptake of methanol was confirmed by gas chromatography and from the optical density at 610 nm the dry weight of cells and yield was calculated (Thomson, 1974).

(c) Effect of N-SERVE on growth (figure 4): four mls of an inoculum were introduced into 250 ml Erlenmeyer flasks fitted with side arms and containing NMS and 20 ml CH_4 and N.M.S. and CH_3OH (0.5 % v/v) respectively. The flasks were incubated at 30°C and after commencement of growth, which was measured by nephelometry, 1 ml of a filter-sterilised, saturated solution of N-SERVE was injected through the "suba-seal" into each flask and the subsequent growth over a six-hour period was measured and compared with a control flask incubated in the absence of N-SERVE and the results recorded in figure 4.

^{18}O-incorporation experiments

Sealed 25 ml "Quickfit" round-bottom flasks with four ml buffer were flushed with oxygen-free nitrogen for ten minutes and, after checking a gas sample by gas chromatography for anaerobicity, 5 ml of the gas contents were removed and replaced with 5 mls of 30% ^{18}O-enriched oxygen. Incubations with 1 ml of washed cell suspensions optical density 100 (610 nm) were set up in the usual way in the presence and absence of CO (3 ml) or CH_4 (3ml). Initially the flasks after incubation were frozen and the gas phase analysed at a later date but as these experiments proved negative the incubations were then carried out with larger amounts of cells in the same room as the mass spectrometer so that the

gas phase could be sampled and analysed throughout the course of the incubation which was carried out at 23°C.

The ^{18}O content of the evolved CO_2 and the total CO_2 content were determined by injecting a 0.2 ml gas sample (or 0.5 ml if the CO_2 contents of the flask did not exceed 0.5 ml) into a Micromass 12 Combined Gas Chromatography/ Mass Spectrometer unit (GC/MS) which consisted of a PYE 104 Gas Chromatograph (1.6 m silica gel column, column temperature 140°C and gas flow rate 40 ml helium min^{-1}) coupled to the mass spectrometer maintained with 70 ev emission. The oxygen and carbon dioxide peaks appeared after 0.6 and 1.6 minutes respectively and the mass spectrum of both peaks was taken with a 3-second scan encompassing mass numbers 26 to 48. At the beginning and end of each run a mass spectrum was taken of the background column effluent and the mean of this figure was subtracted from the measured mass spectra. The mass composition of the ^{18}O source was determined by sampling the oxygen in each flask and averaging, and of the CO source and a CO_2 standard (Distillers Company) by injecting samples directly into the GC/MS. Care had to be taken to avoid dilution of the CO source by air which would lead to the confusion of the ^{28}N and ^{28}CO present. The expression $[\sum X^2/(n-1) - (\sum X)^2/(n-1)n]^{\frac{1}{2}}$ where X was the amount of $C^{18}O^{16}O$ component with mass 46 as a percentage of the $C^{16}O_2$ component with mass 44 and n the number of samples, was used to calculate the standard deviations of the ^{18}O-enrichments of the incubations with and without CO.

the production of hydrogen by methylobacteria

Anaerobic incubations, prepared by flushing flasks with oxygen-free nitrogen for 10 minutes and containing 47 mg dry weight of M. trichosporium OB3B or M. albus BG8 in a total volume of 5 ml buffer, were incubated in the presence of either formate (10 mM.), or CO (3 ml) or in the absence of substrate and in the case of M. trichosporium OB3B, with sodium hydroxybutyrate (10mM.). Similar aerobic incubations were set up in the presence and absence of CO (3 ml). 0.2 ml of the gas contents of each flask were injected into the gas chromatograph at the beginning of the incubation, after one and two hours, and after overnight incubation and the CO_2 production and H_2 evolution determined and shown in table 27. Samples of the liquid phase (2.5 µl) of the anaerobic incubations of M. trichosporium OB3B in the presence and absence of hydroxybutyrate were assayed by gas chromatography for acetone production.

electron paramagnetic resonance studies

One ml aliquots of washed cell suspensions of known optical density of methylobacteria, Brevibacterium JOB5, glucose-grown XX and Escherichia coli B, which were all grown on the same N.M.S. medium supplemented with methane, propane, glucose and nutrient broth respectively, were shaken vigorously for one minute in sealed test tubes in the absence of substrate ('oxidised sample') and in the presence of 20 ml methane or a few crystals of sodium dithionite. Approximately 0.4 ml of each suspension was then introduced into 0.3 mm internal diameter quartz electron paramagnetic resonance tubes (James F. Scanlon Company) and the samples were slowly frozen in liquid nitrogen over a

period of two or three minutes to avoid cracking of the tubes during freezing. Samples were only prepared of the oxidised cell suspensions in the case of the two organisms Escherichia coli B and Brevibacterium JOB5. As controls, samples were prepared of N.M.S. basal salts medium and phosphate buffer. Samples were also prepared of incubations of M. trichosporium OB3B with methanol and formate which were both added before shaking as 100 µl of 100 mM solutions.

Electron paramagnetic resonance spectra were recorded with a Varian E4 spectrophotometer and unless otherwise stated were carried out at -172°C with receiver gain 1.25 x 10^3, modulation amplitude 1.25 x 10^1 Gauss, modulation frequency 100 kHz and microwave power 50 mW. Scans were carried out at fixed frequency 9.140 GHz with a time constant of one second over a magnetic field range of 1000 Gauss with a centre of the field set at 3100 Gauss. 'g-values' were calculated by substituting in the expression $g = h\nu / \beta H$ where h is Planck's constant, β is the Bohr magnetron, H is the magnetic field at resonance and ν is the set frequency (Swarz et al., 1972).

rates of ammonia-stimulated nitrite production

The same method was used to determine rates of nitrite production in the presence of ammonia and hydroxylamine and rates of disappearance of added nitrite: cell suspensions containing 47 mg dry wt cells of M. trichosporium OB3B grown on methane and N.M.S. or A.M.S. basal salts media and buffer were pre-incubated at 30°C with shaking for 20 minutes and the reaction initiated by the addition of 0.5 ml of solutions of NH_4Cl (100 mM), $NH_2OH.HCl$ (100 mM), or $NaNO_2$ (10mM.) with

and without HCOONa (20 mM) such that the final volume of the contents of each flask was 5 ml. Samples (0.2 ml) of the suspensions were drawn from each incubation at measured time intervals that varied from 2 to 8 minutes and added to 0.8 ml buffer at 30°C in microcentrifuge tubes and, after sedimentation of the cells by centrifugation for 1 minute in a "Quickfit 320 Microcentrifuge", the supernatants were decanted and 0.5 ml samples withdrawn and assayed for nitrite according to the diazotisation method of Nicholas and Nason (1957). The time of sampling was taken as 10 seconds after commencement of centrifugation. In the case of the inhibitor studies the cells were pre-incubated for 20 minutes with inhibitor before addition of substrate. With the gaseous inhibitor CO, 0.3 ml samples of the suspensions were withdrawn through the suba-seal by syringe and 0.2 ml of these were diluted, centrifuged and assayed for nitrite as described above.

methanol dehydrogenase assay

This enzyme was assayed according to the method of Anthony and Zatman (1965) with the slight modifications of Davey (1971). Analar methanol (10 μmoles) was added to the sample cuvette to initiate the reaction which was followed at 600 nm. When examining for inhibition by hydrazine, 100 μl of a solution of hydrazine sulphate was added to the cuvette to give a final concentration of 2×10^{-3}M and the reaction mixture was allowed to stand for two minutes in the presence of hydrazine before the reaction was initiated by the addition of methanol.

formate dehydrogenase assay (EC 1.2.1.2. formate:NAD Oxidoreductase)

The assay was adapted from Quayle (1966). The complete reaction system contained sodium phosphate buffer pH 6.8, NAD (1.0 µmole) and 0.1ml of a soluble fraction of *M. trichosporium* OB3B containing 31µg protein. The reaction was initiated by the addition of 20 µmoles sodium formate to the sample cuvette.

preparation of cell-free extracts

In the attempts made to obtain cell-free methane or carbon monoxide oxidation the method of preparation of the extracts were as described by Ribbons and Michalover (1970) and Ferenci (1974). For the preparation of soluble fractions from the cells for the formate and methanol dehydrogenase assay 5 mls of a suspension of *M. trichosporium* OB3B or *M. albus* BG8 containing 47 mg dry wt cells/ml were subjected to two passages through a cooled French pressure cell at 4000 lb in^{-2} and a microscopic examination of the resultant extract confirmed that cell breakage was in excess of 90%. A soluble fraction was prepared by centrifugation of the extract for 1 hr at 30,000 g at 5°C and using the supernatant.

protein and dry weight determinations

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumen as a standard. Dry weights of cells in suspension were estimated from measurement of absorption at 610 nm and comparison with a dry weight calibration curve.

spectrophotometry

A Unicam Series SP 600 spectrophotometer was used for dry weight, protein and nitrite determinations. Measurement

of the rates of NAD reduction and methanol-stimulated dye reduction involved the use of a Unicam SP800 recording spectrophotometer.

gases

Methane (technical grade), Oxygen, Helium, air, nitrogen ("oxygen-free") and hydrogen were obtained from British Oxygen Company Limited, while ethane, propane and butane were purchased at 'C.P.' grade from Air Products Limited. Carbon dioxide was obtained from Distillers Company Limited and Carbon Monoxide, ethylene and dimethyl ether from Matheson Gas Products. ^{18}O-enriched oxygen (30%) was obtained from Miles Laboratory Limited. No impurities in any of the above gases were detected by the gas chromatography systems used in this study.

inhibitors

SKF 525A (β-diethylaminoethyldiphenylpropylacetate) was a gift from Smith, Kline and French Laboratories, Welwyn Garden City and Lilly 18947 (2-4 dichloro-6-phenoxyethyl-diethylamine) and Lilly 53325 (2,4 dicholoro 6-phenylphenoxy ethylamine hydrobromide) were kindly provided by Lilly Research Limited, Windlesham, Surrey. N-SERVE, (2-chloro-6-trichloromethyl pyridine) was a gift from the Dow Chemical Company Limited, Houndslow, Middlesex. All other inhibitors were analytical grade reagents or equivalent and were obtained from B.D.H., Sigma or Eastmans Kodak Limited.

Metal ions used in reversibility studies were all in the form of the following B.D.H. 'Analar' salts: $CoCl_2.6H_2O$, $CrK(SO_4)_2 12H_2O$, $SnCl_2 2H_2O$, $MnSO_4 4H_2O$, $FeSO_4 7H_2O$ and $FeCl_3 6H_2O$.

Substrates and coenzymes

Formaldehyde was prepared by boiling an aqueous suspension of para-formaldehyde ('Analar' BDH) in a screw-capped vial. HCOONa, CH_3OH, NH_4Cl, $NaNO_2$ and all other substrates were B.D.H. 'Analar' grade.

RESULTS AND DISCUSSION

SPECIFIC INHIBITORS OF METHANE OXIDATION

The difficulties encountered in obtaining cell-free extracts capable of oxidising methane have limited investigations of the methane oxygenase to the study of the reaction in whole cells. Faced with a similar situation with the ammonia oxygenase system of Nitrosomonas Hooper and Terry (1973) have attempted to characterise the oxygenase reaction in whole cells by obtaining a number of compounds which inhibit ammonia oxidation but leave hydroxylamine, the presumed product of ammonia oxidation (Hofmann and Lees, 1953), unaffected. Such an approach has not been applied to the methane oxygenase and as Quayle (1972) points out, the published inhibitor work on methane and methanol oxidation has proved difficult to repeat. It has now been well-established, however, that carbon monoxide specifically inhibits methane oxidation leaving the oxidation of methanol unaffected (Davey and Mitton, 1973; Thomson, 1974; Ferenci, 1974) and a similar effect for cyanide has been recorded (Davey, 1971).

In the present study the effect of a number of compounds on the oxidation of methane and methanol was investigated with a view to obtaining specific inhibitors of the methane oxygenase and Methylosinus trichosporium OB3B, a membrane-type II methylotroph, was selected as a test organism on account of its generally higher level of oxidative metabolism, Davey (1971). The effect of a range of concentrations of each potential inhibitor on methane and methanol-stimulated respiration by cell suspensions in the oxygen probe was initially determined and if the compound proved inhibitory

to methane oxidation, the rates of methane and methanol disappearance from incubations in sealed flasks were determined by gas chromatography at the concentration of inhibitor that produced the greatest differential effect between the inhibited methane and uninhibited methanol respiration rates. The effect of a number of compounds found to selectively inhibit methane oxidation is shown in Tables 5 and 6, where the division into metal-binding compounds and miscellaneous inhibitors has been chosen for convenience.

The metal ion chelators fall into three broad classes depending on the chemical nature of the ligands present; nitrogen-nitrogen (o-phenanthroline, aa'dipyridyl), nitrogen-oxygen (8-quinolinol), sulphur-nitrogen (thioacetamide, ethyl xanthate, dithyldithiocarbamate and the structurally related thiourea, allyl thiourea and thiosemicarbazide) and sulphur-sulphur ('British anti Lewisite'or dimercapto propanol). Ethylene diamine tetra acetic acid (E.D.T.A.) at concentrations of up to 10^{-3}M was ineffective as a selective inhibitor suggesting that chelators with oxygen-oxygen ligands lacked the specificity towards methane oxidation that the other chelators possessed. The oxygen-containing analogue of thiourea, urea, also proved ineffective in inhibiting methane stimulated respiration at 10^{-3}M indicating the importance of the sulphur atom in the inhibition of methane oxidation by thiourea. All the inhibitors in part I of table 5 were reversible except British Anti-Lewisite as were the monodentate ligands in part II. Methane oxidation proved less sensitive to the compounds in the second group with the exception of cyanide.

Table 5

Inhibition of methane and methanol metabolism in washed cell suspensions of Methylosinus thrichosporium OB3B by metal-binding compounds

Inhibitor	Concentration (M)	Rates of disappearance (% control)[1] CH_4	CH_3OH	Reversibility[2] of inhibition of CH_4 oxidation
I				
Thiourea	10^{-4}	0	96	++
Allyl thiourea	10^{-5}	28	108	++
Thioacetamide	10^{-3}	0	100	++
Diethyldithio-carbamate	7.5×10^{-4}	0	90	++
8-quinolinol	10^{-4}	0	102	++
o-phenanthro-line	5×10^{-5}	65	90	++
aa'dipyridyl	10^{-4}	0	92	++
Ethyl xanthate (K salt)	10^{-3}	0	80	++
Dimercaptopro-panol (British Anti-Lewisite)	5×10^{-3}	0	100	+
Thiosemi-carbazide	10^{-5}	45	100	++
EDTA	10^{-3}	72	76	N.T.
II				
Sodium cyanide	10^{-6}	38	100	++
Sodium azide	10^{-3}	7	85	++
Histidine	10^{-2}	0	105	++
Pyridine	10^{-3}	36	108	++
Imidazole	10^{-3}	0	109	++
3-amino triazole	10^{-2}	27	91	++

1. The uninhibited rates of methane and methanol disappearance were 20nmoles/min/mg dry cell weight and 100 n moles/min/dry cell weight respectively.

2. Reversibilities: ++ and + indicate restoration to more than 50% activity and less than 50% respectively, and - indicates irreversibility.

N.T. - Not tested.

Table 6

Inhibition of methane and methanol metabolism in washed cell suspensions of M. trichosporium OB3B by miscellaneous compounds

Inhibitor	Concentration (M)	Rates of disappearance (% control) CH_4	CH_3OH	Reversibility of inhibition of CH_4 oxidation
Diethylamino-ethyl-diphenyl-propylacetate (SKF 525A)	5×10^{-3}	36	100	-
2-4 dichloro-6-phenyl-phenoxy ethyldiethyl-amine (Lilly 18947)	10^{-2}	0	100	N.T.
2,4 dichloro (6-phenyl-phenoxy) ethyl-amine hydrobromide (Lilly 53325)	5×10^{-3}	0	100	N.T.
Cysteamine	10^{-2}	0	84	-
Hydrazine sulphate	2×10^{-3}	70	6	-
Hydroxylamine hydrochloride	10^{-3}	23	75	++
Aminoguanidine	10^{-2}	32	96	N.T.
Spermine	2×10^{-3}	41	100	N.T.
Methanol	10^{-2}	5	-	N.T.

The notes for Table 5 apply to Table 6.

In table 6 the finding of Thomson (1974) that methanol inhibits methane oxidation is confirmed and extended to hydroxylamine, the likely product of ammonia oxidation by Nitrosomonas (Hofman and Lees, 1953). This compound is commonly used as a mutagen and the observation that it inhibits methane oxidation provides an explanation for the low viability of hydroxylamine-treated cultures of methylobacteria found by Shimmin (1974). Aminoguanidine at 10^{-2}M inhibited methane oxidation and although not presented in table 3 another mutagen and derivative of guanidine, nitroso guanidine at 0.7×10^{-2}M, inhibited methane respiration to 10% of the control value leaving methanol respiration unaffected. The sensitivity of methane oxidation to these two mutagens may necessitate the use of methanol as carbon source for further studies of mutagenesis in methylobacteria although this would restrict such studies to a small group of Methylobacteria that grow on methanol.

SKF 525A is a known inhibitor of Cytochrome P-450 mediated mono-oxygenase reactions in mammalian systems (Hildebrandt, 1972) and carotene hydroxylation in Staphylococcus aureus (Hammond and Whyte, 1970) and was found to irreversibly inhibit methane oxidation as did Lilly 53325 and Lilly 18947, two other known inhibitors of oxygenase reactions (Hildebrandt, 1972). Cysteamine and aminoguanidine both inhibited methane oxidation irreversibly. The polyamine spermine was originally tried unsuccessfully as a potential protective agent for the preparation of methane oxidising cell-free extracts of methylobacteria in the light of its successful application

in the ammonia oxidising cell-free extracts of Nitrosomonas (Suzuki and Kwok 1970) however it was discovered that it also acted as a selective inhibitor of methane oxidation.

Inhibition of methane oxidation by amino acids

The selective inhibition of methane oxidation by histidine shown in table 5 is likely to be the explanation for the inhibition of growth of Methylococcus capsulatus on methane as carbon source found by Eccleston and Kelly (1972) who were able to explain the inhibition of growth by L-threonine through interference with the feed-back mechanisms of branched amino acid synthetic pathways but were unable to offer an explanation for inhibition by histidine. The results of a survey of the effects of added amino acids on the respiration of methane and methanol by M. trichosporium OB3B is shown in table 7 and it can be seen that only histidine, proline, methionine and valine showed any selective effect

Table 7 Effect of added amino acids on Methane and Methanol-stimulated respiration by Methylosinus trichosporium OB3B

Amino acid[1]	Conc. (M.)	Respiration Rates (% Control[2]) CH_4	CH_3OH
Methionine	10^{-2}	16	79
Cysteine	10^{-3}	7	9
Arginine	10^{-2}	0	22
Proline	10^{-2}	16	68
Valine	10^{-2}	30	80
Tryptophan	10^{-2}	62	84
Glycine	10^{-2}	82	85
Asparagine	10^{-2}	80	80
Ornithine	10^{-2}	80	85
Serine	10^{-2}	80	100

(1) All amino acids were L-isomers. The following amino acids at 10^{-2}M. reduced methane and methanol-stimulated respiration by less than 10% of the control: threonine, phenylalanine, tyrosine, homoserine, lysine, alanine, glutamamate, aspartate.

(2) control rates of methane and methanol respiration were 89 and 53 nmoles O_2/min/mg dry cell wt.

towards methane respiration and cysteine had a non-selective effect on methane and methanol respiration. All of these inhibitory amino acids are known for their ability to bind to metals in enzymes and the significance of this will become apparent when the mechanisms of inhibitions by the selective inhibitors is discussed in a later section. The lack of effect of all of the inhibitory amino acids except histidine on growth of Methylococcus capsulatus may be due to strain differences or more likely the physiological state of the cells since growth of Methylococcus capsulatus was found to be sensitive to a wider range of amino acids when they were added during the lag phase (Eccleston and Kelly, 1972).

The effect of n-octylamine on methane and methanol metabolism

In view of the specific inhibition of methane oxidation by a number of nitrogen-containing compounds, the effect of a primary amine on methane and methanol-stimulated respiration of M. trichosporium OB3B was investigated. The rates of methane and methanol-stimulated respiration were not inhibited by 10^{-4}M. n-octylamine but were reduced by 10^{-3}M to 84 and 85% respectively of the control rates of 89 and 53 nmoles O_2/min/mg cells and to 0 and 8% of the control rates by 10^{-2}M. n-octylamine. This inhibition was found to be irreversible and when cell suspensions of Pseudomonas methanica were also treated by 10^{-2}M. n-octylamine the intense pink colour of the suspension was bleached out.

The lack of specificity of the inhibition by n-octylamine shows that the possession of a free amino group alone is insufficient for specificity and a non-specific mode of inhibition through cellular lysis is suggested by the irreversibility of the inhibition and the bleaching of Pseudomonas methanica.

Effect of temperature

The variation of methanol and methane-stimulated respiration with temperature for M. trichosporium is shown in table 8 and it is evident that the lowering of the temperature of the suspension to 15° has a much greater effect on methane-stimulated respiration, reducing it to 3% of the rate at 30°C.

Table 8 Effect of temperature on Methane and Methanol-stimulated Respiration by M. trichosporium

Temperature °C	Respiration rates (% control) CH_4	CH_3OH*
30	100	100
20	40	58
15	3	33

(1) control respiration rates for CH_4 and CH_3OH at 30°C 115 and 105 nmoles O_2/min/mg dry cell wt.

A similar effect of temperature was found by Jeenes (1972) with the thermo-tolerant organism Methylococcus capsulatus Bath where lowering the temperature from 46°C to 30°C inhibited methane-stimulated respiration completely but only reduced the methanol-stimulated respiration rate by 51%.

Effect of inhibitors on growth on methane and methanol carbon sources

Inherent in the approach adopted in this study was the assumption that compounds that inhibit methane, but not methanol, oxidation are specific inhibitors of the methane oxygenase enzyme(s) and leave the rest of cellular metabolism unaffected. In reality, however, the results merely show that the compounds do not affect methanol oxidation. In an

attempt to establish that these compounds are specific for the methane oxygenase the effect of selected inhibitors on growth on methanol and methane carbon sources was examined. Methylomonas albus BG8, a membrane-type I methylotroph, was used for this study since unlike M. trichosporium OB3B, it is capable of growth on methanol at high concentrations.

Table 9 Effect of Inhibitors on Growth of Methylomonas albus BG8 on Methane and Methanol carbon sources

Inhibitor	Concentration (M.)	Growth after 5 days (% control) CH_4	CH_3OH
Allyl thiourea	10^{-5}	1.5	94
Thiosemicarbazide	10^{-5}	15	112
Hydroxylamine hydrochloride	10^{-5}	0	91
Diethyldithiocarbamate	10^{-4}	0	90
αα'dipyridyl	5×10^{-5}	2	80
8-quinolinol	10^{-5}	15	154
Pyridine	10^{-4}	4	88
CO	25% CO: 75% O_2	0	94

(1) Methane and methanol contents of growth media were 20 mls and 0.3% (v/v) respectively and yields of controls 12 and 13.1 mg dry weight.

The growth of M. albus BG8 after five days in the presence of a selected range of inhibitors is shown in table 9 in which growth is expressed the percentage of the control flasks in the absence of the inhibitor. The complete uptake of methanol substrate was confirmed by gas chromatography. In all of the cases tested growth on methanol was recorded in the presence of concentrations of inhibitor which prevented growth on methane, although ten-fold higher concentrations of inhibitor generally inhibited

growth on both substrates. The assumption that it is the initial step in the metabolism of methane that is affected by the inhibitors is validated by the lack of inhibition of the wide range of cellular reactions associated with cell growth and replication. These results also confirm that methane oxidation in a membrane-type I methylotroph is also affected by the specific inhibitors of methane oxidation in M. trichosporium OB3B and the lower concentrations required for inhibition are likely to be an effect of the lower cell density in a shake culture (approximately 0.2 mg dry wt./ml) than a washed cell suspension (10 mg dry wt./ml). In some cases there was a stimulation of yield on methanol in the presence of inhibitors for which the reason was unknown.

Effect of inhibitors on the metabolism of formate

In view of the sensitivity to inhibition by cyanide of the formate dehydrogenase activity in extracts of Methylococcus capsulatus (Patel and Hoare, 1971) and of the partially-purified enzyme from Pseudomonas AM1 (Johnson and Quayle, 1964) it is possible that these inhibitors may act by inhibiting formate dehydrogenase and the production of NADH for a methane mono-oxygenase reaction. That this is not the case is shown by the results in table 10 in which the effect of a range of inhibitors on both formate-stimulated respiration by cell suspensions and formate-stimulated NAD reduction (formate dehydrogenase) in a supernatant fraction of M. trichosporium are shown. The sensitivity to cyanide found by Patel and Hoare (1971) is confirmed, although in contrast with their results the sensitivity of formate oxidation to inhibition by cyanide was less than methane oxidation, but the low level of inhibition by the other

specific inhibitors of methane oxidation indicates that the formate dehydrogenase enzyme is not the site of action of the inhibitors, pointing again to the specificity of these inhibitors for the methane oxygenase.

Table 10 Effect of Inhibitors on the Metabolism of Formate by Methylosinus trichosporium OB3B

Inhibitor	Concentration (M)	Effect on formate metabolism (% Control)[1]	
		Formate respiration	Formate Dehydrogenase
αα'dipyridyl	10^{-4}	100	86
8-quinolinol	10^{-4}	85	77
thiosemicarbazide	10^{-4}	103	82
Allyl thiourea	10^{-4}	100	80
Thiourea	10^{-4}	100	85
NaCN	10^{-4}	7	0
,,	10^{-5}	24	13
,,	10^{-6}	64	64

(1) Formate metabolism in control: formate-stimulated respiration 10 nmoles O_2/min/mg dry cell weight; formate dehydrogenase activity 0.035 absorbance units (340 nm)/ mg protein.

Mechanism of action of specific inhibitors

The specific inhibition of methane oxidation by such a large number of metal ion chelators and metal-binding compounds would suggest that these compounds inhibit methane oxidation through binding to a metal ion in the methane oxygenase system. Indeed, the presence or absence of inhibition by such compounds is often presented as evidence for or against the involvement of a metal ion in an enzyme and a good discussion of the use of chelators in the study of metallo-enzymes is given by Hughes (1972). Any conclusions based on inhibition by one chelator must be made with caution as the

inhibition could be through a mechanism other than the binding to a metallo-enzyme as is known for the inhibition of some flavoprotein mono-oxygenases by o-phenanthroline (Yamamoto et al., 1969) and inhibition of cytochrome oxidase by British Anti-Lewisite (Cooperstein, 1963). The protection from inhibition by pre-incubation of the enzyme with a metal ion and the restoration of activity after inhibition by the addition of a metal ion are both considered as evidence for the involvement of a metal ion. The satisfaction of the latter criterion by a range of inhibitors is shown in table 11 where the inhibitors were chosen as representatives of the three main categories of inhibitors containing nitrogen-nitrogen, oxygen-nitrogen and sulphur-nitrogen ligands.

It is possible to draw some conclusions as to the nature of the metal involved in an enzyme from the chemical nature of the chelator although as discussed in detail by Hemmerich (1966) the term 'specificity' as applied to metal ion chelators is considerably mis-used in the scientific literature. The thermodynamic specificity of a chelator for a metal is the criterion most relevant to the inhibition of biological redox systems and can be generalised according to the rule that 'hard' metal ions react with 'hard' ligands and 'soft' metal ions with 'soft' ligands where hard means ionic and not easily polarised atoms and molecules such as Fe (III), Mn (II), Cr (III), Na and the ligands H_2O, $RCOO^-$, RNH_2 and 'soft' means easily polarisable molecules such as Cu (I), Ag (I) ions and the ligands CN^-, RSH, R_2S, RS (Hughes, 1972). In addition to these two classes there is an 'inter-

Table 11 Effects of Added Metal Ions on Inhibition of Methane-stimulated Respiration in Methylosinus trichosporium OB3B

Inhibitors	Conc. (M)	% Restoration of activity by added Metal ions (10^{-4}M)			
		Fe(II)	Co(II)	Cu(II)	Zn(II)
αα'dipyridyl	10^{-4}	37	64	62	11
8-quinolinol	10^{-4}	45	14	65	11
thiosemicarbazide	10^{-4}	0	0	14	0
thiourea	10^{-4}	0	0	0	0
allyl thiourea	10^{-4}	0	0	0	0

mediate' class of Zn (II), Cu (II), Ni (II), Fe (II), Sn (II), Pb (II) and the ligands pyridine, N_3^-, NO^-_2. Thus the specific inhibitors of methane oxidation which contain the ligands oxygen-nitrogen, nitrogen-nitrogen and nitrogen-sulphur fall into the categories of intermediate and soft whereas hard ligands such as E.D.T.A., octylamine and urea are not specific inhibitors. Another metal ion chelator with hard ligands, 1,2-dihydroxybenzene 3,5-disulphonic acid disodium salt or 'tiron', has been reported to combine specifically with Fe (III) (Harvey and Manning, 1959; Kojima et al., 1967) and consistent with a role for copper in the methane oxygenase this compound at 10^{-3}M concentrations inhibited methane respiration of suspensions of M. trichosporium OB3B by less than 10%. Purely chemical considerations would suggest that the metal ion in the oxygenase is a 'soft' ion such as Ag (I) or Cu (I) and this is supported by the results in table 11 which show that although th inhibition of methane-stimulated respiration by the 'intermediate' inhibitors αα'dipyridyl and 8-quinolinol is reversed by a range of added metal ions, only Cu (II) reversed

inhibition by the sulphur-containing thiosemicarbazide and none of the added metal ions even at concentrations of 10^{-3}M reversed inhibition by thiourea and allyl thiourea. No reversal of inhibition by any inhibitor was observed on the addition of Mn (II), Fe (III), Cr (III) and Sn (II) at a concentration of 10^{-3}M.

The high thermodynamic stability constant of thiourea for Cu (I) of β_6= 15 (Sillen and Martell, 1971) is approached only by Ag (I) and from the known involvement of copper in oxygenase enzymes a copper component is suggested. The extremely low solubility of cuprous compounds and their tendency to readily undergo disproportionation to Cu (0) and Cu (II) in aqueous solution makes the above reversibility studies difficult (Hammerich, 1966). The addition of a suspension of cuprous chloride to Methylosinus trichosporium in the oxygen probe resulted in a low but detectable 12% restoration of activity of thiourea-inhibited methane respiration which supports a mechanism of inhibition of methane oxidation by thiourea involving the formation of a chelate complex with a cuprous component of the methane oxygenase.

If the mechanism of inhibition by thiourea was distinct from the other compounds in table 11 the total inhibitions by combinations of thiourea and the metal ion-reversible inhibitors need not necessarily be additive. The inhibitions of methane-stimulated respiration by M. trichosporium OB3B in the presence of αα'dipyridyl, 8-quinolinol and thiourea, both individually and in combinations with each other are shown in

table 12. The close agreement between the inhibition by pairs of inhibitors and the arithmetical products of the individual inhibitions is consistent with a single site of inhibition for thiourea, αα'dipyridyl and 8-quinolinol but does not constitute proof.

Table 12 Effect of Combinations of Inhibitors on Methane-stimulated respiration of M. trichosporium

Inhibitors	CH_4 respiration (% Control[1])	Arithmetic products of inhibitions[2]
8-quinolinol (10^{-5}M)	27	
αα'dipyridyl (10^{-5})	44	
thiourea ($5x10^{-6}$)	64	
thiourea ($5x10^{-6}$) +8-quinolinol (10^{-5})	17	17
thiourea ($5x10^{-6}$) +αα'dipyridyl (10^{-5})	27	28
8-quinolinol (10^{-5}) +αα'dipyridyl (10^{-5})	14	12

(1) control rate methane respiration = 57 nmoles O_2/ mg dry wt. cells/min.

(2) i.e. 27 x 64 = 17 etc.

Among the 'intermediate' metals the strength of a metal-ligand bond increases in the order Mn (II) Fe (II) Co (II) Ni (II) Cu (II) Zn (II) and the reversibility of inhibition by added metal ions other than copper is consistent with this. Thus the ability of Fe (II) from a copper-free analytical grade preparation to relieve inhibition by αα'dipyridyl and 8-quinolinol is in no way contradictory with the involvement of a copper atom since this effect is observed in the relief of inhibition by these compounds of the copper-containing dopamine hydroxylase enzyme (Goldstein, 1966). The inhibitory

action of a metal ion chelator may involve a complete removal of the metal from the enzyme leaving the apo-enzyme which may or may not be reactivated by the addition of the original metal ion, or the replacement of some of the protein groups only to form a mixed-enzyme inhibitor complex (Hughes, 1972). If a mixed complex is formed then addition of metal ion should compete with the inhibitor and thus reverse the inhibition. The relief of inhibition by a range of metal ions would suggest that inhibition is through the formation of a mixed complex but kinetic studies on the purified enzyme would be required to prove this conclusively.

Effect of inhibitors on kinetics of methane respiration

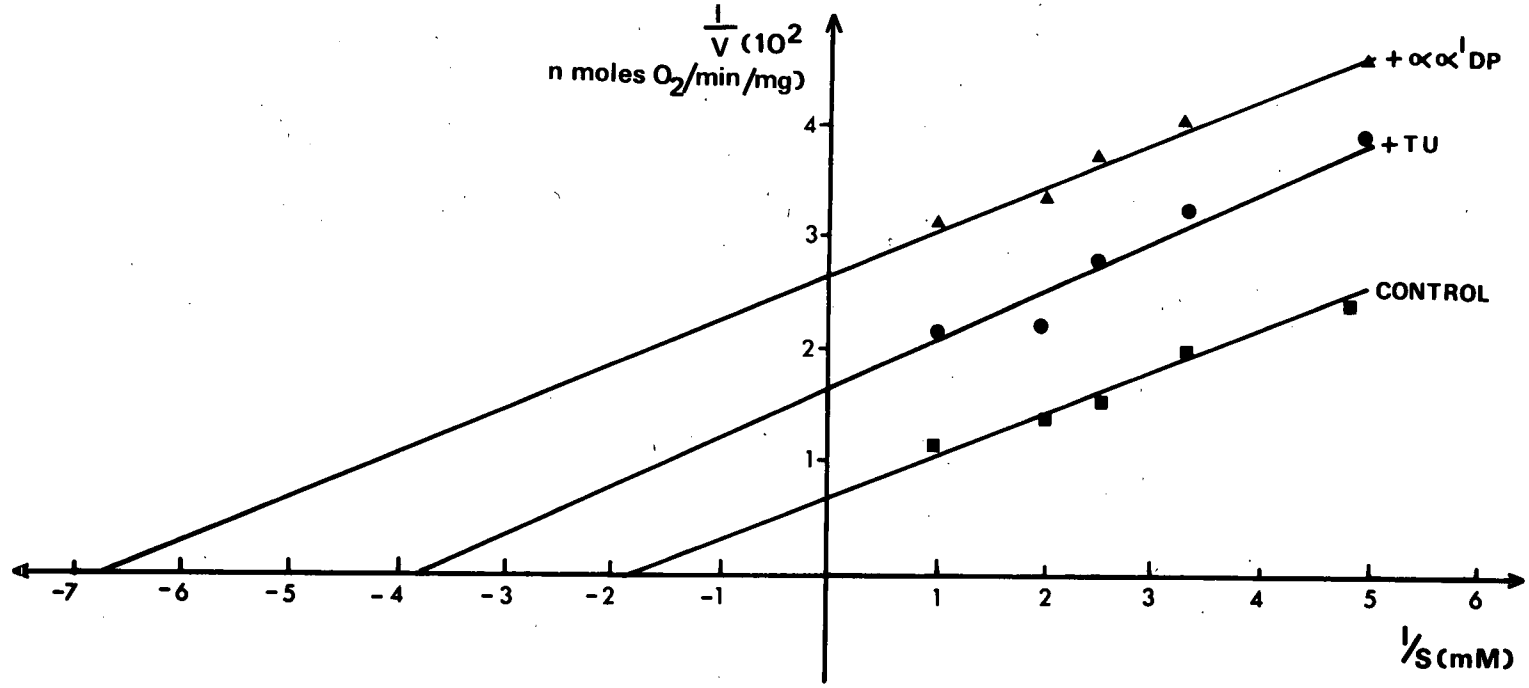
Inhibitors can be classified according to their mode of action as 'competetive' 'non-competitive' and 'uncompetitive' and the kinetic analysis method of Lineweaver and Burk (1934) can in principle be applied to determine the mode of action of any inhibitor provided the inhibition is reversible and the rate of enzyme reaction can be measured as a function of substrate concentration. With a gaseous substrate such as methane it is particularly difficult to determine the substrate concentration in solution and Thomson (1974) gives a detailed critique of the attempt by Phillips (1970) to demonstrate competitive inhibition by ammonia of methane disappearance through the determination of rates of methane disappearance in flasks with varying amounts of ammonia.

An alternative to measuring methane disappearance in flasks is to use an oxygen probe to measure the rate of methane-stimulated respiration and this method has the advantage that the concentration of dissolved methane can be

easily controlled by the addition of different amounts of methane-saturated buffer to the reaction vessel. This method has been used by Harrison (1973) to derive the Km for methane of a methane-oxidising Pseudomonas strain and figure 3 shows that it can also be applied to M. trichosporium OB3B to give a Km for methane of 520 μmolar compared with that of Harrison of 26 μmolar. Indicative of the limitations of working with whole cells, considerable variations were found with different batches of cells and Kms of as low as 50 μmolar have been found. The low Km for methane made it necessary to add to the oxygen probe chamber small amounts of substrate which rapidly becomes used up making steady-state respiration rates difficult to determine accurately and resulting in the scatter of points about the straight line found in this study and in Harrison (1973). Figure 3 also shows double reciprocal plots for methane-stimulated respiration partially-inhibited by thiourea and αα'dipyridyl and the alteration of both V_{max} and Km for methane-stimulated respiration by these inhibitors is typical of the 'uncompetitive' class of inhibitors and is clearly not competitive inhibition. The simplest qualitative interpretation of uncompetitive inhibition is that it is due to the inhibitor binding with a form of the enzyme that differs from that responsible for combination with the free substrate, thus lowering the amount of enzyme available for the reaction (Cleland, 1963) and, although more complex interpretations can be made, this simple mechanism is consistent with inhibition caused by binding of metal ion chelators to a metal in the oxygenase system.

Fig. 3: Kinetics of inhibition of methane-stimulated respiration in M. trichosporium OB3B by thiourea (5×10^{-6}M.) and αα'dipyridyl (10^{-5}M.)

Double-reciprocal plots of respiration rates, determined with 5 mg cells in the oxygen probe and the dissolved methane concentration: in the absence of inhibitor (■), in the presence of 5×10^{-6}M. thiourea (●) and 10^{-5}M αα'dipyridyl (▲)


1/V (10²
n moles O₂/min/mg)
+ ∝∝ ¹DP
+TU
CONTROL
4
3
2
1
-7
-6
-5
-4
-3
-2
-1
1
2
3
4
5
6
1/S (mM)

The similarity between the plots for thiourea and αα'dipyridyl inhibition shown in figure 3 lends further support for a similar mechanism for these two compounds.

In support for a role for copper in the metabolism of methane by methylobacteria, a batch culture study with a mixed culture HR (Vary and Johnson, 1967) and continuous culture studies of Methylococcus capsulatus (Harwood and Pirt, 1972), Methylomonas strain Fl (Fukuoka, 1974) and a mixed culture of methane oxidising Gram-negative rods (Sheehan and Johnson, 1971) have all demonstrated a requirement for copper ions in the growth medium. None of these studies on copper requirement were sufficiently detailed to assess the significance of the requirement for copper over and above that of a normal Gram-negative heterotroph.

Although a mechanism of inhibition involving combination with a copper species is consistent with the properties of many of the inhibitors, it does not explain inhibition by methanol which is not found in Methylomonas albus BG8 (Thomson, 1974), nor is it likely to explain the inhibition by the compounds SKF 525A, Lilly 18947 and Lilly 53325 whose mode of action in inhibiting Cytochrome P-450-mediated oxygenase reactions are little understood (Hildebrandt, 1972). Metapyrone, which inhibits some Cytochrome P-450 hydroxylation reactions (Hildebrandt, 1972) did not inhibit methane oxidation at concentrations of 1mM although limited interpretations can be made of negative results with cell suspensions where permeability barriers may arise.

Comparisons between the inhibition patterns of the ammonia oxygenase in *Nitrosomonas* and methane oxidation in *Methylosinus*

A list of the compounds which were found by Hooper and Terry (1973) to inhibit ammonia but not hydroxylamine oxidation by *Nitrosomonas europaea* and which have also been shown in this study to inhibit methane-,but not methanol-oxidation in *Methylosinus trichosporium* is given in table 13 and it can be seen that seventeen out of the twenty-five specific inhibitors of methane oxidation are also specific for ammonia oxidation. The higher concentrations of inhibitor required for inhibition of methane oxidation in *Methylosinus trichosporium* is readily explained by the higher concentrations of cells used in each assay. The studies of Hooper and Terry (1973) used 0.2 mg dry wt. cells/ml of *Nitrosomonas* and 10 mg/ml of *Methylosinus* was used in this study.

This similarity in the pattern of inhibition suggests that the two oxygenases may resemble each other. Resemblances between methylobacteria and *Nitrosomonas* also extend to the highly fastidious growth requirements for ammonia by *Nitrosomonas* and for methane, methanol and dimethyl ether by methylobacteria (Quayle, 1972), the possession by both groups of complex internal membrane systems (Davies and Whittenbury, 1970; Whittenbury, 1969; Smith and Ribbons, 1970; DeBoer and Hazeu, 1972, Murray and Watson, 1965; Watson and Mandel, 1971) a lesion in the Tricarboxylic acid cycle of *Nitrosomonas* (Hooper, 1969) and membrane-type I methylobacteria (Davey *et al.*, 1972) and the initial metabolism of the energy source by an oxygenase reaction

Table 13 Compounds which inhibit both Methane Oxidation in Methylosinus and Ammonia Oxidation in Nitrosomonas

	Ammonia Oxidation in Nitrosomonas[1]			Methane Oxidation in Methylosinus[2]		
	Conc. (M)	% Control	Reversibility	Conc. (M)	% Control	Reversibility
Metal-binding Compounds						
Allyl thiourea	10^{-6}	18	+	10^{-5}	28	+
o-phenanthroline	$5x10^{-5}$	0		$5x10^{-5}$	65	+
diethyldithio-carbamate	10^{-5}	0	+	$7.5x10^{-4}$	0	+
8-quinolinol	10^{-5}	0		10^{-4}	0	+
αα'dipyridyl	10^{-4}	0		10^{-4}	0	+
KCN	$5x10^{-6}$	22	+	10^{-6}	36	+
Enzyme-and Haeme Protein-Binding Compounds						
Thiosemicarbazide	10^{-5}	5		10^{-5}	45	+
CO	(95%O_2:5%CO)	8		(85%O_2:15%CO)	0	
Ethyl Xanthate	10^{-4}	0		10^{-3}	0	+
Aminoguanidine	10^{-3}	26		10^{-2}	32	
LILLY 18947	10^{-4}	4		10^{-2}	0	
LILLY 53325	10^{-4}	4		$5x10^{-3}$	0	
SKF 525A	$5x10^{-5}$	35	-	$5x10^{-3}$	36	-
3-Aminotriazole	10^{-3}	0		10^{-2}	27	+
Short Chain Alcohols						
Methanol	$5x10^{-3}$	0		10^{-2}	0	+
Ethanol	0.09	0		10^{-2}	0[3]	+
Hydroxylamine				10^{-4}	23	+

(1) figures from Hooper and Terry (1973)

(2) figures from tables 1, 2 and 3 of this section.

(3) Thomson (1974)

(4) Reversibility = +

(Higgins and Quayle, 1970; Rees and Nason, 1966).

It is thus pertinent to consider in detail the analysis made by Hooper and Terry (1973) of their inhibitor data. The conclusion that a copper ion may be involved in ammonia oxidation has been based on the inhibition of ammonia oxidation by thiourea, allyl thiourea and diethyl dithiocarbamate (Hofman and Lees, 1953, Anderson, 1965, Hooper and Terry, 1973) but in reality the evidence is in a similar state as for the involvement of copper in methane oxidation in relying heavily on the chemical properties of the metal ion chelators. Growth on Nitrosomonas in shake cultures has been shown to require copper ions (Loveless and Painter, 1968) and a copper species has been detected in whole cells and extracts using electron paramagnetic resonance spectroscopy (Nicholas et al., 1962) and the significance of the latter study will be discussed in a later section.

Hooper and Terry (1973) reported that the the addition of Cu (II) ions reversed the inhibition of ammonia oxidation by KCN and diethyl dithiocarbamate and not Mg (II), $CeCl_3$, $SnCl_2$ or Cu $(EDTA)_3^{2+}$ but did not try Fe (I)which although not shown in table 11 reversed inhibition by diethyl dithiocarbamate of methane oxidation in M. trichosporium OB3B. As found in this study with M. trichosporium OB3B they reported the absence of inhibition by the heavily substituted derivative of o-phenanthroline, Bathocuproine, at 1 mM. Histidine was not included in their study although it has been shown by Lees (1952a) to inhibit ammonia oxidation by washed cell suspensions of Nitrosomonas and by Clark and Schmidt (1967) to inhibit growth.

The grouping of inhibitors in this present study was made solely on the basis of their chemical properties but Hooper and Terry (1973) classify a number of their compounds as 'Enzyme and haeme protein-binding compounds' on the basis of the known inhibition of catalase by thiosemicarbazide, 3-amino triazole, amino guanidine and carbon monoxide (Nicholls and Schonbaum, 1963), of peroxidase by potassium ethyl xanthate (Anderson, 1968), of diamine oxidases by diethyldithiocarbamate, aminoguanidine, hydroxylamine, Cn^- and thiosemicarbazide (Zeller, 1963) and of cytochrome P-450-mediated oxygenase reactions by Lilly 18947, Lilly 53325 and SKF 525A (Hildebrandt, 1972). However most of these compounds are metal ion chelators and could act by binding to the same site as the first group and thus any attempt to involve specific enzymes on the basis of inhibition data should be made with caution. The suggestion by Hooper and Terry (1973) that a catalase or peroxidase-like enzyme may be involved in ammonia oxidation, either directly through the production of $(OH)^{\cdot}$ or as a hydroxylase or indirectly through the removal of inhibitory levels of H_2O_2 is interesting but must be considered highly speculative. The same qualification also applies to their suggestion that methanol might inhibit by reacting with a peroxide-metabolising enzyme as has been shown for catalase (Nicholls and Schonbaum, 1963) or as a free radical trap.

Valuable though such a comparison between inhibitors of methane and ammonia oxidation may be it must be borne in mind that there are differences such as the insensitivity of methane oxidation to inhibition by 2-4 dinitrophenol at 10^{-3}M and N_2O (90% O_2, 10% N_2O).

The inhibition of ammonia oxidation by lowering the temperature was also noted by Hooper and Terry (1973) who demonstrated that lowering the temperature of incubation of Nitrosomonas from 25°C to 15°C resulted in a reduction in the rate of ammonia oxidation by 77% but only reduced hydroxylamine oxidation by 50%.

In their study of the lipid content of the membranes of the same membrane - type II methylobacterium as used in this study M. trichosporium OB3B, Dugan and Weaver (1974) showed that the fatty acid content consisted predominantly of a $C_{18:1}$ fatty acid and commented that this was the longest fatty acid known to exist in bacteria which is liquid at room temperature. Smith and Ribbons (1970) have also shown that over 90% of the esterified fatty acids in the membranes of another membrane type II methylobacterium Methanomonas methano-oxidans was a $C_{18:1}$ fatty acid. A high content of $C_{16:1}$ fatty acid has been reported for Nitrosomonas (Blumer et al., 1969).

In view of the likely location of the methane oxygenase in the complex internal membrane systems of methylobacteria, an explanation of the inhibition of the methane oxygenase is suggested in which lowering the temperature 'freezes' the $C_{18:1}$ fatty acid. That this 'freezing' is reversible is indicated by the ease of storage of cell suspensions of methylobacteria at 4° without any loss of methane-oxidising activity on restoration of the temperature to 30°C. Lowering the temperature of cell suspensions of methylobacteria below 0°C, however, was found in this study to result in the irreversible loss of

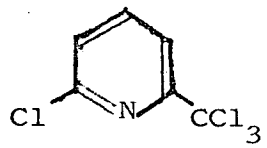
methane-oxidising activity and a loss in ammonia-oxidising activity has also been found on freezing Nitrosomonas (Aleem, 1970).

THE EFFECT OF N-SERVE ON METHANE OXIDATION BY METHYLOBACTERIA

There has been considerable commercial interest in finding compounds which inhibit or even slow down the rate of nitrification of ammonia fertilisers which can lead to the leaching of nitrate and nitrite and their accumulation in rivers and lakes where they can cause both health hazards and eutrophication (review: Prasad et al., 1971). Thus many of the compounds found to be specific inhibitors of ammonia oxidation by Nitrosomonas (Hooper and Terry, 1973) and of methane oxidation by methylobacteria have been investigated as potential "Nitrogen Stabilisers" (Prasad et al., 1971; Bundy and Bremner, 1973).

The ability of a compound to inhibit ammonia oxidation by suspensions of pure cultures does not necessarily indicate its effectiveness in a demanding environment such as soil where it might be washed away, adsorbed onto soil particles or degraded by micro-organisms. The efficiencies of such inhibitors have also been shown to vary with the types of soil. Hence allyl thiourea and thiourea were found to be ineffective in inhibiting nitrification in a range of soils whereas potassium azide proved to be extremely effective (Bundy and Bremner, 1973). Additional criteria such as freedom from toxicity to man, animal and soil organisms as well as manufacturing cost must be superimposed on the biological effectiveness of a nitrogen stabiliser.

A compound which has received considerable attention is 2-chloro,6(trichloromethyl)pyridine (Goring, 1962a; Goring, 1962b) which is commonly known by the trade name "N-SERVE" and whose structure is shown below:



The widespread commercial application in soils of a compound that may be toxic to methylobacteria is sufficient reason to study such a compound, but even more relevant to this present investigation of the methane oxygenase is the mechanism of inhibition of ammonia oxidation by Nitrosomonas proposed by Campbell and Aleem (1965) involving inhibition of cytochrome oxidase by N-SERVE.

The initial studies on N-SERVE by Goring (1962a; 1962b) only demonstrated the retardation of nitrification in soils and it was Campbell and Aleem (1965) who showed the inhibition of growth of Nitrosomonas europaea in pure cultures was completely inhibited by 0.9×10^{-6}M N-SERVE and ammonia-stimulated nitrite production in cell suspensions by 0.43×10^{-5}M N-SERVE was required to produce 90% inhibition of hydroxylamine oxidation. In their comprehensive survey of specific inhibitors of ammonia oxidation by suspensions of Nitrosomonas Hooper and Terry (1973) confirmed the specificity of N-SERVE, showing that a concentration of 5×10^{-5}M caused 86% inhibition of ammonia oxidation and only 4% inhibition of hydroxylamine oxidation.

The effect of N-SERVE on the rate of methane and methanol disappearance from incubations of suspensions of M. trichosporium OB3B is shown in table 14. Concentrations of 1.3 and 0.65 x 10^{-4}M N-SERVE inhibited methane disappearance completely whereas disappearance of methanol was only 10% inhibited at the higher concentration of N-SERVE. The effect of N-SERVE on the disappearance of methane and methanol from suspensions of M. albus BG8 is shown in table 14 and a similar selective effect on methane disappearance is evident. Methane oxidation in M. albus BG8 appeared more sensitive to inhibition by N-SERVE.

At the end of the incubation of M. trichosporium OB3B with 1.3 x 10^{-4}M N-SERVE the cells were washed and sedimented twice with 20 mls buffer, and sedimented and resuspended in the original volume of buffer and re-incubated with methane. After a lag of twenty minutes during which no disappearance of methane occurred the rate of methane disappearance was restored to 57% of the original uninhibited rate of the

Table 14 Effect of N-SERVE on Methane and Methanol Disappearance in Suspensions of Methylobacteria

Organism	Concentration N-SERVE (M.)	Rates of Disappearance (% Control)[1]	
		CH_4	CH_3OH
Methylomonas albus BG8	1.3×10^{-4}	n.t.[2]	59
	1.3×10^{-5}	0	61
Methylosinus trichosporium OB3B	1.3×10^{-4}	0[3]	90
	0.67×10^{-4}	0	100
	1.3×10^{-5}	57	n.t.

(1) Control rates of disappearance in the absence of inhibitor: M. albus BG8 CH_4 11.5, CH_3OH 53 nmoles/min/mg dry cell wt. M. trichosporium OB3B CH_4 21, CH_3OH 82 nmoles/min/mg dry cell wt.

(2) n.t. = not tested

(3) Inhibition on washing cells three times with 20 mls buffer 57% reversible after twenty minute lag.

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control demonstrating the partial reversibility of inhibition of methane oxidation by N-SERVE. In the inhibitor studies with N-SERVE the extremely low solubility of N-SERVE made it necessary to add the compound as a saturated solution in water and the concentrations were calculated from the solubility data supplied by the manufacturer. The addition of N-SERVE as an alcoholic solution in the studies of Campbell and Aleem (1965) was considered unsatisfactory in view of the inhibition by ethanol of methane oxidation (Thomson, 1974) and ammonia oxidation in Nitrosomonas (Hooper and Terry, 1973).

The effect of N-SERVE on formate-stimulated respiration by suspensions of M. albus BG8 and M. trichosporium OB3B was investigated and it was found that formate-stimulated respiration was not inhibited by 2.6×10^{-5}M N-SERVE at which concentration methane-stimulated respiration was totally inhibited.

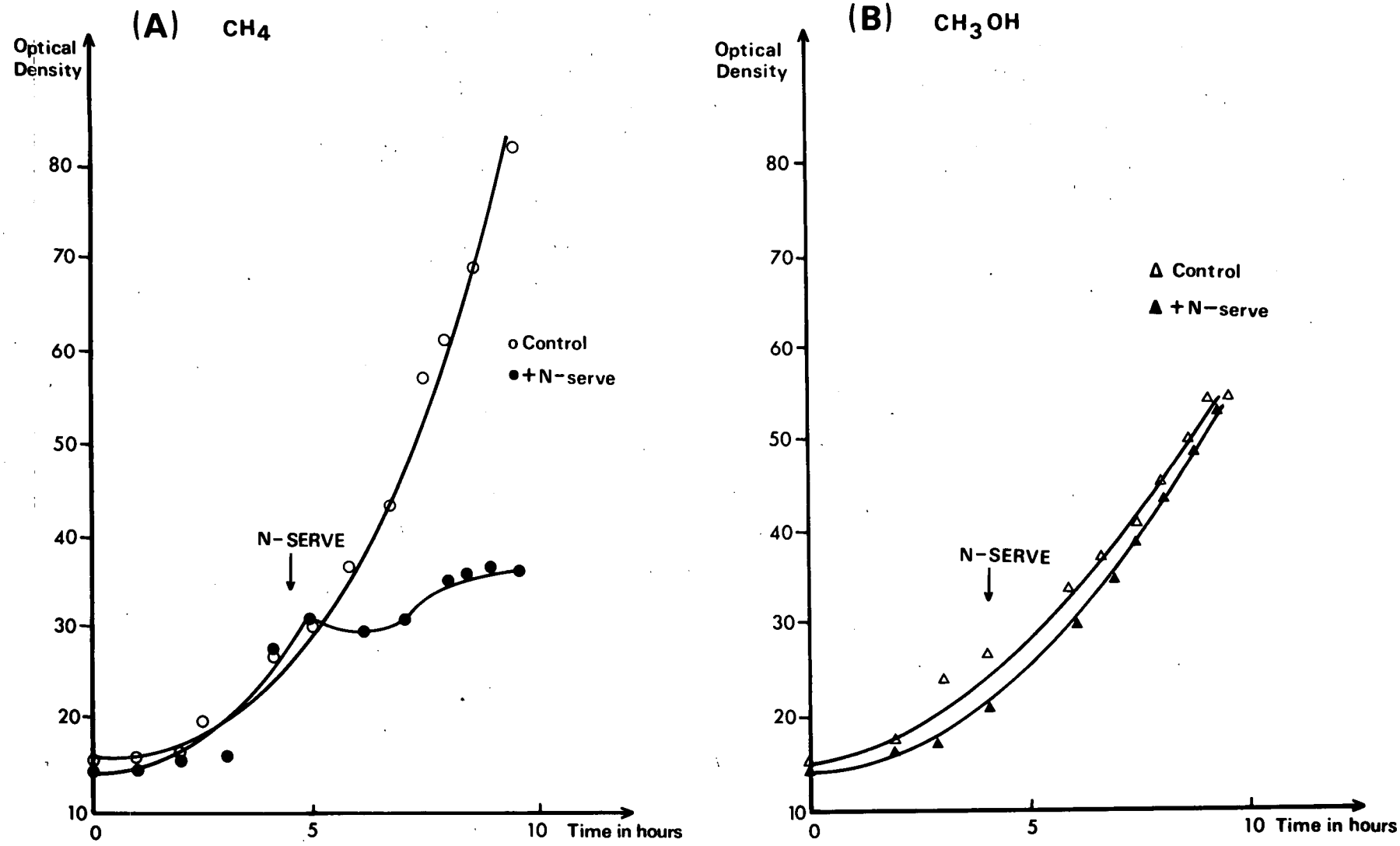
The inhibition of growth of M. albus BG8 with methane as carbon source can be seen from figure 4 which shows the effect of the addition of N-SERVE to cultures in the exponential phase of growth with methanol and methane as carbon sources. Growth was followed for five hours after addition of N-SERVE and although growth on methane was inhibited completely by 0.8×10^{-5}M N-SERVE, growth on methanol was unaffected. From the results in table 14 and figure 4 it is evident that, like the compounds described

Fig. 4: Effect of N-SERVE on growth of M. albus BG8

(A) Growth on methane in the presence (●) and absence (O) of N-SERVE

B) Growth on methanol in the presence (▲) and absence (△) of N-SERVE

Optical densities determined by nephelometry of freshly-inoculated cultures of M. albus with 20 ml methane and 0.3% w/v methanol incubated in 250 ml flasks at 30°C. Control flasks and with 1 ml saturated solution of N-SERVE (0.8×10^{-5}M).


(A) CH_4
Optical Density
80
70
60
50
40
30
20
10
0
5
10 Time in hours
N-SERVE
o Control
● +N-serve
(B) CH_3OH
Optical Density
80
70
60
50
40
30
20
10
0
5
10 Time in hours
N-SERVE
Δ Control
▲ +N-serve

earlier in this study, N-SERVE acts as a specific inhibitor of methane oxidation. In view of the specific inhibition of methane oxidation by pyridine (see table 5) the inhibition of methane oxidation by a pyridine derivative such as N-SERVE should be expected, however N-SERVE inhibits methane oxidation by cell suspensions at 1/15th the concentration of pyridine required for complete inhibition which points to some effect of the -Cl and $-CCl_3$ substituent groups. Although there remains the possibility of the chlorine groups binding directly to a metal, the effect of these electro-negative chlorine substituents on the pyridine ring would be to reduce the metal-binding ability of the nitrogen atom. The greater inhibitory properties of N-SERVE may thus be a result of the extreme insolubility of this compound in water and a possible enhanced solubility in the methylobacterial membranes.

All attempts to demonstrate reversibility by Cu^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+}, Fe^{3+} have been unsuccessful which would suggest that if N-SERVE was inhibiting methane oxidation through combination with a copper enzyme it would have to do so by binding to the enzyme in the cuprous oxidation state or that the high solubility in the methylobacterial membranes prevented reversal by added copper. The inability of added Cu^{2+} to reverse inhibition by N-SERVE contrasts with the results of Campbell and Aleem (1965) who were able to demonstrate complete reversal, however, contradictions even exist between their results and those of Hooper and Terry (1973) who reported the inhibition of ammonia oxidation to be irreversible. The

twenty minute lag observed in this study before restoration of methane uptake by washed N-SERVE-inhibited cells may provide an explanation for the inability of Hooper and Terry (1973) to demonstrate reversibility. Whether this lag was due to the time required to remove the last traces of inhibitor from the cells or the actual metabolism of the bound inhibitor is not certain.

Campbell and Aleem (1965) demonstrated that added reduced cytochrome c was oxidised by suspensions of Nitrosomonas and that this 'cytochrome c oxidase' activity could be followed spectrophotometrically. The contrast between the partial inhibition of this 'cytochrome c oxidase' activity of whole cells and the lack of inhibition of this activity in broken extracts, which did not oxidise ammonia, led Campbell and Aleem (1965) to propose that N-SERVE inhibited a component of the cytochrome oxidase which was involved in ammonia oxidation. The inhibition of 'cytochrome oxidase' activity by N-SERVE was also reversed by the addition of Cu^{2+}.

The relationship between this 'cytochrome c oxidase' and the cytochromes of Nitrosomonas europaea is unclear and it does not necessarily follow that their 'cytochrome c oxidase' activity corresponds to either the a or o-type cytochromes, whose presence in this organism has been well-established (Rees and Nason, 1965; Rees, 1968b; Erickson et al., 1972) or the Cytochrome P-460, the existence of which is disputed (Rees and Nason, 1965; Rees and Nason, 1968b; Erickson and Hooper, 1972). The o-type cytochrome has received little detailed attention but the cytochrome

a has been solubilised and purified (Erickson et al., 1972). Although the specific inhibitors of ammonia and methane oxidation CN^- (10^{-5}M), N_3^{-1} (10^{-3}M.) NH_2OH (5 x 10^{-4}M.) and diethyl dithiocarbamate (10^{-5}M) all inhibited the cytochrome oxidase activity of the purified cytochrome a, aa'dipyridyl, allyl thiourea and hydrazine at 1mM did not inhibit cytochrome c oxidase activity and indeed caused some stimulation, the extent of which was not given in their publication. Unfortunately the effect of N-SERVE on the cytochrome c oxidase activity of their purified enzyme was not investigated.

As with methylobacteria, cytochrome studies of ammonia-oxidising cell-free extracts will be required to establish a functional role for the a-type cytochrome of Nitrosomonas in the oxidation of ammonia.

The Effect of Hydrazine on Methylobacteria

In view of the reported specificity of hydrazine for the ammonia oxygenase in Nitrosomonas (Hooper and Terry, 1973) the effect of the compound on methane and methanol disappearance in suspensions of M. trichosporium OB3B was investigated and the rates of methane and methanol disappearance were found to be 70 and 6% respectively of the control rates. As this was the only compound examined which showed any selectivity for methanol oxidation, the effect of hydrazine on the metabolism of the membrane-type I bacterium M. albus BG8 also was examined and it was found that methane and methanol disappearance were inhibited by 30 and 100% respectively confirming the sensitivity of methanol oxidation in both membrane-types of methylobacteria to inhibition by hydrazine. All attempts to demonstrate by

gas chromatography the accumulation of methanol or any other intermediate in incubations of suspensions of methylobacteria with methane and hydrazine and methane have proved unsuccessful.

It is in this class of inhibition that the controversy in the methylobacterial literature is perhaps the greatest. Brown and Strawinski (1964) had reported that 3mM iodoacetate inhibited methanol but not methane oxidation in cell suspensions of Methanomonas methano-oxidans resulting in 75% of the methane accumulating as methanol and it was this observation, together with yield data, which led Whittenbury et al. (1970) to discount a mono-oxygenase mechanism for the oxidation of methane since they considered that any reducing equivalents for such a reaction would arise from the subsequent metabolism of methanol and hence a situation where methanol oxidation is inhibited but methane oxidation continues would not be expected to arise. Higgins and Quayle (1970) were unable to show inhibition by iodoacetate of methanol oxidation in either Pseudomonas methanica or Methanomonas methano-oxidans and in this study it was found that both methane and methanol disappearance in suspensions of M. trichosporium OB3B were inhibited by 3mM iodoacetate.

In their confirmation of the oxygenase nature of methane oxidation Higgins and Quayle (1970) found that high phosphate concentrations (80mM) inhibited methanol oxidation to the extent that methanol accumulated to a concentration of 2mM showing that methane oxidation can take place when the oxidation of methanol is partially inhibited. Anthony (1975)

has since confirmed the sensitivity of methanol dehydrogenase to phosphate ions.

That confusion and controversy is not limited to inhibitors of methanol oxidation is suggested by the contradiction between the selective inhibition by hydrazine of ammonia oxidation in Nitrosomonas observed by Hooper and Terry (1973) and the selective inhibition of hydroxylamine oxidation leading to the accumulation and identification of this compound during ammonia oxidation observed by Hofman and Lees (1953) and the inhibition of purified hydroxylamine oxidase by hydrazine (Hooper and Nason, 1965).

The contradiction noted by Whittenbury et al. (1970) can be resolved if it is taken into consideration that methanol oxidation, when determined by measuring rates of disappearance of exogenously-added methanol, proceeds at a faster rate than methane, typically 100nmole CH_3OH/min/mg cells compared with 20 nmole CH_4/min/mg cells, thus 90% inhibition of methanol oxidation would have only slight effect on the flow of reducing power to a methane mono-oxygenase. Another factor which must be considered with non-proliferating cells is the possible provision of reducing power from endogenous metabolism such as the formation of reduced pyridine nucleotides from the metabolism of polyhydroxy-butyric acid in M. trichosporium OB3B (Thomson, 1974). Hence both the relative rates of methane and methanol oxidation and the level of endogenous metabolism would determine the extent of accumulation of intermediates and would vary with different batches of cells.

The inhibition of methanol disappearance in cell suspensions of Pseudomonas M27 by a compound similar to hydrazine, phenyl-hydrazine, was noted by Anthony and Zatman (1965) who were unable to detect any inhibition of the methanol dehydrogenase activity of their partially-purified enzyme preparation and this led them to suggest that phenylhydrazine affected some site other than the alcohol dehydrogenase enzyme. The methanol-stimulated reduction of a PMS/DCPIP dye couple by supernatants of cell-free extracts of both M. albus BG8 and M. trichosporium OB3B was followed spectrophotometrically in the presence and absence of 2×10^{-3}M hydrazine sulphate and despite the high activities of ammonia-dependent dye reduction of 0.17×10^{-3} and 0.4×10^{-3} absorbance units (600nm)/min/mg protein respectively the addition of hydrazine had no effect.

The mechanism for the inhibition by hydrazine of methanol oxidation in whole cells is thus likely to be through either the prevention of entry of methanol into the cells, which might explain the inability to detect the accumulation of intermediates, or as proposed for the inhibition of hydroxylamine oxidation in Nitrosomonas by Hooper and Nason (1965), that hydrazine inhibits electron transport from the methanol dehydrogenase enzyme to the cytochrome chain but not to the artificial dye acceptor PMS. The difficulty in obtaining methanol accumulation during methane oxidation may lie in the difference between the oxidation of methanol when that methanol is present as a product of methane oxidation and when methanol is added exogenously.

THE EFFECT OF SPECIFIC INHIBITORS ON ETHANE AND ETHYLENE METABOLISM

Thomson (1974) discussed the difficulties encountered in establishing a common oxygenase mechanism for methane and ethane oxidation by methylobacteria and, despite the sensitivity of both reactions to inhibition by carbon monoxide, the contrast between the inhibition of methane oxidation by dimethyl ether and the insensitivity to this compound of ethane oxidation led him to conclude that methane and ethane were metabolised by separate oxygenase enzymes. Thomson's results could still be accommodated within a single enzyme theory if the rate-limiting step in ethane metabolism was distinct from that of methane and insensitive to dimethyl ether. Support for this suggestion comes from his observation that the oxidation of ethane, but not methane, was stimulated by the addition of formate which would suggest that the provision of reducing power for a possible methane mono-oxygenase was limiting in the case of ethane oxidation but not methane oxidation. An alternative explanation is that inhibition of ethane oxidation may have been superimposed on a stimulation of ethane oxidation by the metabolism of dimethyl ether.

A simple test to verify if one of the above explanations applies is to examine the effect of dimethyl ether on the metabolism of ethane in the presence of formate and the effect of dimethyl ether on ethane disappearance from suspensions of M. trichosporium OB3B and M. albus BG8 in the presence and absence of formate is shown in table 15. Confirming the results of Thomson (1974) the inhibition of

ethane disappearance by dimethyl ether was only slight but a marked inhibition of formate-stimulated ethane disappearance took place with both organisms.

Table 15 Effect of Dimethyl Ether on Ethane Oxidation in the Presence and Absence of Formate

	Rates of Ethane Disappearance (% Control)	
	M. albus BG8	M. trichosporium OB3B
C_2H_6 + CH_3OCH_3 [2]	100	95
C_2H_6 + HCOONa + CH_3OCH_3	50	59

(1) Control rates of ethane disappearance were: M. albus C_2H_6 12, C_2H_6 + HCOONa 29 nmoles C_2H_6/min/mg dry wt. M. trichosporium C_2H_6 12, C_2H_6 + HCOONa 18 nmoles C_2H_6/min/mg dry wt.

(2) concentration of substrates: C_2H_6 1 ml/25 ml flask, HCOONa and dimethyl ether 10mM.

Table 16 Effect of Specific Inhibitors of Methane Oxidation on the Disappearance of Ethane and Ethylene by M. trichosporium OB3B

Inhibitor	Conc. (M.)	Rate of Ethane and Ethylene Disappearance in the presence of formate[1] (% control[2])	
		Ethane	Ethylene
aa'dipyridyl	10^{-4}	0	12
8-quinolinol	10^{-4}	0	0
hydroxylamine	10^{-3}	17	0
Thiosemicarbazide	10^{-4}	0	3
Thiourea	10^{-4}	11	12
N-SERVE	0.67×10^{-4}	21	29

(1) concentration HCOONa 10mM in a total of 5 ml pH 6.8 buffer.

(2) Control rate of formate-stimulated ethane and ethylene disappearance were both 22 nmoles C_2H_6/min/mg dry wt.

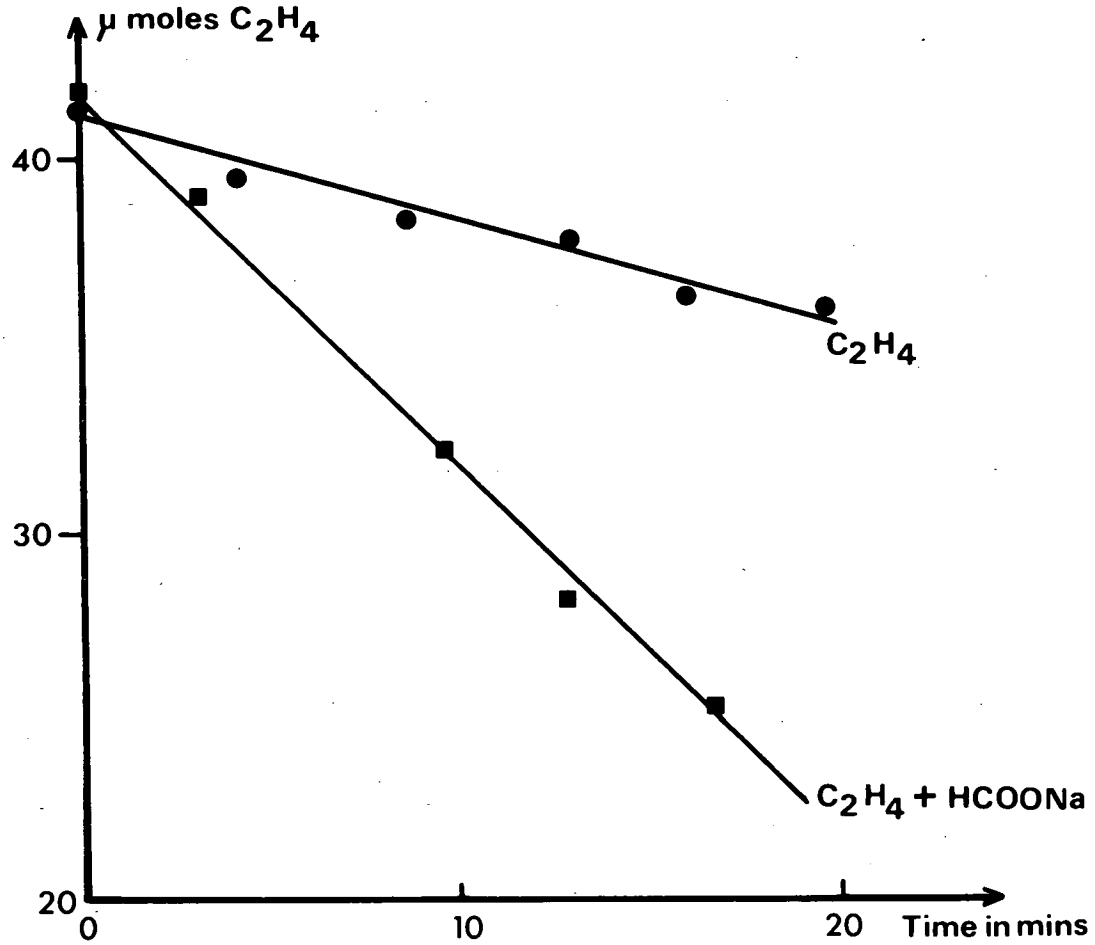
The contradiction between the production of ethylene in the presence of acetylene by methanol- but not methane-grown cultures of a methylobacterial isolate MS led DeBont and Mulder (1974) to propose that ethylene was being oxidised and, although ethylene could not serve as sole carbon source, their suggestion was supported by the demonstration that ethylene disappeared from cultures of MS growing with methane, but not methanol, as sole carbon source.

The disappearance of ethylene from incubation of M. trichosporium OB3B is shown in figure 5 as well as the effects of addition of formate. Ethylene disappeared at a rate of 7 nmole C_2H_4/min/mg cells which was increased to 22.2 in the presence of formate. Ethylene did not disappear from a control incubation with buffer alone in place of a cell suspension or from an incubation of cells that had been boiled for five minutes indicating that ethylene uptake could not be ascribed to the solution of ethylene in the red rubber 'suba-seals' as the solution of ethylene in rubber has been shown to occur (Kavanagh and Postgate, 1970). Although not shown in figure 5 ethylene disappearance was inhibited 45% in the presence of carbon monoxide (25% CO:75% O_2) indicating a lower sensitivity than methane oxidation to inhibition by carbon monoxide.

The rates of ethane disappearance in the presence and absence of formate from incubations of cell suspensions taken from the same batch of cells were 13.4 and 22 nmole C_2H_6/min/mg cells respectively, demonstrating that although the rate of ethane oxidation was higher than ethylene in the absence of

Fig. 5: Ethylene disappearance in suspensions of M. trichosporium OB3B

Standard suspensions (of 47 mg cells in 5 ml pH 6.8 buffer) incubated with 1 ml ethylene in the presence and absence of 50 μmoles formate.


μ moles C_2H_4
40
30
20
0
10
20
Time in mins
C_2H_4
C_2H_4 + HCOONa

formate, the rates of the two reactions were identical in the presence of formate.

The effect of the specific inhibitors of methane oxidation described in the first chapter of this study on the oxidation of ethane and ethylene by M. trichosporium OB3B was then determined. However in view of the lack of inhibition by dimethyl ether of ethane disappearance at the unstimulated rate in the absence of added formate, the effect of inhibitors on the formate-stimulated ethane and ethylene disappearance was determined. The rates of ethane and ethylene disappearance in the presence of formate and a range of inhibitors is shown in table 16 and it can be seen that, in addition to inhibiting methane oxidation, these compounds also inhibited ethane and ethylene disappearance. The inability of DeBont and Mulder (1974) to observe ethylene disappearance by methanol-grown cultures may thus be explained by the inhibition by methanol of methane oxidation by suspensions of M. trichosporium shown in table 6 and in Thomson (1974) and the inhibition of methane uptake by a mixed culture on the addition of methanol (Wilkinson, 1972). Table 16 also shows that N-SERVE inhibits both ethane and ethylene oxidation by M. trichosporium OB3B although the ecological significance of this observation will await a detailed study of the ecology of methane-utilisers in the soil and the role of ethylene which has recently been implicated in the regulation of fungistasis (Smith, 1973; Smith and Cook, 1974).

Whilst not constituting proof, the above data is consistent with a 'one enzyme' theory for methane, ethane and ethylene oxidation by methylobacteria.

THE EFFECT OF SPECIFIC INHIBITORS OF METHANE OXIDATION ON AMMONIA OXIDATION BY METHYLOBACTERIA

The reports by Hutton and Zobell (1953) and Whittenbury *et al.* (1970) of the ability of cultures of Methylobacteria to produce nitrite in the presence of ammonia raise the possibility that methane and ammonia are oxidised in Methylobacteria by the same oxygenase enzyme. Phillips (1970) showed that in the presence of 0.1% NH_4Cl (0.019M) methane uptake by suspensions of a range of Methylobacteria was inhibited 20-50% and, by following the rates of methane uptake at different methane concentrations in the gas phase and applying the analysis of Lineweaver and Burke (1934), attempted to demonstrate competitive inhibition between ammonia and methane. Thomson (1974) criticised this approach which relies not only on knowing the substrate concentration which is difficult for a gaseous substrate, but also requires the determination of the initial rates of methane oxidation at that substrate concentration. Thus the plots of Phillips (1970) though typical of competitive inhibition, may not necessarily represent competition between methane and ammonia for the same enzyme binding site. Inhibition of methane oxidation by ammonia may be through competition for the same oxygenase enzyme but the metal-binding properties of ammonia may allow it to complex with a copper component of the methane oxygenase and inhibit methane oxidation in an analogous manner to the other specific inhibitors of methane oxidation. If NH_2OH, by analogy with ammonia oxidation in *Nitrosomonas* (Lees, 1952b), is the immediate product of ammonia oxidation by Methylobacteria then inhibition of methane oxidation may be through the formation of hydroxylamine which

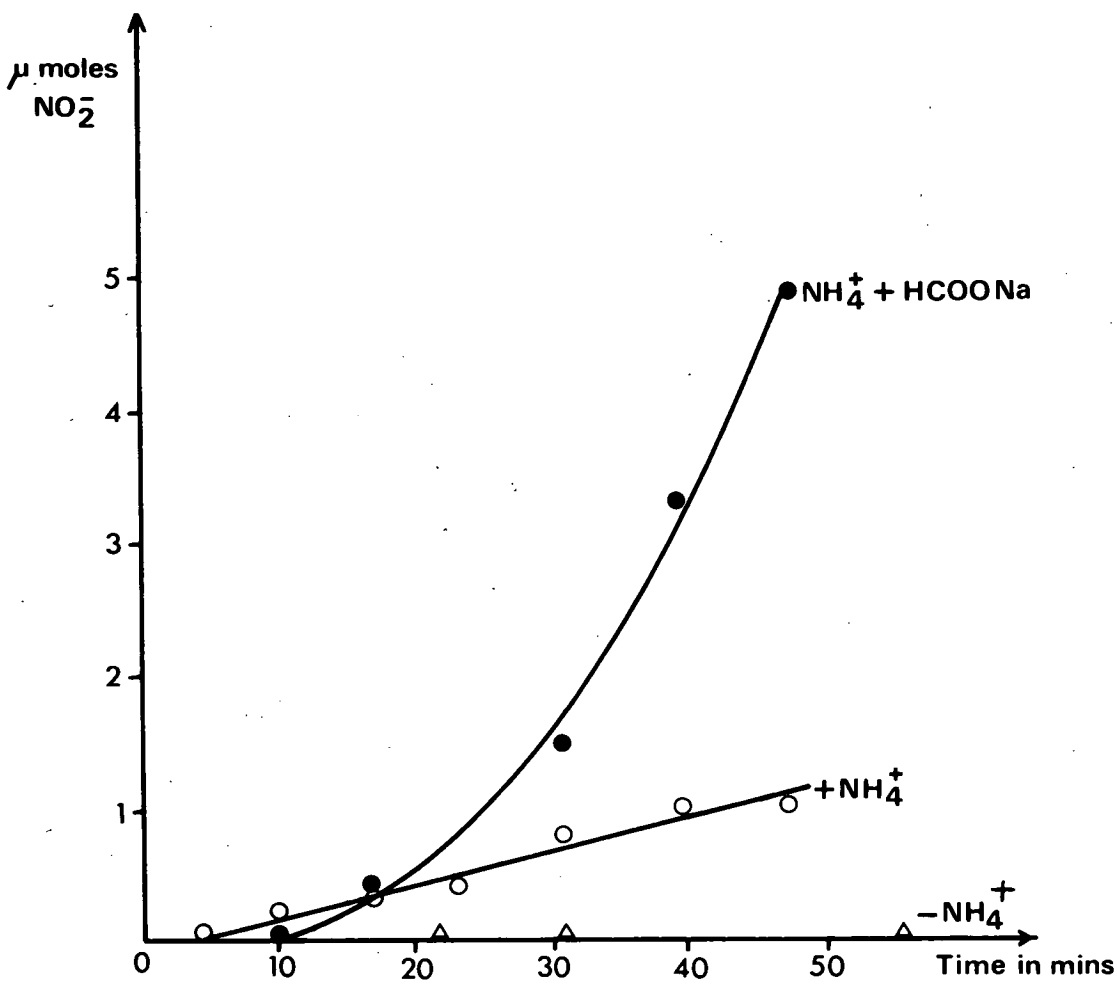
was shown in this study to be a specific inhibitor of methane oxidation. Phillips (1970) had earlier demonstrated that hydroxylamine (0.005%, 1.5×10^{-3}M) inhibited growth of both M. albus and M. trichosporium on methane as carbon source and also inhibited oxidation of methane by cell suspensions of those two methylobacteria but no value was given of the degree of inhibition.

Exploratory experiments were carried out on ammonia oxidation by M. trichosporium. In the determination of the rate of ammonia oxidation by assaying the rate of disappearance of ammonia, complications may arise from the ammonia assimilation enzymes for cellular growth and thus rates of ammonia oxidation are typically determined by taking liquid samples from incubations of cell suspensions with ammonia, centrifuging and performing a nitrite assay on the supernatant (e.g. Hooper and Terry, 1973). This method was used to determine the rate of nitrite formation by washed cell suspensions of M. trichosporium OB3B in the presence of 10mM NH_4Cl and figure 6 shows the linear rate of nitrite formation of 0.4 nmoles NO_2^- /min/mg cells which began after an initial lag of five minutes. There was no nitrite production in the control incubation in the absence of ammonia.

A lag in ammonia respiration, which was more pronounced as cells approached stationary phase, has been observed in Nitrosomonas europeae by Hooper (1969) who demonstrated that the lag could be removed by the addition of hydroxylamine or by pre-incubation and shaking of the cells in dilute suspensions. He interpreted this as indicating that the oxidation of ammonia required a product of the metabolism of hydroxylamine or endogenous metabolism.

Fig. 6: Ammonia-stimulated nitrite production by suspensions of M. trichosporium OB3B and the effect of formate

Nitrite production by standard suspensions (of 47 mg cells in 5 ml pH 6.8 buffer) incubated at 30°C with 50 μmoles ammonium chloride in the presence and absence of 50 μmoles sodium formate. Control incubation of cells with no added substrate.


μ moles
NO_2^-
5
4
3
2
1
0
10
20
30
40
50
Time in mins
$NH_4^+ + HCOONa$
$+NH_4^+$
$-NH_4^+$

The incubation shown in figure 6 had been shaken for 20 minutes at 30°C before the addition of substrate hence the lag could not be ascribed to temperature adjustment or be removed by pre-incubation. The same figure also shows the effect of the addition of formate to the incubation and although the final rate of ammonia-stimulated nitrite production is 600% stimulated at 2.8 nmoles NO_2^- /min/mg cells the lag is still present and is even greater.

The respiration of suspensions of M. albus and M. trichosporium in the presence of NH_4^+ and a range of C_1 and C_2 compounds was investigated and the results are shown in table 17. A marked inhibition of methane-stimulated respiration

Table 17 Effect of C_1 and C_2 Metabolites on Ammonia-stimulated Respiration by Methylobacteria

C_1 and C_2 compound[1]	Respiration Rates[2] (nmoles O_2/min/mg dry wt)			
	M. albus BG8		M. trichosporium OB3B	
	$-NH_4$	$+NH_4^+$	$-NH_4^+$	$+NH_4$
CH_4	33	28	112	58
CH_3OH	30	29	111	85
HCHO	20	21	69	88
HCOONa	1.5	5	15	22.5
C_2H_6	2	1	4	3
C_2H_5OH	21	21	69	72
CH_3CHO	2	2	14	10

(1) Substrate Concentrations: CH_4 and C_2H_6 added as 0.6ml of gas-saturated buffer, NH_4Cl added to a final concentration of 10mM and all other substrates added as 0.1ml of a 100mM solution.

(2) Endogenous respiration rates were 1 and 1 nmoles O_2/min/mg dry wt for M. albus BG8 and M. trichosporium OB3B respectively which were increased to 2 and 4 nmoles O_2/min/mg dry wt by the addition of 10mM NH_4Cl.

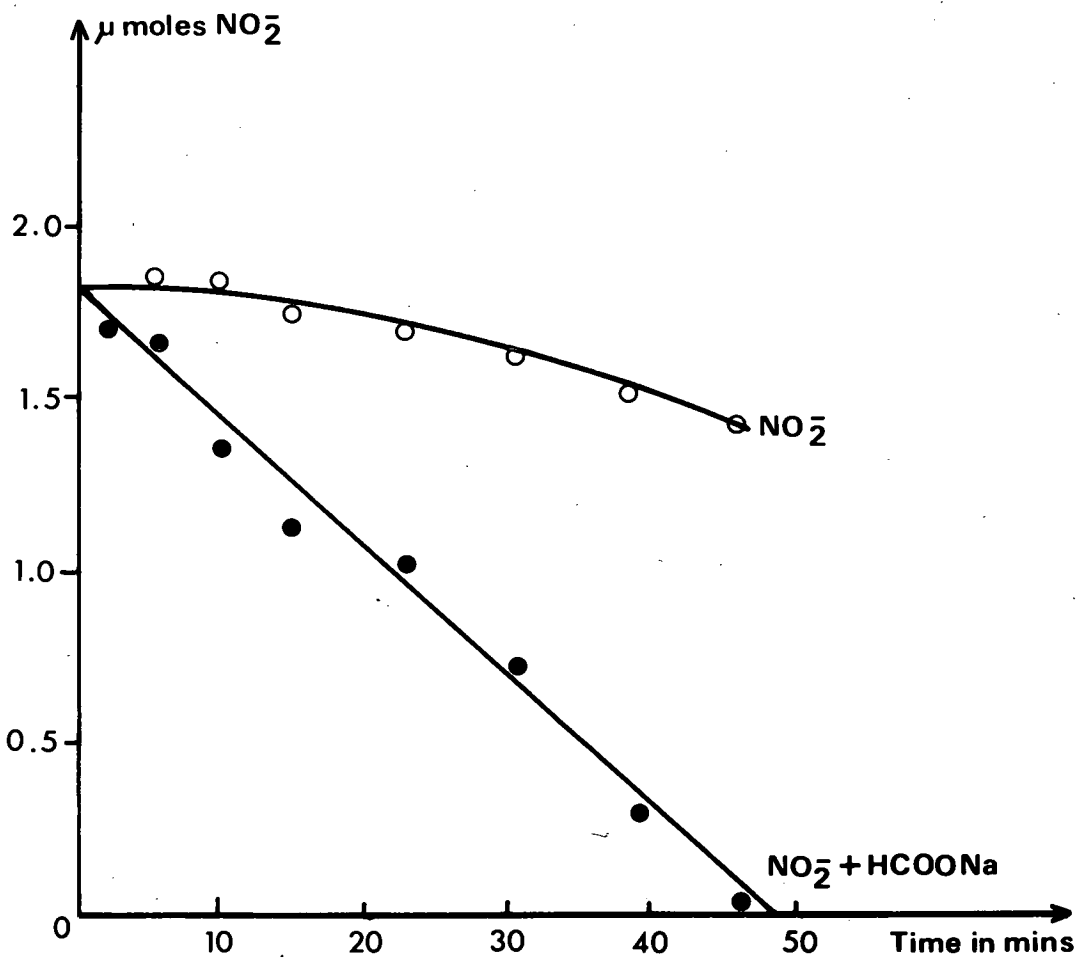
by 10mM NH_4Cl was only apparent with M. trichosporium OB3B. Ammonia-stimulated respiration was higher in the case of M. trichosporium OB3B which may be a reflection of the higher rates of methane respiration of this organism. Consistent with the stimulation in ammonia oxidation by formate in figure 6, respiration of both methylobacteria were considerably enhanced by combinations of formate and ammonia above the sum of the two individual respirations and a similar effect was found, in M. trichosporium only, for the combination of HCHO and ammonia. No lags in ammonia respiration were apparent in the oxygen probe studies which were carried out over time spans of ten minutes.

The washed cell suspension of methylobacteria routinely used in this study were prepared from cells grown on a nitrate mineral salts medium (N.M.S.) medium and the possible effect of the nitrate assimilation enzymes on the uptake of nitrite was investigated by incubating suspensions of M. trichosporium OB3B with 1mM $NaNO_2$ in the presence and absence of formate and the results shown in figure 7. Nitrite was found to disappear from the suspensions at a rate of 0.19 nmole NO_2^-/min/mg cells with no lag and the disappearance was 305% stimulated by formate to give a final rate of 0.77 nmoles NO_2^-/min/mg cells. Although analysis of supernates of the suspensions revealed no ammonia, the lack of stimulation of respiration when nitrite was added to cell suspensions respiring formate in the oxygen probe suggests that the nitrite disappearance was due to reductive assimilation.

The contradiction between the lag in ammonia-stimulated nitrite production and the absence of a lag in the ammonia-

Fig. 7: Disappearance of nitrite from suspensions of Methylosinus trichosporium OB3B and the effect of formate

Standard suspensions of 47 mg cells in 5 ml pH 6.8 buffer incubated at 30°C with 2.5 μmoles sodium nitrite in the presence (●) and absence of 50 μmoles sodium formate (○).


μ moles NO_2^-
2.0
1.5
1.0
0.5
0
10
20
30
40
50
Time in mins
NO_2^-
NO_2^- + HCOONa

stimulated respirations described in table 17 together with the nitrite disappearance found in suspensions of NMS-grown cells suggested that the apparent lag in ammonia oxidation was due to interference from the nitrate assimilation enzymes. Washed cell suspensions were then prepared from cells grown on an Ammonia Mineral Salts Medium (A.M.S.) and the ammonia-stimulated nitrite production and nitrite disappearance in the presence and absence of formate were followed and the results together with the control incubations in the presence and absence of formate are shown in figure 8. No nitrite disappearance was observed in the presence and absence of formate and nor was any lag observed in ammonia-stimulated nitrite production. The rates of nitrite formation in the presence and absence of formate were 4.3 and 9.4 nmoles NO_2^-/min/mg cells. The lag in ammonia-stimulated nitrite production by N.M.S.-grown M. trichosporium OB3B can thus be ascribed to the uptake of nitrite by the inducible nitrate assimilation enzymes.

The production of nitrite by suspensions of A.M.S.-grown M. trichosporium OB3B incubated with ammonia and hydroxylamine is shown in figure 9 and the rate of hydroxylamine-stimulated nitrite production was 6.8 compared with 2.6 nmoles NO_2^-/min/mg for the ammonia-stimulated rate. Although hydroxylamine-stimulated nitrite production was only 48% inhibited by CO (75% O_2, 25% CO) the ammonia-stimulated nitrite production was completely inhibited.

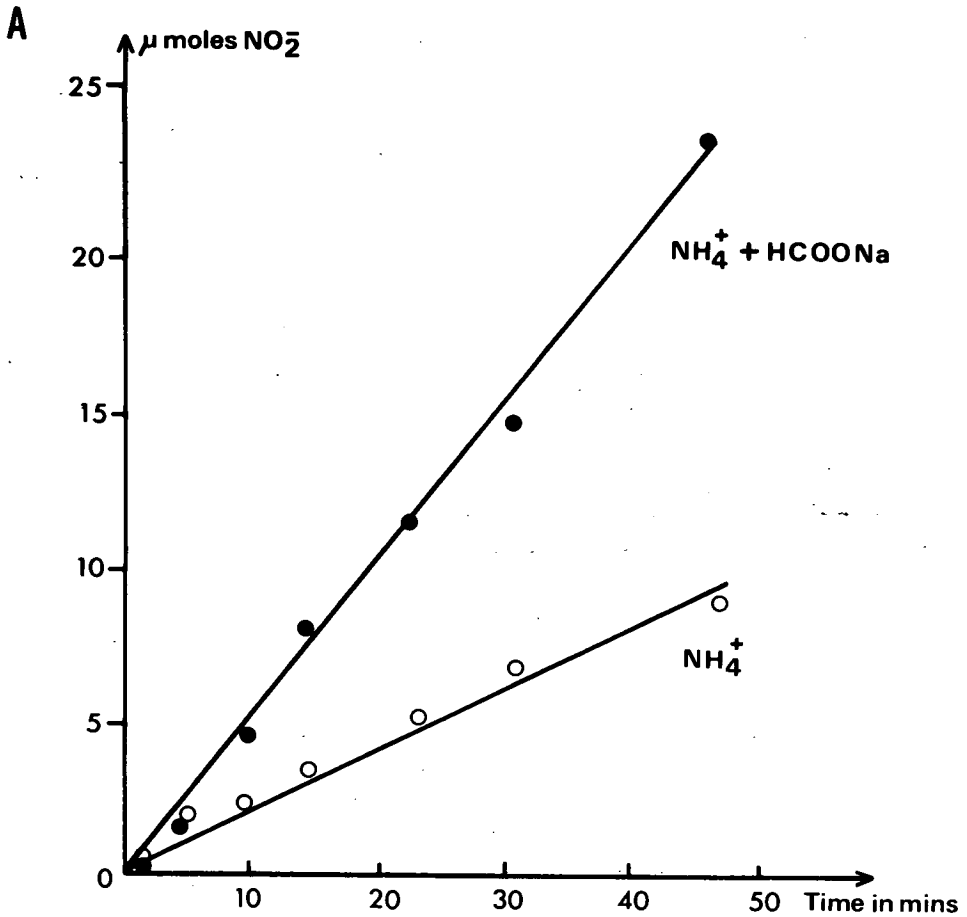
If ammonia is oxidised by the methane oxygenase as suggested by Whittenbury et al. (1970) the specific inhibitors of methane oxidation described in section would be expected to inhibit ammonia-stimulated nitrite production.

Fig. 8: Ammonia-stimulated nitrite production and nitrite disappearance in suspensions of M. trichosporium and the effect of formate

A) Ammonia-stimulated nitrite formation in the presence (●) and absence (○) of formate.

B) Nitrite disappearance in the presence (▲) and absence (△) of formate and control incubations in presence (■) and absence (□) of formate.

Standard suspensions (of 47 mg cells in 5 ml pH 6.8 buffer incubated at 30°C) with 50 μmoles NH_4Cl or 10 μmoles $NaNO_2$ in the presence and absence of 100 μmoles sodium formate. Control incubations of standard suspensions in the presence and absence of 100 μmoles sodium formate.


A
μ moles NO_2^-
25
20
15
10
5
0
NH_4^+ + HCOONa
NH_4^+
10
20
30
40
50
Time in mins

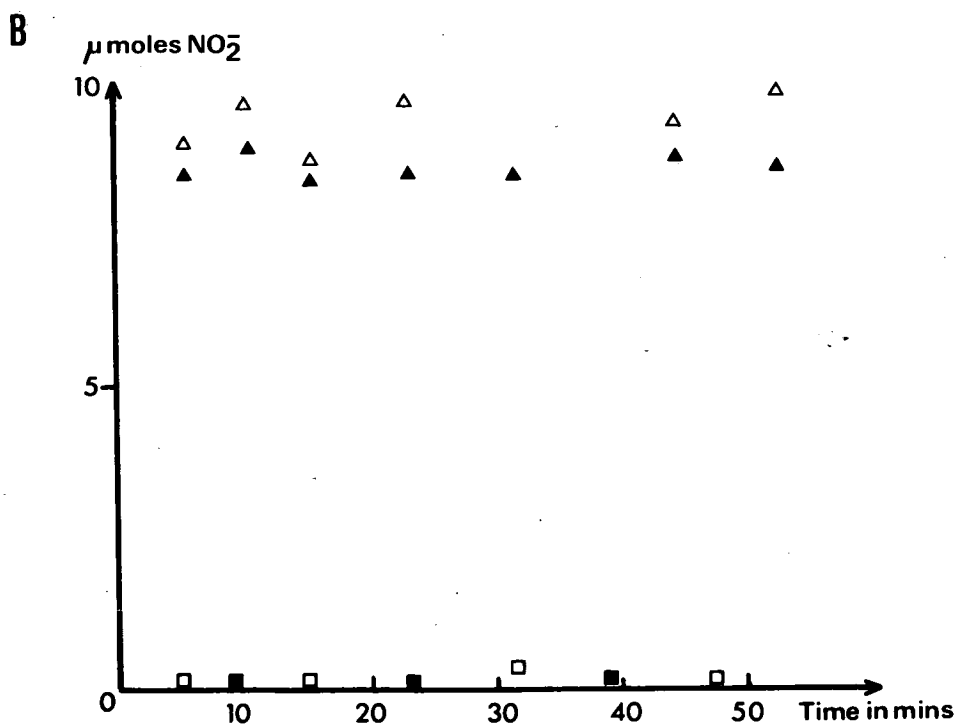
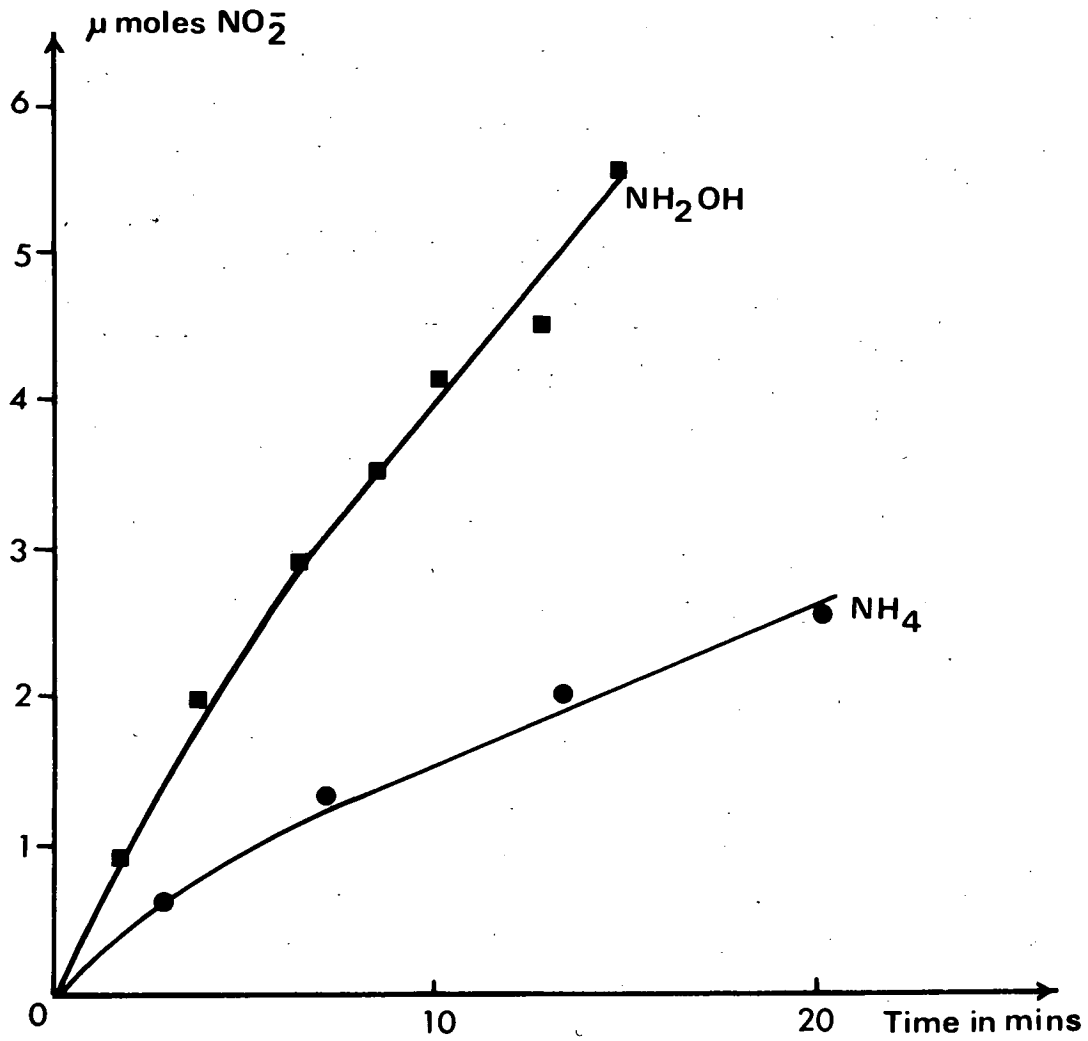

B
μ moles NO_2^-
10
5
0
10
20
30
40
50
Time in mins

Fig. 9: Hydroxylamine and ammonia-stimulated nitrite production by suspensions of A.M.S.-grown Methylosinus trichosporium OB3B.

Nitrite production by standard suspensions of 47 mg cells in 5 ml pH 6.8 buffer incubated at 30°C with 50 μmoles hydroxylamine hydrochloride (■) and 50 μmoles ammonium chloride (●).


μ moles NO_2^-
6
5
4
3
2
1
0
10
20
Time in mins
NH_2OH
NH_4

In view of the problems that arose over the inhibition of ethane oxidation by dimethyl ether, inhibition studies were carried out on incubations of cell suspensions in the presence of formate. The effect of a range of specific inhibitors of methane oxidation on M. trichosporium is shown in table 18 and it is evident that ammonia-stimulated nitrite production in the presence of formate was inhibited by all of the compounds tested. The effect of these compounds on hydroxylamine-stimulated nitrite production is also shown in table 18 and ammonia-stimulated nitrite production was inhibited by all of these compounds

Table 18 Effect of Specific Inhibitors of Methane Oxidation on Ammonia and Hydroxylamine-stimulated Nitrite Production by M. trichosporium OB3B

Compound	Concentration (M)	Rate of nitrite production (% control[3]) ammonia[1]	hydroxylamine[2]
8-quinolinol	10^{-4}	11	22
aa'dipyridyl	10^{-4}	24	81
allyl thiourea	10^{-4}	16	100
thiosemicarbazide	10^{-4}	19	100
diethyldithiocarbamate	7.5×10^{-4}	0	11
N-SERVE	0.67×10^{-4}	26	100

(1) 50 μmoles NH_4Cl and 100 μmoles HCOONa in 5 ml suspension of pH 6.8 buffer.

(2) 50 μmoles $NH_2OH.HCl$ in 5 ml suspension of pH 6.8 buffer.

(3) Control rates of ammonia and formate-stimulated nitrite production and hydroxylamine-stimulated nitrite production were 7.5 and 10 nmoles NO_2^-/min/mg dry wt cells.

more than the hydroxylamine-stimulated nitrite production although the latter reaction was also inhibited by 8-quinolinol and diethyldithiocarbamate. If the production of nitrite from ammonia is assumed analogous

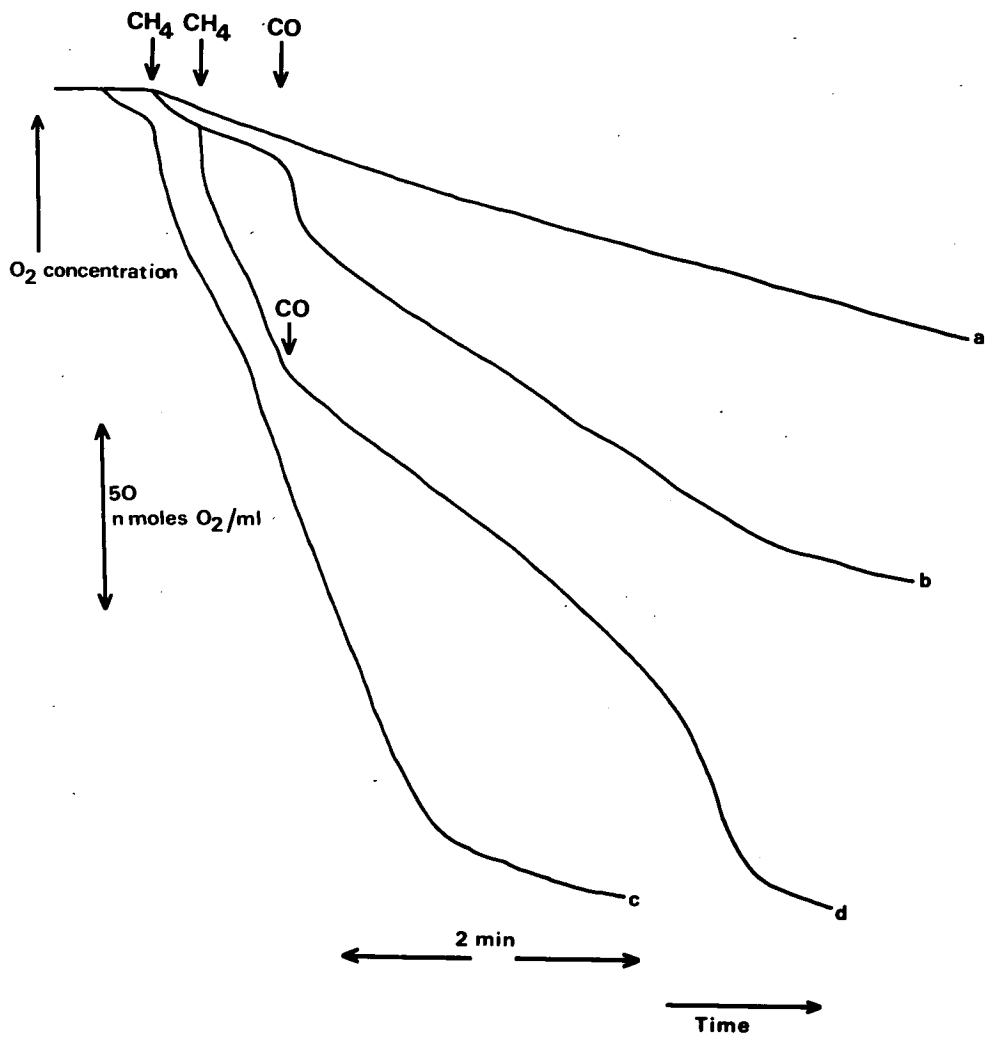
to the same process in Nitrosomonas europaea, hydroxylamine would be the product of the initial oxygenation of ammonia and it is the subsequent metabolism of hydroxylamine, a process poorly understood in Nitrosomonas (Aleem, 1970), which generates nitrite. Thus a number of the specific inhibitors of methane oxidation are also likely to be specific inhibitors of ammonia oxidation in M. trichosporium OB3B, lending support to the suggestion by Whittenbury et al. (1970) and Phillips (1970) of a common oxygenase enzyme system. A detailed study of the mechanism of ammonia oxidation in methylobacteria was beyond the scope of the present investigation but the importance of nitrification in nature would make such a study worthwhile.

CARBON MONOXIDE OXIDATION BY METHYLOBACTERIA

In the course of a study of the inhibition of methane respiration by carbon monoxide (CO) it was observed that in addition to inhibiting methane respiration of M. trichosporium OB3B, carbon monoxide itself stimulated respiration as can be seen from the superimposed oxygen probe traces in figure 10. The endogenous respiration rate, the respiration rates in the presence of carbon monoxide-saturated buffer, methane and both methane and carbon monoxide were 6, 12, 53 and 16 nmoles O_2/minute/mg dry cell weight respectively. The two-fold stimulation of endogenous respiration only lasted for three minutes and in the presence of carbon monoxide and methane the initial inhibited respiration lasted for a similar period as that with carbon monoxide alone before reverting to a rate identical to the uninhibited rate for methane alone. The sharp decrease in oxygen content on addition of carbon

Fig. 10: Respiration of M. trichosporium OB3B in the presence of carbon monoxide and methane

Respiration at 30°C determined in oxygen probe of 5mg cells (a) endogenously in the absence of added substrated (b) with 0.3 ml carbon monoxide-saturated pH 6.8 buffer, (c) with 0,3 ml methane-saturated buffer and (d) with 0.3 ml carbon monoxide and CH_4 saturated buffer. The total volume of the reaction chamber was 2.7 ml.


CH4 CH4 CO
O2 concentration
CO
50
n moles O2/ml
a
b
c
d
2 min
Time

monoxide and methane are merely due to a dilution of the oxygen-saturated buffer in the chamber.

This simple experiment suggested that during the period of inhibition of methane respiration by carbon monoxide the observed respiration was due to oxidation of carbon monoxide and that methane-stimulated respiration only started up again when all of the carbon monoxide was removed.

A survey was then carried out on the ability of washed cell suspensions of a range of membrane-type I and II methylobacteria to carry out carbon monoxide-stimulated respiration and the endogenous, carbon monoxide- and methane-stimulated respiration for a range of methylobacteria are given in table 19. Carbon monoxide-stimulated respiration

Table 19 A Survey of Carbon Monoxide-Stimulated Respiration in Methylobacteria.

Organism	Respiration Rates (nmoles O_2/min/mg dry wt)		
	Endogenous	CH_4	CO
Type I			
Methylomonas albus BG8	1	31	1
Methylomonas agile	0.5	83	0
Pseudomonas methanica	0	80	0
Type II			
Methylosinus trichosporium OB3B	3	53	3
" " OB4	1	112	5
" " PG	4	140	4.5
Methylocystis parvus OBBP	0	114	4
Methylosinus trichosporium 12	6	163	6.5

can be seen to be a property of all membrane-type II methylobacteria examined but only *M. albus* BG8 of the three membrane-type I organisms examined. Methane-stimulated

respiration rates were generally higher with membrane-type II methylotrophs but the ability of M. albus BG8 to carry out CO-stimulated respiration when a suspension of Pseudomonas methanica with a higher rate of methane respiration showed no methane-stimulated respiration suggests that methane and carbon monoxide-stimulated respiration are not necessarily correlated.

Although considerable confusion exists in the early literature, the biological oxidation of carbon monoxide is now a well-established phenomenon and the literature has been discussed fully in two reviews (Chapelle, 1962b; Fenn, 1970) and mentioned briefly in a review by Quayle (1972). The organisms that metabolise carbon monoxide fell into two categories: Gram-negative bacteria which utilise carbon monoxide as sole carbon source and which may also utilise hydrogen (Kistner, 1953; Kistner, 1954; Collins, 1969; Savelieva and Nozhesnikova, 1972; Nozhesnikova and Savelieva, 1972) and a much larger group of organisms which cannot utilise carbon monoxide as sole carbon source but can oxidise carbon monoxide to carbon dioxide and in the process may be able to incorporate some of the evolved carbon dioxide into cell material. The latter category includes groups as diverse as plant leaves (Bidwell and Fraser, 1972; Bidwell and Bebee, 1974), green algae (Chapelle, 1962a), animal tissues (Fenn and Cobb, 1932), photosynthetic bacteria (Hirsch, 1968), Desulphovibrio (Yagi and Tamiya, 1962; Postgate, 1970), Clostridium (Fuchs et al. 1974), methanogenic bacteria (Kluyver and Schnellen, 1947), soil fungi (Inman and Ingersoll, 1971). Even such an ubiquitous enzyme to life as

cytochrome oxidase has been shown to oxidise carbon monoxide (Breckenridge, 1953; Tzagoloff and Wharton, 1965). It is thus likely that carbon monoxide oxidation is a property of all respiring plant and animal tissues. The failure of all attempts to grow methylobacteria with carbon monoxide as carbon source places carbon monoxide oxidation by methylobacteria in the second category but the higher rates of carbon monoxide-stimulated respiration of 3 - 6 nmoles O_2/min/mg dry cell wt. than the carbon monoxide 'burning' rates compiled from the literature by Fenn (1970), which do not exceed 0.3 nmoles CO/min/mg dry wt., suggests that carbon monoxide oxidation in methylobacteria may have some special significance.

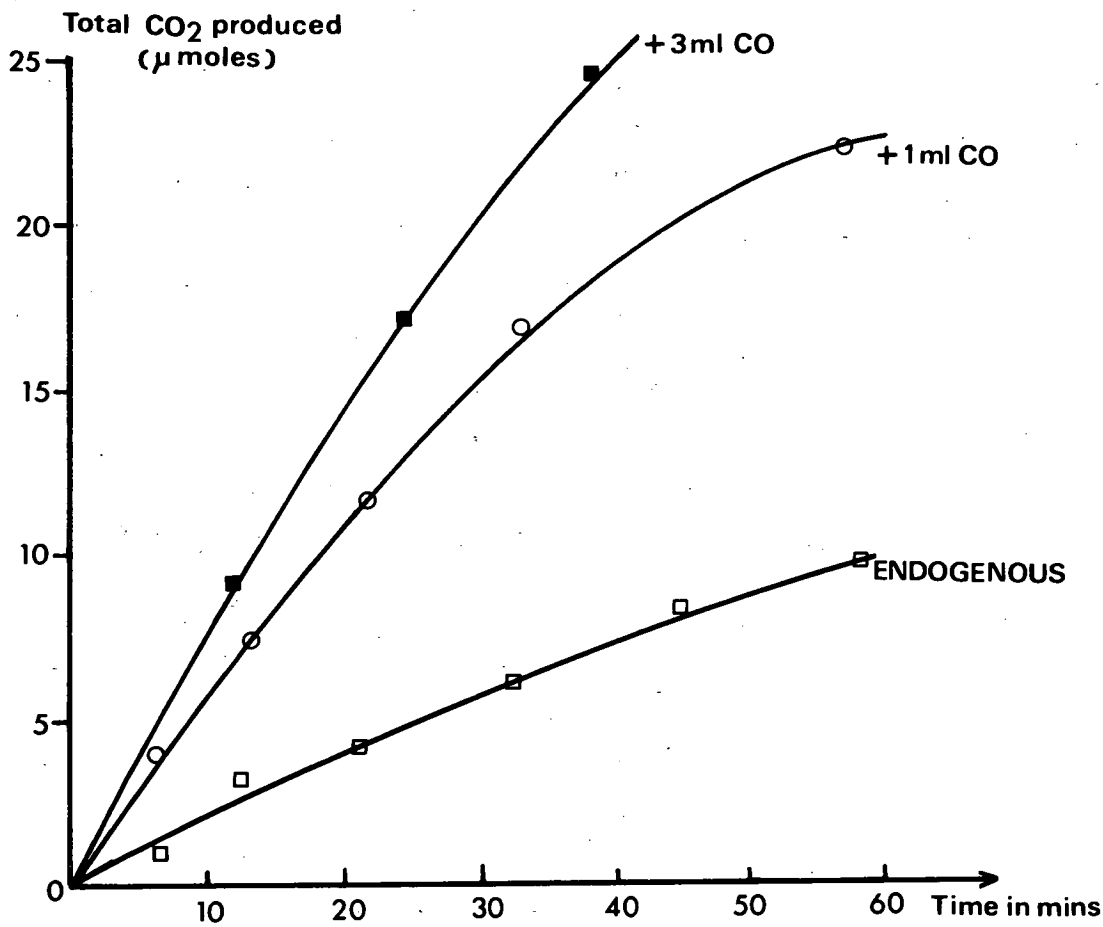
Independent of this study it was shown that respiration of washed cell suspensions of M. trichosporium OB3B was stimulated by carbon monoxide and that respiration of cell-free extracts of Pseudomonas methanica was stimulated by both methane and carbon monoxide and that this stimulation was dependent on the provision of NADH (Ferenci, 1974). Despite the conclusion of Ferenci (1974) that the methane oxygenase was capable of the oxidation of carbon monoxide his study only showed stimulation of respiration and NADH oxidation by carbon monoxide and did not establish the product of carbon monoxide oxidation or offer any proof that carbon monoxide was actually oxidised.

The immediate product of carbon monoxide oxidation, where investigated, has been shown to be carbon dioxide (see Chapelle, 1962b) and the addition of carbon monoxide to incubations of suspensions of both M. albus and M. trichosporium

OB3B resulted in a stimulation of the endogenous carbon dioxide production which was followed by gas chromatography. Figure 11 shows a time course plot of carbon dioxide production by a suspension of M. trichosporium OB3B in the endogenous incubation and in the presence of 1 and 3 mls of CO. Over the initial 30 minutes rates of carbon dioxide production were approximately linear and were 4, 11.5 and 14 nmoles CO_2/min/mg dry wt giving a rate of 'excess CO_2' production of 6.5 and 10 nmoles CO_2/min/mg dry cell wt in the presence of 1 and 3 mls CO respectively. The latter figure was typical although there was some slight variation with each batch and over 5 experiments the figure varied between 6 and 10 nmoles CO_2/min/mg dry cell wt. Similar plots could be obtained for the membrane type I methylotroph M. albus BG8 and the rates of carbon monoxide-stimulated carbon dioxide production were generally lower, falling into the range 3 to 6 nmoles O_2/min/mg dry wt. The stimulation of carbon dioxide production in the presence of carbon monoxide was completely abolished by boiling the cell suspensions for five minutes or incubating the cells with CO anaerobically, indicating that the CO-stimulated CO_2 production was dependent on a biological process and required molecular oxygen. Attempts to obtain stimulation of CO_2 production by CO from cell-free extracts of M. trichosporium OB3B using either the method of Yagi and Tamiya (1962) for Desulphovibrio and Thauer et al. (1974) for Clostridium pasteurianum, which both used methyl viologen as electron acceptor or the method of Ferenci (1974) for Pseudomonas methanica using NADH as electron donor were all unsuccessful.

Fig. 11: Production of carbon dioxide by suspensions of M. trichosporium incubated in the presence of carbon monoxide

Standard suspension (of 47mg cells in 5ml pH 6.8 buffer) incubated at 30°C endogenously in the absence of substrate (□) and in the presence of 1ml (○) and 3ml (■) carbon monoxide.


Total CO_2 produced
(μ moles)
25
20
15
10
5
0
+3ml CO
+1ml CO
ENDOGENOUS
10
20
30
40
50
60
Time in mins

The experiments which demonstrated carbon monoxide-stimulated respiration and carbon dioxide production were all carried out with washed cell suspensions and these activities could be properties of non-proliferating cells, thus before any significance could be ascribed to this process in Methylobacteria it was necessary to establish whether it took place in growing cells. Growth of methylobacteria on methane is inhibited by carbon monoxide hence methanol was used as carbon source. Table 20 shows the carbon dioxide produced and cell yield of M. albus BG8 grown in the presence and absence of 20 ml carbon monoxide and the carbon dioxide produced by each set of cultures on exhaustion of methanol which was verified by gas chromatography. There was a slight inhibition of yield in the presence of carbon monoxide but a considerable enhancement of carbon dioxide production and oxygen uptake. The increase in carbon dioxide production in the presence of carbon monoxide is equivalent to 9 mg of carbon and cannot be explained away in terms of an increased carbon dioxide production from methanol due to a decrease in yield.

Table 20 Carbon dioxide production and yields of M.albus BG8 Grown in the presence and absence of Carbon Monoxide

Growth Conditions	O_2 consumed (μmoles)	CO_2 produced (μmoles)	Yield (mg dry wt) cells
CH_3OH[1]	980	570	16
CH_3OH + CO (20ml)	1490	1330	15

1 0.5% v/v CH_3OH in 20 ml Nitrate mineral salts medium.

The gas chromatography system routinely used for assaying methane, carbon dioxide and oxygen did not resolve carbon monoxide from oxygen and carbon monoxide appeared as a shoulder

on the oxygen peak. Although a decrease in the height of this shoulder during the course of incubations of cell suspensions in the presence of carbon monoxide indicated that carbon monoxide was indeed disappearing, more satisfactory evidence came from the demonstration of the conversion of ^{14}CO to $^{14}CO_2$ by suspensions of M. albus BG8 and M. trichosporium OB3B usin a method which involved incubating cell suspensions with ^{14}CO and the estimation of the evolved $^{14}CO_2$ by the injection of gas samples into the gas chromatograph, thus separating the gases present, trapping the CO_2 by sparging the effluent from the column through hyamine hydroxide, and counting the dissolved $^{14}CO_2$ in a scintillation counter. The results of two trapping experiments are shown in table 21 and it can be seen that the specific activities of the 'excess CO_2', where 'excess' was the amount of evolved CO_2 over and above the endogenous level of CO_2 in the control, were of the same magnitude as the specific activity of the carbon monoxide source which would be expected if the 'excess CO_2' was derived from carbon monoxide. There is close agreement between the specific activities in the case of M. albus BG8 but some discrepancies with M. trichosporium OB3B and the possible significance of the low specific activity for the 'excess' carbon dioxide in terms of a stimulation of endogenous carbon dioxide production in addition to oxidation of carbon monoxide will be discussed in a later section of this study.

The above results unambiguously confirm that carbon monoxide is oxidised to carbon dioxide by M. albus BG8 and M. trichosporium OB3B making it highly likely that the carbon

Table 21 The activities of $^{14}CO_2$ trapped in hyamine hydroxide and the specific activities of the excess CO_2

	Time minutes	CO_2 in endogenous control (μmole)	CO_2 in flask with CO (μmole)	Specific activity of excess CO_2 counts/min/μmole x 10^5
Methylomonas albus (BG8)	57	8	14	2.6
	133	16	26	2.1
	433	43	63	2.6
Methylosinus trichosporium (OB3B)	61	7	17	1.2
	122	14	29	2.3
	187	18	63	3.5

Specific activity of CO source = 2 x 10^5 counts/min/μmole CO

monoxide-stimulated respiration found in other methylobacteria was also due to the oxidation of carbon monoxide.

The trapping method described above was inconvenient for repeated sampling with time and an alternative method was devised whereby gas samples from incubations of cell suspensions with ^{14}CO were injected directly into sealed counting vials containing hyamine hydroxide into which the $^{14}CO_2$ dissolved and could be counted on addition of scintillant. The linear rate of $^{14}CO_2$ production from such an experiment with M. trichosporium OB3B is shown in fig 12 and from knowing the specific activity of the ^{14}CO source a rate of CO oxidation of 8 nmoles CO/min/mg dry cell wt was readily deduced. In the same figure a plot of $^{14}CO_2$ production in the presence of NH_2OH ($10^{-3}M$) is given showing that carbon monoxide oxidation is 88% inhibited by NH_2OH which, in addition to being a selective inhibitor of methane oxidation, has been shown to inhibit the oxidation of carbon monoxide in green algae (Chapelle, 1962a) and purified cytochrome oxidase (Breckenridge, 1953).

Effect of Specific Inhibitors of Methane oxidation on the Carbon-Monoxide-stimulated Respiration by M. trichosporium OB3B

The effect of a range of specific inhibitors on the rate of carbon monoxide-stimulated respiration by M. trichosporium OB3B is shown in table 22 and it is evident that this reaction is inhibited by the specific inhibitors of methane oxidation.

The inhibition of carbon monoxide oxidation in the green algae by 8-quinolinol ($5 \times 10^{-3}M$) and hydroxylamine ($10^{-4}M$) has been reported by Chapelle (1962a) and in purified cytochrome oxidase by hydroxylamine at $10^{-4}M$ (Breckenridge, 1953).

Fig. 12: Production of $^{14}CO_2$ by M. trichosporium incubated with ^{14}CO

Standard suspension (of 47 mg cells in 5 mls pH 6.8 buffer) incubated at 30°C with 3 mls ^{14}CO (890 counts/minute/μmole CO) in the presence (⊙) and absence (●) of hydroxylamine hydrochloride to final concentration 10^{-3}M.

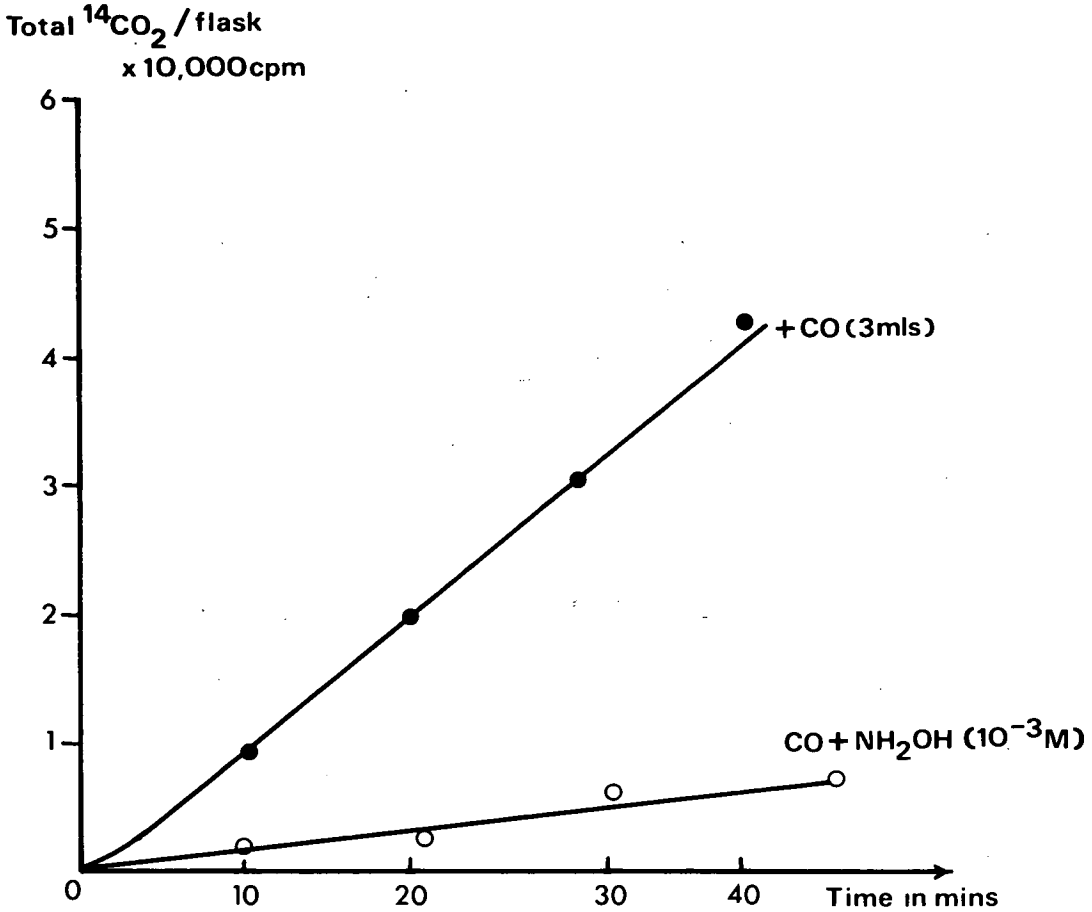

Total $^{14}CO_2$ / flask
x 10,000cpm
6
5
4
3
2
1
0
10
20
30
40
Time in mins
+CO (3mls)
CO + NH_2OH (10^{-3}M)

Table 22 Effect of Specific Inhibitors of Methane Oxidation on Carbon Monoxide-stimulated Respiration by Methylosinus trichosporium OB3B

Compound	Concentration (M)	Rate CO stimulated respiration (% Control)[1]
8-quinolinol	10^{-4}	40
o-phenanthroline	5×10^{-5}	0
αα'dipyridyl	10^{-4}	9
Thioacetamide	10^{-3}	0
diethyl dithiocarbamate	7.5×10^{-4}	0
Thiourea	10^{-4}	0
Allyl thiourea	10^{-4}	0
NH_2OH	10^{-3}	0
Pyridine	10^{-3}	70
3-amino triazole	10^{-2}	80
Thiosemicarbazide	10^{-4}	0

(1) Control rates of carbon monoxide-stimulated respiration were 5 nmoles O_2/min/mg dry cell wt.

The inhibition by the monodentate nitrogen ligands such as 3-amino triazole and pyridine was slight but the marked inhibition by the rest of the metal ion chelators show a close association between the methane oxygenase and the oxidation of carbon monoxide.

The interaction of carbon monoxide and endogenous metabolism

The effect of carbon monoxide on endogenous metabolism was investigated by the converse of the ^{14}CO studies recorded in table 21 and figure 12. ^{14}C-labelled cell suspensions were prepared from exponential phase cultures of M. albus BG8 and M. trichosporium OB3B grown in the presence of $^{14}CH_4$ and incubations of these labelled cells were set up in sealed flasks in the presence and absence of 'cold' carbon monoxide.

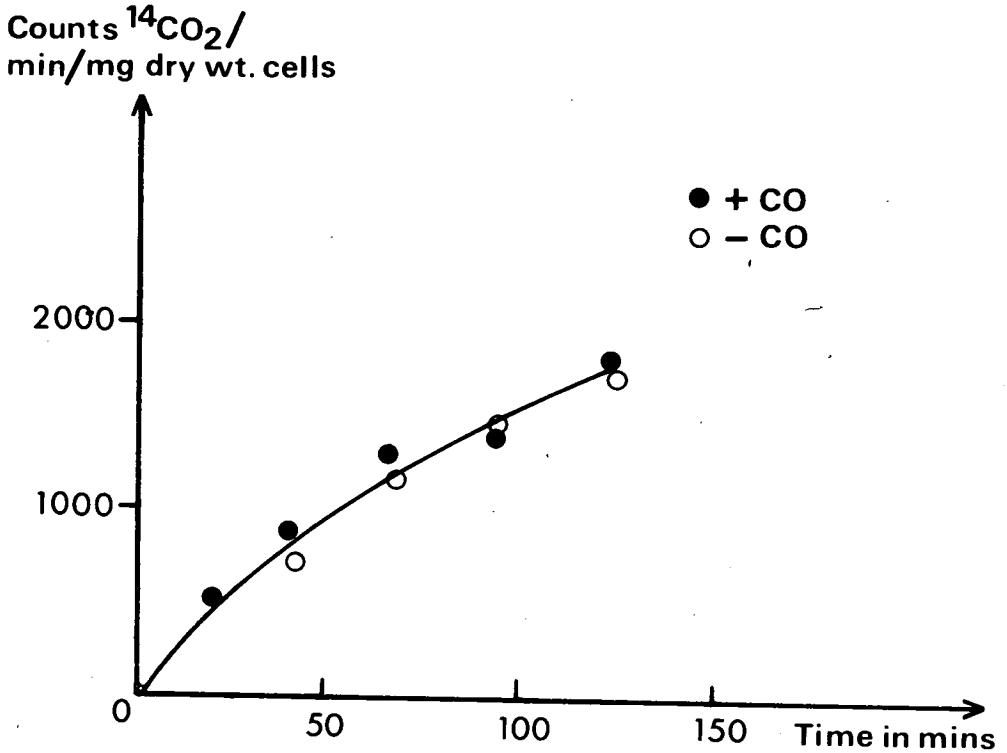
The total carbon dioxide production in each flask was monitored over a two hour period by gas chromatography and the $^{14}CO_2$ production was followed by injection gas samples into sealed vials containing hyamine hydroxide. The results for M. albus BG8 are given in fig. 13 and for M. trichosporium OB3B in fig. 14. Although carbon dioxide production by M. albus BG8 was stimulated in the presence of carbon monoxide the rate of $^{14}CO_2$ production did not increase indicating that all of the increased carbon dioxide production was due to the oxidation of carbon monoxide. The same experiment with ^{14}C-labelled M. trichosporium OB3B revealed a different picture: $^{14}CO_2$ production was stimulated in the presence of carbon monoxide but not enough to account for all the stimulation of carbon dioxide production. The stimulation began after a lag of 10 minutes.

A stimulation of endogenous carbon dioxide production would affect the calculation of 'specific radioactivity of excess CO_2' shown in table 21 and may provide an explanation for the low figure for this compared with the ^{14}CO source in the incubation of M. trichosporium OB3B sampled after one hour.

The difference between M. albus BG8 and M. trichosporium OB3B in the ability of carbon monoxide to stimulate endogenous metabolism may provide an explanation for the greater ability of M. trichosporium OB3B to oxidise carbon monoxide. The contradiction between the inability of cell suspensions of Pseudomonas methanica, a membrane type I methylotroph, to carry out carbon monoxide-stimulated respiration and the NADH dependent carbon monoxide-stimulated respiration found in cell-free extracts of the same organisms led Ferenci (1974) to

Fig. 13: Effect of carbon monoxide on the endogenous $^{14}CO_2$ evolution by suspensions of M. albus BG8

$^{14}CO_2$ and total CO_2 evolved from suspensions of 26mg ^{14}C-labelled cells in 5ml pH 6.8 buffer incubated at 30°C in the presence (●) and absence (O) of 3mls carbon monoxide.


Counts $^{14}CO_2$/
min/mg dry wt. cells
● + CO
○ – CO
2000
1000
0
50
100
150
Time in mins

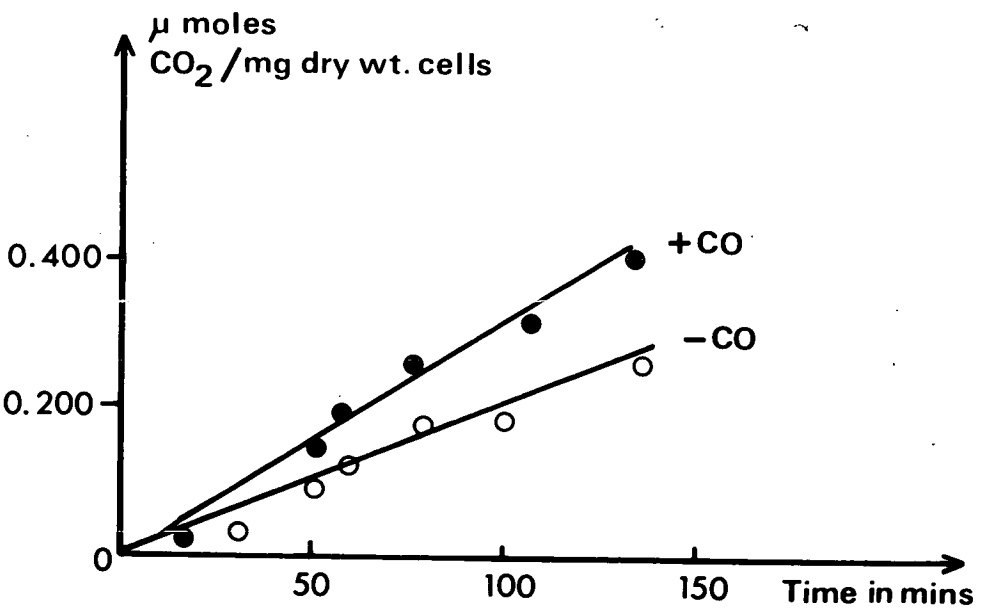
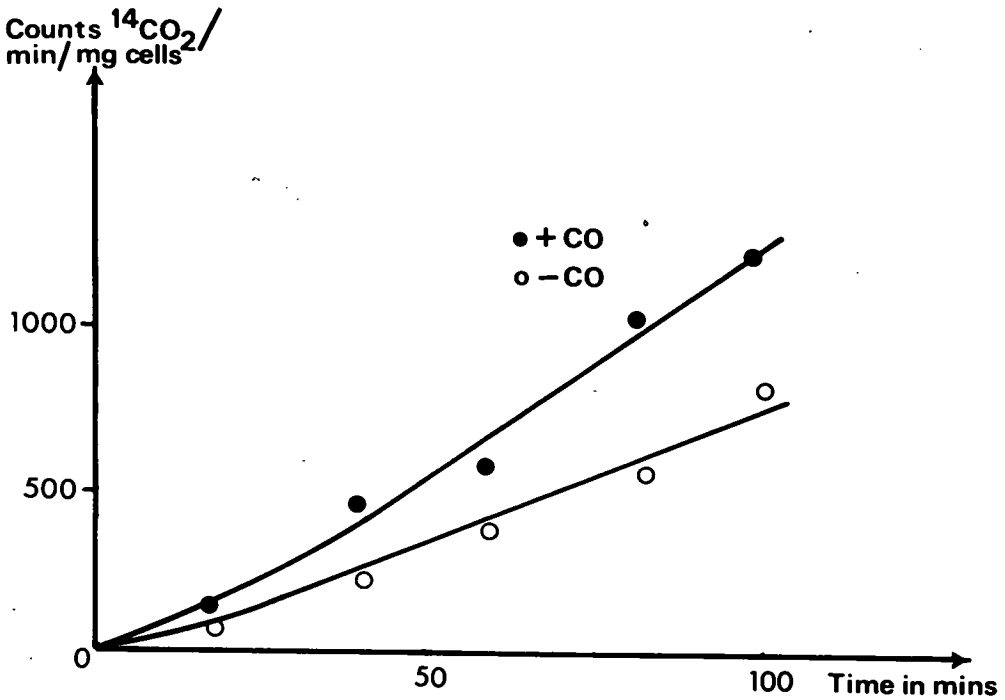
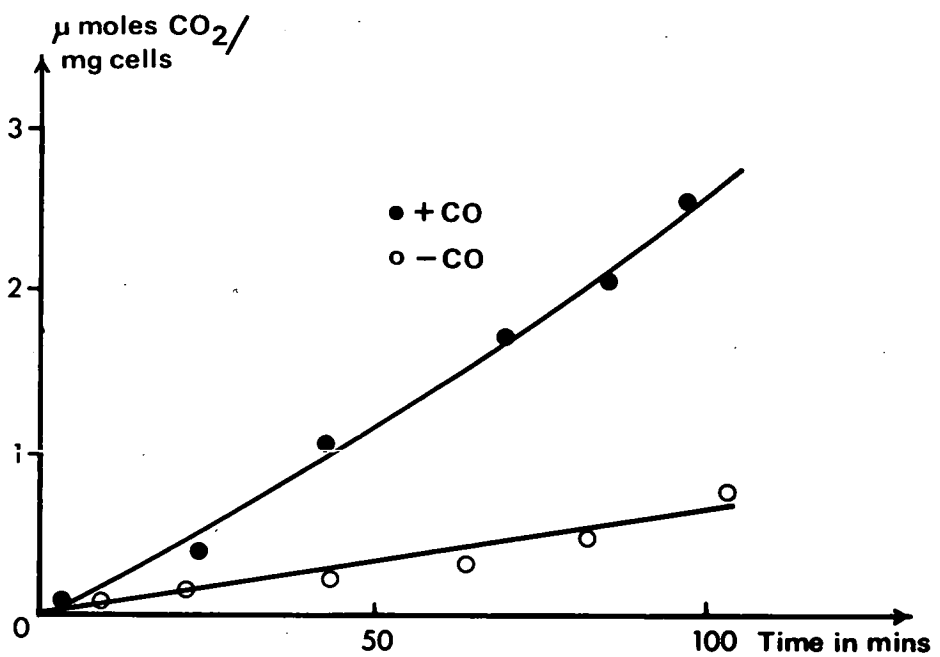

μ moles
CO_2 /mg dry wt. cells
0.400
0.200
0
+CO
–CO
50
100
150
Time in mins

Fig. 14: Effect of carbon monoxide on the endogenous $^{14}CO_2$ evolution by suspensions of M. trichosporium OB3B

$^{14}CO_2$ and total CO_2 evolved from suspensions of 47 mg ^{14}C-labelled cells in 5 ml pH 6.8 buffer incubated at 30°C in the presence (⊗) and absence (O) of 3 mls carbon monoxide.


Counts $^{14}CO_2$/ min/ mg cells
1000
500
0
● + CO
o − CO
50
100
Time in mins


μ moles CO_2/ mg cells
3
2
1
0
● + CO
o − CO
50
100
Time in mins

suggest that the explanation for the ability of suspensions of *M. trichosporium* OB3B, but not *Pseudomonas methanica*, lay in the ability of *M. trichosporium* OB3B to mobilise endogenous reserves of reducing power for the postulated mono-oxygenase reaction of carbon monoxide oxidation and in support of this he demonstrated with *Ps. methanica* an apparent stimulation of formate and formaldehyde respiration on addition of carbon monoxide-saturated buffer to the oxygen probe chamber. That this stimulation was due to carbon monoxide was inferred by the demonstration that the rate of formaldehyde disappearance remained constant during the period of stimulated respiration. He thus concluded that like *M. trichosporium* OB3B, *Pseudomonas methanica* was able to carry out carbon monoxide-stimulated respiration provided a source of reducing power was available.

Another approach to establishing the identity of the stimulated respiration with the carbon monoxide-stimulated respiration would be to use the specific inhibitors of methane oxidation. The effects of added carbon monoxide on the respiration of *M. albus* BG8 and *M. trichosporium* OB3B on some C_1 and C_2 substrates is shown in tables 23 and 24 and an enhancement of formate and formaldehyde respiration was observed which was greater than the carbon monoxide-stimulated respiration alone. The effect of the addition of diethyldithiocarbamate and allyl thiourea to suspensions of the methylobacteria respiring on C_1 and C_2 substrates alone and in the presence of carbon monoxide is also given in Tables 23 and 24 which show that stimulation of respiration by combinations of formate and formaldehyde with carbon monoxide is abolished in the presence of inhibitors. This effect is readily explained in

Table 23 Effect of C_1 and C_2 Compounds on Carbon Monoxide-stimulated Respiration by Methylomonas albus BG8

	Respiration Rates (nmoles O_2/min/mg dry cell wt.)			
	CH_3OH	C_2H_5OH	HCHO	HCOONa
Substrate (S)	37	23	24	2
S + CO	52	38	43	5
S + CO + ATU[1]	18	16	17	1
S + ATU	20	20	22	2
S + DTC[2] + CO	20	17	17	3
S + DTC	26	22	27	2

endogenous respiration rate 1 nmole O_2/min/mg dry cell wt.

CO-stimulated respiration rate 1 nmole O_2/min/mg dry cell wt.

Concentrations of C_1 and C_2 substrates; CO 0.6 ml gas-saturated buffer added to chamber. 0.1 ml of 100 mM solution of CH_3OH, C_2H_5OH, HCHO, HCOONa added to Chamber.

(1) ATU = Allyl thiourea (10^{-4}M)

(2) DTC = Diethyldithiocarbamate (7.5×10^{-4}M)

Table 24 Effect of C_1 and C_2 Compounds on Carbon Monoxide-stimulated Respiration by Methylosinus trichosporium OB3B

	Respiration Rates (nmoles O_2/min/mg dry cell wt.)				
	CH_3OH	C_2H_5OH	HCHO	HCOONa	HCOONa[3]
Substrates	89	49	46	7	7
S + CO	74	71	64	24	31
S + CO + ATU	50	38	36	6	5
S + ATU	49	43	37	6	6
S + CO + DTC	55	43	41	7	8
S + DTC	52	42	42	6	7

Endogenous respiration rate 3 nmoles O_2/min/mg dry cell wt.

CO-stimulated respiration rate 5 nmoles O_2/min/mg dry cell wt.

notes as for table 21.

(3) The same cells left overnight in cold room at 4^oC with endogenous and CO-stimulated respiration rate of 1 and 1 nm O_2/min/mg dry wt respectively.

terms of the enhanced respiration being due to a stimulation of carbon monoxide oxidation. The importance of endogenous metabolism in determining the rate of carbon monoxide oxidation is illustrated by footnote 3 of table 24 which shows that although the rate of carbon monoxide-stimulated respiration is lowered on leaving the cell suspension overnight at 4°C the respiration with formate and CO together is still greater than the sum of the two determined separately and indeed is even more enhanced than the previous day, suggesting that the capacity of M. trichosporium OB3B to oxidise carbon monoxide is not altered by overnight incubation at 4°C, only the reserves of endogenous reducing power.

Ferenci (1974) had demonstrated that although suspensions of Pseudomonas methanica were unable to carry out carbon monoxide-stimulated respiration, in the presence of formate there was an enhancement of respiration. This inability to carry out carbon monoxide-stimulated respiration was shown in this study to apply to another membrane-type I methylobacteria, Methylomonas agile (see table 25) and it can be seen that in the presence of formate and carbon monoxide there is considerable stimulation of respiration over the level with formate alone and the abolition of this stimulation by allyl thiourea suggests that this organism, like Pseudomonas methanica, oxidises carbon monoxide provided a source of reducing power is available.

Table 25 Effect of Formate on respiration of Methylomonas agile in the Presence of Carbon Monoxide

Substrate[1]	Respiration Rate (nmoles O_2/min/mg dry wt)
HCOONa	1.5
CO	0
CO + HCOONa	3.5
CO + HCOONa + ATU	1.2
HCOONa + ATU	1.0
CH_4	83
CH_3OH	67
endogenous	0.5

1 notes as for table 23

An explanation for the stimulation of endogenous CO_2 production during the oxidation of CO by M. trichosporium OB3B but not M. albus BG8 is that the process of CO oxidation leads to the breakdown of a storage compound that is only present in the former organisms. Earlier studies using microscopic staining techniques had suggested the presence of polyhydroxybutyric acid (P.H.B.) in some methylobacteria (Whittenbury et al., 1970) and in a more detailed study Thomson (1974) confirmed by chemical analysis the presence of low levels (1.8 - 6.5 µg/mg cells) in M. trichosporium OB3B, and other membrane-type II methylobacteria but not in the membrane-type I methylobacteria M. albus, Methylomonas agile and Methylococcus capsulatus. The incubation of suspensions of M. trichosporium OB3B with ethane stimulated the breakdown of P.H.B. to hydroxybutyrate, acetoacetate, acetone and CO_2 and the dehydrogenation of hydroxybutyrate to acetoacetate was shown in cell-free extracts to be coupled to NAD reduction. A slightly higher level of P.H.B. of 24 µg/mg cells was independently reported to be present in the same strain of M. trichosporium OB3B by Dugan and Weaver (1974).

The level of P.H.B. found by Thomson (1974) on complete conversion to acetone and CO_2 would yield 0.1 µmol CO_2/mg cells which is too little to account for the stimulated endogenous CO_2 production of 0.5 µmol/mg cells. Assay by gas chromatography of the suspensions of M. trichosporium OB3B incubated in the presence and absence of CO as shown in figure 14 showed that the total acetone produced after 90 minutes in the presence of CO was 1.5 µmoles compared with 0.75 µmoles in the endogenous control. Although there was a stimulation of acetone production the levels were lower than those observed by Thomson (1974), which

were typically 5μmoles, and could not account for the stimulated CO_2 production. The involvement of other storage reserves or an alternative pathway of P.H.B. breakdown in the metabolism of CO is thus likely.

The Stoichiometry of Carbon Monoxide Oxidation

Measurements of the stoichiometry of carbon monoxide oxidation in washed cell suspensions are complicated by the need to take into consideration the endogenous metabolism which itself involves uptake of oxygen and production of carbon dioxide. This problem is illustrated by figure 10 where the reversion to the endogenous respiration rate three minutes after the addition of 0.3 mls CO-saturated buffer was taken to indicate the complete oxidation of the added carbon monoxide. If the oxygen uptake during this period is corrected for the endogenous respiration rate 133 nmoles of oxygen would have been consumed to oxidise 258 nmoles of carbon monoxide resulting in a stoichiometry of O_2:CO of 0.55:1; if the endogenous respiration is completely inhibited during this period the oxygen uptake would be 242 nmoles resulting in a stoichiometry of 0.93:1. The results of similar experiments are shown in table 26 which gives the oxygen uptake during the addition of carbon monoxide to suspensions of *M. trichosporium* OB3B respiring on formate or merely endogenous reserves. The oxygen uptake corrected for endogenous or formate respiration is also given and the resulting stoichiometries. Although the respiration studies with only endogenous respiration and with formate-stimulated respiration were carried out with different batches of cells the results were essentially the same, that when corrections for endogenous or formate metabolism are made the

Table 26 Stoichiometries of Carbon Monoxide-stimulated Respiration of M. trichosporium OB3B in the presence and absence of Formate

	Oxygen Uptake (nmoles) and O_2/CO Stoichiometries[1] on Successive Additions of CO			
Amount of CO:	86	172	258	344 nmoles
1) CO and Endogenous				
(a) total CO-stimulated O_2 uptake	90 (1.0)	176 (1.1)	252 (1.1)	300 (0.9)
(b) (a) - endogenous	59 (0.7)	129 (0.7)	196 (0.7)	242 (0.7)
2) CO for Formate[3]				
(a) total CO-stimulated O_2 uptake	100 (1.2)	210 (1.3)	288 (1.1)	328 (1.1)
(b) (a) - formate	44 (0.5)	120 (0.8)	182 (0.7)	192 (0.55)

1 stoichiometries in brackets expressed as the ratio of oxygen uptake/amount of CO added

2 CO was added as a saturated solution at 30°C and the concentration of 860 nmoles CO/ml was assumed

3 Formate was added as 0.1 ml of a 100 mM solution of sodium formate

stoichiometry for O_2:CO is between 0.5 - 0.7 and when the oxygen uptake is not corrected a value between 1.0 - 1.2 emerges.

This problem has not been overcome by Ferenci (1974) who used a similar method to derive a O_2:CO stoichiometry of 1:1 for Pseudomonas methanica. His method was based on the measurement of the oxygen uptake in an oxygen probe on the addition of 0.1 and 0.2 mls of carbon monoxide-saturated buffer and although it is not stated, the respiration on formaldehyde alone is subtracted from the carbon monoxide-stimulated oxygen uptake. The justification for the subtraction of the formaldehyde respiration was the demonstration that the rate of formaldehyde disappearance, measured colourimetrically, was unaltered in the presence of carbon monoxide. There is some confusion at this point in his report because on the oxygen probe trace enclosed with the paper two successive additions of 0.1 and 0.2 mls carbon monoxide-saturated buffer are clearly marked and referred to in the legend of the figure. These amounts correspond to 172 and 86 nmoles dissolved CO, assuming the concentration of carbon monoxide-saturated buffer to be 0.86 μmole/ml at 30°C which is the value he displays with the figure and is contained in standard tables (Seidell, 1920). In the text however it is stated that 108 and 43 nmoles O_2 were consumed on the addition of 86 and 43 nmoles CO resulting in a 1:1 ratio consistent with Ferenci's thesis that carbon monoxide oxidation by Pseudomonas methanica was a mono-oxygenase reaction. Thus the added amounts of carbon monoxide were twice the amounts used in the calculation and instead of a stoichiometry of

1:1.2 and 1:1.1 for the addition of 0.1 and 0.2 mls carbon monoxide-saturated buffer respectively, the results of Ferenci (1974) would indicate a stoichiometry of 1:0.63 and 1:0.5 bringing them closer to the values obtained in this study with M. trichosporium OB3B. A close examination of the oxygen probe trace reveals that if the formaldehyde respiration is not subtracted from the total respiration a stoichiometry of 1:1 results, again in agreement with values for M. trichosporium OB3B.

A stoichiometry for CO oxidation was also obtained from the CO_2 production and O_2 disappearance rates in sealed flasks of cell suspensions of M. albus BG8, as, unlike M. trichosporium OB3B, stimulation of endogenous CO_2 production by CO does not take place (see figure 13) and the excess CO_2 produced in the presence of CO can be assumed to be the product of CO oxidation. The rates of CO_2 production by an incubation of M. albus BG8 in the presence and absence of 1 ml CO were 5.5 and 1.6 nmole CO_2/min/mg cells respectively and the rates of O_2 disappearance were 4.2 and 2.4 nmole O_2/min/mg cells respectivel resulting in a stoichiometry for O_2:CO of 0.6:1 and 1.2:1 depending on whether or not the oxygen consumption is corrected for the endogenous O_2 disappearance in the control. A value between 0.5 and 0.6:1 has been repeatedly obtained for the corrected stoichiometries in a number of incubations of M. albus BG8.

A stoichiometry for CO oxidation by growing cells can be obtained from the levels of CO_2 production and O_2 consumption in methanol-grown cultures of M. albus BG8 and the data in table 20 would indicate a stoichiometry of 0.7:1 supporting the lower stoichiometry for CO oxidation. The calculation of

this figure required the subtraction of the CO_2 evolved and the O_2 consumed in the control flask.

The assumption that endogenous respiration continues unchanged in the presence of substrate is widely made in metabolic studies and will be adopted in this study with some reservations. The stoichiometry of O_2:CO that results from the respiration studies in the oxygen probe, the incubations of washed cell suspensions and the methanol-grown cultures is thus 0.5 - 0.7:1 suggesting that carbon monoxide in methylobacteria proceeds by the following reaction:

$$2CO + O_2 = 2CO_2 \qquad (12)$$

The mechanism of carbon monoxide oxidation by methylobacteria

Carbon monoxide might be oxidised in methylobacteria by one of the following three mechanisms:

$$CO + H_2O \longrightarrow HCOOH \longrightarrow CO_2 \qquad (a)$$

$$CO + H_2O \rightleftharpoons CO_2 + H_2 \qquad (b)$$

$$CO \xrightarrow[\text{oxygen}]{\text{molecular}} CO_2 \qquad (c)$$

The hydration of carbon monoxide to formate with subsequent conversion to carbon dioxide by formate dehydrogenase has not been shown to occur in any living system and many of the studies reviewed by Chapelle (1962) and Fenn (1970) have taken great pains to eliminate such a mechanism. The involvement of formate as an intermediate in carbon monoxide oxidation by methylobacteria is unlikely for two reasons: as shown in tables 23, 24 and by Ferenci (1974) formate stimulates carbon monoxide respiration by washed cell suspensions which would not be expected if formate itself was intermediate. Ferenci (1974) was also able to demonstrate the cell-free stimulation of respiration by Pseudomonas

methanica using a particulate fraction which would eliminate the involvement of the soluble formate dehydrogenase in this organism (Johnson and Quayle, 1964).

There is good evidence that the oxidation of carbon monoxide by the water gas reaction, mechanism b, takes place in some living organisms (Chapelle, 1962b) and it is undoubtedly significant that the only known micro-organisms which can utilise carbon monoxide as sole carbon and energy source can also utilise hydrogen. The occurrence of this mechanism is also likely during carbon monoxide oxidation by Desulfovibrio and the methanogenic bacteria where the formation of molecular hydrogen has been confirmed (Yagi and Tamiya, 1962; Kluyver and Schnellen, 1947). The real biological significance of non-growth carbon monoxide oxidation by this mechanism is questioned by the largely-overlooked cautionary note by Seitz (1941) who showed that at neutral pH under anaerobic conditions carbon monoxide can be converted to carbon dioxide in the presence of light and an hydrogen acceptor such as methylene blue, and at alkaline pH in the presence of either. It is not at all certain how many of the earlier publications on carbon monoxide oxidation were merely describing this non-physiological process.

This mechanism has been proposed by Yagi and Tamiya (1962) for the anaerobic oxidation of carbon monoxide by Desulphovibrio but all attempts to obtain cell-free oxidation of carbon monoxide using his method have been unsuccessful.

While this study was in progress the evolution of molecular hydrogen by anaerobic incubation of M. albus BG8 in the presence of formate was observed by Dr J.G. O'Neill (personal communication) indicating formate hydrogen lyase activity in

this organism and, although hydrogenase in some micro-organisms is known to be inhibited by carbon monoxide (Gray and Gest, 1965), this observation suggested a way in which oxidation of CO by mechanism b could take place in methylobacteria. In order to determine whether evolution of hydrogen accompanied carbon monoxide oxidation suspensions of M. albus BG8 and M. trichosporium OB3B were incubated anaerobically in the absence of added substrate and in the presence of carbon monoxide and formate and aerobically in the absence of substrate and the presence of carbon monoxide. A suspension of M. trichosporium OB3B was also incubated anaerobically with hydroxybutyrate. The gas phase of each flask was sampled by gas chromatography after one and two hours incubation and overnight incubation and the amounts of hydrogen and carbon dioxide evolved are shown in table 27.

Anaerobic incubations of both organisms evolved hydrogen in the presence of formate although the rate of evolution was considerably slower in the case of M. trichosporium OB3B. Neither the endogenous control nor the incubation with carbon monoxide evolved hydrogen in the initial two hours although hydrogen was present in the overnight incubations. Although there was slight stimulation by carbon monoxide of carbon dioxide production in the anaerobic incubations, the level was considerably lower than the aerobic incubations confirming the requirement of carbon monoxide oxidation for molecular oxygen.

Thomson (1974) demonstrated NADH-linked Hydroxybutyrate Dehydrogenase activity in M. trichosporium OB3B but not M. albus BG8. Hydroxybutyrate metabolism has been proposed by Klucas and Evans (1968) to be linked to ferredoxin reduction in the nitrogenase enzymes in Rhizobium and this compound was found to

Table 27 Hydrogen evolution by suspensions of Methylobacteria in the presence of Carbon Monoxide, Formate and Hydroxybutyrate

	ANAEROBIC INCUBATIONS[1]								AEROBIC INCUBATIONS	
	Endogenous		+ CO		+ Formate		+ hydroxy-butyrate		Endogenous	+ Carbon Monoxide
time (hrs.) μmoles	CO_2	H_2	CO_2	H_2	CO_2	H_2	CO_2	H_2	CO_2	CO_2
M. albus BG8										
1	4.5	0	5	0	7	8	n.t.[3]	n.t.	1.5	4
2	5	0	6	0	8.5	16	n.t.	n.t.	3	5.5
20	10	3.3	11.5	2.7	34	40	n.t.	n.t.	34	53
M. trichosporium OB3B										
1	1.8	0	4.3	0	2.4	0.5	1.4	0.25	7	36
2	2.1	0	5.1	0	3.4	1.5	2.6[2]	1.3[2]	20.5	75
20	4.0	5.4	8.5	3.8	11.5	15.0	12.5	13.3	n.t.	n.t.

1 The amounts of carbon monoxide, formate and hydroxybutyrate were 3 mls, 10 mmolar and 10 mmolar respectively. Incubations contained 47 mg cells.

2 Acetone content 1.4 μmoles

3 Not tested

stimulate hydrogen evolution in M. trichosporium OB3B with the level of hydrogen after two hours corresponding closely to the amount of acetone present of 1.4 μmole in the suspension which was determined by gas chromatography. The stimulation by hydroxybutyrate of hydrogen evolution suggests that the hydrogenase in M. trichosporium OB3B is NADH-linked. The evolution of hydrogen by the endogenous incubations of the two methylobacteria in the absence of formate would support the view that endogenous metabolism can be linked to the reduction of NAD.

Thus there is no evidence for the oxidation of carbon monoxide in methylobacteria by mechanism b. It is also difficult to reconcile a mechanism that requires an oxidised electron acceptor to receive the evolved hydrogen with a definite requirement for reducing power found by Ferenci (1974) and in this study.

The only author to speculate whether molecular oxygen is involved in carbon monoxide oxidation is Chapelle (1962a) who cites no evidence apart from a requirement for molecular oxygen. As with the oxygen requirement for carbon monoxide oxidation by methylobacteria this can be explained as a requirement for oxygen to oxidise a reduced carrier generated through mechanism b. Conclusive proof for the metabolism of carbon monoxide by an oxygenase mechanism would require the demonstration in incubations of carbon monoxide and ^{18}O-enriched molecular oxygen of incorporation of ^{18}O into CO_2 to give levels of $^{46}CO_2$ significantly above the naturally occurring levels. A serious complication to the determination of ^{18}O incorporation into CO_2 is the dilution of ^{18}O content caused by

the reversible hydration of CO_2 to form carbonic acid, H_2CO_3, a reaction which may be non-enzymic (Mills and Urey 1940) or catalysed by the enzyme Carbonic Anhydrase (Silverman, 1973).

Initially suspensions of M. trichosporium OB3B were aerobically incubated for two hours in the presence and absence of CO and ^{18}O-enriched oxygen and the sealed flasks were frozen until it was convenient to assay the contents by combined gas chromatography/mass spectrometry (GC/MS). These early experiments failed to reveal any incorporation of ^{18}O into CO_2 and the procedure was then adopted of carrying out the incubations in the same room as the GC/MS unit in order that samples could be analysed during the course of incubations. The lack of any temperature control facilities such as a hot room necessitated maintaining the incubations at 23°C rather than the usual temperature 30°C.

The isotopic compositions and quantities of the CO_2 evolved in incubations of M. trichosporium OB3B in the presence of CO, CH_4 and an endogenous control are shown in table 28. Samples of the gas phase of each flask were taken at 30, 60 and 90 minutes and the ratios of the mass spectrometer peaks at mass 46 and 44 calculated and it is evident from the results in table 28 that whereas the 46/44% was similar at 0.7% for the incubations with CH_4 and the endogenous control and equal to the ratio for the CO_2 standard, the value in the incubation with CO was slightly higher at between 1.0 - 1.3%. A value for the 46/44% of the endogenous control at 30 minutes could not be determined as the level of CO_2 present was not high enough to give an adequate signal for the GC/MS.

The apparent enrichment above the control of 0.3 - 0.6% was extremely low and a further experiment was carried out with a

Table 28 Carbon Dioxide Production and ^{18}O-incorporation in incubations of Methylosinus trichosporium OB3B and CO and CH_4 in an ^{18}O-enriched atmosphere

Time (minutes)	Endogenous Control			+ Carbon Monoxide			+ Methane		
	CO_2 produced µmoles	mass 45/mass 44%	46/44%	CO_2 produced µmoles	45/44%	46/44%	CO_2 produced µmoles	45/44%	46/44%
Experiment 1[a]									
30	5	-	-	12	1.3	1.3	39	1.45	0.75
60	8	1.4	0.5	25	1.25	1.25	72	1.65	0.75
90	11	1.4	0.7	42	1.8	1.0	77	1.4	0.7
Experiment 2[b]									
30	8)			29)					
60	16)	1.48 ± 0.14[1]	0.74 ± 0.06	57)	1.38 ± 0.18[2]	2.0 ± 0.4			
90	23)			85)					

a 60 mg cells, one flask for each incubation

b from figure 15. 100 mg cells, two replicates of each incubation

1 arithmetic mean and standard deviation of 9 samples from two incubations in absence of CO

2 " " " " " 13 " " " " presence of CO

3 ^{18}O level in oxygen 30%: 26% $^{36}O_2$, 7% $^{34}O_2$

4 Standard CO_2: 1.35% mass 45/44%0.76% mass 46/mass 44%

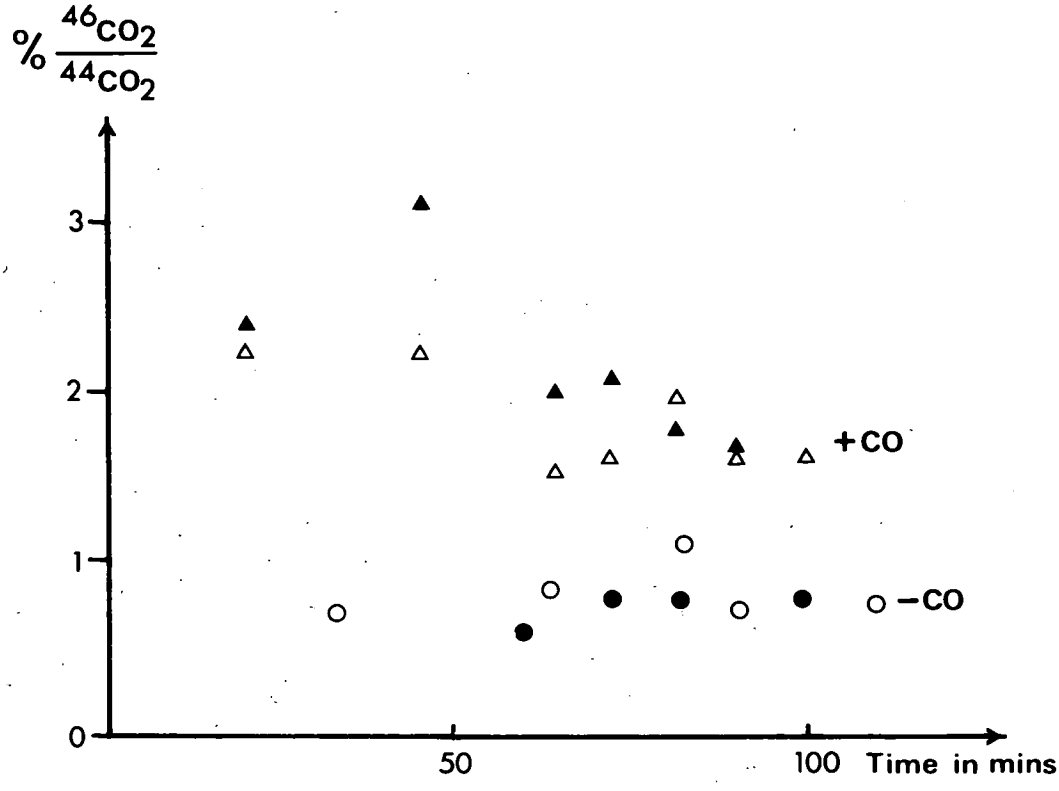
greater amount of cells in each incubation and with two replicates of the endogenous control and the incubation with CO. An incubation with CH_4 was omitted in view of the absence of enrichment of 46/44% in the previous experiment. This lack of enrichment is not surprising since, despite the confirmed oxygenase-mediated metabolism of CH_4, (Higgins and Quayle, 1970) both the hydration of HCHO and CO_2 would be expected to dilute out the ^{18}O label. As soon as the CO_2 contents of the flasks were high enough for the GC/MS assay the gas contents of each pair of incubations were sampled repeatedly. The 46/44% of the thirteen samples of CO_2 produced by the two incubations in the presence of CO and the nine samples of the two control endogenous incubations are shown in figure 15 together with the total amounts present in each flask. The mass 46/mass 44% of the CO_2 formed in the presence of CO were consistently higher than that of the controls and both the CO_2 and CO standards and the mean and standard deviations of the mass 46/mass 44% of the incubations with and without CO were 2.0 ± 0.4 and 0.7 ± 0.06% respectively. The standard deviation of the mass 46/mass 44% in the incubations with CO is larger than that of the control incubations reflecting the scatter in ^{18}O-incorporation shown in figure 15 but the differences between these two means are well outside the standard deviations, pointing unequivocally to the ^{18}O-enrichment of the CO_2 formed in the presence of CO. The ^{18}O contents of CO can vary from 0.3 - 2.2% according to the source (Stevens, 1972) and a high content of ^{18}O in the added CO could provide an alternative explanation for the higher ^{18}O content to an oxygenase mechanism for CO oxidation, however analysis by GC/MS showed that the mass 30 component of the CO source was only 0.3%

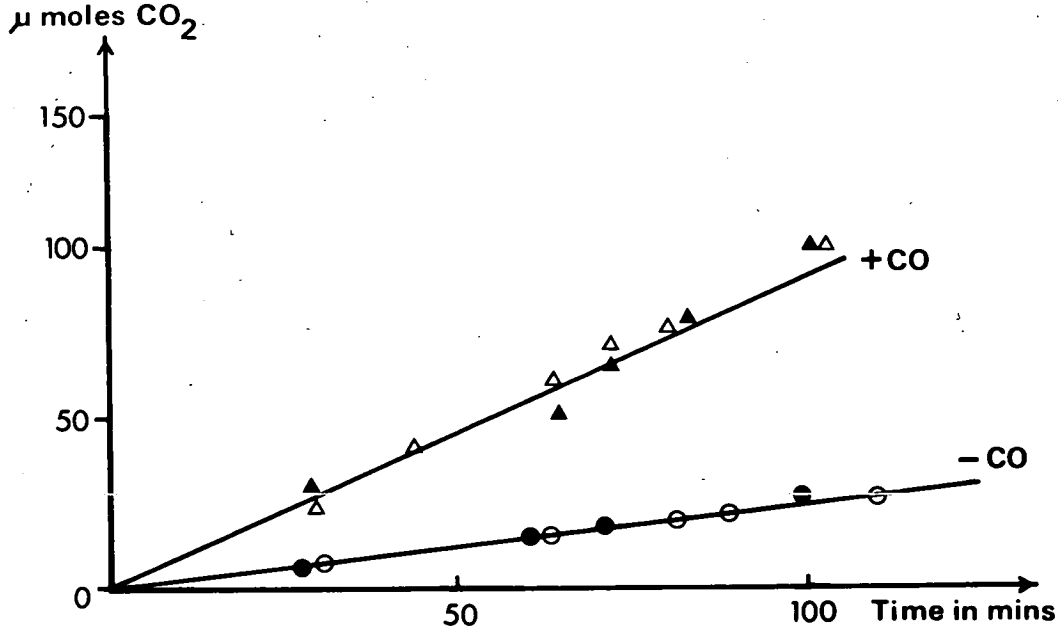
Fig. 15: ^{18}O incorporation into carbon dioxide evolved by incubations of M. trichosporium OB3B in the presence and absence of carbon monoxide and in the presence of ^{18}O-enriched oxygen

A) Percentage mass 46 of mass 44 of evolved carbon dioxide as determined by combined gas chromatography/mass spectrometry

B) Total carbon dioxide production by incubations

5ml suspensions incubated at 23°C with 100mg dry weight of cells and 5ml 30% ^{18}O-enriched molecular oxygen. Two incubations (O and ⊘) in absence of carbon monoxide and two incubations (Δ and ▲) in the presence of 3ml carbon monoxide.


% 46CO2 / 44CO2
3
2
1
0
50
100
Time in mins
+CO
−CO


μ moles CO2
150
100
50
0
50
100
Time in mins
+CO
−CO

of the mass 28 component. Reinforcing the significance of the $^{46}CO_2$ enrichments, the close similarity in the contents of $^{45}CO_2$ which is not due to ^{18}O-enrichment, in the CO_2 standard and the incubations in the presence and absence of CO serves as an internal control.

The difference between the means of the ^{18}O-enrichments in the two controls and the incubations with CO was 1.25% and, as the oxygen source contained 30% ^{18}O, this enrichment corresponded to 4.2% of the theoretical value for an oxygenase-mediated oxidation of CO. The measured mass 46/mass 44% will be diluted by CO_2 produced from endogenous sources and the ^{18}O-enrichment of the CO_2 produced in the presence of CO, corrected using the endogenous CO_2 production in the controls, becomes 5.2% of the theoretical value. A stimulation in endogenous CO_2 production by CO similar to that shown in figure 14 would raise the percentage of the theoretical even further and would explain the slight decline in ^{18}O enrichment with time shown in figure 15.

Both the low percentage of theoretical ^{18}O-incorporation and the higher levels of enrichment in the incubations with the greater amounts of cells with a faster total rate of CO oxidation are consistent with isotopic dilution of ^{18}O-labelled CO_2 by hydration with water. By way of comparison Rees and Nason (1966) proposed an oxygenase mechanism for ammonia oxidation by Nitrosomonas europeaea on the basis of the presence of 6.6 - 7.2% of the theoretical ^{18}O-incorporation in the evolved NO_2^- and also suggested that the reason for their low ^{18}O-enrichment lay in isotopic exchange with water.

An explanation of the low levels of ^{18}O incorporation in terms of exchange of carbon dioxide with $H_2{}^{18}O$ formed when ^{18}O serves as a terminal acceptor can be readily discounted: oxygen could not be quantitatively estimated by the gas

chromatography system used in this particular experiment but if an O_2:CO:CO_2 ratio for carbon monoxide oxidation of 0.5:1:1 is assumed and taking the ^{18}O-enrichment of the oxygen to be 30% the amount of $H_2{}^{18}O$ which would have been formed in 90 minutes incubation with CO would be 96 x 0.5 x 0.3 i.e. 15 μmoles. The total amount of water in 5 mls of a suspension can be taken as 0.28 moles thus the resultant enrichment of the water would be less than 0.005% which is negligible in comparison with the observed enrichments of ^{18}O in the CO_2 produced by incubations of M. trichosporium OB3B with CO.

Lane and Dole (1956) have demonstrated that bacteria utilise $^{32}O_2$ at 1.015 greater rate than $^{36}O_2$ but this isotopic effect is also too small to explain the observed enrichments.

The demonstration of ^{18}O-incorporation into the CO_2 evolved from incubations of M. trichosporium OB3B with CO provides conclusive evidence for an oxygenase mechanism for CO oxidation by this organism and both the common effect of inhibitors on the CO-stimulated respiration of suspensions of M. albus BG8 (table 22) Methylomonas agile (table 24) and cell-free results of Ferenci (1974) with Pseudomonas methanica support a similar oxygenase mechanism for CO oxidation in other methylobacteria. Although the oxidation of CH_4 and CO by mono-oxygenase mechanisms remains to be conclusively established and is inconsistent with the observed stoichiometries in this study, the involvement of the same oxygenase enzyme system in both processes would seem likely in view of the inhibition of CO-stimulated respiration by specific inhibitors of methane oxidation, the ability of cell-free extracts of Pseudomonas methanica to carry out NADH-dependent respiration with both substrates and the possible inhibition of CH_4 oxidation by CO through a competitive mechanism.

On the basis of the sensitivity of methane oxidation to inhibition by CO, Davey and Mitton (1973) had suggested that the a-type cytochrome present in methylobacteria was involved in CH_4 oxidation and the specific inhibition of CH_4 oxidation by other known inhibitors of cytochrome oxidase azide, hydroxylamine, cyanide (Lemberg, 1969), British Anti-Lewisite (Cooperstein, 1963) and diethyl dithiocarbamate (Erickson et al., 1972) would support this view. The known involvement of copper in mammalian and some bacterial cytochrome oxidases (Lemberg, 1969) would make the involvement of the a-type cytochrome in the methane oxygenase reaction consistent with the mechanism by the specific inhibitors proposed in an earlier section. The possibility of CO acting as a substrate for the methane oxygenase led Ferenci (1974) to disagree with the above mechanism of inhibition by carbon monoxide and propose instead that inhibition was by a competitive mechanism. These two mechanisms of inhibition need not necessarily be mutually exclusive as there is evidence from two independent sources that mammalian cytochrome oxidase can bring about the oxidation of carbon monoxide.

Breckenridge (1953) eliminated the involvement of catalase, cytochromes b and c, diaphorase and myoglobin in the conversion of ^{14}CO to $^{14}CO_2$ by pig heart muscle and demonstrated that the activity resided in the cytochrome oxidase from that tissue. He was also able to show that the oxidation of carbon monoxide by purified cytochrome oxidase was stimulated by an increased flow of electrons down the electron transport chain on the addition of lactate or cytochrome c and concluded that cytochrome oxidase must be in the reduced state prior to its combination with, and oxidation of, CO.

Unaware of the earlier work of Breckenridge (1953), Tzagoloff and Wharton (1965) showed that ^{14}CO reacted with purified cytochrome oxidase from beef heart mitochondria to form a spectroscopically distinct complex and that the ^{14}CO could be dissociated from this complex by passing air through the suspension to liberate $^{14}CO_2$. The authors did not speculate as to whether molecular oxygen participated in the reaction and it is impossible to assess from their described experimental method whether oxidation of CO took place before or after the addition of air and thus whether there was an oxygen requirement for CO oxidation or merely for the re-oxidation of cytochrome oxidase. These two studies of CO oxidation by cytochrome oxidase indicate a mechanism for oxidation of CO in which CO combines with the reduced cytochrome and is oxidised either by molecular oxygen or water. Meaningful analogies between methylobacterial and mammalian cytochrome oxidase-mediated CO oxidation must await detailed studies of the properties of the purified methylobacterial a-type cytochrome and the participation of molecular oxygen in the oxidation of CO by mammalian cytochrome oxidase.

ELECTRON PARAMAGNETIC RESONANCE STUDIES WITH METHYLOBACTERIA

The presence of unpaired d electrons in the outer orbitals of the transition metals iron and copper has established Electron Paramagnetic Resonance (E.P.R.) Spectroscopy as a valuable tool in the study of the role of metals in oxygenase reactions (Swartz, 1972). The determination of the two parameters, the 'g-value', and the hyperfine splitting of a signal can give information as to both the nature of the metal involved and the oxidation state. Thus E.P.R. spectroscopy was used to demonstrate that copper changes valency from Cu(II) to Cu(I) during the course of the copper-containing dopamine-hydroxylase mono-oxygenase enzyme reaction (Blumberg et al., 1965). In addition the E.P.R. spectrum of a purified enzyme can confirm the presence of covalent bonding to the metal and the presence of hyperfine splitting in the signal can sometimes help in the characterisation of the ligands.

In an early study of the application of E.P.R. spectroscopy to bacteria Nicholas et al. (1962) showed the presence of a copper resonance of g-value 2.1 with hyperfine splitting in whole cells of Nitrosomonas europaea and in hydroxylamine-oxidising cell-free extracts consisting of particles containing cytochromes b, c and a. The changes in the fine structure of the signal on the addition of hydroxylamine were considered evidence for the involvement of the copper in the metabolism of hydroxylamine. That the spectral change was not due merely to binding to the copper of hydroxylamine, which has been shown to change the E.P.R. copper signal of cytochrome oxidase (Beinert, et al., 1962), was inferred from the observation that these changes

occurred more rapidly in the presence of cytochrome c although no kinetic data was presented.

A role for copper in hydroxylamine oxidation in Nitrosomonas cannot be conclusively established from this brief study of Nicholas et al., (1962) and it is unfortunate that none of the subsequent detailed studies on hydroxylamine oxidation in Nitrosomonas (review: Aleem, 1970) have extended the E.P.R. studies. The presence of copper in the particles is interesting, however, in the light of the proposed involvement of copper in the ammonia oxygenase suggested by the inhibition studies of Hooper and Terry (1973).

A limitation to the application of E.P.R. spectroscopy to the study of whole cells and intact tissues is that the concentrations of biologically-important paramagnetic species present are usually low and measurement of resonance spectra must be carried out at high receiver amplifications with correspondingly low signal-to-noise ratios (Swartz, 1972). The E.P.R. spectra obtained of a dense suspension of Methylomonas albus BG8 shaken in air ('oxidised') and reduced by dithionite are presented in figure 16 and are typical of those repeatedly obtained with this organism. A prominent resonance is present which can be assigned a $g_{\perp}$ value of 2.06 and also three peaks at a lower magnetic field. Both the resonance at g-value 2.06 and the three peaks are abolished on reduction by sodium dithionite and the dithionite-reduced sample also serves as a control for the background E.P.R. spectrum due to the cavity alone.

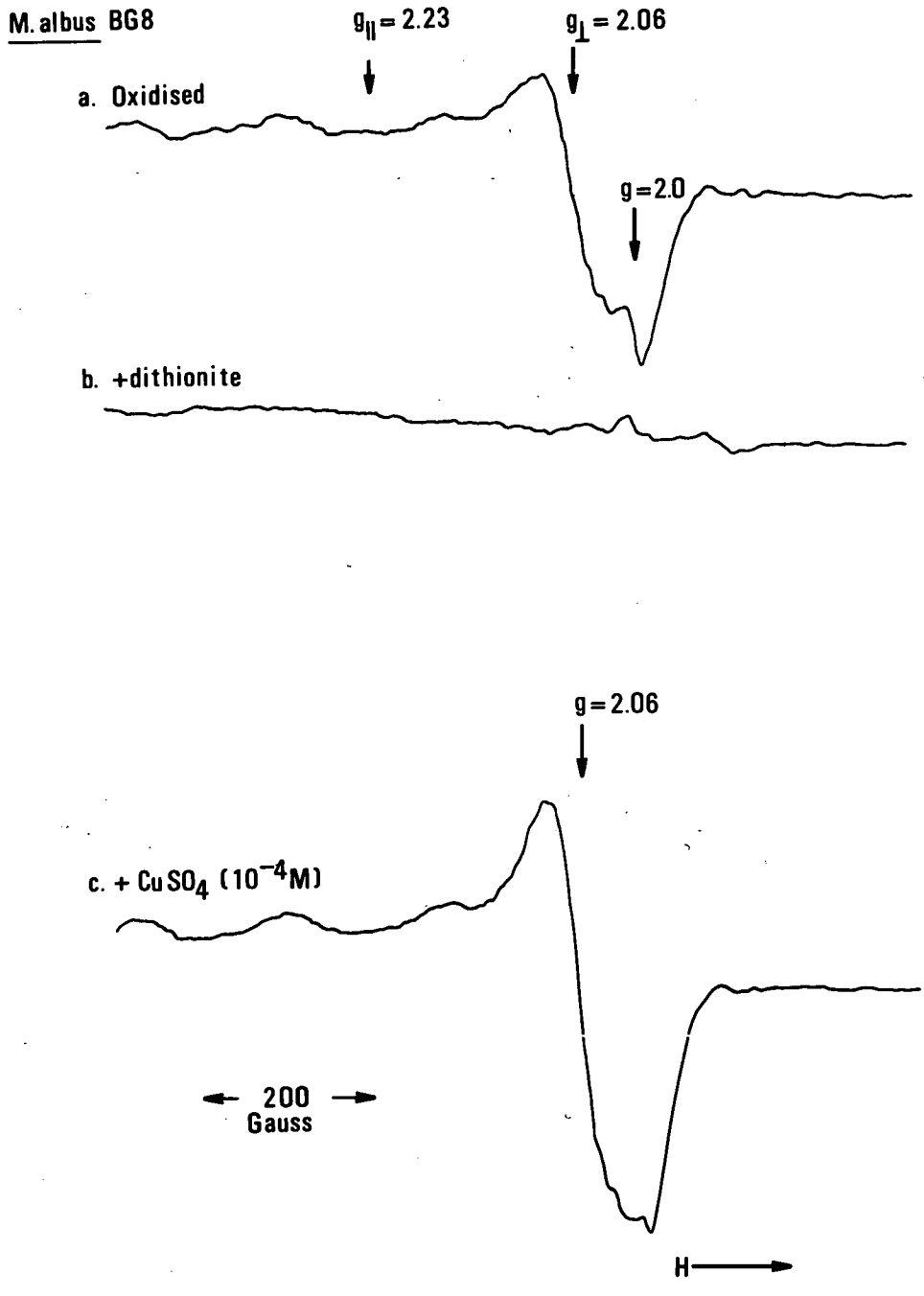
The resonance of g-value 2.06 is typical of Cu(II) and the three low-field peaks can be attributed to the nuclear

Fig. 16: Electron Paramagnetic Resonance spectra of Methylomonas albus BG8 and the effect of the addition of copper

Suspensions containing 143 mg dry wt cells/ml were shaken for one minute: (a) in the absence of substrate, (b) in the presence of a few crystals of sodium dithionite and (c) in the presence of $CuSO_4$ to a final concentration of 10^{-4}M.

Spectra were recorded at -172°C with receiver gain 5×10^2, modulation amplitude 1.25×10^1 Gauss, modulation frequency 100 kHz and microwave power 50 mW. Scans were carried out at fixed frequency 9.140 GHz with a time constant of one second over a magnetic field range of 1000 Gauss with the centre of the field set at 3100 Gauss.

Spectra are reduced to 25% of the original size.


M. albus BG8
$g_{\parallel} = 2.23$
$g_{\perp} = 2.06$
a. Oxidised
g=2.0
b. +dithionite
g=2.06
c. + $CuSO_4$ (10^{-4}M)
200
Gauss
H

hyperfine splitting caused by the interaction of the nuclear spin of the copper atom with the unpaired electron. The fourth component of the nuclear hyperfine signal is likely to be obscured by the g = 2.06 resonance and a $g_{\parallel}$ -value of 2.23 can be assigned to the resonance. Vänngård (1972), in his review of the E.P.R. spectroscopy of copper proteins, lists the $g_{\perp}$ values of over thirty copper complexes as ranging from 2.0 to 2.1 and the $g_{\perp}$ resonance of the E.P.R.-detectable copper species in Nitrosomonas europaea was centred about 2.1 (Nicholas et al., 1962). An assignment of the resonance to two other transition elements of biological importance, Molybdenum and Iron, can be eliminated on the basis of the g-values. E.P.R.-detectable Mn (II) has been found in a wide range of bacteria in the study of Nicholas et al. (1962) but although their observed resonances were centred about g-value 2.01 their spectra were typically a sextet of six equally-spaced components which does not correspond to the observable resonances in the E.P.R. spectrum of oxidised M. albus BG8 in figure 16. The E.P.R. spectra of M. albus BG8 in the presence of $CuSO_4$ (10^{-4}M) is also shown in figure 16. On addition of Cu(II) the resonance of the suspension of M. albus BG8 changed neither in g-value or in shape but only in intensity supporting the assignment of the identity of the E.P.R.-detectable species to Cu(II).

The presence of an analogous E.P.R.-detectable Cu(II) species in three other methylobacteria of both membrane-types and the effect of incubation with methane on all four methylobacteria were then examined. The results are displayed in figures 17 and 18 which show the E.P.R, spectra

Fig. 17: Electron Paramagnetic Resonance spectra of membrane-type I methylobacteria and the effect of methane

I *Methylomonas albus* BG8

II *Pseudomonas methanica*

Suspensions containing 47 mg cells/ml were shaken for one minute: (a) in the absence of substrate, (b) in the presence of methane and (c) in the presence of a few crystals of sodium dithionite. A control blank of pH 6.8 phosphate buffer was included (d).

Spectra were recorded under the same operating conditions as described in the notes for figure 16 with the exception that a receiver gain of 1.25×10^3 was employed. Spectra are reduced to 25% of the original size.

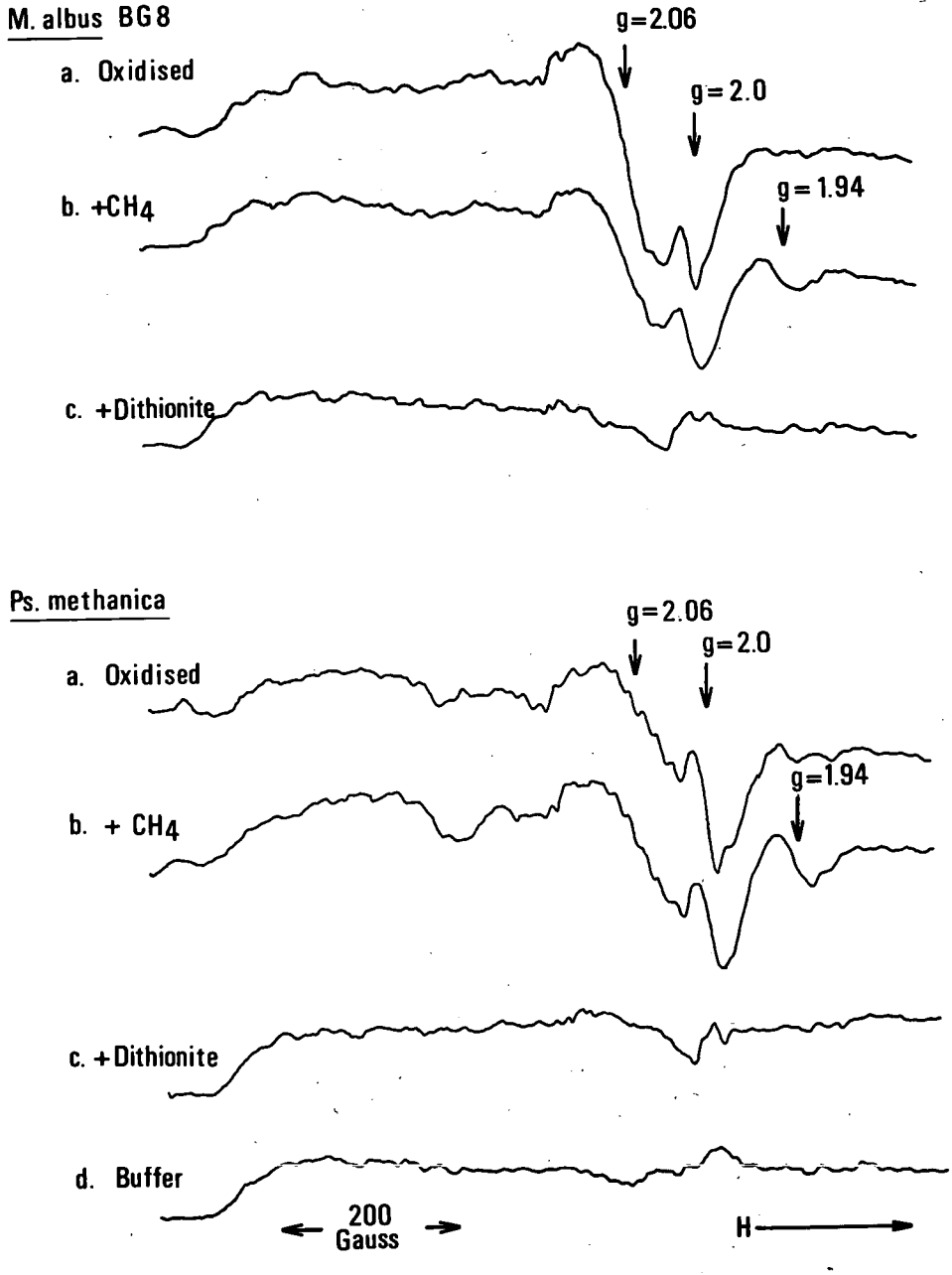

M. albus BG8
a. Oxidised
b. +CH4
c. +Dithionite
g=2.06
g=2.0
g=1.94
Ps. methanica
a. Oxidised
b. + CH4
c. +Dithionite
d. Buffer
g=2.06
g=2.0
g=1.94
200 Gauss
H

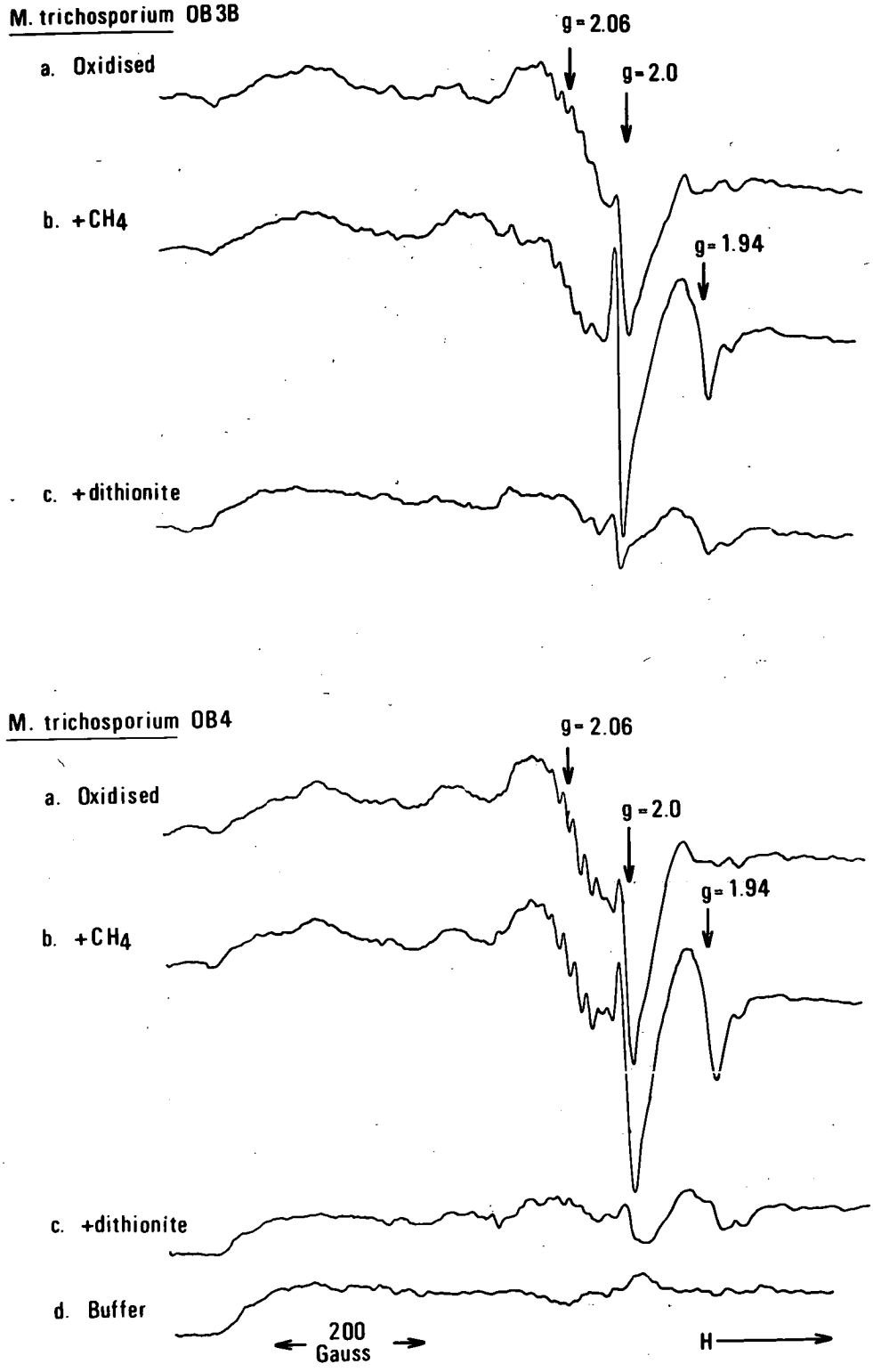
Fig. 18: Electron Paramagnetic Resonance spectra of membrane-type II methylobacteria and the effect of methane

I *Methylosinus trichosporium* OB3B

II *Methylosinus trichosporium* OB4

Suspensions containing 88 and 100 mg dry wt/ml for I and II respectively were shaken for one minute: (a) in the absence of substrate, (b) in the presence of methane and (c) in the presence of a few crystals of sodium dithionite. A control blank of pH 6.8 buffer was included (d).

Spectra were recorded under the same operating conditions as described in the notes for figure 16 with the exception that a receiver gain of 1.25×10^3 was employed. Spectra are reduced to 25% of the original size.


M. trichosporium OB3B
a. Oxidised
b. +CH4
c. +dithionite
g = 2.06
g = 2.0
g = 1.94
M. trichosporium OB4
a. Oxidised
b. +CH4
c. +dithionite
d. Buffer
g = 2.06
g = 2.0
g = 1.94
200 Gauss
H

of cell suspensions of the membrane-type I methylobacteria M. albus BG8 and Pseudomonas methanica and the membrane-type II organisms Methylosinus trichosporium OB3B and Methylosinus trichosporium OB4 shaken in air ('oxidised'), in methane and air and reduced by sodium dithionite. Lower cell concentrations and a correspondingly 2.5 times higher sensitivity of receiver gain were used in these studies. The E.P.R. spectrum of the phosphate buffer in which the cells were washed and resuspended is also displayed with both figures 17 and 18 as a control for both the cavity signal and for the presence of extraneous contaminating metal ions. All spectra in figures 16, 17 and 18 have been reduced by the same amount to 25% of their original size.

The prominent resonance centred at g-value 2.0 and which is also present in the concentrated M. albus BG8 in figure 16 corresponds to the resonance of an unpaired electron in a free radical which is commonly found in biological material (Swartz, 1972) and although in the case of the incubation of M. trichosporium OB3B with methane in figure 18 the resonance was enhanced, this was not typical and no special significance can be attributed to the free radical from this study.

The resonance at g-value 2.06 was again present in Methylomonas albus BG8 and resonances centred about the same g-value were also present in the oxidised spectra of the other methylobacteria although they were not so intense. Although peaks corresponding to nuclear hyperfine structure of Cu(II) were present in both the membrane-type II methylobacteria, the signal-to-noise ratios in the type I methylobacteria were such that no nuclear hyperfine

structure could be resolved. The resonances centred about g-value 2.06 in the suspension of the two membrane-type II methylobacteria and also Pseudomonas methanica showed considerable hyperfine structure which is likely to be due to the interaction of the nuclear spins of the ligand atoms with the unpaired electron of the Cu(II) species (Vänngård, 1972) but a spectrum from a purified enzyme would be required before any further analysis could be made. The abolition by dithionite of the resonances centred about 2.06 in all methylobacteria supports the assignment of these resonances to a Cu(II) species.

On shaking suspensions of methylobacteria in the presence of methane the peak-to-trough heights of the g-value 2.06 resonance were typically reduced by 20% as can be seen in figures 17 and 18. Although this is suggestive of some involvement of this E.P.R.-detectable species in the oxidation of methane the evidence is inconclusive as the reduction in size of the resonance did not always take place and the adjacent free radical resonance made the accurate peak-to-trough heights difficult to determine. As preparation of samples required the slow freezing of methylobacterial suspensions in liquid nitrogen prior to analysis by E.P.R. spectroscopy, the detection of other transient species or a greater reduction of the g-value 2.06 resonance could have been prevented.

In addition to the above changes on incubation with methane, a resonance centred about g-value 1.94 was invariably observed which, although not shown in figure 18, also appeared when M. trichosporium OB3B was incubated with methanol and formate. Such a species is typical of the $g_{\perp}$

resonance of an iron-sulphur protein (Orme-Johnson, 1973; Yoch and Valentine, 1972) and the lack of symmetry of the resonances, although possibly distorted by the adjacent free radical resonance, may indicate the presence of more than one iron-sulphur protein. The $g_{\parallel}$ components of the resonance of iron-sulphur proteins are typically found at g-value 2.01 but the presence of the prominent resonances at g-values 2.06 and 2.0 would be expected to obscure such a component. The reduction by dithionite of cell suspensions did not result in the appearance of the g-value 1.94 resonance in M. albus BG8 of Ps. methanica and only to a slight extent in the two membrane-type II methylobacteria. Reduction by dithionite of whole cells of Pseudomonas putida by dithionite has been shown to generate the E.P.R.-detectable resonance of g-value 1.94 of Putaredoxin (Peterson, 1970) and it must be assumed that sodium dithionite reduces the iron-sulphur protein(s) in methylobacteria to a level that is not paramagnetic. An alternative explanation for the g-value 1.94 resonance which has not been eliminated from this study is that it is an 'overshoot' component of a Cu(II) species (Vänngård, 1972) caused by changes in the electronic environment of the Cu(II) as a result of incubation with methane and the lack of the 'overshoot' signal on treatment with dithionite could then be ascribed to the reduction of Cu(II) to the non-paramagnetic Cu(I) level. Whilst eliminating the involvement of an iron-sulphur protein in methylobacterial metabolism, such an alternative would necessarily involve the participation of E.P.R.-detectable Cu(II) species. However the 'overshoot' phenomenon is ill-characterised

and as the presence of formate-hydrogen lyase activity in methylobacteria by analogy with other bacteria is an indication of the presence of an iron-sulphur protein (Gray and Gest, 1965), the assignment of the g-value 1.94 resonance to a copper 'overshoot' component becomes unlikely.

A crude estimate of the amount of E.P.R.-detectable copper in the methylobacterial strains examined can be obtained by comparisons of the peak-to-trough heights of the resonances with that of a copper standard such as the increase in resonance on the addition of Cu(II) to M. albus BG8 in figure 16. The concentrations of E.P.R.-detectable Cu(II), adjusted to the same cell concentration, in suspensions of M. albus BG8 in figure 16, and M. albus BG8, Pseudomonas methanica (figure 17) M. trichosporium OB3B and M. trichosporium OB4 (figure 18) of 0.5, 0.45, 0.2, 0.2 and 0.3 x 10^{-4}M respectively for suspensions containing 47 mg dry wt cells per ml. The arithmetic means of these concentrations corresponds to 0.6 nmole or 4 x 10^{-5}mg Cu(II) per mg of cells and if it was assumed that the copper was a component of a protein of molecular weight 50,000 and there was only one copper atom per protein molecule, such a protein would represent 3% of the bacterial mass. Difficulties in measuring the peak-to-trough heights because of the adjacent free radical resonances, the possibility that the dense bacterial suspensions were only partially-oxidised and some of the copper is present as the non-paramagnetic Cu(I) and even the existence of non-paramagnetic Cu(II) complexes renders the above crude estimation of value only in obtaining the minimum amount of copper present in these

methylobacteria.

On disruption of Methylomonas albus BG8 in a french pressure cell and subjecting the extract to differential centrifugation 50% of the E.P.R.-detectable copper was present in the soluble fraction.

The mere presence of an E.P.R.-detectable Cu(II) species in suspensions of methylobacteria does not necessarily implicate that copper in the methane oxygenase since the ion may be a contaminant species from the medium or a normal feature of a Gram-negative bacterium. The level of added copper in the trace elements component of the nitrate mineral salts (N.M.S.) medium used to grow the methylobacteria is only 2.6×10^{-6}M and is not detectable by E.P.R. Spectroscopy at the receiver gains employed in this study. Nicholson et al. (1962) in their exploratory E.P.R. study of bacteria did not observe a copper E.P.R.-detectable resonance in particulate extracts of Escherichia coli, Azotobacter vinlandii and Clostridium pasteurianum but did not give any details of results with whole cell suspensions. In this study an examination of the E.P.R. spectrum of a suspension of Escherichia coli B grown on nutrient broth/N.M.S. liquid medium failed to reveal any copper using sensitivities and cell concentrations similar to those employed in figure 16.

As a further control, the presence or absence of the E.P.R.-detectable copper signal in a hydrocarbon-utiliser grown in the same N.M.S. salts medium was investigated. The Gram-positive organism JOB5 was selected on the basis of its proposed ability to grow on hydrocarbons ranging from C_1 to higher alkanes (Perry, 1968). This wide-

substrate specificity would make JOB5 unique among methylobacteria since, although hydrocarbons other than methane can be oxidised by methylobacteria (Leadbetter and Foster, 1960; Davey, 1971; Thomson, 1974), only methane, methanol and dimethyl ether have been shown to act as growth substrates. When this organism was inoculated into flasks containing N.M.S. medium and methane, ethane, propane or butane, however, only propane and butane supported growth. The respiration of propane-grown JOB5 on a variety of hydrocarbon and alcohol substrates is shown in table 29 and

Table 29 Respiration of Bacterium JOB5 on Hydrocarbons, alcohols and Formate

Substrate[1]	Respiration Rate (nmoles O_2/min/mg dry wt[2])
n-butane	11
n-propane	12
ethane	10.5
methane	0
n-butanol	37
n-propanol	61
ethanol	52
methanol	3
formate	2

(1) gases added as 0.6 mls of gas-saturated buffer at 30°C. All other compounds added as 10 µmoles of aqueous solution.

(2) endogenous respiration 4 nmoles O_2/min/mg dry wt.

it is evident that a significant respiration above the endogenous could only be obtained for ethane, propane and butane and that respiration of methanol was extremely low in comparison to the higher alcohols. The respiration results, together with the lack of growth with methane and ethane as substrates, indicate that JOB5 behaves as a

conventional hydrocarbon-utiliser in not metabolising methane. The results of Perry (1968) might be explained by impurities in the methane used, which was quoted as 99% pure, and the heavy reliance on manometric techniques rather than the direct estimation of methane disappearance by gas chromatography. The low rate of methanol-stimulated oxygen uptake by washed-cell suspensions of their 'methane-grown' JOB5, which was 18% of the 'methane-stimulated' rate and 11% of the ethanol respiration rate was in direct contrast to that of a typical methane-utiliser where the methanol respiration rate typically exceeds both the methane and ethanol respiration rate (see Davey, 1971).

Growth of JOB5 on propane-N.M.S. medium was not inhibited by CO (75% O_2:25% CO) and examination of the effects of the specific inhibitors of methane oxidation showed that butane respiration in the presence of the following compounds: diethyldithiocarbamate (7.5×10^{-4}M), $\alpha\alpha'$dipyridyl (10^{-4}M), thiosemicarbazide (10^{-4}M), allyl thiourea (10^{-4}M) and hydroxylamine (10^{-3}M) was 100, 73, 90, 83 and 69% of the control rate of 70 nmoles O_2/min/mg cells and this lack of significant inhibition points to a fundamental difference between hydrocarbon metabolism by JOB5 and methylobacteria. An examination of the E.P.R. spectrum of washed cell suspensions of JOB5 grown on propane in the same N.M.S. medium as used in this study for methylobacteria showed no E.P.R.-detectable copper resonance at receiver gains of four times the sensitivity employed in figure 17. The absence of an E.P.R.-detectable copper species in the cells is consistent with the lack of participation of copper in hydrocarbon

metabolism by JOB5 which is suggested by the lack of inhibition of butane oxidation by the specific inhibitors of methane oxidation.

studies on the proposed facultative methylotroph XX

Patt et al., (1974) have challenged the current assumptions as to the obligate methylotrophy of methane-utilising bacteria in their report of the isolation of a facultative methylotroph capable of growth on methane or complex carbon sources such as sugars and nutrient agar.

The evidence presented in support of their thesis that the bacteria grown on glucose and methane were identical was based on: (1) single cell isolation from colonies, (2) a similar pattern of antibiotic sensitivity, (3) a similar level of heat tolerance, (4) the ability to recover isolates of methane-utilising organisms after 'many' single colony isolations on nutrient agar, (5) an identical DNA density as determined by CsCl gradient studies and (6) the incorporation of $^{14}CH_4$ into glucose-grown cells. Although no details are given, a footnote to the publication acknowleges Professor R. Whittenbury "for his confirmation of the facultative nature of the isolate XX."

In the year prior to the publication of Patt et al. (1974) a study of the properties of the organism XX has been underway in this laboratory by Dr Williams (personal communication). There is an inconsistency between the conclusions based on experiments (1), (2) and (3) which were based on formation of colonies on agar plates and the reported ability of XX to grow on agar, an observation which has been confirmed by Dr Williams for the

heterotrophic-, but not methane-, grown XX. However, in support of the facultative nature of XX, Dr Williams has been able to transfer glucose-grown XX to methane N.M.S. liquid medium even after subculturing single-cell colonies on nutrient agar N.M.S. medium weekly for one year although this transfer required a large inoculum and only took place after a lag of 5 to 7 days. Further transfers took place without any lag. Considerable difficulty has been encountered by Dr Williams in bringing about the reverse transformation from methane-grown cells to glucose-N.M.S. liquid medium and the few successful transfers involved very dense inocula and could be a result of survival of heterotrophic XX in the previous N.M.S.-methane culture.

Washed cell suspensions of glucose and methane-grown XX were prepared and the respiration in the presence of a variety of carbon sources determined in the oxygen probe and shown in table 30.

Table 30 Respiration rates of methane and glucose-grown XX

Substrate[1]	Respiration rates[2] (nm O_2/min/mg cells)	
	CH_4-grown	Glucose-grown
CH_4	57	>1
CH_3OH	58	67
C_2H_6	4.5	0
C_2H_5OH	45	75
HCOONa	3	67
Glucose	<1	6
Sucrose	0	2
Malate	0	18
Succinate	0	30

(1) gases added as 0.6 ml of saturated solution at 30°C and CH_3OH, C_2H_5OH and HCOONa added as 100 µl of 100 mM solution. Glucose, sucrose, malate and succinate added as 100 µl of 10% w/v solution.

(2) endogenous respiration rates 15 and 10 nmoles O_2/min/mg wt cells for CH_4 and Glucose-grown cells.

The pattern of respiration of suspensions of the methane-grown cells was similar to that of a typical obligate methane-utiliser (see Davey, 1971) in that cells respired on C_1 compounds but not tricarboxylic acid cycle intermediates. The low levels of the respirations on methane in comparison with the endogenous respiration rate by glucose-grown cells cannot be considered significant but in addition to respiring on sugars and T.C.A. cycle intermediates the suspensions of glucose-grown cells respired on methanol, formaldehyde and formate. Prompted by these observations it was found that both glucose and methane-grown cultures of XX could grow readily on methanol and this observation was noted and recorded by Patt et al., (1974).

As evidence for the single identity of the methane and glucose-grown XX, Patt et al. (1974) reported the ability of cell suspensions of the latter to incorporate $^{14}CH_4$ and also the stimulation by glucose of $^{14}CH_4$ incorporation by the suspensions of methane-grown cells. However the incorporation of $^{14}CH_4$ into glucose-grown cells was only 5.5% of the $^{14}CH_4$ incorporation by methane-grown cells. Methane respiration equivalent to the slight incorporation of $^{14}CH_4$ by the glucose-grown cells would be difficult to establish conclusively with the oxygen probe. These results show that, if the organism XX is a genuine facultative methylotroph, it must be postulated that the methane-utilising and heterotrophic phase are mutually-exclusive, although residual traces of each may remain when the other carbon source is employed. Consistent with this, the presence of complex internal type II membranes were present in thin sections of methane-grown cells only (Patt et al., 1974). Differences in the physical properties of the two

phases were found in this study where cell suspensions of the glucose-grown cultures were found to be deep red in colour and to clump easily whereas suspensions of the methane-grown culture were cream-coloured and did not clump.

An examination of the effects of the addition of allyl thiourea (10^{-4}M), thiosemicarbazide (10^{-4}M), aa'dipyridyl (10^{-4}M), 8-quinolinol (10^{-4}M) and diethyldithiocarbamate (7.5×10^{-4}M) to the rate of methane-stimulated respiration of suspensions of methane-grown XX showed that methane respiration was over 90% inhibited by all of these compounds. Thus the conclusions drawn earlier in this study that copper is involved in the methane oxygenase of Methylosinus trichosporium OB3B and Methylomonas albus BG8 can be extended to the methane-grown XX. A suspension of glucose and methane-grown XX was then examined by E.P.R. spectroscopy and the spectra obtained are presented in fig 19. An E.P.R. signal centred about $g_{\perp}$ 2.06 similar to the copper-like resonances in figures 16, 17 and 18 is clearly present but no E.P.R.-detectable copper signal was evident in the glucose-grown cultures. Similar results with two further batches of methane and glucose-grown cells confirmed the repeatibility of the observation.

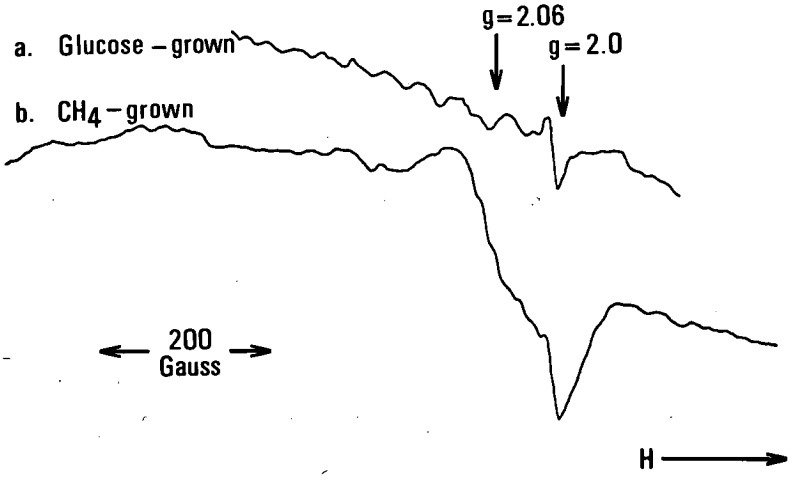
The absence of an E.P.R.-detectable copper signal in suspensions of the Gram-negative bacterium Escherichia coli, the non-methane-utilising hydrocarbon utiliser JOB5 and glucose-grown XX and the presence of E.P.R.-detectable copper in the four methylobacteria examined and methane-grown XX supports the involvement of copper in the metabolism of methylobacteria and, in the light of the inhibitor data presented in this study, in the methane

Fig. 19: Electron Paramagnetic Resonance spectra of the proposed facultative methylotroph XX grown with methane and glucose as carbon sources

Suspensions of methane-grown cells and glucose-grown cells containing 29 and 22 mg dry wt cells/ml respectively shaken in air.

Spectra were recorded under the same operating conditions as described in the notes for figure 16 with the exception that a receiver gain of 1×10^3 was employed. Spectra are reduced to 25% of original size.

Proposed facultative methylotroph XX



oxygenase. An alternative explanation involving a high affinity of the methane-utilising enzyme system or the complex internal membranes for copper from the medium cannot be eliminated by the results in this present study and it is in the elucidation of the possible role of this copper that methane-utilising cell-free systems will be undoubtedly required.

GENERAL DISCUSSION

GENERAL DISCUSSION

This study was initiated in the hope that a deeper understanding of the enigmatic methane oxygenase system could be obtained from studies on the reaction in whole cells and it is appropriate to re-evaluate the previous knowledge of the methane oxygenase in the light of the results presented in this thesis.

The ability of a number of metal-binding compounds to reversibly inhibit the oxidation of methane but not methanol by washed cell suspensions of methylobacteria provides strong evidence for the involvement of at least one species of metal ion in the methane oxygenase system. Purely chemical considerations based on the nature of the inhibitors and the reversibility of the inhibition by metal ions led to the proposed involvement of copper although the participation of other metals cannot be eliminated. Electron Paramagnetic Resonance (E.P.R.) studies of cell suspensions of methylobacteria confirm the presence of a species resembling Cu(II) in the five methylobacteria Methylomonas albus BG8, Pseudomonas methanica, Methylosinus trichosporium OB3B, Methylosinus trichosporium OB4 and CH_4-grown XX and, although the absence of this E.P.R.-detectable species in suspensions of Escherichia Coli B, Brevibacterium JOB5 and the glucose-grown proposed facultative methylotroph XX strongly support a role for copper in methylobacteria, the absence of any significant reduction of the Cu(II) species on treatment with CH_4 prevents the conclusive establishment of a role for this species in methane oxidation. The application of stop-flow E.P.R. spectroscopy methods such as have been developed for the detection of transient species

by Bray and Patterson (1961) to a study of the response of the g-value 2.06 resonance during methane oxidation may confirm such a role.

E.P.R. studies with four methylobacteria also suggested the involvement of an iron-sulphur protein in methane oxidation. In view of the evidence presented in this study for the involvement of copper in the methane oxygenase it would be of considerable interest if in any future continuous culture study of the growth of methylobacteria the role of copper was investigated in detail. Such studies should involve not only the determination, under a variety of copper-limiting conditions, of the total amount of copper detectable by chemical assay but also the amount of E.P.R.-detectable copper.

The results of the present study would suggest that one way in which studies of methane-oxidising cell-free extracts could proceed beyond the limited observations of Ribbons and Michalover (1970) and Ferenci (1974) would be if such extracts were examined for the presence of E.P.R.-detectable copper, sensitivity to metal-ion chelators and iron-sulphur proteins.

Hayaishi (1969) has summarised the roles that a metal may play in an oxygenase as follows: as a component of the electron transport system associated with mono-oxygenase systems e.g. non-haeme iron proteins, in the aggregations of subunits to form a native enzyme or in the activation of substrate, molecular oxygen or both. Copper has been implicated in the activation of oxygen by the dopamine-β-hydroxylase system (Goldstein, 1966) but it would be premature to speculate at this stage on the role of a metal

in the methane oxygenase system or to rule out the involvement of metals other than copper.

The selective inhibition of methane oxidation by carbon monoxide observed by Davey and Mitton (1973), Thomson (1974) and Ferenci (1974) was confirmed and the further observation that carbon monoxide stimulated respiration and CO_2 production by cell suspensions of methylobacteria led to the suggestion that CO was itself metabolised by methylobacteria which was confirmed by the demonstration of the production of $^{14}CO_2$ from ^{14}CO. The inhibition of CO-stimulated respiration by a variety of the specific inhibitors of methane oxidation together with the ability of cell-free extracts of Pseudomonas methanica to catalyse both NADH-dependent CH_4 and CO-stimulated respiration and CH_4 and CO-stimulated NADH oxidase activity (Ferenci, 1974) suggested that CO might be oxidised by the methane oxygenase itself and, although problems of isotope dilution by water were initially encountered, the oxygenase nature of CO oxidation was confirmed by the unambiguous demonstration of the ^{18}O-enrichment of the CO_2 evolved from suspensions of Methylosinus trichosporium OB3B incubated with CO and ^{18}O-enriched oxygen. Although a mechanism for the biological oxidation of CO involving the addition of water has been well-established (review Fenn, 1972), there are no reports of biological oxidation of CO by an oxygenase mechanism.

The conclusion of Davey and Mitton (1973) that the a-type cytochrome of methylobacteria was involved in methane oxidation was based solely on the inhibition of CH_4 oxidation by CO and the known inhibition of cytochrome oxidase by CO (Lemberg, 1969). An oxygenase mechanism for

CO oxidation suggests that CO may inhibit through competing with CH_4 rather than O_2. The two theories may be essentially similar if the observations of Breckenridge (1953) and Tzagoloff and Wharton (1965) that purified cytochrome oxidase can oxidise CO can be extended to the a-type cytochrome of methylobacteria and the involvement of copper in cytochrome oxidase (Lemberg, 1969)would make the inhibitor results consistent with such a mechanism. It would be of considerable interest to duplicate the study of Erickson et al. (1972) with Nitrosomonas and purify the a-type cytochrome from a methylobacterium and examine it for the presence of copper and sensitivity to the specific inhibitors of methane oxidation.

A difficulty in proposing a role for the a-type cytochrome in methane oxidation is the low level present in Methylomonas albus compared with Methylosinus trichosporium OB3B found by Davey (1971). Such a low level might account for the generally higher levels of CO oxidation found in type II methylobacteria but in the light of the results presented in this thesis and by Ferenci (1974) a difference in endogenous metabolism is a more likely reason.

Carbon monoxide can bind with Cu(I) and inhibit copper-containing oxygenase enzymes (Keilin and Mann, 1938; Kaufman and Friedman, 1965) and, although competition with oxygen for the oxygen-binding site of the haeme is the assumed mechanism of inhibition of cytochrome oxidase by CO, it is of interest that a model for the binding of CO to cytochrome oxidase involving bridge formation between the copper and the haeme has been proposed (Lindsay and Wilson, 1974). It is unfortunate that there has been no extension

to the work of Breckenridge (1953) and Tzagoloff and Wharton (1965) on CO oxidation by Cytochrome Oxidase since if the reaction were shown to proceed by an oxygenase mechanism it could be of considerable significance in understanding the mechanism of the terminal oxidation reaction in mammalian electron transport.

The similarity in inhibition pattern of methane oxidation in methylobacteria and ammonia oxidation in Nitrosomonas reinforces the suggestion by Quayle (1972) of an evolutionary link between the two organisms and prompted a brief examination of ammonia and hydroxylamine oxidation in Methylosinus trichosporium OB3B. Although some confusion was initially encountered because of the presence of nitrite uptake in suspensions of N.M.S. but not A.M.S.-grown cells, the production of nitrite by suspensions incubated in the presence of ammonia and hydroxylamine could be readily demonstrated and the rate of the former was stimulated by the addition of formate.

A wide substrate specificity of the methane oxygenase is suggested by the ability of cell suspensions of methylobacteria to oxidise methane, ethane, ethylene, ammonia and carbon monoxide. The suggestion by Thomson (1974) of the existence of distinct oxygenases for methane and ethane oxidation on the basis of the lack of inhibition of disappearance of the former from cell suspensions by dimethyl ether and the demonstration of the inhibition of formate-stimulated ethane oxidation in this study indicates how easily the results of inhibition studies can be mis-interpreted. Thus, although the inhibition of CO-stimulated respiration, ammonia, ethane, and ethylene oxidation by the

specific inhibitors of methane oxidation strongly supports a common oxygenase mechanism, conclusive verification will await kinetic analysis both of the inhibition by the specific inhibitors of methane oxidation and of the inhibition of methane oxidation by ammonia, carbon monoxide, ethane and ethylene. The difficulties inherent in kinetic studies with whole cells have already been discussed and such studies may have to be delayed until reproducible methane-oxidising cell-free extracts can be obtained. The ease in controlling the exact amount of ammonia in solution as NH_3 and NH_4^+, provided the pH is defined, compared with the gaseous substrates, will facilitate such kinetic analysis of the inhibition of ammonia oxidation.

A wide substrate specificity is also a feature of mammalian Cytochrome P-450 mono-oxygenase systems such as the liver microsomal system which has been reported to metabolise steroids and fatty acids as well as a large number of foreign compounds including aliphatic and aromatic hydrocarbons, amines, alcohols, phenols and thiophenes (see Orrenius and Ernster, 1974). It is difficult to envisage a binding site for a small non-polar molecule such as methane as anything but a hydrophobic region of the enzyme and one explanation is that this wide substrate specificity is due to some inherent lack of specificity in a hydrophobic substrate binding site. The effect of temperature on methane oxidation is consistent with a role for the complex internal membranes of methylobacteria and it is undoubtedly significant that only the methane-grown cells of the proposed facultative methylotroph XX possess

membranes (Patt et al., 1974). A role for the hydrophobic environment within the complex membranes as a 'solvent' to concentrate an insoluble compound such as methane is possible and it is interesting that unusually large amounts of long-chain $C_{18:1}$ fatty acids (Dugan and Weaver, 1974) are present in Methylosinus trichosporium as well as hydrocarbons such as squalene which make up 0.55% of the dry weight of Methylococcus capsulatus (Bird et al. 1971b). A role for the membrane in concentrating methane from the aqueous environment would account for the variation in Km for methane-stimulated respiration found with different batches of cells since a variation in the amount of membrane at different stages in harvesting the cells would influence the intra-cellular concentration of methane.

The inhibition of methane oxidation and therefore growth of methylobacteria by ethane (Thomson, 1974), ammonia (Phillips, 1970) and carbon monoxide (Davey and Mitton, 1973; Thomson, 1974; Ferenci, 1974) and the oxidation products acetaldehyde (Thomson, 1974) and hydroxylamine (this study) could support the suggestion advanced by Phillips (1970) that the oxidation of these compounds serves a detoxification role. A detoxification role, however, would be of secondary importance to a mere lack of specificity in the methane oxygenase resulting in the competitive inhibition of methane oxidation by these compounds which themselves are oxidised.

Energy conservation in the initial oxidation of methane would be a novel reaction among oxygenases but if it did take place, the oxidation of CO to CO_2, which can be calculated to occur with an accompanying free

energy change at 25°C of -61.4 Kcal $mole^{-1}$, may provide energy to the cell. The initial oxidation of ammonia to hydroxylamine in Nitrosomonas has been calculated to be an endergonic reaction at 25°C and pH 7 and the oxidation of hydroxylamine to nitrite, which takes place under the same conditions with a calculated free energy change of -69.1 Kcal $mole^{-1}$, has been implicated as the energy-yielding step in the metabolism of ammonia (Anderson, 1964). Thus if the free energy changes associated with the metabolism of these compounds could be coupled with the synthesis of ATP, which remains to be established, the ability to oxidise these compounds could benefit the methylobacterial cell even if they do not provide carbon for growth.

The enhancement of the rates of CO-stimulated respiration, ethane, ethylene and ammonia oxidation by formate lends support to a theory in which reducing power is required by the methane oxygenase and that this reducing power is limiting when the further metabolism of the products of oxygenation of alternative substrates do not yield reducing power or energy. Such a theory is consistent with the requirement for NADH shown by the reported methane-oxidising cell-free systems (Ribbons and Michalover, 1970; Ferenci, 1974) but, although NADPH could not serve as an alternative in these studies, it has not been established that NADH is the only in vivo electron donor to the methane oxygenase. The formate-linked hydrogenase activity in Methylomonas albus BG8 and Methylosinus trichosporium OB3B, which was also linked to hydroxybutyrate in the latter organism, suggests a way in which NADH might be linked to an oxygenase through a

ferredoxin-like compound and it is interesting that a signal in the E.P.R. spectrum of g-value 1.94 has been observed in four of the methane-treated methylobacteria. A detailed study of the hydrogenase enzyme in methylobacteria is required before any role can be established.

Thomson (1974) noted that the relative rates of disappearance of methane and ethane in washed cell suspensions was not constant when different batches of the same organism were used and in this study it was found that the CO- and CH_4- stimulated respiration rates of a range of methylobacteria (see table 14) also do not correlate. These observations, together with the ability of suspensions of Pseudomonas methanica (Ferenci, 1974) and Methylomonas agile (table 25) to carry out CO-stimulated respiration only in the presence of formate, suggests that it is the level of endogenous metabolism which determines the rate of oxygenase metabolism of 'non-growth' substrates. The evolution of hydrogen at a low rate, by suspensions of both Methylomonas albus BG8 and Methylosinus trichosporium OB3B incubated anaerobically in the absence of substrate (table 26) confirms the ability of endogenous metabolism to generate electrons and the stimulation of endogenous $^{14}CO_2$ production from ^{14}C-labelled cell suspensions of Methylosinus trichosporium but not Methylomonas albus suggests that Methylosinus trichosporium is able to mobilise endogenous reserves to provide reducing power for the oxygenase-mediated metabolism of carbon monoxide. The only storage polymer that has been conclusively demonstrated in methylobacteria is Poly Hydroxy Butyrate (P.H.B.) (Whittenbury et al., 1970; Thomson, 1974; Dugan and Weaver, 1974), although Thomson (1974) suggests

the possibility of squalene fulfilling such a function. Acetone production, which was shown by Thomson (1974) to accompany P.H.B. breakdown in Methylosinus trichosporium OB3B, did not take place to any significant extent during CO oxidation, thus another storage compound, the metabolism of P.H.B. by an alternative pathway, or merely the mobilisation of a pool of metabolites must be implicated. Thomson (1974) overlooked the possibility that the possession of a complete Tricarboxylic Acid (T.C.A.) Cycle in membrane-type II methylotrophs (Davey et al., 1972) might enable Methylosinus trichosporium OB3B to metabolise P.H.B. to hydroxybutyrate, acetoacetyl Coenzyme A, acetyl Coenzyme A and CO_2. The role of the complete T.C.A. Cycle in the metabolism of type II methylotrophs was assumed by Davey et al. (1972) to be only that of providing carbon skeletons for cell synthesis and an examination of the role of the T.C.A. Cycle in methylobacteria is likely to provide an interesting field of study.

A corollary of a requirement of the methane oxygenase for reducing power is that, even when methane is the substrate and the products of methane oxidation can provide electrons for the initial oxidation, reducing power from endogenous sources would be required to initiate the oxygenase reaction. This would be particularly important in the natural environment when the cell may have to survive without substrate for long periods and the presence of large amounts of lipid in the resting stages of some of the methylobacteria (Whittenbury et al., 1970) is consistent with such a role. It is unfortunate that the studies which have demonstrated only a low incorporation into cellular

material of acetate have been carried out with the membrane-type I methylobacterium Methylococcus capsulatus (Patel et al., 1969, Eccleston and Kelly, 1973), which has an incomplete T.C.A. Cycle (Patel et al., 1969; Davey et al., 1972), but the absence of any evidence for the incorporation in significant amounts of acetate or CO_2, the ability of cells to respire on acetate (Davey, 1971) or the coupling of NADH oxidation to energy production is consistent with a role for storage polymers in methylobacteria in providing reducing power for the initial oxygenation of methane analogous to a starting handle or battery of a car. A detailed study of the nature and role of endogenous metabolism and storage polymers in methylobacteria would thus be of considerable relevance to the mechanism of methane oxidation.

The requirement for NADH and the 1:1 ratio between NADH disappearance and oxygen uptake found in the cell-free studies of methane oxidation (Ribbons and Michalover, 1970 and Ferenci, 1974) does not necessarily indicate a mono-oxygenase mechanism for methane oxidation and, as discussed in the introduction to this study the stoichiometries of CH_4 and O_2 consumption during growth of methylobacteria are evidence against this mechanism. If carbon monoxide oxidation is assumed to be completely analogous to methane oxidation the observed stoichiometry of between 0.5 - 0.7:1 for O_2:CO uptake would support a ratio of at least 0.5 moles of oxygen for each mole of methane oxidised in the initial step. Although it is uncertain to what extent the mechanism of ammonia oxidation in Nitrosomonas is relevant to ammonia and methane oxidation in methylobacteria, a

stoichiometry of 1.5:1 for oxygen: ammonia for the conversion of ammonia to nitrite in cell-free extracts has been found (Suzuki and Kwok, 1970), whereas an initial oxygenation of ammonia by a mono-oxygenase mechanism, which might be expected on the basis of the elimination of the lag in ammonia-stimulated respiration by the addition of hydroxylamine, (Hooper, 1969b), would require a stoichiometry of 2:1. In neither of the two cell-free studies of ammonia oxidation in Nitrosomonas (Suzuki and Kwok, 1970; Suzuki et al., 1974) or Nitrocystis (Watson et al., 1970) was a requirement showed for NADH although the extracts of Nitrocystis required A.T.P. The absence of such a requirement does not eliminate the involvement of reduced pyridine nucleotides since the crude extracts may have contained endogenous NADH-generating systems.

The contradiction between the manifest requirement for reducing power in the oxidation of substrates other than methane and the stoichiometric data can be resolved by analogy with dioxygenases, some of which have been showed to require reducing power e.g. anthranilate hydroxylase (Hayaishi, 1966). Whereas in a conventional mono-oxygenase (Hayaishi, 1969) the reductant reduces the oxygen atom that is not incorporated to water, in a di-oxygenase the role may be the reduction or activation of the oxygen molecule or the reduction of a copper enzyme to the Cu(I) oxidation state.

A NADH-requiring oxygenase reaction in which both oxygen atoms are incorporated into molecules of methane resulting in the formation of methanol (equation 13)

$$2CH_4 + O_2 \xrightarrow{NADH} 2CH_3OH \qquad (13)$$

would overcome many of the problems encountered with the mono-oxygenase mechanism in equation 10 and is a more favourable alternative to the dimethyl ether pathway proposed by Wilkinson (1971) and Davey (1971) in which the metabolism of dimethyl ether involves a further oxygenase reaction (Mitton et al., in preparation).

Both the mechanism described in equation 13 and the dimethyl ether pathway would show a requirement for reducing power and the problems discussed by Davey (1971) in accounting for yields of greater than 50% incorporation of methane into cell carbon, if the reducing power were derived from the formate dehydrogenase-mediated metabolism of formate, apply to both mechanisms. The fixation of CO_2 must be postulated to account for these higher yields. The need for further continuous culture studies of yield and stoichiometries of pure cultures of methylobacteria under a variety of substrate-limiting conditions has been emphasised and these studies may help distinguish between the following alternatives:

(1) That the non-energy yielding oxygenation of methane takes place by the scheme in equation 13 with a considerable amount of CO_2 fixation.

(2) The oxygenase metabolism of methane is energy-yielding. A possible mechanism for such a novel reaction would involve electron transport from NADH and other electron donors accompanied by phosphorylation to the a-type cytochrome or a copper-containing oxygenase as the terminal electron acceptor and, instead of O_2 being reduced to water in the terminal oxidation reaction, the oxygen atoms are

incorporated into the substrate. A similar mechanism has been proposed by Higgins and Quayle (1970) whereby only one of the oxygen molecules is reduced to water and the other participates in the oxygenase reaction.

(3) The natural acceptor of the methanol and formaldehyde dehydrogenase reaction may be linked to the methane oxygenase either directly or indirectly through the production of NADH from the A.T.P.-mediated reversal of electron transport. This means of generating NADH has been demonstrated in Nitrosomonas europea (Aleem, 1966), where the need for NADH was envisaged not for the initial oxygenase reaction but for the fixation of CO_2.

(4) The reaction might not take place by equation 13: the stoichiometries of oxygen and methane uptake obtained by Harwood and Pirt (1972) from oxygen-limited continuous culture studies with Methylococcus capsulatus are too low to accommodate even a dioxygenase mechanism and an alternative might be a free radical chain reaction, the initiation of which requires reducing power for activation of oxygen but in which water may participate if molecular oxygen is limiting. Such a mechanism may explain the accumulation of dimethyl ether in suspensions under ill-defined conditions (Thomson, 1974). The lack of evidence for such a mechanism places it well in the realms of speculation but in the absence of any evidence for the other mechanism it deserves consideration. It is interesting, but not necessarily relevant,

that studies on the photochemical decomposition of CH_4 and CO in the upper atmosphere have suggested that both can be metabolised by the hydroxyl radical to form HCHO and CO_2 respectively (McConnel et al., 1971).

The possibilities (1) to (4) are by no means mutually exclusive and confirmation of the involvement of any of these mechanisms will require a detailed study of the electron transport system in methylobacteria in both whole cells and methane-oxidising cell-free extracts, precise carbon and energy balances in cells grown in continuous culture and confirmation in cell-free extracts of the initial products and stoichiometry of methane oxygenation. Close attention should be paid to any advances made in the studies of the metabolism of ammonia by Nitrosomonas as these may shed considerable light on the problem in methylobacteria.

As more is discovered about chemolithotrophic bacteria and methylotrophs, many of the myths and folklore surrounding the autotrophic bacteria, which were initiated by the classic studies of Winogradsky (1890), have now been exposed and the internal biochemistry of these organisms have been shown to be essentially similar to heterotrophic bacteria in all but the metabolism of growth substrate (reviews: Rittenberg, 1969; Kelly, 1971). Earlier generalisations of the inhibition by organic compounds per se of growth of autotrophs and methylotrophs have been proved untrue and in cases where inhibition by an organic compound has been demonstrated, explanations

involving interactions with other biochemical pathways have been advanced. Thus Eccleston and Kelly (1973) explained as end-product inhibition of branched amino acid synthetic pathways the inhibition of growth of Methylococcus capsulatus by the amino acids L-threonine, L-phenylalanine, L-tyrosine and L-homoserine but not L-histidine at 10^{-3}M and the specific inhibition of methane oxidation by suspensions of Methylosinus trichosporium supports a mechanism of inhibition by histidine involving the interaction with a metal component of the methane oxygenase.

The considerable difficulties encountered in growing methylobacteria may also be explained by the ease of inhibition of the methane oxygenase by compounds which bind metals such as thiosemicarbazide which can be a component of red rubber (Hooper and Terry, 1973). It would be interesting to know whether methane oxidation is inhibited by another component of red rubber carbon disulphide, which was recently shown to inhibit nitrification (Powlson and Jenkinson, 1971).

The formation of toxic products and the inability to obtain energy from reduced pyridine nucleotides have both been advanced to explain the inability of methylobacteria to grow on organic compounds other than methane and methanol but the simplest explanation is that proposed by Ribbons et al. (1970) and echoed by Davey et al. (1972), Quayle (1972) and Thomson (1974) that obligate methylotrophy is an outcome of the inability of methylobacteria to derive energy from the metabolism of compounds other than methane or methanol.

Current views on obligate autotrophy have been challenged by the substantiated reports of the successful cultivation of autotrophic bacteria in heterotrophic media under carefully controlled conditions (review: Kelly, 1971) and although the notorious ease of contamination of methylobacterial cultures renders the reports in the early literature of heterotrophic methane-utilisers suspect (review: Silverman, 1964) three reports have recently appeared of heterotrophic methane-utilisers which were all isolated from fresh water lakes (Naguib and Overbeek, 1970; Cappenberg, 1972; Patt et al., 1974). Only in the case of the Gram-negative bacterium XX has there been any attempt to rigorously prove that the heterotrophic methane-utiliser was not a contaminant and their evidence together with some of the difficulties encountered in repeating their work in this laboratory has already been discussed. If the facultative nature of the methylotrophy can be established beyond all doubt the nature of the transition between the methane-oxidising stage with membranes and the heterotrophic stage will be an exciting and rewarding field of study and the presence of an E.P.R. Cu(II) species in the methane but not glucose-grown cells will acquire real significance. The existence of an inducible methane oxygenase enzyme will pose no conceptual problems and the growth of the glucose-grown XX on methanol could provide a mechanism by which growth could be achieved with methane as carbon source but although the glucose-metabolising enzymes might also be inducible it is difficult to understand why the methane-grown phase does not respire on Tricarboxylic Acid Cycle intermediates.

The most significant question in the understanding of the nature of obligate methylotrophy will be why these two processes are so mutually exclusive.

There have been no detailed studies reported on the quantitative role of methylobacteria in the natural recycling of methane and because of the additional abilities of these organisms to oxidise hydrocarbons, carbon monoxide, ethylene and ammonia this is a serious omission. The current interest in the possible role of ethylene in controlling fungistasis (Smith, 1973; Smith and Cook, 1974), the toxicity to man of the globe's most common pollutant, carbon monoxide, (Jaffe, 1970), the importance of nitrification and nitrogen fixation in agriculture and pollution are all pressing reasons for embarking on such studies, especially in the light of the proposed widespread application of nitrogen stabilisers such as N-SERVE (Prasad et al., 1971).

ACKNOWLEDGEMENTS

I would like to express my thanks to the following:

Professor J.F. Wilkinson for his supervision of this work and criticism of the manuscript.

Dr J.I. Mason for recording the electron paramagnetic resonance spectra and his valuable advice on their interpretation.

Dr P.C. Bell for carrying out the combined gas chromatography and mass spectrometry analyses.

Dr J.R. Mitton and Dr A.W. Thomson and Dr T. Stephenson for useful discussions during the course of the study.

Dr Sutherland for his helpful advice in the preparation of the manuscript.

Eleanor Clark for her constant vigilance in sub-culturing and maintaining the purity of the cultures of methylobacteria used in this study.

Mr G. Finnie for his assistance in preparation of diagrams.

Mrs P. Cameron for typing the manuscript.

British Petroleum Ltd. who financed the research.

REFERENCES

REFERENCES

ALEEM, M.I.H. (1966). Generation of reducing power in chemosynthesis. II Energy-linked reduction of pyridine nucleotides by the chemoautotroph, Nitrosomonas europaea. Biochim. Biophys. Acta. 113, 216-224.

ALEEM, M.I.H. (1970). Oxidation of inorganic nitrogen compounds. Ann. Rev. Plant Physiol. 21, 67-90.

ALEEM, M.I.H. & LEES, H. (1963). Autotrophic enzyme systems. I Electron transport systems concerned with hydroxylamine oxidation in Nitrosomonas. Can. J. Biochem. Physiol. 41, 763-778.

ANDERSON, J.H. (1964). Studies on the oxidation of ammonia to hydroxylamine by Nitrosomonas. Biochem. J. 92, 1c-3c.

ANDERSON, J.H. (1965). Studies on the oxidation of ammonia by Nitrosomonas. Biochem. J. 94, 688-698.

ANDERSON, J.R., STRUMEYER, D.H. & PRAMER, D. (1968). Purification and properties of peroxidase from Nitrosomonas europaea. J. Bacteriol. 96, 93-97.

ANTHONY, C. (1975). The Microbial Metabolism of C_1 compounds: The cytochromes of Pseudomonas AM1. Biochem. J. in press.

ANTHONY, C. & ZATMAN, L.J. (1964a). The microbial oxidation of methanol. I Isolation and properties of Pseudomonas sp. M27. Biochem. J. 92, 609-613.

ANTHONY, C. & ZATMAN, L.J. (1964b). The Microbial oxidation of methanol. 2 The methanol-oxidising enzyme of Pseudomonas sp. M27. Biochem. J. 92, 614-621.

ANTHONY, C. & ZATMAN, L.J. (1967a). The Microbial oxidation of methanol. Purification and properties of the alcohol dehydrogenase of Pseudomonas sp. M27. Biochem. J. 104, 953-959.

ANTHONY, C. & ZATMAN, L.J. (1967b). The Microbial oxidation of methanol. The prosthetic group of the alcohol dehydrogenase of Pseudomonas sp. M27: a new oxidoreductase prosthetic group. Biochem. J. 104, 960-969.

ANTHONY, C. & ZATMAN, L.J. (1965). The Microbial oxidation of methanol. The alcohol dehydrogenase of Pseudomonas sp. M27. Biochem. J. 96, 808-812.

BAPTIST, J.N., GHOLSON, R.K. & COON, M.J. (1963). Hydrocarbon oxidation by a bacterial enzyme system. I Products of octane oxidation. Biochem. Biophys. Acta. 69, 40-47.

BEINERT, H., GRIFFITHS, D.E., WHARTON, D.C. & SANDS R.H. (1962). Properties of the copper associated with cytochrome oxidase as studied by paramagnetic resonance spectroscopy. J. Biol. Chem. 237, 2337-2346.

BELLION, E. & HERSH, L.B. (1972). Methylamine metabolism in a Pseudomonas spp. Arch. Biochem. Biophys. 153, 368-374.

BIDWELL, R.G.S. & BEBEE, G.P. (1974). Carbon monoxide fixation by plants. Can. J. Botany 52, 1841-1847.

BIDWELL, R.G.S. & FRASER, D.E. (1972). Carbon monoxide uptake and metabolism by leaves. Can. J. Bot. 50, 1435-1439.

BIRD, C.W., LYNCH, J.M., PIRT, S.J. & REID, W.W. (1971a). The identification of Hop-22 (29)-ene in prokaryote organisms. Tetrahedron Letts. 34, 3189-3190.

BIRD, C.W., LYNCH, J.M., PIRT, S.P., REID, W.W., BROOKS, C.J.W. & MIDDLEDITCH, B.S. (1971b). Steroids and squalene in Methylococcus capsulatus grown on methane. Nat. 230, 473.

BLUMBERG, W.E., GOLDSTEIN, M.M., LAUBER, E. & PEISACH, J. (1965). Magnetic resonance studies on the mechanism of the enzymic β-hydroxylation of 3,4-dihydroxyphenolethylamine. Biochem. Biophys. Acta. 99, 187-190.

BLUMER, M., CHASE, T. & WATSON, S.W. (1969). Fatty acids in the lipids of marine and terrestial nitrifying bacteria. J. Bacteriol. 99, 280-282.

BOYD, G.S. (1972). Ed. Biological hydroxylation mechanisms. Biochemical Soc. Symp. 34, Academic Press.

BRAY, R.C. & PETTERSSON, R. (1961). Electron spin resonance methods. Biochem. J. 81, 194-195.

BRECKENRIDGE, B. (1953). Carbon monoxide oxidation by cytochrome oxidase in muscle. Am. J. Physiol. 173, 61-69.

BROBERG, P.L. & SMITH (1967). The cytochrome system of Bacillus megaterium KM: the presence and some properties of two CO-binding cytochromes. Biochem. Biophys. Acta. 131, 479-479.

BROWN, L.R. & STRAWINSKI, R.J. (1958). Intermediates in the oxidation of methane. Bact. Proc. 58, 122.

BROWN, L.R., STRAWINSKI, R.J. & McCLESKY, C.S. (1964). The Isolation and characterisation of Methylomonas methano-oxidans. Can. J. Microbiol. 10, 791-799.

BUNDY, L.G. & BREMNER, J.M. (1973). Inhibition of nitrification in soils. Soil Sci. Soc. Amer. Proc. 37, 396-398.

CAMPBELL, N.E.R. & ALEEM, M.I.H. (1965). The effect of 2-chloro,6-(tricholoromethyl) pyridine on the chemoautotrophic metabolism of nitrifying bacteria. I Ammonia and hydroxylamine oxidation by Nitrosomonas. Ant. van. Leeuwenhoek 31, 124-136.

CAPPENBERG, Th.E.,(1972). Ecological observations on heterotrophic methane-oxidising and sulphate-reducing bacteria in a pond. Hydrobiologia 40, 471-485.

CARDINI, G. & JURTSHUK, P. (1968). Cytochrome P-450 involvement in the oxidation of n-octane by cell-free extracts of Corynebacterium sp; strain 7E1C. J. Biol. Chem. 243, 6070-6072.

CASTOR, L.N. & CHANCE, B. (1959). Photochemical determinations of the oxidases of bacteria. J. Biol. Chem. 234, 1587-1592.

CHAPPELLE, E.W. (1962a). Carbon monoxide oxidation by algae. Biochem. Biophys. Acta. 62, 45-62.

CHAPPELLE, E.W. (1962b). Carbon monoxide metabolism. Dev. Ind. Microbiol. 3, 99-122.

CHOUTEAU, J., AZOULAY, E. & SENEZ, J.C. (1962). Anaerobic formation of n-heptene from n-heptene by resting cells of Pseudomonas aeruginosa. Nature 194, 576-578.

CLARK, C. & SCHMIDT, E.L. (1967). Growth response of Nitrosomonas europaea to amino acids. J. Bacteriol. 93, 1302-1308.

CLELAND, W.W. (1963). The kinetics of enzyme-catalysed reactions with two or more substrates or products. II Inhibition: nomenclature and theory. Biochim. Biophys. Acta. 67, 173-187.

COLBY, J. & ZATMAN, L.J. (1972). Hexose phosphate synthase and tricarboxylic acid cycle enzymes in Bacterium 4B6, an obligate methylotroph. Biochem. J. 128, 1373-1376.

COLLINS, V.G. (1969). Isolation, cultivation and maintainance of autotrophs. In: Methods in microbiology, Norris, J.R., Ribbons, D.W., Eds., 3B, pp. 1-52, Academic Press, London and N.Y.

COON, M.J., STROBEL, H.W. & BOYER, R.F. (1973). On the Mechanism of hydroxylation reactions catalysed by Cytochrome P-450. Drug. Metabol. Disp. 1, 92-97.

COOPERSTEIN, S.J. (1963). Reversible inactivation of cytochrome oxidase by disulphide bond reagents. J. Biol. Chem. 238, 3606-3610.

COTY, V.F. (1967). Atmospheric nitrogen fixation by hydrocarbon-oxidising bacteria. Biotechnol. and Bioeng. IX, 25-32.

DAHL, J.S., MEHTA, R.J. & HOARE, D.S. (1972). New obligate methylotroph. J. Bacteriol. 109, 916-921.

DAVEY, J.F. (1971). A study of methane-oxidising bacteria. Ph.D. Thesis, University of Edinburgh.

DAVEY, J.F. & MITTON, J.R. (1973). Cytochromes of two methane-utilising bacteria. FEBS Letters 37, 335-337.

DAVEY, J.F., WHITTENBURY, R. & WILKINSON, J.F. (1972). The distribution in the methylobacteria of some key enzymes concerned with intermediary metabolism. Arch. Microbiol. 87, 359-366.

DAVIES, S.L. & WHITTENBURY, R. (1970). Fine structure of methane and other hydrocarbon-utilising bacteria. J. Gen. Microbiol. 61, 227-232.

DE BOER, W.E. & HAZEU, W. (1972). Observations on the fine structure of a methane-oxidising bacterium. Antonie van Leewenhoek 38, 33-47.

DE BONT, J.A.M. & MULDER, E.G. (1974). Nitrogen fixation and co-oxidation of ethylene by a methane-utilising bacterium. J. Gen. Microbiol. 83, 113-121.

DOSTALEK, M., HAGGESTROM, L. & MOLIN, N. (1972). Optimisation of biomass production from methanol. Proc. IV International Fermentation Symposium: Fermentation Technology Today, 497-501.

DOW CHEMICAL COMPANY (1973). Technical information bulletin for N-SERVE Nitrogen stabilisers.

DUGAN, P.R. & WEAVER, T.L. (1974). Bacterial methane oxidation and its influence in the aquatic environment, Project A-027-OHIO, Office of water research and technology, United States Department of the Interior.

DWORKIN, M. & FOSTER, J.W. (1956). Studies on Pseudomonas methanica (Söhngen) nov, comb. J. Bacteriol. 72, 646-659.

ECCLESTON, M. & KELLY, D.P. (1972). Assimilation and toxicity of exogenous amino acids in the methane-oxidising bacterium Methylococcus capsulatus. J. Gen. Microbiol. 71, 541-554.

ECCLESTON, M. & KELLY, D.P. (1973). Assimilation and toxicity of some exogenous C_1 compounds, alcohols, sugars and acetate in the methane-oxidising bacterium Methylococcus capsulatus. J. Gen. Microbiol. 75, 211-221.

ERICKSON, R.H. & HOOPER, A.B. (1972). Preliminary characterisation of a variant CO-binding haeme protein from Nitrosomonas. Biochim. Biophys. Acta. 275, 231-244.

ERICKSON, R.H., HOOPER, A.B. & TERRY, K.R. (1972). Solubilisation and purification of Cytochrome a_1 from Nitrosomonas. Biochim. Biophys. Acta. 283, 155-166.

EROSHIN, V.K., HARWOOD, J. & PIRT, S.J. (1968). Influence of amino acids and sugars on the growth of Methylococcus capsulatus on methane. J. Appl. Bacteriol. 31, 560-567.

ESTABROOK, R.W. (1967). Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. In: Methods in Enzymology, Vol. 10, R.W. Estabrook, M.E. Pullman (eds.), pp. 41-47. Academic Press, New York.

FENN, W. (1970). The burning of carbon monoxide in tissues. Ann. N.Y. Academy of Science 174, 64-71.

FENN, W.O. & COBB, D.M. (1932). The stimulation of muscle respiration by carbon monoxide. Am. J. Physiol. 102, 379-392.

FERENCI, T. (1974). Carbon monoxide-stimulated respiration in methane-utilising bacteria. FEBS Letters 11, 94-97.

FERENCI, T., STRØM, T., & QUAYLE, J.R. (1974). Purification and properties of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase from Methylococcus capsulatus. Biochem. J. 144, 477-486.

FOSTER, J.W. & DAVIS, R.H. (1966). A methane-dependent coccus, with notes on classification and nomenclature of obligate methane-utilising bacteria. J. Bacteriol. 91, 1924-1931.

FUCHS, G., SCHNITKER, U. & THAUER, R.K. (1974). Carbon monoxide oxidation by growing cultures of Clostridium pasteurianum. Eu. J. Biochem. 49, 111-115.

FUKUOKA, S. (1974). Relationship between productivity and K_{1a} on cultivation of methane-utilising bacteria. In: the Proceedings of the first International symposium on microbial growth on C_1 compounds, 18.

GEL'MAN, N.S., LUKOYANOVA, M.A. & OSTROVSKI, D.N. (1967). Respiration and phosphorylation of bacteria. Plenum Press, N.Y.

GHOLSON, R.K., BAPTIST, J.N. & COON, M.J. (1963). Hydrocarbon oxidation by a bacterial enzyme system. II Cofactor requirements for octanol formation from Octane. Biochemistry 2, 1155-1159.

GIBSON, D.T. (1971). The microbial oxidation of aromatic hydrocarbons. Crit. Rev. Microbiol. 1, 199-237.

GOLDSTEIN, M. (1966). Dopamine-β-hydroxylase-a copper enzyme. In: The biochemistry of copper, J. Peisach, P. Aisen, W.E. Blumberg, (eds.), pp. 443-454, Academic Press, N.Y.

GORING, C.A.I. (1962a). Control of nitrification of ammonia fertilisers and urea by 2-chloro-6-(trichloro-methyl) pyridine. Soil Sci. 93, 211-218.

GORING, C.A.I. (1962b). Control of nitrification of ammonium fertilisers and urea by 2-chloro-6-(trichloro-methyl) pyridine. Soil Sci. 93, 431-439.

GRAY, C.T. & GEST, H. (1965). Biological formation of molecular hydrogen. Science 148, 186-192.

GUNSALUS, I.C. & MARSHALL, V.P. (1971). Monoterpene dissimilation: chemical and genetic models. Crit. Rev. Microbiol. 1, 291-309.

GUNSALUS, I.C., MEEKS, J.R., LIPSCOMB, J.D., DEBRUNNER, P. & MÜNCK, E. (1974). Mono-oxygenases - the P-450 cytochrome system. In: Molecular Mechanisms of Oxygen Activation, Hayaishi, O. (ed.), pp. 559-613, Academic Press, New York.

HACKING, A.J. & QUAYLE, J.R. (1974). Purification and properties of Malyl-Coenzyme A Lyase from Pseudomonas AM1. Biochem. J. 139, 399-405.

HAMILTON, G.A. (1969). Mechanisms of two and four electron oxidations catalysed by some metallo-enzymes. Adv. in Enzymology 32, 55-96.

HAMMOND, R.K. & WHYTE, D.C. (1970). Inhibition of carotenoid hydroxylation to Staphylococcus aureus by mixed-function oxidase inhibitors. J. Bacteriol. 103, 607-612.

HARRINGTON, A.A. & KALLIO, R.E. (1960). Oxidation of methanol and formaldehyde by Pseudomonas methanica. Can. J. Microbiol. 6, 1-7.

HARRISON, D.E. (1973). Studies on the affinity of methanol- and methane-utilising bacteria for their carbon substrates. J. Appl. Bact. 36, 99-108.

HARRISON, D.E.F., TOPIWALA, H.I. & HAMER, G. (1972). Yield and productivity in single-cell protein production from methane and methanol. In: Fermentation Technology Today 491-495, G. Terini (ed.), Society of fermentation technology, Japan.

HARVEY, A.E. & MANNING, D.C. (1950). Spectrophotometric methods of establishing empirical formulas of coloured complexes in solution. J. Amer. Chem. Soc. 72, 4488-4493.

HARWOOD, J.H. & PIRT, S.J. (1972). Quantitative aspects of growth of the methane-oxidising bacterium Methylococcus capsulatus on methane in shake flask and continuous chemostat culture. J. Appl. Bact. 35, 597-607.

HASH, J.H. (1972). Liquid scintillation counting in microbiology. In: Methods in Microbiology 6B, Norris, J.R. and Ribbons, D.W. (eds.), pp. 109-155, Academic Press, New York and London.

HAYAISHI, O. (1966). Enzymic studies on the mechanism of double hydroxylation. Pharmacological Rev. 18, 71-75.

HAYAISHI, O. (1969). Enzymic Hydroxylations. Ann. Rev. Biochem. 38, 21-44.

HAYAISHI, O. (1974). Ed. Molecular Mechanisms of oxygen activation, Academic Press Inc., N.Y.

HAYAISHI, O., KATAGIRI, M. & ROTHBERG, S. (1955). Mechanism of the pyrocatechase reaction. J. Amer. Chem. Soc. 77, 5450-5451.

HEMMERICH, P. (1966). Model studies on the binding of univalent and redox active copper in proteins. In: The Biochemistry of Copper, Peisach, J., Aisen, P. and Blumberg, W.E. (eds.), pp. 15-34, Academic Press, N.Y.

HEPTINSTALL, J. & QUAYLE, J.R. (1970). Pathways leading to and from serine during growth of Pseudomonas AM1 on C1 compounds or succinate. Biochem. J. 117, 563-572.

HERSH, L.B. & BELLION, E. (1972). Malate cleavage reaction in Pseudomonas species (Shaw strain MA). Biochem. Biophys. Res. Comm. 48, 712-719.

HIGGINS, I.J. & QUAYLE, J.R. (1970). Oxygenation of methane by methane-grown Pseudomonas methanica and Methylomonas methanooxidans. Biochem. J. 118, 201-208.

HILDEBRANDT, A.G. (1972). The binding of metapyrone to cytochrome P-450 and its inhibitory action on microsomal hepatic mixed function oxidation reactions. In: Biological hydroxylation mechanisms, G.S. Boyd, R.M. Smellie (eds.), pp. 79-102. Academic Press, N.Y.

HIRSCH, P. (1968). A photosynthetic bacterium growing under carbon monoxide. Nature (Lond.) 217, 555-556.

HOFMAN, T. & LEES, H. (1953). The Biochemistry of the nitrifying organisms. 4. The respiration and intermediary metabolism of Nitrosomonas. Biochem. J. 54, 579-583.

HOOPER, A.B. (1969a). Biochemical bases of obligate autotrophy in Nitrosomonas europaea. J. Bacteriol. 97, 776-779.

HOOPER, A.B. (1969b). Lag phase of Ammonia oxidation by resting cells of Nitrosomonas europaea. J. Bacteriol. 97, 968-969.

HOOPER, A.B. & NASON, A. (1965). Characterisation of the hydroxylamine-cytochrome c reductase from the chemolithotrophs Nitrosomonas europaea and Nitrocystis oceanus. J. Biol. Chem. 240, 4044-4057.

HOOPER, A.B. & TERRY, K. (1973). Specific inhibitors of ammonia oxidation in Nitrosomonas. J. Bacteriol. 115, 480-485.

HUGHES, M.N. (1972). The inorganic chemistry of biological processes. Wiley-Interscience.

HUTTON, W.E. & ZOBELL, C.E. (1953). Production of nitrite from ammonia by methane-oxidising bacteria. J. Bacteriol. 65, 216-219.

INMAN, R.E. & INGERSOLL, R.B. (1971). Note on the uptake of carbon monoxide by soil fungi. J. Air Pollution Control Association, 21, 646-647.

JAFFE, L.S. (1970). Sources, characteristics, and fate of atmospheric carbon monoxide. Ann. N. Y. Acad. Sci. 174, 76-88.

JEENES, D.J. (1972). Respiration and electron transport in methylotrophs. Honours Thesis. University of Edinburgh.

JOHNSON, P.A. & QUAYLE, J.R. (1964). Microbial growth on C_1 compounds. 6. Oxidation of methanol, formaldehyde and formate by methanol-grown Pseudomonas AM1. Biochem. J. 93, 281-290.

JOHNSON, P.A. & QUAYLE, J.R. (1965). Microbial growth on C_1 compounds. Synthesis of cell constituents by methane- and methanol-grown Pseudomonas methanica. Biochem. J. 95, 859-867.

JURTSHUK, P. & CARDINI, G.E. (1971). The mechanism of hydrocarbon oxidation by a Corynebacterium species, Crit. Rev. Microbiol. 1, 239-289.

KANEDA, T. & ROXBURGH, J.M. (1959). A methanol-utilising bacterium. II Studies on the pathway of methanol assimilation. Can. J. Microbiol. 5, 187-195.

KATAGIRI, M., GANGULI, B.N. & GUNSALUS, I.C. (1968). A soluble cytochrome P-450 functional in methylene hydroxylation. J. Biol. Chem. 243, 3543-3546.

KAUFMAN, S. & FRIEDMAN, S. (1965). Dopamine-β-Hydrolyase. Pharmacological Rev. 17, 71-100.

KAVANAGH, E.P. & POSTGATE, J.R. (1970). Absorption and release of hydrocarbons from rubber closures: a source of errors in biological assays. Lab. Practice 19, 159-160.

KEILIN, D. & MANN, T. (1938). Polyphenol oxidase: purification, nature and properties. Proc. Roy. Soc. B 125, 187-204.

KELLY, D.P. (1971). Autotrophy: Concepts of lithotrophic bacteria and their organic metabolism. Ann. Rev. Microbiol. 25, 177-210.

KEMP, M.B. (1974). Hexose phosphate synthase from Methylococcus capsulatus makes D-arabino-3-hexulose phosphate. Biochem. J. 139, 129-134.

KEMP, M.B. & QUAYLE, J.R. (1966). Microbial growth on C_1 Compounds, Incorporation of C_1 units into allulose phosphate by extracts of Pseudomonas methanica. Biochem. J. 99, 41-48.

KEMP, M.B. & QUAYLE, J.R. (1967). Microbial growth on C_1 compounds. Uptake of ^{14}C formaldehyde and ^{14}C formte by methane-grown Pseudomonas methanica and determination of the hexose labelling pattern after brief incubation with ^{14}C methanol. Biochem. J. 102, 94-102.

KING, T.E., MASON, H.S. & MORRISON, M. (eds.), (1973). Oxidases and Related Redox Systems I, II. Univ. Park Press, Baltimore.

KISTNER, A. (1953). On a bacterium oxidising carbon monoxide. Proc. Roy. Soc. C. (Amsterdam) 56, 443-450.

KISTNER, A. (1954). Conditions determining the oxidation of carbon monoxide and of hydrogen by Hydrogenomonas carboxydomonas. Proc. Roy. Soc. C. (Amsterdam), 57, 186-195.

KLUCAS, R.V. & EVANS, H.J. (1968). An electron donor system for nitrogenase-dependent acetylene reduction by extracts of soybean noducles. Pl. Physiol. 43, 1458-1460.

KLUG, M.J. & MARKOVETZ, A.J. (1971). Utilisation of aliphatic hydrocarbons by micro-organisms. Adv. Microbial. Physiol. 5, 1-43.

KLUYVER, A.J. & SCHNELLEN, C.G.T.P. (1947). On the fermentation of carbon monoxide by pure cultures of methane bacteria. Arch. Biochem. Biophys. 14, 57-70.

KOJIMA, Y., FUJISAWA, H., NAKAZAWA, A., NAKAZAWA, T., KANETSUNA, F., TANIUCHI, H., NOZAKI, M. & HAYAISHI, O. (1967). Studies on pyrocatechase 1. Purification and spectral properties. J. Biol. Chem. 242, 3270-3278.

KUSUNOSE, M., MATSUMOTO, J., ICHIHARA, K., KUSUNOSE, E. & NOZAKA, J. (1967). Requirement of three proteins for hydrocarbon oxidation. J. Biochem. 61, 665-667.

LADNER, A. & ZATMAN, L.J. (1969). Formaldehyde oxidation by the methanol dehydrogenase of Pseudomonas PP. J. Gen. Microbiol. 55, xvi.

LANE, G.A. & DOLE, M. (1956). Fractionation of oxygen isotopes during respiration. Science 123, 574-576.

LAWRENCE, A.J. & QUAYLE, J.R. (1970). Alternative carbon assimilation pathways in methane-utilising bacteria. J. Gen. Microbiol. 63, 371-374.

LEADBETTER, E.R. & FOSTER, J.W. (1958). Studies on some methane-utilising bacteria. Arch. Microbiol. 30, 91-118.

LEADBETTER, E.R. & FOSTER, J.W. (1959). Incorporation of molecular oxygen in bacterial cells utilising hydrocarbons for growth. Nature 184, 1428-1429.

LEADBETTER, E.R. & FOSTER, J.W. (1960). Bacterial oxidation of gaseous alkanes. Arch. Microbiol. 35, 92-104.

LEBEAULT, J.M., ROCHE, B., DUVNJAK, Z. & AZOULAY, K. (1969). General scheme for metabolism of hydrocarbons by Candida tropcalis. A. Van. Leeuwenhoek 35, supplement F33.

LEES, H. (1952a). The Biochemistry of the nitrifying organisms 1. The ammonia-oxidising systems of Nitrosomonas. Biochem. J. 52, 134-141.

LEES, H. (1952b). Hydroxylamine as an intermediate in nitrification. Nature 169, 156-157.

LEMBERG, M.R. (1969). Cytochrome Oxidase. Physiological Rev. 49, 48-121.

LINDSAY, J.G. & WILSON, D.F. (1974). Reaction of cytochrome c oxidase with CO; involvement of invisible copper. FEBS Letters 48, 45-49.

LINEWEAVER, H. & BURKE, D. (1934). The determination of enzyme dissociation constants. J. Amer. Chem. Soc. 56, 658-666.

LOVELESS, J.E. & PAINTER, H.A. (1968). The influence of metal ion concentration and pH value on the growth of a Nitrosomonas strain isolated from activated sludge. J. Gen. Microbiol. 52, 1-14.

LOVENBERG, W. (1973). Ed. The Iron Sulphur Proteins. Academic Press, New York.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.J. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

MARKOVETZ, A.J. (1971). Subterminal oxidation of aliphatic hydrocarbons by micro-organisms. Crit. Rev. Microbiol. 1, 225-237.

MASON, H.S. (1957). Mechanisms of oxygen metabolism. Adv. Enzymol. 19, 79-233.

MASON, H.S. (1965). Oxidases. Ann. Rev. Biochem. 34, 595-634.

MASON, H.S., FOWLKS, S.C. & PETERSON, E. (1955). Oxygen transfer and electron transport by the phenolase complex. J. Amer. Chem. Soc. 77, 2914-2915.

MATELES, R.I. (1974). Process aspects of continuous culture for SCP production with methanol as a substrate. In: the Proceedings of the 1st Intersectional Congress of the International Association of Microbiological Societies, Tokyo. pp. 112.

McCONNEL, J.C., McELROY, M.B. & WOFST, S.C. (1971). Natural Sources of Atmospheric CO. Nature 233, 187-188.

McKENNA, E.J. & KALLIO, R.E. (1965). The biology of hydrocarbons. Ann. Rev. Microbiol. 19, 183-208.

MILLS, G.A. & UREY (1940). Kinetics of isotopic exchange between carbon dioxide, bicarbonate ion and water. J. Am. Chem. Soc. 62, 1019-1026.

MURRAY, R.G. & WATSON, S.W. (1965). Structure of Nitrocystis oceanus and comparison with Nitrosomonas and Nitrobacter. J. Bacteriol. 89, 1594-1609.

NAGUIB, M. & OVERBEEK, J. (1970). On methane-oxidising bacteria in fresh waters. I Introduction to the problem and investigations on the presence of obligate methane-oxidisers, Z. Allg. Mikrobiol. 10, 17-36.

NICHOLAS, D.J.D. & NASON, A. (1957). Determination of nitrate and nitrite. In: Methods in Enzymology III Colwick, S.P. and Kaplan, N.O. (eds.), 981-984, Academic Press, N.Y.

NICHOLAS, D.J.D., WILSON, P.W., HEINEN, W., PALMER, G. & BEINERT, H. (1962). Use of electron paramagnetic resonance spectroscopy in investigations of functional metal components in micro-organisms. Nature 196, 433-436.

NICHOLLS, P. & SCHONBAUM, G.R. (1963). Catalases. In: The Enzymes 8, P.D. Boyer, H. Lardy and K. Myrback (eds.) pp. 147-225, Academic Press, N.Y.

NOZHESNIKOVA, A.N., SAVELIEVA, N.D. (1972). Autotrophic assimilation of carbon dioxide by the bacterium oxidising carbon monoxide. Mikrobiologiya 41, 939-946.

NOZHEVNIKOVA, A.N., ZAVARZIN, G.A. (1973). Symbiotic oxidation of carbon oxide by bacteria. Mikrobiologiya 42, 158-159.

OGATA, K. (1974). Oxidation of Methanol by Yeasts. In: the Proceedings of the First International Symposium on microbial growth on C_1 compounds, 15-17.

OOYAMA, J. & FOSTER, J.W. (1965). Bacterial oxidation of cycloparaffinic hydrocarbons. Antonie van Leeuwenhoek 31, 45-65.

ORME-JOHNSON, W.H. (1973). Iron-sulphur proteins: structure and function. Ann. Rev. Biochem, 43, 159-203.

ORRENIUS, S. & ERNSTER, L. (1974). Microsomal Cytochrome P-450-linked mono-oxygenase systems in mammalian tissues. In: Molecular Mechanisms of Oxygen Activation, Hayaishi, O. (ed.), pp. 614-650, Academic Press, New York.

PATEL, R., HOARE, D.S. & TAYLOR, B.F. (1969). Biochemical basis for the obligate C_1 dependence of Methylococcus capsulatus. Bacteriol. Proc. 128.

PATEL, R.N. & HOARE, D.S. (1971). Physiological studies of methane and methanol-oxidising bacteria; oxidation of C_1 compounds by Methylococcus capsulatus. J. Bacteriol. 107, 187-192.

PATEL, R.N., BOSE, H.R., MANDY, W.J. & HOARE, D.S. (1972). Physiological studies of methane- and methanol-oxidising bacteria: comparison of a primary alcohol dehydrogenase from Methylococcus capsulatus (texas strain) and Pseudomonas species M27. J. Bacteriol. 110, 570-577.

PATEL, R.N., MANDY, W.J. & HOARE, D.S. (1973). Physiological studies on methane- and methanol-utilising bacteria: immunological comparison of a primary alcohol dehydrogenase from Methylococcus capsulatus and Pseudomonas sp. M27. J. Bacteriol. 113, 937-945.

PATT, T.E., COLE, G.C., BLAND, J. & HANSON, R.S. (1974). Isolation and Characterisation of bacteria that grow on methane and organic compounds as sole sources of carbon and energy. J. Bacteriol. 120, 955-964.

PAYNE, W.J. (1970). Energy yields and growth of heterotrophs. Ann. Rev. Microbiol. 24, 17-52.

PERRY, J.J. (1968). Substrate specificity in hydrocarbon-utilising micro-organisms. Antonie van Leeuwenhoek 34, 27-36.

PETERSON, J.A. (1970). Cytochrome content of two Pseudomonads containing mixed-function oxidase systems. J. Bacteriol. 103, 714-721.

PETERSON, J.A., BASU, D. & COON, M.J. (1966). Enzymatic w-oxidation. I Electron carriers in fatty acid and hydrocarbon hydroxylation; J. Biol. Chem. 241, 5162-5164.

PETERSON, J.A., KUSUNOSE, M., KUSUNOSE, E. & COON, M.J. (1967). Enzymatic w-oxidation. II Function of rubredoxin as the electron carrier in w-hydroxylation. J. Biol. Chem. 242, 4334-4340.

PHILLIPS, K.C. (1970). Studies on methane-utilising bacteria. Ph. D. Thesis, University of Edinburgh.

POSTGATE, J.R. (1970). Carbon monoxide as a basis for primitive life on other planets: a comment. Nat. 226, 978.

POWLSON, D.S. & JENKINSON, D.S. (1971). Inhibition of nitrification in soil by carbon disulphide from rubber bungs. Soil Biol. Biochem. 3, 267-269.

PRASAD, R., RAJALE, G.B. & LAKHDIVE, B.A. (1971). Nitrification retarders and slow-release nitrogen fertilisers. Advan. Agron. 23, 337-383.

PRATT, H.K. & GOESCHL, J.D. (1969). Physiological roles of ethylene in plants. Ann. Rev. Plant Physiol. 20, 541-584.

QUAYLE, J.R. (1966). Formate dehydrogenase. In: Methods in Enzymology IX, 360-364, S.P. Colowick and N.O. Kaplan (eds.), Academic Press, New York.

QUAYLE, J.R. (1972). The metabolism of single-carbon compounds. Adv. Microbial Physiol. 7, 119-203.

REES, M.K. (1968a). Studies of the hydroxylamine metabolism of Nitrosomonas europaea. I Purification of the hydroxylamine oxidase. Biochemistry 7, 353-365.

REES, M.K. (1968b). Studies of the hydroxylamine metabolism of Nitrosomonas europaea. II Molecular properties of the electron-transport particle, hydroxylamine oxidase. Biochemistry 7, 366-372.

REES, M. & NASON, A. (1965a). A P-450-like cytochrome and a soluble terminal oxidase identified as Cytochrome o from Nitrosomonas europaea. Biochem. Biophys. Res. Comm. 21, 248-256.

REES, M. & NASON, A. (1966). Incorporation of atmospheric oxygen into nitrite formed during ammonia oxidation by Nitrosomonas europaea. Biochim. Biophys. Acta. 113, 398-402.

RIBBONS, D.W. & MICHALOVER, J.L. (1970). Methane oxidation by cell-free extracts of Methylococcus capsulatus. FEBS Letters 11, 41-44.

RIBBONS, D.W., HARRISON, J.E. & WADZINSKI, A.M. (1970). Metabolism of single carbon compounds. Ann. Rev. Microbiol. 24, 135-158.

RITTENBERG, S.C. (1969). The roles of exogenous organic matter in the physiology of chemolithotrophic bacteria. Adv. in Microbial Physiol. 3, 159-196.

ROBINSON, E. & ROBBINS, R.C. (1967). Sources, abundance and fate of gaseous atmospheric pollutants. Stanford Research Inst. Project PR-6755 report prepared for the American Petroleum Institute, New York.

SALEM, A.R., HACKING, A.J. & QUAYLE, J.R. (1973). Cleavage of malyl-coenzyme A and glyoxylate by Pseudomonas AM1 and other C_1-utilising bacteria. Biochem. J. 136, 89-96.

SAVELIEVA, N.D., NOZHEVNIKOVA, A.N. (1972). Autotrophic growth of Seliberia carboxydohydrogena during oxidation of hydrogen and carbon monoxide. Mikrobiologiya 41, 813-817.

SEIDELL, A. (1920). Solubilities of inorganic and organic substrates, 2nd Edition, Crosby, Lockwood and Son, London.

SEITZ, W. (1941). Über die photo-chemishe oxydoreduktion von kohlenoxyd und wasser zu kohlensäure und wasserstoff, Biochem Zeitschrift 308, 102-108.

SHEEHAN, B.T. & JOHNSON, M.J. (1971). Production of bacterial cells from methane. Appl. Microbiol. 21, 511-515.

SHIMMIN, M. (1974). Genetic studies on methane-oxidising bacteria. Ph.D. thesis, University of Edinburgh.

SILLEN, L.G. & MARTEL, A.E. (1971). Stability constants of metal ion complexes. Chemical Society Publication No. 25.

SILVERMAN, D.N. (1973). Carbonic anhydrase catalysed ^{18}O exchange between bicarbonate and water. Arch. Biochem. Biophys. 155, 452-457.

SILVERMAN, M.P. (1964). Methane oxidising bacteria. U.S. Bur. Mines Inforn. Circular 8246, 1.

SMITH, A.M. (1973). Ethylene as a cause of soil fungistasis. Nature 246, 311-313.

SMITH, A.M. & COOK, R.J. (1974). Implications of ethylene production by bacteria for biological balance of soil. Nat. 252, 703-705.

SMITH, U. & RIBBONS, D. (1970). Fine structure of Methanomonas methano-oxidans. Arch. Microbiol. 74, 116-122.

SÖHNGEN, N.L. (1906). Über bakterion, welche metheu als kohlenstoffnahrung und energiequelle gebrauchen. Zentbl. Bakt. Parasitkde. Abte II 15, 513-517.

SPERL, G.T., FORREST, H.S., FORREST, H.S. & GIBSON, D.I. (1973). Chemical characteristics of the cofactor isolated from the alcohol dehydrogenase of methane and methanol-utilising bacteria. Abstracts of the Annual meeting of the American Society for Microbiology, pp. 151.

SPERL, G.T., FORREST, H.S. & GIBSON, D.T. (1974). Substrate specificity of the purified primary alcohol dehydrogenases from methanol-oxidising bacteria. J. Bacteriol. 178, 541-550.

STANIER, R.Y. (1970). Some aspects of the biology of cells and their possible evolutionary significance, In: Symp. of the Soc. Gen. Microbiol. 20, H.P. Charles and B.C.J.G. Knight (eds.), pp. 1-38, Cambridge University Press.

STEVENS, C.M. (1972). In: "Carbon monoxide: natural sources dwarf man's output." by T.H. Maugh. Science 177, 338-339.

STOCKEN, L.A., THOMPSON, R.H. (1949). Reactions of British Anti-Lewisite with arsenic and other metals in living systems. Physiol. Rev. 29, 168-194.

STRAWINSKI, R.J. & BROWN, L.R. (1957). Isolation criteria and characterisation of a new methane-oxidising bacterium. Bact. Proc. 57, 18.

STRØM, T., FERENCI, T. & QUAYLE, J.R. (1974). The carbon assimilation pathways of Methylococcus capsulatus, Pseudomonas and Methylosinus trichosporium (OB3B) during growth on methane. Biochem. J. 144, 465-476.

SUZUKI, I. & KWOK, S.C. (1970). Cell-free ammonia oxidation by Nitrosomonas europaea extracts: effects of polyamines, Mg^{++} and albumen. Biochem. Biophys. Res. Comm. 39, 950-955.

SUZUKI, I., DULAR, U. & KWOK, S.C. (1974). Ammonia or ammonium ion as substrate for oxidation by Nitrosomonas europaea cells and extracts. J. Bacteriol. 120, 556-558.

SWARTZ, H.M. (1972). Cells and tissues. In: Biological applications of ESR, Swartz, H.M., Bolton, J.R. & Borg, D.C. (eds.), 115-195, Wiley.

SWARTZ, H.M., BOLTON, J.R. & BORG, D.C. (1972). Biological applications of ESR, Wiley.

THAUER, R.K., FUCHS, G., KAÜFER, B. & SCHNITKER, U. (1974). Carbon monoxide oxidation in cell-free extracts of Clostridium pasteurianum. Eu. J. Biochem. 45, 343-349.

THOMSON, A.W. (1974). Aspects of ethane metabolism in methylobacteria. Ph.D. Thesis, University of Edinburgh.

TONGE, G.M., KNOWLES, C.J., HARRISON, D.E.F. & HIGGINS I.J. (1974). Metabolism of one carbon compounds: cytochromes of methane-and methanol-utilising bacteria. FEBS Letters 44, 106-110.

TZAGALOFF, A. & WHARTON, D.C. (1965). Studies on the electron transfer system LXII. The reaction of cytochrome oxidase with carbon monoxide. J. Biol. Chem. 240, 2628-2633.

URUSHIBARA, T. & FORREST, H.S. (1970). Identification of 2-amino-4hydroxy-6-methyl pyeridine as a naturally-occurring compound in two methane-oxidising bacteria. Biochem. Biophys. Res. Comm. 40, 1189-1193.

VAN DER LINDEN, A.C. & THIJSSE, G.J.E. (1965). The mechanisms of microbial oxidation of petroleum hydrocarbons. Adv. Enzym. 27, 469-546.

VÄNNGÅRD, T. (1972). Copper proteins. In: Biological applications of Electron Spin Resonance, Swartz, H.M., Bolton, J.R. & Borg, D.C. (eds.), 411-417, Wiley.
VARY, P.S. & JOHNSON, M.J. (1967). Cell Yields of bacteria grown on methane. Appl. Microbiol. 15, 1473-1478.
WATSON, S.W. ASBELL, M. & VALOIS, F.W. (1970). Ammonia oxidation by cell-free extracts of Nitrocystis oceanus. Biochem. Biophys. Res. Comm. 38, 1113-1119.
WATSON, S.W. & MANDEL, M. (1971). Comparison of the morphology and deoxyribonucleic acid composition of 27 strains of nitrifying bacteria. J. Bacteriol. 107, 563-569.
WHITTENBURY, R.W. (1969). Microbial utilisation of methane. Process Biochem. 4, 51-56.
WHITTENBURY, R., PHILLIPS, K.C. & WILKINSON, J.F. (1970). Enrichment, isolation and some properties of methane-utilising bacteria. J. Gen. Microbiol. 61, 205-218.
WILKINSON, J.F. (1971). Hydrocarbons as a source of single-cell protein. In: Symp. of the Soc. Gen Microbiol. 21, Hughes, D.E. and Rose, A.H. (eds.), pp. 15-46, Cambridge Univ. Press.
WILKINSON, T.G. (1972). Interactions in a mixed bacterial population growing on methane in continuous culture. Ph.D. Thesis, University of London.
WILKINSON, T.G., TOPIWALA, H.H. & HAMER, G. (1974). Interactions in a mixed bacterial population growing on methane in continuous culture. Biotechnol. Bioeng. XVI, 41-59.
WILLIAMS, E. & BAINBRIDGE, B.W. (1974). Mutation, repair mechanisms and transformation in the methane-utilising bacterium Methylococcus capsulatus. Proceedings of the 2nd international symposium of the genetics of industrial micro-organisms. Academic Press Inc., N.Y.
WINOGRADSKY, S. (1890). Recherches sur les organisms de la nitrification. Ann. Inst. Pasterur 4, 213-231.
YAGI, T. & TAMIYA, N. (1962). Enzymic oxidation of carbon monoxide III. Reversibility. Biochem. Biophys. Acta. 65,,508-509.
YAMAMOTO, S., TAKEDA, H., MAKI, Y. & HAYAISHI, O. (1969). Studies on mono-oxygenases. III Examinations of metal participation in flavoprotein mono-oxygenases of Pseudomonads. J. Biol. Chem. 244, 2951-2955.
YOCH, D.C. & VALENTINE, R.C. (1972). Ferredoxins and Flavodoxins of bacteria. Ann. Rev. Microbiol. 26, 139-162.
ZELLER, E.A. (1963). Diamine oxidases. In: The enzymes 8, P.D. Boyer, H. Lardy and K. Myrback (eds.), pp. 313-335, Academic Press, N.Y.

Arch. Microbiol. 95, 365—368 (1974)

The Oxidation of Carbon Monoxide by Methane-Oxidizing Bacteria

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Received October 24, 1973

Summary. Carbon monoxide induces both respiration and carbon dioxide production in washed cell suspensions of the two methane-oxidizing bacteria *Methylomonas albus* (BG8) and *Methylosinus trichosporium* (OB3B). The ability of these organisms to oxidise carbon monoxide to carbon dioxide was confirmed by the use of tracer techniques.

There are few reports of the biological oxidation of CO in the literature but the fact that this property is attributed to sources as diverse as hydrogen oxidizing bacteria (Kistner, 1954; Savelieva and Nozhesnikova, 1972; Nozhesnikova and Savelieva, 1972), photosynthetic bacteria (Hirsch, 1968), *Desulfovibrio* (Yagi and Tamiya, 1962), soil fungi (Inman and Ingersoll, 1971), green algae (Chappelle, 1962), animal tissues (Breckenridge, 1953) and plant leaves (Bidwell and Fraser, 1972) suggest this may be a widespread phenomenon.

In the course of a study of the respiration of washed cell suspension of methylotrophs it was observed that the addition of carbon monoxide-saturated buffer apparently stimulated respiration and that time course plots of CO_2 production by washed cell suspensions in the presence of CO showed an enhancement of CO_2 production. These observations suggested that these two methylobacteria were capable of oxidizing CO to CO_2 or that CO might be stimulating endogenous CO_2 production.

Materials and Methods

Organisms. The organisms were used as representatives of the two main types of methylotrophs. These were *Methylomonas albus (BG8)* and *Methylosinus trichosporium (OB3B)* (Whittenbury *et al.*, 1970).

Preparation of Washed Cell Suspensions. Two liter Erlenmeyer flasks with 400 ml of a nitrate mineral salts medium (NMS) (Whittenbury *et al.*, 1970) and fitted with bladders filled with methane (British Oxygen Company, technical grade) such that the CH_4: air ratios were approximately 1:1, were inoculated from

slope cultures and after 3 days on a reciprocal shaker at 30°C were harvested by centrifugation (6000 g, 10 min), resuspended in fresh NMS and centrifuged again. The pellet was taken up in NMS to give a suspension of optical density approximately 100 at 610 nm measured with a Unicam SP600 series 2 spectrophotometer.

Respiration Studies. The respiration of the organisms at 30°C was determined with a Clark-type oxygen electrode with a reaction chamber of capacity 2.7 ml (Estabrook and Pullman, 1967). CO-saturated buffer (0.4 ml) was added 1 min after the addition of the cell suspensions.

Standardization of the ^{14}CO Source. An ampoule of ^{14}CO (500 μCi, Radiochemical Centre, Amersham) was broken in unlabelled CO (70 ml, Matheson Gas products) and helium (10 ml, British Oxygen Company) and the specific activity of the resulting mixture determined using the following method: a known amount of the ^{14}CO (1 ml) was introduced into a 25 ml round bottomed flask fitted with a gas-tight "Suba-seal" whose volume had been accurately determined by weighing empty and filled with water; 0.2 ml samples from the flask were injected into a gas chromatograph (PYE 104 series, silica gel column, carrier gas flow rate 15 ml N_2 min^{-1}, 50°C) fitted with a flame ionization detector (FID). The $^{14}CO_2$ produced by the burning of the CO in the FID flame was trapped by sparging the exhausted gas through 10 ml hyamine hydroxide (1 M solution in methanol, Nuclear Enterprises Ltd.). The resulting trapped radioactivity was measured by making a 1:9 dilution of the $^{14}CO_2$-hyamine hydroxide with scintillator (PPO, POPOP and toluene) and measuring the counts in a Beckman liquid scintillation counter. No quenching due to the presence of hyamine hydroxide was detected.

Measurement of CO_2 Production in the Presence of CO and Trapping of $^{14}CO_2$. Three round bottomed flasks whose exact volumes had been measured as described above, were set up with 4 ml of NMS medium and, as a control against injection errors, 1 ml helium. Flask A was a control to measure the endogenous CO_2 production. The diluted ^{14}CO (1 ml) was added to flask B. An equal amount of washed cell suspension containing 47 mg dry wt/ml was added to each flask and they were mounted on a wrist action shaker in a room thermostatically maintained at 30°C. The gas phase in each of the flasks was analysed by injecting 0.2 ml samples at timed intervals over a 3-h period or longer in some cases into a gas chromatograph (PYE 104, silica gel column, flow rate 30 ml N_2 min^{-1}, 150°C) with a katharometer detector. Helium, oxygen and methane gave line peaks of retention times 0.5, 0.7 and 1.3 min respectively and hence the peak heights could be taken as proportional to the amounts present and the rates of CH_4 oxidation calculated. However, CO_2 gave a broad peak of retention time 4.5 min and the peak heights were standardised against known samples of CO_2 in order to determine CO_2 production rates.

At different times during the incubations (from 1 min before the appearance of the CO_2 peak until 1 min after) the gas stream of the gas chromatograph was sparged through 10 ml hyamine hydroxide and counted as described above.

Results

Typical rates of respiration on CO for the organisms used were between 6 and 8 nmoles O_2/min/mg dry wt cells compared with endogenous respiration rates of between 2 and 3 nmoles O_2/min/mg dry wt cells in the presence of cells alone. Rates of CO_2 production were doubled in the presence of CO to give CO_2 production rates of the order of 5 nmoles CO_2/min/mg dry wt cells.

Table 1. The activities of $^{14}CO_2$ trapped in hyamine hydroxide and the specific activities of the excess CO_2

	Time min	CO_2 in endogenous control (μmole)	CO_2 in flask with CO (μmole)	Specific activity of excess CO_2 counts/min/μmole × 10^5
Methylomonas albus (BG8)	57	8	14	2.6
	133	16	26	2.1
	433	43	63	2.6
Methylosinus trichosporium (OB3B)	61	7	17	1.2
	122	14	29	2.3
	187	18	63	3.5

Specific activity of CO source 2.1 count/min/μmole × 10^5.

The expression $x/(y-z)$ was used to calculate the "specific activity of the excess CO_2" where x was the total counts per min of $^{14}CO_2$ in the flask with ^{14}CO, y the amount of CO_2 in nmoles in the flask with ^{14}CO, and z the amount of endogenously produced CO_2 in the control flask.

If the excess CO_2 above the endogenous level is derived from the ^{14}CO then the "specific activity" of the excess CO_2, i.e. the counts/min/μmole excess CO_2, must equal the specific activity of the original ^{14}CO. The specific activities of the excess CO_2 as well as the amounts of CO_2 present in both flask are shown in Table 1. The total counts of $^{14}CO_2$ trapped were high and in the range 1 to 4 × 10^4 c/min. The specific activity of the CO source was 2.1 × 10^5 (mean of three determinations 2.1, 2.2, 2.1 × 10^5) counts ^{14}C/min/μmole CO and in the experiment with *Methylomonas albus (BG8)* this corresponds closely with the specific activities of the excess CO_2. In the case of *Methylosinus trichosporium OB3B* the mean specific activity over the complete incubation time corresponded with the activity of the added CO but there was a lower activity in the early phase and a higher activity in the final phase.

It is evident that the respiration studies and the CO_2 evolution data indicate that CO is oxidized by suspensions of the two methylobacteria and the alternative hypothesis that the CO may be merely stimulating endogenous CO_2 production has been eliminated by the demonstration of the formation of $^{14}CO_2$ from ^{14}CO.

References

Bidwell, R. G. S., Fraser, D. E.: Carbon monoxide uptake and metabolism by leaves. Canad. J. Bot. **50**, 1435—1439 (1972)

Breckenridge, B.: Carbon monoxide oxidation by cytochrome oxidase in muscle. Amer. J. Physiol. **173**, 61—69 (1953)

Chappelle, E. W.: Carbon monoxide oxidation by algae. Biochim. biophys. Acta (Amst.) **62**, 45–62 (1962)

Estabrook, R. W., Pullman, M. E. (Eds.): Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios; Vol. X, p. 41. New York: Academic Press 1967

Hirsch, P.: A photosynthetic bacterium growing under carbon monoxide. Nature (Lond.) **217**, 555–556 (1968)

Inman, R. E., Ingersoll, R. B.: Note on the uptake of carbon monoxide by soil fungi. J. Air Poll. Control Ass., **21**, 646–647 (1971)

Kistner, A.: Conditions determining the oxidation of carbon monoxide and of hydrogen by *Hydrogenomonas carboxydomonas*. Proc. roy. Soc. Cp. (Amst.) **57**, 186–195 (1954)

Nozhesnikova, A. N., Savelieva, N. D.: Autotrophic assimilation of carbon dioxide by the bacterium oxidizing carbon monoxide. Mikrobiologiya **41**, 939–946 (1972)

Savelieva, N. D., Nozhesnikova, A. N.: Autotrophic growth of *Seliberia carboxydohydrogena* during oxidation of hydrogen and carbon monoxide. Mikrobiologiya **41**, 813–817 (1972)

Whittenbury, R., Phillips, K. C., Wilkinson, J. F.: Enrichment, isolation and some properties of methane-utilizing bacteria. J. gen. Microbiol. **61**, 205–218 (1970)

Yagi, T., Tamiya, N.: Enzymic oxidation of carbon monoxide III. Reversibility. Biochim. biophys. Acta (Amst.) **65**, 508–509 (1962)

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Arch. Microbiol. 102, 199–202 (1975)

Specific Inhibitors of Methane Oxidation in *Methylosinus trichosporium*

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Received August 29, 1974

Abstract. Methane oxidation by washed cell suspensions of *Methylosinus trichosporium* OB3B was selectively inhibited by 25 compounds, including metal binding components such as carbon monoxide (85% O_2 : 15% CO), KCN (10^{-6} M), $\alpha\alpha'$-dipyridyl (10^{-4} M), 8-quinolinol (10^{-4} M), thiosemicarbazide (10^{-5} M), thiourea (10^{-5} M), hydroxylamine (10^{-4} M), histidine (10^{-2} M), British Anti-Lewisite (5×10^{-3} M), and miscellaneous known inhibitors of other oxygenases. A role for copper in the methane oxygenase system was suggested by the pattern of inhibition and relief of inhibition by added metal ions.

Key words: Methylosinus trichosporium — Methane Oxidation — Metal Ions — Metal Binding Components — Role of Copper — Specific Inhibitors.

Methane-utilising bacteria have an obligate growth requirement for methane or methanol as carbon source. Oxidation of methane leading to the formation of methanol in *Pseudomonas methanica* has been shown to involve the incorporation of molecular oxygen (Higgins and Quayle, 1970), and the involvement of a monooxygenase has been implied by the methane-stimulated $NADH_2$ oxidation observed in cell-free extracts of *Methylococcus capsulatus* (Ribbons and Michalover, 1970) and *Pseudomonas methanica* (Ferenci, 1974). Further characterisation of the methane-oxidising enzyme(s) has been impeded by the lack of success experienced in this and other laboratories in the preparation of cell-free extracts capable of methane oxidation. We have therefore attempted to characterise the enzyme system(s) in whole cells by studying the effect of selected compounds on methane oxidation, noting that this approach has proved informative in studies on the ammonia oxygenase system in *Nitrosomonas* (Hooper and Terry, 1973).

Materials and Methods

The organisms used were *Methylosinus trichosporium* OB3B and *Methylomonas albus* BG8, isolated by Whittenbury *et al.* (1970). Growth of organisms and preparation of washed cell suspensions was as described by Hubley *et al.* (1974) with the exception that cells were suspended in phosphate buffer (1 g/l Na_2HPO_4; 0.8 g/l KH_2PO_4; pH 6.9). Rates of methane and methanol disappearance were determined by gas chromatography of samples taken periodically during a 20 min incubation, which was initiated by the injection of a volume of cells corresponding to 50 mg dry weight into a sealed 25 ml flask containing required reagents dissolved in phosphate buffer to give a final volume of 5.4 ml. Where appropriate, the amount of methane present was 1 ml, while methanol was added to 10 mM. The incubation temperature was 30° C. Methane was measured with a Pye Series 104 katharometer detector with a 2.8 m silica gel column at 140° C and nitrogen (30 ml/min) as carrier gas. 0.2 ml gaseous samples were taken from incubation flasks for this analysis. Methanol was measured in solution with a Pye Series 104 flame ionisation detector, using a 1.6 m Chromosorb 101 column at 150° C with nitrogen as carrier at 30 ml/min. The sample size in this case was 2.5 µl. Respiration rates were measured at 30° C with a Clark-type oxygen electrode (Estabrook, 1967) with a reaction chamber of 2.7 ml capacity. Methane saturated buffer (0.6 ml) or methanol (0.1 ml of 100 mM solution in buffer) were added 1 min after the addition of a volume of cells equivalent to 5 mg dry weight and inhibitor, where required, the total volume being 2.7 ml. Reversibility of inhibition was tested as follows. Cell suspensions were held for 30 min at 30° C with inhibitor present at a concentration known to cause 100% inhibition of methane oxidation. Cells were sedimented and resuspended in 20 volumes of buffer, sedimented again, and finally resuspended in the original volume. Methane-stimulated respiration rates were measured and compared with a control which had been treated similarly in the absence of inhibitor. Relief by metal ions of inhibition was also tested with the oxygen electrode system described above, where metal ion solution (0.1 ml) was added after full inhibition had been reached. The resultant rate was compared with an uninhibited control.

SKF 525A (β-diethylaminoethyldiphenylpropylacetate) was a gift from Smith, Kline and French Laboratories, Welwyn Garden City, U.K.; and Lilly 18947 (2,4-dichloro-6-phenylphenoxyethyldiethylamine) and Lilly 53325 (2,4-dichloro(6-phenylphenoxy)ethylamine hydrobromide) were kindly provided by Lilly Research Limited, Windlesham, Surrey, U.K. All other chemicals were analytical grade reagents or equivalent. Metal ions were in the form of the following B.D.H. Analar grade salts: $CoCl_2 \cdot 6\,H_2O$, $CrK(SO_4)_2 \cdot 12\,H_2O$, $SnCl_2 \cdot 2\,H_2O$, $MnSO_4 \cdot 4\,H_2O$, $FeSO_4 \cdot 7\,H_2O$, $FeCl_3 \cdot 6\,H_2O$.

Results and Discussion

The concentration of each inhibitor which gave the greatest difference between inhibition of methane and methanol-stimulated respiration by washed cell suspensions of *Methylosinus trichosporium* was determined. The effects of these concentrations on methane and methanol disappearance are presented in Tables 1 and 2, in which the division into metal-binding compounds and other inhibitors has been chosen for convenience.

In Table 1 the involvement of a metal ion(s) in methane oxidation is suggested by the specific inhibition of methane oxidation by chelators (Group I) and the monodentate strongly nucleophilic ligands included in Group II (pyridine, imidazole, histidine, 3-amino-triazole). Histidine has previously been shown to inhibit growth of *Methylococcus capsulatus* on methane (Eccleston and Kelly, 1972). It appears that EDTA lacks the specificity towards methane oxidation shown by the nitrogen- and sulphur-containing chelators.

The significance of carbon monoxide inhibition has been discussed in terms of the participation of a CO-binding cytochrome in methane oxidation by Davey and Mitton (1973) who tentatively suggested a role for cytochrome *a* in methane oxidation, relegating the cytochrome *o* to the role of terminal acceptor. Another explanation has been provided by Ferenci (1974) based on a possible role for the methane oxygenase system in CO oxidation, a phenomenon originally observed in *M. trichosporium* and *Methylomonas albus* (Hubley *et al.*, 1974). In support of this explanation, we have found carbon monoxide oxidation in *M. trichosporium* to be inhibited to a similar extent as methane oxidation by the inhibitors detailed in Tables 1 and 2. The two theories of inhibition by carbon monoxide may be essentially similar if the observation that purified mamallian cytochrome oxidase can oxidise carbon monoxide (Breckenridge, 1953; Tzagaloff and Wharton, 1965) can be extended to the *a*-type cytochrome of methylobacteria. The evidence for participation of an *a*-type cytochrome in methane oxidation must be considered highly speculative and there remains the distinct possibility that both CH_4 and CO oxidation are brought about by a metal containing oxygenase unrelated to the cytochromes.

The relief by metal ions of the inhibition of methane-stimulated respiration by some of the more effective inhibitors at 10^{-4} M was investigated, with inhibitors being chosen to represent different ligand combina-

Table 1. Inhibition of methane and methanol metabolism in washed cell suspensions of *Methylosinus trichosporium* OB3B by metal-binding compounds

Inhibitor	Concentration (M)	Rates of disappearance (% control) [a]		Reversibility [b] of inhibition of CH_4 oxidation
		CH_4	CH_3OH	
I				
Thiourea	10^{-4}	0	96	++
Allyl thiourea	10^{-5}	28	108	++
Thioacetamide	10^{-3}	0	100	++
Diethyldithiocarbamate	7.5×10^{-4}	0	90	++
8-Quinolinol	10^{-4}	0	102	++
o-Phenanthroline	5×10^{-5}	65	90	++
$\alpha\alpha'$Dipyridyl	10^{-4}	0	92	++
Ethyl xanthate (K salt)	10^{-3}	0	80	++
Dimercaptopropanol (British Anti-Lewisite)	5×10^{-3}	0	100	+
Thiosemicarbazide	10^{-5}	45	100	++
EDTA	10^{-3}	72	76	N.T.
II				
Carbon monoxide	(85% O_2 : 15% CO)	0	100	N.T.
Sodium cyanide	10^{-6}	38	100	++
Sodium azide	10^{-3}	7	85	++
Histidine	10^{-2}	0	105	++
Pyridine	10^{-3}	36	108	++
Imidazole	10^{-3}	0	109	++
3-Amino triazole	10^{-2}	27	91	++

[a] The uninhibited rates of methane and methanol disappearance were 20 nmoles/min/mg dry cell weight and 100 nmoles/min/mg dry cell weight respectively.

[b] Reversibilities: ++ and + indicate restoration to more than 50% activity and less than 50% respectively, and − indicates irreversibility.

N.T. = not tested.

Table 2. Inhibition of methane and methanol metabolism in washed cell suspensions of *Methylosinus trichosporium* OB3B by miscellaneous compounds

Inhibitor	Concentration (M)	Rates of disappearance (% control)		Reversibility of inhibition of CH_4 oxidation
		CH_4	CH_3OH	
Diethylaminoethyldiphenyl-propylacetate (SKF 525A)	5×10^{-3}	36	100	−
2,4 Dichloro-6-phenyl-phenoxyethyldiethylamine (Lilly 18947)	10^{-2}	0	100	N.T.
2,4 Dichloro(6-phenylphenoxy)-ethylamine hydrobromide (Lilly 53325)	5×10^{-3}	0	100	N.T.
Cysteamine	10^{-2}	0	84	−
Hydrazine sulphate	2×10^{-3}	70	6	−
Hydroxylamine hydrochloride	10^{-4}	23	75	++
Aminoguanidine	10^{-2}	32	96	N.T.
Spermine	2×10^{-3}	41	100	N.T.
Methanol	10^{-2}	5	−	N.T.

The notes for Table 1 apply to Table 2.

tions, *i.e.* nitrogen-nitrogen ($\alpha\alpha'$dipyridyl), oxygen-nitrogen (8-quinolinol), and sulphur-nitrogen (thiourea, allyl thiourea, thiosemicarbazide). Fe^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+} (10^{-4} M) relieved the inhibition by $\alpha\alpha'$dipyridyl and 8-quinolinol. Cu^{2+} was the only metal ion tested which relieved inhibition by thiosemicarbazide, and none of the metal ions even at concentrations of 10^{-3} M were able to relieve inhibition by thiourea and allyl thiourea. No effect of Mn^{2+}, Fe^{3+}, Sn^{2+} or Cr^{3+} was observed on inhibition by any of the compounds tested. Assuming these inhibitors to act as chelators, these results suggest that copper in some form may be a component of the methane oxygenase system. Thiourea has a high affinity for Cu^{1+}, with a stability constant β_6 for the formation of the $[Cu_2(\text{thiourea})_6]^{2+}$ complex of 15.4, which is approached in magnitude only by silver (Sillen and Martel, 1971). The ability of Fe^{2+} to relieve inhibition by $\alpha\alpha'$dipyridyl and 8-quinolinol is not contradictory since this effect has been observed in the copper-containing dopamine hydroxylase (Goldstein, 1966). The low solubility of cuprous compounds and their tendency to undergo disproportionation in aqueous solution precluded its use in the above reversibility studies.

SKF 525A and Lilly compounds 18947 and 53325 (Table 2) have been shown to inhibit other oxygenase systems (Hammond and Whyte, 1970; Hildebrandt, 1972) although in this respect their effect on methane oxidation should be qualified by the high concentrations required for inhibition. Amongst all the compounds tested, hydrazine sulphate was unique in inhibiting methanol oxidation more than methane oxidation. A mechanism of inhibition through combination with a Cu^{2+} or Cu^{1+} species, either in cytochrome oxidase or a distinct oxygenase, is compatible with the chemical properties of all the compounds in Table 1 and many of those in Table 2. It is of course acknowledged that some compounds may act in various ways; for example British Anti-Lewisite can be a chelator (Stocken and Thompson, 1949), or can interact with disulphide bonds (Cooperstein, 1963). Nor do our results rule out the additional involvement of metal ions other than copper.

Unlike *M. trichosporium*, *M. albus* can grow on methanol as sole carbon source. Consistent with the specificity of inhibition we have observed, growth of *M. albus* on methanol (but not methane) can occur in the presence of selected inhibitors (10^{-5} M allyl thiourea, 10^{-5} M thiosemicarbazide, 10^{-5} M hydroxylamine, 7.5×10^{-5} M diethyldithiocarbamate, 5×10^{-5} $\alpha\alpha'$dipyridyl, 10^{-5} M 8-quinolinol, 10^{-3} M pyridine, 10^{-5} M azide). These organisms represent the two groups of methane-utilising bacteria which differ in their pathway of carbon assimilation (Lawrence and Quayle, 1970) and internal membrane organisation (Davies and Whittenbury, 1970). It is therefore likely that the inhibitory effects we have observed are applicable to both groups. The inability of these compounds to inhibit all the cellular reactions associated with growth of *M. albus* in methanol also reinforces the

hypothesis that these inhibitors act on the initial step in the metabolism of methane.

In conclusion attention is drawn to the similar pattern of inhibition of methane oxidation in *Methylosinus* and ammonia oxidation in *Nitrosomonas* (Hooper and Terry, 1973) and the possibility of the involvement of a copper species in both oxygenases. This may support the suggestion of an evolutionary link between these two groups of microorganisms (Quayle, 1972).

Acknowledgements. This work was financed by grant to J.F.W. from the Science Research Council and British Petroleum Limited. J.H.H. was in receipt of a British Petroleum Studentship. We thank Miss E. Clark for her technical assistance.

References

Breckenridge, B.: Carbon monoxide oxidation by cytochrome oxidase in muscle. Ann. J. Physiol. **173**, 61–69 (1953)

Cooperstein, S.J.: Reversible inactivation of cytochrome oxidase by disulphide bond reagents. J. biol. Chem. **238**, 3606–3610 (1963)

Davey, J.F., Mitton, J.R.: Cytochromes of two methane-utilizing bacteria. FEBS Letters **37**, 335–337 (1973)

Davies, S.L., Whittenbury, R.: Fine structure of methane and other hydrocarbon utilizing bacteria. J. gen. Microbiol. **61**, 227–232 (1970)

Eccleston, M., Kelly, D.P.: Assimilation and toxicity of exogenous amino acids in the methane-oxidizing bacterium *Methylococcus capsulatus*. J. gen. Microbiol. **71**, 541–554 (1972)

Estabrook, R.W.: Mitochondrial respiratory control and the polarographic measurement of ADP:0 ratios. In: Methods in enzymology, Vol. 10, R. W. Estabrook, M. E. Pullman, eds., pp. 41–47. New York: Academic Press 1967

Ferenci, T.: Carbon monoxide-stimulated respiration in methane-utilizing bacteria. FEBS Letters **11**, 94–97 (1974)

Goldstein, M.: Dopamine-β-hydroxylase-a copper enzyme. In: The biochemistry of copper, J. Peisach, P. Aisen, W. E. Blumberg, eds., pp. 443–454. New York: Academic Press 1966

Hammond, R.K., Whyte, D.C.: Inhibition of carotenoid hydroxylation in *Staphylococcus aureus* by mixed-function oxidase inhibitors. J. Bact. **103**, 607–612 (1970)

Higgins, I.J., Quayle, J.R.: Oxygenation of methane by methane-grown *Pseudomonas methanica* and *Methylomonas methanooxidans*. Biochem. J. **118**, 201–208 (1970)

Hildebrandt, A.G.: The binding of metapyrone to cytochrome P-450 and its inhibitory action on microsomal hepatic mixed function oxidation reactions. In: Biological hydroxylation mechanisms, G. S. Boyd, R. M. Smellie, eds., pp. 79–102. New York: Academic Press 1972

Hooper, A.B., Terry, K.: Specific inhibitors of ammonia oxidation in *Nitrosomonas*. J. Bact. **115**, 480–485 (1973)

Hubley, J.H., Mitton, J.R., Wilkinson, J.F.: The oxidation of carbon monoxide by methane-oxidizing bacteria. Arch. Microbiol. **95**, 365–368 (1974)

Lawrence, A.J., Quayle, J.R.: Alternative carbon assimilation pathways in methane-utilizing bacteria. J. gen. Microbiol. **63**, 371–374 (1970)

Quayle, J.R.: The metabolism of single-carbon compounds. Advanc. Microbial Physiol. **7**, 119–203 (1972)

Ribbons, D.W., Michalover, J.L.: Methane oxidation by cell-free extracts of *Methylococcus capsulatus*. FEBS Letters **11**, 41–44 (1970)

Sillen, L.G., Martel, A.E.: Stability constants of metal ion complexes. Chemical Society Publication No. 25 (1971)

Stocken, L.A., Thompson, R.H.: Reactions of British Anti-Lewisite with arsenic and other metals in living systems. Physiol. Rev. **29**, 168–194 (1949)

Tzagaloff, A., Wharton, D.C.: Studies on the electron transfer system LXII. The reaction of cytochrome oxidase with carbon monoxide. J. biol. Chem. **240**, 2628–2633 (1965)

Whittenbury, R., Phillips, K.C., Wilkinson, J.F.: Enrichment, isolation, and some properties of methane-utilizing bacteria. J. gen. Microbiol. **61**, 205–218 (1970)

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