OXIDATION OF CARBON COMPOUNDS BY *METHYLOCOCCUS CAPSULATUS*

by

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TO MY MOTHER AND FATHER

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'Aunque sepa los caminos
Yo nunca llegaré a Cordoba' *

Lorca.

* Although I may know the roads, I will never arrive at Cordoba.

Declaration

The work contained in this thesis was the result of original research conducted by myself with the exception of the hexulose-phosphate synthase and hexulose-phosphate isomerase activities quoted in Section VI which were kindly provided by Professor J.R. Quayle. Also the work contained in Section IV concerning the substrate specificity studies of the methane mono-oxygenase was done in conjunction with Dr. J. Colby of the University of Warwick.

None of the work contained in this thesis has been previously submitted for examination.

SUMMARY

The effect of various potential inhibitors on methane oxidation was tested on whole-cell suspensions of Methylococcus capsulatus (strain Texas) (TRMC) and Methylococcus capsulatus (strain Bath) (MC). Methane oxidation by TRMC was specifically inhibited by a large number of metal-chelating/binding agents and suggested the involvement of a metal ion(s) with the methane mono-oxygenase. The whole-cell results of MC showed a much more restricted specific inhibitor pattern for methane oxidation, which was confirmed by cell-free studies. The inhibitor pattern of MC was compared with that of TRMC and with reported patterns for other methane-utilisers. The effect of a number of acetylenic compounds on methane oxidation by MC was tested and the results discussed.

The substrate specificity of the methane mono-oxygenase from MC was determined and found to be very non-specific. It catalysed the oxidation of various substituted methane derivatives, including methanol. C_1-C_8 n-alkanes were hydroxylated to the corresponding 1- and 2-alcohols, carbon monoxide to carbon dioxide, terminal alkenes to the corresponding 1, 2-epoxides and internal alkenes to a variety of products. Ethers, alicyclic, aromatic and heterocyclic compounds were also oxidised. The significance of the various oxidations are discussed. Only NADPH could replace NADH as electron donor for methane mono-oxygenase activity.

The subject of non-growth substrate oxidation by micro-organisms is discussed and the terminology of the area critically reviewed. Whole-cell oxidation studies with MC revealed five fortuitously oxidised non-growth substrates (chloromethane, bromomethane, dimethyl ether, ethene and propene) and seven cometabolic non-growth substrates (carbon monoxide, diethyl ether, ethane, propane, but-1-ene, cis but-2-ene and trans but-2-ene). From these, dimethyl ether, bromomethane and carbon monoxide were selected to study in detail their effect on cellular metabolism of MC.

During the oxidation studies it was suspected that an $NAD(P)^+$-linked aldehyde dehydrogenase was present. Confirmative tests proved positive. Activity in crude cell-free extracts was lost on dialysis, but could be restored by supplementing with inactive, heat-treated extract. The non-dialysable, heat-sensitive component was isolated and purified. The heat-stable component/co-factor was presumed to be a low molecular weight protein or polypeptide.

The enzymic potential for an $NAD(P)^+$-linked cyclic scheme for the complete oxidation of formaldehyde was detected in crude cell-free extracts of MC. The relative importance of the different formaldehyde oxidation systems found is discussed.

SECTION I. General Introduction

1. The Concept of Methylotrophy

The almost universally accepted definition of a methylotrophic organism was originally proposed by Colby and Zatman (1972). They described methylotrophs as organisms which possess the ability to grow non-autotrophically at the expense of carbon compounds containing one or more carbon atoms but containing no carbon-carbon bonds. This group of organisms could be further sub-divided into obligate and facultative methylotrophs, the latter class having the additional ability to grow and replicate on a variety of other organic compounds.

Methylotrophs, as defined above, are organisms (both eukaryotic and prokaryotic) which can utilise methane, methanol, N-methyl compounds and S-methyl compounds as sole sources of carbon for growth and replication. The extensive and varied physiology and biochemistry of all the various types of methylotrophs have been comprehensively covered in two excellent reviews, by Quayle (1972) and more recently by Anthony (1975a), and so the general introduction to this thesis will concern primarily the biochemistry and physiology of obligate methane-utilising bacteria.

Recently, Whittenbury and Kelly (1977) revised and expanded the previously narrow definitions of the term autotrophy (Kelly, 1971; Schlegel, 1975) to incorporate methylotrophs and other C_1-utilising organisms such as anaerobic methane and acetic acid producers, ammonia oxidisers, etc. They have defined autotrophic organisms as micro-organisms which can synthesise all their cellular constituents from one or more C_1 compounds. These organisms can be divided into three groups, specified by their pathway of C_1 compound assimilation, i.e. (1) those possessing the ribulosebisphosphate pathway; (2) those possessing the ribulose monophosphate pathway; (3) those possessing the serine pathway. This new, attractive noumenon of autotrophy, although relegating terms like chemolithotrophy, photolithotrophy and methylotrophy to subsidiary positions in the autotrophic régime, does not diminish the value of such terms in specifiying a closely-related group of organisms.

2. Occurrence and Isolation of Methane-Utilising Bacteria

The isolation of all types of methylotrophs has been extensively described by Quayle (1972). Until recently, the only methylotrophic organisms isolated were bacteria, but a number of methylotrophic yeasts have now been isolated and identified, e.g. Candida N-16 (Fujii and Tonomura, 1972), Candida boidiniii (Sahm, 1975), Kloeckera sp. (Kato, Tamaoki, Tani and Ogata, 1972), Hansenula polymorpha (van Dijken, Otto and Harder, 1975; van Dijken, Oostra-Demkes, Otto and Harder, 1976) and Pichia pinus (van Dijken et al., 1976).

Methane is produced naturally in a number of diverse situations; coal and oil deposits, anaerobic sewage digesters, rumens of ruminant animals, and in lakes and ponds. Isolation of methane-utilising bacteria is generally accomplished using samples from lakes or ponds. The anaerobic decomposition of organic matter in the sedimented mud/silt layer at the bottom of lakes and ponds gives rise to methane, but due to the oxygen requirement of methane-utilising bacteria, these organisms are rarely found in and around the sediment. Large bodies of water, such as lakes or deep ponds, undergo seasonal stratification of microbial activity resulting in a concentration layer or lens of methane-oxidising activity (Cappenberg, 1972).

Although the presence and wide distribution of methane-oxidising bacteria had been known for many years and some fairly extensive surveys of methane-utilisers from various locations had been performed by various workers at the turn of the century, e.g. Söhngen (1906), Kasener (1906), very few species of methane-oxidising bacteria had been isolated and characterised until 1970. Prior to 1970 only three species of methane-utilising bacteria had been successfully isolated and characterised, Pseudomonas methanica (Söhngen, 1906; Dworkin and Foster, 1956), Methanomonas methano-oxidans (Brown, Strawinsky and McClesky, 1964; Stocks and McClesky, 1964) and Methylococcus capsulatus (Foster and Davis, 1966). A fourth species, Pseudomonas methanitrificans, was isolated by Davis, Coty and Stanley (1964) and is included in the latest edition of Bergey's Manual. However, it is now

thought that this isolate was in fact a mixed-culture (Malashenko, Romanovskaya,Bogachenko, Khotyan and Voloshin, 1973; Whittenbury, Phillips and Wilkinson, 1970a; Whittenbury, Colby, Dalton and Reed, 1976).

This apparent difficulty in isolating pure cultures of methane-oxidisers was almost certainly due to the lack of a reliable enrichment and isolation technique for these organisms. This situation was dramatically transformed in 1969-70 by Whittenbury and his colleagues who devised simple and effective techniques for the enrichment and isolation of methane-utilising bacteria (Whittenbury, 1969; Whittenbury et al., 1970a). Using their techniques they successfully isolated more than 100 methane-utilising organisms (Whittenbury et al., 1970a).

Since 1970 a number of studies concerning the isolation and characterisation of obligate methane-utilising bacteria havebeen done. New Methylococcus species were described in a taxonomical study by Malashenko, Romanovskaya and Kvasnikov (1972),and Hazeu (1975) described a number of different methane-utilising bacteria and grouped them according to pigmentation, cell morphology and internal membrane structure. Recently, Malashenko (1976) described the isolation and characterisation of several new species of thermophilic and thermo-tolerant methane-oxidisers and Galchenko (1977) has isolated and characterised a number of Methylocystis species. Probably the most interesting methane-oxidising bacterium isolated in recent years is Methylobacterium organophilum which can utilise not only methane or methanol as sole source of carbon and energy, but a variety of organic compounds such as acetate, mono- and di-saccharides and amino acids (Patt, Cole, Bland and Hanson, 1974; Patt, O'Connor, Cole, Day and Hanson, 1976). This is the first facultative methylotroph which can utilise methane as sole source of carbon and energy to be isolated and characterised,although it is reported that such organisms are ubiquitous in nature (Patt et al., 1976).

The ability of Methylobacterium organophilum to utilise a number of organic substrates presented the first good opportunity to do extensive genetic and enzyme regulation studies on a methane-utilising bacterium and a number of reports concerning these topics have recently been published (O'Connor and Hanson, 1977; O'Connor, Wopat and Hanson, 1977; O'Connor and Hanson, 1978).

3. Grouping and Classification of Obligate Methane-Utilising Bacteria

On examination under the electron microscope of sections of numerous methane-utilising bacteria, Whittenbury and his co-workers decided that the isolates could be divided into two groups based on the internal membrane arrangement (Davies and Whittenbury, 1970). The two different types were designated Type I and Type II. Type I organisms were described as possessing bundles of stacked, disc-shaped membrane vesicles which were randomly distributed throughout the interior of the cell, whereas Type II organisms possessed layers of paired membranes situated around the periphery of the cell. These membrane arrangements were confirmed for a number of species by other workers using similar techniques (Proctor, Norris and Ribbons, 1969; Smith, Ribbons and Smith, 1970; Smith and Ribbons, 1970; Weaver and Dugan, 1975).

This major difference in internal membrane morphology plus a number of other divergent properties between the two types, i.e. nature of the resting stage produced by the organism (Whittenbury, Davies and Davey, 1970b), cell and colony morphology, prompted Whittenbury and his associates to propose a provisional classification of their isolates into 5 main groups (Whittenbury et al., 1970a). These 5 groups, 'Methylosinus', 'Methylocystis', 'Methylomonas', 'Methylobacter' and 'Methylococcus' were then further subdivided into 15 subgroups on the basis of a further 8 characteristics: growth temperatures, growth on methanol, enhancement of growth on methane by yeast extract, malate, acetate or succinate, shortest replication time, motility and flagellation, capsule formation, colony colour, production of water soluble pigments (Whittenbury et al., 1970a). The above authors designated only group and subgroup names to the 15 types of organism, but many workers have

subsequently adopted these names as genus/species titles although these names have no formal nomenclatural standing in Bergey's Manual (8th edition) (Leadbetter, 1974). Leadbetter (1974) only recognised two genera, Methylomonas and Methylococcus. The genus Methylomonas contained three species, M. methanica (Dworkin and Foster, 1956), M. methano-oxidans (Brown et al., 1964) and M. methanitrificans (Davis et al., 1964), whereas the genus Methylococcus contains only 1 species, M. capsulatus (Foster and Davis , 1966).

Lawrence and Quayle (1970) found a correlation between the carbon assimilation pathways (see section 4D of General Introduction) and the intracytoplasmic membrane arrangements of a number of methane-utilising bacteria. The Type I membrane system, as defined by Davies and Whittenbury (1970),correlated with the ribulose monophosphate cycle of formaldehyde fixation, whereas organisms possessing a Type II membrane system generally used the serine pathway. Davey, Whittenbury and Wilkinson (1972) examined cell-free extracts of various methane-utilising bacteria for activity of a number of key enzymes concerned with intermediary metabolism and found more biochemical disparities between Type I and Type II organisms. These observations combined with the data previously reported and mentioned above provided Whittenbury and co-workers with the information to construct a new classification scheme of obligate methane-utilising bacteria (Table I) (Whittenbury, Dalton, Eccleston and Reed, 1975; Whittenbury et al., 1976).

This detailed scheme appears to clearly separate two types of methane-utilisers; however a number of recent reports cast doubts on the validity of some of the suggested divergent characteristics. It has been shown that a number of Type I and Type II methane-utilisers and other methylotrophs can possess both hexulose phosphate synthase and hydroxypyruvate reductase activity, which are indicative of the ribulose monophosphate cycle and the serine pathway of formaldehyde fixation respectively (Lawrence and Quayle, 1970; Mateles and Goldberg, 1975; Whittenbury et al., 1976; Malashenko, 1976; Shishkina, Yurchenko, Romanovskaya, Malashenko and Trotsenko, 1976). Obviously, the

presence of these enzymes in cell-free extracts does not constitute irrefutable proof that the two assimilation pathways were operational in vivo, but these results do suggest that perhaps carbon assimilation pathways cannot be correlated usefully with internal membrane arrangements. This suggestion is supported by the fact that some methylotrophs which do not utilise methane but assimilate carbon by the ribulose monophosphate cycle and serine pathway are devoid of internal membrane structures, such as those found in obligate methane-utilisers (Dahl, Mehta and Hoare, 1972; Quayle, 1972; Malashenko, 1976). Also the presence of ribulose 1,5-bisphosphate carboxylase and phosphoribulokinase in cell-free extracts of Methylococcus capsulatus (Bath) (Taylor, S., 1977) suggested the possibility of a ribulose bisphosphate pathway of carbon assimilation being operative in Methylococcus capsulatus (Bath) thereby possibly separating the organism from other Type I organisms.

Recent work on the enzymology of certain methane-utilising bacteria has provided more anomalies to the scheme in Table 1. It was reported that 2-oxoglutarate dehydrogenase was present in the Type I organism, Methylomonas rubra, and that some members of the genus Methylococcus possessed only $NADP^+$-linked isocitrate dehydrogenase (Romanovskaya, 1977). Finally, there is now some doubt as to whether methane-utilising bacteria can be precisely separated into two specific groups on the basis of intracytoplasmic membrane arrangement. Galchenko and Suzina (1977) have reported 8 different arrangements of internal membrane structures in various methane-utilisers and although they still referred to Types I and II, it appeared that for Type I organisms at least, some diversity within one type was evident. Similar diversity in intracytoplasmic membrane arrangement had been previously reported for Type I organisms (Tyurin and Galchenko, 1976). Brannan and Higgins (1978) have reported that, under certain conditions, Methylosinus trichosporium OB3b was devoid of intracytoplasmic membrane structures, but was still able to grow on methane, whereas previously it had been shown to possess a Type II membrane system (Davies and Whittenbury, 1970; Weaver and Dugan, 1975). Although this does not dispute the fact that usually a typical Type II

TABLE 1. Classification of Obligate Methane-Utilising Bacteria

(from Whittenbury et al., (1975, 1976))

	Type I		Type II
	Methylococcus, Methylomonas sp		Methylosinus, Methylocystis sp
Membrane arrangement	Bundles of vesicular discs		Paired membranes ; layers around periphery
Resting stage	Cysts (Azotobacter-like)		Exospores or 'lipid' cyst-unique structures
Carbon assimilation pathway	All have a ribulose monophosphate pathway, and some have a serine pathway		A serine pathway only
TCA cycle	Incomplete (2-oxoglutarate dehydrogenase negative)		Complete
Glucose 6-phosphate and 6-phosphogluconate dehydrogenase	+		–
Nitrogen fixation	Some fix, 'oxygen sensitive'		All fix, 'oxygen tolerant'
	Methylococcus capsulatus	Methylomonas sp	
Cell shape	coccus	rod	rod and vibrios
Isocitrate dehydrogenase	NAD^+ specific	NAD^+ and $NADP^+$	$NADP^+$ specific
Malate dehydrogenase	low activity	high activity	high activity
G + C (%)	62.5	50-54	62.5

internal membrane arrangement is found in this organism, it does suggest that the membranes are not essential for growth on methane and are therefore probably not a reliable characteristic for classification purposes.

It appears from the above evidence that intracytoplasmic membrane arrangement and the pathways of carbon assimilation should not be linked in a scheme for the classification of methane-utilising bacteria. To preserve a Type I and Type II form of grouping, either membrane structure or assimilation pathway could be used separately to divide the organisms, but in view of recent findings regarding internal membrane arrangements, it is suggested that the C_1-assimilation pathways are used to group the organisms. Bacteria shown to definitely possess more than one C_1-assimilation pathway could be grouped together to form other Types as required. This form of grouping could be extended to cover all methylotrophs and indeed all autotrophs (as defined by Whittenbury and Kelly (1977)) if organisms possessing the ribulose-bisphosphate pathway of C_1-assimilation were included as a third Type.

4. Physiology and Biochemistry of Obligate Methane-Utilising Bacteria

A. Basic growth requirements

All methane-oxidisers are strictly aerobic due to their requirement for gaseous oxygen for the initial oxidation of methane (Higgins and Quayle, 1970) and can utilise either methane or methanol as a sole source of carbon and energy. The growth of methane-utilisers is inhibited by normal heterotrophic metabolites at usual heterotrophic concentrations (Eccleston and Kelly, 1972; Eccleston and Kelly, 1973). No growth factors are required and they are normally grown on a mineral salts medium containing a nitrogen source, divalent cations (Ca^{++} and Mg^{++}), sulphate, phosphate, iron and trace elements (Dalton and Whittenbury, 1976a). The trace elements can be omitted from the medium normally, as sufficient trace elements are present in the distilled water and the medium ingredients, but growth is enhanced when

the medium is supplemented with additional trace elements, especially in continuous culture where high cell densities are sometimes required (Phillips, 1970; Harwood and Pirt, 1972). Whittenbury et al. (1970a) found minimum generation times of between 3.5 and 5.5 hours with their isolates from batch culture experiments.

B. Nitrogen metabolism

(i) Introduction.- All the isolates of Whittenbury et al. (1970a) could use ammonium salts as their nitrogen source for growth. Most organisms could use nitrate and appeared to prefer this to ammonium salts as a nitrogen source, as Leadbetter and Foster (1958) found with their methane-utilising organisms. A few organisms could use urea, casamino acids and yeast extract. More recent isolates have varied in their ability to use ammonium salts as a source of nitrogen. A number of methane-utilisers isolated by Hazeu (1975), in particular all the brown pigmented group of isolates, were unable to use ammonium chloride as a nitrogen source. Similarly a number of thermotolerant methane-utilisers isolated by Malashenko (1976) were only able to use nitrate initially as their nitrogen source, but gradually developed the ability to use ammonium salts during months of subculturing.

(ii) Dinitrogen fixation.- Relatively few reports of dinitrogen (N_2) fixation by methane-oxidising bacteria have been published in recent years, although the phenomenon was originally observed with methane-oxidisers in 1964 (Davis, Coty and Stanley, 1964). Coty (1967) showed $^{15}N_2$ uptake with his methane-utilising culture thus unequivocally showing dinitrogen fixation by a methane-utiliser. However, the above cultures could grow on nutrient agar and although the growth was very poor, the purity of the culture must be doubted.

The techniques used by the above authors to assay for dinitrogen fixation, i.e. Kjeldahl analysis and $^{15}N_2$ uptake are both tedious, and in the latter case expensive, and it was not until 1966 that a cheap, rapid and relatively easy technique for assaying nitrogenase activity was developed, i.e. the acetylene reduction test. This assay utilises the ability of nitrogenase to reduce acetylene to ethylene (Dilworth, 1966; Schöllhorn and Burris, 1967), and is now an established assay for nitrogenase activity (Hardy, Holsten, Jackson and Burns, 1968;

Postgate, 1972). Even with the advent of this new assay, only a few reports of methane-oxidising bacteria fixing dinitrogen have subsequently appeared.

Whittenbury et al. (1970a) isolated the strain Methylomonas trichosporium PG from Coty's original culture and found that cultures of it would actively reduce acetylene during prolonged incubations. More recently, de Bont and Mulder (1974) isolated a methane-utilising bacterium which resembled the Whittenbury et al. (1970a) isolate Methylosinus sporium, and the strain Methylovibrio söhngenii isolated by Hazeu and Steenis (1970), and they found that it would fix dinitrogen as assayed by the $^{15}N_2$ uptake technique. The organism could grow slowly on nitrogen-free mineral salts medium under a methane/air atmosphere, and the rate of growth was dependent upon the partial pressure of oxygen in the gas phase. If the partial pressure of oxygen was reduced, growth was markedly increased as previously observed with Azotobacter chroococcum (Dalton and Postgate, 1969). Curiously, they found that no ethylene was produced from acetylene by cultures in the presence of methane. They also found that ethylene disappeared progressively in the presence of cell suspensions which suggested to the authors that the failure of the acetylene reduction test could be attributed to the 'co-oxidation' of the product ethylene. However, they did not show that acetylene was disappearing during the assays in the presence of methane, and ethylene was produced during similar acetylene reduction assays in the presence of methanol instead of methane.

The real reason for the failure of the acetylene reduction tests in the presence of methane was revealed during a similar study of dinitrogen fixation by the obligate methane-utiliser, Methylococcus capsulatus (Bath) by Dalton and Whittenbury (1976a). They found that 0.5 ml of acetylene injected into the gas-stream supplying a continuous culture of nitrogen-fixing, methane-oxidising Methylococcus capsulatus (Bath) caused an immediate cessation of methane and oxygen uptake and carbon dioxide production. Ethylene, the expected product of

acetylene reduction, was not detected in the culture or effluent. Similar tests were repeated in a closed batch system and gave the same results, but on the addition of methanol, growth, oxygen uptake and carbon dioxide production were restored. When acetylene was replaced by ethylene , only 55% inhibition of the normal gas uptake and production occurred and no complete termination of cellular metabolism was produced. Oxygen electrode studies with whole-cell suspensions of Methylococcus capsulatus (Bath) confirmed that acetylene was indeed a potent inhibitor of methane oxidation yet was totally ineffective towards methanol oxidation. This was recently verified using cell-free, methane-oxidising systems from Pseudomonas methanica (Colby, Dalton and Whittenbury, 1975) and Methylococcus capsulatus (Bath) (Colby and Dalton, 1976).

No ethylene 'co-oxidation'would occur during acetylene reduction by nitrogen-fixing whole-cell suspensions of Methylococcus capsulatus (Bath) as it is thought that ethylene is oxidised by the methane mono-oxygenase and therefore ethylene oxidation will be completely inhibited by the acetylene present (see Section IV). Therefore it appeared that the reason for the failure of the acetylene reduction assays performed by de Bont and Mulder (1974) was not the 'co-oxidation' of the product ethylene but the inhibition of methane oxidation by acetylene , methane oxidation being required to generate the requisite energy and reducing power for nitrogenase activity (Dalton and Whittenbury, 1976b). de Bont later reported that the explanation above was , in fact, the most probable interpretation of the problem (de Bont, 1976a).

Dalton and Whittenbury (1976a) demonstrated that the acetylene reduction assay could, in fact, be used for cultures of methane-oxidising bacteria as long as a suitable electron donor (other than methane or any other oxygenase substrate) was present, and a suitably low partial pressure of oxygen employed. They found methanol, formaldehyde, potassium formate, ethanol or hydrogen could act as a suitable electron donor for nitrogenase activity.

Two different assays for nitrogenase activity have been suggested for methane-oxidising bacteria using substrates (other than acetylene) which allow methane to be used as electron donor; nitrous oxide, which

is reduced to dinitrogen (Dalton and Whittenbury, 1976a) and acrylonitrile, which is reduced to propylene, propane and ammonia (de Bont, 1976a). The latter assay has the disadvantage of producing products which could be oxidised.

(iii) Ammonia oxidation. - Ammonia ($NH_3 + NH_4^+$) has been shown to inhibit the growth of methane-utilising bacteria and competitively inhibit the oxidation of methane by these organisms (Whittenbury et al., 1970a; Wilkinson, 1971; Colby et al., 1975; Ferenci, Strøm and Quayle, 1975; O'Neill and Wilkinson, 1977; Drozd, Godley and Bailey, 1978). Ammonia oxidation by whole-cell suspensions of methane-oxidising bacteria was first reported by Whittenbury et al. (1970a) and since then a similar phenomenon has been reported for whole-cell suspensions of a Methylococcus sp. (Drozd, Bailey and Godley, 1976; Drozd et al., 1978), Methylosinus trichosporium OB3b and Methylomonas albus BG8 (O'Neill and Wilkinson, 1977) and in cell-free extracts of Methylococcus capsulatus (Bath) (Dalton, 1977). In each case it was suspected that ammonia was in fact oxidised by the methane oxygenase. There is some controversy over which active species was in fact oxidised, i.e. whether NH_3 or NH_4^+ was the substrate for the oxygenase. Whittenbury et al. (1970a) originally claimed NH_4^+ was the species oxidised, but provided no data to support the claim. Both Dalton (1977) and O'Neill and Wilkinson (1977) claimed that ammonia was the species oxidised, based on the increasing ammonia oxidation observed with increasing assay pH over pH 7.0 and would therefore be consistent with results obtained with Nitrosomonas europaea cells and extracts (Suzuki, Dular and Kwok, 1974). However, Drozd et al. (1978) suggested from their results that there was little evidence to support this view, even though they found that the V_{max} value for ammonia oxidation increased four-fold on raising the assay pH from pH 6 to pH 9.

All the reports suggested that the final oxidation product of ammonia oxidation is nitrite (Whittenbury et al., 1970a; Dalton, 1977; O'Neill and Wilkinson, 1977) except for the isolate of Drozd et al. with which they found nitrate was the major product (Drozd et al., 1976; Drozd et al., 1978).

The most comprehensive investigation into ammonia oxidation by a methane-oxidising bacterium was peformed by Dalton (1977) using cell-free extracts of Methylococcus capsulatus (Bath). He found that ammonia was oxidised to hydroxylamine (NH_2OH) which was subsequently oxidised to nitrite (NO_2^-). Hydroxylamine was oxidised to nitrite, catalysed by an enzyme system that used phenazine methosulphate (PMS) as electron acceptor in vitro but was not the methanol oxidase/dehydrogenase system found in extracts of this organism. He concluded that ammonia was oxidised by the methane mono-oxygenase based on four lines of evidence. Both ammonia and methane oxidation required reduced nicotinamide nucleotide (NADH) and gaseous oxygen for activity. The oxidations of methane to methanol and ammonia to hydroxylamine were specifically inhibited by acetylene, 8-hydroxyquinoline or methanol. A number of metal-chelating agents had no inhibitory effect on either methane or ammonia oxidation. Finally, methane was a good inhibitor of ammonia oxidation and vice versa. The inhibitor pattern obtained for ammonia oxidation by cell-free extracts of Methylococcus capsulatus (Bath) was much more restricted than that obtained for ammonia oxidation by whole-cell suspensions of Nitrosomonas europaea (Hooper and Terry, 1973) and possibly suggests a diversity between the two enzyme systems.

(iv) Ammonia assimilation .- The biochemistry and enzyme regulation of ammonia assimilation have been found to be very complex in enteric bacteria, i.e. Escherichia coli, Klebsiella aerogenes (Tempest, Meers and Brown, 1970; Foor, Janssen and Magasanik, 1975; Senior, 1975; Bender, Janssen, Resnick, Blumenberg, Foor and Magasanik, 1977). In these organisms it is thought that glutamine synthetase acts as a positive controller for nitrogen assimilation (Tubb, 1974; Streicher, Shanmugam, Ausubel, Morandi and Goldberg, 1974; Magasanik, 1977). When ammonia was present in excess of growth requirements, the level of glutamine synthetase (GS) was low and nitrogenase activity was undetectable, but the level of glutamate dehydrogenase (GDH) was high. Low concentrations of ammonia caused derepression of nitrogenase synthesis with a concomitant

rise in the level of GS and decrease in the level of GDH. Under nitrogen-fixing conditions, GS was high and GDH low (Nagatani, Shimazu and Valentine, 1971).

It is only very recently that any attempts to study this aspect of nitrogen metabolism have been made with methane-utilising bacteria. Dalton and Whittenbury (1976b) reported similar fluctuations in enzyme activity (GS, GDH and nitrogenase) as found with enteric organisms when Methylococcus capsulatus (Bath) was grown on methane in oxygen-limited continuous culture with different nitrogen sources (ammonia, nitrate or dinitrogen). However, these results are now thought to be erroneous in the light of current research being undertaken in our laboratory. It has been found recently that the level of GDH activity remains very low irrespective of the nitrogen source (or concentration) and a high level of GS activity appears to be constitutive (Orchard, personal communication). Also, high levels of alanine dehydrogenase (ADH) (approximately 80 m units (mg protein)$^{-1}$) have been found in crude cell-free extracts of Methylococcus capsulatus (Bath) and like GS appears to be constitutive (Orchard, personal communication). The presence of ADH could mean that Methylococcus capsulatus (Bath) has two constitutive routes for ammonia assimilation, i.e. glutamine synthetase/glutamate synthase mediated production of glutamate from ammonia and 2-oxoglutarate, and alanine dehydrogenase mediated alanine production from ammonia and pyruvate. There would appear to be no elaborate enzymic control mechanism for ammonia assimilation in Methylococcus capsulatus (Bath) as found in enteric organisms (Magasanik, 1977).

It is presently thought that ADH does not play an important role in primary ammonia assimilation in either prokaryotes or eukaryotes (Rowell and Stewart, 1976) but its constitutive presence in Methylococcus capsulatus (Bath) could mean that it plays an important role in either removing excess ammonia by the amination reaction involving pyruvate or providing ammonia by deamination under conditions of nitrogen starvation.

Similar results to those of Orchard in our laboratory were found with continuous culture studies using Methylococcus NCIB 11083 by Drozd et al. (1978). They too found GS constitutively produced irrespective of nitrogen source (ammonia, nitrate or dinitrogen) or ammonia concentration (up to 14 mM) and very low levels of GDH under all growth conditions. However, no significant levels of ADH were detected under any growth conditions unlike the results obtained with Methylococcus capsulatus (Bath).

A recent report by Shishkina and Chetina (1977) suggested that the two types of methane-utilising bacteria, as originally defined by Davies and Whittenbury (1970), showed differences in their nitrogen metabolism pathways based upon evidence obtained with seven methane-utilisers (4 Type I, 3 Type II). They claimed that Type I organisms assimilated ammonia mainly via the anabolic dehydrogenases of glutamate and alanine (GDH and ADH), and Type II organisms only assimilated ammonia via glutamine synthetase (GS) and glutamate synthase (GOGAT). On the evidence of Orchard and Drozd et al. above the correlation between organism Types (I and II) and ammonia assimilation pathways does not appear to be valid. Both Methylococcus capsulatus (Bath) and Methylococcus NCIB 11083 are Type I organisms and apparently possess high levels of GS constitutively, which is contrary to the pattern expected of Type I methane-utilising bacteria as proposed by Shishkina and Chetina (1977).

Obviously the area of nitrogen assimilation in methane-utilising bacteria has only undergone preliminary investigation and requires a great deal more work to dispel present anomolies and produce a clear outline of the ammonia assimilation routes involved in these organisms.

C. Methane Oxidation Pathway

(i) Introduction.- The pathway for the complete oxidation of methane has been generally assumed to involve the following series of reactions, originally proposed by Dworkin and Foster (1956):

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

The evidence for this pathway has been extensively reviewed by several workers (Ribbons, Harrison and Wadzinski, 1970; Wilkinson, 1971; Quayle, 1972; Wilkinson, 1975), therefore only a brief summary will be presented and for original references the above reviews should be consulted.

The proposed intermediates, methanol, formaldehyde and formate have been shown on numerous occasions to be oxidised by whole-cell suspensions of methane-utilising bacteria. One or more of the postulated intermediates have been detected, usually only in small quantities, on various occasions during the oxidation of methane or methanol by different methane-utilisers under environmental stress. However, it should be noted that some experiments involving the use of trapping agents such as iodoacetate or sodium sulphite to enhance the accumulation of intermediate (Brown et al., 1964) have proved difficult to repeat (Higgins and Quayle, 1970; Hazeu, 1975). The enzymes which catalyse the oxidation of methanol, formaldehyde and formate have been found active in cell-free extracts of various methane-utilising organisms and in some cases have been isolated and purified. Other circumstantial evidence for the series of reactions (1) includes the ability of methanol to act as an alternative carbon source and the fact that the majority of carbon is assimilated at the level of formaldehyde in obligate methane-utilising bacteria.

(ii) Initial oxidation of methane.- The early studies on the oxidation of methane were concerned with the question of the origin of the oxygen atom found in methanol. The first indirect evidence for the involvement of gaseous oxygen was obtained by Leadbetter and Foster (1959) who found that Pseudomonas methanica grown on methane in the presence of $^{18}O_2$ contained sixteen times the incorporation of $^{18}O_2$ into cell material than cells grown on methanol in the presence of $^{18}O_2$. These results led both Leadbetter and

Foster (1959) and Johnson (1967) to suggest that the oxidation of methane to methanol was catalysed by a mono-oxygenase (mixed function oxidase).

Conclusive evidence for the incorporation of gaseous oxygen into methane was obtained by Higgins and Quayle (1970), again using $^{18}O_2$. Although unable to repeat the experiments of Brown et al. (1964) where methanol accumulated in the presence of iodoacetate, they found that a high phosphate concentration (80 mM) caused the accumulation of methanol (up to 2.5 mM) with whole-cell suspensions of Pseudomonas methanica and Methanomonas methanooxidans oxidising methane.

These important findings suggested that one of two oxygenase enzyme mechnisms catalysed the oxidation of methane to methanol:

$$CH_4 + O_2 + XH_2 \longrightarrow CH_3OH + H_2O + X \qquad (2)$$

$$2CH_4 + O_2 \longrightarrow 2CH_3OH \qquad (3)$$

Reaction (2) is catalysed by a mono-oxygenase (mixed function oxidase) and reaction (3) by a di-oxygenase. The results virtually excluded the possibility that a hydroxylase enzyme mechanism (4) was operative, as suggested by Whittenbury (1969).

$$CH_4 + H_2O \longrightarrow CH_3OH + 2H \qquad (4)$$

He based his hypothesis on two main observations. Firstly, the inhibition of methanol oxidation by iodoacetate, which had no effect on methane oxidation (Brown et al., 1964). This suggested that a mono-oxygenase could not be involved as theprovision of the requisite reducing power for such an enzyme would be terminated if the further oxidation of methanol was inhibited. Secondly, molar growth yields on methane were reported to be higher than those obtained with methanol which suggested that the initial oxidation step of methane to methanol is energy productive.

The specific inhibition of methanol oxidation by iodoacetate reported by Brown et al. (1964) could not be repeated by Higgins and Quayle (1970) and therefore the result must be regarded with some doubt. The molar growth yields can still be interpreted in terms of a methane mono-oxygenase system if either a completely novel coupling of ATP synthesis to the mono-oxygenase existed which, thermodynamically, is perfectly feasible, or methanol-uptake is energy-dependent but methane is not (Quayle, 1972).

The fundamental problem concerning the elucidation of the mechanism of initial enzymic attack on methane by methane-utilisers has historically been the difficulty in obtaining stable cell-free extracts which showed methane-oxidising activity. The first report of such activity by cell-free extracts of a methane-utilising organism was by Ribbons and Michalover (1970) who demonstrated methane-stimulated respiration and methane-stimulated NADH oxidation by cell-free particulate preparations of Methylococcus capsulatus (Texas). Subsequently, similar results were obtained by Ferenci using cell-free particulate preparations of Pseudomonas (Methylomonas) methanica (Ferenci, 1974; Ferenci et al., 1975). Both systems gave tentative stoichiometric relationships which suggested a mono-oxygenase mechanism for methane oxidation but none of this work could be considered as conclusive evidence for such a mechanism as neither substrate disappearance nor product accumulation was demonstrated, and the cell-free methane oxidation activity was notoriously unstable.

The lack of evidence regarding substrate disappearance and product formation was partially fulfilled with the demonstration of simultaneous NADH oxidation, methane and oxygen uptake by particulate extracts of Methylococcus capsulatus (Texas) (Ribbons, 1975). No initial product of methane oxidation was detected due to the presence of methanol- and formaldehyde- oxidising activities in the crude, particulate extracts however, formate did accumulate but only 60% of the expected yield (Ribbons, 1975; Wadzinski and Ribbons, 1975).

Two other reports of cell-free methane oxygenase activity were published in 1975, one using extracts of Methylosinus trichosporium OB3b (Tonge, Harrison, Knowles and Higgins, 1975), and the other using extracts of Methylomonas methanica (Colby, Dalton and Whittenbury, 1975). Sonicated, crude extracts of Methylosinus trichosporium OB3b occasionally showed methane-dependent oxygen consumption, but this activity was always lost on storage at 4^{o} C for 24 hours. It was found that a high phosphate concentration (150 mM) completely inhibited methanol oxidase activity in the sonicated extracts, verifying the earlier findings of Higgins and Quayle (1970) with washed, cell-suspensions of other methane-utilising organisms. When 150 mM phosphate was incorporated in the assay for methane oxygenase activity, it was found that methanol accumulated at a rate seven times that of the concomitant methane-stimulated, NADH-dependent oxygen consumption (Tonge et al., 1975). Although this was the first clear evidence that methanol was the initial product of methane oxidation, it appeared that the stoichiometric relationship beween oxygen consumption and product formation (1.3:0.9) was inconsistent with a mono-oxygenase mechanism for methane oxidation. However, it was found that a number of electron donors as well as NADH (ascorbate, methanol, formate plus NAD^{+}) could support methane oxidation by crude, cell-free extracts and it was suggested that the oxidation of methane may cause a redirection of pre-existing electron flow to oxygen into the methane oxygenase system (Tonge et al., 1975; Higgins, Knowles and Tonge, 1976).

Crude, particulate extracts of Methylomonas methanica were found to catalyse the oxygen- and NAD(P)H-dependent disappearance of bromomethane (Colby et al., 1975). The stoichiometric relationship between the specific activities of bromomethane-dependent NADH oxidation and bromomethane disappearance suggested a mono-oxygenase catalysed reaction. Colby et al. (1975) also proposed that in Methylomonas methanica the enzyme that catalysed bromomethane disappearance in vitro was in fact the enzyme that catalysed methane oxidation in vivo,

based on the following evidence: similar inhibition patterns, using various metal-binding agents, for bromomethane oxidation by extracts of Methylomonas methanica and for methane oxidation by whole-cell suspensions of Methylosinus trichosporium OB3b (Hubley, Thomson and Wilkinson, 1975) plus the inhibition of bromomethane oxidation caused by substrate analogues and acetylene. The use of a substrate analogue to assay for methane oxidation in cell-free extracts had been shown previously by Ferenci using carbon monoxide (CO) as a substrate for the cell-free methane oxygenase in extracts of Pseudomonas methanica (Ferenci, 1974; Ferenci et al., 1975; see Section V). He measured oxygen consumption and carbon dioxide (CO_2) production concomitantly in an oxygen electrode and the stoichiometries of CO-dependent CO_2 formation, O_2 consumption and NADH oxidation, plus the partial stoichiometries of methane-dependent NADH oxidation, suggested the involvement of a mono-oxygenase in these reactions (Ferenci et al., 1975). Strong circumstantial evidence was presented to suggest that CO mono-oxygenase activity was a secondary activity of the methane mono-oxygenase, including similar enzyme inhibition patterns, the same pH optimum for activity and similar stability on storage (Ferenci et al., 1975).

A soluble, methane oxygenase was obtained from crude, cell-free extracts of Methylococcus capsulatus (Bath) when centrifuged at 160 000 g for one hour (Colby and Dalton, 1976). This was the first report of a totally soluble methane oxygenase, although Tonge et al. (1975) were able to obtain active, soluble preparations of methane mono-oxygenase by treatment of the particulate membrane extracts of Methylosinus trichosporium OB3b with detergents, phospholipase D or or ultrasonic radiation. Colby and Dalton (1976) found that methanol oxidase activity, present in the soluble extract, was not inhibited by high phosphate concentrations (cf. Tonge et al., 1975) but was totally inhibited by 0.5 mM potassium cyanide. The soluble methane oxygenase, unaffected by the presence of potassium cyanide, could catalyse both

methane oxidation with the subsequent accumulation of methanol and bromomethane disappearance. A number of control assays(requirement for gaseous oxygen, no acitivty in absence of NADH, inhibited by acetylene)suggested that the enzyme which catalysed methane oxidation in Methylococcus capsulatus (Bath) was a mono-oxygenase. The enzyme was fractionated into two components by ion-exchange chromatography (DEAE-cellulose) and activity was only restored on mixing the two components, suggesting that the methane mono-oxygenase of Methylococcus capsulatus (Bath) was a multi-component enzyme (Colby and Dalton, 1976).

The methane oxygenase present in crude extracts of Methylosinus trichosporium OB3b (Tonge et al., 1975),mentioned above, has recently been purified and shown to be a three-component enzyme system (Tonge, Harrison and Higgins, 1977). The components are a soluble CO-binding cytochrome c , a copper-containing protein and a small protein with molecular weights of 13 000, 470 00 and 9 400 respectively. The purified enzyme used ascorbate, or methanol in the presence of partially purified methanol dehydrogenase from the same organism, as electron donor,but is unable to use NADH or NAD(P)H. It appears that in vivo electrons are recycled from the non-NAD(P)H-linked methanol dehydrogenase to the cytochrome c_{CO} component which is the immediate electron donor for the oxygenase system (Tonge et al., 1977). NAD(P)H could presumably serve indirectly as an electron donor in vivo, via the electron transport chain. It was found that the soluble cytochrome c_{CO} component possessed high ascorbate oxidase activity (50 μmol of oxygen consumed min^{-1} (mg protein)$^{-1}$), but on the addition of the other two methane oxygenase components plus methane most of the electron flow to oxygen became involved in the oxygenase reaction, based on the rates of methanol accumulation. The overall rate of oxygen consumption was the same in the presence or absence of methane, therefore it appeared that the addition of methane to the purified components plus

ascorbate caused a change in the reaction responsible for oxygen consumption, i.e. oxidase to oxygenase, but not in the overall rate of oxygen consumption. A stoichiometric realtionship of 5:4:7 was obtained for methane utilisation/methanol accumulation/oxygen consumption and suggested that the change from an oxidase to an oxygenase function was not complete, and approximately 40% of electron flow was still via the oxidase mechanism (Tonge et al., 1977). If this assumption was correct it would appear that the enzyme responsible for methane oxidation in Methylosinus trichosporium OB3b was almost certainly a mono-oxygenase.

All the reports of cell-free methane oxygenase activity prepared from various methane-utilising bacteria have suggested that the enzyme responsible for methane oxidation in these organisms was a mono-oxygenase (Ribbons and Michalover, 1970; Ferenci, 1974; Colby et al., 1975; Ferenci et al., 1975; Ribbons, 1975; Tonge et al., 1975; Colby and Dalton, 1976; Higgins et al., 1976; Tonge et al., 1977) and therefore is analogous to microbial enzyme systems involved in higher alkane oxidation (see Section IV). Although from the studies of purified or partially purified enzymes all the methane-oxidising bacteria appear to possess a methane mono-oxygenase, there seems to be at least two different types based on the form of reductant required for activity (Colby and Dalton, 1976; Tonge et al., 1977; Colby and Dalton, personal communication). The methane mono-oxygenase system from Methylosinus trichosporium OB3b uses a reduced cytochrome c_{-CO} as its immediate electron donor, which is reduced by electrons recycled from methanol oxidation (Tonge et al., 1977), whereas the methane mono-oxygenase system from Methylococcus capsulatus (Bath) uses NAD(P)H directly as its electon donor (Colby and Dalton, 1976; Colby and Dalton, personal communication). The prevalence of a particular methane mono-oxygenase system, using either of the above electron donor schemes or the presence of another novel scheme(s) in methane-utilising bacteria generally, remains to be

seen, but the type of mono-oxygenase present in an organism could prove to be of some taxonomic use in the future.

Two other speculative mechanisms of methane oxidation have been suggested in recent years: (i) the oxidation of methane via dimethyl ether, (ii) the oxidation of methane via free radical intermediates.

The unpublished results of Bryan-Jones and Wilkinson (Wilkinson, 1971),supposedly showing that dimethyl ether was a growth substrate for methane-utilising bacteria and was oxidised by whole-cell suspensions, led to the suggestion that instead of the oxygenation of methane producing methanol directly, the initial product was dimethyl ether according to the following equation:

$$2CH_4 + O_2 \longrightarrow CH_3OCH_3 + H_2O \qquad (5)$$

It was reported that dimethyl ether had been shown to accumulate when whole-cell suspensions of methane-oxidising bacteria were incubated with methane, but no data was provided and it was stated that the conditions required for such an accumulation had proved difficult to reproduce, so the phenomenon remains doubtful (Thomson, 1974; Hubley, 1975; Wilkinson, 1975). These results/observations plus other unpublished work from the same laboratory in Edinburgh led to the proposal of two different schemes for the oxidation of methane involving dimethyl ether as an intermediate (Davey, 1971; Thomson, 1974; Hubley, 1975).

The first consisted of the oxidation of methane to dimethyl ether, which was subsequently oxidised to methyl formate. Methyl formate was then hydrolysed by an esterase to give methanol and formate (Davey, 1971). The second scheme was a modified version of the first, where methane was oxidised to the unstable hemi-acetal methoxymethanol via dimethyl ether. The hemi-acetal could then either spontaneously break down to give methanol and formaldehyde or form methyl formate as before (Thomson, 1974; Hubley, 1975). The validity of these two schemes and the involvement of dimethyl ether in methylotrophic metabolism is discussed in more detail in Section V.

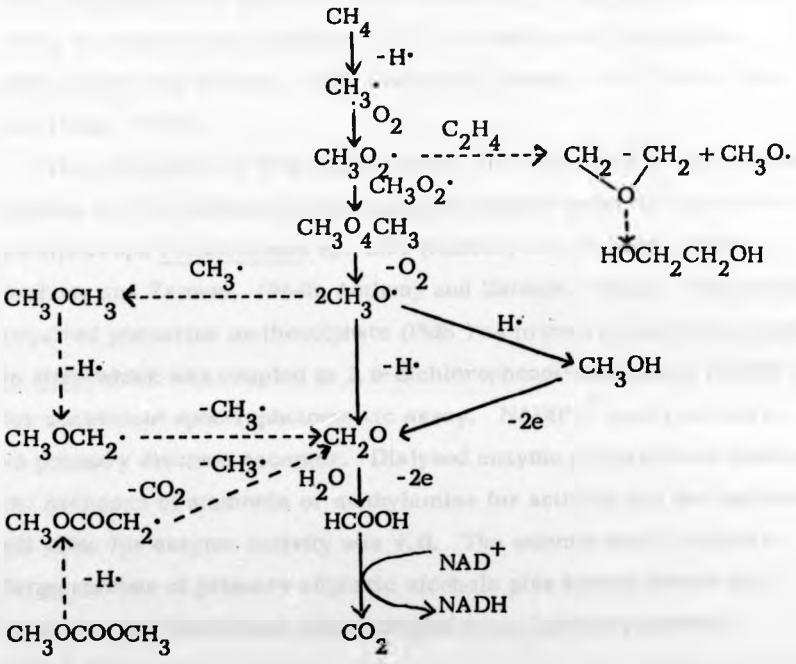
Current evidence suggests that dimethyl ether is not an intermediate in the oxidation of methane, i.e. the demonstration of stoichiometric methanol accumulation during methane oxidation by various cell-free preparations of methane mono-oxygenase (Tonge et al., 1975; Colby and Dalton, 1976; Tonge et al., 1977), the observation that purified methane mono-oxygenase was unable to oxidise dimethyl ether (Tonge et al., 1977) plus the dubious nature of some of the above evidence.

Hutchinson, Whittenbury and Dalton (1976) proposed that methane was oxidised by Methylococcus capsulatus (Bath) by a mechanism involving free radicals. The pathway they proposed is shown in Fig. 1, the first stable intermediate being formaldehyde which can undergo normal enzymic catalysis (see below). The complex metallochemistry involved in this elegant hypothesis is too extensive to cover in this introduction and the reader is referred to the original paper for coverage of this aspect. Current knowledge of the enzyme responsible for methane oxidation in Methylococcus capsulatus (Bath) would suggest that the above hypothetical mechanism, however elegant, is not operative in this organism. Methanol has been shown to accumulate during methane oxidation by crude, soluble cell-free extracts of Methylococcus capsulatus (Bath) and this activity required NAD(P)H (Colby and Dalton, 1976; Colby and Dalton, personal communication). These cell-free extract preparations can, in addition to methane, oxidise di- and tri- halogenated methane analogues (see Section IV) and such reactions could 'poison' the proposed free-radical mechanism (Hutchinson et al., 1976). The enzyme responsible for the initial oxidation of methane in Methylococcus capsulatus (Bath) has been found to be a multi-component enzyme and is nearing purification (Colby and Dalton, 1976, Colby and Dalton, 1978; Colby and Dalton, personal communication).

(iii) Oxidation of methanol - Methanol dehydrogenase activity has been demonstrated in cell-free extracts of both obligate and facultative methylotrophs (Johnson and Quayle, 1964; Anthony and Zatman, 1964a; Anthony and Zatman, 1964b; Ladner and Zatman, 1969; Heptinstall and

Fig. 1. Possible pathways involved in the free radical oxidation of methane.

——— main pathways --------- ancillary pathways



(from Hutchinson et al., 1976)

Quayle, 1970; Davey, 1971; Patel and Hoare, 1971; Dahl *et al.*, 1972; Patel, Bose, Mandy and Hoare, 1972; Mehta, 1973; Patel, Mandy and Hoare, 1973; Sperl, Forrest and Gibson, 1974; Colby and Zatman, 1975; Wadzinski and Ribbons, 1975; Goldberg, 1976; Patel and Felix, 1976; Ben-Bassat and Goldberg, 1977; Yamanaka and Matsumoto, 1977; Yordy and Weaver, 1972; Ghosh and Quayle, 1978; Patel, Hou and Felix, 1978).

The properties of this enzyme were first described by Anthony and Zatman for the methanol dehydrogenase isolated from the facultative methylotroph *Pseudomonas* sp. M27 (Anthony and Zatman, 1964a; Anthony and Zatman, 1964b; Anthony and Zatman, 1965). The enzyme required phenazine methosulphate (PMS) as primary electron acceptor *in vitro* which was coupled to 2, 6-dichlorophenol-indophenol (DCPIP) for convenient spectrophotometric assay. $NAD(P)^+$ would not serve as primary electron acceptor. Dialysed enzyme preparations required the presence of ammonia or methylamine for activity and the optimum pH value for enzyme activity was 9.0. The enzyme could oxidise a large number of primary aliphatic alcohols plus formaldehyde and therefore has sometimes been referred to as 'primary alcohol dehydrogenase'.

An NAD^+-linked dehydrogenation of methanol involving a catalase-linked peroxidase was reported in a methanol-utilising organism identified as *Pseudomonas methanica* (Harrington and Kallio, 1960). However, Johnson and Quayle (1964) suggested that this phenomenon was probably an *in vitro* artefact, as a known inhibitor of catalase (3-amino-1, 2, 4-triazole) had no effect on methanol oxidation in cell-free extracts of that organism, and the absence of such activity in *Pseudomonas* sp. 1727 (Anthony and Zatman, 1964b) and *Pseudomonas* AM1 (Johnson and Quayle, 1964) would support this view. Harrington and Kallio (1960) did not examine their cell-free extracts of *Pseudomonas methanica* for the presence of PMS-dependent methanol dehydrogenase activity.

Methanol dehydrogenase has been purified from a number of methylotrophs (Table 2). All the enzymes purified to date possess similar properties as described for the enzyme from Pseudomonas sp. M27 by Anthony and Zatman (1964a; 1964b; 1965; 1967a; 1967b). They all have a molecular weight of around 120 000 with the exception of the enzyme isolated from Methylosinus sporium which had a molecular weight of 60 000 (Patel and Felix, 1976).

Table 2. Methylotrophs from which methanol dehydrogenase has been purified

Organism	Reference
Pseudomonas M27	Anthony and Zatman (1967a) Patel et al. (1972)
Methylococcus capsulatus (Texas)	Patel et al. (1972) Wadzinski and Ribbons (1975)
Pseudomonas RJ1	Mehta (1973)
Hyphomicrobium WC	Sperl et al. (1974)
Pseudomonas TP1	Sperl et al. (1974)
Pseudomonas W1	Sperl et al. (1974)
Pseudomonas C	Goldberg (1976)
Methylosinus sporium	Patel and Felix (1976)
Pseudomonas 2941	Yamanaka and Matsumoto (1977)
Methylophilus methylotrophus	Ghosh and Quayle (1978)
Methylomonas methanica	Patel et al. (1978)

There is now good evidence that methanol dehydrogenase can catalyse the PMS-dependent oxidation of formaldehyde to formate in the presence of ammonia as an activator. This phenomenon was originally reported by Ladner and Zatman (1969), but the validity of their report is questionable because of their discovery at a late stage that their formaldehyde was contaminated with methanol. Further circumstantial evidence for the dual specificity of the enzyme was provided by Heptinstall and Quayle (1970) who showed that extracts of Pseudomonas AM1, under normal methanol dehydrogenase assay conditions, oxidised formaldehyde at a rate approximately a quarter of that observed with methanol, while a mutant strain M-15A, which lacked methanol dehydrogenase, was unable to oxidise formaldehyde.

More conclusive evidence came from the demonstration that purified methanol dehydrogenase from Methylococcus capsulatus (Texas) oxidised both formaldehyde and methanol, and the ratios of specific activity remained constant over a wide range of pH and ammonia concentrations(Patel et al., 1972). Similar studies with the purified methanol dehydrogenases from three methanol-utilisers, Hyphomicrobium WC, Pseudomonas TP1 and Pseudomonas W1,supported the dual specificity of the enzyme and also showed that acetaldehyde could be oxidised at a low rate by the enzyme when assayed manometrically over a 15 minute period (Sperl et al., 1974). The failure of other laboratories to detect acetaldehyde oxidation was ascribed to the short time interval over which the normal spectrophotometric dye reduction assays were performed (Sperl et al., 1974). The fact that formaldehyde and acetaldehyde can undergo hydration to the respective diols prompted Sperl et al. (1974) to suggest that this was the reason why these two aldehydes alone can act as substrates for the primary alcohol dehydrogenase.

A detailed study of the distribution and nature of methanol/formaldehyde dehydrogenation activity in Methylococcus capsulatus (Texas) by Wadzinski and Ribbons (1975) revealed two apparently distinct enzymes: a methanol/formaldehyde oxidase which was PMS-independent and the normal PMS-dependent dehydrogenase. They found that approximately 60% of the methanol oxidising activity found in extracts resided in the particulate fraction, although this was easily solubilised by various phospholipases or detergents. Due to the probable association of methane mono-oxygenase activity with the membrane fraction (Ribbons, 1975) they proposed that in Methylococcus capsulatus (Texas) the complete oxidation of methane to formate, with concomitant carbon fixation, occurs in a membrane-bound environment.

During methanol oxidation in whole cells, PMS is presumably replaced by an unknown physiological electron acceptor which is part of the organism's electron transport chain. This unknown component is almost certainly not $NAD(P)^+$ considering the in vitro observations above and the fact that the standard redox potential for the methanol/formaldehyde

half reaction at pH 7.0 (E_o' - 0.182 volts) is thought insufficient for the reduction of $NAD(P)^+$ (E_o' - 0.320 volts) (Ribbons et al., 1970; Thauer, Jungermann and Decker, 1977).

It has been reported that methanol dehydrogenase is not a flavoprotein but does contain a pteridine derivative (Anthony and Zatman, 1967a; Anthony and Zatman, 1976b; Sperl et al., 1974). Sperl et al. (1974) suggested that the prosthetic group is probably a lumazine (2, 4-dihydroxypteridine) derivative based on the effects of chemical treatment on the fluorescence and absorption spectra of the pteridine derivative from the purified alcohol dehydrogenase from various methylotrophs.

Two mechanisms have been proposed by which pteridines might be involved in methanol oxidation (Anthony and Zatman, 1967b). The first involves a folic acid type of function where the alcohol binds to the $N_{(5)}$ atom of the pteridine ring with the subsequent formation of a 5, 10-methylene derivative. The second mechanism involves the pteridine derivative functioning in a manner analogous to flavin adenine dinucleotide (FAD) or flavin mono-nucleotide (FMN) in flavoproteins and acting as the natural electron acceptor for methanol dehydrogenase activity.

The first mechanism is attractive because the 5, 10-methylene derivative produced could be assimilated directly by the serine pathway via serine hydroxymethylase, hydrolysed to free formaldehyde or oxidised to a 5,10-methylidyne derivative and subsequently to formate. The advantage of the second mechanism is the possible role such a reduced co-factor could play in supplying electrons for methane oxidation or in electron transfer to the cytochrome chain. The results of Sperl et al. (1974), tentatively identifying a lumazine derivative, would tend to favour the second of the above mechanisms as such a compound could act as an electron carrier.

A role for cytochrome c in the oxidation of methanol by methylotrophs was originally suggested as a result of the inability of cells of a cytochrome c-deficient mutant of Pseudomonas AM1 (containing normal

levels of methanol dehydrogenase activity) to oxidise methanol (Anthony , 1975b). Since then it has been shown that a CO-binding cytochrome c purified from Methylosinus trichosporium OB3b could act as an electron acceptor for methanol dehydrogenase activity, and the reduced form could then act as an immediate electron donor for methane mono-oxygenase, purified from the same organism (Tonge et al., 1975; Tonge et al., 1977). It was proposed that in Methylosinus trichosporium OB3b the electrons derived from the oxidation of methanol were passed from the pteridine derivative of the methanol dehydrogenase to the cytochrome c from which the electrons could then be recycled into the methane oxygenation reaction (Tonge et al., 1975; Higgins et al., 1976).

(iv) Oxidation of formaldehyde and formate.- Formaldehyde can be oxidised to carbon dioxide (CO_2) by two routes in methylotrophs: (i) oxidised to formate then CO_2, (2) oxidised to CO_2 by a cyclic enzyme scheme involving hexulose phosphate synthase (Strøm, Ferenci and Quayle, 1974; Colby and Zatman, 1975). The former route involves formaldehyde dehydrogenase of which there are seven reported types in methylotrophs (see Section VI). No methane-oxidising bacterium, with the exception of Pseudomonas methanica (Harrington and Kallio, 1960; Johnson and Quayle, 1964) have been shown to possess an $NAD(P)^+$-linked formaldehyde dehydrogenase. A number of methane-oxidising bacteria have been shown to possess the enzymic potential for the second route of formaldehyde oxidation, i.e. the cyclic scheme involving hexulose phosphate synthase, but it is generally unknown what percentage, if any, this route contributes to the overall oxidation of formaldehyde in vivo. The oxidation of one molecule of formaldehyde by this route can theoretically produce two molecules of NAD(P)H (Strøm et al., 1974; Colby and Zatman, 1975). It appears that the majority of methane-utilising bacteria are possibly dependent on the dual specificity of methanol dehydrogenase for the bulk of their formaldehyde oxidation, although this assumption is based on the possibly artefactual in vitro properties of the enzyme.

The whole area of formaldehyde oxidation by methylotrophs is extensively covered in Section VI.

Formate oxidation in methylotrophs appears to be universally catalysed by an NAD^+-linked formate dehydrogenase, first described by Johnson and Quayle (1964). The enzyme has been reported present in numerous methane-utilising bacteria (Johnson andQuayle, 1964; Davey, 1971; Patel and Hoare, 1971; Quayle, 1972; Wadzinski and Ribbons, 1975; Ben-Bassat and Goldberg, 1977). Recently, low formate dehydrogenase activity (15 m units (mg protein)$^{-1}$) was reported in a methane-utilising bacterium, Methylophilus methylotrophus, for the first time (Taylor,L,1977; Beardsmore and Quayle, 1978). It was suggested that this organism oxidised most of its formaldehyde by the cyclic scheme mentioned above.

D. Carbon Assimilation Pathways in Methylotrophs

Due to the extensive literature concerning this topic and because it is not directly relevant to the work contained in this thesis, this area of methylotrophic biochemistry will be summarised. Further knowledge of the elucidation of the pathways concerned and the experimental techniques employed in doing so can be found in the following reviews: Quayle (1972) and Anthony (1975a).

(i) Distribution of carbon assimilation pathways. - All methylotrophs (as defined by Colby and Zatman (1972))can be considered to assimiliate carbon at the oxidation level of formaldehyde, but some organisms, which are not methylotrophs as defined above, can grow on methanol autotrophically using the ribulosebisphosphate pathway of carbon fixation (Cox and Quayle, 1975; Sahm, Cox and Quayle, 1976).

In general, methane-utilising bacteria with aType I membrane system (Davies and Whittenbury, 1970) all use the ribulose monophosphate cycle of formaldehyde fixation, whereas organisms with a Type II membrane system use the serine pathway, Anomalies to this general rule are known and were discussed earlier. All obligate methylotrophs isolated to date which cannot utilise methane as a sole source of carbon, appear to possess

the ribulose monophosphate cycle (Colby and Zatman, 1972; Dahl et al., 1972; Colby and Zatman, 1975; Sahm and Wagner, 1975) as do a very few facultative methylotrophs (Stieglitz and Mateles, 1973; Colby and Zatman, 1975). The vast majority of facultative methylotrophs use the serine pathway of formaldehyde fixation, usually the isocitrate lyase variant (Anthony, 1975a).

(ii) The serine pathway of formaldehyde assimilation. - Virtually all the work done in elucidating this pathway was done with facultative methanol-utilising bacteria as these organisms are considerably easier to work with due to their versatility of growth substrate. This ability to grow on multi-carbon compounds permitted the isolation of mutants for C_1-assimilation pathways whereas such mutations would probably be lethal to an obligate methylotroph.

The serine pathway, as it operates in most of the methylotrophs, is outlined in Fig. 2 and can be seen to be a cyclic scheme of reactions which effectively condenses formaldehyde and CO_2 to produce one molecule of acetyl-CoA (Anthony, 1975a):

$$HCHO + CO_2 + 2NADH_2 + 2ATP + CoA \longrightarrow CH_3CO\text{-}CoA + 2NAD^+ + 2ADP + 2Pi + H_2O \qquad (6)$$

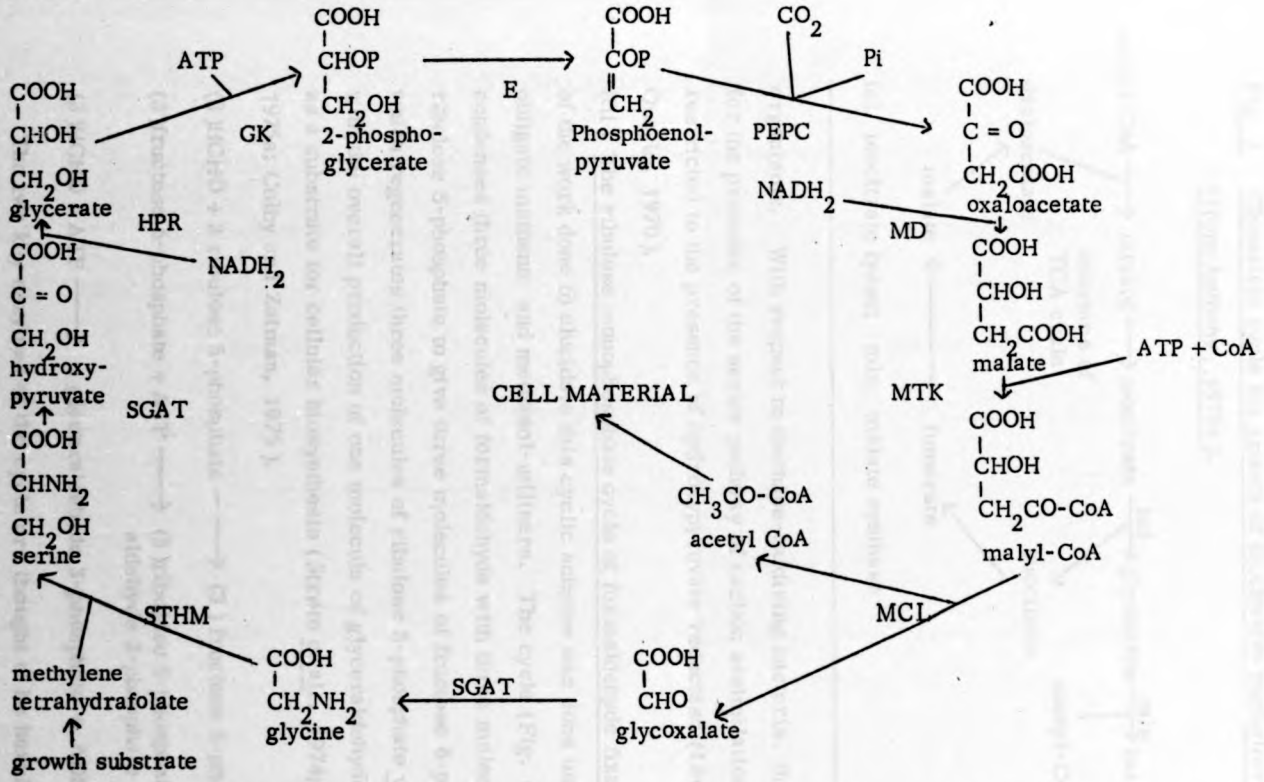
The cycle is initiated by serine transhydroxymethylase which catalyses serine synthesis from glycine and $N^{5,10}$-methylenetetrahydrofolate (originally derived from methanol). There are considered to be four key enzymes operating in the cycle, i.e. those enzymes that are thought to exclusively function in the serine pathway, serine-glyoxalate aminotransferase, hydroxypyruvate reductase, glycerate kinase and malyl-CoA lyase (Anthony, 1975a). Hydroxypyruvate reductase is usually the enzyme which is assayed for in cell-free extracts of methylotrophs as an initial indicator of the serine pathway being operative in the organism, although as reported by Bamforth and Quayle (1977) working with Paracoccus denitrificans the presence of this enzyme alone does not constitute conclusive proof of the existence of the complete serine pathway.

The next step in the overall pathway is the net conversion of acetyl-CoA to the C_3- and C_4-compounds required for cellular biosynthesis. These compounds are available from the cyclic scheme itself, but to maintain the cycle operative, acetyl-CoA must be converted to glyoxalate to replenish the cycle. This is probably accomplished by one of two routes, depending on the presence or absence of the enzyme isocitrate lyase. The organisms which possess this enzyme appear to generate glyoxalate from acetyl-CoA via the glyoxalate cycle (Fig. 3), the two key enzymes being isocitrate lyase and malate synthase. However, if the glyoxalate cycle was operative during methylotrophic growth, i.e. the serine pathway was also operative, there would appear to be an antithesis of function, one catalysing the synthesis of malate (malate synthase), the other the cleavage of malate (malyl-CoA lyase). It was shown that during methylotrophic growth, Hyphomicrobium X did not synthesise malate synthase although isocitrate lyase was present, whereas during growth on acetate or ethanol both enzymes were present suggesting that during methylotrophic growth the complete glyoxalate cycle was not operative (Harder, Attwood and Quayle, 1973). To accommodate this, Harder et al. (1973) proposed an overall pathway for methanol assimilation involving isocitrate lyase but omitting malate synthase (Fig. 4). The resultant biosynthetic intermediate is a C_3-compound, 3-phosphoglycerate.

Glyoxalate formation in organisms with no isocitrate lyase, e.g. Pseudomonas AM1 and other pink pseudomonads, is thought to be connected with a malate synthase cycle found operative during growth on ethanol and 8-hydroxybutyrate (Anthony, 1975a). This pathway is similar to the glyoxalate pathway, differing only in the generation of glyoxalate from acetyl-CoA. It is thought that glycollate could possibly be an intermediate between acetyl-CoA and glyoxalate, therefore permitting two possible schemes for the malate synthase cycle (Fig. 5)(Anthony, 1975a).

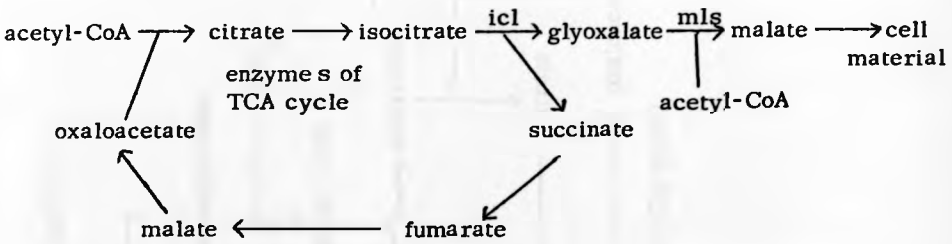
Obviously much more research is required to resolve the problem of glyoxalate regeneration in both variants of the serine pathway, i.e. the isocitrate lyase positive organisms and the isocitrate lyase negative

Fig. 2. The serine pathway for methylotrophic growth (from Anthony, 1975a).



STHM, serinetranshydroxymethylase; SGAT, serine-glyoxalate aminotransferase; HPR, hydroxypyruvate reductase; GK, glycerate kinase; E, enolase; PEPC, phosphoenolpyruvate carboxylase; MD, malate dehydrogenase; MTK, malate thiokinase; MCL, malyl-CoA-lyase.

Fig. 3 Glyoxalate cycle for growth of facultative methylotrophs (from Anthony, 1975a).



icl, isocitrate lyase; mls, malate synthase

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organisms. With respect to methane-oxidising bacteria, the evidence for the presence of the serine pathway of carbon assimilation is usually restricted to the presence of hydroxypyruvate reductase (Lawrence and Quayle, 1970).

(iii) The ribulose monophosphate cycle of formaldehyde fixation. - Most of the work done to elucidate this cyclic scheme was done using both obligate methane- and methanol-utilisers. The cycle (Fig. 6) effectively condenses three molecules of formaldehyde with three molecules of ribulose 5-phosphate to give three molecules of fructose 6-phosphate which regenerates three molecules of ribulose 5-phosphate via the cycle with the overall production of one molecule of glyceraldehyde 3-phosphate as a substrate for cellular biosynthesis (Strøm et al., 1974; Anthony, 1975a; Colby and Zatman, 1975).

(3) HCHO + 3 ribulose 5-phosphate ⟶ (3) fructose 6-phosphate (7)

(3) fructose 6-phosphate + ATP ⟶ (3) ribulose 5-phosphate + glyceraldehyde 3-phosphate + ADP (8)

(3) HCHO + ATP ⟶ 1 glyceraldehyde 3-phosphate + ADP (7) + (8)

The two key enzymes of the cycle are thought to be hexulose phosphate synthase and hexulose phosphate isomerase, the enzymes responsible for condensing formaldehyde and ribulose 5-phosphate and converting the

Fig. 4. Pathway of C_1 assimilation in methanol-grown Hyphomicrobium X (from Harder et al., 1973).

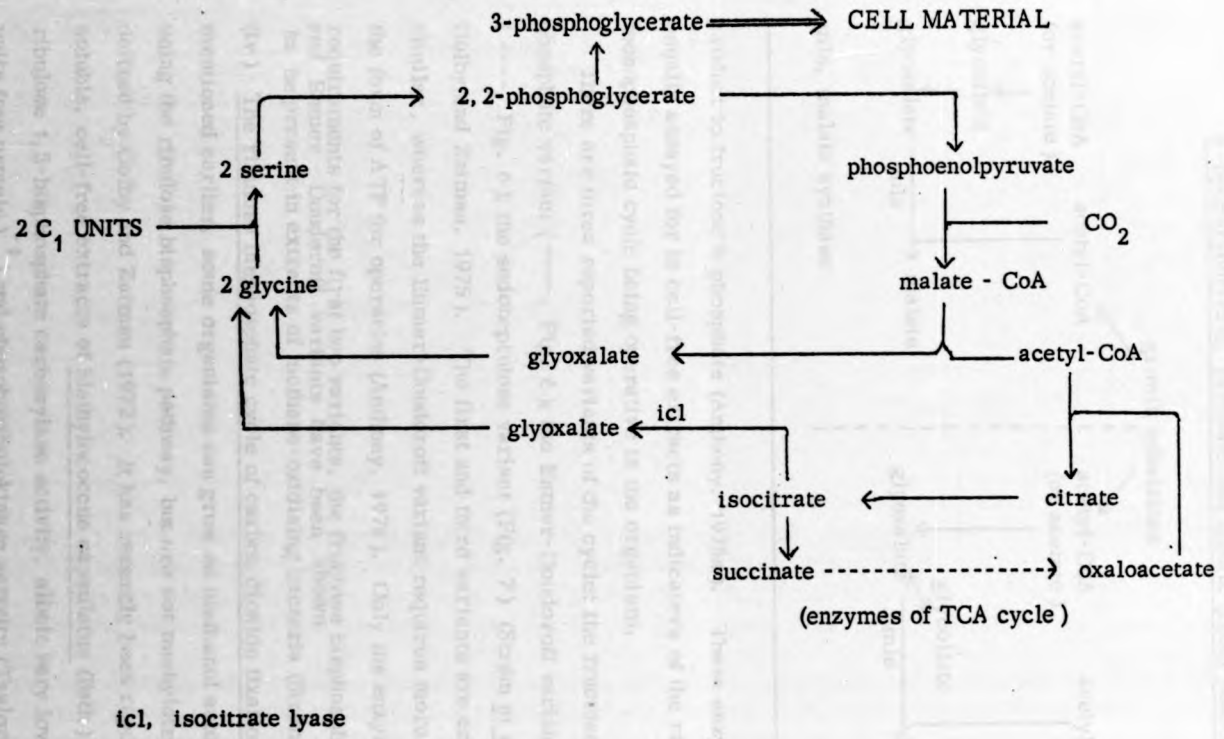
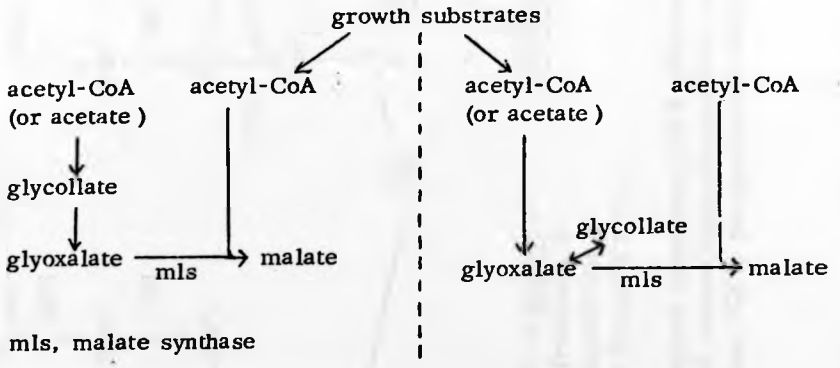


Fig. 5. Malate synthase pathway for bacterial growth on ethanol, malonate, β-hydroxybutyrate, pyruvate and lactate (from Anthony, 1975a).

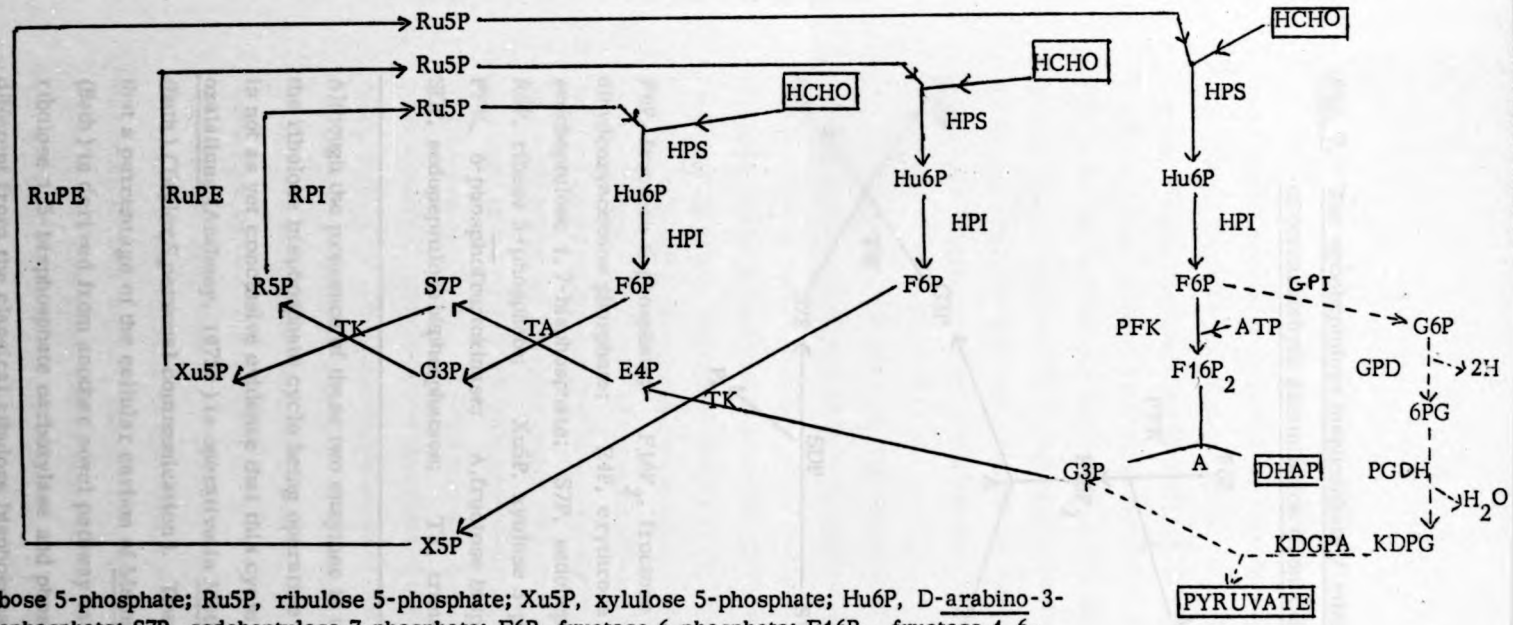


product to fructose 6-phosphate (Anthony, 1975a). These enzymes are usually assayed for in cell-free extracts as indicators of the ribulose monophosphate cycle being operative in the organism.

There are three reported variants of the cycle: the fructose bisphosphate variant (——, Fig. 6); the Entner-Doudoroff variant (----, Fig. 6); the sedoheptulose variant (Fig. 7) (Strøm et al., 1974; Colby and Zatman, 1975). The first and third variants are energetically similar, whereas the Entner-Doudoroff variant requires more energy in the form of ATP for operation (Anthony, 1978). Only the enzymic requirements for the first two variants, the fructose bisphosphate and Entner-Doudoroff variants have been shown to be present in extracts of methane-oxidising bacteria (Strøm et al., 1974).

(iv) The ribulose bisphosphate cycle of carbon dioxide fixation.- As mentioned earlier, some organisms can grow on methanol autotrophically using the ribulose bisphosphate pathway, but are not methylotrophs as defined by Colby and Zatman (1972). It has recently been reported that soluble, cell-free extracts of Methylococcus capsulatus (Bath) contained ribulose 1,5-bisphosphate carboxylase activity, albeit very low (10.5 m units (mg protein)$^{-1}$) and phosphoribulokinase activity (Taylor,S., 1977).

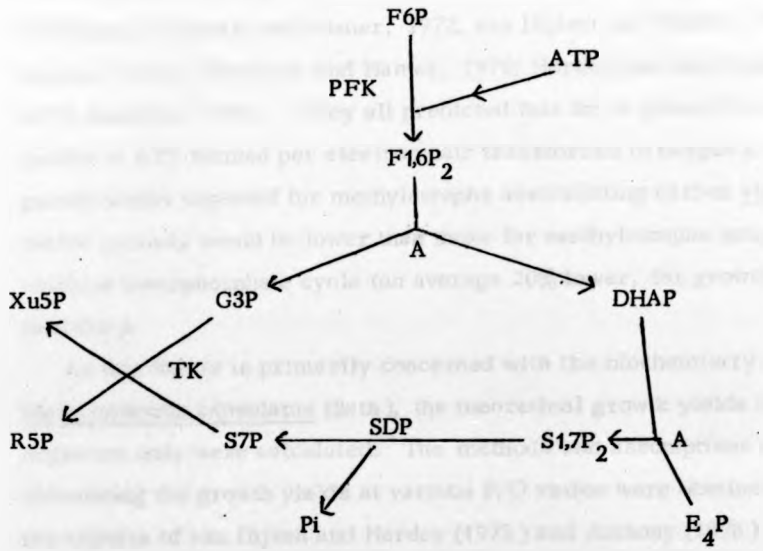
Fig. 6. Ribulose monophosphate cycle of formaldehyde assimilation showing the fructose bisphosphate (—) and the Entner-Doudoroff (---) variants (from Colby and Zatman, 1975)



R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; Xu5P, xylulose 5-phosphate; Hu6P, D-arabino-3-hexulosephosphate; S7P, sedoheptulose 7-phosphate; F6P, fructose 6-phosphate; $F1,6P_2$, fructose 1, 6-biphosphate; E4P, erythrose 4-phosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; 6PG, 6-phosphogluconate; G6P glucose 6-phosphate; KDPG, phospho-2-keto-3-deoxygluconate; HCHO, formaldehyde.

HPS, hexulose phosphate synthase; HPI, hexulose phosphate isomerase; RPI, ribose phosphate isomerase; RuPE ribulose phosphate epimerase; TA, transaldolase; TK, transketolase; A, fructose biphosphate aldolase; HDP, hexose biphosphatase; PFK, 6-phospho-fructokinase ; GPI, glucose phosphate isomerase; GDP, glucose 6-phosphate dehydrogenase; PGDH, phosphgluconate dehydratase; KDPGA, KDPG aldolase.

Fig. 7. The sedoheptulose bisphosphate / ribulose monophosphate cycle of formaldehyde assimilation (from Colby and Zatman, 1975).



F6P, fructose 6-phosphate; $F1,6P_2$, fructose 1, 6-bisphosphate; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; $S1,7P_2$, sedoheptulose 1, 7-bisphosphate; S7P, sedoheptulose 7-phosphate; R5P, ribose 5-phosphate; Xu5P, xyulose 5-phosphate. PFK, 6-phosphofructokinase; A, fructose bisphosphate aldolase; SDP, sedopeptulose bisphosphatase; TK, transketolase.

Although the presence of these two enzymes is thought to be indicative of the ribulose bisphosphate cycle being operative (Anthony, 1975a), there is not as yet conclusive evidence that this cycle, as found in Pseudomonas oxalaticus (Anthony, 1975a) is operative in Methylococcus capsulatus (Bath) (Taylor, S, personal communication). There is also the possibility that a percentage of the cellular carbon of Methylococcus capsulatus (Bath) is derived from another novel pathway involving the two enzymes ribulose 1, 5-bisphosphate carboxylase and phosphribulokinase which is different from the classical ribulose bisphosphate cycle.

E. Theoretical Growth Yields of Methylococcus capsulatus (Bath)

The potential growth yields of methylotrophs growing on methane or methanol have been theoretically calculated by several workers (Harrison, Topiwala and Hamer, 1972; van Dijken and Harder, 1975; Barnes, Drozd, Harrison and Hamer, 1976; Harder and van Dijken, 1976; Anthony, 1978). They all predicted that for a given P/O ratio (moles of ATP formed per electron pair transferred to oxygen), the growth yields expected for methylotrophs assimilating carbon via the serine pathway would be lower than those for methylotrophs using the ribulose monophosphate cycle (on average 20% lower, for growth on methane).

As this thesis is primarily concerned with the biochemistry of Methylococcus capsulatus (Bath), the theoretical growth yields for this organism only were calculated. The methods and assumptions used in calculating the growth yields at various P/O ratios were obtained from the reports of van Dijken and Harder (1975) and Anthony (1978).

The following assumptions were made: (1) the formula for cellular material was $C_4H_8O_2N$, (2) assimilation of C_1-compounds proceeded via 3-phosphoglycerate (3-PGA), (3) the Y_{ATP} (molar growth yield for ATP) value for the assimilation of 3-PGA was 10.5 g dry weight per mole of ATP, (4) the reductant required for the assimilation of 3-PGA was NAD(P)H, (5) the nitrogen source for bacterial growth was ammonia, (6) no active transport of substrates for cell growth occurred, (7) the complete oxidation of formaldehyde yields two molecules of NADH per molecule of formaldehyde, (8) formaldehyde is assimilated via the fructose bisphosphate variant of the ribulose monophosphate cycle. The effect of some of these assumptions being invalid will be discussed later.

Based on the assumptions above the equation for the assimilation of methane into cell material can be calculated (Fig. 8). From these equations the cell yields can be estimated if the energy requirment (29 molecules of ATP) is accounted for by substituting the amount of

carbon substrate (CH_4) whose complete oxidation is sufficient to satisfy the energy deficit remaining after subtracting the ATP generated from the oxidation of 'surplus' reducing equivalents (XH_2) in the right-hand side of the equation.

The estimated growth yields of Methylococcus capsulatus (Bath) growing on methane are listed in Table 3, showing the effect of different P/O ratios for the oxidation of methanol and formaldehyde. The oxidation of formate is known to generate 1 molecule of NADH per molecule of formate which is required for methane mono-oxygenase (Colby and Dalton, 1976). Similarly, as mentioned in the assumptions, the oxidation of formaldehyde has to be $NAD(P)^+$-linked if the methane mono-oxygenase requires NAD(P)H for activity (Anthony, 1978) as is the case with Methylococcus capsulatus (Bath) (Colby and Dalton, 1976; Colby and Dalton, personal communication). However, no $NAD(P)^+$-linked formaldehyde dehydrogenase activity has been reported present in Methylococcus capsulatus (Bath) (see Section VI). This NAD(P)H deficit, could be satisfied if, as suggested by van Dijken and Harder (1975), reversed-electron transport was present in Methylococcus capsulatus (Bath). Although preliminary results suggested that reversed-electron flow could occur in Methylococcus capsulatus (Bath) (see Dalton, 1977) no conclusive evidence for such activity in this organism has as yet been obtained.

The growth yields of organisms like Methylococcus capsulatus (Bath) which possess an NAD(P)H-dependent methane mono-oxygenase could be totally or substantially NAD(P)H-limited, unlike most typical heterotrophic bacteria whose growth yields are predominantly determined by the ATP supply (apart from the obvious fact that growth yields are determined by the supply ofcarbon also) (Anthony, 1978). This situation arises primarily as a result of the requirement for NAD(P)H for the initial oxygenation of methane but is accentuated if the P/O ratio for other reducing equivalents (other than NAD(P)H), e.g. XH_2, generated

Fig. 8. Equations for estimation of cell yields of Methylococcus capsulatus (Bath) growing on methane (adapted from Anthony, 1978).

(9) $4PGA + 29ATP + 5.5\ NADH + 3\ NH_3 \longrightarrow 306$ g cell material $(C_4H_8O_2N)_3 + 10H_2O$ — Assimilation of PGA into cell material

(10) $12\ HCHO + 4H_2O \longrightarrow 4PGA + 4NADH$ — Assimilation of formaldehyde by RMP cycle

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(9) + (10) $12HCHO + 1.5\ NADH + 29ATP + 3NH_3 \longrightarrow 306$ g cell material $+ 6H_2O$ — Assimilation of formaldehyde into cell material

(11) $25.5\ CH_4 + 25.5O_2 \longrightarrow 12HCHO + 1.5\ NADH + 13.5\ CO_2 + 25.5\ XH_2$ — Oxidation of methane

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[(9) + (10)] + (11) $25.5\ CH_4 + 25.5\ O_2 + 29\ ATP + 3NH_3 \longrightarrow 306$ g cell material $+ 25.5\ XH_2 + 13.5\ CO_2 + 18H_2O$ — Assimilation of methane into cell material

Table 3. The predicted effect of variations in P/O ratios on cell yields of Methylococcus capsulatus (Bath) during growth on methane

P/O ratio		Cell yields	
X	Y	Y_{Sg}	$\%CO_2$
2	3	0.75	53
1	3	0.73	55
1	2	0.72	55
1	1	0.70	56
2	0	0.75	53
1	0	0.66	59
0	3	0.54	66
0	2	0.48	70
0	1	0.35	78

The P/O ratios represent the number of molecules of ATP formed per atom of oxygen during the oxidation of methanol to formaldehyde (X) and formaldehyde to formate (Y). Y_{Sg} is the yield (g dry weight cells) per g of substrate. $\%CO_2$ is the percentage of carbon substrate which is completely oxidised to carbon dioxide.

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during the complete oxidation of methane is high, effectively cancelling the energy requirement for carbon assimilation (final equation, Fig. 8). Therefore, for Methylococcus capsulatus (Bath) growing on methane if the P/O ratio for XH_2 is 2, the growth yield is totally NAD(P)H-limited and only when the P/O ratio for XH_2 is 0 does the growth yield become substantially determined by the ATP supply. If reversed-electron flow does occur in Methylococcus capsulatus (Bath), then a potential high ATP yield could be converted to NAD(P)H and theoretically give greater growth yields than those predicted in Table 3.

The nitrogen source for the above calculations was assumed to be ammonia; if, however, the nitrogen source was nitrate or dinitrogen the theoretical growth yields would be lower due to energy and reducing power being required to reduce these nitrogen sources to the level of ammonia.

The values of the predicted growth yields in Table 3 will also have to be revised if it is conclusively shown that a percentage of the cellular carbon of Methylococcus capsulatus (Bath) is derived from the fixation of CO_2 either by the ribulose bisphosphate cycle of CO_2 assimilation or by a novel but similar system involving ribulose 1,5-bisphosphate carboxylase (Taylor, S., 1977). This would have the effect of lowering the predicted yields as a system like the ribulose bisphosphate cycle is energetically less favourable than the ribulose monophosphate cycle of formaldehyde assimilation (Anthony, 1978).

The above use of Y_{ATP} values, originally measured in heterotrophic organisms, to estimate the ATP requirement for cellular biosynthesis from 3-PGA is probably acceptable because it is not unreasonable to assume that the efficiency of 3-PGA utilisation in methylotrophs is similar to typical heterotrophs. Even if the Y_{ATP} value was lower or higher, it would have little or no effect on the estimated growth yield values for Methylococcus capsulatus (Bath) due to the effect of NAD(P)H limitation. For example, if the Y_{ATP} value was 14, then 22 molecules of ATP would be required for the assimilation of 3-PGA into cellular material as opposed to 29 (Fig. 8) (Anthony, 1978). This would mean that if the P/O ratio for XH_2 was 1 or 2, the theoretical growth yield (Y_{S_g}) would be the maximum, 0.75 g dry weight cells per g of substrate, and growth would be effectively NAD(P)H-limited.

The concept of Y_{ATP} obviously assumes that growth yields are dependent on ATP yields from carbon substrate oxidation and therefore clearly the concept is not valid for methylotrophs, such as Methylococcus capsulatus (Bath) where the cell yield is dependent on the NAD(P)H yield from substrate oxidation. Harder and van Dijken (1976) have recently estimated the ATP required for cellular biosynthesis by means of calculated Y_{ATP} values. The Y_{ATP} values were calculated for a specific growth rate and were corrected for the energy required for maintenance and energy 'lost' due to a certain amount of uncoupling of energy generation and growth (Harder and van Dijken, 1976). Their

method for calculating Y_{ATP} values could be valuable for estimating growth yields in heterotrophs and possibly some methylotrophs, but is unsuitable for methylotrophs whose growth is NAD(P)H-limited, as the energy 'lost' due to uncoupling of energy generation and growth could be considerable (Anthony, 1978).

The growth yields determined experimentally for methane oxidising bacteria possessing the same formaldehyde assimilation pathway as Methylococcus capsulatus (Bath) and grown on methane, have ranged from 0.63 to 1.11 g cellular material per g carbon substrate (Barnes et al., 1976; Harder and van Dijken, 1976). On the basis of the theoretical maximum of 0.75 g cellular material per g carbon substrate for Methylococcus capsulatus growing on methane calculated above the organisms with higher yields reported must either have a methane mono-oxygenase that does not require NAD(P)H for activity, like that purified from Methylosinus trichosporium OB3b (Tonge et al., 1977) or possess reversed-electron flow which converts 'excess' ATP to NAD(P)H. Careful determination of experimental growth yields of Methylococcus capsulatus (Bath) is required to evaluate the predicted growth yields determined above.

Very little is known about the actual P/O ratios for the oxidation of XH_2YH_2 or NADH but recent reports suggest that a P/O ratio of 1 is likely for these oxidations in methane-grown Methylosinus trichosporium OB3b (Tonge et al., 1977) and Methylococcus NCIB 11083 (Drozd, Linton, Downs, Stephenson, Bailey and Wren, 1977). If this value was correct for Methylococcus capsulatus (Bath), then the theoretical maximum growth yield would only be 0.70 g cellular material per g carbon substrate.

It is worth noting that the predicted cell yields for Methylococcus capsulatus (Bath) growing on methanol were estimated to be similar or lower to those for growth on methane for a given P/O ratio (Anthony, 1978).

Obviously with certain aspects of the fundamental biochemistry of methane-utilising bacteria not yet fully understood, and the very limited data available on growth energetics of these organisms, the predicting of accurate and valid growth yields is not realistically possible. Although the quantitative values estimated may not be as reliable as those predicted for typical heterotrophs, interesting facts have emerged from such exercises, such as the limitation of growth by intracellular NAD(P)H levels.

SECTION II MATERIALS AND METHODS

Organisms

Two obligate methane-utilising bacteria were used during these studies, Methylococcus capsulatus (Texas) (Foster and Davies, 1966) and Methylococcus capsulatus (Bath) (Whittenbury et al., 1970a).

Media

A basic mineral salts medium (MS), described previously (Dalton and Whittenbury, 1976a), was used throughout these studies for the routine growth of the organisms. The medium was either supplemented with 1 g litre^{-1} potassium nitrate, giving nitrate mineral salts medium (NMS) or 1 g litre^{-1} ammonium chloride, giving ammonium mineral salts medium (AMS).

For solid media, 17 g litre^{-1} of Difco bacto-agar was added to the basic mineral salts medium (minus phosphates) prior to sterilisation. Sterile phosphate solution was added aseptically to the sterile mineral salts medium on cooling.

Maintenance and growth of organisms

Cultures were maintained by sub-culturing every two to three weeks on AMS agar plates. The plates were incubated in 5 litre 'Tupperware' containers fitted with airtight lids. Prior to closing the lid, methane was injected into the container by means of a football bladder, inflated with methane, to give a concentration of approximately 50% v/v with air. The containers were incubated at 45^{o} C for Methylococcus capsulatus (Bath) and 37^{o} C for Methylococcus capsulatus (Texas).

Whole cells of Methylococcus capsulatus (Texas) for respiration and cometabolism studies were grown as batch cultures at 37^{o} C in a 5 litre fermenter (L.H. Engineering Ltd., Stoke Poges, Bucks., U.K.) on AMS medium as described, with methane (20% v/v, in air) as the carbon source. Whole cells of Methylococcus capsulatus (Bath) for respiration and cometabolism studies were grown in continuous culture in a similar fermenter (2.5 l working volume) to that above at a dilution rate of 0.05 h^{-1} on AMS medium with methane (20% v/v, in air) as carbon source.

Large quantities of whole cells of Methylococcus capsulatus (Bath) for preparing cell-free extracts were grown at 45° C in batch culture on AMS medium in a 100 litre fermenter (L.H. Engineering Ltd.). The fermenter was inoculated with 15 litres of a continuous culture overflow and after 18 hours, when the optical density (A_{540}) of the culture was approximately 8, the culture was harvested using a Westfalia continuous centrifuge (Westfalia Separator Ltd., Wolverton, Bucks., U.K.). The outflow of the fermenter was attached to the centrifuge via a stainless steel cooling-coil immersed in ice.

To ascertain whether Methylococcus capsulatus (Bath) could grow on bromomethane as sole source of carbon and energy, several 250 ml flasks were set up containing 25 ml of sterile NMS, to which varying amounts of bromomethane were added (25 µmol, 125 µmol, 250 µmol and 1.25 mmol. Bromomethane was injected into the flasks through Suba Seal stoppers due to its volatile nature. Appropriate controls, using methane as sole source of carbon and no carbon source, were prepared, then all the flasks were inoculated with 0.5 ml of a chemostat culture of Methylococcus capsulatus (Bath) in steady state, giving an initial optical density (A_{540}) of 0.24 in the flasks. The flasks were incubated on an orbital shaker at 45° C for 10 days. Methylococcus capsulatus (Bath) was also inoculated on to NMS agar plates and incubated in 'Tupperware' containers with 20% or 30% (v/v, in air) bromomethane. Again suitable controls were prepared and the containers were incubated at 45° C for 10 days.

Similar experiments to those above were done to test whether Methylococcus capsulatus (Bath) could utilise dimethyl ether or carbon monoxide as carbon sources. Flasks were set up as above except that bromomethane was replaced with varying amounts (0.5-1.2 mmol) of dimethyl ether or carbon monoxide. Again suitable controls were prepared and all the flasks were inoculated and incubated as before. These experiments were repeated with some flasks being supplemented with potassium formate (50-125 µmol) as a potential energy and reducing power source. The formate was added aseptically as small volumes of a sterilised 200 mM solution of potassium formate.

Preparation of cell-free extracts

Whole cells of Methylococcus capsulatus (Bath) grown in 100 litre batch culture were harvested as described above. After washing the cells once with ice-cold, 20 mM sodium phosphate buffer, pH 7.0, cell-free extracts were prepared by a single passage of the cell suspension through a French pressure cell at 137 MP_a (20 000 lb in^{-2}), followed by centrifugation at 80 000 g for 1 hour. This gave a clear, red supernatant, which was decanted off, and an intensely red pellet which was discarded. The soluble extract was immediately frozen in pellet form by drop-wise addition into liquid nitrogen, and the pellets were stored at -80° C.

Dry weight estimations of cultures and cell suspensions

Dry weight estimations were measured by filtration of suitable volumes of culture through previously dried and weighed 'Oxoid' membrane filters (0.4 µm pore size) (Oxoid Ltd., London, U.K.). The membranes, along with fresh control membranes, were then dried in an oven at 60° C and then weighed, with equal time intervals between removal from the oven and weighing for each membrane. Curves were constructed of optical density (A_{540}) versus dry weight for chemostat-grown cells of Methylococcus capsulatus (Bath) and batch-grown cells of Methylococcus capsulatus (Texas).

Respiration Studies

Respiration studies were carried out in a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) with a reaction vessel of 3 ml capacity. The reaction mixture was stirred by means of a magnetic stirrer and suitable flea. The correct assay temperatures (45° C for Methylococcus capsulatus (Bath) and 37° C for Methylococcus capsulatus (Texas)) were maintained by means of a water jacket around the electrode and a heating circulator pump (± 0.1° C). The output from the electrode was measured on a chart recorder (Servoscribe, Smith Industries, London, U.K.) with 100% oxygen saturation adjusted

to give 90% deflection on the recorder scale to maximise base-line accuracy. The electrode was frequently calibrated by the method of Robinson and Cooper (1970) and gave values for the dissolved oxygen concentration very similar to the theoretical value obtained using Henry's Law.

Whole-cell suspensions for respiration studies were grown as previously mentioned, then centrifuged at 5 000 g for 10 minutes, washed once with ice-cold, 20 mM potassium phosphate buffer, pH 6.8, and resuspended in a small volume of the same buffer, so that only microlitre quantities of suspension had to be added to the reaction vessel for each assay. The assays contained 20 mM potassium phosphate buffer, pH 6.8, and sufficient whole-cell suspension to give a final optical density (A_{540}) of 2. Once the buffer and cells were present in the electrode, the top was placed on the reaction vessel leaving a maximum airspace of 0.4 ml for the addition of substrates, inhibitors, etc. The buffer and cells were allowed to equilibrate until the temperature reached 45° C (usually 2-3 minutes). This equilibrium time was kept to a minimum by pre-heating the buffer to 45° C in a water bath. Substrates, inhibitors, etc., were injected into the reaction vessel by syringe taking care to expel the airspace in doing so. The volume of the addition was kept to a maximum of 0.4 ml and ideally was 50-100 µl, and where possible the compounds were pre-heated to 45° C in a water bath. Compounds which were virtually insoluble, e.g. imidazole, were subjected to ultrasonic radiation to aid dispersion. Gaseous compounds were added as saturated buffers, the compound concentration being calculated using Henry's Law and the relevant Henry's constant (International Critical Tables, vol. III, pp. 255-261). Where necessary, temperature versus solubility curves were constructed from the tabulated constants to enable concentrations at temperatures not quoted to be approximated.

The reaction vessel was cleaned after each assay by means of suction aspiration and repeated washings with distilled water to ensure

the electrode was free from any residual inhibitor etc. The final reaction rates were calculated after subtracting any endogenous rate obtained from the whole cells and buffer after equilibration, prior to substrate addition.

Gas chromatography procedures

Gas chromatography was used to identify and quantify a large number of compounds throughout these studies. In all cases a Pye series 104 gas chromatograph (Pye Unicam, Cambridge, U.K.) fitted with a flame ionization detector was used (except for the detection of carbon dioxide). Gas (50-100 µl) and liquid (5 µl) samples were injected into 2.1 m glass columns packed with Porapak Q (Waters Associates, Milford, Ma., U.S.A.) with Chromosorb 102 (Johns-Manville, Denver, Colorado, U.S.A.) or with 5% (w/w) of Carbowax 20M on Chromosorb W (60-80 mesh). Trimethylamine hydrochloride was estimated on a column of Porapak Q which incorporated a pre-column (4 in) of soda-lime housed in the heated-injection block. These three columns used in conjunction and operated isothermally at temperatures ranging between 50° C and 230° C, with dinitrogen carrier-gas flow rates of 15-60 ml min^{-1} achieved the separation of all the volatile compounds tested. Compounds were identified by comparing their retention times on one or more columns with those of authentic standards and quantitatively estimated by establishing a linear relationship between peak height (or in some cases, area) and concentration for each compound.

Carbon dioxide was identified and estimated by using a similar Pye series 104 gas chromatograph as above, but fitted with a katharometer (thermal conductivity) detector. A glass column (2.1 m x 4 mm internal diameter) packed with Porapak R (Waters Associates) was used with helium (30 ml min^{-1}) as the carrier gas and an oven temperature of 50° C. The katharometer was calibrated using carbon dioxide/air mixtures of known composition.

Confirmation of identification of some products from oxidation reactions was obtained by treatment of the reaction mixture with 20 µl of hydrochloric acid or 5 µl of bromine for five minutes at 45° C, followed by gas chromatography of the mixture as described to determine whether the product remained after treatment (cf. May and Abbot, 1973). Hydrochloric acid catalysed the hydrolysis of epoxides and bromine complexes with unsaturated compounds by simple addition reactions.

Analytical determinations

Formaldehyde was determined by the dimedon method of Frisell and MacKenzie (1963) and routinely assayed by the acetylacetone method of Nash (1953). Formate was determined by the method of Lang and Lang (1972). Tyrosine was estimated colorimetrically as described by Kaufman (1970).

Pyridine N-oxide was identifed by thin-layer chromatography on silica-gel plates as follows. After incubation, reaction mixtures,which initially contained 90 µmol of pyridine as test substrate , were treated with an equal volume of dichloromethane to extract any pyridine N-oxide if present. The dichloromethane layer was drawn off and evaporated to dryness. The residue was redissolved in 0.1 ml of fresh dichloromethane and spotted on to the chromatograms, together with authentic pyridine N-oxide samples prepared in the same way with dichloromethane. The chromatograms were developed in either acetone or methanol and the R_f values for the extracted reaction product compared with those for the pyridine N-oxide standards, in each solvent.

Protein was measured by the method of Lowry, Rosebrough, Farr and Randall (1951) using dried, crystalline bovine plasma albumin as the standard.

Syntheses and specially prepared chemicals

trans 2, 3-epoxybutane and cis 2, 3-epoxybutane were made by forming epoxides from the corresponding cis or trans but-2-ene with 3-chloroperbenzoic acid at room temperature. A conical flask (25 ml) sealed with a Suba Seal stopper contained 3 ml of a 10% (w/v) solution of 3-chloroperbenzoic acid in dichloromethane. But-2-ene gas (cis or trans) was passed through the flask for approximately five minutes

by means of syringe needles inserted through the stopper. During the reaction a heavy precipitate was formed which was resuspended by the addition of a further 0.5 ml fresh dichloromethane. The suspension was centrifuged (2 000 g for 10 minutes) to remove any precipitate and a 0.5 ml sample of the supernatant removed, washed twice with 0.5 ml ice-cold saturated sodium bicarbonate, then 'dried' with the addition of a few crystals of anhydrous sodium sulphate. The resulting dichloromethane solution of cis or trans 2, 3-epoxybutane was used as an authentic standard for identification purposes during gas chromatographic analyses of the oxidation products from cis or trans but-2-ene by whole cells and cell-free extracts of Methylococcus capsulatus (Bath).

Contamination-free formaldehyde was prepared by heating aqueous solutions of paraformaldehyde (May and Baker. Ltd., Dagenham, Essex, U.K.) at 100° C for two to three hours. Acetylene was prepared by reacting a small amount (20-25 ml)of distilled water with chips of calcium carbide in a sealed flask. The resulting acetylene gas was collected in a football bladder connected to a side arm on the flask. Commercial dimethyl ether (Cambrian Chemicals, Croydon, Surrey, U.K.) was found to contain an unknown contaminant which appeared to be totally removed or at least greatly reduced in concentration by bubbling the gas through a glass gas-jar containing distilled water in which a few grammes of potassium permanganate were dissolved. The 'clean' dimethyl ether was collected in a football bladder. DL-glyceraldehyde-3-phosphate, diethylacetal, barium salt (Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.) was converted to DL-glyceraldehyde-3-phosphate in accordance with the suppliers instructions.

The stabilising agent was removed from styrene by vacuum distillation at 40° C immediately prior to use. Contaminating ethanol (10-20 mol per 100 mol NADH) was removed from 100 mM NADH solutions in 20 mM sodium phosphate buffer, pH 7.0, by washing with diethyl ether to extract the ethanol. The ether was removed by

evaporation under vacuum and two or three washings were required to reduce the ethanol to a negligible concentration (0.1-0.2 mol per 100 mol NADH).

Spectrophotometric procedures

All spectrophotometry, e.g. enzyme assays, adsorption spectra, etc. was done in a Unicam SP1800 UV recording spectrophotometer (Pye Unicam) except routine optical density readings of liquid cultures which were measured, at a wavelength of 540 nm, in a Unicam SP500 spectrophotometer (Pye Unicam). The SP1800 instrument incorporated a cuvette carriage which could be heated by means of a water circulator to temperatures suitable for enzyme assays, e.g. 45° C. Cuvettes of 1 cm light path were used throughout.

Enzyme assays

Methane mono-oxygenase: Activity was routinely measured in cell-free extracts of Methylococcus capsulatus (Bath) by one of three different assays, (1) bromomethane disappearance, (2) methanol accumulation, (3) epoxyethane production.

(1) Bromomethane disappearance. In this assay the substrate analogue of methane, bromomethane, was oxidised by the mono-oxygenase at rates similar to, but less than, those obtained with methane (Colby and Dalton, 1976). The assays were done in conical flasks (7 ml internal volume) containing 1 ml of reaction mixture and sealed with Suba Seal stoppers. Reaction mixtures (1 ml) contained : 50 μmol sodium phosphate buffer, pH 7.0; 5 μmol NADH; 0.5 μmol potassium cyanide; soluble extract (usually around 5 mg of extract protein); 1 μmol bromomethane. Bromomethane was added as an ice-cold solution, the reaction flask being immediately sealed with the stopper to prevent any evaporation of the substrate. Any other additions, e.g. inhibitors, to the reaction mixtures were as indicated in the Tables concerned. The flasks were incubated at 45° C in a shaking water bath (90 oscillations min^{-1}) for 5 minutes, when freshly thawed soluble extract was injected through the stopper to initiate the assay. Samples (50 μl gas

phase) were removed at appropriate time intervals, e.g. 0, 5 and 10 minutes, and injected into the gas chromatograph. The concentration of bromomethane was calculated as described previously. Activities were determined from the amount of substrate oxidised after 10 minutes (Colby et al., 1975).

(2) Methanol accumulation. This assay measured the amount of methanol produced from methane. The assays were done in similar flask arrangements to the bromomethane assays. Reaction mixtures (1 ml) contained: 50 µmol sodium phosphate buffer, pH 7.0; 5 µmol NADH; 0.5 µmol potassium cyanide; soluble extract (usually around 5 mg extract protein). Methane was added after the flasks were stoppered by removing 3 ml of the gas phase with a syringe and replacing it with 3 ml of methane. Any other additions or omissions to the reaction mixtures were as indicated in the Tables concerned. The flasks were incubated at the temperatures indicated and the reaction initiated as above. Methanol production was monitored by removing 5 µl liquid samples at timed intervals and injecting them into the gas chromatograph. The chromatograph was calibrated with freshly prepared methanol solutions of known concentrations and measuring the peak heights observed. Activities were calculated from the amount of methanol accumulated with time (Colby and Dalton, 1976).

(3) Epoxyethane production. In this assay, methane mono-oxygenase oxidised ethene to the single product, epoxyethane, and at a specific rate nearly twice that for methane oxidation (see Section IV). The assays were done in the same flask arrangements as above. Reaction mixtures (1 ml) contained: 50 µmol sodium phosphate buffer, pH 7.0; 5 µmol NADH; 0.5 µmol potassium cyanide, soluble extract (usually around 5 mg extract protein). Ethene (3 ml gas phase) was added to the reaction flask in a similar fashion to methane in the previous assay. Any other addition or omissions to the reaction mixtures were as indicated in the Tables concerned. The reaction flasks were incubated and the reaction initiated as with the bromomethane assays. Liquid

samples (5 µl) were removed at timed intervals and injected into the gas chromatograph. The chromatograph was calibrated with freshly prepared epoxyethane solutions of known concentration and measuring peak heights. Activities were calculated from the amount of epoxide produced with time.

Substrate specificity. Assays were done in similar flask arrangements as for the previous assays. Reaction mixture (1 ml) contained: 50 µmol sodium phosphate buffer, pH 7.0; 5 µmol NADH; 0.5 µmol potassium cyanide; soluble extract; test substrate (amount as indicated in Tables, Section IV). Liquid substrates were incorporated into the reaction mixtures and gaseous substrates were added to the reaction flasks as with methane and ethene above. The reaction flasks were incubated and the reactions started as with the previous assays.

The rate of oxidation of most test compounds was measured by following the appearance of products, exceptions being the substituted methane derivatives (minus carbon monoxide), dimethyl ether and pyridine, where substrate disappearance was measured. Samples (5 µl liquid) of reaction mixture were injected into the gas chromatograph immediately after the addition of the soluble extract (zero time) and after 12 minutes of incubation. Preliminary experiments had shown that product formation from each test substrate was approximately linear with time over this incubation period. Samples (0.5 ml) from flasks containing carbon monoxide as the test substrate were taken from the gas phase and injected into the gas chromatograph / katharometer for product detection.

As the products of n-octane oxidation were insoluble, they were first extracted with 1 ml of dichloromethane and then 5 µl of the dichloromethane layer was injected into the gas chromatograph. Specific activities were calculated from the total amount of products formed after 12 minutes incubation.

Formaldehyde dehydrogenase: All assays, except where specified, were done in 1.5 ml semi-micro quartz cuvettes at 45° C. The reaction mixture (1.5 ml) contained: 30 µmol potassium phosphate buffer, pH 7.2;

1 μmol $NAD(P)^+$; test protein. When crude, soluble extract was assayed for formaldehyde dehydrogenase activity, 4 μmol potassium cyanide was added to the reaction mixture to completely inhibit formate dehydrogenase activity and to partially inhibit NADH oxidase activity. Once these two activities were removed during the purification of formaldehyde dehydrogenase,potassium cyanide was omitted from the reaction mixture. After 3 minutes pre-incubation, the reaction was started by the addition of 4 μmol formaldehyde. Activity was measured by following the reduction of $NAD(P)^+$ at 340 nm.

After step 1 of the purification of the heat-sensitive component of the formaldehyde dehydrogenase, the partially purified or purified heat sensitive component required the presence of heat-treated, soluble extract to regain activity. Heat-treated, soluble extract was prepared by heating crude, soluble extract to 70° C in a water bath for 12 minutes, then centrifuging the extract for 3 minutes in a Quickfit Instrumentation micro-centrifuge (J.A. Jobling and Co. Ltd., Stone, Staffs., U.K.) to remove the precipitated protein. The heat-treated soluble extract showed no activity when assayed alone.

Formate dehydrogenase (formate: NAD^+ oxidoreductase, EC 1.2.1.2): the assay for this enzyme was based on the method of Quayle (1966). The reaction mixture (1.5 ml) contained: 30 μmol sodium phosphate buffer, pH 7.0; 1 μmol NAD^+; test protein. The reaction was started by the addition of 4 μmol potassium formate. Assays were done at 45° C in 1.5 ml semi-micro quartz cuvettes and measured the reduction of NAD^+ at 340 nm.

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49): the assay for this enzyme was based on the method of Kornberg and Horecker (1955). The reaction mixture (1.5 ml) contained: 100 μmol glycylglycine buffer, pH 8.0; 15 μmol $MgCl_2$; 0.5 μmol NAD^+ or $NADP^+$; test protein (usually about 5 mg extract protein). The reaction was started by the addition of 3 μmol glucose

6-phosphate. Assays were done at 30° C or 45° C in 1.5 ml semi-micro quartz cuvettes and measured the reduction of $NAD(P)^+$ at 340 nm.

Phosphogluconate dehydrogenase (6-phospho-D-gluconate: $NAD(P)^+$ oxidoreductase, EC 1.1.1.43): the assay for this enzyme was based on the method of Horecker and Smyrniotis (1955). The reaction mixture (1.5 ml) contained: 100 µmol glycylglycine buffer, pH 7.6; 15 µmol $MgCl_2$; 0.5 µmol NAD^+ or $NADP^+$; test protein (usually about 5 mg extract protein). The reaction was started by the addition of 3 µmol 6-phosphogluconate. Assays were done at 30° C or 45° C in 1.5 ml semi-micro quartz cuvettes and measured the reduction of $NAD(P)^+$ at 340 nm.

Glucose phosphate isomerase (D-glucose 6-phosphate ketolisomerase, EC 5.3.1.9): the assay for this enzyme was based on the method of Wu and Racker (1959). The reaction mixture (1.5 ml) contained: 75 µmol Tris/HCl buffer, pH 7.4; 15 µmol $MgCl_2$; 0.5 µmol $NADP^+$; 2 units of glucose 6-phosphate dehydrogenase; test protein (usually about 5 mg extract protein). The reaction was initiated by the addition of 2 µmol fructose 6-phosphate. Assays were done at 45° C in 1.5 ml semi-micro quartz cuvettes and measured the reduction of $NADP^+$ at 340 nm.

NADH-oxidase (Reduced NAD^+ dehydrogenase, NADH; (acceptor) oxidoreductase, EC 1.6.99.3): the assay for this enzyme was based on the method of Smith, London and Stanier (1967). The reaction mixture (1.5 ml) contained: 30 µmol sodium phosphate buffer, pH 7.0; 10 µmol $MgCl_2$; test protein (usually around 2 mg extract protein). The reaction was initiated by the addition of 0.25 µmol NADH. Assays were done at 45° C in 1.5 ml semi-micro quartz cuvettes and measured the oxidation of NADH at 340 nm.

Assay of molecular weight standards

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) (E. coli): Enzyme activity was measured by the method of Garen and Levinthal (1960).

Alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) (yeast): Enzyme activity was measured by the method of Racker (1955).

Catalase (hydrogen-peroxide: hydrogen-peroxide oxidoreductase. EC 1.11.1.6) (bovine liver): Enzyme activity was measured by the method of Chance and Maehly (1955).

Malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37) (pig heart): Enzyme activity was measured by the method of Ochoa (1955).

Enzyme units: One unit of activity was defined as the amount of enzyme required to transform 1 μmol of substrate per minute.

Gel filtration and molecular weight determination

The gel filtration procedures were based on the techniques of Reiland (1971) and all gel filtration was done at 4^o C. The molecular weight of the purified, heat-sensitive component of the formaldehyde dehydrogenase was estimated by Sephadex gel filtration (G-200) as described by Andrews(1964). The elution volume (V_e) for the purified protein and various protein standards of known molecular weight were determined and the K_{av} values calculated for each. $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume, V_o is the void volume as determined by blue dextran, and V_t is the bed volume.

Purification of the heat-sensitive component of formaldehyde dehydrogenase from Methylococcus capsulatus (Bath)

Step 1: 15 ml of crude, soluble cell-free extract was taken to 30% saturation with ammonium sulphate by the slow addition of solid $(NH_4)_2SO_4$ at 4^o C. After equilibration at 4^o C for 20 minutes, the mixture was centrifuged (38 000 g, 10 minutes) at 4^o C and the pellet discarded. The supernatant was brought to 55% saturation with $(NH_4)_2SO_4$, allowed to equilibrate, then centrifuged as before. This time the supernatant was discarded and the pellet was redissolved in the minimum volume (2.7 ml) of 20 mM potassium phosphate buffer, pH 7.2, 4^o C.

Step 2: The redissolved pellet from the initial salt precipitation step of the purification was applied to a column (3.8 x 90 cm) of Sephadex G-75, previously equilibrated with 20 mM potassium phosphate buffer, pH 7.2. The column was eluted with the same buffer. Fractions (3 ml) were collected and those showing significant formaldehyde dehydrogenase

activity were pooled (28 ml). The pooled fractions were then concentrated in an Amicon ultrafiltration unit (Model 12) fitted with a PM 10 Diaflo ultrafiltration membrane (Amicon Ltd., High Wycombe, Bucks., U.K.) to give a final volume of 6 ml.

Step 3: The concentrated 6 ml pool from the Sephadex G-75 step was applied to a column (3 x 90 cm) of Sephadex G-200, previously equilibrated with 20 mM potassium phosphate buffer, pH 7.2. The column was eluted with the same buffer and fractions (3 ml) were collected as for step 2 (Sephadex G-75) and pooled (44 ml).

Step 4: Approximately 25 ml diethylaminoethyl (DEAE)-cellulose slurry was mixed with the pooled fractions from Step 3 (44 ml) at 4^{o} C. The DEAE-cellulose was previously extensively washed with 20 mM potassium phosphate buffer, pH 7.2, and adjusted to pH 7.2. The mixture was slowly stirred and allowed to equilibrate for 20 minutes at 4^{o} C, then centrifuged (4^{o} C) at 38 000 for 10 minutes. The pellet was discarded and the supernatant frozen by dropwise addition into liquid nitrogen, the pellets being stored at -80^{o} C.

Polyacrylamide gel electrophoresis

The procedure was based in general on the techniques outlined by Gabriel (1971). Separating gels of 7.5% (w/v) acrylamide were used at pH 8.8, to which 0.2 ml of stacking gel (1.25%, w/v, acrylamide) was added. 1 ml of bromophenol blue tracking dye (0.01% w/v) was added to the reservoir (electrode) buffer (50 mM Tris/glycine, pH 8.3). Electrophoresis was done at 2.5 mA per tube until the tracking dye had almost reached the base of the gel. The gels were fixed and stained for two to four hours with 0.1% (w/v) Coomassie brilliant blue R in 7.5% (v/v) acetic acid/46% (v/v) methanol and destained by soaking overnight with the same solvent.

The molecular weights of the formaldehyde dehydrogenase heat-sensitive component subunits were estimated by sodium dodecyl sulphate (SDS) gel electrophoresis in a 13% (w/v) gel system. The gel buffer was 300 mM Tris/HCl buffer, pH 8.8, and the reservoir

buffer 50 mM Tris/glycine, pH 8.3, both containing 0.1% (w/v) SDS. The samples were prepared by mixing 0.4 ml (0.18 mg) sample protein with 0.5 ml 10% (w/v) SDS in 50 mM Tris/HCl buffer, pH 7.8, containing 0.1% (v/v) 2-mercaptoethanol and 0.1 ml of 60% (w/v) sucrose solution. The mixture was then heated at 100° C in a boiling water bath for 10 minutes and afterwards cooled to 4° C. Electrophoresis and staining were then done as before and the relative mobilities of the sample protein and standard marker proteins compared.

Activity stain

Formaldehyde dehydrogenase activity was located in unstained (7.5% w/v) gels by incubating them in the dark with a mixture (100 ml) containing: 1.4 mmol Tris/HCl buffer, pH 7.2; 100 μmol nitro blue tetrazolium; 45 μmol NAD^+; 4.5 μmol phenazine methosulphate; 4 mmol formaldehyde; 160 mg heat-treated soluble extract. Two gels were incubated at 45° C for 30 minutes, one being a control gel without formaldehyde, and a third gel was incubated at 30° C for 90 minutes. Purified formaldehyde dehydrogenase, heat-sensitive component (20 μg) was applied to each gel.

Trypsin treatment of the heat-treated soluble extract

Samples of heat-treated soluble extract (0.75 ml, 12 mg protein) were dispensed into four micro-centrifuge tubes (1.5 ml capacity). Tube 1 was kept in ice for 30 minutes and tubes 2, 3 and 4 were incubated at 37° C in a shaking water bath. Before incubation, 2 mg trypsin (280 BAEE units) was added to tube 3, and 2 mg trypsin plus 4 mg trypsin inhibitor were added to tube 4. After the 30 minutes incubation period, 4 mg trypsin inhibitor was added to tube 3, and then all four tubes were stored in ice.

Photography

Gels were photographed from above by immersing them in destaining solution in a 20 cm diameter petri dish placed on the illuminated surface of an X-ray illuminator ('Industrex' X-ray illuminator, Model 2, Kodak Ltd., London, U.K.). Photographs were taken with a Pentax SP 500 camera fitted with a Hoya Y (K2) yellow filter and Kodak Panatomic X film.

Chemicals

Most compounds, inhibitors, enzymes, substrates, products, media components, etc. were obtained from the following manufacturers: B.D.H. Chemicals, Poole, Dorset, U.K.; Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; Fisons Scientific Apparatus, Loughborough, Leics., U.K.; Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.; Hopkins and Williams, Chadwell Heath, Essex, U.K.; Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; May and Baker Ltd., Dagenham, Essex, U.K.; Polaron Equipment, Watford, Herts., U.K.; Kodak Ltd., Kirby, Liverpool, U.K.

Gases

Methane (technical grade), carbon monoxide (research grade) were obtained from British Oxygen Co., London, U.K. Chloromethane (99.8%),but-1-ene (99%), propene (99%), cis but-2-ene (99%), trans but-2-ene (99%), propyne (96%) and but-1-yne (95%) were obtained from B.D.H. Chemicals Ltd. Ethane (CP), n-propane (CP), ethene (99.85%), ethyne (99.6%) and dimethyl ether (99%) were obtained from Cambrian Chemicals, Croydon, Surrey, U.K. But-2-yne was obtained from Kodak (ICN) Ltd.

Gifts

Ferron (8-hydroxy-7-iodo-5-quinoline-sulphonic acid) and o-aminophenol were gifts from Dr. J.R. Dilworth, ARC Unit of Nitrogen Fixation, University of Sussex. 2-Hexanol, 3-hexanol, 1-heptanol, 2-heptanol, 3-heptanol, 4-heptanol, 1-octanol, 2-octanol, 3-octanol and 4-octanol were gifts from British Petroleum Ltd., Sunbury, Middlesex, U.K.

SECTION III

Inhibition of Methane Oxidation

1. Introduction

The study of the role of inhibitors in enzymic reactions has contributed greatly to an understanding of enzyme mechanisms, particularly in situations where enzyme purification has proved difficult. Indeed one method often used to provide evidence for or against the involvement of a metal ion in an enzyme is the presence or absence of inhibition by chelating or other metal-binding agents. It is often assumed, however, that any inhibitory effect caused by such compounds is due solely to binding of a metal ion and not to any other interaction between the inhibitor and the enzyme, cases of which have been reported. For example, the inhibition of some flavoprotein mono-oxygenases, yeast alcohol dehydrogenases and a pseudomonad meta-pyrocatechase by o-phenanthroline was thought to be due to hydrophobic interaction between the enzyme protein and the inhibitor (Yamamoto, Takeda, Maki and Hayasishi, 1969). Therefore the interpretation of inhibition data where only one or two metal-chelating/binding agents were effective must be made with caution.

Even with the possibility of such problems, great value can come from a study of metalloenzyme-inhibitor interactions. If a reasonably comprehensive inhibitor pattern can be established for a particular enzyme, a sensible deduction of the possible metal ion involved can be made by examining both the detailed formation-constant data available and the generalised 'hard-soft' classification of metal ions and ligands.

Formation constants provide a quantitative measure of the extent to which a metal will complex with any particular group or ligand. Used alone, formation constant data can be misleading with enzyme complexes, as structural features in the protein may reduce the strength of any metal-inhibitor binding, normally produced in model systems, or even prevent the operation of the chelating agent. The 'hard-soft' classification of ligands and metal ions is based on their

thermodynamic specificity and is probably the criterion most relevant to the inhibition of biological redox systems. A general rule is that 'hard' metal ions complex with 'hard' ligands and 'soft'metal ions complex with 'soft' ligands, where 'hard' means ionic and not easily polarised, and 'soft' means easily polarised atoms and molecules (Hughes, 1972). In addition to these two classes there is an 'intermediate' class, examples of which are listed along with examples of 'hard' and 'soft' metal ions and ligands in Table 4.

Metals such as iron and copper have often been directly implicated in a number of enzymes which catalyse oxygen incorporation into substrates, including the involvement of copper in a methane mono-oxygenase (Tonge et al., 1977). Metals are also believed to be involved in other methane mono-oxygenases from numerous methane-utilising bacteria as a result of inhibitor data obtained (Colby et al., 1975; Hubley, Thomson and Wilkinson, 1975; Patel, Hou and Felix, 1976). Such data can play an important role in understanding the nature of the reaction mechanism of an enzyme like methane mono-oxygenase, as invariably it proves difficult to purify and hence characterise. Therefore, a comprehensive study of the inhibitor pattern of methane oxidation in Methylococcus capsulatus (Texas) and Methylococcus capsulatus (Bath) was undertaken, with the latter organism being of particular interest as preliminary inhibitor studies (Colby and Dalton, 1976) suggested a conflicting pattern to those observed with other methane-oxidising bacteria (Colby et al., 1975; Hubley et al., 1975; Ribbons, 1975; Patel et al., 1976; Tonge et al., 1977).

2. Effect of Various Potential Inhibitors on Methane and Methanol Oxidation by Whole-cell Suspensions of Methylococcus capsulatus (Texas).

The results of the inhibitor studies with whole-cell suspensions of M. capsulatus strain Texas (TRMC) (Table 5) correlate well with similar studies on TRMC by Patel et al. (1976) (Table 7). Only

TABLE 4

'Hard' and 'soft' metal ions and ligands

METAL IONS						LIGANDS				
Hard		Intermediate		Soft		Hard		Intermediate	Soft	
H^+	Mn^{2+}	Zn^{2+}	Sn^{2+}	Cu^+	Pd^{2+}	H_2O	RNH_2	Pyridine	$-SCN^-$	R_3P
Li^+	Cr^{3+}	Cu^{2+}	Pb^{2+}	Ag^+	Pt^{2+}	ROH	$-NCS^-$	Br^-	$-CN^-$	R_3As
Na^+	Fe^{3+}	Ni^{2+}		Au^+	Pt^{4+}	R_2O	Cl^-	N_3^-	RSH	H^-
K^+	Co^{3+}	Fe^{2+}		Tl^+	Cd^{2+}	OH^-	PO_4^{3-}	NO_2^-	R_2S	$S_2O_3^{2-}$
						OR^-	SO_4^{2-}		RS^-	
						NH_3				

(Taken from Hughes, 1972)

four compounds, amobarbital, phenol, pyridine and aniline, and to a lesser extent, acriflavin, imidazole and antimycin A, caused little or no inhibition to methane oxidation, whereas all the remaining compounds gave virtually total inhibition. Most of the potent inhibitors were metal ion chelators with different types of 'intermediate' and 'soft' ligand combinations represented, i.e. nitrogen-nitrogen (e.g. 2, 2'-bipyridine), oxygen-nitrogen (e.g. 8-hydroxyquinoline) and sulphur-nitrogen (e.g. thiourea).

In order to confirm that the inhibition pattern observed was due to the specific inhibition of methane oxidation and not to a general inhibition of respiration, the effect of the various compounds was tested on methanol oxidation. A specific inhibitor of methane oxidation would not be expected to inhibit methanol oxidation.

Only three of the compounds tested significantly inhibited methanol oxidation by whole-cell suspensions of TRMC, o-amino-phenol (36% inhibition), ferron (65% inhibition) and potassium cyanide (100% inhibition). The remaining compounds showed at best a 19% inhibition. This suggested that the majority of the inhibitors were specific for the initial enzyme oxygenation of methane to methanol.

The large number of metal ion chelators and other metal-binding compounds that appeared to specifically inhibit methane oxidation in TRMC strongly suggested the involvement of at least one metal ion in the enzyme responsible for the oxidation of methane, thus supporting the viewpoint of Patel et al. (1976). Inhibition data such as that in Table 5 is often used as good evidence for predicting the actual metal ion involved, e.g. Cu(II), Fe(III), etc., but can often be misleading, as discussed in the introduction to this Section. However, with this in mind, on examination of the metal complex stabilisation constants (O'Sullivan, 1969) for the various inhibitors and the classification in Table 4, it appears that both copper and iron are the metals most readily complexed by the reagents used.

TABLE 5

The effect of various potential inhibitors on methane and methanol oxidation by whole-cell suspensions of Methylococcus capsulatus (Texas)(A) and Methylococcus capsulatus (Bath)(B)

Inhibitor	Rate of methane oxidation (% control) A[a]	Rate of methane oxidation (% control) B[b]	Rate of methanol oxidation (% control) A[c]	Rate of methanol oxidation (% control) B[d]
None	100	100	100	100
Thiourea	3	50	86	82
Thioacetamide	0	89	117	93
Thiosemicarbazide	5	70	106	100
Diethyldithiocarbamate	0	0	115	60
2, 2'-Bipyridine	3	42	97	98
Neocuproine	0	57	81	94
Amobarbital	96	81	120	92
8-Hydroxyquinoline	0	0	100	80
1, 10-Phenanthroline	0	62	120	68
Imidazole	37	79	100	112
Acriflavin	52	77	100	97
Antimycin A	65	100	115	98
Aniline	100	100	100	100
Phenol	80	70	100	100
o-Aminophenol	18	0	64	60
Ferron	0	0	35	0
Potassium cyanide	0	0	0	0
Carbon monoxide[e]	0	0	200	193
Pyridine	92	71	100	100
Acetylene[e]	0	0	100	100

[a] The uninhibited rate of methane oxidation was 273 nmol oxygen consumed min^{-1} (mg dry cell weight) $^{-1}$.

[b] The uninhibited rate of methane oxidation was 225 nmol oxygen consumed min^{-1} (mg dry cell weight) $^{-1}$.

[c] The uninhibited rate of methanol oxidation was 219 nmol oxygen consumed min^{-1} (mg dry cell weight) $^{-1}$.

[d] The uninhibited rate of methanol oxidation was 170 nmol oxygen consumed min^{-1} (mg dry cell weight) $^{-1}$.

[e] These gaseous compounds were added (0.2 ml) as saturated phosphate buffer (0.2 μmoles CO; 7.5 μmoles C_2H_2).

The stimulation of oxygen consumption during methanol oxidation in the presence of carbon monoxide was thought to be caused by methanol acting as an electron donor for carbon monoxide oxidation, thus resulting in an additive effect with respect to oxygen uptake (cf. Ferenci, 1974).

3. Effect of Various Potential Inhibitors on Methane and Methanol Oxidation by Whole-cell Suspensions of Methylococcus capsulatus (Bath).

In contrast to the results observed with TRMC, the inhibition pattern obtained with whole-cell suspensions of M. Capsulatus strain Bath (MC) was much more restricted (Table 5). Of the metal chelators tested, only 8-hydroxyquinoline and diethyldithiocarbamate gave 100% inhibition, with thiourea (50% inhibition), 2,2'-bipyridine (58% inhibition) and neocuproine (43% inhibition) being the only other chelators to cause any significant inhibition. Another five compounds gave complete inhibition, i.e. o-aminophenol, ferron, potassium cyanide, carbon monoxide and acetylene, of which both ferron and potassium cyanide caused complete inhibition of methanol oxidation also, and so could not be considered specific inhibitors of the methane mono-oxygenase.

Before drawing conclusions from the inhibitor data of MC in Table 5 the same range of compounds was tested on crude cell-free extracts of MC which contained good methane mono-oxygenase activity. The data from these assays should provide a more accurate specific inhibitor pattern for the methane mono-oxygenase as only the initial oxidation step of methane to methanol was monitored.

4. Effect of Various Potential Inhibitors on Methane Oxidation by Cell-free Extracts of Methylococcus capsulatus (Bath)

Methane mono-oxygenase activity was measured by methanol accumulation as described in the Methods, and the results of the effect of the various potential inhibitors on methane oxidation are shown in Table 6. Potassium cyanide (0.5 μmol) was present in all the assays, as it had been previously shown that cyanide inhibited residual methanol

TABLE 6

The effect of variouspotential inhibitors on methane oxidation by cell-free extracts of Methylococcus capsulatus (Bath)

Inhibitor	Rate of methanol accumulation (%control)[a]
None	100
Thiourea	90
Thioacetamide	93
Thiosemicarbazide	92
Diethyldithiocarbamate	100
2, 2'-Bipyridine	99
Neocuproine	100
Amobarbital	100
8-Hydroxyquinoline	29
Imidazole	81
Acriflavin	84
Aniline	100
Phenol	84
o-Aminophenol	88
Ferron	62
Carbon monoxide[b]	95
Pyridine	84
Acetylene[c]	0

[a] The uninhibited rate of methane oxidation as determined by methanol accumulation (see Materials and Methods) was 53 nmol methanol produced min^{-1} (mg of extract protein)$^{-1}$.

[b] Carbon monoxide was present in the gas phase as 15% v/v in air.

[c] Acetylene was present in the gas phase as 3% v/v in air. Reaction mixture (final vol. 1 ml): 20 µmol phosphate, 5 µmol NADH, 0.5 µmol KCN, 5 mg extract protein, 3 ml gas phase CH_4, 0.1 µmol inhibitor (where present).

oxidase activity in the cell-free extracts but did not inhibit cell-free methane mono-oxygenase activity (Colby and Dalton, 1976). Methanol dehydrogenase, which is present in this organism, was completely inactive in the assay system.

Only two compounds significantly inhibited methane oxidation, acetylene (100% oxidation) and 8-hydroxyquinoline (71% inhibition), while the rest gave very little or no inhibition. Comparing these results observed with cell-free extracts of MC with the results from the MC whole-cell suspensions tests (Table 5), only acetylene and 8-hydroxyquinoline were potent inhibitors in both cases. Therefore, five out of the seven compounds which strongly inhibited whole-cell methane oxidation in MC no longer gave significant inhibition of methane oxidation with cell-free extracts.

Diethyldithiocarbamate appeared to be a potent, relatively specific inhibitor of methane oxidation in whole cells of MC (Table 5). However, no inhibition of methane oxidation occurred with cell-free extracts revealing that diethyldithiocarbamate did not inhibit the methane mono-oxygenase. As the requisite reducing power (NAD(P)H) for the oxidation of methane to methanol was exogenously supplied in all the cell-free extract assays, it is possible that the inhibition shown by diethyldithiocarbamate with whole-cell suspensions of MC was due to an interruption in the regeneration of NAD(P)H in the cell rather than a direct inhibition of the methane mono-oxygenase.

A similar effect could explain the results obtained with o-amino-phenol, whereas a more general cellular disruption may have occurred with ferron as it completely inhibited both methane and methanol oxidation by whole-cells. Alternatively, the results observed with ferron could be explained by the specific inhibition of the methanol oxidising enzyme(s), preventing the regeneration of reducing power therefore inhibiting whole-cell methane oxidation but permitting cell-free methane oxidation in the presence of exogenous reducing power.

Potassium cyanide, as mentioned previously, was found to be non-inhibitory to cell-free methane mono-oxygenase activity of MC, and therefore presumably inhibited methane and methanol oxidation in whole cells as a result of its ability to complex with cytochrome oxidase.

Acetylene remained a potent, specific inhibitor of methane oxidation in cell-free extracts supporting the report that acetylene was a potent inhibitor of methane oxidation in whole cells of MC and appeared to act specifically on the initial oxidation step of methane to methanol (Dalton and Whittenbury, 1976a).

Carbon monoxide (CO) appeared from the whole-cell data to be a potent, specific inhibitor of methane oxidation, but when tested with cell-free preparations of MC gave negligible inhibition. An attempt to explain this phenomenon is complicated further by the fact that CO can be oxidised to carbon dioxide (CO_2) by whole cells and cell-free extracts of MC, but only in the presence of a suitable electron donor, such as methanol, formaldehyde or formate (see Section V). These observations plus the evidence reported by Hubley, Mitton and Wilkinson (1974) and Ferenci et al. (1975) suggested that the oxidation of CO to CO_2 is probably catalysed by a mono-oxygenase. Assuming this to be the case, the specific inhibition of methane oxidation by CO in whole cells of MC could possibly be explained in three ways. (1) If CO was oxidised by an NAD(P)H-requiring enzyme other than the methane mono-oxygenase and the former enzyme had a lower K_m for NAD(P)H, then any endogenous reducing power available in the cell would very rapidly become depleted. Therefore, as CO oxidation does not regenerate any reducing power, all NAD(P)H-dependent mono-oxygenase activity would stop. (2) CO could inactivate the methane mono-oxygenase either by competing with methane for the substrate binding site of the enzyme complex or by binding to another site

(allosteric inhibitor) and thus inactivating the multi-component methane mono-oxygenase, present in MC (Colby and Dalton, 1976; Colby and Dalton, 1978). (3) CO could interrupt the regeneration of NAD(P)H during the complete oxidation of methane, therefore starving the methane mono-oxygenase of the requisite reducing power.

The inability of CO to inhibit methane oxidation in cell-free systems of MC could suggest that CO was not actively competing with methane for the methane mono-oxygenase, hence favouring the first or third explanation above. However, as CO is 25% less soluble than methane at 45° C and three times more methane than CO was present in the assay, the K_m for CO would have to be considerably lower than the K_m for methane for CO to effectively compete with methane for the active site of the methane mono-oxygenase. No possibility can be excluded until the methane mono-oxygenase system is purified and tested for CO oxidation, CO inhibition of methane oxidation, etc.

Similar results to those with MC, concerning the effect of CO on methane oxidation and CO oxidation, have been reported by Ferenci (1974) with Methylomonas (Pseudomonas) methanica. Although CO was reported to be a potent inhibitor of methane oxidation in whole cells but ineffective on cell-free methane-oxidising extracts, Ferenci concluded in a later paper that CO was in fact oxidised by the methane mono-oxygenase (Ferenci et al., 1975), using a similar cell-free system. They obtained a 68% inhibition of methane mono-oxygenase activity by CO when using bromomethane as the substrate for the enzyme.

THE SITUATION WAS MADE EVEN MORE AMBIGUOUS IN METHYLOMONAS METHANICA BY COLBY ET AL. (1975)

8-Hydroxyquinoline remained the only metal chelator to significantly inhibit both whole-cell and cell-free methane oxidation in MC. This could suggest that if in fact a metal ion complex was involved in the methane mono-oxygenase system, it must have been well shielded from attack by most of the metal-binding compounds. However, as pointed out in the introduction, any conclusion based on the inhibition of one chelator must be made with caution. Therefore,

the interpretation of the inhibitor data of MC, with respect to the presence or absence of a metal ion(s) must remain unresolved until the methane mono-oxygenase system is sufficiently purified to allow metal analyses of the components.

When the results reported here are combined with additional inhibitor studies on cell-free extracts of MC by Colby and Dalton (1976), it provides an overall inhibition pattern for Methylococcus capsulatus (Bath) which differs greatly from that observed with Methylococcus capsulatus (Texas). As similar inhibitor studies on whole-cell suspensions of various methane-oxidising bacteria have been reported, it would be useful to compare the emergent patterns to see if any common trends arise.

5. Comparisons Between the Inhibition Patterns of Methane Oxidation by Whole-cell Suspensions of Various Methane-Oxidising Bacteria

On examination of the whole-cell suspension inhibitor pattern for MC and TRMC (Table 5) it can be clearly seen that there are major differences, particularly with respect to the effect of metal chelators. TRMC was far more susceptible to inhibition by the range of compounds tested, and on this evidence is the more likely of the two organisms to have metal ion(s) involvement with the methane mono-oxygenase.

Table 7 shows the effect of a number of potential inhibitors on whole-cell suspensions of three different methane-oxidising bacteria. The two sets of results for Methylococcus capsulatus (Texas) give very similar patterns as do the two sets of results for Methylosinus trichosporium (OB3b), and interestingly the cumulative patterns of both organisms are strikingly similar. It appears that both organisms were very susceptible to inhibition by metal chelators and other metal-binding compounds. The authors of the reports from which these results were taken concluded that there was metal ion(s) involvement with the methane mono-oxygenase in both these organisms (Hubley et al., 1975; Patel et al., 1976).

TABLE 7

Inhibitor	Methane oxidation by whole-cell suspensions										Ammonia oxidation by whole-cell suspensions of Nitrosomonas europaea[4]	
	MC[1]		TRMC[1]		TRMC[2]		OB3b[2]		OB3b[3]			
	conc. M	%control	conc. M	%control	conc. M	%control	conc. M	%control	conc. M	%control	conc. M	%control
Thiourea	10^{-3}	50	10^{-3}	3	10^{-3}	0	10^{-3}	0	10^{-4}	0		NT
Thiosemicarbazide	10^{-3}	70	10^{-3}	5	10^{-3}	20	10^{-3}	0	10^{-5}	45	10^{-5}	5
Diethyldithiocarbamate	10^{-3}	0	10^{-3}	0		NT		NT	7.5×10^{-4}	0	10^{-5}	0
Imidazole	10^{-3}	79	10^{-3}	37	10^{-3}	0	10^{-3}	10	10^{-3}	0		NT
2, 2'-Bipyridine	10^{-3}	42	10^{-3}	3	10^{-3}	0	10^{-3}	10	10^{-4}	0	10^{-4}	0
8-Hydroxyquinoline	10^{-3}	0	10^{-3}	0	10^{-3}	0	10^{-3}	0	10^{-4}	0	10^{-5}	0
1, 10-Phenanthroline	10^{-3}	62	10^{-3}	0	10^{-3}	0	10^{-3}	8	5×10^{-5}	65	5×10^{-5}	0
KCN	10^{-3}	0	10^{-3}	0	10^{-4}	0	10^{-4}	0	10^{-6}	38	5×10^{-6}	22
CO	7×10^{-5}	0	7×10^{-5}	0	ND	20	ND	11	15%(v/v, in air)	0	5%(v/v, in air)	8

MC, Methylococcus capsulatus (Bath)

TRMC, Methylococcus capsulatus (Texas)

OB3b, Methylosinus trichosporium (OB3b)

Ref. 1 Table 5 of this thesis
Ref. 2 Patel et al., (1976)
Ref. 3 Hubley et al., (1975)
Ref. 4 Hooper and Terry, (1973)

100% control represents the uninhibited methane (ammonia) oxidation rate.

NT, not tested
ND, not determined

Similar results to those obtained with the above two organisms were found with cell-free extracts of Methylomonas methanica (Colby et al., 1975) suggesting perhaps a uniformity of sensitivity to metal-binding agents and therefore possibly a universal involvement of a metal ion(s) in methane mono-oxygenase activity. This similarity in mono-oxygenase would appear to cover both types of methane-oxidising bacteria (Types 1 and 2 as originally defined by Whittenbury et al. (1970a)). It is also possible that this apparent uniformity in inhibitor pattern may hold for oxygenases other than those which oxidise methane, as a very similar pattern was found by Hooper and Terry (1973) for ammonia oxidation by whole-cell suspensions of Nitrosomonas europaea. They found that a large number of compounds, like those listed in Tables 5 and 7, inhibited ammonia oxidation but not hydroxylamine oxidation by whole-cell suspensions of Nitrosomonas europaea. This similarity in inhibitor patterns suggests that the two types of oxygenases (methane and ammonia) resemble each other. Combine this observation with other similarities listed below and the possibility arises that these two types of organisms, i.e. ammonia-oxidising bacteria and methane-oxidising bacteria, are very closely related and have, from an evolutionary standpoint, only very recently diverged from each other. Other similarities between the two types of organisms include: fastidious growth requirements (Quayle, 1972); the possession of complex internal membrane arrangements (Murray and Watson, 1965; Davies and Whittenbury, 1970; Smith and Ribbons, 1970; Watson and Mandel, 1971; De Boer and Hazeu, 1972); a lesion in the TCA cycle (Hooper, 1969; Davey et al., 1972); the energy source is initially metabolised by an oxygenase reaction (Rees and Nason, 1966; Higgins and Quayle, 1970); the apparent oxidation of ammonia by cell-free methane mono-oxygenase preparations (Dalton, 1977); methane can replace ammonia in ammonia-stimulated NADH oxidation by cell-free preparations of Nitrosomonas europaea (Suzuki, Kwok and Dular, 1976); methane oxidation by cell-free extracts of methane-utilisers is inhibited by ammonia (Colby et al., 1975; Ferenci et al., 1975), and

ammonia oxidation by cell-free extracts of Nitrosomonas europaea is inhibited by methane (Suzuki et al., 1976); possession of ribulose 1,5-Hsphosphate carboxylase in a methane-utilising organism (Taylor, S., 1977) and Nitrosomonas (Nicholas and Rao, 1964; Hooper, 1969).

However, Methylococcus capsulatus (Bath) appears to be the exception to the rule as it has a much more restricted inhibitor pattern (Tables 5 and 7) than other methane-oxidisers tested (Table 7). This difference in patterns is accentuated when the results of the cell-free extract tests of Methylococcus capsulatus (Bath) are examined (Table 6). Therefore on the available evidence of the inhibitor patterns of methane oxidation, Methylococcus capsulatus (Bath) appears divergent from the main group of methane-utilising bacteria due to its lack of susceptibility towards metal-binding agents. Whether this proves to be of any physiological or evolutionary importance remains to be seen.

It is obvious from the inhibitor data of Methylococcus capsulatus (Bath) in Tables 5 and 6 that a much more accurate inhibition profile of the specific reaction in question was obtained when the effect of the compounds was tested on cell-free extracts, as opposed to whole-cell suspensions. Therefore, it is suggested, where possible, that inhibitor data should be obtained using cell-free extracts to ensure greater validity to the results. Unfortunately, very little inhibitor data for cell-free methane mono-oxygenase activity has been reported, mainly as a result of the difficulties found in producing from most methane-utilisers cell-free extracts which contained good methane mono-oxygenase activity. Tonge et al. (1977) showed that a large variety of metal chelators and other compounds potently inhibited the purified methane mono-oxygenase of Methylosinus trichosporium (OB3b), thus substantiating the previously reported whole-cell inhibitor data (Hubley et al., 1975; Patel et al., 1976). Ribbons (1975) reported limited inhibitor data for methane mono-oxygenase activity in cell-free extracts of Methylococcus capsulatus (Texas) and the results seem to confirm the whole-cell inhibition results reported in Table 7. Therefore,

the limited cell-free extract information available supports the view that methane mono-oxygenase enzymes (with the exception of the enzyme found in Methylococcus capsulatus (Bath)) have a common inhibitor pattern and probably have a metal ion(s) involved with the enzyme.

6. Effect of Acetylenic Compounds on Methane Oxidation by Methylococcus capsulatus (Bath)

As acetylene was found to be the most potent specific inhibitor of methane oxidation in M. capsulatus strain Bath, a range of acetylenic compounds were tested as potential inhibitors to see whether any correlation could be observed between the size, chemical composition and arrangement of the compounds with the extent of inhibition shown. The compounds were initially tested on methane oxidation by whole-cell suspensions of M. capsulatus (Bath) and the results obtained are summarised in Table 8. Four of the eight acetylenic compounds, acetylene, propyne, but-1-yne and but-2-yne potently inhibited methane oxidation specifically, whereas the remaining four (with the possible exception of propyn-1-ol) had no effect. The apparent oxidation of propyn-1-ol was not due to auto-oxidation of the compound or oxidation by the methane mono-oxygenase, as the oxidation was not inhibited by acetylene or 8-hydroxyquinoline. As propyn-1-ol is a primary alcohol, it was almost certainly the result of oxidation by methanol dehydrogenase/oxidase.

To confirm the results from the whole-cell experiments, similar tests were done using cell-free extracts of M. capsulatus (Bath) (Table 9). Methane mono-oxygenase activity was assayed by two methods, (i) measuring methanol accumulation or (ii) meauring bromomethane disappearance, thereby hopefully revealing any erroneous results obtained with either method. The results obtained with the two methods correlate well, and showed both acetylene and propyne to be potent inhibitors of the methane mono-oxygenase. The small amounts of methanol which accumulated in the presence of acetylene and propyne were thought to be due to the release of methanol, previously bound to methane mono-oxygenase, during the assay, as similar amounts were produced in the absence of substrate. The results suggested that the inhibition efficiency of the acetylenic compounds decreases not only with increasing carbon chain length but with the positioning of the acetylenic bond in a sub-terminal location.

TABLE 8 The effect of acetylenic compounds on methane oxidation by whole-cell suspensions of Methylococcus capsulatus (Bath)

Inhibitor (final conc. 1 mM)	Chemical structure	Inhibitor oxidation	Rate of methane oxidation (%control)[a]	Rate of methanol oxidation (%control)[b]
Acetylene[c]	$HC \equiv CH$	–	0	100
Propyne[c]	$HC \equiv CCH_3$	–	0	100
But-1-yne[c]	$HC \equiv CCH_2CH_3$	–	0	100
But-2-yne	$CH_3C \equiv CCH_3$	–	10	100
Propyn-1-ol	$HC \equiv CCH_2OH$	+	nd[d]	100
Propargylamine	$HC \equiv CCH_2NH_2$	–	100	100
Dimethylamino propyne	$HC \equiv CCH_2N(CH_3)_2$	–	100	100
Pargyline	$HC \equiv CCH_2NC_6H_5CH_2CH_3$	–	100	100

a The uninhibited rate of methane oxidation was 225 nmol oxygen consumed min^{-1} (mg dry cell weight)$^{-1}$

b The uninhibited rate of methanol oxidation was 170 nmol oxygen consumed min^{-1} (mg dry cell weight)$^{-1}$

c These gaseous compounds were added as saturated solutions in phosphate buffer (0.2 ml)

d Not determined due to the masking of true methane oxidation rate by propyn-1-ol oxidation rate

TABLE 9 The effect of acetylenic compounds on methane mono-oxygenase activity in cell-free extracts of Methylococcus capsulatus (Bath) as determined by (a) measuring methanol accumulation (b) bromomethane disappearance

Inhibitor	Rate of methanol accumulation (% control)[a]	Rate of bromomethane disappearance (% control)[b]
Acetylene[c]	9	0
Propyne[c]	11	0
But-1-yne[c]	52	50
But-2-yne	88	79
Propyn-1-ol	76	60
Propargylamine	100	100
Dimethylaminopropyne	92	100
Pargyline	100	100

[a] The uninhibited rate of methanol accumulation from methane was 53 nmol methanol produced min^{-1} (mg protein)$^{-1}$

[b] The uninhibited rate of bromomethane disappearance was 25 nmol bromomethane min^{-1} (mg protein)$^{-1}$

[c] These gaseous compounds were added as saturated solutions in phosphate buffer (0.1 ml).

Reaction mixture (final vol. 1 ml): 20 µmol phosphate, 5 µmol NADH, 0.5 µmol KCN, 5 mg extract protein, 2.5 µmol inhibitor (except gaseous compounds), 3 ml gas phase CH_4 (a), 1 µmol bromomethane (b).

The partial inhibition of methane mono-oxygenase activity by propyn-1-ol (Table 9) was obtained in the presence of 0.5 mM potassium cyanide, and as KCN has been shown to inhibit any methanol oxidase activity present in soluble cell-free extracts of M. capsulatus (Bath) (Colby and Dalton, 1976), this eliminated the possibility of the inhibition being caused by a product of propyn-1-ol oxidation by methanol oxidase. However, this small but significant inhibition by propyn-1-ol was not surprising when one considers that methanol is in fact a better substrate than methane for the methane mono-oxygenase of M. capsulatus (Bath) (see Section IV). It appears from Table 8 and Table 9 that propargylamine, dimethylaminopropyne and pargyline had no effect on methane oxidation in M. capsulatus (Bath). One might have expected propargylamine to have shown some inhibitory effect like that of but-1-yne, but-2-yne or propyn-1-ol as structurally they are very similar. Their close similarity in structure and molecular size would suggest that rather than a simple steric effect, an electro-steric effect was the cause of the differences in their inhibitor potential.

During the experiment concerning the effect of acetylene on bromomethane oxidation by cell-free extracts of M. capsulatus (Bath), it was noticed that the concentration of acetylene diminished during the course of the assay. Similar assays were done in the absence of bromomethane and confirmed acetylene disappearance in the presence of cell-free extract, NADH and gaseous oxygen. Further tests showed that the rate of acetylene disappearance was dependent on extract concentration and required the presence of NADH and gaseous oxygen. Attempts to identify a product for this apparent acetylene oxidation proved unsuccessful, therefore it remains uncertain whether acetylene was in fact truly oxidised by cell-free extracts of M. capsulatus (Bath) or was simply bound to an enzyme complex in the requisite presence of NADH and gaseous oxygen. Nevertheless, this result suggested the possibility of a methane mono-oxygenase of unusual substrate specificity, and a detailed study of the substrate specificity of the enzyme was undertaken.

SECTION IV Substrate Specificity Studies of the Methane Mono-oxygenase from Methylococcus capsulatus (Bath)

1. Introduction

Historically cell-free extracts of methane-oxidising bacteria capable of active methane oxidation have proved very difficult to obtain, as discussed in Section I. However, there have been reports of NADH- and oxygen-dependent methane oxidation by cell-free preparations from Methylococcus capsulatus (Texas) (Ribbons and Michalover, 1970; Ribbons, 1975), Methylomonas (Pseudomonas) methanica (Ferenci, 1974; Colby et al., 1975; Ferenci et al., 1975), Methylosinus trichosporium OB3b (Tonge et al., 1975; Tonge et al., 1977) in addition to Methylococcus capsulatus (Bath) (Colby and Dalton, 1976). All these studies, with the possible exception of Methylosinus trichosporium OB3b, were consistent with an NADH-linked mono-oxygenase being responsible for methane oxidation.

Active particulate membrane preparations of Methylomonas methanica have been reported to oxidise carbon monoxide (Ferenci, 1974; Ferenci et al., 1975), ethane (Ferenci et al., 1975), bromomethane (Colby et al., 1975) and ammonium chloride (Colby et al., 1975; Ferenci et al., 1975). Similar cell-free preparations of Methylococcus capsulatus (Texas) and Methylosinus trichosporium OB3b have been reported to oxidise ethane (Ribbons, 1975) and ethane, n-propane, n-butane and carbon monoxide (Tonge et al., 1977) respectively. Soluble cell-free extracts of Methylococcus capsulatus (Bath) have been reported to oxidise bromomethane (Colby and Dalton, 1976). All the above reports concluded that oxidation of the methane analogues and homologues was probably catalysed by the methane mono-oxygenase.

Nothing further is known about the substrate specificity of methane mono-oxygenases, although a comprehensive knowledge of the catalytic abilities of these enzymes would be a valuable asset in the elucidation of their mechanisms. This approach has proved very informative during studies of the terminal hydroxylase of Pseudomonas oleovorans (Abbott and Hou, 1973; May and Abbott, 1973; May, Schwartz, Abbott and Zaborsky, 1975). This Section (IV) presents the results of the investigations into the substrate specificity of the soluble methane mono-oxygenase of Methylococcus capsulatus (Bath).

2. Validity of Results

Since the methane mono-oxygenase from Methylococcus capsulatus (Bath) has so far resisted purification, due to instability of some components, crude soluble extract was used as the source of mono-oxygenase. Precautionary measures were undertaken to ensure the validity of the results. The further metabolism of primary alcohol products, including methanol, was prevented by virtue of the methanol oxidase activity being almost exclusively particulate in nature, and by inhibiting any residual methanol oxidase activity with 0.5 mM potassium cyanide (Colby and Dalton, 1976). Even in the absence of cyanide, the methanol oxidase activity of the soluble extracts used in these studies was negligible, if any. Under the conditions of the methane mono-oxygenase assays (see Methods) no methanol dehydrogenase activity was detected, although the soluble extracts used for the tests contained high specific activity of methanol dehydrogenase when measured under its optimum in vitro conditions (Anthony and Zatman, 1964b). All other detectable products, with the exception of formaldehyde, did not appear to be metabolised further than the initial oxidation. Therefore all but one of the products observed were the result of a single oxidation step involving the incorporation of one oxygen atom into the substrates, consistent with the operation of a mono-oxygenase. Formaldehyde was found to be rapidly oxidised by soluble extracts of Methylococcus capsulatus (Bath) in the presence of NAD^+ and under the normal assay conditions for methane mono-oxygenase, including the presence of potassium cyanide (see Section VI).

3. Control Assays.

A number of essential controls were done with each substrate tested to ensure that any oxidations observed were catalysed by the methane mono-oxygenase. Five control assays were used: (1) and (2) assays done in the absence of NADH or gaseous oxygen to ensure that both these co-substrates were required; (3) assay done in the absence of potassium cyanide because many mono-oxygenases are inhibited by cyanide (see later), unlike the enzyme from Methylococcus capsulatus (Bath); (4) assay done in the presence of 0.2 ml ethyne (acetylene), previously shown to be a potent,

specific inhibitor of methane mono-oxygenase activity in Methylococcus capsulatus (Bath) (Colby and Dalton, 1976; Dalton and Whittenbury, 1976a; see Section III); (5) boiled soluble extract replaced fresh extract in assays to ensure any oxidation observed was enzyme catalysed. The results obtained using these controls are included in the relevant Tables and are given in parentheses. For all the substrates listed in the Tables, the control values were consistent with the oxidation being catalysed by methane mono-oxygenase. Until the enzyme is purified, however, there is always the possibility that any of the substrates (other than methane) could have been oxidised by another enzyme(s) similar to the methane mono-oxygenase.

4. Oxidation of Substituted Methane Derivatives and of Carbon Monoxide

The oxidation rates of the various substituted methane derivatives (Table 10) was measured by following their disappearance because no volatile products could be detected by gas chromatography. This apparent lack of detectable oxidation products could be explained by the production of highly unstable substituted methanol derivatives as the reaction products.

Regarding the monohalogenated methane analogues, chloromethane was oxidised at a rate comparable to that observed with methane (Table 11). Bromomethane was oxidised at a rate approximately 75% of that found with the above two compounds, while iodomethane was not oxidised at all. The decline in rate of oxidation corresponded with an increase in size of substituting halogen, suggesting a simple steric hindrance effect. This conclusion is supported when the results obtained with all four chlorinated methane analogues are compared. Chloromethane and dichloromethane were oxidised as rapidly as methane, whereas the oxidation rate of trichloromethane was less than half that of methane, and tetrachloromethane was not oxidised at all. Again, an increase in the molecular size of the substrate caused a decrease in the rate of oxidation. It is interesting, however, that two hydrogen atoms of methane can be replaced by chlorine atoms with apparently no effect on the rate of substrate oxidation.

TABLE 10 Oxidation of substituted methane derivatives and of CO by soluble extracts of M. capsulatus (Bath)

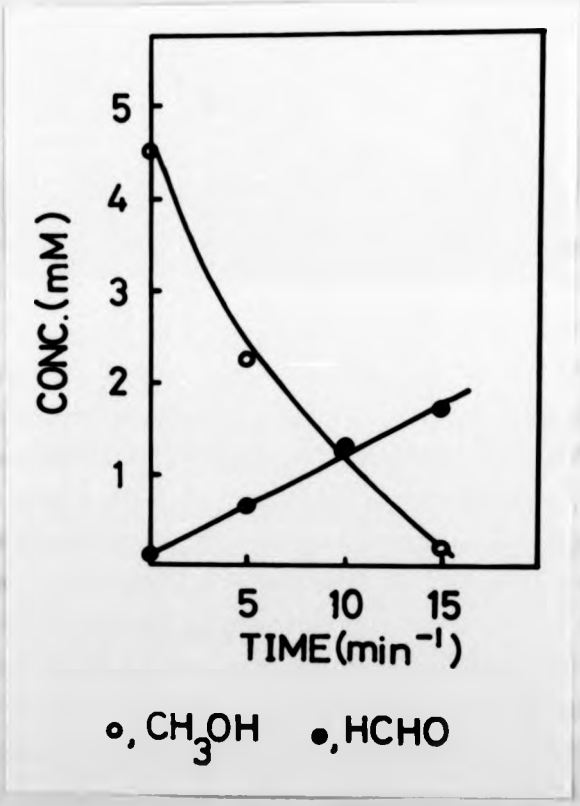
Substrate (μmol per reaction flask)	Specific activity [munits (mg of protein)$^{-1}$]
Chloromethane (1)	84 (0, 0, 80, 0, 0)
Bromomethane (1)	66 (0, 0, 66, 0, 0)
Iodomethane (1-3)	0
Dichloromethane (1)	82 (0, 0, 76, 0, 0)
Trichloromethane (1)	35 (0, 0, 38, 0, 0)
Tetrachloromethane (1-3)	0
Cyanomethane (1)	33 (0, 0, 25, 0, 0)
Nitromethane (2)	45 (0, 0, 41, 0, 0)
Methanethiol (2)	64 (0, 0, 64, 0, 0)
Methanol (5)	246 (0, 20, nd, 16, 0)
Trimethylamine (2-4)	0
CO (134)	61 (12, 10, 56, 12, nd)

Specific activities with CO as substrate were calculated from the rate of CO_2 formation. Otherwise specific activities were calculated from the rate of disappearance of substrate and the products were not identified. Full experimental details are given in the Materials and Methods section. Specific activity values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2 ml of ethyne, or with boiled extract. [nd, not done.]

Cyanomethane (39%), nitromethane (54%) and methanethiol (76%) were oxidised more slowly than methane; the rates expressed as percentages of the rate observed with methane. Trimethylamine was not oxidised. Methanol was oxidised approximately three times as rapidly as methane, which is interesting considering it is the product of methane oxidation. However, the apparent K_m values for methanol and methane were 0.45 mM and 0.16 mM respectively, suggesting that methanol has a relatively poor affinity for the methane mono-oxygenase. Formaldehyde was the expected product of methanol oxidation, but because of the presence of formaldehyde dehydrogenase activity in soluble extracts, no product was observed.

In an attempt to show formaldehyde accumulation from methanol, a sample of crude soluble extract was subjected to ion-exchange chromatography (DEAE-cellulose) to produce a partially purified methane mono-oxygenase preparation which retained good methane mono-oxygenase activity, but poor formaldehyde-oxidising activity. The methane mono-oxygenase was resolved into two fractions, one of which passed straight through the DEAE-cellulose column and the other which bound to the DEAE-cellulose and was subsequently eluted off with 0.5 M sodium chloride. When combined, the fractions constituted good methane mono-oxygenase activity, but possessed poor formaldehyde dehydrogenase activity. The partially purified mono-oxygenase preparation was then used for a similar methanol oxidation assay as in Table 10, but this time samples were removed after 0, 5, 10 and 15 minutes, and assayed for methanol (gas chromatography) and formaldehyde (diacetylacetone method of Nash (1953)). The results (Fig. 9) showed a fall in methanol concentration with a concomitant rise in formaldehyde concentration, even though the relationship was not stoichiometric. The usual controls were done and gave results consistent with methanol oxidation by the methane mono-oxygenase, producing formaldehyde. Although a stoichiometric production of formaldehyde from methanol was not shown, the results

Fig. 9. The Production of Formaldehyde from Methanol Oxidation by the Methane Mono-oxygenase from M. capsulatus (Bath)



in Fig. 9 suggested that formaldehyde was the true product of methanol oxidation by the methane mono-oxygenase.

Carbon monoxide was oxidised by the methane mono-oxygenase to carbon dioxide at approximately 75% the rate observed with methane. Similar activity has been shown with cell-free preparations of Methylomonas methanica (Ferenci et al., 1975) and Methylosinus trichosporium OB3b (Tonge et al., 1977), although the former report only showed that carbon monoxide stimulated respiration in the presence of oxygen and NADH, and did not show that carbon monoxide disappeared or carbon dioxide was produced.

5. Oxidation of C_1-C_8 n-Alkanes

The methane mono-oxygenase from Methylococcus capsulatus (Bath) catalysed the oxidaton of n-alkanes of 1 to 8 carbons (Table 11). C_1 to C_5 all gave comparable rates of oxidation but there was a rapid decline in the rates from n-hexane (C_6) to n-octane (C_8). Secondary alcohols were produced from n-propane through to n-octane, indicating that both primary and secondary alkyl (C-H) bonds can be hydroxylated. Therefore the enzyme is not a terminal hydroxylase like the rubredoxin, reductase-containing hydroxylase system found in Pseudomonas oleovorans, which catalyses the ω-hydroxylation of fatty acids and the terminal oxidation of alkanes and 1-alkenes (McKenna and Coon, 1970; May and Abbott, 1972; Abbott and Hou, 1973; May et al., 1975; Ruettinger, Griffith and Coon, 1977) and the cytochrome P-450, alkane hydroxylase system from a Corynebacterium species (Cardini and Jurtshuk, 1968; Jurtshuk and Cardini, 1971). A terminal hydroxylase (mono-oxygenase) has also been implicated in two methane-utilising bacteria. When whole-cell suspensions of Methylomonas albus BG8 or Methylosinus trichosporium OB3b were incubated with n-propane or n-butane, only terminal oxidation products were formed (Thomson, 1974). The purified methane mono-oxygenase from Methylosinus trichosporium OB3b produced only the corresponding alcohols from n-propane and n-butane (Tonge et al., 1977).

TABLE 11 Oxidation of C_1-C_8 n-alkanes by soluble extracts of *M. capsulatus* (Bath)

Substrate (μmol per reaction flask)	Products (μmol formed in 12 min)	Specific activity [m units (mg of protein)$^{-1}$]
Methane (134)	Methanol (2.02)	84 (0, 0, 85, 13, 0)
Ethane (134)	Ethanol (1.64)	68 (0, 0, 63, 13, 0)
Propane (134)	1-Propanol (0.65)	69 (0, 0, 68, 0, 0)
	2-Propanol (1.00)	
Butane (134)	1-Butanol (1.10)	77 (0, 5, 68, 0, 0)
	2-Butanol (0.92)	
Pentane (150)	1-Pentanol (0.49)	73 (0, 0, 69, 0, 0)
	2-Pentanol (1.26)	
	3-Pentanol (< 0.06)	
Hexane (150)	1-Hexanol (0.60)	40 (0, 0, 39, 0, 0)
	2-Hexanol (0.36)	
	3-Hexanol (<0.01)	
Heptane (150)	1-Heptanol (0.14)	27 (0, 0, 27, 0, 0)
	2-Heptanol (0.51)	
	3-Heptanol (<0.01)	
	4-Heptanol (<0.01)	
Octane (300)	1-Octanol (0.04)	9 (0, 0, 9, 0, 0)
	2-Octanol (0.39)	
	3-Octanol (<0.01)	
	4-Octanol (<0.01)	

Reaction mixtures contained 2 mg of extract protein except for those with n-octane as substrate which contained 4 mg of extract protein. Specific activities were calculated from the total amount of products formed after 12 min incubation. The values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2 ml of ethyne, or with boiled extract. Full details of the experimental methods are given in the Materials and Methods section.

Interestingly, the methane mono-oxygenase from Methylococcus capsulatus (Bath) is apparently specific for the 1- and 2-alkyl carbons as there was only negligible formation of 3- or 4-alcohols from n-pentane, n-hexane, n-heptane or n-octane. However, the methane mono-oxygenase from Methylococcus capsulatus (Bath) is by no means unique in its ability for sub-terminal oxidation of n-alkanes as this is a well-known phenomenon in micro-organisms (Markovetz, 1971), although the vast majority of reports are of sub-terminal oxidation of higher n-alkanes, i.e. C_{10} and over, by whole-cell suspensions. In most reports a random mixture of secondary alcohols (2, 3, 4 etc.) and their oxidation products, together with the primary alcohols were formed, whereas with Methylococcus capsulatus (Bath) only mixtures of 1- and 2-alcohols were produced from the oxidation of n-alkanes. For example, Fredricks (1967) showed that whole-cell suspensions of Pseudomonas aeruginosa oxidised n-decane to produce a mixture of 1, 2, 3, 4 and 5-decanols plus the analogous ketones and acids. Similar results have been obtained with two different Arthrobacter species, where one species transformed n-hexadecane to a mixture of the isomeric ketones (Klein, Davis and Casida, 1968), and the other again transformed n-hexadecane but to a mixture of ketones and internal hexadecanols (Klein and Henning, 1969).

Sub-terminal oxidation has been reported with whole-cell oxidation studies of other methane-oxidising bacteria. Leadbetter and Foster (1960) observed the formation of 1-propanol, propionic acid and propanone (acetone) when suspensions of Methylomonas (Pseudomonas) methanica were incubated with n-propane. Similarly, 1-butanol, butyric acid and butan-2-one were formed from n-butane. Considering the alcohol dehydrogenase present in this organism is specific for primary alcohols, the mechanism of ketone formation is obscure (Johnson and Quayle, 1964; Ferenci et al., 1975) however, the fact that such ketones were produced suggests that sub-terminal oxidation can occur.

A few instances of the specific oxidation of long-chain fatty acids and n-alkanes at the ω-1 and ω-2 carbon atoms have been reported,

e.g. a Torulopsis species of yeast (Tulloch, Spencer and Gorlin, 1962; Heinz, Tulloch and Spencer, 1970) and the rat liver microsomal cytochrome P-450 system (Bjorkhem and Danielsson, 1970). However, in the latter case it has recently been suggested (Ellin and Orrenius, 1975; Gustafsson and Bergman, 1976) that the ω-1 and ω-2 hydroxylations were catalysed by different cytochrome P-450 species, based on their differing inhibitor sensitivities (Ellin and Orrenius, 1975) and differing susceptibilities towards oxidation agents (Gustafsson and Bergman, 1976).

6. Oxidation of Internal and Terminal Alkenes

The results of the various alkene oxidations are listed in Table 12. Soluble extracts of Methylococcus capsulatus (Bath), containing active methane mono-oxygenase, catalysed the oxidation of the terminal alkenes, ethene (ethylene), propene (propylene) and but-1-ene to the corresponding 1,2-epoxides. Surprisingly, no unsaturated alcohols (2-propen-1-ol, 3-buten-1-ol) were formed from propene or but-1-ene. The oxidation rate obtained with ethene was twice that observed with methane, but for the other alkenes the rate of oxidation decreased with increasing carbon-chain length.

Both configurations of but-2-ene were oxidised. trans-But-2-ene yielded a mixture of trans-2,3-epoxybutane and trans-but-2-en-1-ol, indicating that both the internal double bond and the terminal methyl group could be oxygenated, even though just over three times as much unsaturated alcohol than epoxide was produced. Significantly, the trans-configuration was retained in both products, showing that the reaction mechanism precluded the formation of racemisable intermediates.

The oxidation of cis-but-2-ene produced three products, two of which were positively identified as cis-2,3-epoxy-butane and butan-2-one. The third product could not be conclusively identified as cis-but-2-en-1-ol because the authentic standard was not available for a reference marker.

TABLE 12 Oxidation of C_2-C_4 n-alkenes by soluble extracts of *M. capsulatus* (Bath)

Substrate (μmol per reaction flask)	Products (μmol formed after 12 min)	Specific activity [munits (mg of protein)$^{-1}$]
Ethene (134)	Epoxyethane* (3.54)	148 (0, 13, 122, 12, 0)
Propene (134)	1,2-Epoxypropane* (2.10)	83 (0, 0, 83, 0, 0)
But-1-ene (134)	1,2-Epoxybutane* (1.19)	49 (0, 0, 49, 0, 0)
cis-But-2-ene (134)	cis-2,3-Epoxybutane* (0.61)	57 (0, 0, 51, 0, 0)
	cis-2-Buten-1-ol† (0.57)	
	Butan-2-one‡ (0.20)	
trans-But-2-ene (134)	trans-2,3-Epoxybutane* (0.77)	141 (0, 0, 128, 0, 0)
	trans-2-Buten-1-ol† (2.52)	

Full experimental details are given in the Materials and Methods section. Specific activities were calculated from the total amount of products formed after 12 min incubation. The values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2 ml of ethyne, or with boiled extract.

† Product disappears from gas chromatograms after treating the reaction mixture with 5 μl of bromine, but remains after treatment with 20 μl of HCl.

‡ product remains after treating the reaction mixture with either bromine or HCl.

* Product disappearsfrom gas chromatograms after treating the reaction mixture with 20 μl of HCl but remains after treatment with 5 μl of bromine.

However, its retention times on gas-chromatographic analysis, which were very similar but not identical to trans-but-2-en-1-ol, and its reaction with bromine but no reaction with dilute hydrochloric acid (see legend, Table 12), make its identification virtually certain. Like trans-but-2-ene, the cis-configuration was retained in the epoxide and most likely in the alken-1-ol.

The production of butan-2-one from cis-but-2-ene is difficult to explain and requires further study. However, it is unlikely that the ketone arose from the oxidation of some contaminating compound in the starting substrate because the amount of cis-but-2-ene present in the assay had to be reduced to a level where the production of the other two products was affected before the rate of butan-2-one production decreased. Also, no superfluous peaks were observed on gas chromatography analysis of the substrate alone. It was not possible to distinguish whether butan-2-one was formed as a direct enzymic product of the methane mono-oxygenase or whether it was a subsequent chemical or enzymic breakdown product of either the cis-epoxide or cis-alken-1-ol.

Interestingly, there was a marked difference in the oxidation rates of cis- and trans-but-2-ene. trans-But-2-ene was oxidised more rapidly than any other of the alkenes tested, with the exception of ethene. This suggests that the trans-configuration is sterically favoured for oxidation by the methane mono-oxygenase from Methylococcus capsulatus (Bath). n-Alkene oxidation by whole-cell suspensions of several bacteria has been reported (van der Linden, 1963; Huybregtse and van der Linden, 1964; Markovetz, Klug and Forney, 1967; Buswell and Jurtshuk, 1969; Jurtshuk and Cardini, 1971; Abbott and Hou, 1973; Watkinson, 1973; de Bont, 1976b; de Bont and Albers, 1976; de Bont and Harder, 1978). In all of the above reports, only terminal alkenes were oxidised, with the exception of whole-cell suspensions of a Corynebacterium sp. which could oxidise hex-2-ene and hept-2-ene (Buswell and Jurtshuk, 1969).

Oxidation of terminal n-alkenes by purified hydroxylase systems or crude cell-free extracts has been reported in a few instances (Jurtshuk and Cardini, 1971; May and Abbott, 1973), however, it appears from all the reports consulted that the only bacterial, cell-free oxygenase preparation shown to oxidise the double bond of internal n-alkenes is that of Methylococcus capsulatus (Bath) (Table 12).

7. Oxidation of Dimethyl Ether and Diethyl Ether

The specific activities obtained with these two substrates are shown in Table 13. No products were identified for dimethyl ether oxidation even though it was oxidised approximately three times as rapidly as methane, at a rate comparable to that observed with methanol as substrate. Two products were detected and indentified from diethyl ether oxidation, but the oxidation rate was only half that observed with methane. The products, ethanol and ethanal (acetaldehyde) were formed in approximately equimolar amounts and indicated that diethyl ether was only oxidised sub-terminally, i.e. only the C atoms bonded to the oxygen atom were oxidised because 2-ethoxyethanol would have been produced if terminal oxidation had occurred.

Oxidation of, or growth on, dimethyl ether by whole-cell suspensions of a number of methane-oxidising bacteria has been reported (Davey, 1971; Wilkinson, 1971; Thomson, 1974; Hazeu, 1975; Wilkinson, 1975; Patel et al., 1976) and cell-free, particulate preparations of Methylococcus capsulatus (Texas) have also been shown to oxidise dimethyl ether (Ribbons, 1975). It has been suggested that dimethyl ether is oxidised by the methane mono-oxygenase and is in fact an early intermediate during methane oxidation (Thomson, 1974; Hubley, 1975; Wilkinson, 1975; Patel et al., 1976). On close inspection, however, the majority of the reported data and postulated metabolic pathways

TABLE 13 Oxidation of ethers by soluble extracts of *M. capsulatus* (Bath)

Substrate (μmol per reaction flask)	Products (μmol formed after 12 min)	Specific activity [m units (mg of protein)$^{-1}$]
Dimethyl ether (4.5)	Not known	248 (0, 0, 227, 0, 0)
Diethyl ether (4.5)	Ethanol (0.51)	45 (0, 0, 45, 0, 0)
	Ethanal (0.57)	

Specific activities were calculated from the rate of disappearance of the substrate. However, the formation of ethanol and ethanal from diethyl ether was detected by analysing samples (5 μl) of reaction mixtures before and after incubation. Full experimental details are given in the Materials and Methods section. Specific activity values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2 ml of ethyne, and with boiled extract.

concerning dimethyl ether oxidation appears very dubious in nature. This conclusion is based on three main observations. Firstly, very little actual data on dimethyl ether oxidation by methane-oxidising bacteria has been published, as a number of papers concerning this topic, which were reportedly 'in press', 'submitted for publication' or 'in preparation', have in fact never appeared in published form (see Wilkinson, 1971; Thomson, 1974; Hubley, 1975; Wilkinson, 1975). Secondly, conflicting conclusions have arisen from independent studies, for example, Patel *et al.* (1976) on the basis of inhibitor patterns of methane and dimethyl ether oxidation with whole-cell suspensions of *Methylosinus trichosporium* OB3b, suggested that dimethyl ether was oxidised by the methane mono-oxygenase. However, Tonge *et al.* (1977) have shown that the

purified methane mono-oxygenase from Methylosinus trichosporium OB3b does not oxidise dimethyl ether. Lastly, it appears from results presented in this thesis (see Section V) that the commercial grade of dimethyl ether used by our laboratory, and apparently by others, contains an unknown contaminant which was rapidly oxidised by whole-cell suspensions of methane-oxidising bacteria. In an attempt to clarify the situation, for Methylococcus capsulatus (Bath) in particular, a more detailed study than in Table 13 was done, using both whole-cell suspensions and cell-free preparations (see Section V) and a more comprehensive review of the literature concerning dimethyl ether oxidation accompanies the results of this study.

Results of ether oxidation by the methane mono-oxygenase of Methylococcus capsulatus (Bath) resembled those obtained by Heydeman (1974) using soil bacteria isolated from diethyl ether enrichments. He found that diethyl ether was oxidised to the hemiacetal 1-ethoxyethanol ($CH_3CH_2OCHOHCH_3$), i.e. a sub-terminal carbon was hydroxylated. This hemiacetal then dismutated to form equimolar amounts of ethanol and ethanal. A similar mechanism of oxidation was expected by Thomson (1974) for diethyl ether oxidation by whole-cell suspensions of Methylosinus trichosporium (OB3b) and Methylomonas albus BG8, his assumption based on a similar pathway proposed by Mitton et al. (Hubley, 1975) for dimethyl ether oxidation. However, he found that whole-cell suspensions of these two organisms oxidised only the terminal carbons of diethyl ether, yielding 2-ethoxyethanol ($CH_3CH_2OCH_2CH_2OH$), 2-ethoxyethanal and 2-ethoxyacetate. He concluded from this data that diethyl ether could not be regarded as an intermediate of ethane oxidation as previously proposed for dimethyl ether and methane oxidation (Davey, 1971; Wilkinson, 1971). It also appears from the above information that these two organisms may contain methane mono-oxygnases which are specific for terminal carbons.

8. Oxidation of Alicyclic, Aromatic and Heterocyclic Compounds

The results obtained with the above compounds are given in Table 14. Cyclohexane was oxidised to cyclohexanol with a specific activity of approximately 1.5 times that observed with hexane. The ability of the methane mono-oxygenase to oxidise cycloalkanes presumably reflects its ability to oxidise methylene C atoms.

Benzene was oxidised at the same rate found with cyclohexane, yielding phenol, proving that the methane mono-oxygenase from Methylococcus capsulatus (Bath) can oxidise aromatic compounds. Toluene was an interesting substrate as it tested the enzyme with two analogues of known oxidisable compounds, the phenyl derivative of methane and the methyl derivative of benzene. A mixture of benzyl alcohol and cresol was produced when the enzyme was incubated with toluene, indicating that both the methyl group and aromatic ring were hydroxylated. Unfortunately, gas chromatography analysis did not distinguish between o, m or p cresols, and therefore prevented conformational analysis of the products. Styrene is analogous to toluene in being the phenyl derivative of ethene and the vinyl derivative of benzene. Styrene was oxidised to styrene epoxide only, yielding no hydroxylated aromatic ring products as found with toluene.

Pyridine oxidation yielded no volatile products which could be detected by gas chromatography. Reaction mixtures were examined for non-volatile products by thin-layer chromatography as described in the Methods. A spot corresponding to pyridine N-oxide was produced by t.l.c. of complete reaction mixtures after 12 minutes incubation. No 2-, 3- or 4-hydroxypyridine was detected, although these compounds could be easily distinguished from pyridine N-oxide by t.l.c. No pyridine N-oxide was found in complete reaction mixtures at zero time or after 12 minutes incubation of the appropriate control reaction mixtures.

TABLE 14 Oxidation of some alicyclic, aromatic and heterocyclic compounds by soluble extracts of M.capsulatus (Bath)

Substrate (μmol per reaction flask)	Products (μmol formed after 12 min)	Specific activity [munits (mg of protein)$^{-1}$]
Cyclohexane (460)	Cyclohexanol‡ (3.0)	62 (0, 0, 62, 0, 0)
Benzene (450)	Phenol† (3.0)	62 (0, 0, 62, 0, 0)
Toluene (460	Benzyl alcohol† (1.5)	53 (0, 0, 52, 0, 0)
	Cresol† (1.0)	
Styrene (90)	Styrene epoxide* (2.3)	47 (0, 0, 37, 0, 0)
Pyridine (3, 90)	Pyridine N-oxide (nd)	29 (0, 4, 25, 0, 0)
L-Phenylalanine (2-16)	Tyrosine (0)	0

Full details of the methods used are given in the Materials and Methods section. All reaction mixtures contained 4 mg of extract protein. Except when pyridine was the substrate, specific activities were calculated from the total amount of products formed after 12 min incubation. The rate of pyridine oxidation was determined by following pyridine disappearance from reaction flasks containing 3 μmol of pyridine. Pyridine N-oxide was identified as the product of pyridine oxidation by t.l.c.; in this case reaction flasks contained 90 μmol of pyridine as substrate. Specific activity values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2 ml of ethyne, or with boiled extract.

nd Not done.

* Product disappears from gas chromatograms after treating the reaction mixture with 20 μl of HCl or with 5 μl of bromine.

† Product disappears after treating the reaction mixture with 5 μl of bromine, but remains after treatment with 20 μl of HCl.

‡ Product remains after treating reaction mixtures with either 20 μl of HCl or 5 μl of bromine.

Methane mono-oxygenase was tested for its ability to hydroxylate the aromatic ring of L-phenylalanine to give L-tyrosine by assaying the product colorimetrically (Kaufman, 1970). No tyrosine was produced after 12 minutes incubation. The absence of assayable product was not due to further metabolism of tyrosine because crude soluble extracts of Methylococcus capsulatus (Bath) did not catalyse the disappearance of tyrosine under the usual methane mono-oxygenase assay conditions.

Reports in the literature suggest that micro-organisms with the capability to grow on cyclic or aromatic hydrocarbons are rare. A recent review (Perry, 1977) concerning the microbial metabolism of cyclic hydrocarbons and other related compounds reported that the vast majority of hydrocarbon-oxidising micro-organisms isolated are unable to grow on cyclic hydrocarbons and suggested that many of the earlier positive isolates were probably mixed cultures. In recent years, however, an ever-growing number of reports have appeared, describing the oxidation of many cyclic and aromatic compounds by pure or mixed cultures of micro-organisms in the requisite presence of their normal growth substrate (Horvath, 1972a). This ability of organisms to 'co-oxidise' non-growth compounds appears to be widespread and has been reported for a number of methane-utilising bacteria (Leadbetter and Foster, 1960; Ferenci, 1974; Hubley et al., 1974; Reed, 1976). Many organisms probably have the enzymic capability, like Methylococcus capsulatus (Bath), to oxidise cyclic or aromatic hydrocarbons, but the potential will remain unknown until the appropriate cell-free studies are done. An example of this is the hydroxylating system found in Pseudomonas aeruginosa (strain 473) which could only oxidise/grow on n-heptane. However, it was found that cell-free extracts could oxidise a wide range of hydrocarbons, including cyclic and aromatic compounds (van Ravenswaay Claasen and van der Linden, 1971).

9. Effect of Replacing NADH with Other Potential Electron Donors and Hydroxylating Agents

In addition to the results presented in Table 15, the following compounds gave no activity when tested at 5 and at 20 mM in place of NADH with methane as the substrate: sodium ascorbate, quinol, sodium dithionite, sodium borohydride, sodium chlorite, sodium periodate. From the results in Table 15 it can be seen that only NADPH can replace NADH as electron donor. The broad specificity of the methane mono-oxygenase from Methylococcus capsulatus (Bath) suggested that the enzyme mechanism might involve the generation of a non-specific hydroxylating species such as H_2O_2 or superoxide anion. However, the failure of catalase or superoxide dismutase to prevent activity and the failure of H_2O_2 or superoxide anion (generated in situ from xanthine and xanthine oxidase) to support methane or benzene oxidation effectively excludes these possibilities. Sodium chlorite ($NaClO_2$) and sodium periodate ($NaIO_4$), by serving as oxygen donors, have been shown to support steroid hydroxylation in a number of partially purified preparations of cytochrome P-450 systems (Gustafsson and Bergman, 1976). Neither of these compounds supported methane oxidation by the methane mono-oxygenase of Methylococcus capsulatus (Bath).

10. Final Conclusions from the Substrate Specificity Studies of the Methane Mono-oxygenase from M. capsulatus (Bath)

The methane mono-oxygenase from Methylococcus capsulatus (Bath) is a very non-specific enzyme and can oxidise a large number of substrates with apparently little or no structural resemblance to its in vivo substrate, methane. It catalyses the hydroxylation of primary and secondary alkyl C-H bonds, the epoxidation of both terminal and internal alkenes, the hydroxylation of cyclic and aromatic compounds, the N-oxidation of pyridine, and the oxidation of carbon monoxide to carbon dioxide. In some cases, e.g. cis or trans but-2-ene and toluene, substrates can be oxygenated in more than one position.

TABLE 15 Effect of replacing NADH with other potential electron donors and hydroxylating agents

Test compound	Specific activity [m units (mg of protein)$^{-1}$]	
Substrate	Methane	Benzene
NADH (5mM)	85	62
NADPH (5 mM)	49	nd
NADH (5 mM)+ superoxide dismutase (100 units)	87	62
Xanthine (5 mM) + xanthine oxidase (0.1, 1 unit)	0	0
H_2O_2 (5, 20 mM)	0	0
NADH (5 mM) + catalase (100 units)	80	68

Methanol formation from methane, and phenol formation from benzene, were measured as described in the Materials and Methods section, except that cyanide was omitted from the reaction mixtures and NADH was replaced with the compounds indicated.
nd = not done.

It is impossible to predict the mechanism of oxygen activation and addition for the methane mono-oxygenase from Methylococcus capsulatus (Bath) on the evidence to date. However, the data suggests that neither peroxide nor superoxide species are involved. Cytochrome P-450 does not appear to be involved as no inhibition of methane oxidation was obtained with carbon monoxide (Colby and Dalton, 1976; see Section III), which is known to potently inhibit cytochrome P-450 systems (Gunsalus, Meeks, Lipscomb, Debrunner and Münck, 1974).

On comparison of three characteristics (sensitivity to carbon monoxide, sensitivity to cyanide and involvement of cytochrome P-450) the four most studied n-alkane, bacterial mono-oxygenases, i.e. the enzymes from a Corynebacterium species, Pseudomonas oleovorans, Methylosinus trichosporium OB3b and Methylococcus capsulatus (Bath), appear to be different (Table 16). Although the

TABLE 16 Comparison of the characteristics of n-alkane oxidation systems from four hydrocarbon-utilising bacteria

	Involvement of cytochrome P-450	Inhibition by CO	Inhibition by cyanide
Corynebacterium sp. (Jurtshuk and Cardini, 1971)	+	+	–
Pseudomonas oleovorans (McKenna and Coon, 1970)	–	–	+
Methylosinus trichosporium OB3b (Tonge et al., 1977)	–	nd	+
Methylococcus capsulatus (Bath)	–	–	–

nd = not done

inhibition of methane oxidation by carbon monoxide for Methylosinus trichosporium OB3b has not been tested, it is most likely that such an inhibition would occur for one of two reasons. A CO-binding cytochrome is involved in the enzyme reaction and also CO is itself a substrate for the methane mono-oxygenase (Tonge et al., 1977) and would therefore probably competitively inhibit methane oxidation.

It is thought the n-alkane oxidation system of the Corynebacterium species will be found to be representative of the corynebacterium-mycobacterium-nocardia-streptomyces group of organisms and analogous to the hydrocarbon oxidation system of eukaryotic organisms, such as yeasts and moulds (Jurtshuk and Cardini, 1971).

Closer inspection of the available information revealed that the properties of the n-alkane oxidation systems in Pseudomonas oleovorans and Methylosinus trichosporium OB3b were even more fundamentally different to the system present in Methylococcus capsulatus (Bath). Methylosinus trichosporium OB3b appears to contain a terminal n-alkane mono-oxygenase (Thomson, 1974; Tonge et al., 1977) unlike Methylococcus capsulatus (Bath), and

on the basis of their vastly contrasting substrate, electron donor and inhibitor specificities, the two enzymes appear to be very different in their reaction mechanism and component composition. However, it is still possible that a CO-binding cytochrome c of the type involved with the mono-oxygenase of Methylosinus trichosporium OB3b is a component of the mono-oxygenase of Methylococcus capsulatus (Bath). Pseudomonas oleovorans has been shown to possess a terminal specific hydrocarbon mono-oxygenase as it is unable to sub-terminally oxidise n-alkanes or internal alkenes (McKenna and Coon, 1970; May and Abbott, 1972; May et al., 1975; Ruettinger et al., 1977).

SECTION V Oxidation of non-growth substrates by Methylococcus capsulatus (Bath)

1. Introduction

In 1960, Leadbetter and Foster reported that whole-cell suspensions of Methylomonas (Pseudomonas) methanica actively growing on methane could concomitantly oxidise ethane to acetic acid, n propane to propionic acid and propan-2-one, and butane to butanoic acid and butan-2-one (Leadbetter and Foster, 1960). This phenomenon, whereby a micro-organism oxidises a compound but cannot utilise any carbon or energy derived from the oxidation, was later described by Foster (1962) as 'co-oxidation'.

Jensen (1963) expanded the definition to include not only oxidation reactions but other reactions such as dehalogenations, etc. as well, but dropped the obligate requirement for a co-substrate. He coined the term 'cometabolism' to describe this amended definition, i.e. the phenomenon in which micro-organisms can metabolise a compound but are unable to utilise subsequent products for growth. However, due to the ambiguous use of both terms (co-oxidation and cometabolism) in the literature over the past few years, the terms have become virtually synonymous.

Numerous examples of co-oxidation and cometabolism have been published as well as many other reports which describe co-oxidative/cometabolic events, but the authors have failed to recognise the phenomenon as such or have chosen not to distinguish the event from 'ordinary' metabolic events. A review by Horvath (1972a) lists many examples of co-oxidation or cometabolism published up until 1972. Since then, the frequency of reports concerning the above phenomena has steadily increased, the main area of attention being the co-oxidation/cometabolism of pesticides, either by mixed soil cultures or pure cultures of micro-organisms. For example, it has been shown that the complete degradation of DDT (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane) can occur in vitro using pure and mixed culture combinations. DDT can be metabolised to a variety of products

(DDD, DDMS, DBP, DDMU and DDE), none of which are ring cleavage products, either by cell-free extracts of a Hydrogenomonas species anaerobically (Pfaender and Alexander, 1972) or by sewage flora aerobically (Pfaender and Alexander, 1973). Whole cells of the Hydrogenomonas species in the presence of oxygen cometabolically produced p-chlorophenylacetic acid (pCPA) from the initial products (Pfaender and Alexander, 1972), hence one of the two aromatic rings of DDT had been cleaved. pCPA can then be further degraded by an Arthrobacter species which could utilise pCPA as a sole source of carbon (Pfaender and Alexander, 1972). Therefore DDT was cometabolised to pCPA which could be subsequently used as a growth substrate by an Arthrobacter strain.

Although the biodegradation of DDT is possible in the laboratory, the validity of such a process in natural environments may be questionable considering the apparent persistence of DDT in nature. It has been suggested that the insecticide is only metabolised by a few micro-organisms, none of which can utilise the compound as a sole source of carbon (Pfaender and Alexander, 1972). Therefore it is just as likely that DDT persists because no organism, however active in the cometabolism of the compound, has any necessary selective advantage over the remaining microflora and so its population density would not increase in response to DDT applications.

Various other herbicides/pesticides have been reported to undergo cometabolism by micro-organisms: 2,3,6-trichlorobenzoate (Horvath, 1971; Horvath, 1972b), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Horvath, 1970), dieldrin (Matsumura, Boush and Tai, 1968), endrin (Patil, Matsumura and Boush, 1970), diazinon (Sethunathan and Pathak, 1971). In addition to the above compounds, other persistent organo-chemicals have been reported to undergo cometabolic attack by micro-organisms, e.g. halogenobenzoates (Horvath and Alexander, 1970; Spokes and Walker, 1974; Horvath, Dozlaf and Kreger, 1975; Horvath and Flathman, 1976), alkyl benzene sulphonate (Horvath and Koft, 1972), methylcatechols (Knackmuss, Hellwig, Lackner and Otting, 1976), substituted phenols (Engelhardt, Rast and Wallnöfer, 1977)

and coumarin (Kunc, 1974). The cometabolism/co-oxidation of drugs has proved very important, not only from the developmental approach but for the prediction of the metabolic fate of a drug in mammals (Smith and Rosazza, 1975; Smith, Acosta and Rosazza, 1977).

The argument concerning microbial infallibility versus the existence of molecules totally recalcitrant to microbial degradation (reviewed Alexander, 1965) still remains unresolved, and probably always will. The number of compounds previously considered recalcitrant to biodegradation is diminishing gradually with the development of cometabolism/co-oxidation studies in the laboratory, sustaining the belief, held by many biologists, in the biochemical omnipotence of natural microfloras. However, data concerning the persistence of many organochemicals under natural conditions (Edwards, 1973) show that many compounds reported to be biodegradable in the laboratory are very resistant to microbial attack. Therefore it seems that a form of environmental recalcitrance exists for some compounds.

Horvath (1972a) attempted to form a biochemical mechanism for cometabolic events from the various reasons suggested by workers to explain such events. Foster (1962) suggested that the phenomenon was a result of an organism's inability to assimilate the products of oxidation, but Horvath argued that this did not explain the incomplete oxidation of a substrate or the accumulation of end products. Hughes (1955) suggested that the phenomenon was due to an organism's inability to cleave the halogen substituent from the benzene ring, thereby preventing the progress of metabolism and carbon assimilation, but this does not account for the cometabolism/co-oxidation of alkyl or non-halogenated aromatic compounds. Tranter and Cain (1967) proposed that the accumulation of toxic products from halogenated compounds resulted in the inability of organisms to grow on such compounds; however, this did not account for the production of toxic

conditions due to the excessive accumulation of an oxidation product not normally toxic to the organism. Again, this explanation appeared to only consider halogenated compounds. Gibson et al. suggested that certain halogenated benzene derivatives caused chelation of enzymes involved in the further metabolism of these compounds, therefore preventing the complete metabolism of these substrates (Gibson, Koch, Schuld and Kallio, 1968). This explanation would not account for a large number of cometabolic/co-oxidative events, e.g. ethane oxidation by Methylomonas methanica (Leadbetter and Foster, 1960).

Horvath (1972a) suggested that m-chlorobenzoate cometabolism by an Arthrobacter species (Horvath and Alexander, 1970) and 2,3,6-trichlorobenzoate cometabolism by a Brevibacterium species (Horvath, 1971) resulted because of an initial oxidative attack by the same enzyme which catalyses the oxidation of the growth substrate, i.e. non-specific enzymic attack. The further metabolism of these compounds was then restricted by the specificity of the ring-cleavage enzyme.

This biochemical mechanism of cometabolic/co-oxidative events would seem the most plausible explanation of the phenomenon. The occurrence of a cometabolic/co-oxidative event will almost certainly arise due to the initial action of a non-specific enzyme, normally involved in growth substrate metabolism. The explanation of why these compounds cannot then support growth and replication of the organism will sometimes be, as Horvath (1972a) suggested, the result of a key enzyme of growth substrate metabolism being specific for a particular substrate/metabolite. However, other reasons for the inability of a metabolised compound to support growth and replication are possible. The metabolism of halogenated compounds could easily give rise to very toxic derivatives if the halogen is lost by enzymic or spontaneous cleavage (see the oxidation of bromomethane). Also, compounds could be oxidised to such a level that it is no longer energetically favourable for the organism to assimilate the compound (see the oxidation of carbon monoxide).

The whole concept of cometabolism/co-oxidation has recently received strong criticism (Hulbert and Krawiec, 1977). These authors suggested

that the above terms describe metabolic events which are easily encompassed by the existing terms for metabolism, anabolism and catabolism. Although some of their criticisms are trivial and often simply arguments of semantics, there does seem to be valid reasons for dropping the term cometabolism as it is presently defined. The metabolism of a compound which is unable to support cell replication in the absence of another transformable substrate does not involve a new metabolic event but is merely a reflection of the non-specific nature of the particular enzyme initiating the fortuitous metabolism of that compound, but for one reason or another the metabolism of the compound is incomplete, as discussed above. This type of incomplete metabolism easily conforms with the original definition of catabolism by Foster in 1888 (Hulbert and Krawiec, 1977) and essentially constitutes the partial fulfilment of a normal catabolic pathway.

Co-oxidation as originally defined by Foster (1962) does describe an unusual metabolic phenomenon, although the actual enzymic route of any particular co-oxidative event still operates within the confines of the normal anabolic and catabolic pathways of the organism involved. However, it is felt that such phenomena are sufficiently interesting and unusual metabolic events to merit a term to describe them. It is proposed that the term cometabolism be redefined as below:

the transformation of a compound, which is unable to support cell replication, in the requisite presence of another transformable compound (co-substrate) Def. 1.

This definition remains true to the original definition of co-oxidation by Foster (1962), but encompasses other reactions as well as oxidations and extends the range of co-substrates to include compounds which cannot support cell replication as well as growth substrates.

It is also suggested that the term 'non-growth substrate' is used to describe compounds which do not support cell replication, i.e. cannot act as a sole source of carbon for the cell. It is possible that such compounds could be assimilated into the cellular biosynthetic pathways but remain unable to support cell replication.

The term co-oxidation could still be used within the confines of cometabolism as a sub-type concerning purely oxidation reactions, but it is suggested that this term should be phased out to avoid confusion. The transformation of non-growth substrates in the absence of a co-substrate should simply be referred to as 'fortuitous' oxidations, dehalogenations, etc.

Examples of cometabolism, as redefined above, by methane-oxidising bacteria, are shown in Table 17. The oxidations reported by Leadbetter and Foster (1960) with Methylomonas methanica required methane as co-substrate, whereas the other three examples all required the presence of a primary alcohol. Numerous examples of fortuitous oxidations of non-growth substrates by methane-oxidising bacteria have been reported, and examples are shown in Table 18.

An interesting aspect of these cometabolic and fortuitous oxidative events, as yet unexplained, is the evaluation of the benefit, if any, which an organism receives from such metabolic activities. It is possible that although a substrate is unable to support cell replication it may contribute to the economy of the cell by producing energy and/or reducing power in some form. In some cases the substrate could possibly contribute assimilable carbon but still be unable to sustain cell replication. Ethanol and formate, for example, have been shown to be suitable electron donors and energy sources for nitrogenase activity in cell suspensions of Methylococcus capsulatus (Bath) (Dalton, and Whittenbury, 1976a) but both compounds cannot support cell replication.

With these possibilities in mind, all the compounds which were apparently oxidised by the cell-free methane mono-oxygenase preparations of Methylococcus capsulatus (Bath) in Section IV were tested for oxidation by whole-cell suspensions of the same organism. Those found to be oxidised in the absence of a co-substrate were tested to see if they could support cell replication, the remaining compounds were assayed for cometabolic potential.

TABLE 17 Examples of cometabolism by methane-oxidising bacteria

Organism	Cometabolic substrate	Reference
Methylomonas methanica	Ethane n-Propane n-Butane	Leadbetter and Foster (1960)
Methylomonas agile	Ethane	Whittenbury et al. (1970a)
Methylomonas methanica	Carbon monoxide	Ferenci (1974)
Methylomonas agile	Carbon monoxide	Hubley (1975)

TABLE 18 Examples of fortuitous oxidation of non-growth substrates by methane-oxidising bacteria

Organism	Non-growth substrate	Reference
Methylomonas methanica	Bromomethane	Colby et al., (1975)
Methylococcus capsulatus (Bath)	Bromomethane	Colby and Dalton (1976)
Methylosinus trichosporium OB3b	Ethane	Thomson (1974)
Methylomonas agile A30		
Methylomonas albus BG8		
Methylomonas agile A30		
Methylocystis parvus OBBP		
Methylosinus trichosporium OB3b	Carbon monoxide	Ferenci (1974); Hubley et al. (1974)
Methylomonas albus BG8	Carbon monoxide	Hubley et al. (1974)
Methylococcus capsulatus (Texas)	Ethanol	Patel and Hoare (1971)
Methylococcus capsulatus (Texas)	Formate	Patel and Hoare (1971)
Various un-named methane-oxidising organisms	Dimethyl ether	Hazeu (1975)

2. Oxidation of Various Carbon Compounds by Whole-cell Suspensions of Methylococcus capsulatus (Bath)

All the compounds which were oxidised by the soluble methane mono-oxygenase preparations of Methylococcus capsulatus (Bath) in Section IV were tested for oxidation by whole-cell suspensions of the same organism. The results are shown in Table 19, with the oxidation rates calculated either from the amount of substrate consumed or from the amount of product formed. Only five of the extensive list of compounds (chloromethane, bromomethane, dimethyl ether, ethene and propene) previously oxidised by cell-free extracts of M. capsulatus (Bath) appeared to be oxidised by the methane mono-oxygenase in vivo. This may not be as surprising as first appears when one considers that the initial oxidation by the methane mono-oxygenase requires one molecule of NADH or reducing equivalent per molecule of substrate oxidised and as many compounds are probably not further oxidised, preventing the regeneration of reducing power, no oxidation will occur. Other possible reasons for negative results could be the formation of oxidation products toxic to the cell or the substrate itself being toxic to the cell, also some compounds may not be able to enter the cell.

None of the compounds, tested at various concentrations (1 mM, 5 mM, 10 mM and 50 mM), would support growth of M. capsulatus (Bath) when incubated at 45^{o} C for 10 days. Therefore, these five compounds appear to be fortuitously oxidised, non-growth substrates as defined in the introduction to this Section.

The same range of compounds were again tested for oxidation by whole-cell suspensions of M. capsulatus (Bath), but this time in the presence of 4 mM formaldehyde. The formaldehyde was included in an attempt to generate reducing power which could be used for methane mono-oxygenase activity. Therefore any potential substrate which was not oxidised in the previous assays (Table 19) due to its inability to regenerate reducing power would now be oxidised. The results are listed in Table 20 and show that seven previously unoxidised compounds

TABLE 19 Oxidation of Various Carbon Compounds by Whole-cell Suspensions of *Methylococcus capsulatus* (Bath)

Compound	Oxidation rate: µmol substrate consumed min^{-1} (mg dry weight cells)$^{-1}$	Oxidation rate: µmol product formed min^{-1} (mg dry weight cells)$^{-1}$
Chloromethane	0.17	–
Bromomethane	0.088	–
Iodomethane	0	–
Dichloromethane	0	–
Trichloromethane	0	–
Tetrachloromethane	0	–
Cyanomethane	0	–
Nitromethane	0	–
Methanethiol	0	–
Trimethylamine	0	–
Carbon monoxide	–	0
Dimethylether	0.007	–
Diethylether	0	–
Ethane	–	0
n-Propane	–	0
n-Butane	–	0
n-Pentane	–	0
n-Hexane	–	0
n-Heptane	–	0
n-Octane	–	0
Ethene	–	0.004 (epoxyethane)
Propene	–	0.006 (1,2-epoxypropane)
But-1-ene	–	0
cis But-2-ene	–	0
trans But-2-ene	–	0
Cyclohexane	–	0
Benzene	–	0
Toluene	–	0
Styrene	–	0
Pyridine	0	–
L-phenylalanine	–	0

TABLE 20 Oxidation of Various Carbon Compounds by Whole-cell Suspensions of Methylococcus capsulatus (Bath) in the Presence of 4 mM Formaldehyde

Compound	Oxidation rate: μmol substrate consumed min^{-1} (mg dry weight cells)$^{-1}$	Oxidation rate: μmol product formed min^{-1} (mg dry weight cells)$^{-1}$
Chloromethane	0.170	–
Bromomethane	0.088	–
Iodomethane	0	–
Dichloromethane	0	–
Trichloromethane	0	–
Tetrachloromethane	0	–
Cyanomethane	0	–
Nitromethane	0	–
Trimethylamine	0	–
Carbon monoxide	–	0.520 (carbon dioxide)
Dimethyl ether	0.125	–
Diethyl ether	0.016	–
Ethane	–	0.025 (ethanal)
Propane	–	0.004 (propan-1-al)
Butane	–	0
n-Pentane	–	0
n-Hexane	–	0
n-Heptane	–	0
n-Octane	–	0
Ethene	–	0.022 (epoxyethane)
Propene	–	0.022 (1,2-epoxypropane)
But-1-ene	–	0.023 (1,2-epoxybutane)
cis But-2-ene	–	0.014 (cis-2,3-epoxybutane)
		0.013 (cis-2-buten-1-ol)
trans But-2-ene	–	0.017 (trans-2,3-epoxybutane)
		0.035 (trans-2-buten-1-ol)
Cyclohexane	–	0
Benzene	–	0
Toluene	–	0
Styrene	–	0
Pyridine	0	–
L-Phenylalanine	–	0

(carbon monoxide, diethyl ether, ethane, propane, but-1-ene, cis but-2-ene, trans but-2-ene) were oxidised in the presence of exogenous formaldehyde. These seven compounds cannot support growth of M. capsulatus (Bath) and require the presence of a co-substrate for oxidation, therefore they can be described as cometabolic substrates as redefined (Def. 1) in the introduction to this Section.

Of the five compounds which were oxidised in the absence of formaldehyde, chloromethane and bromomethane gave identical oxidation rates in the presence of formaldehyde, whereas dimethyl ether, ethene and propene were oxidised approximately three, five and four times more rapidly, respectively, in the presence of formaldehyde.

This suggests that in the absence of an exogenous supplier of reducing power, the oxidation rates of dimethyl ether, ethene and propene were limited by the poor regeneration of reducing power from the further oxidation of these compounds. The inability of formaldehyde to stimulate the oxidation rates of either chloromethane or bromomethane suggests that the supply of reducing power is not the rate-determining factor in these cases.

From the twelve oxidisable compounds listed in Table 20, three compounds, dimethyl ether, bromomethane and carbon monoxide, were chosen for more detailed study in an attempt to elucidate their true role in the cellular metabolism of M. capsulatus (Bath).

3. Oxidation of Dimethyl Ether

A. Historical background

The ability of methane-oxidising bacteria to oxidise and utilise dimethyl ether as a sole source of carbon and energy was first reported by Wilkinson (1971). In his review article he refers to a a paper 'in press' by Bryan-Jones and Wilkinson for the results concerning dimethyl ether oxidation, however to the best of my

knowledge this communication has never appeared in any scientific journal. Since then, the oxidation of dimethyl ether by methane-oxidising bacteria has only been positively reported on a few occasions (Davey, 1971; Ribbons, 1975; Hazeu, 1975; Patel et al., 1976), although a number of reviews and papers refer to the remarks of Wilkinson (1971; 1975) as evidence of dimethyl ether oxidation.

The unpublished observations of Bryan-Jones and Wilkinson (Wilkinson, 1971) that dimethyl ether could act as a growth substrate for various methane-oxidising bacteria and can be oxidised by whole-cell suspensions of these organisms, led to the suggestion that instead of methane being directly oxidised to methanol , the initial product formed is dimethyl ether, according to the following reaction:

$$2CH_4 + O_2 \longrightarrow CH_3OCH_3 + H_2O \qquad (12)$$

Other unpublished results by Mitton involving $^{18}O_2$ incorporation were reported as showing that dimethyl ether was oxidised to methyl formate by a carbon monoxide-sensitive oxygenase (Hubley, 1975; Wilkinson, 1975). It was also shown that methyl formate could be hydrolysed to formate and methanol by means of an esterase. Davey (1971) on the strength of these observations proposed a pathway of methane oxidation as shown in Fig. 10(a).

However, subsequent to the proposal of the above scheme, certain conflicting evidence arose. If ethane was oxidised by the same enzyme as methane as thought, and dimethyl ether was indeed an intermediate of methane oxidation, it would be expected that the oxidation of diethyl ether would produce a common product to ethane oxidation. Unfortunately, Thomson (1974) showed that diethyl ether was oxidised by whole-cell suspensions of Methylosinus trichosporium OB3b and Methylomonas albus BG8 via 2-ethoxyethanol and 2-ethoxyacetaldehyde to 2-ethoxyacetic acid. Also work by Mitton and co-workers 'submitted for publication' (Wilkinson, 1975)

and 'in preparation' (Hubley, 1975), was reported as showing that no ^{14}C-incorporation into methyl formate was obtained with whole-cell suspensions of Methylosinus trichosporium OB3b and Methylomonas albus BG8 incubated with $^{14}CH_4$, although $^{14}CH_3OH$ was readily detectable (Hubley, 1975; Wilkinson, 1975).

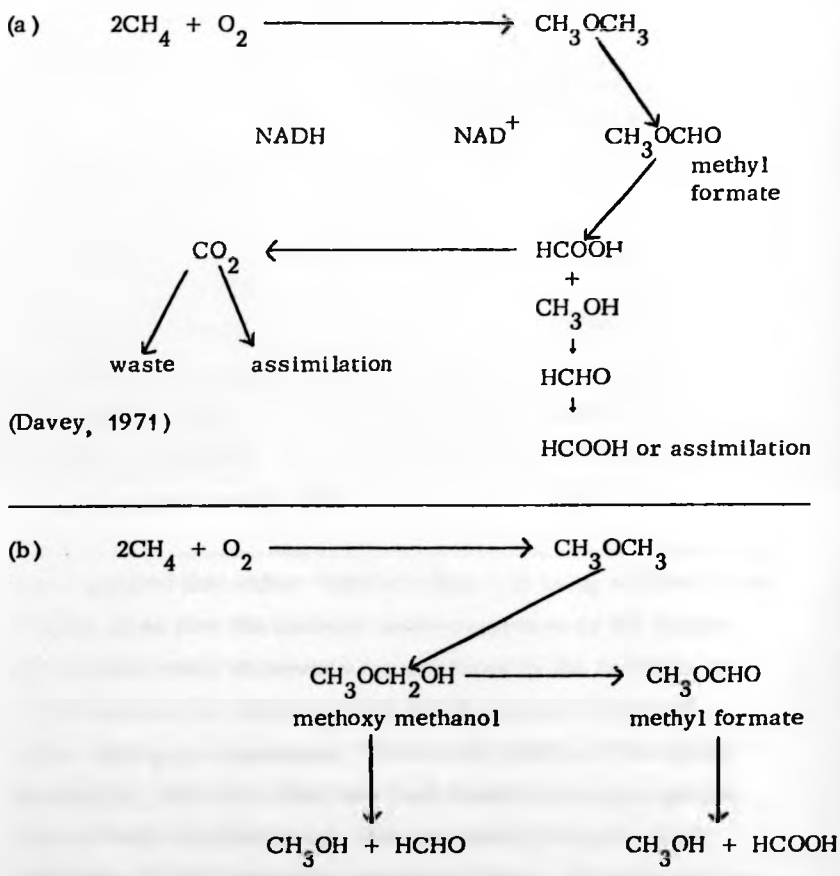
To incorporate these findings and still retain a role for dimethyl ether as an intermediate in methane oxidation, a new scheme (Fig. 10(b)) was suggested in which the initial product of dimethyl ether oxidation was the unstable hemi-acetal, methoxymethanol. This could either spontaneously decompose to give formaldehyde and methanol or form methyl formate (Thomson, 1974; Hubley, 1975). Obviously the route resulting in the production of methanol and formaldehyde is more attractive, as the route via methyl formate would require either the fixation of carbon dioxide arising from formate oxidation or the reduction back to formaldehyde to obtain cellular yields above 50% (Wilkinson, 1975).

Even with this revised, hypothetical pathway there is no direct evidence for the involvement of dimethyl ether as an intermediate in methane oxidation. This, combined with the fact that the two most studied, cell-free methane mono-oxygenase systems, i.e. Methylosinus trichosporium (Tonge et al., 1975; Tonge et al., 1977) and Methylococcus capsulatus (Bath) (Colby and Dalton, 1976), have indicated that methanol is the direct product of methane oxidation, means that the possibility of dimethyl ether being a normal intermediate in methane oxidation is very remote. Even though dimethyl ether may eventually be conclusively shown not to be involved in the oxidation of methane, it remains an interesting compound with regard to its fortuitous oxidation.

B. Whole-cell studies

The respiration rates observed in an oxygen electrode with whole-cell suspensions of M. capsulatus (Bath) using dimethyl ether as substrate are shown in Table 21. Very rapid oxygen

Fig. 10 Pathways of methane oxidation with dimethyl ether as an intermediate



consumption rates were obtained with high concentrations of dimethyl ether, even in the presence of acetylene and 8-hydroxyquinoline. Only 30% inhibition was caused by acetylene at a final concentration of 5 mM, which was five times the concentration required to completely inhibit methane oxidation. As acetylene and and 8-hydroxyquinoline were shown to be potent and apparently specific inhibitors of the methane mono-oxygenase (see Section III),

TABLE 21 Dimethyl ether (CH_3OCH_3) respiration studies using whole-cell suspensions of M. capsulatus (Bath)

Substrates (final conc.)	Oxidation rate (nmol oxygen consumed min^{-1} (mg dry weight cells)$^{-1}$)
CH_3OCH_3 (100 mM)	473
CH_3OCH_3 (3 mM)	0
CH_2OCH_3 (0.1 mM)	0
CH_3OCH_3 (100 mM) + acetylene (1.4 mM)	468
CH_3OCH_3 (100 mM) + acetylene (5 mM)	334
CH_3OCH_3 (100 mM) + 8-hydroxyquinoline (1 mM)	405

this suggested that either dimethyl ether was being oxidised by an enzyme other than the methane mono-oxygenase or the oxygen consumption rates observed were produced by the oxidation of a contaminant in the dimethyl ether which accumulated in the buffer during gas saturation. To test the validity of the latter explanation, dimethyl ether was first bubbled through a gas jar half-full with distilled water, then rebubbled through a flask containing 20 mM potassium phosphate buffer. It was hoped that the water in the gas jar would 'scrub' out any contaminating compound in the gas and consequently allow the production of a contaminant-free, saturated dimethyl ether buffer solution. The oxygen consumption rates obtained with whole-cell suspensions of M. capsulatus (Bath) using the new dimethyl ether saturated buffer were as follows: 100 mM (final concentration) dimethyl ether - 8 nmol oxygen consumed min (mg dry weight cells)$^{-1}$; 1 mM (final concentration) dimethyl ether - 0 nmol oxygen consumed min^{-1} (mg dry weight cells)$^{-1}$. Therefore it appeared that results shown in Table 21 could be due to the oxidation of a contaminant instead of dimethyl ether. It was thought that a likely contaminant would be methanol and the results in Table 21 would be as expected if this was the case.

An attempt to identify this possible contaminant from the original saturated buffer solution was done, using the gas chromatograph system described in the Methods. Unfortunately the concentration of saturated dimethyl ether (approx. 1.5 M) produced a large peak of such a size and retention time that it would obscure any smaller peaks from compounds of similar retention times, e.g. methanol. Hence different chromatography column packings to those used would be required to differentiate between dimethyl ether and the probable contaminant by gas chromatography. When dimethyl ether-saturated buffer was tested as a potential substrate for methanol dehydrogenase in crude, soluble extracts of M. capsulatus (Bath), using the optimal conditions for the enzyme (Anthony and Zatman, 1964b), a rapid reduction of dichlorophenol-indophenol was observed. This supports the conclusion that a primary alcohol, most likely methanol, was contaminating the dimethyl ether-saturated buffer.

With the probability of a contaminant creating erroneous results during respiration studies, it was decided to monitor dimethyl ether oxidation by following dimethyl ether disappearance by gas chromatography, as described for the substrate specificity assays of Section IV. This method would give a more accurate appraisal of dimethyl ether oxidation by whole-cell suspensions as it directly monitors carbon substrate disappearance. The results of these assays are shown in Table 22, and are all corrected for endogenous rates.

From the first result it appears that dimethyl ether was very poorly oxidised by whole-cell suspensions of M. capsulatus (Bath). Increasing the concentration of dimethyl ether in the assay had very little effect on the disappearance rate. Lowering the concentration caused a decline in the observed disappearance rate. Bearing in mind the rapid oxidation rate obtained with cell-free extracts of M. capsulatus (Bath) (Section IV), it was thought that the apparantly poor oxidation rate by whole cells was due to either

the inability of the substrate to enter the cell or the further oxidation (if any occurred) not regenerating the reducing power required for the initial oxidative attack by the methane mono-oxygenase. The other results contained in Table 22 show that marked increases, up to elevenfold, in dimethyl ether disappearance rates were obtained in the presence of co-substrate which could generate reducing power. These results suggested that dimethyl ether oxidation in whole cells of M. capsulatus (Bath) did not regenerate the requisite reducing power for methane mono-oxygenase activity. However, the possibility cannot be excluded of some active transport mechanism for the entry of dimethyl ether into the cell which requires reducing power or energy, which is not regenerated by dimethyl ether oxidation.

TABLE 22 Rates of dimethyl ether disappearance with whole-cell suspensions of M. capsulatus (Bath)

Substrate(s)	Rates of dimethyl ether disappearance (nmol CH_3OCH_3 min^{-1} (mg dry weight cells)$^{-1}$)
CH_3OCH_3	19
$CH_3OCH_3 + CH_3OH$	175
$CH_3OCH_3 + HCHO$	125
$CH_3OCH_3 + KCOOH$	223

Reaction mixture: (final vol. 1 ml)	20 µmol potassium phosphate 0.53 mg dry weight cells 10 µmol dimethyl ether
(where present)	4 µmol methanol 4 µmol formaldehyde 4 µmol potassium formate

C. Conclusions

Dimethyl ether was rapidly oxidised by cell-free extracts of M. capsulatus (Bath) with the initial oxidation apparently catalysed by the methane mono-oxygenase (see Section IV). Whole-cell suspensions of M. capsulatus (Bath) oxidised dimethyl ether very poorly and this appeared to be due to the inability of the cells to regenerate reducing power required for methane mono-oxygenase activity during dimethyl ether oxidation. M. capsulatus (Bath) could not grow on dimethyl ether when supplied as sole carbon and energy source and this combined with the above information confirms that dimethyl ether is a non-growth substrate which can be fortuitously oxidised, but whether any carbon assimilation can occur from the oxidation of dimethyl ether requires incorporation studies using ^{14}C dimethyl ether.

The erroneous results obtained for dimethyl ether oxidation when assayed by oxygen consumption provided a possible doubt about the validity of the three other reports of dimethyl ether oxidation by Davey (1971), Ribbons (1975) and Patel et al., (1976) who all monitored dimethyl ether oxidation by oxygen consumption. None of these workers referred to the purity of gas used or on the possibility of contamination. It is therefore suggested that all future oxidation studies involving dimethyl ether should, where possible, measure either dimethyl ether diappearance or the formation of products, if detectable, and thereby negate the nced to monitor oxygen consumption.

4. Oxidation of Bromomethane

Crude cell-free extracts of two organisms, Methylomonas methanica and Methylococcus capsulatus (Bath), have been reported capable of catalysing the disappearance of bromomethane (Colby et al., 1975; Colby and Dalton, 1976). In both instances bromomethane oxidation was thought to be catalysed by the methane mono-oxygenase. The results of Section IV confirmed that the

oxidation of bromomethane as observed in the cell-free extract studies with Methylococcus capsulatus (Bath) by Colby and Dalton (1976) was almost certainly due to the action of the methane mono-oxygenase.

Whole-cell suspensions of M. capsulatus (Bath) can also catalyse bromomethane disappearance (Table 19), the rate of which was not increased by the presence of formaldehyde (Table 20). Whole-cell suspensions of M. capsulatus (Bath) also gave an oxygen consumption rate of 48 nmol min^{-1} (mg dry weight cells)$^{-1}$ on the addition of bromomethane (Table 24).

The results of attempts to grow M. capsulatus (Bath) on bromomethane as sole source of carbon and energy were negative, therefore in conjunction with the results of the above whole-cell studies suggests that bromomethane is a non-growth substrate which is fortuitously oxidised. Nevertheless, preliminary evidence suggests that carbon from bromomethane can, to a limited extent, be assimilated into cellular material.

It was found that cell-free extracts of Methylococcus capsulatus (Bath) containing good methane mono-oxygenase activity, when incubated at 45° C in the presence of bromomethane and NADH produced carbon dioxide at twice the rate found with a similar assay minus bromomethane (Table 26). The increased production of CO_2 appeared to be the result of bromomethane oxidation as virtually no increases in CO_2 production over the control values were obtained in the absence of NADH or oxygen or in the presence of acetylene (all indicators of methane mono-oxygenase involvement). This suggested that bromomethane was initially oxidised by the methane mono-oxygenase to some, as yet unknown, intermediary product which was enzymically broken down, spontaneously decayed, or a combination of both, to eventually produce carbon dioxide. The inhibition by potassium cyanide supports the involvement of at least one cyanide sensitive enzyme, such as formate dehydrogenase or methanol oxidase, both active in

soluble, cell-free extracts of M. capsulatus (Bath). Therefore, it is possible that during the complete oxidation of bromomethane by cell-free extracts of M. capsulatus (Bath), formaldehyde is produced. From this indirect evidence it is conceivable that carbon assimilation, via formaldehyde, could occur during bromomethane oxidation by whole cells of M. capsulatus (Bath).

Further circumstantial evidence supporting this hypothesis was obtained by incubating partially purified methane mono-oxygenase from M. capsulatus (Bath) with bromomethane and NADH, then subsequently assaying the reaction mixture for formaldehyde. Methane mono-oxygenase activity was resolved into two fractions by passage through a column of DEAE-cellulose (Colby and Dalton, 1976). These two component fractions were incubated with 8 μmol bromomethane, 10 μmol NADH and 1 μmol potassium cyanide. After 10 minutes, only 0.25 μmol of formaldehyde was found present in the reaction mixture. However, this may not have been a true reflection of the amount of formaldehyde produced during the incubation period as the partially purified methane mono-oxygenase preparation was found to contain cyanide insensitive formaldehyde dehydrogenase activity. Nevertheless it appears that formaldehyde could be an intermediate in bromomethane oxidation. The production of any formaldehyde in the presence of potassium cyanide suggests that if bromomethanol is the initial product of bromomethane oxidation, then it must dismutate to give formaldehyde rather than be enzymically oxidised, as any methanol oxidase activity would be inhibited by the cyanide.

Preliminary direct evidence has been obtained by qualitatively measuring ^{14}C incorporation from ^{14}C bromomethane. A resting cell suspension of M. capsulatus (Bath) was incubated at 45° C in a sealed flask, into which a small quantity (50 μmol/0.1 m Ci) of ^{14}C bromomethane was released. Samples were taken at various time intervals from zero time to 30 minutes and injected straight

into ethanol (98%, v/v) to immediately stop any cellular metabolism. Soluble extracts were then prepared from each extract and then subjected to 2-dimensional paper chromatography and autoradiography as outlined by Reed (1976). On development of the autoradiograms, black spots corresponding to the radioactive spots on the chromatograms were observed. Although these spots were not properly identified and the experiment requires repeating quantitatively, these preliminary results suggest that carbon from bromomethane can in fact be incorporated into the cellular biosynthetic pathways.

If carbon assimilation can occur as a result of bromomethane oxidation, then this substrate can be clearly defined as a non-growth substrate, the fortuitous oxidation of which can provide assimilable carbon. The question arises, why can carbon from such a substrate be assimilated but no subsequent cell replication occur ? One possible answer for bromomethane would be the inhibition of growth caused by toxic bromine residues produced in the cell during the oxidation of bromomethane.

5. Oxidation of Carbon Monoxide

A. Reports of carbon monoxide (CO) oxidation by bacteria

There are very few reports of bacteria oxidising carbon monoxide. The first irrefutable proof of CO oxidation was obtained by Kistner (1953; 1954) with a hydrogen-utilising bacterium. Since then CO oxidation by various types of organisms has been reported and they can be split into two categories ; bacteria which can utilise CO as a sole carbon source and may also utilise hydrogen (Kistner, 1953; Kistner, 1954; Nozhevnikova and Savelieva, 1972; Savelieva and Nozhevnikova, 1972; Zavarzin and Nozhevnikova, 1976; Meyer and Schlegel, 1977; Romanova and Nozhevnikova, 1977) and a much larger group of organisms which cannot utilise CO as a sole carbon source but can oxidise CO to CO_2, which could possibly be incorporated into cell material. This latter category includes photosynthetic bacteria (Hirsch, 1968), Desulfovibrio (Yagi and Tamiya, 1962; Postgate, 1970),

Clostridium (Fuchs, Schnitker and Thauer, 1974), methanogenic bacteria (Kluyver and Schnellen, 1947) and methane-oxidising bacteria (Hubley et al., 1974; Ferenci, 1974; Ferenci et al., 1975; Hubley, 1975; Tonge et al., 1977).

B. Reported evidence of CO oxidation by methane-utilising bacteria

Ferenci (1974) first reported CO-stimulated respiration in whole-cell suspensionsof Methylosinus trichosporium OB3b and whole-cell suspensions plus cell-free extracts of Methylomonas methanica. However, as he was monitoring respiration by measuring oxygen consumption and carbon dioxide production polarographically, plus NADH oxidation spectrophotometrically, he had no direct evidence of CO oxidation to CO_2. The increased respiration rates in the presence of CO could have been due to CO stimulation of endogenous CO_2 production. The first direct evidence for CO oxidation to CO_2 was reported by Hubley et al. (1974) who showed that ^{14}C-labelled CO when incubated in the presence of cell suspensions of either Methylosinus trichosporium OB3b or Methylomonas albus BG8 gave rise to ^{14}C-labelled CO_2. The likelihood of CO stimulating endogenous CO_2 production was reduced further after Hubley (1975) showed that ^{14}C-labelled cell suspensions of Methylomonas albus BG8 and Methylosinus trichosporium OB3b, prepared by growing the cells on ^{14}C-labelled methane, did not show increased ^{14}C-labelled CO_2 production in the presence of 'cold' CO, even though CO_2 production in total had increased significantly.

Mono-oxygenase involvement with CO oxidation has been implicated in a number of reports (Ferenci, 1974; Ferenci et al., 1975; Hubley, 1975; Tonge et al., 1977). Ferenci (1974) reported that whole-cell suspensions of Methylomonas methanica oxidised CO with a 1:1 molar ratio between presumed CO disappearance and oxygen consumed. The same author also reported an approximate 1:1:1:1 molar ratio for CO added, CO_2 produced, O_2 consumed and NADH oxidised, when following oxidation by cell-free extracts of Methylomonas methanica

(Ferenci et al., 1975). In both situations the author assumed that the endogenous respiration rate observed prior to CO addition was maintained during CO oxidation, suggesting the following mono-oxygenase reaction:

$$CO + O_2 + NADH + H^+ \longrightarrow CO_2 + NAD^+ + H_2O \qquad (13)$$

Hubley (1975) did similar oxygen electrode stoichiometric examinations with whole-cell suspensions of Methylosinus trichosporium OB3b and Methylomonas albus BG8 and calculated values for the stoichiometry of CO added:O_2 consumed as between 1.0 and 1.2, when the endogenous oxygen consumption rates after CO addition were assumed to be completely inhibited. When the values were adjusted on the assumption that after the addition of CO the endogenous respiration rate observed prior to CO addition was still operative, then values of between 0.5 and 0.7 were obtained. This latter range of values was probably the more correct because, as previously mentioned, Hubley (1975) has shown that ^{14}C-labelled cell suspensions of both Methylosinus trichosporium OB3b and Methylomonas albus BG8 maintained a constant rate of $^{14}CO_2$ production upon the addition of 'cold' CO.

Therefore the whole-cell studies of Hubley (1975) appear to contradict those of Ferenci (1974), but on close inspection of the data presented by Ferenci, it transpires that he calculated his CO-saturated buffer additions to the oxygen electrode of 0.2 and 0.1 ml to contain 86 and 43 nmol CO respectively, when in fact these amounts correspond to 172 and 86 nmol of dissolved CO respectively. The correct stoichiometry ratios between CO added and O_2 consumed should therefore be 0.63:1 and 0.5:1 and not 1.2:1 and 1.1:1 as reported.

A possible explanation why the above whole-cell suspensions CO-oxidation results do not appear consistent with mono-oxygenase involvement is that, on addition of the CO-saturated buffer to the oxygen electrode, CO is oxidised at a maximal rate until the concentration falls towards the K_m value for CO, whereupon the respiration rate decreases rapidly. Therefore a percentage of the added CO remains

unoxidised in the electrode after the apparent cessation of CO oxidation as observed from the recorder trace (see Ferenci, 1974) and as Ferenci (1974) and Hubley (1975) have both assumed that the degeneration of the linear rate of oxygen consumption subsequent to the addition of CO represented the complete oxidation of all the CO added, then it is likely that their respective stoichiometry values were underestimated.

Although the above whole-cell studies are not consistent with a mono-oxygenase catalysed CO oxidation, the cell-free extract studies of Ferenci et al. (1975) support a mono-oxygenase involvement.

Further evidence for a mono-oxygenase involvement in CO oxidation was provided by Hubley (1975) who showed that CO oxidation by whole-cell suspensions of Methylosinus trichosporium OB3b required gaseous oxygen by means of $^{18}O_2$ tracer studies. Ferenci (1974) and Hubley (1975) showed that whole-cell suspensions of Methylomonas methanica and Methylomonas agile respectively required the presence of a co-substrate in order to achieve CO oxidation. These co-substrates, e.g. formaldehyde, formate, were all capable of generating reducing power intracellularly, suggesting the requirement of NADH or reducing equivalent for CO oxidation by these organisms and consequently implicating mono-oxygenase involvement in CO oxidation. This requirement for reducing power for CO oxidation was substantiated by the reports showing NADH or reducing equivalent requirement for CO oxidation by cell-free extracts of various methane-oxidisers (Ferenci, 1974; Ferenci et al., 1975; Tonge et al., 1977; see Section IV).

The first direct evidence for CO oxidation being catalysed by a mono-oxygenase was reported by Tonge et al. (1977) who purified the methane mono-oxygenase from Methylosinus trichosporium OB3b and found that the purified enzyme catalysed CO oxidation. This suggested that CO oxidation by other methane-utilising bacteria could also be due to the presence of a non-specific methane mono-oxygenase. Evidence

for the presence of a mono-oxygenase in methane-utilising bacteria that can oxidise both methane and carbon monoxide is summarised in Table 23. The evidence as yet is very circumstantial, but the possibility of a common mono-oxygenase for methane and carbon monoxide oxidation (as found in Methylosinus trichosporium OB3b (Tonge et al., 1977))being found in methane-utilising bacteria generally, cannot be excluded.

C. Oxidation of CO by Methylococcus capsulatus (Bath)

(i) Whole-cell studies.- Respiration studies were done in an oxygen electrode using whole-cell suspensions of M. capsulatus (Bath). When 0.3 ml or 0.4 ml of CO (commercial purity)-saturated buffer was added to the electrode containing an equilibrated whole-cell suspension (A_{540} of 2),oxygen consumption rates of 293 and 332 nmol min^{-1} (mg dry weight cells)$^{-1}$ were obtained respectively. Similar CO oxidation assays were done in the presence of 1.2 µmol acetylene and approximately 20% inhibition of the oxygen consumption rates observed previously was observed. 8-Hydroxyquinoline caused little inhibition (30%) also. It was expected that, if carbon monoxide was oxidised by resting-cell suspensions of M. capsulatus (Bath), the oxidation would be possibly catalysed by the methane mono-oxygenase. Consequently, it was expected that extensive inhibition of CO oxidation would have occurred in the presence of acetylene and 8-hydroxy-quinoline, previously shown to be potent, specific inhibitors of methane oxidation in M. capsulatus (Bath) (see Section III). Therefore to eradicate the possibility of a contaminant in the cylinder of CO (commercial purity) causing erroneous results, as with dimethyl-ether oxidation, CO-saturated buffer prepared from research grade CO was used in similar respiration tests to those above. The results obtained are shown in Table 24. It was evident that in fact CO was not oxidised by resting-cell suspensions of M. capsulatus (Bath). No oxygen consumption rate in addition to the endogenous rate was obtained over a range of CO final concentrations, i.e. 0.03 - 0.3 mM.

TABLE 23

Evidence for a common mono-oxygenase catalysing methane and carbon monoxide oxidation in methane-utilising bacteria

Studies with cell suspensions		Organism* (reference)
(1)	Similar specific inhibitor patterns	PM (Ferenci et al., 1975) OB3b (Hubley et al., 1975; Hubley, 1975)
(2)	Similar K_m values for oxygen	PM (Ferenci, 1976)
(3)	Possible competitive inhibition of CH_4 oxidation by CO	PM (Ferenci, 1974) TRMC (Patel et al., 1976) OB3b (Patel et al., 1976)
Studies with cell-free extracts		
(4)	Similar stoichiometries	PM (Ferenci, 1974; Ferenci, et al., 1975)
(5)	Similar enzyme stabilities	PM (Ferenci et al., 1975)
(6)	Similar pH optima	PM (Ferenci et al., 1975)
(7)	Similar inhibitor patterns	PM (Ferenci et al., 1975)
(8)	Oxidation of CO by purified CH_4 mono-oxygenase	OB3b (Tonge et al., 1977)

* Organisms:

PM - Methylomonas methanica

OB3b - Methylosinus trichosporium OB3b

TRMC - Methylococcus capsulatus (Texas)

TABLE 24

Rates of oxygen uptake by cell suspensions of Methylococcus capsulatus (Bath)

Substrates	Oxygen uptake rate [nmol O_2 min^{-1} (mg dry weight cells)$^{-1}$]
CH_4	463
$CH_4 + C_2H_2$	0
CH_3Br	48
$CH_3Br + C_2H_2$	0
CO	0
CH_3OH	312
$CH_3OH + CO$	604
$CH_3OH + C_2H_2$	312
$CH_3OH + CO + C_2H_2$	310
HCHO	228
HCHO + CO	520
$HCHO + C_2H_2$	228
$HCHO + CO + C_2H_2$	227
HCOOK	106
HCOOK + CO	212
$HCOOK + C_2H_2$	106
$HCOOK + CO + C_2H_2$	106
CH_3CH_2OH	155
$CH_3CH_2OH + CO$	157
CH_3CHO	35
$CH_3CHO + CO$	35
CH_3COONa	13
$CH_3COONa + CO$	13

Reaction vessel: 60 µmol phosphate
(Final vol. 3 ml) Whole cells, 0.825 mg DCW

(Where present) CH_4 (0.12 µmol), CH_3Br (2 µmol), CO (0.16 µmol), C_2H_2 (1.2 µmol), CH_3OH (1 µmol), HCHO (1 µmol), HCOOK (10 µmol), CH_3CH_2OH (1 µmol), CH_3CHO (1 µmol), CH_3COONa (10 µmol)

The rates of oxygen uptake obtained with methanol, formaldehyde and potassium formate were approximately doubled in the presence of 0.16 μmol CO (Table 24). As mentioned, a similar phenomenon was found with whole-cell suspensions of Methylomonas methanica (Ferenci, 1974) and the results with M.capsulatus(Bath) support Ferenci's suggestion that CO was oxidised by a mono-oxygenase, possibly the methane mono-oxygenase, with the co-substrates (methanol, formaldehyde and formate) producing the requisite reducing power.

These CO-stimulated co-substrate respiration rates could be explained by the stimulation of the co-substrate oxidation rather than the actual oxidation of CO. In an attempt to eradicate this possibility, a similar experiment to one done by Ferenci (1974) with Methylomonas methanica whole cells was done. Oxygen uptake and formaldehyde disappearance rates were measured concomitantly during a respiration assay which was initiated with formaldehyde (1 μmol), then after a suitable time (1 min) CO-saturated buffer (0.2 ml) was added. The rate of formaldehyde disappearance, as determined by the acetylacetone method of Nash (1953), remained constant throughout the assay even after the addition of CO, whereas the oxygen consumption rate doubled on the addition of CO. This strongly suggests that the increased oxygen uptake after the addition of CO was the result of CO oxidation. There is still the slight possibility that even though the rate of formaldehyde disappearance remained constant, the percentage completely oxidised to CO_2, as opposed to assimilated, was greatly increased in the presence of carbon monoxide.

Further evidence supporting the oxidation of CO by whole-cell suspensions of M.capsulatus (Bath) in the presence of a co-substrate was obtained when the oxygen uptake of whole-cell suspensions was measured with CO plus co-substrate (methanol, formaldehyde and formate) in the presence of acetylene. The oxygen uptake rates (Table 24) were virtually identical to those obtained with the

co-substrate alone. Acetylene had no effect on co-substrate oxidation, therefore not only did this strongly support the oxidation of CO by a mono-oxygenase, but increased the probability of the methane mono-oxygenase being responsible, as acetylene is thought to be a specific inhibitor of the methane mono-oxygenase (see Section III).

CO did not stimulate oxygen consumption by whole-cell suspensions of M. capsulatus with any of the C_2 homologues of the co-substrates, i.e. ethanol, acetaldehyde or sodium acetate (Table 24). This suggested that none of these C_2 compounds could support CO oxidation by whole-cell suspensions of M. capsulatus (Bath), possibly due to their inability to generate reducing power of the form required for the CO mono-oxygenase.

Whole-cell suspensions of Methylococcus capsulatus (Texas) gave similar results to those obtained with M. capsulatus (Bath) when incubated with CO alone or in the presence of a co-substrate (Table 25). Although all the oxygen uptake rates are slower than those observed with cell suspensions of M. capsulatus (Bath), it appears that the salient points were the same, as CO was only oxidised by whole-cell suspensions of M. capsulatus (Texas) in the presence of a suitable electron donor, e.g. methanol.

Summarising the whole-cell data, it appears that resting-cell suspensions of M. capsulatus (Bath) were unable to oxidise CO, probably due to the cells being unable to regenerate the requisite reducing power for mono-oxygenase activity. It is interesting to note that some methane-oxidising bacteria can oxidise CO in the absence of any co-substrate. Resting-cell suspensions of Methylosinus trichosporium OB3b have been shown to oxidise CO in the absence of any co-substrate (Ferenci, 1974; Hubley, 1975), as have four other Type II methane-utilisers (Hubley, 1975). Only one Type I methane-oxidiser has been shown to oxidise CO in the absence of other co-substrates, i.e. Methylomonas albus BG8 (Hubley, 1975), but the oxygen consumption rate was negligible

TABLE 25

Rates of oxygen uptake by whole-cell suspensions of Methylococcus capsulatus (Texas)

Substrates	Oxygen uptake rate [nmol O_2 min^{-1} (mg dry weight cells)$^{-1}$]
CH_4	106
$CH_4 + C_2H_2$	0
CO	0
CH_3OH	53
$CH_3OH + CO$	104
$CH_3OH + C_2H_2$	53
$CH_3OH + CO + C_2H_2$	56
HCHO	43
HCHO + CO	64
$HCHO + C_2H_2$	43
$HCHO + CO + C_2H_2$	43

Reaction vessel: 60 µmol phosphate
(Final vol. 3 ml) Whole cells, 0.78 mg DCW
(Where present) CH_4 (0.12 µmol)
CO (0.16 µmol)
C_2H_2 (1.2 µmol)
CH_3OH (1.0 µmol)
HCHO (1.0 µmol)

(1 nmol min (mg dry weight cells)$^{-1}$). It was thought that the ability of the Type II organisms to oxidise CO in the absence of a co-substrate was due to the breakdown of a storage compound, polyhydroxybutyric acid (PHB), known to be present in Type II methane oxidisers (Hubley, 1975). Thomson (1974) showed that whole - cell suspensions of Methylosinus trichosporium OB3b incubated with ethane (another non-growth substrate) stimulated the breakdown of PHB to acetone and carbon dioxide via hydroxybutyrate and acetoacetate, with the penultimate step coupled to the reduction of NAD^+. If a similar stimulation of PHB breakdown occurred when cells were incubated with CO, then the resultant NADH could be used for CO oxidation.

(ii) Cell-free extract studies.- In an attempt to determine more about the nature and properties of the enzyme responsible for CO oxidation in M. capsulatus (Bath), cell-free extracts were prepared and assayed for CO_2 production (see Methods) in the presence of CO and other compounds. The results of M. capsulatus (Bath) cell-free extract CO oxidations are shown in Table 26.

The complete reaction mixture plus CO gave a CO_2 production rate over 3 times that observed with the control. In the absence of NADH or gaseous oxygen, very little CO_2 was produced. These results combined with the fact that only one atom of oxygen is required by CO to produce CO_2 strongly suggests the involvement of a mono-oxygenase, i.e. see equation 13).

The potent inhibition of CO oxidation by acetylene and the very poor inhibition exhibited by potassium cyanide strongly suggested that CO oxidation in M. capsulatus (Bath) was catalysed by the methane mono-oxygenase (see Section III).

The effect of some potential inhibitors on methane and CO oxidation by cell-free extracts of M. capsulatus (Bath) are shown in Table 27 and presents further circumstantial evidence for the oxidation of carbon monoxide by the methane mono-oxygenase. Only

TABLE 26

Rates of CO_2 production by cell-free extracts of Methylococcus capsulatus (Bath)

	Control (no substrate)	substrates CH_4	CO	CH_3Br
Reaction mixture	15.0	20.1	46.0	30.0
- NADH	8.0	–	11.2	8.6
+ KCN	9.2	–	49.2	5.8
+ C_2H_2	6.4	–	10.8	8.6
Anaerobic	6.4	–	10.4	5.4

Rates expressed as nmol CO_2 produced min^{-1} $(mg\ protein)^{-1}$

Reaction mixture:	20 µmol phosphate
(Final vol. 1 ml)	5 µmol NADH
	5 mg extract protein
(Where present)	3 ml gas phase CH_4
	3 ml gas phase CO
	1.2 µmol CH_3Br
	1.5 µmol KCN
	0.2 ml gas phase C_2H_2

one of the six potential inhibitors tested, 8-hydroxyquinoline, caused significant inhibition of both methane and CO oxidation. The other compounds had at best 16% inhibition of either substrate oxidation. These results support the possibility of CO oxidation by the methane mono-oxygenase.

Methane mono-oxygenase activity in crude, cell-free extracts of M. capsulatus (Bath) could be separated into two fractions by DEAE-cellulose chromatography, as described previously. CO oxidation, like bromomethane oxidation also required the presence of both fractions for activity further supporting the possibility of the methane mono-oxygenase having the ability to oxidise CO.

TABLE 27

The effect of various potential inhibitors on methane oxidation and carbon monoxide oxidation by cell-free extracts of Methylococcus capsulatus (Bath).

Inhibitor	Rate of methanol accumulation (% control*)	Rate of carbon dioxide production (% control†)
None	100	100
Thiourea	90	100
Diethyl dithiocarbamate	100	125
2, 2-Bipyridine	99	105
Neocuproine	100	85
8-Hydroxyquinoline	29	30
Acriflavin	84	90

* The uninhibited rate of methane oxidation as determined by methanol accumulation (see Methods) was 53 nmol methanol produced min^{-1} (mg extract protein)$^{-1}$. These results are taken from Table 6, Section III.

† The uninhibited rate of carbon monoxide oxidation as determined by carbon dioxide production (see Methods) was 32 nmol carbon dioxide produced min^{-1} (mg extract protein)$^{-1}$. The results were corrected for a minus CO control.

The comparatively poor rate of CO_2 production observed with methane as substrate (Table 26) was probably due to the negligible methanol oxidase activity found in cell-free extracts of M. capsulatus (Bath) prepared as described in the Methods. No primary alcohol dehydrogenase (PAD) activity was detectable under the assay conditions used for CO_2 production, although the soluble extracts containedhigh specific activity of PAD when assayed under the optimum in vitro conditions (Anthony and Zatman, 1964b).

Since it was established that CO oxidation by cell-free extracts of M. capsulatus (Bath) required NADH, it was decided to test whether NADH could be generated in situ by the oxidation of certain co-substrates and concomitant reduction of NAD^+. The results are shown in Table 28.

Considering first the results of the C_1 co-substrates, all three compounds, methanol, formaldehyde and potassium formate gave rates of CO_2 production approximately 4 times that of the control. The rate obtained with formate was not unexpected as all the formate dehydrogenase enzymes reported present in methane-oxidising bacteria have been soluble in nature (see Section I), therefore good formate dehydrogenase activity was expected in the soluble, cell-free extracts of M. capsulatus (Bath). The rates observed with methanol and formaldehyde were much higher than first anticipated, due to the absence of primary alcohol dehydrogenase activity, the enzyme thought responsible for the oxidation of both methanol and formaldehyde.

The presence of potassium cyanide drastically reduced the rates of CO_2 production from the co-substrates as expected, because the final step of the complete oxidation of methane is catalysed by formate dehydrogenase which in many methane-utilisers is known to be very sensitive to cyanide (Johnson and Quayle, 1964; Patel and Hoare, 1971) and was found to be a potent inhibitor of formate dehydrogenase activity in M. capsulatus (Bath). The results of the co-substrate

TABLE 28 Rates of CO_2 production by cell-free extracts of Methylococcus capsulatus (Bath)

	Control (no substrate)	Co-substrates CH_3OH	HCHO	HCOOK	CH_3CH_2OH	CH_3CHO	CH_3COONa
Reaction mixture	11.2	40.4	41.6	38.0	11.6	11.8	9.6
+ KCN	8.6	7.6	14.0	12.8	-	-	-
+ CO	19.2	21.4	81.4	70.0	13.8	22.4	19.4
+ CO + KCN	12.8	12.8	51.4	17.0	-	24.6	20.6
+ CO - NAD^+	11.2	17.1	33.0	19.3	-	-	-
- NAD^+	8.0	12.8	14.4	15.0	-	-	-

Rates expressed as nmol CO_2 produced min^{-1} $(mg\ protein)^{-1}$

Reaction mixture: 20 µmol phosphate
(Final vol. 1 ml) 2 µmol NAD^+
5 mg extract protein

(Where present)

3 ml gas phase CO
1.5 µmol KCN
2 µmol CH_3OH
2 µmol HCHO
2 µmol HCOOK
2 µmol CH_3CH_2OH
2 µmol CH_3CHO
2 µmol CH_3COONa

oxidations incubated in the presence of CO showed very rapid rates of CO_2 production with formaldehyde or formate. The rates obtained with these two co-substrates plus CO were approximately twice those observed in the absence of CO, strongly suggesting the oxidation of CO as a result of *in situ* generation of NADH, presumably arising from the activity of the NAD^+-linked formate dehydrogenase. The rate of CO_2 production from methanol and CO was approximately half that observed in the absence of CO, suggesting an inhibition of methanol oxidation by CO, contradicting the whole-cell inhibition studies (see Section III).

The results of the reaction mixture assays containing co-substrate plus CO and potassium cyanide when compared with the expected results contain one anomolous yet interesting result. It was expected that cyanide would inhibit virtually all co-substrate oxidation and all CO oxidation as formate dehydrogenase would be rendered totally inactive by the presence of potassium cyanide, therefore no CO_2 could be produced from the co-substrate and no NADH generated. Methanol and formate gave poor rates of CO_2 production as expected, however a rate of 51.4 nmol CO_2 produced min^{-1} (mg extract protein)$^{-1}$ was obtained when the reaction mixture was incubated with formaldehyde, CO and potassium cyanide. This rate was approximately 20% faster than that observed with the reaction mixture and formaldehyde alone, suggesting that at least a part of the CO_2 produced resulted from CO oxidation. Obviously any oxidation of formaldehyde to CO_2 could not proceed *via* formate, nevertheless it was possible that formaldehyde was being completely oxidised to CO_2 and consequently generating NADH by a cyclic scheme involving hexulose phosphate synthase, similar to the scheme reported by Strøm *et al.* (1974) and Colby and Zatman (1975). However, the result of the assay containing reaction mixture, formaldehyde and cyanide showed a very poor rate of CO_2 production implying that no such scheme was operative or indeed that very little formaldehyde was oxidised to CO_2 in the presence of cyanide

at all. It appears that the vast majority of CO_2 produced from the assay containing reaction mixture, formaldehyde, CO and cyanide resulted from CO oxidation and not from formaldehyde oxidation. If this was indeed the case, then the NADH required for CO oxidation was most probably produced by a hitherto undiscovered NAD^+-linked formaldehyde dehydrogenase. Further tests were done to prove the existence of such an enzyme in crude, soluble cell-free extracts of M. capsulatus (Bath) and the results are presented in Section VI.

The results of the assays containing the reaction mixture plus CO and cosubstrates but minus exogenous NAD^+ showed relatively poor rates of CO_2 production with methanol and formate but formaldehyde gave a rate approximately 37% of that observed with the same assay plus NAD^+. It was thought that the reason any rates higher than the control were obtained due to the presence of a small quantity of endogenous NAD^+ in the extract. If this assumption was correct, then the result of the increased rate observed with formaldehyde could be explained if the K_m for NAD^+ was much lower than the K_m for the NAD^+-linked formate dehydrogenase.

The final set of assays containing the C_1 co-substrate showed that for maximal CO_2 production from the co-substrates exogenous NAD^+ was required, although relatively small increases in CO_2 production, with respect to the control value, were obtained supporting the presence of a small quantity of enogenous NAD^+ in the extracts.

If an NAD^+-linked formaldehyde dehydrogenase was present in soluble cell-free extracts of M. capsulatus (Bath) as was eventually proved (see Section VI), then this would explain a number of anomalous results obtained with the C_1 co-substrates, i.e. the unexpected rapid CO_2 production rates from methanol and formaldehyde with the reaction mixture alone, and the apparent CO inhibition of CO_2 production from methanol. The presence of a formaldehyde dehydrogenase would explain the oxidation of formaldehyde in the absence of primary

alcohol dehydrogenase activity. In thelight of the methane mono-oxygenase substrate specificity tests (see Section IV) where methanol was shown to be an excellent substrate for the mono-oxygenase, it is conceivable that methanol was oxidised by the methane mono-oxygenase to formaldehyde, the requisite NADH being generated by the subsequent oxidation of formaldehyde and formate. An initial primer of NADH would be required to initiate the mono-oxygenase activity, but this could be supplied from the oxidation of methanol by the small but detectable methanol oxidase activity present in soluble cell-free extracts of M. capsulatus (Bath).

Similarly, the inhibition of methanol oxidation by CO could be explained in terms of competitive inhibition of the methane mono-oxygenase. CO might have competitively inhibited methanol oxidation by the methane mono-oxygenase and therefore interrupted the regeneration of NADH because unlike methanol, CO could not generate NADH by subsequent oxidation of the initial product.

The oxidation of the C_2 co-substrates in the presence and absence of CO gave very different results to those obtained with the C_1 compounds. All three C_2 co-substrates, ethanol, acetaldehyde and sodium acetate were poorly oxidised if at all, as the rates of CO_2 production were only slightly higher than the control or, in the case of acetate, lower than the control. These results were not unexpected as the results of the whole-cell oxidation tests (Table 24) implied a very poor enzymic capability with regards to acetaldehyde and acetate oxidation. In the presence of CO, slightly increased rates of CO_2 production over the control rate were observed for acetaldehyde and acetate, whereas ethanol plus CO only gave a rate approximately 65% of that of the control. These results suggested that either acetaldehyde and acetate were capable of generating a small quantity of NADH for CO oxidation or CO simply stimulated CO_2 production from the C_2 compounds or other endogenous

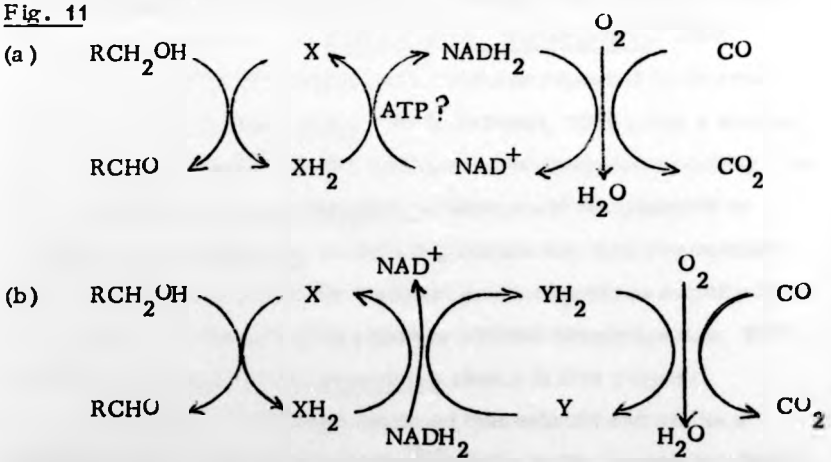
substrates. These acetaldehyde and acetate plus CO assays repeated in the presence of potassium cyanide gave very similar results to those obtained in the absence of cyanide. This supports the view that CO probably stimulated CO_2 production from the co-substrates. The whole-cell data (Table 24) would appear to confirm this view as no apparent CO oxidation was obtained with either acetaldehyde or acetate. Also it was later shown that neither acetaldehyde nor acetate were substrates for the NAD^+-linked formaldehyde dehydrogenase (see Section VI).

The results of the C_2 compound oxidation studies in the presence of CO, both with whole-cell suspensions and cell-free extracts of M. capsulatus (Bath) provided some interesting data, especially the results obtained with ethanol and CO. No NAD^+-linked enzyme was found that could oxidise ethanol, and the complete oxidation of ethanol was found to be limited by the negligible oxidation of acetaldehyde and acetate, similar to the results found with M.capsulatus (Texas) (Patel and Hoare, 1971). Therefore it would appear from the whole-cell and cell-free data that ethanol cannot support CO oxidation as a result of its inability to generate NADH during oxidation to acetaldehyde. This implies that the reducing equivalents (other than NADH) produced during the oxidation of ethanol to acetaldehyde, catalysed by the primary alcohol dehydrogenase, were unable to directly or indirectly supply the electrons required for CO oxidation by M. capsulatus (Bath). If indeed CO was oxidised by the methane mono-oxygenase in M. capsulatus (Bath) as is suggested from the data presented above, then the same implication regarding ethanol would hold for methane oxidation.

Very different results were obtained with whole-cell suspensions of Methylomonas methanica by Ferenci (1974; Ferenci et al., 1975). He found that ethanol could generate suitable reductant for CO oxidation and could be oxidised to acetate, but no acetate oxidation to CO_2 was detected. Therefore the reductant required for CO oxidation, thought to be catalysed by the methane mono-oxygenase,

was being generated from either ethanol or acetaldehyde oxidation. No enzyme other than primary alcohol dehydrogenase was found capable of oxidising ethanol or acetaldehyde (Ferenci et al., 1975), thus no NADH generating enzymes appeared involved. However, he demonstrated that crude, particulate cell-free extracts could catalyse CO oxidation in the presence of NADH (Ferenci, 1974; Ferenci et al., 1975) and to accommodate both findings he proposed two possible schemes for the channeling of reductant from alcohol oxidation fo the CO mono-oxygenase (Fig. 11 (a) and (b)). The difference between the two schemes is whether NADH is the immediate reducing agent for the mono-oxygenase or acts through another carrier(s), Y.

Fig. 11



(from Ferenci et al., 1975)

From the data presented above, it is impossible to determine whether NADH is the immediate reducing agent for the CO (CH_4) mono-oxygenase in M. capsulatus (Bath), but there does appear to be a fundamental difference between M. capsulatus (Bath) and Methylomonas methanica in the recycling of electrons for mono-oxygenase activity. Although both organisms are classified as Type I organisms (Whittenbury et al., 1970a), the scheme for recycling electrons in Methylomonas methanica would appear to be more akin to

the scheme proposed present in Methylosinus trichosporium OB3b (Tonge et al., 1975; Higgins et al., 1976; Tonge et al., 1977). In Methylosinus trichosporium OB3b it was found that NADH or NADPH could act as electron donors for the methane mono-oxyganese in crude cell-free extracts or when partially purified (Tonge et al., 1975). However, in purified methane mono-oxygenase preparations NAD(P)H could no longer act as a reducing agent for mono-oxygenase activity (Tonge et al., 1977). It was found that only ascorbate, or methanol plus partially purified primary alcohol dehydrogenase were suitable as reducing agents for the purified methane mono-oxygenase (Tonge et al., 1977) and it appears that the enzyme is neither directly or obligatorily NAD(P)H-linked. Higgins et al.(1976) put forward a tentative scheme incorporating these results for electron transport and energy transduction in Methylosinus trichosporium OB3b.

It appears from the preliminary evidence reported by Ferenci (Ferenci, 1974; Ferenci et al., 1975; Ferenci, 1976) that a similar electron donor mechanism for methane mono-oxygenase activity as found in Methylosinus trichosporium OB3b could be operative in Methylomonas methanica, as both organisms are able to generate sufficient reducing power for methane mono-oxygenase activity from the oxidation of an alcohol by primary alcohol dehydrogenase. Both organisms differ from M. capsulatus (Bath) in this respect.

Interestingly, it has been reported that ethanol can act as a suitable electron donor for nitrogenase activity in M. capsulatus (Bath) (Dalton and Whittenbury, 1976a; Dalton and Whittenbury, 1976b). This proves that useful reducing power can be generated from the oxidation of ethanol, but in the light of the data presented above it would appear that NADH cannot be the immediate reducing agent for nitrogenase activity.

In conclusion, with respect to CO oxidation by M. capsulatus (Bath), it appears that CO is a cometabolic non-growth substrate, i.e. it requires the presence of a co-substrate for oxidation. The

question of possible assimilation of the oxidation product, CO_2, requires further study, and in the light of the results reported by Taylor, S., (1977) concerning the presence of ribulose 1,5-bisphosphate carboxylase and phosphoribulokinase activities in cell-free extracts of M. capsulatus (Bath) (see Section I) could prove an interesting problem.

There have also been reports suggesting the presence of the serine pathway of formaldehyde assimilation in M. capsulatus (Bath) when grown at 45° C (Whittenbury et al., 1975; Reed, 1976; Whittenbury et al., 1976). Therefore, although no direct evidence exists for carbon assimilation resulting from CO oxidation, the enzymic apparatus for CO_2 fixation by one form or another appears to be present in M. capsulatus (Bath) suggesting that CO is a cometabolic, non-growth substrate from which carbon can be assimilated.

6. Final Conclusions

Although there is a great deal of circumstantial evidence to suggest that the non-growth substrates described in this Section are indeed oxidised by the methane mono-oxygenase, obviously oxidation by a purified preparation of the methane mono-oxygenase is required for conclusive proof.

From all the non-growth substrates tested for oxidation by M. capsulatus (Bath) there appear to be examples for four types of unusual transformation done by whole cells of M. capsulatus (Bath). Ethene and propene are non-growth substrates which on fortuitous oxidation form epoxyethane and epoxypropane respectively which probably consist of non-assimilable carbon. Bromomethane, however, is a non-growth substrate which probably forms the assimilable product formaldehyde on fortuitous oxidation.

Ethane, propane and the three butene derivatives are all cometabolic substrates which on oxidation form products which are probably non-assimilable. Carbon monoxide is also a cometabolic substrate, but on oxidation forms the potentially assimilable product, carbon dioxide.

SECTION VI Oxidation of Formaldehyde by Methylococcus capsulatus (Bath)

1. Introduction

Two different routes for the complete oxidation of formaldehyde in C_1-utilising bacteria have been reported: (i) the oxidation of formaldehyde to carbon dioxide with formate as an intermediate; (ii) the cyclic scheme involving hexulose phosphate synthase for the oxidation of formaldehyde to carbon dioxide.

The first route involves a formaldehyde dehydrogenase of which seven different types have been reported in C_1-utilising bacteria (Table 29). These various types can be roughly divided into two groups, (1) $NAD(P)^+$-linked enzymes and (2) $NAD(P)^+$-independent enzymes. The $NAD(P)^+$-linked enzymes comprise the first four of the seven enzyme categories shown in Table 29.

The first type of $NAD(P)^+$-linked formaldehyde dehydrogenase is formaldehyde specific and requires glutathione for activity. This enzyme has been found in many different C_1-utilising bacteria (Table 29) including both facultative and obligate methyltrophs. It is not known whether these enzymes catalyse the glutathione-dependent oxidation of formaldehyde to S-formylglutathione which is subsequently oxidised by formate dehydrogenase, as suggested for similar enzymes found in a number of methanol-utilising yeasts (Fig. 12) (Kato et al., 1972; van Dijken et al., 1976; Schütte et al., 1976).

The second type of $NAD(P)^+$-linked formaldehyde dehydrogenase is again formaldehyde-specific but this time is glutathione-independent. The only example of this type of enzyme was reported by Kung and Wagner (1970) who found such an enzyme present in extracts of Pseudomonas MS. However, these authors did not determine the substrate specificity of the enzyme and so this enzyme could be included in the third type of $NAD(P)^+$-linked formaldehyde dehydrogenase, i.e. glutathione-independent aldehyde dehydrogenase. This type of enzyme can oxidise other aldehydes in addition to formaldehyde and has

TABLE 29 Different types of formaldehyde dehydrogenase enzymes found in C_1-utilizing bacteria

Type	$NAD(P)^+$-linked: Glutathione dependent	$NAD(P)^+$-linked: Glutathione independent	Artificial electron acceptor*	Substrate specificity: Formaldehyde	Substrate specificity: Other aldehydes	Substrate specificity: Other compounds	Activator	Organism	Ref.†
Group 1									
1	+	-	-	+	-	-	-	Methylomonas methanica (Texas)	1
								Methylomonas methanica (Iowa)	2
								Pseudomonas extorquens	1
								Bacterium 5H2	3
								Protaminobacter ruber	1
								Pseudomonas aminovorans	4
2	-	+	-	+	-	-	-	Pseudomonas MS	5
3	-	+	-	+	+	-	-	Bacterium 4B6	6
4	-	+	-	+	-	-	Tetrahydrofolate	Pseudomonas AM1	7
								Methylobacterium organophilum	8
Group 2									
5	-	-	DCPIP or $K_3Fe(CN)_6$	+	-	-	-	Pseudomonas RJ1	9
6	-	-	DCPIP or PMS	+	+	-	-	Pseudomonas AM1	1
								Pseudomonas aminovorans	4
7	-	-	PMS	+	± (CH_3CHO)	+	NH_3 or CH_3NH_2	Methylococcus capsulatus (Texas)	10
								Pseudomonas M27	11
								Hyphomicrobium WC	12
								Pseudomonas TP1	12
								Pseudomonas W	12
								Methylosinus sporium	13
								Pseudomonas RJ1	14
								Methylobacterium organophilum	8

* DCPIP, 2,6-Dichlorophenol-indophenol; PMS, phenazine methosulphate.

† (1) Johnson and Quayle, 1964; (2) Harrington and Kallio, 1960; (3) Hampton and Zatman, 1973; (4) Boulton and Large, 1977; (5) Kung and Wagner, 1970; (6) Colby and Zatman, 1973; (7) Large and Quayle, 1963; (8) O'Connor and Hanson, 1977; (9) Mehta, 1975; (10) Patel and Hoare, 1971; (11) Anthony and Zatman, 1964b; (12) Sperl et al., 1974; (13) Patel and Felix, 1976; (14) Mehta, 1973.

been shown to be present in an obligate methylotroph named bacterium 4B6 (Colby and Zatman, 1973).

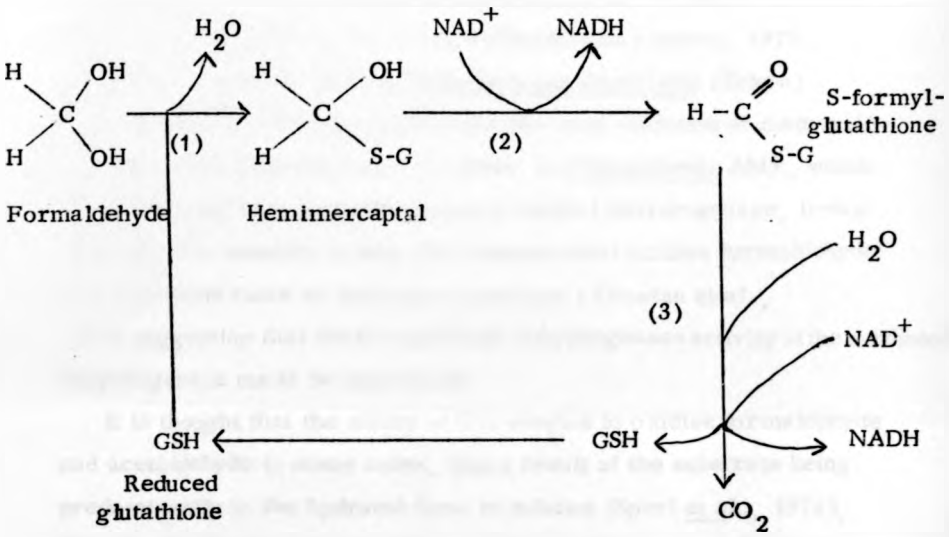
The fourth and last type of $NAD(P)^+$-linked formaldehyde dehydrogenase is glutathione-independent and formaldehyde-specific but requires the presence of tetrahydrofolate. Formaldehyde reacts non-enzymically with tetrahydrofolate to give $N^{5,10}$-methylenetetrahydrofolate which is then oxidised by $N^{5,10}$-methylenetetrahydrofolate dehydrogenase to give $N^{5,10}$-methenyl-tetrahydrofolate, this latter enzymic step being equivalent to the oxidation of formaldehyde to formate (Large and Quayle, 1963).

The second group of formaldehyde dehydrogenases, the $NAD(P)^+$-independent enzymes are composed of three types of enzymes, as shown in Table 29, all of which require the presence of an artificial electron acceptor for in vitro assay. The first type can be assayed by using either 2,6-dichlorophenol-indophenol (DCPIP) or potassium ferricyanide ($K_3Fe(CN)_6$) as the initial electron acceptor and is formaldehyde specific. Only one organism has been reported to possess this type of formaldehyde dehydrogenase, i.e. Pseudomonas RJ1 (Mehta, 1975) but the author tested a very limited range of potential substrates and it is possible that this enzyme may not be formaldehyde specific.

The second type of $NAD(P)^+$-independent formaldehyde dehydrogenase can use either DCPIP or phenazine methosulphate (PMS) as its initial electron acceptor for assay, and can oxidise other aldehydes than formaldehyde. This enzyme has been reported present in a number of organisms, e.g. Pseudomonas AM1 (Johnson and Quayle, 1964) and Pseudomonas aminovorans (Boulton and Large, 1977) but the specific activity was generally low, casting doubts on the significance of such an enzyme considering the rapid rate of formaldehyde oxidation shown by most C_1-utilising bacteria.

The last type of $NAD(P)^+$-independent formaldehyde dehydrogenase is methanol dehydrogenase (see Section I), first described by Anthony and Zatman (1964b) and found in all methylotrophs tested. The

Fig. 12



Scheme for formaldehyde oxidation in methanol-utilising yeasts (adapted from van Dijken et al. (1976)).

(1) Spontaneous formation of a hemimercaptal between formaldehyde and reduced glutathione.

(2) Oxidation of formaldehyde to S-formylglutathione catalysed by formaldehyde dehydrogenase.

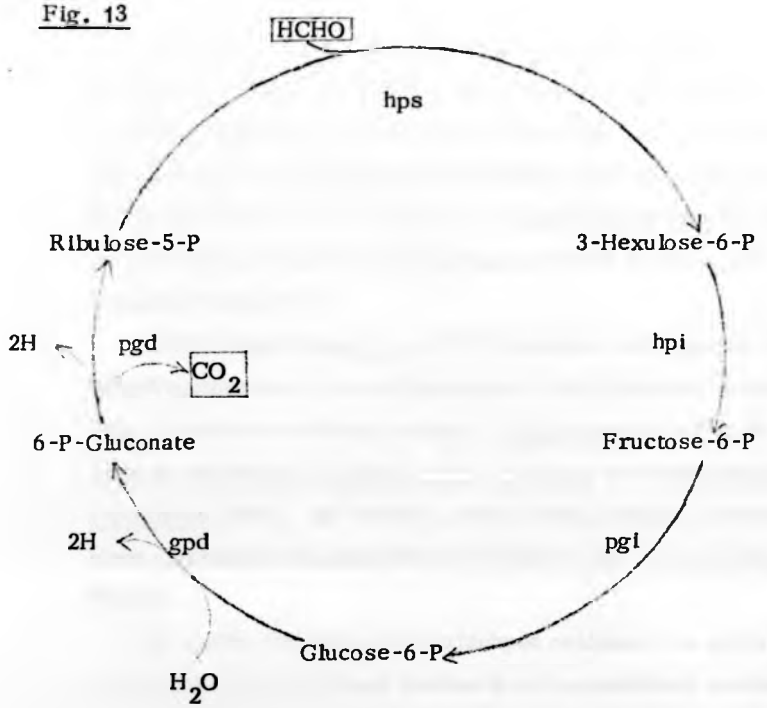
(3) Oxidation of the formyl group of S-formylglutathione by formate dehydrogenase.

importance of this enzyme with respect to formaldehyde oxidation *in vivo* appears to vary from organism to organism. *Methylococcus capsulatus* (Texas) possesses none of the formaldehyde dehydrogenases mentioned above with the exception of the primary alcohol dehydrogenase (Patel et al., 1972; Wadzinski and Ribbons, 1975). Therefore it appears that for *Methylococcus capsulatus* (Texas) methanol dehydrogenase catalyses the two-step oxidation of methanol *via* formaldehyde to formate. However, in *Pseudomonas* AM1, which normally possesses a similar primary alcohol dehydrogenase, it was observed that mutants lacking this enzyme could oxidise formaldehyde at comparable rates to wild type organisms (Dunstan et al., 1972) suggesting that the formaldehyde dehydrogenase activity of the methanol dehydrogenase could be superfluous.

It is thought that the ability of this enzyme to oxidise formaldehyde, and acetaldehyde in some cases, was a result of the substrate being predominantly in the hydrated form in solution (Sperl *et al.*, 1974), thus the dual nature of the primary alcohol dehydrogenase in these organisms could be purely coincidental.

The second route for the complete oxidation of formaldehyde in methylotrophs, *i.e.* the cyclic scheme involving hexulose phosphate synthase, was first proposed by Strøm *et al.* (1974) working with *Methylococcus capsulatus* (Texas) and *Methylomonas methanica*. Almost simultaneously but independently Colby and Zatman (1975), working with various obligate and restricted facultative methylotrophs, proposed a similar scheme for the complete oxidation of formaldehyde, based on various enzyme activities observed with crude cell-free extracts. The scheme envisaged by both groups of workers is shown in Fig. 13 and comprises five enzymes, hexulose phosphate synthase (hps), hexulose phosphate isomerase (hpi), phosphoglucoisomerase (pgi), glucose-6-phosphate dehydrogenase (gpd) and 6-phosphogluconate dehydrogenase (pgd).

Fig. 13



Cyclic pathway for oxidation of formaldehyde (Strøm et al., 1974; Colby and Zatman, 1975).

hps, hexulose phosphate synthase ; hpi, hexulose phosphate isomerase; pgi, phosphoglucoisomerase; gpd, glucose-6-phosphate dehydrogenase; pgd, 6-phosphogluconate dehydrogenase.

Both Strøm et al. and Colby and Zatman proposed this cyclic scheme on the basis of circumstantial enzymic evidence, but it has been shown recently that ^{14}C-formaldehyde oxidation to $^{14}CO_2$ by extracts of Pseudomonas C (Ben-Bassat and Goldberg, 1977) and Pseudomonas oleovorans (Trotsenko and Sokolov, 1977) required not only the presence of either NAD^+ or $NADP^+$ but also ribulose-5-phosphate, therefore providing direct evidence for a hexulose phosphate synthase mediated cyclic scheme of formaldehyde oxidation.

Organisms using the ribulose monophosphate (RMP) cycle of formaldehyde fixation (see Section 1) obviously possess some of the enzymes required for the cyclic scheme (hps, hpi, pgi in fructose bisphosphate and sedoheptulose variants; hps, hpi, pgi, gpd in Entner-Doudoroff variant), but even these organisms require the presence of 6-phosphogluconate dehydrogenase at least for the cycle to be potentially operative.

Interestingly Davey et al. (1972) detected both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity in 4 Type I methane-oxidising bacteria, Methylomonas albus BG8, Methylomonas methanica, Methylococcus minimus and Methylococcus capsulatus (Bath), but failed to realise the potential of these enzymes when combined with those from the RMP cycle of formaldehyde fixation.

The cyclic scheme of formaldehyde oxidation can generate 2 molecules of NAD(P)H per molecule of formaldehyde oxidised, from the $NAD(P)^+$-linked glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase steps. Two other cyclic pathways of formaldehyde oxidation have been proposed for Pseudomonas MA growing on methylamine and assimilating formaldehyde by the serine pathway, but these schemes produce 1 molecule of NADH and 1 molecule of reduced flavoprotein (Newaz and Hersh, 1975).

Strøm et al. (1974) speculated that as no enzymes which oxidise C_1-compounds were known to be $NADP^+$-linked, the cycle might serve as a source of NADPH for biosynthetic purposes. Colby and Zatman (1975) suggested that the cycle was probably responsible for the generation of energy during methylotrophic growth of type L restricted facultative methylotrophs, as these organisms apparently have no other mechanism for formaldehyde oxidation. Similarly, it has recently been reported that Methylophilus methylotrophus possesses the enzymic apparatus for a cyclic pathway of formaldehyde oxidation, which predominantly generates NADPH, but lacks either an $NAD(P)^+$ or DCPIP-linked formaldehyde dehydrogenase. In addition, the organism only

possesses a very low level of formate dehydrogenase activity, suggesting that this organism is also dependent on a cyclic pathway for formaldehyde oxidation (Taylor, I., 1977; Beardsmore and Quayle, 1978).

The objective of this Section is to determine what routes of formaldehyde oxidation are present in Methylococcus capsulatus (Bath) and what enzymes are involved.

2. Presence of $NAD(P)^+$-linked Formaldehyde Dehydrogenase in Crude Soluble-Extracts of Methylococcus capsulatus (Bath)

The initial circumstantial evidence for the presence of an $NAD(P)^+$-linked formaldehyde dehydrogenase being present in M. capsulatus (Bath) was obtained during the studies concerning carbon monoxide oxidation by cell-free extracts of M. capsulatus (Bath). It was found that formaldehyde plus exogenous NAD^+ in the presence of cyanide (1.5 mM) could supply reducing power for CO-mono-oxygenase activity. The possibility of a cyclic scheme of formaldehyde oxidation being responsible for the production of reducing power in these assays was ruled out (see Section V), although the enzymic apparatus for such a scheme is present in M. capsulatus (Bath) as described later.

In an attempt to augment the above circumstantial evidence, the potential formaldehyde dehydrogenase in crude cell-free extracts was tested to see if it could support methane oxidation by the cell-free methane mono-oxygenase. The results are shown in Table 30. The rate of methane oxidation was estimated by measuring methanol accumulation as described in the Methods. The rate obtained with the control assay containing potassium cyanide and NADH shows the rate of methanol accumulation possible in the presence of excess reducing power. Formaldehyde and formate in the absence of cyanide gave similar rates to the positive control described above, but when cyanide was present the formate-dependent rate was only equivalent to the appropriate control, whereas the formaldehyde-dependent rate was more than twice the control rate. The results of the assays incubated in the absence of exogenous NAD^+ show that exogenous NAD^+ was required for maximal rates. These results supported the idea that an NAD^+-linked

TABLE 30

Rates of CH_3OH accumulation from CH_4 by cell-free extracts of Methylococcus capsulatus (Bath)

	Control (no co-substrate)	Co-substrate	
		HCHO	HCOOK
Reaction mixture	4.2	21.2	21.3
+ KCN	6.0	13.3	5.7
+ KCN - NAD^+	2.3	6.8	4.0
+ KCN + NADH	24.3	-	-

Rates expressed as n mol CH_3OH accumulated min^{-1} (mg protein)$^{-1}$

Reaction mixture:
(final vol. 1 ml) 20 µmol phosphate (when present): 1.5 µmol KCN
2 µmol NAD^+ 2 µmol HCHO
5 mg extract protein 2 µmol HCOOK
3 ml gas phase CH_4 5 µmol NADH

formaldehyde dehydrogenase was present in crude, soluble extracts of M. capsulatus (Bath).

During the substrate specificity tests detailed in Section IV, it was found that ethylene (ethene) was apparently oxidised by the methane mono-oxygenase to yield the single product ethylene oxide (epoxyethane). The rate of ethylene oxidation was about twice that of methane, probably as a result of the increased solubility of the C_2 compound. Hence it was decided to repeat the assay performed in Table 30 following ethylene oxidation to ethylene oxide as a monitor of methane mono-oxygenase activity. The technique of the assay was virtually identical to that of the methanol accumulation assays and is described in the Methods. The results are shown in Table 31. The advantage of this assay was not only the increased oxidation rates obtained, but methanol could also be tested as a co-substrate.

TABLE 31

Rates of ethylene oxide production from ethylene by cell-free extracts of Methylococcus capsulatus (Bath)

	Control (no co-substrate)	Co-substrate		
		CH_3OH	HCHO	HCOOK
Reaction mixture	13.4	13.2	58.0	39.2
+ KCN	6.6	8.0	51.6	13.4
- NAD^+	8.0	4.4	27.6	4.0
-NAD^+ + NADH	52.4	-	-	-

Rates expressed as nmol ethylene oxide produced min^{-1} (mg protein)$^{-1}$

Reaction mixture:
(final vol. 1 ml) 20 µmol phosphate (where present): 1.5 µmol KCN
2 µmol NAD^+ 2 µmol CH_3OH
5 mg extract protein 2 µmol HCHO
3 ml gas phase C_2H_4 2 µmol HCOOK
5 µmol NADH

The control assay in the presence of NADH gave a guide to the possible rate obtainable in the presence of adequate reducing power for methane mono-oxygenase activity. The presence of methanol did not stimulate the rate of ethylene oxide production over the control rate. This could result from one or more of three reasons: (i) there was no methanol oxidase actively present in the extract, (ii) methanol cannot act as an electron donor for methane mono-oxygenase activity in M. capsulatus (Bath), (iii) methanol competitively inhibited methane mono-oxygenase activity, as methanol has been shown to be a good substrate for this enzyme (see Section IV).

Formate and especially formaldehyde gave greatly enhanced rates of ethylene oxide production in the absence of cyanide, compared with the control, but when cyanide was present the formate-dependent rate was dramatically reduced whereas the formaldehyde-dependent rate was

reduced by only 10%. These results confirm the presence of an NAD^+-linked formaldehyde dehydrogenase in M. capsulatus (Bath). The rates of ethylene oxide produced in the absence of exogenous NAD^+ suggested that formaldehyde could, to a certain extent, support methane mono-oxygenase activity without exogenous NAD^+. However, it is thought that this rate obtained with formaldehyde was due to the presence of a small quantity of endogenous NAD^+, and that the reason no similar stimulated rate was observed with formate could possibly be the result of vastly differing K_m values for NAD^+, i.e. the formate dehydrogenase K_m for NAD^+ is much greater than the formaldehyde dehydrogenase K_m for NAD^+.

It was found that exogenous NAD^+ was rapidly reduced in the presence of formaldehyde, potassium cyanide and aliquots of crude, soluble extract of M. capsulatus (Bath) by following its reduction spectrophotometrically at 340 nm (see Methods). Specific activities of around 100 m units $(mg\ protein)^{-1}$ were obtained using different extract preparations. Similar assays were done, with $NADP^+$ replacing NAD^+, and rates of $NADP^+$ reduction were obtained that were approximately 80% of the rates found with equivalent assays using NAD^+. No NAD^+-linked formate dehydrogenase activity was detectable in the presence of similar concentrations of cyanide as used in the formaldehyde dehydrogenase assays of crude extracts, although in the absence of cyanide formate dehydrogenase activity was high.

The disappearance of formaldehyde was then followed colorimetrically, either by the method of Nash (1953) or Chrastil and Wilson (1975), in assays containing aliquots of crude, soluble extract of M. capsulatus (Bath), formaldehyde, potassium cyanide and NAD^+ (see Methods). Similar specific activities to those obtained following the reduction of NAD^+ were obtained. These formaldehyde disappearance assays were then repeated, replacing $NADP^+$ for NAD^+ and it was found that $NADP^+$ supported formaldehyde disappearance with a reduction in rates of approximately 20%. Very little formaldehyde disappeared in assays containing no nicotinamide nucleotide.

These spectrophotometric and colorimetric assay results confirmed the presence of an $NAD(P)^+$-linked formaldehyde dehydrogenase in soluble cell-free extracts of M. capsulatus (Bath). Fig. 14 shows the relationship between formaldehyde dehydrogenase activity and soluble extract concentration when determined in the presence of NAD^+ or in the presence of $NADP^+$.

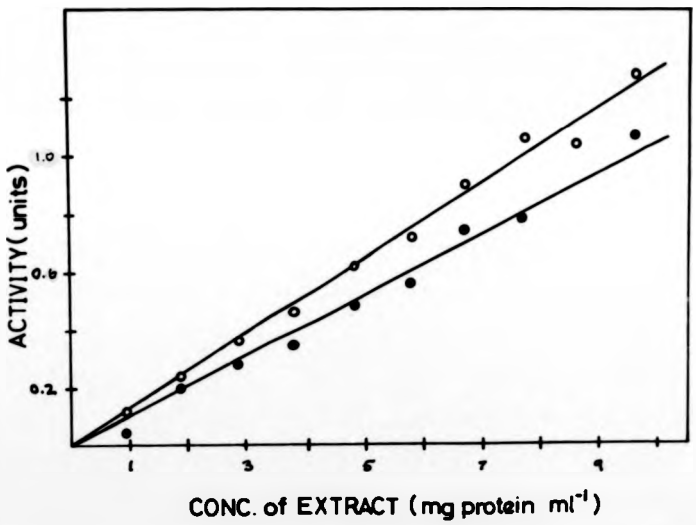
3. Purification of the Formaldehyde Dehydrogenase from M. capsulatus (Bath)

When crude, soluble extract of M. capsulatus (Bath) was dialysed for 24 hours at 4^o C against 20 mM potassium phosphate buffer, pH 7.0, it was found that formaldehyde dehydrogenase activity was lost. However, activity could be restored to the dialysed extract when supplemented with heat-treated soluble extract (prepared as described in the Methods) which alone showed no formaldehyde dehydrogenase activity. Therefore, as neither the dialysed nor heat treated extract had any formaldehyde dehydrogenase activity when assayed separately but activity was restored when assayed together, it was thought that either the formaldehyde dehydrogenase was composed of at least two components, one of which was heat stable at 70^o C, or the enzyme required a heat-stable co-factor, present in the crude soluble extract.

It was decided to attempt to purify the non-dialysable, heat-sensitive component of the enzyme, and the purification of this protein is summarised in Table 32. Formaldehyde dehydrogenase activity was detected at each stage of the purification by supplementing the test samples with heat-treated soluble extract and measuring the reduction of NAD^+ spectrophotometrically. The four steps in the purification procedure are described in the Methods and Fig. 15 shows the various stages of purification monitored by polyacrylamide gel electrophoresis, with gels five and six containing the purified protein.

Fig. 14

The relationship between formaldehyde dehydrogenase activity and soluble extract concentration with Methylococcus capsulatus (Bath)



* Formaldehyde dehydrogenase activity was determined by measuring formaldehyde disappearance, as described in Methods.

○, Activity measured in the presence of NAD^+.

●, Activity measured in the presence of $NADP^+$.

TABLE 32 Purification of heat-sensitive component of the formaldehyde dehydrogenase

Formaldehyde dehydrogenase activity was measured spectrophotometrically as described in Methods.

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity [m units (mg protein)$^{-1}$]	Yield	Purification factor
Soluble extract	15.0	720	60.8	84.4	100	1.0
$(NH_4)_2SO_4$ (30 to 55% saturation precipitate)	2.8	466	45.3	97.2	74.5	1.2
Sephadex G-75	6.0	370	48.6	131.5	79.9	1.6
Sephadex G-200	44.0	88	89.1	1012.5	146.5	12.0
DEAE-cellulose	39.0	17.6	75.3	4278.4	123.8	50.7

The presence of potassium cyanide in the formaldehyde dehydrogenase assay prevented any formate dehydrogenase activity and partially prevented the NADH oxidase activity present in crude soluble extracts of M. capsulatus (Bath). A specific activity of 45 m units (mg protein)$^{-1}$ for NADH oxidase activity was obtained with crude soluble extracts of M. capsulatus (Bath) in the absence of cyanide. In the presence of 2.5 mM potassium cyanide, the concentration regularly used in the formaldehyde dehydrogenase assays, the NADH oxidase specific activity was reduced by only 50%. Similar cyanide insensitive NADH oxidase activity has been reported for a number of methane-utilising bacteria (Davey et al., 1972). It is thought that the apparent increase in the yield during the purification of the heat-sensitive component was largely due to the removal of the cyanide-resistant NADH oxidase activity, originally present in the crude, cell-free extract.

Attempts were also made to purify the component/co-factor present in heat-treated, soluble extract (HTSE). However, no activity was restored to the purified heat-sensitive component when tested with HTSE fractionated by gel filtration (Sephadex G-50) or ion-exchange chromatography (DEAE-cellulose). Similarly, no

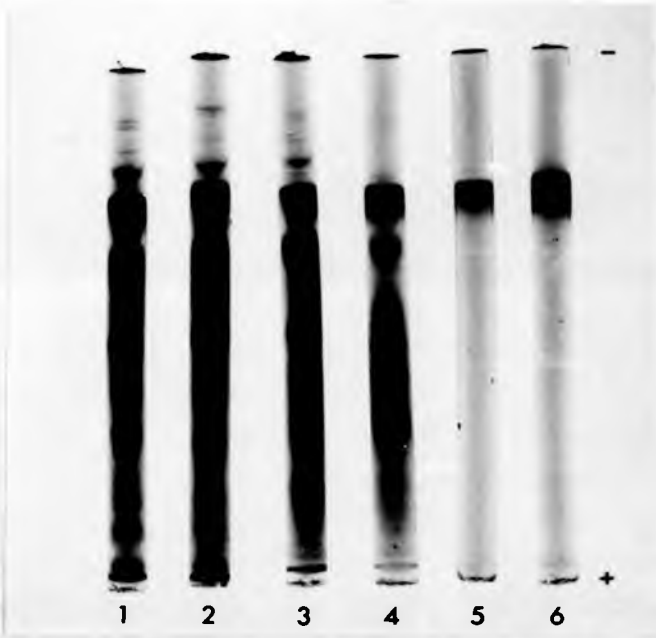
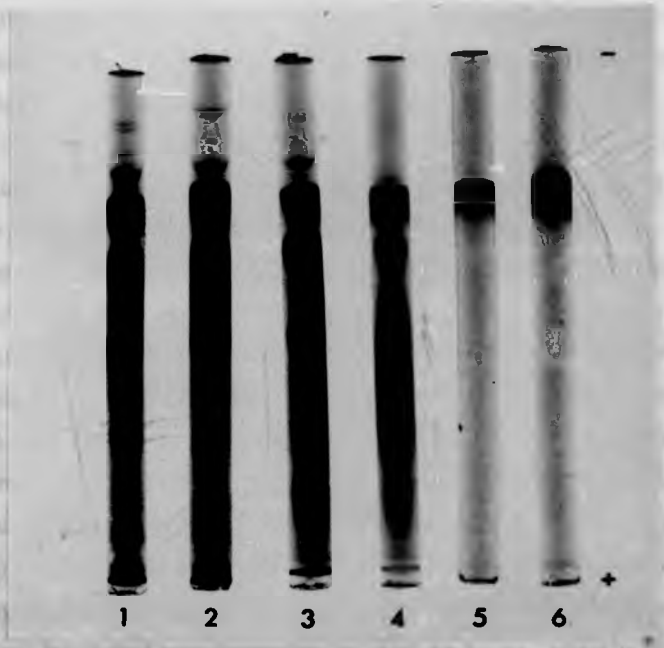


Fig. 15 Polyacrylamide gel electrophoresis of various preparations obtained during purification of the formaldehyde dehydrogenase heat-sensitive component:

(1) crude soluble extract (200 μg protein);
(2) after ammonium sulphate treatment (step 1)(200 μg protein);
(3) after Sephadex G-75 chromatography (step 2) (200 μg protein);
(4) after Sephadex G-200 chromatography (step 3) (100 μg protein);
(5) after DEAE-cellulose treatment (step 4) (20 μg protein);
(6) after DEAE-cellulose treatment (step 4) (50 μg protein). All samples were applied in 100 μl and contained 6% (w/v) sucrose. Tracking dye is at the base (anodic end) of the gels.



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(4) after Sephadex G-200 chromatography (step 3) (100 μg protein);
(5) after DEAE-cellulose treatment (step 4) (20 μg protein);
(6) after DEAE-cellulose treatment (step 4) (50 μg protein). All samples were applied in 100 μl and contained 6% (w/v) sucrose. Tracking dye is at the base (anodic end) of the gels.

separation was obtained with salt or acid precipitation, therefore these preliminary attempts to purify the component/co-factor present in HTSE have so far proved unsuccessful. With the purification of the HTSE component/co-factor proving difficult, it was decided to ascertain more about the nature of the compound by determining whether it was trypsin sensitive. This would either confirm that the component/co-factor was a polypeptide of some kind or suggest that it was probably a small, non-proteinaceous co-factor of some description.

The trypsin treatment of the heat-treated, soluble extract and the various controls done are described in the Methods. The HTSE which was kept in ice for 30 minutes (tube 1, see Methods), when supplemented with 11 μg of purified, heat-sensitive formaldehyde dehydrogenase component in 0.4 ml, gave a specific activity of 4.28 m units $(\text{mg protein})^{-1}$, i.e. no activity was lost as compared with fresh HTSE. Incubation of HTSE at 37° C for 30 minutes (tube 2) had no effect on its ability to give the same specific activity as for tube 1 when combined with purified, heat-sensitive component (11 μg). Incubation of HTSE at 37° C for 30 minutes in the presence of trypsin (tube 3) completely abolished its ability to restore formaldehyde dehydrogenase activity to the purified, heat-sensitive component. It was found that supplementing an inactive assay, containing HTSE from tube 3 and purified, heat-sensitive component, with fresh HTSE restored normal activity (4.28 units $(\text{mg protein})^{-1}$). This confirmed that the addition of trypsin inhibitor to tube 3 after the 30 minutes incubation at 37° C had completely inhibited residual trypsin activity and the initial inactivity of the assay was not due to the effect of trypsin on the purified, heat-sensitive component of the formaldehyde dehydrogenase. The HTSE that had been incubated at 37° C for 30 minutes in the presence of trypsin plus an excess of trypsin inhibitor (tube 4) restored full formaldehyde dehydrogenase activity to the purified, heat-sensitive component.

These results suggested that the component/co-factor in heat-treated, soluble extract was sensitive to the action of the endopeptidase, trypsin. Therefore, the enzyme component/co-factor present in HTSE is either a protein or polypeptide. As HTSE restores formaldehyde dehydrogenase activity to dialysed, crude soluble extract, it is likely that the component/co-factor present in HTSE is dialysable, thus increasing the probability that it is a polypeptide rather than a protein. A number of enzymes are known to require polypeptides as activators or stabilising agents (Lin, Liu and Cheung, 1974; Dunaway and Segal, 1976). Whether this probable polypeptide is shown to be simply a co-factor of the enzyme or an actual component of formaldehyde dehydrogenase obviously will require the purification of the compound.

4. Properties of the Purified Formaldehyde Dehydrogenase from M. capsulatus (Bath)

Throughout the remainder of this Section, purified, non-dialysable, heat-sensitive component of the formaldehyde dehydrogenase from M. capsulatus (Bath) will be referred to as purified formaldehyde dehydrogenase to reduce the awkwardness of the terminology.

A. Products and stoichiometry of formaldehyde oxidation

Using purified formaldehyde dehydrogenase, supplemented with heat-treated, soluble extract, formate and NAD(P)H were the only products detected from formaldehyde oxidation by the colorimetric and spectrophotometric methods described in the Methods. Quantitatively, 3.90 µmol formaldehyde gave rise to 3.91 µmol formate and 3.36 µmol NADH, suggesting a 1:1:1 molar ratio.

B. Substrate specificity

The substrate specificity of the purified formaldehyde dehydrogenase was determined by the spectrophotometric assay for NADH, as described in the Methods. The following potential substrates were tested for oxidation at final assay concentrations of 0.25 mM, 1 mM, 2.5 mM and 20 mM: methanol, ethanol, 1-propanol, acetaldehyde (ethanal), propionaldehyde (propanal), butyraldehyde

(butanal), ethylene glycol, glycolaldehyde, glyoxal, glyoxylic acid, DL-glyceraldehyde and DL-glyceraldehyde 3-phosphate. Three of the compounds tested, glycolaldehyde, glyoxal and DL-glyceraldehyde were oxidised, suggesting that the enzyme is not formaldehyde specific. There was no evidence for the occurrence of the reverse reaction with 2.5 mM potassium formate and 0.5 mM NADH at pH 7.2.

When determined at 0.67 mM NAD^+, apparent K_m values of 0.68 mM, 7.0 mM, 0.075 mM and 2.0 mM were obtained for formaldehyde, glycolaldehyde, glyoxal and DL-glyceraldehyde respectively, as determined by the direct linear plot method of Eisenthal and Cornish-Bowden (1974). Similarly V_{max} values were calculated for the four enzyme substrates, formaldehyde (570 units), glycolaldehyde (73 units), glyoxal (281 units) and DL-glyceraldehyde (15 units).

These results show that the $NAD(P)^+$-linked formaldehyde dehydrogenase from M. capsulatus (Bath) is non-specific and can oxidise other aldehydes. However, as only formaldehyde, glyoxal, glycolaldehyde and glyceraldehyde are oxidised, the range of substrate aldehydes is limited compared with the variety of aldehydes oxidised by similar enzymes isolated from other methylotrophic bacteria, e.g. Pseudomonas AM1 (Johnson and Quayle, 1964), Pseudomonas aminovorans (Boulton and Large, 1977) and bacterium 4B6 (Colby and Zatman, 1973). The formaldehyde dehydrogenases from these other organisms could oxidise other n-alkane aldehydes like acetaldehyde, propionaldehyde, etc.

The apparent substrate specificity of the formaldehyde dehydrogenase of M. capsulatus (Bath) could be further restricted if the activity obtained with glyceraldehyde was the result of possible contamination by formaldehyde, since a 25 mM solution of DL-glyceraldehyde was found to contain approximately 60 μM formaldehyde, as determined by the method of Chrastil and Wilson (1975). The authors claim that no reactivity with DL-glyceraldehyde is obtained using their colorimetric assay.

The K_m and V_{max} values obtained with glyoxal could be greater than quoted because of the very narrow range of substrate concentrations which gave activity, probably due to potent substrate or product inhibition. Therefore the apparent greater affinity shown by the enzyme for glyoxal as compared with formaldehyde could be reduced or even reversed. On the evidence of K_m values, glycolaldehyde and glyceraldehyde appear to be much poorer substrates for the enzyme than formaldehyde.

C. Electron acceptors

Various artificial and physiological electron acceptors were tested with the purified formaldehyde dehydrogenase supplemented with heat-treated, soluble extract. Phenazine methosulphate (PMS) 0.7 mM, 2,6-dichlorophenol-indophenol (DCPIP) 0.2 mM, PMS 0.7 mM plus DCPIP 0.2 mM, potassium ferricyanide 2.0 mM, potassium ferricyanide 2.0 mM plus PMS 1.0 mM, potassium ferricyanide 2.0 mM plus NAD^+ 0.67 mM, and cytochrome c 0.1 mM did not act as electron acceptors. NAD^+ and $NADP^+$ both acted as electron acceptors as mentioned earlier, and when determined at 2.5 mM formaldehyde gave apparent K_m values of 0.063 mM and 0.155 mM respectively, as determined by the method of Eisenthal and Cornish-Bowden (1974).

D. Restoration of activity by thiol and other compounds

A number of thiol compounds were tested at different final concentrations (0.1 mM, 2.0 mM and 20 mM) to see if they could restore activity to the purified formaldehyde dehydrogenase, i.e. cancel the requirement for heat-treated, soluble extract. The assays were done as before, following the reduction of NAD^+ spectrophotometrically as described in the Methods, with the thiol compounds being preincubated with the enzyme protein (11 μg). The specific activity of the purified enzyme supplemented with heat-treated soluble extract (0.4 ml) was 4.28 units $(mg\ protein)^{-1}$. The thiol compounds tested were: reduced glutathione, sodium thioglycollate, dithiothreitol, British Anti-Lewisite, DL-cysteine and 2-mercaptoethanol. None of these compounds restored any activity to the purified formaldehyde dehydrogenase.

This lack of activity observed when the purified enzyme was supplemented with various thiol compounds suggested that there was no requirement for a thiol compound for enzyme activity and it is unlikely that any formyl/thiol compound is produced during formaldehyde oxidation in M. capsulatus (Bath). Hence M. capsulatus (Bath) appears dissimilar to the yeast Hansenula polymorpha which required reduced glutathione for activity and produces 5-formylglutathione from the oxidation of formaldehyde (van Dijken et al., 1976).

Various other compounds were tested to see if they could restore formaldehyde dehydrogenase activity to crude, soluble extract of M. capsulatus (Bath) which had been dialysed at 4^{o} C for 24 hours against potassium phosphate buffer (20 mM). The compounds and final assay concentrations were: adenosine 5'-monophosphate, sodium salt (1.0 mM); adenosine 5'-diphosphate, sodium salt (1.0 mM); adenosine 5'-triphosphate, disodium salt (1.0 mM); adenosine 3',5'-cyclic monophosphate, sodium salt (1.0 mM); $N^{6},O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphate, sodium salt (1.0 mM); pyridoxal 5-phosphate (0.1 mM); dl-L-tetrahydrofolic acid (0.1 mM); coenzyme B_{12} (0.05 mM). The specific activity of the dialysed soluble extract supplemented with heat-treated, soluble extract (0.4 ml) was 84.4 m units (mg protein)$^{-1}$. None of the above potential co-factors restored activity to dialysed extract of M. capsulatus (Bath), suggesting that it is unlikely that any of the common, low-molecular weight physiological co-factors, such as AMP, ATP, cyclic AMP, etc. are required for NAD(P)$^{+}$-linked formaldehyde dehydrogenase activity.

A number of metals were also tested as potential co-factors for formaldehyde dehydrogenase activity. Dialysed, soluble extract was assayed in the presence of various metal salts for formaldehyde dehydrogenase activity using similar spectrophotometric assays to those above. The following salts were tested at a final concentration of 1 mM: cobalt sulphate, nickel chloride, magnesium chloride, zinc sulphate, manganese chloride, calcium chloride, copper chloride,

ferrous chloride, disodium molybdate and ammonium sulphate. None of the metals restored formaldehyde dehydrogenase activity to the dialysed extract and in fact a few, e.g. Co^{++}, Mn^{++}, Cu^{++}, partially inhibited formaldehyde dehydrogenase activity when tested in assays containing crude, soluble extract which normally showed good formaldehyde dehydrogenase activity. These results suggest that the requisite component/co-factor for formaldehyde dehydrogenase activity, lost on dialysis of crude, soluble extract is probably not a metal, or at least not a metal alone.

E. Effect of pH and temperature

The optimum pH for $NAD(P)^+$-linked formaldehyde dehydrogenase activity was found to be pH 7.2 (Fig. 16). The enzyme appears equally active in phosphate buffer or Tris/HCl buffer at pH 7.2. Maximum enzyme activity occurred at 45° C (Fig. 17) with the reaction rate deteriorating rapidly at higher temperatures. After preincubating an assay mixture to 45° C as usual (3 minutes) no enzyme activity was lost if the mixture was then further incubated for at least 10 minutes prior to formaldehyde addition. Therefore it appears that the enzyme is relatively thermostable.

F. Molecular weight and subunit size

The molecular weight of the purified formaldehyde dehydrogenase from M. capsulatus (Bath) was determined by gel filtration using Sephadex G-200, as described in the Methods, and was estimated to be 115 000 (Fig. 18). SDS-polyacrylamide gel electrophoresis, as outlined in the Methods, gave a value of 57 000 (Fig. 19) for the estimation of the molecular weight of the enzyme subunits, suggesting that the enzyme is a dimer.

G. Absorption spectrum

The visible and ultraviolet absorption spectrum of the purified formaldehyde dehydrogenase revealed no characteristic absorption peaks other than the absorption at 280 nm, and a concentrated solution of the enzyme had no apparent colour.

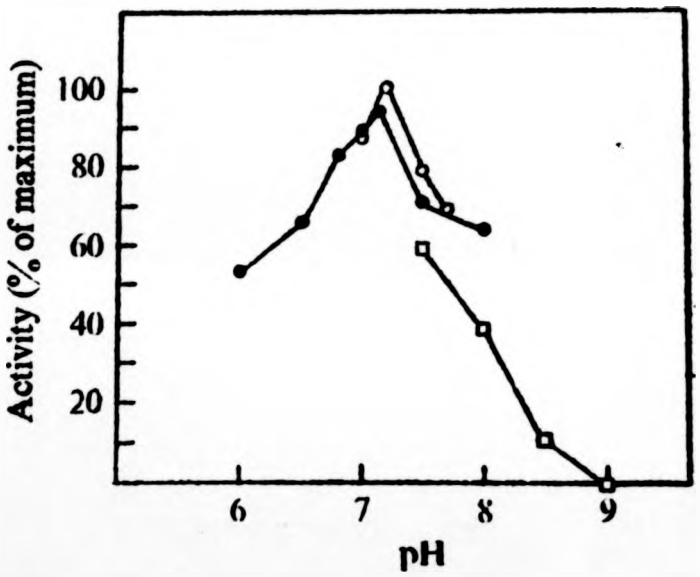
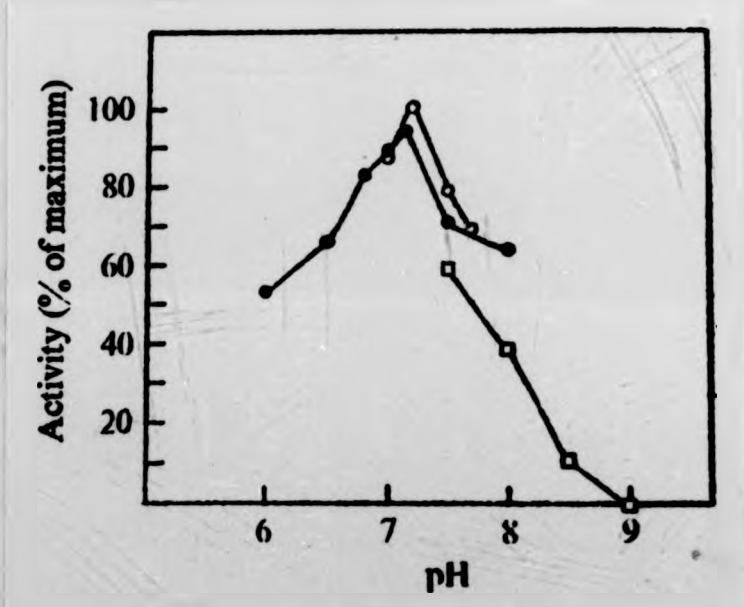


Fig. 16 Effect of pH on formaldehyde dehydrogenase activity. Activity was measured spectrophotometrically as described in Methods; the maximum specific activity was 4.28 units (mg protein)$^{-1}$.
All buffers were 20 mM: (●) potassium phosphate, (○) Tris/HCl, (□) tetrasodium pyrophosphate/HCl.



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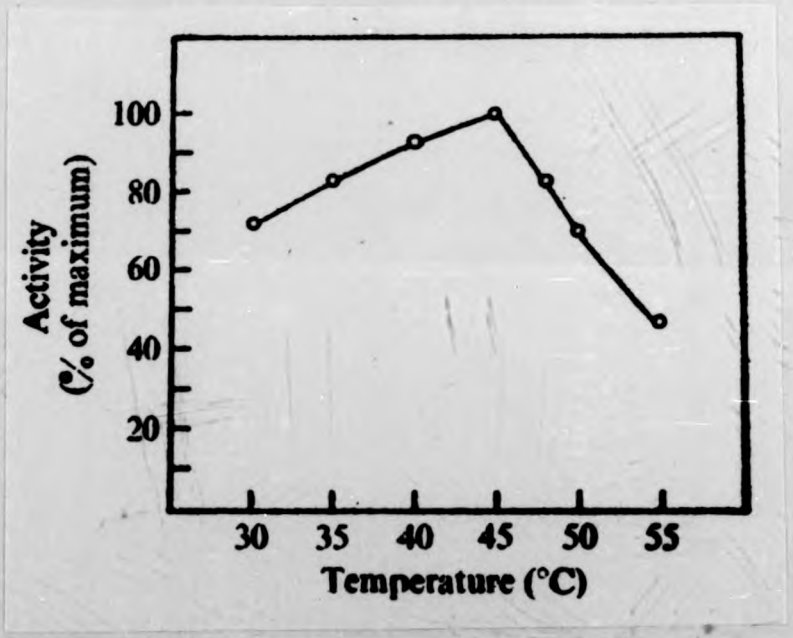

Fig. 17 Effect of temperature on formaldehyde dehydrogenase activity. Activity was measured spectrophotometrically as described in Methods; maximum specific activity was 4.28 units (mg protein)$^{-1}$.

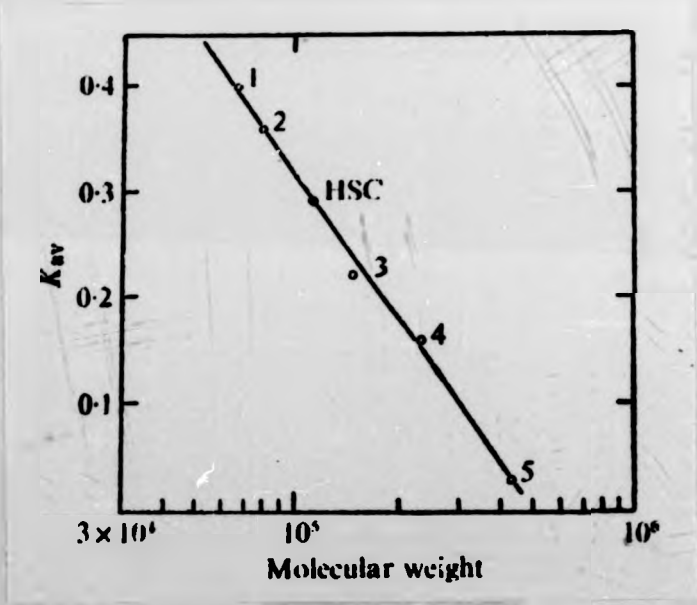

Fig. 18 Molecular weight estimation of the heat-sensitive component of formaldehyde dehydrogenase on a Sephadex G-200 column (see Methods for details). Molecular weight standards were: (1) malate dehydrogenase (67 000); (2) alkaline phosphatase (80 000); (3) alcohol dehydrogenase (150 000); (4) catalase (232 000); (5) ferritin (443 000).

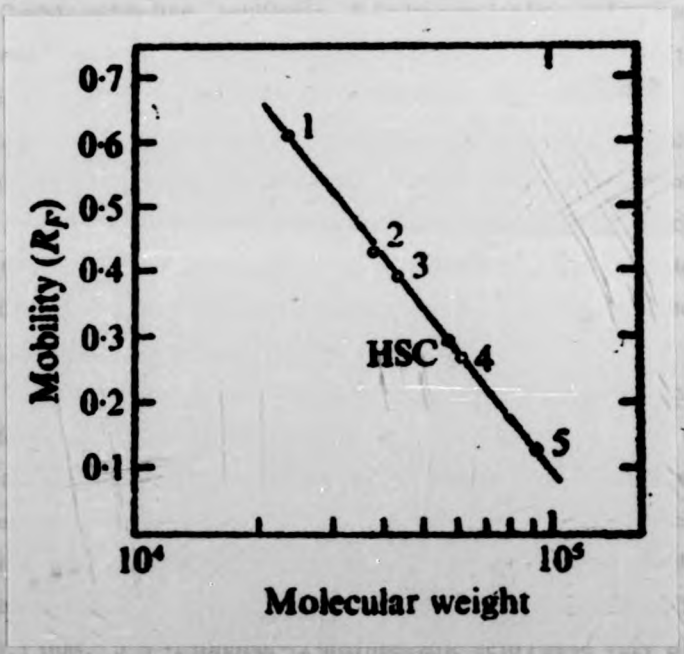

Fig. 19 Sodium dodecyl sulphate gel electrophoresis of the heat-sensitive component of formaldehyde dehydrogenase (see Methods for details). Molecular weight standards were (1) α-chymotrypsinogen A (23 700); (2) alcohol dehydrogenase (375 000); (3) ovalbumin (43 000); (4) catalase (60 000); (5) phosphorylase a (92 500).

H. Inhibition studies

Various metal-binding/metal chelating agents were tested as potential inhibitors of enzyme activity at a final concentration of 2.5 mM in routine spectrophotometric assays containing purified formaldehyde dehydrogenase, heat-treated soluble extract, NAD^+ etc. The compounds tested were: thiourea, thioacetamide, diethyldithiocarbamate, 2,2'-bipyridine, neocuproine, imidazole, 1,10-phenanthroline, acriflavin, 8-hydroxyquinoline and potassium cyanide. Only 2,2'-bipyridine (10% inhibition) and thiourea (10% inhibition) caused any inhibition, suggesting either the non-involvement of a metal(s) in the enzyme or that the enzyme contains a metal-ion complex, well shielded from attack by such agents.

A number of substrate analogues (methanol, ethanol, 1-propanol, acetaldehyde and propionaldehyde) were also tested as potential inhibitors at a final concentration of 2.5 mM, but only methanol (5% inhibition) had any inhibitory effect.

Table 33 shows the effect of thiol reagents at various concentrations on formaldehyde dehydrogenase activity. Iodoacetic acid and iodoacetamide caused little inhibition at the concentrations used, whereas N-ethylmaleimide caused around 60% inhibition at a final concentration of 2.0 mM. p-Hydroxymercuribenzoate completely inhibited activity at a final concentration of 1.0 mM but was totally ineffective at 0.1 mM. 5,5'-Dithiobis-(2-nitrobenzoic acid) gave very little inhibition at a concentration of 0.1 mM, but could not be tested at a higher concentration due to absorption by the compound at 340 nm.

The effect of thiol reagents on formaldehyde dehydrogenase activity was variable, as the results show. The total inhibition obtained with p-hydroxymercuribenzoate at a final concentration of 1.0 mM was not surprising, for this potent thiol reagent is known to inhibit enzymes at μM concentrations (Trudinger, 1969). The weak inhibitions observed in the presence of the less potent thiol reagents, iodoacetic acid and iodoacetamide, support the view suggested previously by the negative thiol co-factor results, that no formyl/thiol

TABLE 33

Inhibition of formaldehyde dehydrogenase activity by thiol reagents

	Final concentration (mM)	Inhibition %
Iodoacetic acid	2.0	14
	1.0	8
	0.1	0
Iodoacetamide	2.0	14
	1.0	28
	0.1	0
N-ethylmaleimide	2.0	59
	1.0	50
	0.1	0
p-Hydroxymercuribenzoate	1.0	100
	0.1	0
5,5'-Dithiobis-(2-nitrobenzoic acid)	0.1	9

Activity was measured spectrophotometrically as described in Methods; each potential inhibitor was preincubated with the enzyme for 5 minutes. The uninhibited specific activity of the enzyme was 4.28 units (mg protein)$^{-1}$.

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compound is involved as an intermediate during the oxidation of formaldehyde, since one would expect potent inhibition of a reaction involving such a compound to occur with the final concentrations of reagents tested. However, considering the results with the thiol reagents in general, there are obviously thiol groups present in the formaldehyde dehydrogenase enzyme which are vulnerable to the action of thiol reagents, but this would not be unexpected with the enzyme having a molecular weight of over 10^5.

I. Activity stain

After polyacrylamide gel electrophoresis of the purified enzyme, the unstained gels were immersed in an active stain or Coomassie brilliant blue (R) stain as described in the Methods. Both the gel incubated at 45° C for 30 minutes in the complete activity stain and the gel incubated in similar stain at 30° C for 90 minutes showed a single actively stained band (Fig. 20). The actively stained bands had identical electrophoretic mobility to the single band found on identical gels stained with Coomassie blue. The gel incubated with the activity stain at 30° C required three times as long as a similar gel incubated at 45° C to develop a band of equal intensity. The gel incubated at 45° C with the activity stain minus formaldehyde gave no equivalent band.

J. Concluding remarks on the nature of the formaldehyde dehydrogenase from M. capsulatus (Bath)

It appears from the preceding results that M. capsulatus (Bath) contains an $NAD(P)^+$-linked formaldehyde dehydrogenase which requires the presence of a low molecular weight polypeptide for activity. It can oxidise a few other aldehyde compounds, other than formaldehyde, and no thiol intermediate appears to be involved during formaldehyde oxidation. The enzyme is resistant to a wide range of potential inhibitors.

5. Enzymic Evidence for a Cyclic Scheme for the Complete Oxidation of Formaldehyde in M. capsulatus (Bath)

The introduction to this Section explained that five specific enzymes were required by an organism for it to possess the enzymic potential for a cyclic scheme for the complete oxidation of formaldehyde to carbon dioxide, as described by Strøm et al. (1974) and Colby and Zatman (1975). These enzymes are glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glucose 6-phosphate isomerase, hexulose-phosphate synthase and hexulose-phosphate isomerase, of which the latter two are always present in methane-utilising bacteria possessing any variant of the

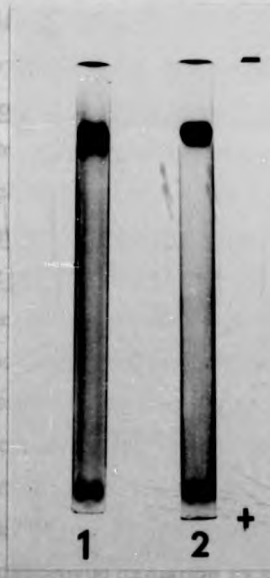

Fig. 20 **Polyacrylamide gel electrophoresis of thepurified formaldehyde dehydrogenase heat-sensitive component. Both gels were actively stained (see Methods for details): (1) 20 µg protein, incubated for 30 minutes at 45° C; (2) 20 µg protein, incubated for 90 minutes at 30° C.**

ribulose monophosphate (RMP) cycle of formaldehyde fixation. Therefore, as M. capsulatus (Bath) possesses the RMP cycle of formaldehyde assimilation, it was decided to assay crude, soluble extracts for activity of the other three enzymes which comprise the cyclic scheme. The assays were done as described in the Methods and the results are incorporated into Table 34 which also shows the reported activities for the three enzymes as found in extracts of other methane-oxidising bacteria. The results obtained for M. capsulatus (Bath) during these studies are listed at the foot of the Table.

The enzymic activities previously reported for a number of methane-utilisers show that Methylomonas methanica, Methylomonas albus (BG8) and Methylococcus capsulatus (Texas), which use the RMP cycle of formaldehyde assimilation, all possess the enzymic potential for a cyclic scheme of formaldehyde oxidation. Extracts of Methylococcus minimus and Methylococcus capsulatus (Bath) were shown by Davey et al. (1972) to contain glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity, but unfortunately they did not test for glucose 6-phosphate isomerase activity as the presence of this enzyme would have shown that both these organisms (RMP cycle positive) were potentially capable of the above cyclic scheme for formaldehyde oxidation. Methylosinus trichosporium (OB3b) does not possess the enzymic apparatus for a cyclic scheme of formaldehyde oxidation as expected because, irrespective of the three enzymes listed in Table 34, all methane-oxidising bacteria which use the serine pathway for formaldehyde assimilation almost certainly lack hexulose-phosphate synthase and hexulose-phosphate isomerase.

The results obtained for extracts of M. capsulatus (Bath) during these studies show that when assayed at 45^{o} C with $NADP^{+}$ as electron acceptor, all three enzymes were present in high activity. When $NADP^{+}$ was replaced by NAD^{+} approximately 63% of the glucose 6-phosphate dehydrogenase activity observed with $NADP^{+}$

TABLE 34 Specific activities of key enzymes in cyclic scheme for complete oxidation of formaldehyde in a number of obligate methane-oxidising bacteria

	Ref.	Glucose-6-phosphate dehydrogenase* NADP+	Glucose-6-phosphate dehydrogenase* NAD+	6-Phosphogluconate dehydrogenase* NADP+	6-Phosphogluconate dehydrogenase* NAD+	Glucose-6-phosphate isomerase*
Methylomonas methanica	1	21	-	13	-	31
Methylomonas methanica	2	14	n.d.	14	n.d.	-
Methylosinus trichosporium (OB3b)	1	2	-	n.d.	-	17
Methylosinus trichosporium (OB3b)	2	n.d.	n.d.	n.d.	n.d.	6
Methylomonas albus (BG8)	2	12	n.d.	36	n.d.	15
Methylococcus minimus	2	10	n.d.	10	n.d.	-
Methylococcus capsulatus (Texas)	1	11	-	15	-	74
Methylococcus capsulatus (Bath)	2	7	n.d.	28	n.d.	-
	Inc. temp.					
Methylococcus capsulatus (Bath)	45°C	257 (pH 8.0)	161 (pH 8.0)	81 (pH 7.6)	8	434 (pH 7.4)
	30°C	16	20	8	n.d.	-

* Enzymes assayed as described in Methods. Specific activities expressed as nmol NAD(P)H formed min^{-1} (mg protein)$^{-1}$.

n.d., not detected. -, not tested.

† Reference 1, Strøm et al. (1974); Reference 2, Davey et al. (1972).

was obtained, whereas only 10% of the 6-phosphogluconate dehydrogenase activity observed with $NADP^+$ was obtained in the presence of NAD^+. The relatively poor activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase obtained with extracts of M. capsulatus (Bath) by Davey et al. (1972) could possibly be explained by the fact that they did their assays at 35^o C as opposed to 45^o C, the optimum growth temperature for the organism. This idea is supported by the enzyme activities obtained with extracts of M. capsulatus (Bath) when incubated at 30^o C during these studies (Table 34).

The following values of hexulose-phosphate synthase and hexulose-phosphate isomerase specific activities in crude, soluble extracts of M. capsulatus (Bath) were kindly provided by Professor Quayle and co-workers: hexulose-phosphate synthase, 5.08 units $(mg\ protein)^{-1}$; hexulose-phosphate isomerase, 0.28 units $(mg\ protein)^{-1}$. The assays were done as described by Bamforth and Quayle (1977). These results in conjunction with the data presented in Table 34 show that M. capsulatus (Bath) possesses the enzymic potential to operate a cyclic scheme for the complete oxidation of formaldehyde as shown in Fig. 13.

6. The Potential Importance of the Routes Available for the Complete Oxidation of Formaldehyde in M. capsulatus (Bath)

If M. capsulatus (Bath) did not possess the capability to generate two molecules of NAD(P)H from the complete oxidation of methanol, then the organism would be unable to generate sufficient NAD(P)H for cellular biosynthesis or to allow formaldehyde to be assimilated, as all methane would have to be completely oxidised to carbon dioxide to generate the requisite NAD(P)H for the initial oxidation, from the NAD^+-linked formate dehydrogenase. Therefore, as M. capsulatus (Bath) grows well on methane as sole source of carbon and energy, it is assumed that at least two molecules of NAD(P)H are generated from

the complete oxidation of 1 molecule of methane to carbon dioxide. Even if two molecules of NAD(P)H are generated per molecule of methane oxidised to carbon dioxide, M. capsulatus (Bath), when growing on methane can be exclusively or substantially NAD(P)H-limited (see Section I). This situation arises due to the organism possessing an NAD(P)H-dependent methane mono-oxygenase and is accentuated if the P/O ratio of the reducing equivalent (other than NAD(P)H) derived from methanol oxidation is 1 or more, thereby relieving the cell of any energy deficiency resulting from cellular biosynthesis (see Section I).

There are three ways in which M. capsulatus (Bath) could derive the two molecules of NADH required for growth on methane: (i) by possessing an NAD^+-linked formaldehyde dehydrogenase, (ii) by possessing an operative cyclic scheme for formaldehyde oxidation and (iii) by generating NADH via reversed electron flow. Both (i) and (ii) have been shown to be present in cell-free extracts of M. capsulatus (Bath) (see above), whereas no conclusive evidence for the third possibility has been obtained. This leaves (i) and (ii) as the likely routes for the generation of the requisite NADH.

Although the enzymic potential for the cyclic scheme has been shown to be present in cell-free extracts of M. capsulatus (Bath) (see above), no direct evidence for the operation of such a scheme in vivo has been shown. Also the two enzymes responsible for NAD^+ reduction in the cycle, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were approximately 2 and 10 times more active respectively with $NADP^+$ than with NAD^+. Therefore, in view of the fact that crude cell-free preparations of methane mono-oxygenase from M. capsulatus (Bath) were half as active with NADPH as with NADH (Colby and Dalton, 1976), it would appear that a cyclic scheme, if operative in vivo, would not be particularly efficient in generating reductant for methane mono-oxygenase activity. Such a scheme could be useful in supplying NADPH for biosynthetic purposes. The requisite reducing power for methane oxidation is probably primarily generated from the complete oxidation of formaldehyde to carbon dioxide, catalysed by an $NAD(P)^+$-linked formaldehyde dehydrogenase and NAD^+-linked formate dehydrogenase.

SECTION VII Final Comments

The primary aim of the research contained in this thesis was to extend the understanding of the biochemistry of the methane-utilising bacterium, Methylococcus capsulatus (Bath), both by conventional biochemical techniques and by developing a new approach to the problem using non-growth substrate oxidations. The research was concentrated on the pathway of methane oxidation (eqn. 1) and the enzymes involved, of which the methane-oxidising enzyme was of particular interest.

The results obtained have provided useful data on the nature and mechanism of the enzyme responsible for the initial oxidation of methane to methanol, and have shown almost conclusively that this enzyme is in fact a mono-oxygenase. Other properties of this enzyme outlined in this thesis have shown it to be an enzyme of remarkable versatility with respect to substrates and possibly with a reaction mechanism which could prove to be of great interest.

The oxidation studies of the non-growth substrates proved very valuable not only in understanding the nature of the methane mono-oxygenase but in the uncovering of an $NAD(P)^+$-linked formaldehyde dehydrogenase in Methylococcus capsulatus (Bath), which would have remained undetected but for the experiments done on reductant regeneration for non-growth substrate oxidation.

Probably the most rewarding aspect of the research was the creation of new areas of both academic and applied research. The discovery of a number of cometabolic substrates and substrates fortuitously oxidised by the methane mono-oxygenase has presented an important biochemical tool for the study of the fundamental biochemistry of methane oxidation in Methylococcus capsulatus (Bath). These compounds could undoubtedly play an important role in the elucidation of the mechanism of the methane mono-oxygenase by studying product stereochemistry, enzyme kinetics, electron recycling for mono-oxygenase activity and by simply providing convenient assays for the enzyme. This versatility of

substrate oxidation has caused the need for a reappraisal of the role methane-utilising organisms play in the environment. For example, the importance and the potential of this group of organisms in environmental situations to degrade or detoxify pollutants such as pesticides, now requires revaluation.

The discovery of an $NAD(P)^+$-linked formaldehyde dehydrogenase meant that there are now three known enzymes in Methylococcus capsulatus (Bath) which can utilise formaldehyde as a substrate, i.e. methanol dehydrogenase, formaldehyde dehydrogenase and hexulose phosphate synthase. Obviously the way in which these enzymes are regulated and controlled (if at all) will be very important with respect to growth yields and energetics. The inter-relationship of these formaldehyde-utilising enzymes poses a very interesting problem and one which requires more research.

The ability of the methane mono-oxygenase from Methylococcus capsulatus (Bath) to oxidise propylene to propylene oxide led to the initiation of new lines of applied research. Propylene oxide is presently produced industrially by relatively expensive chemical processes, therefore it was thought that a simple biological system could be of value. This idea is now being developed in our laboratory and various biological systems, using Methylococcus capsulatus (Bath) and other methane-oxidisers, are presently being evaluated as potential biotransformation systems. These include systems incorporating immobilised whole cells, cell-free extracts or purified enzymes and continuous culture cometabolic studies.

One problem that arose concerning the use of a biotransformation based on the methane mono-oxygenase from Methylococcus capsulatus (Bath) was the fact that any mono-oxygenase oxidation would require NADH as a co-substrate. The cost of supplying stoichiometric amounts of NADH for any such process would be prohibitive, hence systems have to be developed which incorporate either enzyme generation of NADH from a catalytic amount of NAD^+ or a novel

system for reducing the reductase of the methane mono-oxygenase. Research into the generation of reductant for the methane mono-oxygenase has already started, e.g. enzymic recycling of electrons, electrochemical reduction, immobilised co-factors, etc.

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APPENDIX 1

Cometabolism by an Obligate Methanotrophic Bacterium, *Methylococcus capsulatus*. By D.I. STIRLING and H. DALTON (Department of Biological Sciences, University of Warwick, Coventry CV4 7AL).

The term "cometabolism" was designated by Jensen (1) to describe the phenomenon in which micro-organisms can metabolise a compound without having the ability to utilise, for growth, any products arising. The definition of cometabolism has since been restricted to imply the oxidation of a non-growth substrate while actively growing on a utilisable carbon and energy source (2). Unfortunately, both terms are used rather ambiguously in the literature to cover either one or both of the above phenomena. The relevance and inadequacies of these terms, with respect to obligate methanotrophic bacteria will be discussed.

A few non-growth substrates for obligate methanotrophic bacteria have been reported ie. ethane, ethanol, acetaldehyde (3), carbon monoxide (4), ethylene (5), methyl bromide (6). Other potential non-growth substrates together with the existing list were tested for cometabolism by the methane mono-oxygenase system of *Methylococcus capsulatus* (Bath). A number of possible non-growth substrates of interest were found ie. methyl chloride, methyl bromide, dichloromethane, ethyl bromide and carbon monoxide, of which methyl bromide and carbon monoxide were chosen for a more detailed study.

Initial whole-cell studies with *M. capsulatus* (Bath) strongly indicated that both carbon monoxide and methyl bromide are oxidised by the methane mono-oxygenase system. Although methyl bromide was oxidised by resting-cell suspensions, carbon monoxide was only oxidised in the presence of a growth substrate eg. methanol, formaldehyde, potassium formate or with the non-growth substrate, ethanol. Methane could not be used as the growth substrate since both carbon monoxide and methyl bromide are inhibitory to methane oxidation. To ascertain whether methyl bromide was in fact a true non-growth substrate and if so, the true metabolic nature of both it and carbon monoxide, further studies were undertaken using additional whole-cell and cell-free extracts studies.

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Effect of Metal-Binding Agents and Other Compounds on Methane Oxidation by Two Strains of *Methylococcus capsulatus*

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Abstract. Inhibition studies of methane mono-oxygenase activity in whole cell suspensions of *Methylococcus capsulatus* (Texas) and *M. capsulatus* (Bath) were performed and the results compared. The inhibition pattern for *M. capsulatus* (Bath) was not only substantially different from the pattern obtained with *M. capsulatus* (Texas) but also very limited in the number of potent inhibitors specific for methane oxidation. To confirm the whole cell results of *M. capsulatus* (Bath) similar experiments were done using cell-free extracts. It was found that only acetylene (100% inhibition) and 8-hydroxyquinoline (71%) significantly inhibited methane oxidation, verifying the restricted inhibition pattern found with the whole cell suspensions. Eight acetylenic compounds were tested for specific inhibition of methane oxidation by whole cells and cell-free extracts of *M. capsulatus* (Bath). Only two compounds (acetylene and propyne) gave 100% inhibition in both cases with three other compounds (but-1-yne, but-2-yne and propyn-1-ol) giving weaker inhibitions. The inhibition pattern of methane oxidation by whole cell suspensions and cell-free extracts of *M. capsulatus* (Bath) is discussed and reasons for the prominent results are suggested.

Key words: *Methylococcus capsulatus* – Methane oxidation – Methanol oxidation – Metal binding compounds – Acetylenic compounds – Specific inhibitors.

Obligate methylotrophic bacteria have been shown to incorporate gaseous oxygen during the oxidation of methane to methanol (Higgins and Quayle, 1970). The possible involvement of a mono-oxygenase in this reaction was first postulated after the demonstration of methane-stimulated NADH oxidation by crude, cell-free extracts of *Methylococcus capsulatus* (Texas) (Ribbons and Michalover, 1970) and *Methylomonas methanica* (Ferenci, 1974; Colby et al., 1975). Recent attempts with *Methylosinus trichosporium* OB3B (Tonge et al., 1975) and *M. capsulatus* (Bath) (Colby and Dalton, 1976) to purify the methane mono-oxygenase system from crude cell-free extracts has met with some success. Interestingly, only the methane mono-oxygenase system from *M. capsulatus* (Bath) was found in the soluble fractions (Colby and Dalton, 1976), whereas the rest, including *M. capsulatus* (Texas), were found associated with the particulate membrane fractions.

Preliminary experiments concerning the effect of various inhibitors on the soluble mono-oxygenase system from *M. capsulatus* (Bath) suggested a conflicting pattern of results (Colby and Dalton, 1976) compared with similar experiments performed not only on different species of methane-oxidising organisms (Hubley et al., 1975; Patel et al., 1976) but on the Texas strain of *M. capsulatus* (Ribbons, 1975; Patel et al., 1976).

This report distinguishes between the inhibition patterns of *M. capsulatus* (Bath) and *M. capsulatus* (Texas), discusses the differences obtained, and attempts to elucidate reasons for the various inhibitions of methane oxidation shown by *M. capsulatus* (Bath).

MATERIALS AND METHODS

Materials. Thiourea, thioacetamide, thiosemicarbazide, 2,2-dipyridyl, neocuproine, amobarbital, imidazole, acriflavin, 1,10-phenanthroline, antimycin A, diethyldithiocarbamate, and NADH (grade 3) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., 8-hydroxyquinoline, potassium cyanide, pyridine (analar), methanol (aristar), propyn-1-ol, bromomethane, propyne (96%) and but-1-yne (95%) were obtained from BDH Chemicals, Poole, Dorset, U.K. Phenol (AR), aniline (AR) were obtained from Fisons Scientific Apparatus, Loughborough, Leicestershire. Methane (technical grade) and carbon monoxide (research grade) were obtained from British Oxygen Co., London, S.W.19, U.K. Acetylene (99.6%) was obtained from

Cambrian Chemicals, Croydon, Surrey, U.K. But-2-yne was obtained from Kodak (ICN), Ltd., Kirby, Liverpool, U.K. Propargylamine-HCl and dimethylaminopropyne were obtained from Aldrich Chemical Co. Ltd., Wembley, Middlesex, U.K. o-Amino-phenol and ferron (8-hydroxy-7-iodo-5-quinoline-sulphonic acid) were gifts from Dr. J. R. Dilworth, ARC Unit of Nitrogen Fixation, University of Sussex, U.K.

Growth of Organisms and Preparation of Extract. Methylococcus capsulatus (TRMC) (Foster and Davis, 1966) was grown as a batch culture at 37° C in a 5 l fermenter on ammonium mineral salts (AMS) medium (Whittenbury et al., 1970) with methane (20%, v/v in air) as the carbon source. During late exponential growth phase 25–50 ml samples were removed and centrifuged (5000 × g for 10 min). Pellets were washed once with 20 mM sodium phosphate buffer, pH 6.8, resuspended in the same buffer and kept on ice, prior to use.

Methylococcus capsulatus (Bath) (Whittenbury et al., 1970) was grown at 45° C in continuous culture at a dilution rate of 0.05 h^{-1} on AMS medium, with methane (20%, v/v in air) as the carbon source. Whole cell suspensions were prepared as above for strain TRMC. Cell-free extracts (S_{160} fraction) were prepared as described by Colby and Dalton (1976). The protein concentration of the extract used was approximately 50 mg/ml as determined by the Lowry et al. (1951) technique using dried crystalline bovine plasma albumin as the standard.

Whole Cell Experiments. Respiration studies were carried out in a Clark type oxygen electrode (Rank Bros., Bottisham, Cambridge) with a reaction chamber of 3 ml capacity. The correct assay temperatures (45° C for strain Bath and 37° C for strain TRMC) were maintained by means of a water jacket and circulator. All inhibitors were suspended in 20 mM sodium phosphate buffer, pH 6.8, and when added to the electrode gave a final concentration of 1 mM, except when mentioned. Methane was added as a saturated buffer solution to give a final concentration of 0.12 mM and methanol was added to give a final concentration of 1 mM. The reaction vessel contained: 60 µM sodium phosphate buffer, pH 6.8; cell suspension to give a final optical density of 2 (0.815 mg dry cell weight) prepared as described above; and the substrates and inhibitors at the final concentrations indicated.

Cell-Free Extract Experiments. Two techniques were used to evaluate the activity of the cell-free methane mono-oxygenase system at 45° C in the presence of various inhibitors: (1) methanol accumulation from methane; (2) bromomethane disappearance. Both were monitored on a gas chromatograph (Pye series 104, flame ionization detection) equipped with a 2.1 m column (internal diameter 4 mm) of Poropak Q (Water Associates, Milford, Massachusetts, U.S.A.) with N_2 (30 ml/min) as carrier gas. The experimental techniques were essentially as described by Colby et al. (1975) and Colby and Dalton (1976).

RESULTS AND DISCUSSION

The results of our *Methylococcus capsulatus* strain Texas (TRMC) whole cell inhibition studies (Table 1) correlate well with those of Patel et al. (1976). Only amobarbital, phenol, pyridine, aniline and to a lesser extent acriflavin, imidazole and antimycin A gave little inhibition of methane oxidation while virtually total inhibition was shown by all the remaining compounds. The potent inhibitors are mostly metal ion chelators with all major types of ligand combination being represented, i.e. sulphur-nitrogen, oxygen-nitrogen, nitrogen-nitrogen. This pattern of inhibition supports the viewpoint of Patel et al. (1976) that the oxidation of methane by TRMC involves at least one metal ion. Very similar inhibition patterns have been found for other methane-oxidising organisms i.e. *Methylosinus trichosporium* OB3B (Hubley et al., 1975; Patel et al., 1976) and *Methylomonas methanica* (Colby et al., 1975) suggesting the involvement of a similar type of mono-oxygenase in these organisms.

In order to verify that the inhibition patterns observed were due to a specific inhibition of methane mono-oxygenases and not to a general inhibition of respiration, it was necessary to test the effect of the compounds on the oxidation of methanol. A specific inhibitor of methane mono-oxygenase would not be expected to inhibit methanol oxidation also.

Of the inhibitors tested only o-aminophenol (36% inhibition), ferron (65% inhibition) and potassium cyanide (100% inhibition) affected methanol oxidation in whole cells of TRMC. The remaining compounds showed at best a 19% inhibition. This suggested that the majority of the inhibitors do act on the initial enzymatic oxygenation of methane to methanol in TRMC and that the enzyme contains at least one metal ion. It is not possible, however, to identify the specific metals involved in the microbial oxidation of methane, but on examination of the metal complex stabilisation constants for the various compounds it appears that both copper and iron are the metals most readily complexed by the reagents used.

The observed stimulation of methanol oxidation by carbon monoxide is thought to be due to methanol acting as an electron donor for CO oxidation thus giving rise to an additive effect with respect to oxygen uptake (cf. Ferenci, 1974).

In contrast to the results observed with TRMC the inhibition pattern of *M. capsulatus* strain Bath (MC) whole cells shows a much more restricted effect by the compounds (Table 1). Of the metal chelators tested only 8-hydroxyquinoline and diethyldithiocarbamate showed 100% inhibition whereas thiourea, α,α-dipyridyl and neocuproine were the only other chelators to show any significant inhibition. Another five compounds gave complete inhibition, i.e. o-aminophenol, ferron, potassium cyanide, carbon monoxide and acetylene, of which both ferron and potassium cyanide completely inhibited methanol oxidation also, and so cannot be considered as specific inhibitors of the methane mono-oxygenase.

Before drawing any conclusions from these whole cell results we tested the effect of the same potential inhibitors on cell-free extracts of MC, containing good methane mono-oxygenase activity (Table 2). Potassium cyanide (0.5 µmol) was present in all the assays as it

Table 1. The effect of various potential inhibitors on methane and methanol oxidation by whole cell suspensions of *Methylococcus capsulatus* (Texas) (A) and *Methylococcus capsulatus* (Bath) (B)

Inhibitor	Rate of methane oxidation (% control)	Rate of methane oxidation (% control)	Rate of methanol oxidation (% control)	Rate of methanol oxidation (% control)
	A[a]	B[b]	A[c]	B[d]
None	100	100	100	100
Thiourea	3	50	86	82
Thioacetamide	0	89	117	93
Thiosemicarbazide	5	70	106	100
Diethyldithiocarbamate	0	0	115	60
α,α-Dipyridyl	3	42	97	98
Neocuproine	0	57	81	94
Amobarbital	96	81	120	92
8-Hydroxyquinoline	0	0	100	80
1,10-Phenanthroline	0	62	120	68
Imidazole	37	79	100	112
Acriflavin	52	77	100	97
Antimycin A	65	100	115	98
Aniline	100	100	100	100
Phenol	80	70	100	100
o-Aminophenol	18	0	64	60
Ferron	0	0	35	0
Potassium cyanide	0	0	0	0
Carbon monoxide[e]	0	0	200	193
Pyridine	92	71	100	100
Acetylene[e]	0	0	100	100

[a] The uninhibited rate of methane oxidation was 273 nmoles oxygen consumed · min^{-1} (mg dry cell weight)$^{-1}$

[b] The uninhibited rate of methane oxidation was 225 nmoles oxygen consumed · min^{-1} (mg dry cell weight)$^{-1}$

[c] The uninhibited rate of methanol oxidation was 219 nmoles oxygen consumed · min^{-1} (mg dry cell weight)$^{-1}$

[d] The uninhibited rate of methanol oxidation was 170 nmoles oxygen consumed · min^{-1} (mg dry cell weight)$^{-1}$

[e] These gaseous compounds were added (0.2 ml) as saturated phosphate buffer (0.2 μmoles CO; 7.5 μmoles C_2H_2)

Table 2. The effect of various potential inhibitors on methane oxidation by cell-free extracts of *Methylococcus capsulatus* (Bath)

Inhibitor	Rate of methanol accumulation (% control[a])
None	100
Thiourea	90
Thioacetamide	93
Thiosemicarbazide	92
Diethyldithiocarbamate	100
α,α-Dipyridyl	99
Neocuproine	100
Amobarbital	100
8-Hydroxyquinoline	29
Imidazole	81
Acriflavin	84
Aniline	100
Phenol	84
o-Aminophenol	88
Ferron	62
Carbon monoxide[b]	95
Pyridine	84
Acetylene[c]	0

[a] The uninhibited rate of methane oxidation as determined by methanol accumulation (see "Materials and Methods") was 53 nmoles methanol produced · min^{-1} (mg of extract protein)$^{-1}$

[b] Carbon monoxide was present in the gas phase as 15% v/v in air

[c] Acetylene was present in the gas phase as 3% v/v in air Reaction mixture (final vol. 1 ml): 20 μmoles phosphate; 5 μmoles NADH; 0.5 μmoles KCN; 5 mg extract protein; 3 ml gas phase CH_4; 0.1 μmoles inhibitor (where present)

had been previously shown that cyanide inhibits residual methanol oxidase activity but does not inhibit cell-free methane mono-oxygenase activity (Colby and Dalton, 1976). Methanol dehydrogenase, which is present in this organism and requires ammonium chloride, phenazine methosulphate and pH 9 for maximum activity, was completely inactive in the assay system.

Only 2 compounds significantly inhibited methanol formation, acetylene (100%) and 8-hydroxyquinoline (71%), while the others gave generally very little inhibition. Comparing these cell-free results with the whole cell results (Table 1), only the above 2 compounds still gave strong inhibition out of the seven compounds which strongly inhibited whole cell methane oxidation. Diethyldithiocarbamate completely inhibited methane oxidation and showed relatively poor inhibition of methanol oxidation in whole cells (Table 1) but gave no inhibition of methane oxidation in cell-free extracts (Table 2). As the requisite reducing power (NADH) for methane oxidation to methanol was supplied in all the cell-free assays it is possible that the inhibition shown by diethylthiocarbamate in whole cells is due to an interruption in the re-generation of NADH rather than a direct inhibition of the mono-oxygenase. A similar explanation could be given for the o-aminophenol results, whereas a less specific cellular disruption occurs with ferron, which, with respect to the methane mono-oxygenase, can be partly

overcome using cell-free extracts plus exogenous NADH. Cyanide, as mentioned above, has been found to be non-inhibitory to the cell-free methane mono-oxygenase activity of MC, but presumably acts as an inhibitor of oxygen uptake with either methane or methanol as a substrate through its ability to complex with cytochrome oxidase. Acetylene remains a potent inhibitor with cell-free extracts and has been previously reported as a strong, specific inhibitor of methane oxidation in MC (Dalton and Whittenbury, 1976). Carbon monoxide poses an interesting problem as it appears from the whole cell data to be a potent, specific inhibitor of methane oxidation but when tested with cell-free preparations gave negligible inhibition. An attempt to explain this problem is complicated further by the fact that CO can be oxidised to carbon dioxide by whole cells and cell-free extracts of MC but only in the presence of a suitable electron donor, such as methanol, formaldehyde or formate (unpublished work). This observation coupled to the evidence given by Hubley et al. (1974) and Ferenci et al. (1975) suggests that the oxidation of CO to CO_2 is probably due to a mono-oxygenase. With this knowledge the inhibition of methane oxidation by CO in whole cells can possible be explained in three ways. Firstly, if CO was being oxidised by an NADH-dependent enzyme other than the methane mono-oxygenase, it would very rapidly deplete the cell of any endogenous reducing power available therefore terminating all mono-oxygenase activity. Secondly, CO could inactivate the methane mono-oxygenase either by competing with methane for the binding site of the enzyme complex or by binding to and inactivating another component of the multi-component methane mono-oxygenase system reported to be present in MC (Colby and Dalton, 1976). Thirdly, by interrupting the re-generation of NADH during the complete oxidation of methane, therefore starving the methane mono-oxygenase of the required reducing power. The inability of CO to inhibit methane oxidation in cell-free systems suggests that CO is not actively competing with methane for the methane mono-oxygenase, therefore favouring the first and third explanation above. However, no possibility can be ruled out until the methane mono-oxygenase is purified and tested for CO oxidation and its inhibition of methane oxidation. Similar results concerning the effect of CO on methane oxidation and CO oxidation by *Methylomonas methanica* have been reported by Ferenci (1974). Although CO is again a potent inhibitor of methane oxidation by whole cells but ineffective on cell-free systems Ferenci concluded in a later paper that CO is in fact oxidised by the methane mono-oxygenase (Ferenci et al., 1975). However, Colby et al. (1975) using a similar *Methylomonas methanica* cell-free system obtained 68% inhibition of methane mono-oxygenase activity by CO using bromomethane as substrate. Therefore the situation in *M. methanica* is even less clear.

8-Hydroxyquinoline remains the only metal chelator to show significant inhibition of methane oxidation in cell-free systems of MC. This suggests that if indeed any metal ion complex is directly involved in the methane mono-oxygenase system it must be well shielded from attack by most metal-binding compounds.

The results reported here in conjunction with the additional inhibition studies on cell-free extracts of MC by Colby and Dalton (1976) provide an overall inhibition pattern for *M. capsulatus* (Bath) which greatly differs from that of *M. capsulatus* (Texas) and indeed all the other methane-oxidising bacteria similarly studied. Even the inhibition pattern of ammonia oxidation in *Nitrosomonas europaea* by metal chelators (Hooper and Terry, 1973) and by CO (Suzuki et al., 1976; Hooper and Terry, 1973) resembles very closely that of TRMC and the other organisms. Therefore on the evidence of the inhibition pattern of methane oxidation, MC appears divergent from the main group of methane-oxidising bacteria but whether this proves to be of any physiological or evolutionary importance remains to be seen.

As acetylene was found to be the most potent inhibitor a number of acetylenic compounds were tested for inhibition of methane oxidation by whole cells of *M. capsulatus* (Bath) to see whether any correlation could be detected between the size, chemical composition and arrangement of the compounds with the extent of inhibition observed. The results obtained are summarised in Table 3 and it can be seen that four acetylenic compounds, acetylene, propyne, but-1-yne and but-2-yne strongly inhibit methane oxidation specifically, whereas the rest (with the possible exception of propyn-1-ol) appear to have no effect. The oxidation of propyn-1-ol was not due to auto-oxidation of the compound or oxidation by the methane mono-oxygenase as the oxidation was not inhibited by acetylene or 8-hydroxyquinoline, but was almost certainly due to methanol dehydrogenase/oxidase activity.

To confirm the above results and to possibly clarify the situation, similar experiments to those performed on whole cells were done using cell-free extracts (Table 4). Methane mono-oxygenase activity was monitored by two methods, i.e. measuring methanol accumulation and bromomethane disappearance, in an attempt to avoid any possible erroneous results obtained with either method. The results from the two methods correlate well except with acetylene and propyne. It is thought, however, that the trace amounts of methanol accumulating in the presence of the first

Table 3. The effect of acetylenic compounds on methane oxidation by whole cell suspensions of *Methylococcus capsulatus* (Bath)

Inhibitor (final conc. 1 mM)	Chemical structure	Inhibitor oxidation	Rate of methane oxidation (% control[a])	Rate of methanol oxidation (% control[b])
Acetylene[c]	$HC \equiv CH$	–	0	100
Propyne[c]	$HC \equiv CCH_3$	–	0	100
But-1-yne[c]	$HC \equiv CCH_2CH_3$	–	0	100
But-2-yne	$CH_3C \equiv CCH_3$	–	10	100
Propyn-1-ol	$HC \equiv CCH_2OH$	+	nd[d]	100
Propargylamine	$HC \equiv CCH_2NH_2$	–	100	100
Dimethylamino propyne	$HC \equiv CCH_2N(CH_3)^2$	–	100	100
Pargyline	$HC \equiv CCH_2NC_6H_5CH_2CH_3$	–	100	100

[a] The uninhibited rate of methane oxidation was 225 nmoles oxygen consumed · min^{-1} (mg dry cell weight)$^{-1}$
[b] The uninhibited rate of methanol oxidation was 170 nmoles oxygen consumed · min^{-1} (mg dry cell weight)$^{-1}$
[c] These gaseous compounds were added as saturated solutions in phosphate buffer (0.2 ml)
[d] Not determined due to the masking of true methane oxidation rate by propyn-1-ol oxidation rate

Table 4. The effect of acetylenic compounds on methane-mono-oxygenase activity in cell-free extracts of *Methylococcus capsulatus* (Bath) as determined by (a) measuring methanol accumulation, (b) bromomethane disappearance

Inhibitor	Rate of methanol accumulation (% control[a])	Rate of bromomethane disappearance (% control[b])
Acetylene[c]	9	0
Propyne[c]	11	0
But-1-yne[c]	52	50
But-2-yne	88	79
Propyn-1-ol	76	60
Propargylamine	100	100
Dimethylaminopropyne	92	100
Pargyline	100	100

[a] The uninhibited rate of methanol accumulation from methane was 53 nmoles methanol produced · min^{-1} (mg protein)$^{-1}$
[b] The uninhibited rate of bromomethane disappearance was 25 nmoles bromomethane · min^{-1} (mg protein)$^{-1}$
[c] These gaseous compounds were added as saturated solutions in phosphate buffer (0.1 ml)
Reaction mixture (final vol. 1 ml): 20 µmoles phosphate; 5 µmoles NADH; 0.5 µmoles KCN; 5 mg extract protein; 2.5 µmoles inhibitor (except gaseous compounds); 3 ml gas phase CH_4 (a); 1 µmole bromomethane (b)

two compounds is due to the release of methanol, previously bound to the methanol dehydrogenase, during the assay. The results suggest that the inhibition efficiency of the acetylenic compounds decreases not only with increasing carbon chain length but with the shifting of the acetylenic bond away from the terminal carbon to a sub-terminal position. The inhibitory effect of propyn-1-ol on the methane mono-oxygenase as shown in Table 4 was confirmed in the presence of 0.5 mM potassium cyanide. As KCN has been shown to inhibit any methanol oxidase activity which may be present in a cell-free extract of MC (Colby and Dalton, 1976) this eliminates the possibility of the inhibition being caused by a product of propyn-1-ol oxidation by the methanol oxidase. This small but significant inhibition by propyn-1-ol is not surprising when one considers that methanol is in fact a better substrate for the methane mono-oxygenase in MC than methane (Colby et al., 1977). It appears from Tables 3 and 4 that propargylamine, dimethylamino-propyne and pargyline have no effect on the methane mono-oxygenase of MC. One might have expected propargylamine to have shown some inhibitory effect like that of but-1-yne, but-2-yne and propyn-1-ol as structurally they are very similar. Their close similarity in structure and molecular size would suggest that rather than being a simple steric effect an electro-steric effect is the cause of the differences in inhibitory potential.

During the experiment concerning the effect of acetylene on bromomethane disappearance by cell-free extracts of MC it was noticed that the concentration of acetylene diminished during the course of the assay. Similar assays were performed without bromomethane and confirmed acetylene disappearance in the presence of cell-free extract, NADH and oxygen. Further tests showed that acetylene disappearance was dependent on extract concentration, the presence of NADH and gaseous oxygen. Attempts to identify a product for this apparent acetylene oxidation have so far been unsuccessful, therefore it is as yet uncertain whether acetylene is in fact truly oxidised by the cell-free extract or is simply bound to an enzyme complex in the requisite presence of NADH and gaseous oxygen.

The vastly different inhibitor pattern between MC and TRMC in conjunction with a number of other significantly differing properties listed below, suggest that these two organisms may not be as closely related

as presently thought. MC has an optimum growth temperature of 45° C unlike TRMC which optimally grows at 37° C (Patel and Hoare, 1971). MC has little or no capsule whereas TRMC produces large quantities of capsular material. Resting cell suspensions of MC when left at room temperature very quickly (approximately 1 h) lose their methane mono-oxygenase activity unlike TRMC which remains stable for much longer periods, suggesting a possible difference in mono-oxygenase systems. Indeed, on preparation of cell-free extracts with MC all the methane mono-oxygenase activity appears to reside in the soluble fraction upon centrifugation of crude extracts whereas the activity is found exclusively in the particulate fraction with TRMC (Ribbons and Michalover, 1970).

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The Soluble Methane Mono-oxygenase of *Methylococcus capsulatus* (Bath)

ITS ABILITY TO OXYGENATE *n*-ALKANES, *n*-ALKENES, ETHERS, AND ALICYCLIC, AROMATIC AND HETEROCYCLIC COMPOUNDS

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1. Methane mono-oxygenase of *Methylococcus capsulatus* (Bath) catalyses the oxidation of various substituted methane derivatives including methanol. 2. It is a very non-specific oxygenase and, in some of its catalytic properties, apparently resembles the analogous enzyme from *Methylomonas methanica* but differs from those found in *Methylosinus trichosporium* and *Methylomonas albus*. 3. CO is oxidized to CO_2. 4. C_1–C_8 *n*-alkanes are hydroxylated, yielding mixtures of the corresponding 1- and 2-alcohols; no 3- or 4-alcohols are formed. 5. Terminal alkenes yield the corresponding 1,2-epoxides. *cis*- or *trans*-but-2-ene are each oxidized to a mixture of 2,3-epoxybutane and but-2-en-1-ol with retention of the *cis* or *trans* configuration in both products; 2-butanone is also formed from *cis*-but-2-ene only. 6. Dimethyl ether is oxidized. Diethyl ether undergoes sub-terminal oxidation, yielding ethanol and ethanal in equimolar amounts. 7. Methane mono-oxygenase also hydroxylates cyclic alkanes and aromatic compounds. However, styrene yields only styrene epoxide and pyridine yields only pyridine *N*-oxide. 8. Of those compounds tested, only NADPH can replace NADH as electron donor.

The methane mono-oxygenase of *Methylococcus capsulatus* strain Bath is a multi-component enzyme that catalyses the NADH- and oxygen-dependent oxidation of methane to methanol. It is soluble in that enzyme activity is entirely associated with the supernatant fraction after centrifugation of bacterial extracts at 160000*g* for 1h (Colby & Dalton, 1976). Similar activities have been demonstrated in extracts of three other methane-oxidizing bacteria. However, in its entirely soluble nature and in its resistance to inhibition by cyanide and most chelating agents, the enzyme from *M. capsulatus* strain Bath can be distinguished from those enzymes that have been reported in *M. capsulatus* strain Texas (Ribbons, 1975), *Methylomonas methanica* (Colby *et al.*, 1975; Ferenci *et al.*, 1975) and *Methylosinus trichosporium* (Tonge *et al.*, 1977). Moreover, the methane mono-oxygenases from *Methylomonas methanica* (Colby *et al.*, 1975) and from *M. capsulatus* strain Bath (Colby & Dalton, 1976) require NAD(P)H as electron donor and therefore differ from the NAD(P)H-independent three-component enzyme system purified from *Methylosinus trichosporium* by Tonge *et al.* (1977). The available evidence therefore suggests that the methane mono-oxygenases from different methane-oxidizing bacteria have somewhat different properties. The enzyme from *M. capsulatus* strain Bath is at present unique in being entirely associated with the soluble fraction of bacterial extracts, although this does not exclude the possibility that one or more components of the enzyme system are membrane-bound *in vivo*.

The methane mono-oxygenase from *M. capsulatus* strain Bath has been reported to oxidize bromomethane (Colby & Dalton, 1976), the enzyme from *Methylomonas methanica* has been reported to oxidize CO (Ferenci *et al.*, 1975), bromomethane and NH_4Cl (Colby *et al.*, 1975), whereas the purified methane mono-oxygenase system of *Methylosinus trichosporium* oxidizes CO, ethane, propane and butane (Tonge *et al.*, 1977). Nothing further is known about the substrate specificity of methane mono-oxygenases although a better understanding of the catalytic abilities of these enzymes would obviously be a valuable aid in the elucidation of their mechanism of action. The present paper gives the results of our investigations into the substrate specificity of the soluble methane mono-oxygenase of *M. capsulatus* strain Bath. The enzyme apparently possesses a very broad substrate specificity and catalyses a variety of different oxygen-incorporation reactions.

Materials and Methods

Materials

Hexan-2-ol, hexan-3-ol, heptan-1-ol, heptan-2-ol, heptan-3-ol, heptan-4-ol, octan-1-ol, octan-2-ol, octan-3-ol and octan-4-ol were gifts from British

Petroleum Ltd., Sunbury, Middx., U.K. Methane (technical grade) and CO (research grade) were obtained from British Oxygen Co., London S.W.19, U.K. Other potential enzyme substrates were the best grades available. Ethane, *n*-propane, *n*-butane, ethene (ethylene), ethyne (acetylene) and dimethyl ether were obtained from Cambrian Chemicals, Croydon, Surrey, U.K. Fisons Scientific Apparatus, Loughborough, Leics., U.K., supplied *n*-octane, benzene, phenol and diethyl ether; and ethanal (acetaldehyde) and butan-1-ol were obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. Pentan-3-ol, styrene, styrene epoxide, 1,2-epoxybutane and *trans*-2-buten-1-ol (crotyl alcohol) were obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.; 2-ethoxyethanol was from May and Baker Ltd., Dagenham, Essex, U.K. Styrene was obtained free of stabilizer by vacuum distillation at about 40°C immediately before use. Toluene and 2-, 3- and 4-hydroxypyridines were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and 1,2-epoxypropane from Polaron Equipment, Watford, Herts., U.K. Bromine (AristaR), 3-chloroperbenzoic acid and all other potential enzyme substrates and products were obtained from BDH Chemicals, Poole, Dorset, U.K. Merck t.l.c. glass plates precoated with silica gel F-254 were supplied by Anderman and Co. Ltd., East Molesey, Surrey, U.K. Xanthine oxidase (grade 1, EC 1.2.3.2), catalase (ox liver, EC 1.11.1.6), superoxide dismutase (ox blood, EC 1.15.1.1) and NADH (grade 3) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Contaminating ethanol (10–20mol/100mol) was removed from 100mM-NADH solutions in 20mM-sodium phosphate buffer, pH7, by washing with diethyl ether followed by evaporation under vacuum to remove the ether. Two or three washings were necessary to decrease the ethanol to negligible concentration (0.1–0.2mol/100mol).

Syntheses

trans-2,3-Epoxybutane and *cis*-2,3-epoxybutane were made by forming epoxides from the corresponding *cis*- or *trans*-but-2-ene with 3-chloroperbenzoic acid at room temperature (20°C). A conical flask (25ml volume) sealed with a Suba-Seal stopper contained 3ml of a 10% (w/v) solution of 3-chloroperbenzoic acid in dichloromethane. But-2-ene gas (*cis* or *trans*) was passed through the flask for a few minutes by means of syringe needles inserted through the stopper. A heavy precipitate was formed during the reaction and this was resuspended by the addition of a further 0.5ml of dichloromethane when the reaction was completed. The suspension was centrifuged (2000*g* for 10min) to remove the precipitate and a 0.5ml sample of the supernatant removed, washed twice with 0.5ml of ice-cold satd. $NaHCO_3$ solution and then dried by adding a little anhydrous Na_2SO_4. The resulting dichloromethane solution of *cis*- or *trans*-2,3-epoxybutane was diluted with dichloromethane as necessary and then injected into the gas chromatograph for purposes of identification.

Growth of bacteria and preparation of soluble extracts

M. capsulatus strain Bath (Whittenbury *et al.*, 1970) was grown at 45°C in batch culture on ammonium/mineral-salts medium (Dalton & Whittenbury, 1976) in a 100-litre fermenter (L. H. Engineering Ltd., Stoke Poges, Bucks., U.K.). The fermenter was inoculated with 10 litres of a continuous culture, which had been grown as described previously (Colby & Dalton, 1976), and then harvested after 18h (when the A_{540} of the culture was about 8) using a Westfalia continuous centrifuge (Westfalia Separator Ltd., Wolverton, Bucks., U.K.). The centrifuge was connected to the outflow of the fermenter via a stainless-steel cooling-coil immersed in ice. The organisms were washed once with ice-cold 20mM-sodium phosphate buffer, pH7, and then soluble extracts prepared as described previously (Colby & Dalton, 1976) except that the crude bacterial extract was centrifuged at 80000*g* for 1h instead of at 160000*g* for 1h. The soluble extract was immediately frozen in pellet form by dropwise addition to a Dewar flask containing liquid nitrogen, and the pellets were stored at −70°C.

Enzyme assays

Assays were done in conical flasks (7ml internal volume) containing 1ml of reaction mixtures and sealed with Suba-Seal stoppers. Liquid substrates were incorporated into the reaction mixtures, whereas gaseous substrates were added to the reaction flasks by replacing part of the gas phase with the same volume of substrate. Reaction mixtures (1ml) contained: 50μmol of sodium phosphate buffer, pH7; 5μmol of NADH; 0.5μmol of KCN; soluble extract (2mg of protein unless stated otherwise); test substrate (amount as indicated in the Tables). The flasks were incubated in a 45°C reciprocating water bath at 90 oscillations/min for 5min and then the reaction was started by injecting freshly thawed soluble extract through the stopper.

The rate of oxidation of most test compounds was measured by following the appearance of products. Samples (5μl) of reaction mixture (or, in the case of reaction flasks containing CO as substrate, 0.5ml of gas phase) were injected into the gas chromatograph immediately after the addition of the soluble extract (zero time) and after 12min of incubation (preliminary experiments showed that product formation from each test substrate was more or less linear with time over this incubation period). However, because the products of *n*-octane oxidation were insoluble, reaction mixtures incorporating this substrate were

first extracted with 1 ml of dichloromethane and then 5 μl of the dichloromethane layer was injected into the gas chromatograph. Specific activities were calculated from the total amount of products formed after 12 min of incubation (1 unit of activity is 1 μmol of product formed/min).

The oxidation of some other compounds was measured by following their disappearance from the gas phase or (with nitromethane, cyanomethane, methanol, trimethylamine and pyridine) from the liquid phase. Samples (either 5 μl of liquid or 50 μl of gas) were injected into the gas chromatograph at zero time, after 6 min of incubation and after 12 min of incubation. Specific activities were calculated from the amount of substrate removed after a 12 min incubation (1 unit of activity is 1 μmol of substrate used/min).

Each substrate was tested at various concentrations so as to determine the optimum conditions for its oxidation and the data in the Tables represent the highest activities observed for each. The values in the Tables were obtained by using the same soluble extract (protein concentration 40 mg/ml determined with the Folin–Ciocalteu reagent) for each test substrate, although the results were confirmed by using another extract prepared from a different batch of organisms.

Identification and estimation of oxidation products

Most products were identified and estimated by using a Pye series 104 flame-ionization gas chromatograph fitted with 2.1 m glass columns (internal diameter 4 mm) packed with Porapak Q (Waters Associates, Milford, MA, U.S.A.), with Chromosorb 102 (Johns–Manville, Denver, CO, U.S.A.) or with 5% (w/w) of Carbowax 20 M on Chromosorb W (60–80 mesh). Trimethylamine hydrochloride was estimated on a column of Porapak Q which incorporated a soda-lime pre-column housed in the injection heating block. Products were identified by comparing their retention times on each column with that of authentic standards and estimated by establishing a linear relationship between peak height (or in some cases peak area) and concentration for each compound. These three columns used in conjunction and operated isothermally at temperatures between 50° and 230°C with N_2 carrier-gas flow-rates of 15–60 ml/min achieved the separation and preliminary identification of all the volatile products observed except for CO_2 (see below).

The identification of some products was confirmed by treatment of reaction mixtures with 20 μl of HCl or with 5 μl of bromine for 5 min at 45°C followed by gas chromatography to determine whether the products still remained (cf. May & Abbott, 1973). Under these conditions bromine reacts with unsaturated compounds by addition whereas dilute HCl catalyses the hydrolysis of epoxides.

CO_2 formed from the oxidation of CO was estimated by using a Pye series 104 katharometer gas chromatograph fitted with a 2.1 m glass column (internal diameter 4 mm) packed with Porapak R (Waters Associates). The oven temperature was 50°C and the helium carrier-gas flow-rate was 30 ml/min. The katharometer was calibrated by using CO_2/air mixtures of known composition.

Tyrosine was estimated colorimetrically as described by Kaufman (1970). Pyridine *N*-oxide was identified by t.l.c. on silica gel as follows. After incubation, reaction mixtures containing 90 μmol of pyridine as test substrate were extracted with an equal volume of dichloromethane. The dichloromethane layer was taken off and evaporated to dryness. The residue was then redissolved in 0.1 ml of dichloromethane and spotted on to the chromatograms together with dichloromethane solutions of authentic pyridine *N*-oxide. The chromatograms were developed in either methanol or acetone and the R_F values for the reaction product in each solvent compared with those for authentic pyridine *N*-oxide.

Experimental and Results

Precautions taken to ensure the validity of the results

Crude soluble extract was used as the source of methane mono-oxygenase for the substrate-specificity and product-analysis studies because the instability of the enzyme system has so far precluded its purification. The further metabolism of methanol and of other primary alcohol products was prevented by using a soluble extract (methanol oxidase activity is particulate) and by inhibiting residual methanol oxidase activity with 0.5 mM-KCN (Colby & Dalton, 1976). In practice the particular soluble extract used in these studies had negligible methanol oxidase activity even in the absence of KCN. Primary alcohol dehydrogenase, which was present in the soluble extract in high specific activity when measured at pH 9 with added phenazine methosulphate and NH_4Cl (Anthony & Zatman, 1964), was not active under the conditions of the methane mono-oxygenase assays. The further metabolism of secondary alcohols and of other non-alcoholic products except formaldehyde was not observed; the latter is rapidly metabolized by crude soluble extracts even in the presence of KCN and in the absence of added cofactors other than NAD^+. In practice all the products observed were the result of a single oxidation step involving the incorporation of one oxygen atom into the substrate; they are therefore consistent with the operation of a mono-oxygenase.

Adequate controls were essential to ensure that the oxidations observed were catalysed by methane mono-oxygenase. The following controls were used: (i) reaction mixtures containing boiled extract to ensure that the oxidations were enzyme-catalysed;

(ii) reaction mixtures lacking oxygen or NADH to ensure that both these substrates were required i.e. to indicate that a mono-oxygenase was involved; (iii) reaction mixtures with and without KCN because many mono-oxygenases, unlike the methane mono-oxygenase of *M. capsulatus* (Bath), are inhibited by KCN; (iv) reaction mixtures containing ethyne (0.2ml) because the latter appears to be a specific inhibitor of methane mono-oxygenase in *M. capsulatus* strain Bath (Colby & Dalton, 1976; Dalton & Whittenbury, 1976). The results obtained using these controls are given in parentheses in the Tables and are in each case consistent with the oxidations being catalysed by methane mono-oxygenase.

Oxidation of substituted methane derivatives and of CO

The oxidation of the substituted methane derivatives was measured by following their disappearance from reaction flasks (Table 1) because no volatile products were detected by gas chromatography. This absence of detectable products can be accounted for by the instability of the corresponding 1-substituted methanol derivatives. Of the monohalogenated derivatives, chloromethane was oxidized as rapidly as methane (Table 2), bromomethane was oxidized less rapidly and iodomethane was not oxidized at all; i.e. there was a decline in the rate of oxidation with increasing size of the substituting halogen. Of the chlorinated derivatives, chloromethane and dichloromethane were oxidized as rapidly as methane itself, whereas trichloromethane was oxidized more slowly and tetrachloromethane was not oxidized. Thus up to two hydrogen atoms in methane can be replaced with chlorine atoms with no decrease in the observed rate of oxidation and complete substitution is required to prevent oxidation altogether.

The cyano, nitro and thio derivatives of methane were oxidized more slowly than methane, whereas trimethylamine was not oxidized. Methanol was oxidized three times as rapidly as methane even though it is itself the product of methane oxidation. The apparent K_m for methanol was 0.95mM compared with 0.16mM for methane, indicating that methanol has a relatively poor affinity for the enzyme. Formaldehyde would be the expected product of methanol oxidation but its accumulation was not observed presumably because of its rapid metabolism by crude soluble extracts (see above).

Methane mono-oxygenase from *M. capsulatus* strain Bath also catalysed the oxidation of CO to CO_2 and this activity has also been observed with the enzymes from *Methylomonas methanica* (Ferenci *et al.*, 1975) and *Methylosinus trichosporium* (Tonge *et al.*, 1977). The small amounts of CO_2 formed in the control reaction flasks were also formed in controls lacking CO. This suggests that these low amounts of CO_2 were produced from endogenous substrates present in the extract.

Table 1. *Oxidation of substituted methane derivatives and of CO by soluble extracts of M. capsulatus (Bath)*

Specific activities with CO as substrate were calculated from the rate of CO_2 formation. Otherwise specific activities were calculated from the rate of disappearance of substrate and the products were not identified. Full experimental details are given in the Materials and Methods section. Specific activity values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2ml of ethyne, or with boiled extract. nd, Not done.

Substrate (μmol per reaction flask)	Specific activity (munits/mg of protein)
Chloromethane (1)	84 (0, 0, 80, 0, 0)
Bromomethane (1)	66 (0, 0, 66, 0, 0)
Iodomethane (1–3)	0
Dichloromethane (1)	82 (0, 0, 76, 0, 0)
Trichloromethane	35 (0, 0, 38, 0, 0)
Tetrachloromethane (1–3)	0
Cyanomethane (1)	33 (0, 0, 25, 0, 0)
Nitromethane (2)	45 (0, 0, 41, 0, 0)
Methanethiol (2)	64 (0, 0, 64, 0, 0)
Methanol (5)	246 (0, 20, nd, 16, 0)
Trimethylamine (2–4)	0
CO (134)	61 (12, 10, 56, 12, nd)

Oxidation of C_1–C_8 n-alkanes to the corresponding primary and secondary alcohols

Methane mono-oxygenase catalysed the oxidation of *n*-alkanes of 1–8 carbon atoms (Table 2). C_1–C_8 alkanes were oxidized at comparable rates but there was a rapid decline in oxidation rate between pentane and octane. The enzyme is not a terminal hydroxylase: both 1-alcohols and 2-alcohols were produced from *n*-propane and *n*-butane indicating that both primary and secondary alkyl C–H bonds can be hydroxylated. The enzyme is apparently specific for the 1- and 2-alkyl carbon atoms, however, as there was negligible formation of 3- or 4-alcohols from pentane, hexane, heptane or octane.

Oxidation of internal and terminal n-alkenes

Methane mono-oxygenase catalysed the oxidation of the terminal alkenes ethene, propene and but-1-ene to the corresponding 1,2-epoxides (Table 3). Neither 2-propen-1-ol nor 3-buten-1-ol were formed from propene and but-1-ene respectively. The rate with ethene was almost twice that for methane oxidation but thereafter the rate of oxidation decreased with increasing carbon-chain length.

Internal alkenes were also oxidized. Thus *trans*-but-2-ene yielded a mixture of *trans*-2,3-epoxybutane and *trans*-2-buten-1-ol indicating that both the internal double bond and the terminal methyl group

Table 2. *Oxidation of C_1–C_6 n-alkanes by soluble extracts of M. capsulatus (Bath)*

Reaction mixtures contained 2 mg of extract protein except for those with *n*-octane as substrate which contained 4 mg of extract protein. Specific activities were calculated from the total amount of products formed after 12 min incubation. The values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2 ml of ethyne, or with boiled extract. Full details of the experimental methods are given in the Materials and Methods section.

Substrate (μmol per reaction flask)	Products (μmol formed in 12 min)	Specific activity (munits/mg of protein)
Methane (134)	Methanol (2.02)	84 (0, 0, 85, 13, 0)
Ethane (134)	Ethanol (1.64)	68 (0, 0, 63, 13, 0)
Propane (134)	Propan-1-ol (0.65) Propan-2-ol (1.00)	69 (0, 0, 68, 0, 0)
Butane (134)	Butan-1-ol (1.10) Butan-2-ol (0.92)	77 (0, 5, 68, 0, 0)
Pentane (150)	Pentan-1-ol (0.49) Pentan-2-ol (1.26) Pentan-3-ol (<0.06)	73 (0, 0, 69, 0, 0)
Hexane (150)	Hexan-1-ol (0.60) Hexan-2-ol (0.36) Hexan-3-ol (<0.01)	40 (0, 0, 39, 0, 0)
Heptane (150)	Heptan-1-ol (0.14) Heptan-2-ol (0.51) Heptan-3-ol (<0.01) Heptan-4-ol (<0.01)	27 (0, 0, 27, 0, 0)
Octane (300)	Octan-1-ol (0.04) Octan-2-ol (0.39) Octan-3-ol (<0.01) Octan-4-ol (<0.01)	9 (0, 0, 9, 0, 0)

could be attacked. It is significant that the *trans* configuration was retained in both products precluding the formation of racemizable intermediates.

Three products were formed from *cis*-but-2-ene. Two were positively identified as *cis*-2,3-epoxybutane and 2-butanone but the third could not be conclusively identified as *cis*-but-2-en-1-ol because the authentic compound was not available as a reference marker. However, its retention times on gas-chromatographic analysis, which were similar to but not identical with those for *trans*-but-2-en-1-ol, and its reaction with bromine but not with dil. HCl (see Table 3), make its identification virtually certain. Again the *cis* configuration was retained in both the epoxide and the alken-1-ol. The explanation for the production of butanone only from the *cis*-isomer must await further study. It is unlikely to arise from contamination of the starting substrate with some other enzyme substrate because the amount of *cis*-but-2-ene present must be decreased to a value that affects the production of the other two products before the rate of butanone production is decreased. At present it is not possible to distinguish between its formation as a direct enzyme product and its subsequent formation by chemical or enzymic breakdown of one or the other products.

There was a marked difference between the rates of oxidation of the *cis*- and *trans*-but-2-enes: indeed *trans*-but-2-ene was oxidized more rapidly than any of the other alkenes tested except ethene. This suggests that the *trans* configuration is sterically favoured for oxidation by methane mono-oxygenase.

Oxidation of dimethyl ether and diethyl ether

Dimethyl ether oxidation was followed by measuring its disappearance from reaction flasks (Table 4)

Table 3. *Oxidation of C_2–C_4 n-alkenes by soluble extracts of M. capsulatus (Bath)*

Full experimental details are given in the Materials and Methods section. Specific activities were calculated from the total amount of products formed after 12 min incubation. The values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2 ml of ethyne, or with boiled extract. *, Product disappears from gas chromatograms after treating the reaction mixture with 20 μl of HCl but remains after treatment with 5 μl of bromine; †, product disappears after treating the reaction mixture with 5 μl of bromine but remains after treatment with 20 μl of HCl; ‡, product remains after treating the reaction mixture with either bromine or HCl.

Substrate (μmol per reaction flask)	Products (μmol formed after 12 min)	Specific activity (munits/mg of protein)
Ethene (134)	Epoxyethane* (3.54)	148 (0, 13, 122, 12, 0)
Propene (134)	1,2-Epoxypropane* (2.10)	83 (0, 0, 83, 0, 0)
But-1-ene (134)	1,2-Epoxybutane* (1.19)	49 (0, 0, 49, 0, 0)
cis-But-2-ene (134)	*cis*-2,3-Epoxybutane* (0.61) *cis*-2-Buten-1-ol† (0.57) Butan-2-one‡ (0.20)	57 (0, 0, 51, 0, 0)
trans-But-2-ene (134)	*trans*-2,3-Epoxybutane* (0.77) *trans*-2-Buten-1-ol† (2.52)	141 (0, 0, 128, 0, 0)

and the products were not identified. It was oxidized three times as rapidly as methane at a rate similar to that for methanol oxidation. Diethyl ether was oxidized much more slowly, but in this case the products were identified as ethanol and ethanal which were formed in approximately equimolar amounts (Table 4). No 2-ethoxyethanol was formed, indicating that oxidation of diethyl ether occurs only at the carbon atom adjacent to the oxygen.

Oxidation of alicyclic, aromatic and heterocyclic compounds (Table 5)

Cyclohexane was oxidized to cyclohexanol with a specific activity somewhat higher than that for *n*-hexane. The ability of methane mono-oxygenase to hydroxylate such cyclic alkanes presumably reflects its ability to hydroxylate secondary alkyl C–H bonds. The aromatic ring of benzene was also hydroxylated, yielding phenol.

Toluene was tested as a potential enzyme substrate because it is the phenyl derivative of methane and the methyl derivative of benzene and therefore contains two different groups that could be hydroxylated. A mixture of benzyl alcohol and cresol was produced when the enzyme was incubated with toluene, indicating that both the aromatic ring and the methyl group were hydroxylated. The gas-chromatographic analysis did not distinguish between *o*-, *m*- and *p*-cresol. Styrene is analogous to toluene in being the phenyl derivative of ethane and also the vinyl derivative of benzene. Styrene was oxidized to styrene epoxide and, unlike toluene, yielded no ring-hydroxylated products.

Methane mono-oxygenase was tested for its ability to hydroxylate the aromatic ring of L-phenylalanine yielding L-tyrosine by assaying the product colorimetrically (Table 5). No tyrosine production was observed. This was not due to subsequent metabolism of the product because extracts did not catalyse the disappearance of tyrosine under the usual assay conditions.

Pyridine oxidation was detected and its rate estimated by observing the disappearance of pyridine from reaction flasks. However, no volatile products were detected by gas chromatography. Reaction mixtures were examined for non-volatile products by t.l.c. as described in the Materials and Methods section. A spot corresponding to pyridine *N*-oxide was found in complete reaction mixtures after 12min incubation. No 2-, 3- or 4-hydroxypyridine was found although these compounds could be readily

Table 4. *Oxidation of ethers by soluble extracts of M. capsulatus (Bath)*

Specific activities were calculated from the rate of disappearance of the substrate. However, the formation of ethanol and ethanal from diethyl ether was detected by analysing samples (5μl) of reaction mixtures before and after incubation. Full experimental details are given in the Materials and Methods section. Specific activity values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2ml of ethyne, and with boiled extract.

Substrate (μmol per reaction flask)	Products (μmol formed after 12min)	Specific activity (munits/mg of protein)
Dimethyl ether (4.5)	Not known	248 (0, 0, 227, 0, 0)
Diethyl ether (4.5)	Ethanol (0.51) Ethanal (0.57)	45 (0, 0, 45, 0, 0)

Table 5. *Oxidation of some alicyclic, aromatic and heterocyclic compounds by soluble extracts of M. capsulatus (Bath)*

Full details of the methods used are given in the Materials and Methods section. All reaction mixtures contained 4mg of extract protein. Except when pyridine was the substrate, specific activities were calculated from the total amount of products formed after 12min incubation. The rate of pyridine oxidation was determined by following pyridine disappearance from reaction flasks containing 3μmol of pyridine. Pyridine *N*-oxide was identified as the product of pyridine oxidation by t.l.c.; in this case reaction flasks contained 90μmol of pyridine as substrate. Specific-activity values in parentheses refer respectively to assays done in the absence of NADH, anaerobically under N_2, in the absence of KCN, in the presence of 0.2ml of ethyne, or with boiled extract. nd, Not done; *, product disappears from gas chromatograms after treating the reaction mixture with 20μl of HCl or with 5μl of bromine; †, product disappears after treating the reaction mixture with 5μl of bromine but remains after treatment with 20μl of HCl; ‡, product remains after treating reaction mixtures with either 20μl of HCl or 5μl of bromine.

Substrate (μmol per reaction flask)	Products (μmol formed after 12min)	Specific activity (munits/mg of protein)
Cyclohexane (460)	Cyclohexanol‡ (3.0)	62 (0, 0, 62, 0, 0)
Benzene (450)	Phenol† (3.0)	62 (0, 0, 62, 0, 0)
Toluene (460)	Benzyl alcohol† (1.5) Cresol† (1.0)	53 (0, 0, 52, 0, 0)
Styrene (90)	Styrene epoxide* (2.3)	47 (0, 0, 37, 0, 0)
Pyridine (3, 90)	Pyridine *N*-oxide (nd)	29 (0, 4, 25, 0, 0)
L-Phenylalanine (2-16)	Tyrosine (0)	0

Table 6. *Effect of replacing NADH with other potential electron donors and hydroxylating agents*

Methanol formation from methane, and phenol formation from benzene, were measured as described in the Materials and Methods section except that cyanide was omitted from the reaction mixtures and NADH was replaced with the compounds indicated. The following compounds gave no activity when tested at 5 and 20mM in place of NADH with methane as substrate: sodium ascorbate, quinol, sodium dithionite, sodium borohydride, sodium chlorite, sodium periodate. n.d., Not done.

Test compound	Specific activity (munits/mg of protein)	
Substrate ...	Methane	Benzene
NADH (5mM)	85	62
NADPH (5mM)	49	nd
NADH (5mM)+superoxide dismutase (100 units)	87	62
Xanthine (5mM)+xanthine oxidase (0.1, 1 unit)	0	0
H_2O_2 (5, 20mM)	0	0
NADH (5mM)+catalase (100 units)	80	68

distinguished from pyridine *N*-oxide by t.l.c. No pyridine *N*-oxide was found in complete reaction mixtures at zero time, neither was it found after 12 min incubation of the usual control reaction mixtures.

Electron donor specificity of methane mono-oxygenase

A variety of reducing agents was tested as electron donor but only NADPH could replace NADH (Table 6). The broad substrate specificity of methane mono-oxygenase suggests that the enzyme might act by generating a non-specific hydroxylating species such as H_2O_2 or superoxide anion. However, the failure of superoxide dismutase or catalase to prevent activity and the failure of H_2O_2 or superoxide (generated *in situ* from xanthine oxidase and xanthine) to support methane or benzene oxidation effectively excludes this possibility. Sodium chlorite and sodium periodate have been shown to support steroid hydroxylation by replacing NADPH in the cytochrome *P*-450 system of liver microsomal preparations (Hrycay *et al.*, 1975). Neither of these compounds supported methane oxidation by the methane mono-oxygenase of *M. capsulatus* (Bath).

Discussion

Methane mono-oxygenase of *M. capsulatus* (Bath) is not a terminal alkane hydroxylase as are, for example, the rubredoxin-containing alkane hydroxylase of *Pseudomonas oleovorans* (May & Abbott, 1973) and the cytochrome *P*-450 alkane hydroxylase of a diphtheroid bacterium (Cardini & Jurtshuk, 1970). Instead, methane mono-oxygenase oxidizes *n*-alkanes to mixtures of the corresponding 1- and 2-alcohols. Similarly the methane mono-oxygenase will form an epoxide from both terminal and internal alkenes, whereas the alkane hydroxylase from *Ps. oleovorans* will form epoxides from terminal alkenes only (May & Abbott, 1973). The enzyme systems from other methane-oxidizing bacteria have not been examined for these properties, but whole-cell studies suggest that the enzyme from *Methylomonas methanica* is also able to hydroxylate subterminal alkyl C–H bonds. Thus Leadbetter & Foster (1960) observed the formation of propan-1-ol, propionic acid and acetone when suspensions of *Methylamonas methanica* were incubated with *n*-propane; similarly butan-1-ol, butyric acid and 2-butanone were formed from *n*-butane. The mechanism for the production of the ketones is obscure, bearing in mind that the alcohol dehydrogenase present in this organism is specific for primary alcohols (Johnson & Quayle, 1964; Ferenci *et al.*, 1975). Nevertheless, the formation of the ketones does suggest that sub-terminal oxidation of the alkanes occurs. Different results were obtained by Thomson (1974) using two other methane-oxidizing bacteria *Methylomonas albus* and *Methylosinus trichosporium*. When incubated with *n*-propane or *n*-butane these organisms formed products characteristic of terminal oxidation only.

The sub-terminal oxidation of higher *n*-alkanes by micro-organisms is well-known (Markovetz, 1971). However, in most cases a random mixture of secondary alcohols and their oxidation products together with the primary alcohol are formed. For instance, *Ps. aeruginosa* transforms *n*-decane into a mixture of decan-1-ol and decan-2, 3-, 4- and 5-ols together with the corresponding ketones (Fredericks, 1967). Similar results were obtained by Klein *et al.* (1968) with an *Arthrobacter* sp. oxidizing *n*-hexadecane or *n*-pentadecane. Oxidation of long-chain fatty acids and *n*-alkanes specifically at the ω1 and ω2 carbon atoms has been observed, however, in the yeast *Torulopsis* sp. (Tulloch *et al.*, 1962; Heinz *et al.*, 1970). The hydroxylation of long-chain fatty acids at the ω1 and ω2 positions is also catalysed by the rat liver microsomal cytochrome *P*-450 system (Bjorkhem & Danielsson, 1970). However, in the latter case the different inhibitor sensitivities of the ω1 and ω2 hydroxylations (Ellin & Orrenius, 1975) and their different requirements for hydroxylating agents (Gustafsson & Bergen, 1976) suggest that the two types of hydroxylation are catalysed by different cytochrome *P*-450 species. There is no evidence at present to suggest that the 1- and 2-hydroxylations of C_5–C_8 *n*-alkanes observed in the present study are not due to the same enzyme system.

The mechanism for the oxidation of diethyl ether

by the methane mono-oxygenase of *M. capsulatus* (Bath) resembles that observed by Heydeman (1974) in bacteria isolated from diethyl ether enrichments. The sub-terminal carbon is hydroxylated yielding 1-ethoxyethanol as a hypothetical intermediate that dismutates to form ethanol and ethanal in equimolar amounts. This apparently differs from the mechanism of diethyl ether oxidation by two other methane-oxidizing bacteria *Methylomonas albus* and *Methylosinus trichosporium* (Wilkinson, 1975). Whole-cell studies indicated that these organisms oxidize diethyl ether solely at the terminal methyl group, yielding 2-ethoxyethanol, 2-ethoxyethanal and eventually 2-ethoxyacetate. This evidence, together with the observation that these two methane-oxidizing bacteria catalyse only terminal oxidation of *n*-propane and *n*-butane (Thomson, 1974), strongly suggests that they, unlike *M. capsulatus* (Bath) and probably *Methylomonas methanica*, contain an alkane hydroxylase that is specific for terminal methyl groups.

The methane mono-oxygenase of *M. capsulatus* (Bath) is a very non-specific enzyme system and many of its substrates show little or no structural resemblance to its substrate *in vivo*, methane. It catalyses the hydroxylation of primary and secondary alkyl C–H bonds, the formation of epoxides from internal and terminal alkenes, the hydroxylation of aromatic compounds, the *N*-oxidation of pyridine, and the oxidation of CO to CO_2. Moreover, some substrates can be attacked at more than one position e.g. *trans*- and *cis*-but-2-ene and toluene. Such behaviour resembles that of some cytochrome *P*-450-containing mono-oxygenases (Orrenius & Ernster, 1974), but the resistance of the methane mono-oxygenase to inhibition by CO (Colby & Dalton, 1976) suggests that it does not contain cytochrome *P*-450. The possibility that it contains a CO-binding cytochrome *c* of the type involved in the analogous methane mono-oxygenase system from *Methylosinus trichosporium* (Tonge *et al.*, 1977) cannot yet be excluded. However, these two enzyme systems do have very different electron-donor and inhibitor specificities (Colby & Dalton, 1976; Tonge *et al.*, 1977) and, as discussed above, also appear to have different substrate specificities. Investigations into the physicochemical properties of the enzyme system from *M. capsulatus* (Bath) will have to await the purification of its components.

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