Regulation of C_1 Metabolism in *Methylococcus capsulatus* (Bath)

by

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DEDICATION

To Mum, Dad, Debby

and Blue

ABBREVIATIONS

AMP,ADP,ATP	Adenosine 5 -mono,di and triphosphates
C.C.E.	Carbon conversion efficiency
C_{rec}	Carbon recovery determination
DCPIP	2,6-Dichlorophenolindophenol
DEAE Cellulose	Diethylaminoethyl cellulose
EDTA	Ethylenediamine tetracetate
FAD, $FADH_2$	Flavin-adenine dinucleotide and its fully reduced form
FDH	Formate dehydrogenase
FMDH(DYE-linked)	NAD^+-Independent formaldehyde dehydrogenase
FMDH (NAD^+-linked)	NAD^+- dependent formaldehyde dehydrogenase
G6PD	Glucose -6- phosphate dehydrogenase
6PGD	6-Phosphogluconate dehydrogenase
HPR	Hydroxypyruvate reductase
HPS	Hexulose phosphate synthase
MDH	Methanol dehydrogenase
MMO	Methane monooxygenase
mM	Millimolar
µM	Micromolar
M.wt.	Molecular weight
µmol	Micromoles
NAD^+, NADH	Nicotinamide adenine dinucleotide (oxidised and reduced forms)
$NADP^+$, NADPH	Nicotinamide adenine dinucleotide phosphate (oxidised and reduced forms)
Pi	Inorganic phosphate
Pipes	Piperazine-N,N' - bis (2 ethanesulfonic acid); 1,4 Piperazine diethane sulfonic acid
PMS	Phenazine methosulphate
PQQ, $PQQH_2$	Pyrrolo-quinoline quinone (oxidised and reduced forms)
SDS	Sodium dodecyl sulphate
Tris	Tris (hydroxymethyl) aminomethane
Y_{max}	Cell yield on methane

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Finally I would like to thank the SERC for funding this research project.

DECLARATION

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Warwick or any other university or institute of learning.

Steven C. Hay 2/9/90

S. C. Hay

SUMMARY

The aim of this project was to examine aspects of the regulation of C_1 metabolism in M.capsulatus (Bath). To achieve this, steady-state cultures were set up under defined conditions using a gas-limited chemostat, coupled to an on-line mass spectrometer. During periods of steady-state growth, the conditions of culture were perturbed via the addition of various C_1 metabolites and the response of the cells monitored with respect to their capacity to attain a new steady-state.

An initial series of continuous cultivation experiments were carried out to determine the physiological state of the cells prior to making such perturbations. Gas exchange rates, carbon distribution and levels of in vitro enzyme activity monitored within the culture during these periods provided a base-line with which to compare the effect of the addition of C_1 metabolites.

Examination of the response of cells to the addition of formate showed that under carbon-limiting conditions, added formate was oxidised to CO_2. Under oxygen-limiting conditions cells were capable of assimilating low levels of formate carbon, although under such circumstances the cultures were also susceptible to formate-induced uncoupling of oxidative phosphorylation. Additional formate oxidation also resulted in an observed increase in cell yield on methane, especially when the cells expressed soluble MMO. It was concluded that this was a consequence of extra NAD(P)H being generated by the $NAD(P)^+$-linked oxidation of formate, which in turn relieved the apparent NAD(P)H-limitation of cells growing on methane.

The ability of cells to metabolize exogenously supplied formaldehyde was linked to the type of MMO expressed by the cells. Soluble MMO-containing cells showed an increased sensitivity to formaldehyde accumulation compared with particulate MMO- containing cells. At one stage it was possible to maintain particulate MMO- containing cells on formaldehyde as their sole source of carbon, albeit for a limited period of time. This period of time appeared to be linked to the cell's ability to maintain an active MMO. Results showed that the synthesis of soluble MMO was repressed in the presence of additional formaldehyde metabolism, the loss of enzyme coinciding with formaldehyde accumulation and ultimately cell death. Subsequent analysis of the intracellular levels of NAD^+ and NADH in the culture implied that the MMO played an active role in the regulation of the NAD^+ : NADH ratio within the cell. Loss of MMO activity in the presence of additional formaldehyde metabolism effectively compromised the cell's ability to regulate it's intracellular NAD^+ : NADH ratio.

It was shown during this study that cultures of M.capsulatus (Bath) could be transferred directly from methane-limited growth to growth on methanol as a sole source of carbon, without any prior period of physiological adaptation. During this switch in carbon sources, the MMO appeared to be actively involved in the in vivo metabolism of methanol.

Studies concerning the environmental regulation of methanol metabolism showed that the level of methanol dehydrogenase activity in the culture was inversely related to the standing concentration of methanol in the culture. Similarly, higher levels of methanol dehydrogenase activity were recorded at lower dilution rates, during methanol-limited conditions. It was concluded that the methanol dehydrogenase enzyme is subject to regulation via catabolic repression and at low dilution rates the synthesis of the enzyme is effectively derepressed.

CHAPTER 1

Introduction

INTRODUCTION

1.1 The Concept of Methylotrophy

The universally acknowledged definition of methylotrophic organisms was originally proposed by Colby and Zatman (1972). They described methylotrophs as organisms capable of growing non-autotrophically on carbon compounds containing one or more carbon atoms, but no carbon to carbon bonds. Methylotrophic organisms therefore utilize compounds such as methane, methanol, N-methyl and S-methyl compounds as a source of carbon for growth and replication by metabolic routes other than the Calvin cycle.

Methylotrophs that utilize these compounds as their sole carbon and energy source are termed obligate methylotrophs, and methylotrophs that possess the additional ability to grow and replicate on a variety of other multicarbon compounds are called facultative methylotrophs. If the carbon source is methane, then the organisms are termed methanotrophs. The vast majority of methylotrophic organisms are prokaryotes, although eukaryotic methylotrophs have been isolated, namely yeasts capable of growth on methanol. For the purpose of this introduction, only methylotrophic bacteria will be discussed. The reader is referred to a number of comprehensive reviews available which consider the extensive biochemistry and physiology of all the different types of methylotrophic organisms studied, (Quayle, 1972; Colby *et al*, 1981; Large and Bamforth, 1988).

1.2 Occurrence and Isolation of Methane-utilizing Bacteria

1.2.1 Ecology of Methane Formation and Methane Oxidation in Nature.

It has been estimated that 50% of all the total organic carbon degraded anaerobically is converted to methane, (Ehhalt, 1976). Methane generated under such conditions is normally termed "biological methane" and is produced in a variety of diverse environments (table 1.1). In addition to biogenic methane production there exists a number of non-biological sources from which methane is

Table 1.1 Sources of Atmospheric Methane
(from Ehhalt, 1976)

Source		Global Production in 10^6 tonnes of methane/year
1. Biogenic*		
Enteric fermentation in animals		100-220
Paddy fields		280
Swamps, marshes		130-260
Freshwater lakes		1.3-25
Upland fields		10
Forests		0.4
Tundra		0.8-8
Ocean	a) Open	4-6.7
	b) Continental Shelf	0.7-1.4
	Total Biogenic	528-812
2. Other Sources		
Coal mining		6.3-22
Lignite mining		1.6-5.7
Industrial Losses		7-21
Automobile exhaust		0.5
Volcanic emissions		0.2
	Total other sources	15.6-49.4
	Total ALL sources	544-862

Other relevant figures for comparison:
Output of natural gas wells for consumption (1965) 520×10^6 tonnes/yr.
Annual production of dry organic matter 1.65×10^{11} tonnes/yr.
Amount of atmospheric methane (about 1.4 ppm) 4×10^9 tonnes

* These figures do not include biogenic methane oxidized by methanotrophs

evolved. It has been estimated that the levels of non-biological methane may be as high as 20 -100% of that produced by biological means (Gold,1979).

Public awareness concerning the relative levels of methane in the atmosphere has been heightened by the knowledge that methane is a greenhouse gas. It is 25 times more efficient in trapping the sun's infra-red radiation than the more abundant CO_2. This, coupled with the estimate that methane levels in the atmosphere are increasing at a rate of 1% every year and have being doing so since 1950, (Pearce,1989) is a cause for some concern.

As noted in table 1.1, the vast percentage of biological methane evolved, does not reach the atmosphere. This is due mainly to the activities of methane-oxidising bacteria that are widely distributed throughout the environment.

1.2.2. Occurrence of Other Reduced C_1 Compounds in Nature

C_1 compounds such as CO_2 and methane are ubiquitous in the natural environment, because they are products of biological processes. Some C_1 compounds on the other hand occur as a direct consequence of man's activity and their presence is limited to a number of specific environments. Such compounds include formaldehyde, formate and carbon monoxide, and are often regarded as pollutants, although they do occur naturally. The major sources of these compounds are listed in table 1.2.

1.2.3. Isolation of Methanotrophs

Considering the known diversity of carbon compounds that can act as growth substrates for microorganisms, and the relative abundance of gaseous methane in the environment, it is perhaps surprising that prior to 1970 relatively few species of methane-utilizing bacteria had been successfully isolated and characterized. The first of these was at the turn of the century, when Söhngen (1906) isolated an organism which he named Bacillus methanicus. This was later renamed first to Pseudomonas methanica and finally to Methylomonas methanica by Foster and Davis (1966). Two other species defined by 1970 were

Table 1.2 C-1 Compounds of Environmental Significance

(from Higgins et al., 1985)

Compound	Formula	Comments
Methane	CH_4	End product of anaerobic fermentation processes. Generated by free-living and rumen-inhabiting methanogens.
Methanol	CH_3OH	Generated during the breakdown of methyl-esters and ethers (e.g. pectin) and released by methanotrophs.
Formaldehyde	HCHO	Common combustion product, intermediate microbial oxidation of other C-1 compounds and methylated biochemicals (e.g. lignin)
Formate ion	$HCOO^-$	Present in plant and animal tissues. Common product of carbohydrate fermentation.
Formamide	$HCONH_2$	Natural product formed from plant cyanides.
Carbon dioxide	CO_2	Combustion, respiration and fermentation end product. A major reservoir of carbon on Earth.
Carbon monoxide	CO	Combustion product, common pollutant. Product of plant, animal and microbial respiration, highly toxic.
Cyanide ion	CN^-	Generated by plants, fungi and bacteria. Industrial pollutant, highly toxic.

Methylomonas methanoxidans, (Stocks and McCleskey, 1964) and Methylococcus capsulatus, (Foster and Davis, 1966).

The major problem in isolating and characterising methanotrophs lay in the lack of reliable isolation and enrichment techniques. Bacterial colonies formed on agar plates, under an atmosphere of methane and air were often found to be non-methane utilizing bacteria, scavenging dissolved organic materials in the agar component of the medium and thus obscuring the growth of true methylotrophs. This problem was dramatically reduced by Whittenbury et al (1970) through the introduction of a much reduced period of enrichment (3 to 4 days) and the careful use of the plate microscope to identify methane oxidising colonies at an early stage. This helped reduce overgrowth and predation by non-methanotrophic bacteria. This technique facilitated the isolation and classification of over a hundred different strains of methanotrophic bacteria, many of which have been subsequently classified and subjected to a comprehensive analysis of their biochemical and morphological properties.

1.2.4. Classification of Methane-utilizing Bacteria

There has been a considerable amount of debate concerning the introduction of a formal classification scheme which would encompass all forms of methanotrophic bacteria. Although a number of sub-groups have been proposed, based on biochemical and morphological characteristics; it has proved very difficult to apply any strict classification scheme to these organisms for two reasons.

1) The diverse nature of the biochemistry of these organisms. Whilst certain isolates may have a high proportion of similar characteristics, certain isolates within one group may exhibit characteristics commonly associated with another group.
2) Physiological and morphological properties may vary depending on the conditions of growth.

To date the most widely accepted classification scheme for methanotrophs was that proposed by Whittenbury et al (1970), who found a correlation between the type of membrane arrangement, and the biochemisty of carbon

assimilation within the cells. Table 1.3. shows the classification of obligate methane-oxidising bacteria proposed by the above authors.

M.capsulatus (Bath) has been classified as a type I methylotroph since it possesses stacked bundles of intracellular membranes. However, it also possesses both hexulose phosphate synthase and hydroxypyruvate reductase activity; two key enzymes associated the ribulose monophosphate pathway and serine pathway repectively. Therefore it has been speculated that M.capsulatus (Bath) should head a third group of methanotrophs, the type X group (Whittenbury and Dalton, 1981).

Galchenko and Andreev (1984), examined characteristics such as phospholipid and fatty acid composition, antigenic characteristics, DNA homology and protein electrophoretic patterns. Their classification produced results which coincided with that of the scheme devised by Whittenbury et al (1970). However, more detailed examination of intracellular morphology and protein electrophoretic patterns have shown that the above characteristics vary depending on growth conditions (Hyder et al, 1979; Othomo et al, 1977; Takeda and Takana, 1980; Scott et al, 1981 and Prior, 1985).

1.3. Physiology and Biochemsitry of Methylotrophs

1.3.1. Basic Growth Requirements

The unique problems facing any organism growing solely on C_1 compounds are those of energy transduction and the synthesis of C_3 compounds such as pyruvate or phosphoenolpyruvate (PEP). Once these intermediary metabolites have been produced, the synthesis of macromolecules such as proteins, lipids, polysaccarides and nucleic acids required for cellular growth, may be accomplished by the established metabolic routes.

The inability of obligate methylotrophs to use multicarbon compounds as their sole carbon and energy source has not yet been fully explained, however Anthony (1982) proposed 5 possible reasons for this observation.

1) Possible lesions in a metabolic pathway, crucial to the further metabolism of multicarbon compounds.
2) The lack of a system that permits ATP synthesis to be coupled to NADH oxidation.

Table 1.3 Classification of Obligate Methane-utilising Bacteria

Character	Type I	Type II
Membrane arrangement	Bundles of vesicular discs	Paired membranes in layers around periphery
Resting stage	Cysts (Azotobacter-like)	Exospores or "lipid-cysts"
Carbon Assimilation	Ribulose monophosphate pathway	Serine pathway
TCA cycle	Incomplete (lacks 2-oxoglutarate dehydrogenase)	Complete
Glucose-6-phosphate and 6-phosphogluconate dehydrogenase	Present	Present
Examples	Methylococcus Methylomonas Methylobacter	Methylosinus Methylocystis Methylobacterium

3) The lack of transcriptional control mechanisms
4) The lack of suitable transport systems for multicarbon compounds.
5) Toxicity of multicarbon compounds or their products of metabolism.

A possible solution to this dilemma may lie in a combination of these reasons rather than any particular one. The last two proposals are likely to be involved in limiting the nutritional ranges of all types of bacteria and may be the reason for the lack of growth of some methylotrophs on some substrates.

The vast majority of methylotrophic bacteria are strictly aerobic, requiring gaseous oxygen for the initial oxidation of methane, (Higgins and Quayle, 1970). Anaerobic methane oxidation by cells using sulphate as an electron acceptor has been reported by Zehnder and Brock (1979). It is widely believed that anaerobic methane oxidisers may exist deep within marine sediments.

No growth factors are required for the growth of the organisms and they are normally grown on a mineral salts medium containing a nitrogen source, divalent cations (Ca^{2+} and Mg^{2+}), sulphate, phosphate, iron and trace elements (Dalton and Whittenbury, 1976). Murrell and Dalton (1983) demonstrated that under nitrogen-limiting conditions certain species of methanotrophs were capable of fixing atmospheric nitrogen. Although these were mainly confined to type II methanotrophs, M.capsulatus was shown to possess the capacity for nitrogen fixation.

Methane taken up by the organisms is oxidised to CO_2, by way of methanol, formaldehyde and formate, as shown in fig 1.1. It is at the oxidation level of formaldehyde that carbon can either be assimilated into cellular material or dissimilated to CO_2, the latter reactions generating the necessary energy and reducing power for the biosynthetic reactions. To enable cells to grow efficiently both carbon assimilation and dissimilation routes must function simultaneously.

1.3.2. C_1 Assimilation Pathways

Within obligate methylotrophs, carbon may be assimilated into cellular material by one of two distinct pathways; the ribulose monophosphate pathway (RuMP) and the serine pathway. Some methylotrophs do appear to

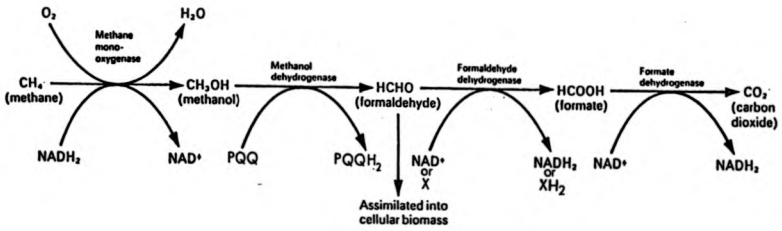


Fig 1.1 The complete oxidation of methane to CO_2 in methylotrophic bacteria.

possess the capacity to utilize CO_2 as a carbon source using the ribulose bisphosphate pathway (Calvin cycle), but the relative importance of carbon assimilation by this route is minimal when compared with the serine and the RuMP pathways.

1.3.2.1. Ribulose Monophosphate Pathway (RuMP)

This pathway is responsible for the cyclic condensation of three molecules of formaldehyde to produce either one molecule of pyruvate or one molecule of dihydroxyacetone phosphate. Although there are four potential variants of this cycle dependent on the cleavage and rearrangement reactions: only two variants (KDPG aldolase/transaldolase and FBP aldolase/sedoheptulose variant) are well represented in the described methylotrophs. The basis of this cycle is outlined in fig 1.2.

1.3.2.2. Serine Pathway

This pathway effects the condensation of two molecules of formaldehyde with one of CO_2 to give one molecule of 3-phosphoglycerate. The cycle (fig 1.3) uses amino acid and carboxylic acid intermediates instead of the carbohydrate carriers in the RuMP pathway. Tetrahydrofolate is also required to act as a "protective" carrier of formaldehyde prior to the initial condensation reaction (Jordon and Akhtar, 1970). There exist two potential variants of the pathway (Icl^- and Icl^+) which differ in the method by which glycine is regenerated.

Comparison of the serine pathway with the RuMP pathway shows that the energy requirement of the latter is less than that of the serine pathway. Consequently cells that utilize the RuMP pathway for carbon assimilation could in theory grow more efficiently than those utilizing the serine pathway. This was confirmed by Goldberg *et al* (1976) who examined the growth yield on methanol of a variety of methylotrophs.

Figure 1.2 The ribulose monophosphate pathway.

Ru5P	Ribulose-5-phosphate
Hu6P	D-erythro-3-hexulose-6-phosphate
F6P	Fructose-6-phosphate
FDP	Fructose-1,6-diphosphate
G3P	Glyceraldehyde-3-phosphate
E4P	Erythrose-4-phosphate
S7P	Sedoheptulose-7-phosphate
SDP	Sedoheptulose-1,7-diphosphate
R5P	Ribose-5-phosphate
G6P	Glucose-6-phosphate
6PG	6-phosphogluconate
PYR	Pyruvate

1. 3-hexulosephosphate synthase
2. Phospho-3-hexuloisomerase
3. 6-phosphofructokinase
4. Fructose diphosphate aldolase
5. Transketolase
6. Transaldolase
7. Ribulose phosphate epimerase
8. Ribulose phosphate isomerase
11. Glucose phosphate isomerase
12. Glucose-6-phosphate dehydrogenase
13. 6-phosphogluconate dehydratase plus phospho-2-keto-3-deoxygluconate aldolase

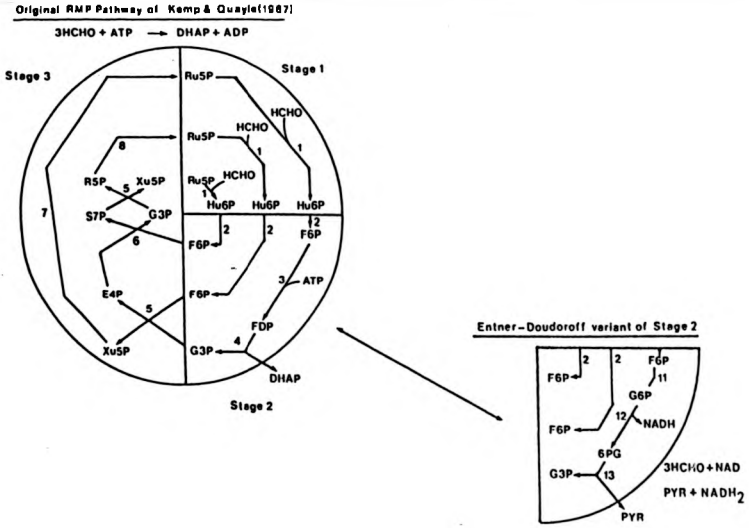

Original RMP Pathway of Kemp & Quayle(1987)
3HCHO + ATP → DHAP + ADP
Stage 3
Stage 1
Stage 2
Ru5P
HCHO
Hu6P
R5P
Xu5P
S7P
G3P
F6P
E4P
FDP
DHAP
ATP
Entner-Doudoroff variant of Stage 2
G6P
NADH
6PG
PYR
3HCHO+NAD
PYR + NADH2

Figure 1.3

The Serine Pathway.

From Colby et al., 1979.

a. Serine transhydroxymethylase.

b, Serine glyoxylate amino-trasferase.

c, Hydroxypyruvate reductase.

d, Glycerate kinase.

e. Phosphopyruvate hydratase.

f, Phosphoenol-pyruvate carboxylase.

g, Malate dehydrogenase.

h, Malate thiokinase.

i, Malyl-CoA lyase.

j, Isocitrate lyase.

----, Unknown reactions.

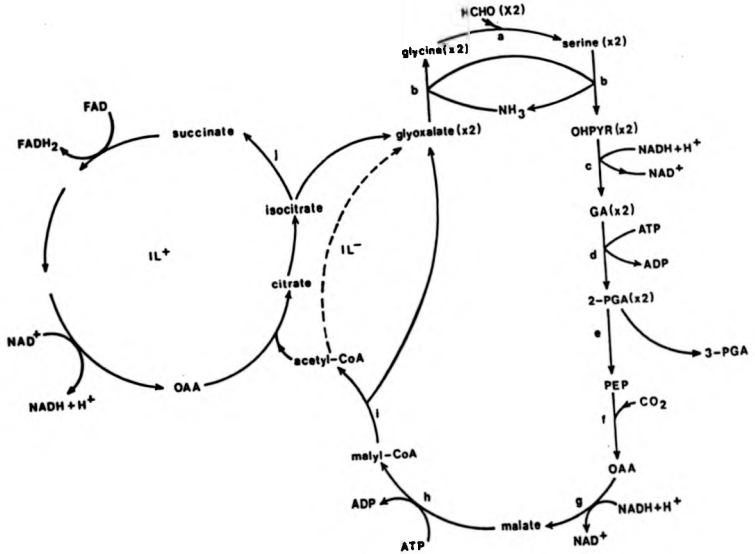
OHPYR, Hydroxypyruvate.

GA, Glycerate.

PGA, Phosphoglycerate.

PEP, Phosphoenol pyruvate.

OAA, Oxaloacetate.


HCHO (X2)
a
glycine(x2)
serine (x2)
b
b
NH3
FAD
FADH2
succinate
glyoxalate (x2)
OHPYR(x2)
NADH+H+
c
NAD+
GA(x2)
ATP
d
ADP
2-PGA(x2)
e
3-PGA
PEP
f
CO2
OAA
g
NADH+H+
NAD+
malate
h
ADP
ATP
malyl-CoA
i
acetyl-CoA
citrate
isocitrate
j
IL+
IL−
NAD+
NADH+H+
OAA

1.3.2.3. Carbon Assimilation Pathways Operating in M.capsulatus (Bath)

The presence of a particular pathway enzyme has been used frequently to determine whether or not a particular metabolic pathway operates within a particular strain of bacteria . Such a technique was used by Lawrence and Quayle (1970) for the initial screening of assimilatory pathways operating in a series of methylotrophic strains. The demonstrated *in vitro* activity of one or two key enzymes does not however offer definitive proof that such a metabolic pathway operates *in vivo*. This has been pointed out in several instances, (Whittenbury *et al*. 1975; Trotsenko, 1976; Bamforth and Quayle, 1977).

The limitations of such an approach were demonstrated by Taylor (1977) and Taylor *et al* (1981) who showed that M.capsulatus (Bath) possessed the enzymes ribulose bisphosphate carboxylase/oxygenase and phosphoribulokinase. These enzymes are normally associated with CO_2 assimilation by the Calvin cycle. However attempts to grow the cells autotrophically failed (Taylor 1977; Stanley and Dalton, 1982) and the fixation of CO_2 could only be demonstrated in the presence of methane, the latter being required as a source of energy. Taylor *et al* (1981) estimated that the level of carbon assimilated at the oxidation level of CO_2 was approximately 2.5% ($^{w}/_{w}$) total cell carbon. It has been postulated that in the absence of FBP aldolase, the presence of RuBP carboxylase/oxygenase may provide an alternative cleavage pathway for the synthesis of 3-phosphoglycerate during growth on methane (Quayle 1979; Stanley and Dalton, 1982).

M.capsulatus (Bath) has been classified as a type I methylotroph; a classification based partially on its ability to assimilate carbon by the RuMP pathway, (Lawrence and Quayle, 1970). Reed (1975) however showed that the organism also possessed hydroxypyruvate reductase activity, a key enzyme in the serine pathway. Eccleston and Kelly (1973) and Reed (1975) reported that the addition of ^{14}C-labelled formate resulted in the detection of labelled glycine and serine with only a limited amount of label appearing in the sugar phosphates. These results implied that M.capsulatus does possess the potential to assimilate carbon by the serine pathway in addition to the RuMP pathway.

It is feasible that such auxiliary C_1 assimilation enzymes operate under specific conditions such as the scavenging of carbon under carbon-limitation or may effectively fix carbon in times of energy excess. Alternatively such routes of C_1 assimilation may have once played a significant role in the cell's metabolic activity, but have lost their original function through evolution.

1.3.3 Dissimilatory Pathways for Energy Generation

To maintain a biosynthetic capacity, methylotrophs are required to oxidise reduced C_1 compounds to CO_2, thereby making both energy and reducing power available to the cell. In the majority of methylotrophs this occurs via a step-wise, linear sequence of oxidation reactions. This oxidation sequence was first proposed by Dworkin and Foster (1956) and is out-lined in fig 1.1. One important feature of this pathway is that many of the dehydrogenases involved do not use nicotinamide nucleotides as electron acceptors. The true nature of the prosthetic groups and of the electron acceptors of some of these enzymes is not known with certainty. It is assumed that such electron acceptors in the cell are more positive in electrode potential than the nicotinamide nucleotides. Consequently, less energy in the form of ATP is generated when these electron acceptors are re-oxidized via the electron transport chain. In addition several enzymes are capable of fulfilling more than one oxidation step. For example the MMO has the capacity to oxidise methanol, as well as methane. Similarly the methanol dehydrogenase is capable of oxidising formaldehyde as well as methanol.

The efficient operation of the oxidation pathway is essential to the cells survival, since not only does it provide the necessary energy and reducing power for the biosynthetic reactions, but it also involves potentially toxic intermediates, notably formaldehyde. Attwood and Quayle (1984) pointed out that the effective operation of this sequence of reactions would be dependent upon the relative intracellular concentrations of the intermediate metabolites. If the metabolite pool sizes were to drop too low, then limitations in substrate availability would arise. This in turn would reduce the efficiency of the pathway since a period of induction would be required to allow the metabolites to regain their optimum levels.

Correspondingly, should such intracellular metabolite concentrations become too high, then the toxicity of such compounds may damage the cell irreversibly.

In addition to formaldehyde being extremely toxic it also occupies the metabolic branch point between carbon assimilation and dissimilation. One might expect that the carbon flux between these two pathways would be flexible enough to accommodate change in the cell's external environment and yet be sufficiently regulated to maintain the correct balance between energy generation and biosynthesis. This must be achieved without upsetting the intracellular concentrations of the intermediate metabolites.

The individual reactions in the linear oxidation pathway will be discussed in more detail in the following sections.

1.3.3.1 Methane Oxidation

The enzyme methane monooxygenase (MMO) is responsible for the NAD(P)H- dependent hydroxylation of methane to methanol. This enzyme can exist in either soluble form (soluble MMO) or in a membrane-associated form (particulate MMO). Some bacteria such as Methylomonas Methanica or Methylomonas albus BG8 express only the particulate MMO, whereas others such as M. capsulatus (Bath) and Methylosinus trichosporium OB3b may express either form, depending on the conditions of cell growth. The factor that determines the nature of the enzyme is the copper: biomass ratio under which the cells are grown, (Stanley et al, 1983). When the ratio is low and the cells are effectively grown under copper-stressed conditions, the cells express predominately soluble MMO. When the copper:biomass ratio is raised, the particulate MMO predominates within the cells.

It is widely believed that this ability to form a soluble MMO under copper-stressed conditions provides the cells with a selective advantage compared with organisms that synthesise only the particulate enzyme. Under low copper conditions, organisms such as Methylomonas albus BG8 are unable to synthesise the soluble enzyme and consequently become copper-limited.

1.3.3.1a Soluble Methane Monooxygenase

To date, the soluble methane monooxygenase (MMO) found in M.capsulatus (Bath) is the most widely studied of all the MMO systems available. Purification and characterisation of the enzyme has been the subject of numerous scientific papers, with much of the earlier work having been reviewed by Dalton (1981). The enzyme has been resolved into three protein components (A, B and C), by DEAE cellulose chromatography. These protein sub-units have been subsequently purified by various chromatographic techniques (Colby and Dalton, 1978; Dalton, 1980, Woodland and Dalton, 1984 a, b).

Protein A has a total molecular weight of 210,000 and comprises of three polypeptides; α , β and γ whose individual molecular weights have been estimated as being 54,000; 42,000 and 17,000 respectively. The native protein contains both non-haem iron (2-3 atoms $mole^{-1}$) and zinc (0.2 - 0.5 atoms $mole^{-1}$) but no acid labile sulphur (Woodland and Dalton, 1984 a). Protein A has no obvious independent catalytic activity, however electron paramagnetic resonance studies (EPR) indicate that the non-haem iron component plays an active role in the binding of the substrate (Dalton, 1980; Woodland and Dalton 1984a).

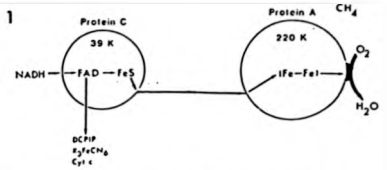
Protein B has a molecular mass of around 16,000 and appears to be devoid of any form of prosthetic group (Green and Dalton, 1985). Like protein A, it has no discernible, independent catalytic activity.

Protein C in contrast to proteins A and B does possess a measurable degree of independent catalytic activity. It acts as an acceptor for NAD(P)H catalysing the transfer of electrons from NAD(P)H to a variety of electron acceptors such as cytochrome c, potassium ferricyanide, DCPIP, oxygen and protein A (Colby and Dalton, 1979). Protein C therefore functions as a NAD(P)H reductase. The protein structure consists of a single polypeptide chain of molecular weight between 39,000 and 44,000, and contains one mole FAD, one mole non-haem iron and one mole of acid labile sulphur for every mole of protein.

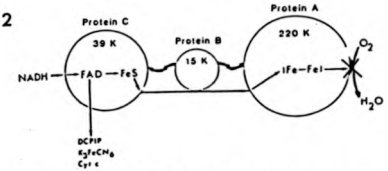
Studies carried out using reconstituted soluble MMO, prepared from purified fractions of proteins, A B and C have made it possible to propose a tentative scheme

concerning the electron transfer and substrate hydroxylation in the enzyme (Lund and Dalton, 1985: Lund et al 1985). Steady-state Kinetic analysis revealed a concerted substitution mechanism in which methane binds to the enzyme followed by NADH to give an initial ternary complex which reacts to yield reduced enzyme and NAD^+. The reduced enzyme-methane complex then binds oxygen to give a second ternary complex, which breaks down to release water and methanol. Green and Dalton (1985) showed that although protein B did not appear to possess a prosthetic group, its presence was essential for the enzyme to fulfil a hydroxylase function. A more detailed analysis of the role of protein B within the MMO enzyme complex indicated that the protein was capable of fulfilling a regulatory role, possessing the capacity to convert the MMO enzyme from an oxidase to an oxygenase. The role of protein B in determining the catalytic function of the soluble MMO from M.capsulatus (Bath) is outlined in fig 1.4. In the presence of proteins A and C, the enzyme catalyses the reduction of oxygen to water in the presence or absence of a hydroxylatable substrate. The addition of protein B switches the enzyme from an oxidase to an oxygenase in the presence of a hydroxylatable substrate. In the absence of substrate, the electron flow between proteins A and C is shut down preventing the reduction of oxygen to water. This would effectively prevent the potentially wasteful oxidation of NADH, a co-factor estimated to be present in vivo in limiting supply when methanotrophs are grown on methane (Anthony, 1978). The addition of hydroxylatable substrate to the complete MMO complex restores the electron flow between proteins A and C, and the oxygenase reaction is catalysed to the complete exclusion of the oxidase reaction.

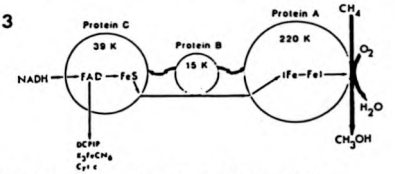
It has been suggested that the role of protein B in uncoupling the oxidase capacity of the enzyme from its oxygenase capacity, may play an important role in the cells need to regulate the relative levels of NADH and NAD^+ (Green and Dalton, 1985; Dalton and Higgins, 1987). Effective regulation of NADH and NAD^+ levels will be essential if the cells are to balance the carbon flux between the assimilatory and dissimilatory pathways without the accumulation of toxic intermediates. The independent oxidase capacity of the MMO may prove important in the removal of excess NADH produced through



1. Components A and C catalyse the four electron reduction of oxygen to water in the presence or absence of a hydroxylatable substrate.



2. The addition of component B switches the enzyme from an oxidase to an oxygenase. In the absence of a hydroxylatable substrate electron flow between components A and C is shut down preventing the reduction of oxygen to water.



3. The addition of a hydroxylatable substrate (methane) to the complete soluble MMO complex restores electron flow between components A and C and the oxygenase reaction is catalysed to the complete exclusion of the oxidase reaction.

Fig 1.4 The mechanism of the soluble MMO from Methylococcus capsulatus (Bath)

the oxidation of C_1 compounds since the level of NADH oxidase within methylotrophic bacteria is assumed to be low (Anthony, 1982). Green and Dalton (1985) reported that the addition of high (possibly non-physiological) levels of formaldehyde to re-constituted MMO proteins did cause uncoupling of the enzyme *in vitro*.

1.3.3.1 b Particulate Methane monooxygenase.

The purification and characterisation of the membrane-bound form of the MMO has been attempted by several workers (Tonge *et al*, 1975; 1977; Higgins *et al*. 1981; Prior, 1985; Smith and Dalton, 1989), with a limited degree of success. Tonge *et al* (1975, 1977) reported the purification of the particulate MMO from *Methylosinus trichosporium* OB3b, however this result has proved impossible to repeat (Higgins *et al*. 1981). To date, the most successful attempt at characterisation of the particulate MMO from *M.capsulatus* (Bath) was reported by Smith and Dalton (1989). In this study the authors reported that the enzyme could be effectively solubilized and reactivated using the non-ionic detergent dodecyl maltoside followed by the addition of lecithin. The role of lecithin in the reactivation of the enzyme suggested that the enzyme required a phospholipid environment in which to function. Further attempts to purify the re-activated, solubilized particulate MMO have been unsuccessful with activity being lost after several stages of purification. Studies concerning the substrate and inhibitor specificities of the different forms of the MMO indicated that although the solubilized form of the particulate MMO and the membrane-bound form possessed similar properties, both differed significantly with repect to the soluble MMO found in cells grown under low copper conditions. Therefore it would appear that the soluble MMO and the particulate MMO are distinct and unrelated; the particulate MMO is not a form of the soluble MMO associated with a membrane.

1.3.3.1 c Regulation of the MMO by Copper Ions and the Supply of Electrons to the MMO

As yet the role of copper ions as the regulator of the physical manifestation of the MMO has not been

explained. Results suggest that copper levels not only effect the physical form of the enzyme (Stanley et al, 1983) but also the enzymes specific activity (Prior and Dalton, 1985; Green et al, 1985; Smith and Dalton, 1989).

The addition of copper ions to chemostat cultures of M.capsulatus (Bath) resulted in the rapid inhibition of the soluble MMO and the synthesis of the particulate enzyme (Dalton et al, 1984). Sodium dodecylsulphate (SDS) polyacrylamide gradient gels showed that this loss of soluble MMO activity was not due to soluble MMO degradation, since the α, β and γ subunits of protein A could be clearly seen up to two hours after the loss of soluble activity. The nature of this inhibition was shown by Green et al, (1985) to involve the inactivation of protein C, preventing the electron flow from C to A. Reports of enzyme inhibition by copper are not unique to the MMO. Several enzymes have been shown to be sensitive to copper ions e.g. phenylalanine hydroxylase (Kaufman, 1962), glycine oxidase (Ratner, 1955) and succinate dehydrogenase (Bonner, 1955). In most cases however, the mechanism of copper inactivation has not been elucidated.

In addition to the disappearance of the α, β and γ subunits of protein A as visualized by SDS polyacrylamide gels, the addition of copper to chemostat cultures of M.capsulatus (Bath) also stimulated the increased or de novo synthesis of at least three polypeptides associated with the particulate fractions, (Leak et al, 1985). Prior and Dalton (1985) also reported that the addition of copper (II) ions to cell-free, membrane preparations of M.capsulatus (Bath) stimulated particulate MMO activity. It was therefore suggested that copper ions may act as a co-factor or prosthetic group for a protein present in the extract. A similar result was obtained when copper (II) ions were added to the solubilized form of the enzyme (Smith and Dalton 1989).

It has still to be established whether the copper affects the particulate MMO directly or is involved in the synthesis or activity of other proteins associated with methane oxidation, in particular a protein involved in electron transport. Studies by Ribbons (1975), Colby and Dalton, (1976); Stirling and Dalton (1977); Scott et al, (1981) and Patel et al (1982); showed that when MMO was associated in vitro with membrane components, its sensitivity to metal chelators, thiol chelators and

electron transport inhibitors was greatly enhanced, compared to its soluble form.

Although both the soluble and particulate MMO require NADH as an electron donor when measured in cell extracts, the situation may be different in whole cells. Ferenci et al (1975) reported that carbon monoxide oxidation by whole cells of Methylomonas methanica was stimulated by ethanol, even though there was no NAD^+-linked alcohol dehydrogenase present in the cell extract. These authors concluded that ethanol oxidation was indirectly coupled to the reduction of NAD^+ via reversed electron transport; the resultant NADH produced, supplying electrons to the MMO. A second alternative was proposed by Prior (1985) who mentioned that NADH might not be the electron donor for the particulate MMO in vivo. Instead the reductant for the MMO may be a component of a membrane-bound electron transport chain, for example a copper-containing protein that was only synthesised when the cells were grown under conditions of copper excess. Leak and Dalton (1986) proposed that active methanol dehydrogenase may be capable of electron transfer to the particulate MMO. This was based on their results which showed that the oxidation of C_2 to C_4 primary alcohols and their corresponding aldehydes by a variety of methanotrophs appeared to stimulate methane monooxygenase activity. To date no one has demonstrated the existence of an NAD^+-linked oxidation route for these higher alcohols and aldehydes. The effect of copper and the role of electron donors in determining the energetics of methane oxidation will be discussed later in this chapter.

1.3.3.2. Methanol Oxidation

1.3.3.2.a. Methanol Dehydrogenase

It is widely assumed that relatively non-polar compounds such as methanol and formaldehyde enter the cell by means of passive diffusion (Bellion et al 1983). Once in the cell, the process of methanol oxidation appears to be largely mediated by an NAD^+-independent methanol dehydrogenase. This enzyme was first described by Anthony and Zatman (1964a, b) in Pseudomonas M27, but has now been isolated and characterized in approximately 30 other methylotrophic bacteria (Anthony, 1982).

The enzyme is assayed *in vitro* at its pH optimium of 9, in the presence of ammonia activator and the artificial electron acceptor, phenazine methosulphate. The electron acceptor *in vivo* is widely believed to be cytrochrome *c* by anaerobically - prepared methanol dehydrogenase (Duine *et al*. 1979; O'Keeffe and Anthony, 1980).

Anthony and Zatman (1967) described the *in vitro* fluorescence characteristics of methanol dehydrogenase and showed that it possessed a novel prosthetic group which is a feature of all methanol dehydrogenases. This prosthetic group was subsequently purified and characterized by Duine *et al*. (1980) and was later named "Pyrrollo-quinoline quinone (PQQ)". PQQ has now been shown to be the prosthetic group in a wide range of dehydrogenases and has been isolated from both prokaryotic and eukaryotic systems. For a review of the properties and occurrence of such quinoproteins in nature, the reader is referred to articles by Duine (1989) and Anthony (1989).

Although the methanol dehydrogenase from most bacteria is found in the soluble fraction after cell breakage, membrane-bound activity has been demonstrated in *M.capsulatus* and in *Paracoccus denitrificans* by Wadzinki and Ribbons (1975) and Bamforth and Quayle (1978) repectively. Observations by O'Keefe and Anthony (1978) and Dawson and Jones (1981a) implied that the methanol dehydrogenase was located on the outer side of the cytoplasmic membrane. Consequently if the methanol dehydrogenase is weakly bound to the cytoplasmic membrane, then the distribution of the enzyme between soluble and membrane fractions of cell extract will be dependent upon the method by which the cell extract is prepared.

In general the substrate specificity of methanol dehydrogenase is restricted to primary alcohols, hence the alternative name of primary alcohol dehydrogenase. The affinity of the enzyme for substrate decreases with increasing chain length, hence methanol dehydrogenase has a high affinity for methanol with a Km value for methanol of 10-20μM (Anthony, 1982). Another common characteristic of the methanol dehydrogenase is its ability to oxidise formaldehyde to formate.

This characteristic of the methanol dehydrogenase will be discussed in greater detail in section 1.3.3.3.a. It is suffice to say that the capacity of the methanol dehydrogenase to catalyse the oxidation of methanol via

formaldehyde to formate could have important implications concerning the regulation of C_1 metabolism in methylotrophs.

Recently a novel NAD^+-dependent methanol dehydrogenase was isolated by Duine et al (1984). Although the above enzyme was orginally isolated in Nocardia sp.237, it has also been reported to occur in M.capsulatus (Bath), (Duine, unpublished). Characterisation of the NAD^+-linked methanol dehydrogenase by Duine et al (1984) indicated that the enzyme activity resided in a multienzyme complex which could be resolved into 3 components. These components consisted of a PQQ-containing methanol dehydrogenase, an NAD^+-dependent aldehyde dehydrogenase and an NADH dehydrogenase. The implications of the discovery of NAD^+-linked dehydrogenase in M.capsulatus (Bath) are important as this enzyme could possibly provide reducing power for MMO systems in the form of NADH.

1.3.3.2.b. Methanol Oxidation by the MMO

Soluble MMO from M.capsulatus(Bath) has been shown to be capable of utilizing methanol as a substrate in vitro. (Colby et al, 1977). Anthony (1982) proposed that growth yields of methanotrophs on methanol were lower than expected, as a consequence of methanol oxidation by the MMO instead of the energetically more favourable methanol dehydrogenase. Direct evidence for methanol oxidation by the MMO in vivo was offered by Cornish et al (1984) using ^{13}C NMR in whole cells of Methylosinus trichosporium OB3b. Using a pulsed feed Cornish et al showed that the rate of methanol utilization and formaldehyde formation could be reduced by inactivating the MMO capacity of the cells. The role of the MMO in the oxidation of methanol in M.capsulatus (Bath) will be examined in greater detail in chapter 8.

1.3.3.3. Formaldehyde Oxidation

Formaldehyde occupies the key position in the methane oxidation pathway since it exists at the branch point between carbon assimilation and dissimilation. There is a greater diversity of proposed enzyme routes by which formaldehyde may be metabolized than any other C_1

compound. It is also a very reactive compound, reacting not only with water but also with thiol and amide groups. Such reactions are not enzyme-catalysed but occur spontaneously and are very rapid in their nature. Consequently formaldehyde is a potentially very toxic substance. Attwood and Quayle (1984) estimated that an interruption of one minute in the metabolism of formaldehyde would be sufficient to cause the accumulation of the metabolite to levels of almost 100mM. Since formaldehyde is normally toxic to cells at concentrations as low as 1mM (Hirt et al, 1978; Attwood and Quayle, 1984) the regulation of the intracellular levels of this metabolite is crucial to the cells survival .

In methylotrophic bacteria, the complete oxidation of formaldehyde to CO_2 results from successive dehydrogenase action or from a cyclic series of reactions involving C_1 assimilation enzymes. Within the linear route of C_1 oxidation there exist several enzymes capable of oxidising formaldehyde to formate. The capacity of the methanol dehydrogenase to oxidise formaldehyde has been widely acknowledged (Sperl et al, 1974) In addition to the methanol dehydrogenase, cells often possess various formaldehyde dehydrogenases, which can be classified into two types:-

1) $NAD(P)^+$-linked formaldehyde dehydrogenase
2) $NAD(P)^+$-independent formaldehyde dehydrogenase

Stirling and Dalton (1978), purified an $NAD(P)^+$-linked formaldehyde dehydrogenase from M.capsulatus (Bath) which also required the presence of a heat-stable proteinaceous co-factor for activity. In addition, M.capsulatus (Bath) has also been shown to possess the capacity to oxidise formaldehyde via a dehydrogenase that does not require $NAD(P)^+$ as a co-factor. Instead the activity of the latter enzyme can be demonstrated in vitro using non-physiological dyes similar to those used to monitor methanol dehydrogenase activity. However, unlike the methanol dehydrogenase, the $NAD(P)^+$-independent formaldehyde dehydrogenase does not require ammonium ions to act as an activator.

Whether or not NAD(P)H is produced during formaldehyde oxidation within the cell is an important consideration, since in some methylotrophs this reductant

is available in limiting concentrations, as a consequence of the amounts required for assimilation and the NAD(P)H-dependent hydroxylation of methane. Furthermore, relatively less ATP is likely to be produced during formaldehyde oxidation if the process is linked to the electron transport chain by way of a flavoprotein dehydrogenase or methanol dehydrogenase. The various methods by which M.capsulatus (Bath) can oxidise formaldehyde will be discussed in greater detail in the following sections.

1.3.3.3.a. Formaldehyde Oxidation by Methanol Dehydrogenase and the Role of the Modifier Protein.

It is widely assumed that methanol dehydrogenase does not oxidise formaldehyde directly, but instead oxidises the hydrated (diol) form of the metabolite, since this is formed spontaneously from formaldehyde and water. Although Anthony (1982) pointed out that the affinities of the methanol dehydrogenase for both methanol and formaldehyde are similar, there is still considerable controversy as to whether this enzyme plays a major role in the oxidation of formaldehyde in vivo. Attwood and Quayle (1984) claimed that it would be uncommon for one enzyme such as the methanol dehydrogenase to catalyse two successive reactions, particularly when the product of the first reaction has such an important metabolic position. Furthermore, mutants of Pseudomonas sp. strain AMI and Hyphomicrobium sp. strain X which lack methanol dehydrogenase, are capable of oxidising formaldehyde at a rate equivilent to wild-type bacteria (Dunstan et al. 1972; Heptinstall and Quayle, 1970; Marison and Attwood, 1982).

The most recent work concerning formaldehyde metabolism by the methanol dehydrogenase has been carried out by Page and Anthony, (1986). These authors implicated the involvement of a regulatory protein in formaldehyde oxidation in Methylophilus methylotrophus. This emanated from the observation by Bolbot and Anthony (1980) that Pseudomonas AMI was capable of oxidising 1,2 propanediol via methanol dehydrogenase, This result was unexpected since it had previously been assumed that a second substituent on the C-2 atom would prevent the binding of a substrate such as 1,2 propandiol to the methanol

dehydrogenase (Anthony and Zatman, 1965). Further *in vitro* analysis by Bolbot and Anthony (1980), and Ford *et al* (1985) showed that although the affinity of the methanol dehydrogenase for propan 1,2 diol was poor, it could be increased dramatically via the addition of a high molecular weight protein (m.wt. of 140,000) termed a modifier protein. In the presence of this protein propan-1,2 diol was oxidised to lactaldehyde. Page and Anthony (1986) subsequently demonstrated that *Methylophilus methylotrophus* also possessed the modifier protein and that not only did its addition to *in vitro* preparations of the methanol dehydrogenase result in an increased affinity of the enzyme for propan 1,2 diol; but it also effectively reduced the affinity of the methanol dehydrogenase for formaldehyde by over 97%. Consequently in the presence of modifier protein the end product of *in vitro* oxidation of methanol was formaldehyde, whereas in the absence of the protein, the end product was formate. Based on these observations Page and Anthony concluded that the primary function of the modifier (M) protein *in vivo* was the regulation of formaldehyde oxidation by the methanol dehydrogenase.

Such a regulatory mechanism would prove very important to cells, since it has the potential not only to prevent formaldehyde oxidation by an energetically less favourable route, but may also act as a "safety valve" for the removal of excess formaldehyde. Preliminary evidence for the latter role of the M protein was offered by Page and Anthony (1986). The authors noted that when the cells were grown under oxygen-limited conditions the ratio of M protein to methanol dehydrogenase was approximately 1:15 whereas when the cells were subjected to carbon limitation the equivalent ratio was only 1:2. Under oxygen-limited conditions, the cells would be more liable to accumulate the toxic formaldehyde. Therefore it would be in the cells interest to synthesise less M protein and permit the methanol dehydrogenase to oxidise excess formaldehyde to formate.

1.3.3.3.b. $NAD(P)^+$-linked Formaldehyde Dehydrogenase

The enzymes in this group include both formaldehyde-specific and non-specific aldehyde dehydrogenases, and many require glutathione for activity. Stirling and

Dalton (1978) purified an $NAD(P)^+$-linked formaldehyde dehydrogenase from M.capsulatus (Bath) which required the presence of a heat-stable co-factor from cell extracts for activity. Recent work by Green, Millet and Dalton (unpublished) has led to the purification of this heat-stable co-factor, preliminary evidence suggesting that it is proteinaneous in origin with a molecular weight of around 10,000. The purified protein (named protein F) appears to be devoid of prosthetic groups but does possess the capacity to alter the substrate specificity of the $NAD(P)^+$-linked formaldehyde dehydrogenase. In the presence of protein F, the enzyme utilizes formaldehyde as its sole substrate. When the protein F is removed, the enzyme loses its capacity to oxidise formaldehyde and aquires the potential to utilize higher aldehydes such as ethanal, propanal and butanal.

The ability of the heat-stable component to alter the substrate specificity of the enzyme may well be an important survival mechanism for M.capsulatus (Bath) in its natural environment since aldehydes are not only toxic but also ubiquitous in their occurrence in nature. Such compounds may also arise as a result of the organism's own metabolic processes, due to the wide substrate specificity of both the MMO and the methanol dehydrogenase. Therefore the $NAD(P)^+$-linked formaldehyde dehydrogenase may fulfil not only a energetically-favourable role in the methane oxidation pathway but in addition may also fulfil a detoxifying role.

NAD^+-linked formaldehyde dehydrogenase that requires a low molecular weight factor has also been isolated in the non-methylotroph Rhodococcus erythropolis (Eggeling and Sahm, 1985) and in methanol-grown Nocardia sp.239 (Ophem and Duine, 1989). The exact nature of the above factors has yet to be established, as has their role in regulating formaldehyde metabolism.

1.3.3.3.c. $NAD(P)^+$-Independent Formaldehyde Dehydrogenase

This group of enzymes require the use of artificial electron acceptors to assay their activity in vitro and normally show a broader substrate specificity than their $NAD(P)^+$-linked counter-parts. Marison and Attwood (1980) compared the activity of such 'dye-linked' formaldehyde dehydrogenases in a number of bacteria growing on C_1

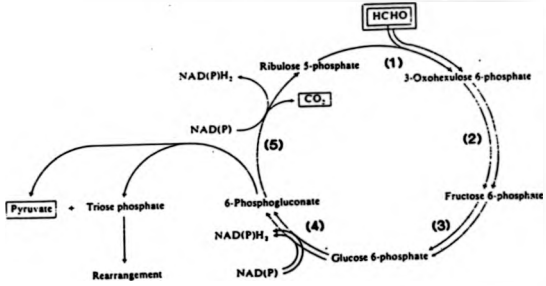
compounds. Their results showed that activity levels of such "dye-linked" formaldehyde dehydrogenases were consistently low and were not induced during cell growth on C_1 compounds. Therefore it was concluded that such enzymes did not play a major role in the dissimilation of formaldehyde. It should however, be borne in mind that non-physiological dyes were used to measure the level of enzyme activity *in vitro* and therefore the activities obtained may be an underestimation of the true level of activity *in vivo*.

The nature of the prosthetic group of the NAD^+-independent formaldehyde dehydrogenase is of considerable importance when considering the bioenergetics of cell growth. This will determine the point at which electrons enter the cytochrome chain and hence the ATP yield during formaldehyde oxidation. One would expect less ATP to be produced if the process is coupled to the electron transport chain by way of flavoprotein dehydrogenase or methanol dehydrogenase than if NADH is produced.

1.3.3.3d. Cyclic Dissimilation of Formaldehyde

Strom *et al* (1974) and Colby and Zatman (1975) proposed the existence of a cyclic scheme for the complete oxidation of formaldehyde to CO_2. This involved the enzymes associated with the RuMP pathway plus 6-phosphogluconate dehydrogenase (6PGD). The cycle is outlined in fig 1.5.

Some bacteria using the RuMP pathway for formaldehyde assimilation have little or no formaldehyde dehydrogenase, or formate dehydrogenase activity and hence, rely entirely on this cyclic route for formaldehyde dissimilation. Although *M.capsulatus* (Bath) has been shown to possess both formaldehyde and formate dehydrogenase activity; Stirling (1978) showed that the above organism also had the enzymic capacity to oxidise formaldehyde by the cyclic route. Examination of the activities of the key enzymes (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) showed the levels of these enzymes to be low compared with the equivalent activities of the formaldehyde and formate dehydrogenases associated with the linear oxidation pathway (Dalton and Leak, 1985). It has been suggested that the operation of the cyclic route might be used to fulfil the cells requirement of NADPH for



Enzymes

1) hexulose phosphate synthase
2) hexulose phosphate isomerase
3) glucose phosphate isomerase
4) glucose - 6 - phosphate dehydrogenase
5) 6 - phosphogluconate dehydrogenase

Fig 1.5 The cyclic route for the dissimilation of formaldehyde

biosynthetic purposes rather than NADH since the 6 PGD has been shown to be $NADP^+$-specific (Davey *et al* 1972).

Besides providing a route for the oxidation of formaldehyde, the cycle also provides a means of controlling the fate of carbon between the assimilatory and the cyclic oxidation routes. Beardsmore *et al* (1982) showed that the 6 PGD, the enzyme occupying the branch point between the cyclic dissimilatory route and the RuMP pathway is inhibited by its "end products", NADH and ATP. Whether or not a similar regulatory system operates in the linear oxidation pathway has yet to be established.

1.3.3.3e. Regulation of Formaldehyde Metabolism

The regulation of the fate of endogenously generated formaldehyde is crucial to the efficient operation of the metabolic processes in methylotrophic bacteria. In spite of this relatively little is known concerning how these organisms regulate their metabolism in order to maintain strict intracellular levels of formaldehyde.

One organism in which the effect of formaldehyde metabolism has been examined in some detail is the facultative methylotroph *Arthrobacter* P1. Dijkhuizen and Levering (1987) showed that formaldehyde generated from the metabolism of methylamine or added directly resulted in the induction of the RuMP pathway enzymes, hexulose phosphate synthase and hexulose phosphate isomerase. This occurred even during growth on heterotrophic substrates in the presence of formaldehyde. Dijkhuizen and Levering also suggested that formaldehyde was capable of regulating its own rate of synthesis from methylamine by inhibiting both the methylamine transport system and the amine oxidase.

The identification of potential regulatory mechanisms controling formaldehyde levels in obligate methylotrophs has been limited to a number of observations. These were listed by Dalton and Higgins (1987).

1) Formaldehyde accumulation has been recorded in cells of *Methylosinus trichosporium* OB3b containing particulate MMO, but not in cells containing the soluble form of the enzyme, (Cornish *et al*, 1984).

2) Under some conditions methanotrophs are difficult to grow in pure culture or to adapt to growth on methanol, probably due to the accumulation of formaldehyde (Linton

and Vokes,1978).

3) It has been shown that the protein B of the soluble MMO modulates the oxidase and oxygenase activities of this enzyme and that formaldehyde via its effect on protein B causes stimulation of the NADH oxidase activity of the MMO.

4) Dalton *et al* (1984) reported that formaldehyde effectively repressed the synthesis of soluble MMO in *M.capsulatus* (Bath).

A theory based on the above observations was presented by Dalton and Higgins (1987), suggesting that the level of formaldehyde in *M.capsulatus* (Bath) was linked to the NAD^+:NADH ratio in cells. In cells possessing soluble MMO, formaldehyde regulates the supply of NAD^+ available for the NAD^+-linked oxidation of formaldehyde. This is achieved by the stimulation of the oxidase function of the MMO, in the presence of formaldehyde, which in turn leads to the generation of NAD^+. This NAD^+ can then be used to allow the NAD^+-linked oxidation of the excess formaldehyde. In contrast, cells containing the particulate MMO utilize alternative electron donors to the NADH for methane oxidation and therefore effectively limit the availablity of NAD^+, required for NAD^+-linked oxidation of formaldehyde. To test this hypothesis, a detailed analysis of the respective levels of NAD^+ and NADH in *M.capsulatus* (Bath) under different conditions of growth is required.

It has yet to be established whether or not the M and F proteins isolated by Page and Anthony (1986) and Green, Millet and Dalton (unpublished) play a role in the *in vivo* regulation of formaldehyde metabolism in methylotrophic bacteria. The mere fact that they exist and they appear to be very specific in their mode of action might imply that formaldehyde metabolism in such organisms is highly regulated.

1.3.3.4 Formate Oxidation

Two types of formate dehydrogenase have been described in bacteria; one is a soluble, NAD^+-linked enzyme which is specific for formate and the other is a membrane bound, NAD^+-independent enzyme which donates electrons to the cytochrome chain at the level of cyctochrome *b* (Dijkhuizen *et al*, 1978, 1979; Rodinov

and Zakharova, 1980). The distribution and specific activities of formate dehydrogenases in a variety of bacteria are detailed by Zatman (1981). In many methylotrophs the formate dehydrogenase appears to be the only enzyme capable of providing NADH for biosynthesis during growth on C_1 compounds.

Attempts to purify formate dehydrogenase from a number of bacterial sources has proved difficult due to the apparent instability of the purified form of the enzyme (Muller et al, 1978; Egorov et al 1979; Jollie, unpublished).

Studies concerning the regulation of formate oxidation have been limited to a number of individual observations. For example, Attwood and Harder (1978) reported that higher levels of NAD^+-linked formate dehydrogenase were induced in Hyphomicrobium X when the cells were grown in the presence of formate or methanol. Marison (1980) reported that the formate dehydrogenase from the above organism was inhibited by both ATP and NADH. This latter observation would imply that the formate dehydrogenase in Hyphomicrobium X may play a role in regulating the carbon flux between assimilation and dissimilation. Under conditions of high biosynthetic capacity (high ATP and high NAD(P)H), the complete oxidation of C_1 compounds would be restricted and the assimilation of carbon presumably increased.

1.3.4 Energetics of Methylotrophic Growth

To grow efficiently, methylotrophic bacteria must be capable of balancing the amount substrate carbon fed into the energy-generating oxidation reactions with that required by the energy-demanding biosynthetic reactions. Consequently the measured efficiency of cell growth will be intimately linked to the cell's ability to regulate the fate of substrate carbon.

In theory, it should be possible to predict methylotrophic growth yields for a given substrate provided that the metabolic pathways involved in the utilization of the substrate are known and certain assumptions are made. Included in these assumptions are that bacterial biomass contains elements in the proportions of C_4 H_8 O_2 N and that all substrate, during cell growth is converted into either cellular

material or CO_2. It is also assumed that the synthesis of cellular material from 3-phosphoglycerate requires approximately the same amount of ATP irrespective of whether the original carbon source was methane or glucose. Consequently to predict the cell yield on methane we need only estimate the energy requirement to make 3-phosphoglycerate from reduced C_1 compounds.

In practice, there is one limitation in accurately predicting cell growth yields on C_1 compounds. This concerns our limited knowledge of how the transport of electrons is linked to energy transduction during the oxidation of C_1 compounds. Due to the unfamiliar nature of some of the electron acceptors involved in the above processes it is difficult to estimate the efficiency with which C_1 oxidation is linked to ATP production. Despite this, theoretical methylotrophic growth yields have been predicted by several workers (Harrison et al, 1972; van Dijken and Harder, 1975; Barnes et al,1976; Harder and van Dijken, 1976; Anthony, 1978; Leak and Dalton 1986b).

Anthony (1982) acknowledged that predicting the effects of physiological variables on cell yield was considerably easier than actually measuring them. Published yields for bacterial growth on methane vary widely even when the studies concerned have used the same or similar organisms. Various authors have estimated that between 19-70% substrate carbon may be incorporated into cell material (Whittenbury et al, 1970; Harwood and Pirt, 1972; Stanley, 1977; Linton and Vokes, 1978). One of the major reasons for such discrepancies concerns the inherent difficulties in accurately measuring rates of substrate utilization when the substrate is in the gaseous phase. Other parameters that must be considered when measuring cell yield include the nature of the nitrogen source used and maintenance requirements of the cells.

A recent study by Leak and Dalton (1986a) concluded that one other factor that determined the efficiency of growth of M.capsulatus (Bath) on methane was the energetic requirements of the soluble and particulate MMO. The authors estimated that when the cells contained the particulate form of the MMO, the cells were capable of assimilating up to 8% more of the total carbon utilized, compared to cells containing soluble MMO. Leak and Dalton concluded that the observed differences in growth yield were a consequence of the different reductant requirements

of the two enzyme systems, the soluble MMO having an absolute requirement for NADH while the particulate enzyme was capable of ultilizing reductants other than NADH. The role of the MMO in determining the efficiency of methylotrophic growth will be re-examined during the course of this study.

1.3.5 The Environmental Regulation of Methylotrophic Growth

The efficiency of microbial growth is intimately linked to the cell's environment. Pirt (1975), stated that metabolic pathways, end products, and ATP yields of carbon and energy source metabolism were all regulated by environmental factors such as dissolved oxygen concentration, pH and temperature. Other factors that influence cell metabolism include specific growth rate and whether the carbon source is available in limiting concentration or is present in excess.

Recent studies by Bussineau and Papoutsakis (1986), and Jones *et al* (1987) have shown that the levels of specific C_1 oxidising enzymes in *Methylomonas* L3 and *Methylophilus methylotrophus* are closely linked to the specific growth rate of the cells. In particular, higher levels of the methanol dehydrogenase activity were obtained when the cells were grown at a lower dilution rate. In addition, Jones *et al* showed that both the methanol dehydrogenase and formate oxidation were repressed in the presence of high concentrations of methanol in the medium. The derepression of enzyme synthesis at low growth rates and under carbon-limiting conditions is not uncommon among catabolic enzymes, (Harder and Dijkhuizen, 1983). It is viewed as a survival mechanism for bacteria growing in nature where because of extremely low concentrations of nutrients (probably below the Km of the catabolic enzyme), a flux sufficient for growth can only be generated by increasing the amount of enzyme present.

Using *in situ* radioisotopic tracer techniques, Bussineau and Papoutsakis (1986) examined the rates of substrate carbon flow *in vivo*, along with the corresponding steady-state levels of several key RuMP and methane oxidation pathway enzymes in *Methylomonas* L3. Their results however led them to conclude that an

absolute correlation between the *in vivo* carbon flux and the *in vitro* specific activity of the enzymes studied could not be established.

The capacity of methylotrophic bacteria to alter their composition and metabolism in response to environmental changes would suggest that specific regulatory mechanisms operating in these organisms are key to their survival. As yet little is known about the actual mechanisms that operate within the cells to regulate key enzyme activity.

Three possible mechanisms by which the activity of specific enzymes may be regulated include the reversible binding of effector molecules, covalent modification and the alteration in the level of enzyme synthesis. Work by Page and Anthony (1986), Green and Dalton (1985) and Green, Millet and Dalton (unpublished) indicated that there exists a series of regulatory proteins (proteins M,B and F) which are capable of modifying the activity of specific enzymes. A summary of the properties of these proteins is given in table 1.4. One would expect this type of regulatory control to give a very rapid response, although it may be limited in the extent to which it is capable of modifying the activity.

A second alternative involves the covalent modification of an enzyme by a means such as phosphorylation, adenylation or glycosylation. This type of reponse is normally slower than equivilent regulation by effectors, although in most cases is normally complete within minutes (Martin, 1987). Over the past ten years there has been an increased realisation of the relative importance of phosphorylation as a means of regulating prokaryotic metabolism. Several phosphorylated protein-systems have been identified in bacteria as diverse *Myxococcus xanthus* (Komano *et al*, 1982), *Escherichia coli* (Nimmo, 1984), *Clostridium sphenoides* (Antranikian *et al* 1985); and *Rhodomicrobium vannielli* (Turner and Mann, 1986, 1988). This form of regulation however requires a second enzyme to catalyse the modification step and is itself subject to control.

The most obvious means by which cells can regulate enzyme activity is via controlling the level of enzyme expressed. This can be achieved by altering the relative rates of enzyme synthesis and enzyme degradation by processes of induction and repression. Co-ordinate

Table 1.4 Comparison of the properties of effector proteins associated with the methane oxidation pathway.

Effector Protein	Heat-stable component of formaldehyde dehydrogenase	protein M	protein B
associated enzyme	NAD^+- linked formaldehyde dehydrogenase	methanol dehydrogenase	MMO
Organism	M.capsulatus (Bath)	Methylophilus methylotrophus	M.capsulatus (Bath)
Mr	10,000	Originally isolated as 140,000; but now estimated to be 40,000 (Anthony, personal communication)	16,000
Mechanism of action	Changes the substrate specificity of the formaldehyde dehydrogenase such that it is specific for formaldahyde	Changes the substrate specificity of the methanol dehydrogenase such that it is no longer capable of oxidising formaldehyde	Changes the type of reaction catalysed by MMO, such that it is converted from an oxidase to an oxygenase reaction
ref.	Green, Millet and Dalton (unpublished)	Page and Anthony (1986)	Green and Dalton (1985)

induction or repression of enzymes involved in C_1 metabolism has been observed in several facultative methylotrophs that use the serine pathway (O'Connor, 1981).

In contrast to regulation by effector or covalent modification, induction and repression of enzyme synthesis is slower in its operation. Consequently it is often associated with long term changes in the physiological state of the cell. Such changes are often in response to changes in the cells environment similar to those observed by Bussineau and Papoutsakis (1986) and Jones *et al* (1987). In conclusion, it is often possible to predict the means of regulation by monitoring the rate at which levels of enzyme activity change within cells.

1.4 Aims of the Present Work

In spite of all the research that has been carried out on C_1 metabolism in methylotrophic bacteria, relatively little is known about how the cells are able to apportion carbon between the required assimilation and energy-yielding reactions; particularly with repect to preventing the accumulation of toxic metabolites. The recent isolation of what appear to be specific regulatory proteins associated with specific steps in the C_1 oxidation sequence has led to a great deal of speculation concerning the potential co-ordination and control of C_1 metabolism.

The problem with such speculation is that it is based predominately on results generated using *in vitro* enzyme systems. Ideally the presence of such regulatory proteins should be demonstrated *in vivo* such that observed changes in the metabolic activity of cells may be related to the presence or absence of such proteins. The problem of studying the role of these proteins *in vivo* is that the turnover of metabolites in the cell is both rapid and continuous. Consequently there is always the danger that the techniques used to study these systems *in vivo*, may create artefacts that are not a true representation of what is actually occurring within the system at a given moment of time.

One technique that has been used to monitor changes in metabolite levels that is both rapid and non-invasive is that of "nuclear magnetic resonance", (NMR). Such a

technique has been used by Cornish et al (1984) and Jones et al (1987) to study in vivo metabolism in methylotrophic bacteria, with some revealing results. The limitations of this technique are that it requires the removal of cells from an actively growing culture and the introduction of significant levels of labelled metabolite (mM concentration). The sensitivity of the technique is also poor, and it requires high levels of metabolite (>2mM) to be made before it will detect them.

The other non-invasive analytical technique available that provides rapid and sensitive analysis of metabolite changes in a microbial culture is mass spectrometry. By coupling a chemostat to an on-line mass spectrometer it is possible to grow organisms under defined and reproducible conditions in which one can readily monitor the concentrations of dissolved gases and volatiles. With repect to the study of methanotrophic growth, changes in the gas exchange rates (methane, oxygen and carbon dioxide) can be monitored and the results used as indirect indices of changes in culture physiology, (Wang et al, 1979; Buckland et al 1985).

The aim of this project is to examine how carbon metabolism is regulated in M.capsulatus (Bath). A way to achieve this is to develop techniques that allow you to follow the changes that occur when cells are grown under specific conditions and then perturbed. Such perturbations made to the cells external environment may include the introduction of methane oxidation pathway intermediates to determine whether or not cells can tolerate raised levels of such metabolites. The effect of such changes may be reflected in variations in the levels of key enzymes, changes in their substrate specificity or the accumulation of intracellular metabolites involved in C_1 metabolism. One would expect such changes to be closely linked to changes in the carbon flux between the various metabolic pathways operating within the cell, co-ordination of these pathways being essential to permit cell growth.

Results obtained from these studies can then be compared with results and theories discussed earlier in this chapter with a view to obtaining a clearer picture as to how C_1 metabolism is regulated in both M.capsulatus (Bath) and methylotrophic bacteria in general.

CHAPTER 2

Materials and Methods

2 Materials and Methods

2.1 Organism

The organism used throughout this study was Methylococcus capsulatus (Bath), which was originally isolated by Whittenbury et al (1970) and which was maintained as a stock culture in our laboratory.

2.2 Media

A nitrate mineral salts medium (NMS) based on that described by Whittenbury et al (1970) was used throughout for the routine growth of this organism. The composition of this medium is given in table 2.1. To prevent precipitation of the phosphates in the medium, these were sterilized separately and added aseptically to the sterile medium when the temperature of the medium was less than 60°C.

For solid medium, $15gl^{-1}$ Difco bacto-agar was added to the nitrate mineral salts medium (minus phosphates) prior to sterilization. Sterile phosphate solution was added to sterile mineral salts medium when the agar was cooling.

2.3 Maintenance and Growth

Cell cultures were maintained on NMS agar plates as described previously (Whittenbury et al, 1970). These were incubated at 45°C in an anaerobic jar, flushed with methane to give a final concentration of approximately 50% $^{v}/_{v}$ methane in air. Cultures were routinely sub-cultured (approximately every two weeks) to prevent fungal contamination and to maintain fresh stocks.

Routine growth on liquid medium was achieved using narrow-necked conical flasks (250ml volume) containing 50ml sterile NMS medium. Flasks were inoculated with small amount of M.capsulatus (Bath) and sealed with a Suba-Seal (William Freeman and Co. Ltd., Barnsley, U.K.) 50ml of air was then removed from the flask and replaced with 50ml methane, before incubating the flasks at 45°c, on a rotary shaker.

Studies on continuous culture of the organisms were

Table 2.1 Composition of Trace Element Solution used in Nitrate Mineral Salts (NMS) Medium

Compound	mg l^{-1}
$CuSO_4.5H_2O$	200
$FeSO_4.7H_2O$	500
$ZnSO_4.7H_2O$	400
H_3BO_4	15
$CoCl_3.6H_2O$	50
EDTA	250
$MnCl_2.4H_2O$	20
$NiCL_2.6H_2O$	10
$NaMoO_4.2H_2O$	500

N.B. This is the trace element solution for low copper medium. In order to produce high copper medium an additional 1 g l^{-1} (1000 mg l^{-1}) should be added to the above list.

Nitrate Mineral Salts Medium

KNO_3	1g l^{-1}
$MgSO_4$	1g l^{-1}
$CaCl_2$	0.26 g l^{-1}
Fe/EDTA	4 mg l^{-1}
Trace element soln.	1 ml l^{-1}

Separate addition phosphate soln. (10X) 10 ml l^{-1}.

10X Phosphate Solution

$Na_2HPO_4.12H_2O$	644.4 g
KH_2PO_4	254.0 g
in 9 l of H_2O	Final pH = 6.8

performed in either an LKB Ultroferm (LKB Bromma, Sweden) or in a L.H. 2000 vessel (L.H. Engineering Ltd, Stoke Poges, U.K.), with working volumes of between 1.5 and 3 litres, dependent on the fermenter size. The dilution rate used was $0.05h^{-1}$ unless stated. The continuous culture techniques used are discussed in greater detail in chapter 3.

2.4 Culture Purity

The purity of cultures were checked regularly by plating cells out on nutrient agar and then incubating at 45°C and 30°C. The presence of colonies on the plates after two to three days was indicative of contamination since obligatemethanotrophs will not grow under such conditions. The possibilty of contamination by methanotrophic bacteria other than M.capsulatus was checked by plating culture samples on NMS agar and incubating in a 50% $^{v}/_{v}$ methane/air environment. Contaminants were identified by different colony morphology and microscopic analysis.

2.5 Estimation of microbial Biomass

This was performed routinely using three methods

1) Optical density measurements
2) Dry weight estimation
3) Total carbon analysis

2.5.1 Optical Density Measurements

For rapid estimation of culture biomass levels, the optical density of the culture was measured at 540nm,, using a Pye SP 1800 spectrophotometer. Water was used as a reference and cell suspensions were diluted with water to give an absorbance value of between 0 and 0.4 (540nm)

2 Dry weight Estimations

A known volume of culture was filtered through a preweighed 0.45µm filter (Courtaulds, Coventry), the filter having been previously heated 102°C/48 hours to remove moisture. The filter plus cells was then heated at

102°C for a minimum of 48hours, before being re-weighed. The dry weight was expressed as g cells/litre.

3 Carbon Analysis

The total carbon content of cells was estimated using a Beckman Total Carbon Analyser (Model 915B, Beckman Instruments Inc., California, U.S.A.). Solutions of known carbon concentration (0-100mgl^{-1}) were prepared using potassium hydrogen phthalate and CO_2-free water. This was then used to estimate the level of carbon in culture samples. The level of carbon in supernatant samples was also estimated. The supernatant was prepared by separating the majority of solids from the culture sample using a microcentrifuge (Quickfit Instrumentation, U.K.) and subsequently filtering the liquid phase through a 0.45 μm filter.

2.6 Gas-Phase Analysis

The composition of the gas-phase entering and leaving the culture was determined by on-line mass spectometry. This was performed by a MM8-80 mass spectrometer (VG Gas Analysis System Ltd, Middlewich, U.K.) as described in chapter 3.

2.7 Estimation of Nitrite

The concentration of nitrite was estimated using the method described by Nicholas and Nason (1957). 1ml of test sample was added to 0.5ml of 1% sulfanilamide and 0.5ml Of 0.02% N-(1-naphthyl) ethylenediamine hydrochloride. The combination of reagents and sample were left to stand at room temperature, before being centrifuged for two minutes in a microcentrifuge to remove any precipitate. The curve of nitrite concentration versus absorbance (540nm) was made using standard solutions of nitrite (2-35nmol nitrite/assay). These were prepared in alkaline conditions (25mg NaOH/100ml) to prevent the liberation of nitrous oxide from the reaction of nitrite with carbon dioxide.

2.8 Estimation of Nitrate

The method used to monitor nitrate levels was similar to that described by Hooper et al (1977). A sample of culture supernatant (1ml) was first adjusted to pH10 using 4M NH_4OH. To this was added 50mg of powdered zinc. After mixing, aliquots of 0.2ml were removed at 2 minute intervals and assayed for nitrite, using the method described previously. The maximum nitrite value obtained was used and adjusted for recovery by comparison with the equivalent nitrite value obtained from standard solutions of nitrate (0-50 μM), treated in the same way.

2.9 Extraction and Estimation of NAD^+/NADH levels

1 Extraction

To obtain a representative sample of cells, an initial sample of culture, two or three times the dead volume of the fermenter sampling port was removed and discarded. A further 4.5ml of culture was removed, rapidly by a syringe containing either 1ml of 0.1N HCl (extracting NAD^+) or 1ml of 3.0N KOH (extracting NADH, destroying NAD^+). The process of extraction used was similar to that discribed by Wimpenny and Firth (1972).

Samples removed from the fermenter were then incubated for 10 minutes at 50°C, before being cooled to 0°C and neutralized cautiously with either 0.1N NaOH or 3N HCl. To avoid localized high concentrations of the acid or alkali, these were added in about 0.05ml volumes, mixing thoroughly after each addition. The neutralized samples were centrifuged at 3,000g for 10 minutes. The samples for NADH analysis were drop frozen in liquid nitrogen and stored at -70°c as recommended by Lowry et al (1961).

Estimation of the NAD^+ levels required the conversion of the NAD^+ in the sample to NADH. This was achieved by incubating 1ml of the extract in 20 μl (500U/ml) alcohol dehydrogenase (sigma No. A3263) at 30°C/15minutes, the enzyme solution having been prepared in 0.1M phosphate buffer, pH7. At the end of the incubation period, 20μl NaOH(10M) was added and the sample was heated at 50°c for 10 minutes. The contaminating protein was then removed by

centrifugation and the resultant supernatant stored in liquid nitrogen at -70°C till required.

2 Estimation of NAD^+/NADH Levels

The levels of NAD^+ and NADH in the treated culture samples were estimated using an LKB-Wallac 1251 luminometer with a 1243-103 NADH monitoring Kit (Wallac Oy,Turku, Finland). The method of estimation was based on the quantitative measurement of light produced as a result of coupling two specific enzyme reactions. The first reaction (1) involved the use of an NADH-specific FMN oxidoreductase

$$NADH + FMN + H^+ \longrightarrow NAD^+ + FMNH_2 \quad -1$$

The second reaction (2) utilized the light producing capacity of bacterial luciferase

$$FMNH_2 + RCHO + O_2 \longrightarrow FMN + RCO_2H + H_2O + Light \quad -2$$

The amount of light produced in (2) is proportional to the NADH used in (1)

2.10 Estimation of Formaldehyde

Formaldehyde in solution was measured colorimeterically using Nash reagent (Nash 1953). This required the measurement of absorbance of reagent plus sample at 412nm against a blank containing water instead of sample.

2.11 Estimation of Formate

The estimation of formate was performed using the method described by Lang and Lang (1972). Calibration graphs were constructed whenever fresh reagents were prepared and the assay was linear between 0 and 5mM formate.

2.12 Estimation of Methanol

Methanol was estimated using a Pye series 104 gas chromatograph (Pye Unicam, Cambridge, U.K.) with a Porapak

Q (Waters Associates, Millford, Mass U.S.A.) column (2.1m x 4mm i.d.) operating at 140°c.

2.13 Chemicals

The majority of chemical were obtained from the following manufacturers. Sigma Chemicals, Poole, Dorset, U.K.; BDH Chemicals, Poole, Dorset, U.K.; Aldrich Chemical Co. Ltd, Gillingham, Kent, U.K.

2.14 Preparation of Formaldehyde

Formaldehyde solutions were prepared by repeatedly heating suspensions of 10% $^{w}/_{v}$ paraformaldehyde at 100°c for 2 to 3 hours, till all the paraformaldehyde had disappeared. The concentration of the resultant formaldehyde was determined by a combination of Nash reagent and total carbon analysis.

2.15 Gases

Methane, chemically produced (technical grade) was obtained from Electrochem Ltd, Stoke-on-Trent, U.K. Oxygen, oxygen/carbon dioxide (5%), acetylene, argon, nitrogen, hydrogen and propylene were obtained from British Oxygen Company Ltd, London, U.K.

2.16 Collection of Cells and Preparation of Cell Extracts

Cells from continuous culture were collected via the over-flow to preserve the operating culture volume and maintain steady-state conditions. Cells were normally collected into an ice-cooled, sterile bottle over a period of up to four hours. At the end of the collection period the cells were immediately centrifuged at 10,000g for 10minutes/4°c. The pellet was then washed and resuspended in cold 25mM Pipes buffer (pH7). Sodium thioglycollate (5mM) was added to cell suspensions that were to be prepared for soluble MMO assays. The resultant cell suspensions were drop-frozen in liquid nitrogen and stored at -70°C till use.

Cell extracts required for enzyme assays were prepared the day of the assay by first thawing out the required amount of frozen cells and breaking them by two

passages through an ice-cooled french pressure cell (20,000 p.s.i.g.). After breakage, the membrane fraction was separated from the soluble extract by centrifugation at 80,000g for one hour.

2.17 Enzyme Assay Conditions and Units

All enzyme activities, except the MMO and the hexulose phosphate synthase were measured continuously using a Pye Unicam SP1800 double beam spectrophotometer. This was fitted with a constant temperature housing and was coupled to a Pye Unicam AR-25 linear recorder. Quartz cuvettes (1ml or 3ml) with a 1cm light path were used. The amount of extract was added such that the reaction rate was linear with respect to time, for at least 3 minutes and was proportional to the amount of protein added.

Activity values were expressed as specific activities [μmol min^{-1}(mg protein in extract)$^{-1}$] and all assays were performed at 45°C.

Enzyme Assays

2.17.1 Ammonia-dependent Methanol Dehydrogenase [E.C.1.1.9.9.8]

1) Methanol + $DCPIP_{(ox)}$ ⟶ Formaldehyde + $DCPIP_{(red)}$

Methanol dehydrogenase activity was measured using the method described by Anthony and Zatman (1967) which used DCPIP and PMS as *in vitro* primary and secondary electron acceptors respectively.

The reaction mix contained:

	μmol ml^{-1}
Glycine/NaOH buffer (pH9)	120
PMS	2
DCPIP	0.2
NH_4Cl	10
Methanol or formaldehyde	10

Extract plus water to a final volume of 1ml.

The reaction was started by the addition of substrate and the resulting decrease in absorbance at 600nm recorded. 10μmol ml^{-1} KCN was initially added to maintain the enzyme conformation and activity, but after several assays it was found that the removal of KCN did not compromise the activity over the initial 3 minutes of the reaction and was therefore left out of future assays.

2.17.2 Formaldehyde dehydrogenase (NAD^+-independent, Ammonia-independent), (No E.C. number exists)

$$\text{Formaldehyde} + \text{DCPIP}_{(ox)} \longrightarrow \text{Formate} + \text{DCPIP}_{(red)}$$

Measured by the method described by Johnson and Quayle (1964) using the same electron acceptors as were used in the methanol dehydrogenase assay.

Reaction mix contained:

	μmol ml^{-1}
Potassium phosphate buffer (pH7)	80
PMS	2
DCPIP	0.2
Formaldehyde	10

Extract plus water to a final volume of 1ml

The reaction was started by the addition of formaldehyde and the resulting decrease in absorbance (600nm) measured.

2.17.3 Formaldehyde Dehydrogenase (NAD^+-linked), (E.C.1.2.11)

$$\text{Formaldehyde} + NAD^+ + H_2O \longrightarrow \text{Formate} + NADH + H^+$$

The above reaction was followed <u>in vitro</u> using the method out-lined by Stirling and Dalton (1978).

The reaction mix contained:

potassium phosphate buffer (pH7.2)	30 μmol
NAD^+	1 μmol
KCN	3.75 μmol
Formaldehyde	3.75 μmol
Extract plus water to a final volume of	1.5 ml

The reaction was initiated by the addition of substrate and monitored by the reduction of NAD^+ at 340 nm.

An alternative method of identifying NAD^+-linked formaldehyde dehydrogenase activity involved the measurement of formaldehyde disappearance within the same assay mix, using Nash reagent, (Nash, 1953). HTSE was prepared by heating crude soluble extract at 70°C for 12 mins; before centrifuging to remove precipitated protein.

2.17.4 Formate Dehydrogenase (E.C. 1.2.12)

$$\text{Formate} + NAD^+ \longrightarrow CO_2 + NADH + H^+$$

The assay system used was similar to that described by Johnson and Quayle (1964); containing the following

Tris/HCl buffer, pH8.4	20µmol
NAD^+	1µmol
Sodium formate	10µmol
Water plus extract to a final volume of	1ml

The activity was monitored by the observed increase in absorbance at 340 nm on the addition of formate to the assay mix.

2.17.5 Glucose-6-phosphate Dehydrogenase (E.C. 1.1.1.49)

$$\text{Glucose-6-phosphate} + NAD(P)^+ \longrightarrow \text{6-phosphogluconate} + NAD(P)H + H^+$$

The assay was based on that described by Kornberg and Horecker (1955), the reaction mix containing:

Glycylglycine buffer, pH8	100µmol
$MgCl_2$	15µmol
NAD(P)	0.5µmol
Glucose-6-phosphate	3µmol
Water plus extract to a final volume	1.5ml.

The reaction was initiated by the addition of glucose-6 phosphate and increased in absorbance measured at 340nm.

2.17.6. 6-Phosphogluconate Dehydrogenase (E.C. 1.1.1.43)

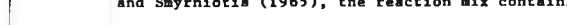
This assay was based on that described by Horecker and Smyrniotis (1965), the reaction mix containing :

Glycylglycine buffer, pH 7.6	100 µmol
$MgCl_2$	15 µmol
$NAD(P)^+$	0.5 µmol
6-phosphogluconate	3 µmol

Water plus extract to a final volume of 1.5 ml

The reaction was initiated with the addition of substrate and the increase in absorbance measured at 340 nm.

2.17.7. Hydroxypyruvate Reductase (E.C. 1.1.1.29)

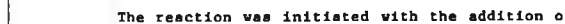


The activity of this enzyme was measured by the method described by Large and Quayle (1963), the reaction mix containing:

Potassium Phosphate buffer (pH7)	100 µmol
NADH	0.4 µmol
Lithium hydroxypyruvate	2 µmol

Hydroxypyruvate reductase activity was measured continuously by recording the reduction in absorbance (340nm) which corresponded to the oxidation of $NADH_2$. Before Lithium hydroxypyruvate was added to the reaction mix, the endogenous NADH oxidation capacity of the extract was measured and the value for this subtracted from the reaction rate generated in the presence of Lithium hydroxypyruvate.

2.17.8. NADH-Oxidase (E.C. 1.6.99.3)



The method used to measure NADH-oxidase activity was based on that described by Smith, London and Stanier (1967). The reaction mix contained:

Sodium Phosphate buffer (pH7)	30 μmol
$MgCl_2$	10 μmol
NADH	0.25 μmol

Extract plus water to a final volume of 1.5ml

The reaction was initiated by the addition of NADH and the reduction in absorbance (340nm) measured.

2.17.9. 10-methylene THF dehydrogenase (E.C. 1.5.1.5)

$$\text{Formaldehyde} + \text{THF} \xrightarrow{\text{Non-enzymic}} \text{5,10- methylene THF}$$

$$\text{5,10 methylene THF} + NADP^+ \longrightarrow \text{5,10 methenyl THF} + NADPH$$

This assay was based on that described by Scrimgeour and Huennekens (1963). The first reaction is non enzymic and 5,10-methylene THF is generated in situ. The second reation was measured spectrophotometrically, following the increase in absorbance (340nm). The reaction mix consisted of:

malic acid/KOH buffer (pH6.9)	40 μmol
$NADP^+$	1.0 μmol
2-mercaptoethanol	20 μmol
DL-tetrahydrofolate	0.25 μmol
formaldehyde	2.5 μmol

extract plus water to a volume of 1ml

The reaction mix was pre-incubated at 30°C/3minutes to permit the non-enzymic formation of the substrate 5,10-methylene THF. The reaction was started by the addition of extract or $NADP^+$ and the increase in absorbance (340nm) monitored. A mix containing all the reagents except cell extract was used as a reference blank.

2.17.10. Formyl-THF synthase (E.C. 6.3.4.3)

$$\text{Formate} + \text{THF} + \text{ATP} \longrightarrow \text{10-formyl THF} + \text{ADP} + P_i$$

$$\text{10 formyl THF} + H^+ \longrightarrow \text{5,10 methenyl THF}$$

Formyl THF synthase was measured by the method described by Rabinowitz and Pricer (1963). The 10-formyl THF formed in the enzyme reaction shown above is

quantitatively converted to 5,10 methenyl-THF by the addition of acid. The resulting product is determined spectrophotometrically by its characteristic absorption maximum at 350nm.
The reaction mix consisted of:

triethanolamine/HCl (pH8) buffer	100	μmol ml^{-1}
THF	2	"
ATP	5	"
2-mercaptoethanol	100	"
$MgCl_2$	10	"
Sodium formate (pH8)	40	"

Water plus extract to a final volume of 1ml

A series of tubes containing the reaction mix (minus cell extract) were preincubated for 2minutes/30°C. The reaction was started by the addition of cell extract and stopped by the addition of 3.6N HCl(2ml). After incubation (10 minutes) at room temperature, the absorbance at 355nm was measured, the extinction coefficient for 5,10 methenyl THF (pH1) being 22.0cm^{-1} mmol. A tube containing the reaction mix without the extract served as a reference blank.

2.17.11. Hexulose phosphate Synthase

Formaldehyde + ribulose 5-phosphate⟶hexulose 6-phosphate

Hexulose phosphate synthase activity was assayed by the discontinuous method described by Jones *et al* (1987), which measured the rate of disappearance of formaldehyde in the presence of ribulose 5-phosphate generated *in situ* from ribose 5-phosphate (Ferenci *et al*, 1974). The reaction mix (1ml) contained 50mM sodium/potassium phosphate buffer (pH7.2), 2.5mM magnesium chloride, 5mM formaldehyde and 5mM ribose-5-phosphate. This mix was incubated in the presence of 1.75 units of phosphoribo isomerase (sigma No. P-1780) at 30°C for 15minutes, to convert the ribose 5-phosphate to ribulose 5-phosphate. Cell extract was then added to the mix to initiate the reaction. Samples (0.05ml) were removed at 30 second intervals and mixed with 0.45ml 10% ($^{w}/_{v}$) trichloroacetic acid to terminate the reaction. The concentration of formaldehyde in the mix was then

estimated using Nash reagent (Nash, 1953). A control assay was done in the absence of ribose 5-phosphate to monitor formaldehyde disappearance in the absence of hexulose phosphate synthase activity.

2.17.12 Methane Monooxygenase (E.C. 1.14.13.25)

$$CH_4 + O_2 + NADH + H^+ \longrightarrow CH_3OH + H_2O + NAD^+$$

In crude cell extracts, addition of methane and NADH in air does not lead to the quantitative accumulation of methanol, since the methanol is futher metabolized by both the methanol dehydrogenase and the methane monooxygenase. Instead, the MMO- catalysed conversion of propylene to propylene oxide is measured since this reaction occurs at a comparible rate to that of the conversion of methane to methanol and the product is not subject to further metabolism by the MMO or other enzymes in the extract.

The amount of propylene oxide generated in the reaction was measured by gas chromatography, using the method described by Prior and Dalton (1985). The assays were performed in conical flasks (7ml), sealed with a rubber closure (Suba Seal, No 37, W.H. Freeman, Barnsley, U.K.). The assay volume was 1ml, which consisted of 25mM Pipes (pH7), (0.2 ml); water and extract. 3ml of air was removed from the sealed vial and replaced with 3ml propylene. This mix was incubated at 45°C/1minute in a gyratory water bath. After one minute 50μl of 100mM NADH was added to initiate the reaction. The level of propylene oxide produced after 3 minutes was measured by gas chromatography. This involved the use of a glass column (1mx4mm) containing Porapak Q (Water Associates, Milford, MA, U.S.A.); maintained isothermally at 190°c with nitrogen as a carrier gas (30ml min^{-1}). A flame ionisation detector was used to detect the propylene oxide produced, the relative amount of which was measured using a Hewlett Packard 3390 reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

When the particulate form of the enzyme was assayed, the reaction mix was supplemented with 0.4mM $CuSO_4 \cdot 5H_2O$ (Prior and Dalton, 1985).

The specific activity value was expressed in terms of Units mg^{-1} (one unit = 1 μmol propylene oxide formed min^{-1} mg^{-1})

2.18 Protein Determination

The estimation of protein levels in soluble cell extracts was performed using commercially available Bio-rad reagent (Bio-rad Ltd, Watford, Herts, U.K.) using bovine serum albumin as a protein standard. The assay of protein levels in particulate fractions involved the use of the method described by Peterson (1977), which in turn was modification of that described by Lowry et al (1951).

2.19 Whole Cell Methane Oxidation Capacity

The conversion of propylene to propylene oxide by whole cells was measured using gas chromatography as described in the methane monooxygenase assays. The reaction vial contained 0.9ml cell suspension, usually diluted to an optical density of approximately 3 (540 nm). The electron donor was supplied in the form of 0.1ml of 1M potassium formate, the oxidation of which generated the required NADH.

2.20 SDS - Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was performed using a linear gradient vertical slab gel and discontinuous buffering system, (Laemlli, 1970). Polypeptide bands were visualized by staining with coomassie Blue R250 stain (BDH chemicals, Poole, Dorset, U.K.).

2.21 Purification of proteins

The soluble MMO proteins and the methanol dehydrogenase were purified by Dr. J. Green and Dr. D. Smith using the methods described by Pilkington and Dalton (in print).

CHAPTER 3

Gas-Limited Continuous Culture

3.1 Introduction

The usefulness of continuous culture as a tool in the study of microbial metabolism has been widely acknowledged. The great advantage of this technique is that it permits the study of the effect of changing one variable while keeping the rest of the environment constant. Consequently, it allows the physiology of microbial growth to be studied under defined and reproducible conditions.

A continuous culture operated under gas-limited conditions can neither be described as a chemostat nor a turbidostat, since the availability of the limiting nutrient is not determined by the medium flow rate. Under the above conditions, the supply of the limiting substrate is independent of dilution rate and is determined by factors such as the partial pressure of the gas, mixing characteristics and the overall gas transfer characteristics of the fermenter. In gas-limited continuous culture the microorganism concentration is highest at low dilution rates and falls off steeply as the dilution rate increases, (Harrison, 1972; Barnes *et al*, 1976). A comprehensive review of the kinetics of gas-limited continuous culture was given by Stanley (1977).

One factor that must be considered during the operation of a gas-limited continuous culture is the gas transfer rate of the limiting nutrient. Unlike a true chemostat not all the limiting nutrient entering the vessel will be consumed by cells, but only that which is transferred from the gas to the aqueous phase of the culture (gas transfer rate). This in turn will be dependent upon not only the flow rate and the solubility of the gas but also the design of the vessel in which the cells are grown. Bearing this in mind it was decided to limit all steady-state physiological studies to one particular fermenter, (LKB 1601 Ultroferm).

Both oxygen and methane are relatively insoluble in water, (1mmole l^{-1} at 40°C/760mmHg pressure for both gases). A methane/oxygen mixture is also potentially explosive when in proportions of 5 to 15% methane in air. Therefore it is important to avoid such a mixture of gas when growing bacteria on methane.

3.2 Analysis of the Gas Phase

The accurate measurement of substrate that exists in the gas phase is considerably more difficult than measuring either a miscible liquid or soluble solid substrate, (van Dijken and Harder, 1975; Leak and Dalton, 1986a). The estimation of cell yield on methane requires the accurate measurement of both the gas flow rate and the amount of methane consumed by the culture. Consequently it has proved difficult in the past to obtain reproducible cell yields on methane (Leak and Dalton, 1986a).

The system used for gas analysis in this project was similar to that described by Richards *et al* (1987). Modifications to the above system were introduced as a direct result of problems that were encountered during the initial attempts at monitoring the metabolic activity of *M.capsulatus* (Bath). The system, including modifications is outlined in fig 3.1 and consists of a gas-limited chemostat coupled to an on-line mass spectrometer. The operation of the system can be split into four stages: 1) gas supply, 2) fermentation, 3) sample processing and transport, 4) analysis. These are described below.

1 Gas Supply

M.capsulatus (Bath) requires both methane and oxygen for growth. The oxygen was supplied as air, the flow rate of which was controlled independently of the methane supply. Gas flow rates were calibrated using needle valves connected to a flowmeter (Platon Flowbits Ltd., U.K.). Initial attempts at generating gas-limited, steady-state growth in the chemostat proved difficult due to fluctuations in the partial pressure of the gases in the supply line. Most of these fluctuations were a result of the consumption of the common gas supply by other fermenters. This problem was overcome by the addition of an extra pressure regulator in both the air and methane lines at a position shown in fig 3.1.

The gases were mixed after passing through the flow controllers. A 5 litre round bottomed flask was added to the system at this mixing stage to minimise the effect of any minor fluctuations that may occur in the total gas flow rate during periods of steady-state growth. After

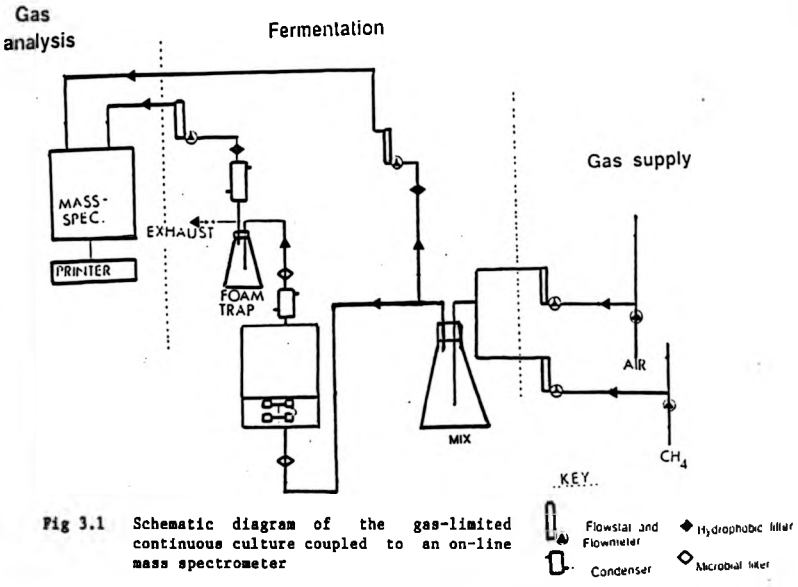


Fig 3.1 Schematic diagram of the gas-limited continuous culture coupled to an on-line mass spectrometer

the gases were mixed, the flow was split with 10 - 15% of the gas flow being diverted into the mass spectrometer. The remainder of the gas flow was fed into the fermenter. The mass spectrometer requires a continuous gas flow of at least $100cm^3min^{-1}$, this flow being controlled and monitored by a flowstat coupled to a flowmeter.

2) Fermentation

The gases fed into the fermenter were initially filtered to remove contaminating microorganisms, before entering the vessel via a ring sparger situated near the base of the fermenter. The total volume of the LKB fermenter was 6 litre and the steady-state working volume used was 3 litre. Consequently there existed a 3 litre head-space in which the gases produced by cells and the inlet gases that were not consumed could mix before leaving the vessel.

3) Sample Processing and Transport

One of the major concerns using this type of system for gas analysis was the possible contamination of the mass spectrometer by foam, culture or condensation which may enter the fermenter's exhaust line. Precautions taken to reduce this problem included the inclusion of hydrophobic filters in both the sample lines connected to the mass spectrometer. In addition to these the gas outlet line also contained an extra condenser along with a water trap to reduce the level of moisture reaching the hydrophobic filter. These filters remained functional throughout their routine use, although problems did arise when a mechanical antifoam device was operated in a similar system; the filter blocking as a direct result of aerosols formed during the operation of the device (Stanley, personal communication). An additional foam trap (5 litre round bottomed flask) was incorporated into the exhaust gas line prior to the hydrophobic filter. This also allowed for extra mixing of the exhaust gases prior to sampling by the mass spectrometer and therefore provided a more representative sample of exhaust gas during steady-state growth. Both the mixing vessels situated in the inlet and exhaust gas lines were removed during experiments that involved monitoring the cell's

immediate response to environmental changes during non steady-state conditions of growth.

The back pressure caused by the inlet gas entering the fermenter broth meant that there was a sufficient gas flow to allow the sampling of the inlet gas line by the mass spectrometer. It was however necessary to restrict the exhaust line to achieve sufficient pressure (0.05bar) to sample the head space gas.

4) Analysis

The mass spectrometer used throughout this project was a V.G. MM8-80F (V.G. Gas Systems Ltd., Middlewich U.K.). Details of its mode of operation have been covered by Winter (1987).

Precautions

The efficient operation of such a system requires several precautions:

1) The accuracy of measurement of the components in the gas phase is dependent upon the accurate monitoring of the gas flow rates. As a result of this the flowmeters in the system were calibrated regularly using a bubble flowmeter. This technique although simple in its operation, has the disadvantage of being discontinuous. Therefore it is open to the possibility that the gas flow rate measured by this method may not be representative of that which was in operation at the time of mass spectrometer sampling. The probability of such a discrepancy occurring can however be minimized by the repeated calibration of the flow meters and checking that the gas composition fed into the fermenter was consistent over a set period of time. Any fluctuation in either the air or methane flow rates would be reflected in a change in the relative percentages of the constituent gases in the gas inlet stream.

2) The transfer line between the fermenter and the mass spectrometer was kept as short as possible to facilitate a rapid response between gas evolution in the fermenter and the sampling of the gas. The gas lines into the fermenter

were routinely checked for gas leaks, since these not only provide a means by which contaminating gases may enter the system but also a route by which potentially explosive gas mixtures may escape. The transfer lines consisted of nylon tubing which is both flexible and impermeable to gases. Silicone tubing attached to the fermenter was kept to a minimum since it is extremely permeable to gases.

3) As was pointed out by Richards et al (1987), the reliability of the calibration of the mass spectrometer is dependent on the accuracy of the composition of the calibration gases. Errors in these gases may arise through a) incorrect formulation b) inaccurate preparation by the manufacturer or c) mixing with extraneous gases, notably air that may enter the mass spectrometer via the gas inlet valve system.

3.3 Carbon Balance Calculations During Methanotrophic Growth

The ability of M.capsulatus (Bath) to utilize methane as its sole carbon and energy source means that the distribution of carbon entering and leaving the fermenter during continuous culture may be summarized in fig 3.2. By coupling the gas-limited continuous culture to the on-line mass spectrometer, the distribution of carbon within the system can be monitored and carbon recovery determinations carried out on each physiological steady-state achieved within the culture. Balancing the carbon fed into the system with that leaving not only quantifies the distribution of substrate between the gaseous and aqueous phases of the culture, but also acts as a check on the analytical accuracy of the system. The amount of carbon being fed into the vessel must equal that leaving to within $\pm$ 5%. To achieve such carbon balances the following must be known:

1) The relative proportions of individual gas constituents entering and leaving the fermenter; these being determined by the mass spectrometer.
2) The total flow rate of the gases entering the fermenter.

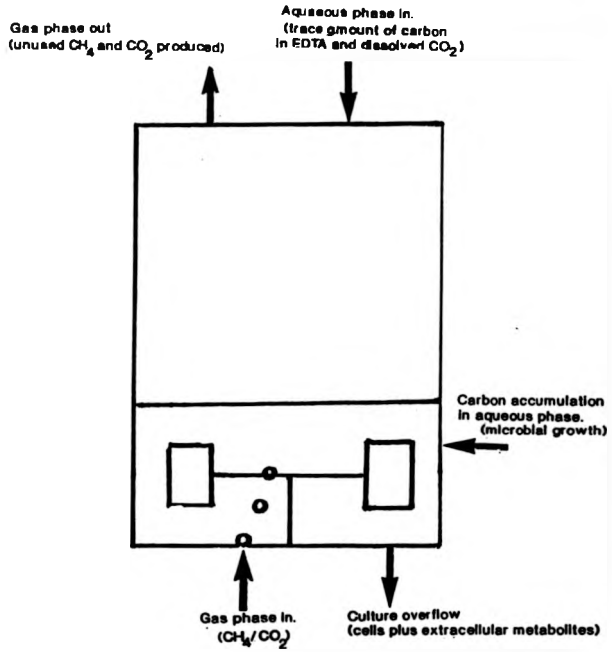


Fig 3.2 Distribution of carbon in a gas-limited continuous culture, during growth on. methane

3) The amount of carbon accumulating within the fermenter.

The flow rate of gases leaving the fermenter may also be measured, however this is not essential as this value may be calculated using an internal standard. The results generated by the mass spectrometer concerning the composition of the gas entering and leaving the fermenter is expressed as a percentage. The mass spectrometer automatically sums up all the gas phase constituents to 100%, but does not take into account any changes in the water vapour concentration which will differ between gases entering and leaving the vessel. Consequently it is necessary to correct for this by using what is termed an "internal standard". An inert gas such as nitrogen is normally used as the internal standard since it is assumed that this gas is neither consumed nor produced by the cells and therefore acts as a correction factor for the differences caused by the presence of water vapour. The total gas flow out of the fermenter can thus be calculated using the following equation.

$$F_{(exhaust)} = F_{(inlet)} \times \frac{\%N_2\ (inlet)}{\%N_2\ (exhaust)}$$

where F = gas flow rate ($l min^{-1}$)

It should be noted that nitrogen can only be used as an internal standard during M.capsulatus (Bath) growth studies when the cells are not nitrogen-limited, since under the latter conditions these organisms are capable of fixing atmospheric nitrogen, (Murrell and Dalton, 1983). Under such circumstances argon would be used as the internal standard.

If the total gas flow rates entering and leaving the vessel are known, along with the relative proportions of the molecular components of these gases, then it is possible to calculate the rate at which the individual components enter or exit the system, using the following equations:-

$$\text{moles of gas } l^{-1}h^{-1} = \%gas \times F \times \frac{1}{22.4} \times \frac{1}{V} \times 60 \times \frac{P}{760} \times \frac{273}{T+273}$$

where F = total gas flow rate (inlet or exhaust), ($1 min^{-1}$)
V = operating volume of the fermenter (l)

$\frac{P}{760} \times \frac{273}{T+273}$ = the correction factor for standard temperature and pressure, where P = pressure (mmHg) and T = temperature (°C) measured close to the point where the gas flow is measured.

This equation is based on the assumption that one mole of gas occupies 22.4 litres at standard temperature and pressure.

The bulk of carbon fed into the system will be in the form of methane, although trace levels of CO_2 will be present in the air being fed in. The amount of carbon in the aqueous phase being fed into the vessel will consist of dissolved CO_2 and trace compounds such as the chelating agent EDTA. The low levels of such carbon sources, combined with the low dilution rate used ($0.05 h^{-1}$) meant that the level of carbon fed into the fermenter in the aqueous phase was negligible (<1ppm) compared with the level of carbon in the gaseous phase.

The rate of carbon accumulation within the culture was measured discontinuously using a total carbon analyser. Such carbon includes cells, extracellular metabolites and a small amount of inorganic carbon (<0.5% total carbon). The rate at which carbon accumulated during steady-state periods of growth was calculated using the following equation

Rate of biomass accumulation	=	medium flow rate into the fermenter	x	steady-state total carbon concentration in the culture
(g carbon h^{-1})		($1 h^{-1}$)		(g carbon 1^{-1})

This can then be converted into moles carbon 1^{-1} h^{-1} via the following:

$$\text{moles carbon } 1^{-1}h^{-1} = \frac{\text{g carbon } h^{-1}}{\text{culture volume (l) x 12}}$$

The exhaust gas phase consisted of both unused methane and CO_2 produced by cells. From the measured levels of carbon in both the aqueous and gaseous phases of the culture it was possible to express the distribution of carbon as follows:

$$CH_{4(inlet)} + CO_{2(inlet)} = CH_{4(exhausted)} + CO_{2(exhausted)} + \text{Biomass (produced)}$$

3.4 Conclusion

The measurement of gas uptake or output by organisms plays a key role in the investigation of biological activities (Lloyd and Scott, 1985). Gas analysis by mass spectrometry provides a sensitive and rapid means of monitoring changes in the exchange rates of gases within a microbial culture. When the cells are allowed to grow under a series of defined conditions as in the case of gas-limited continuous culture, the composition of the gas phase within the fermenter should remain constant during periods of steady-state growth. By altering these conditions of growth it should be possible to induce changes in the physiology of the culture. Such physiological changes will in turn be reflected in changes in the composition of the gaseous phase in the culture's head-space. Such changes can be monitored directly by on-line mass spectrometry using the set-up described in this chapter. The accuracy of the analytical system can then be checked by performing a complete carbon balance to ensure all carbon entering and leaving the system can be accounted for.

Using such a system it should be possible to grow M.capsulatus (Bath) under a defined set of conditions and record the physiological state of the cells. Only after this has been achieved is it then possible to alter the growth conditions and monitor the cells response

CHAPTER 4

Continuous Cultivation of M.capsulatus (Bath), Control Experiments

Chapter 4

Continuous Culture of M.capsulatus (Bath), Control Experiments

4.1 Introduction

The physiological state of any microorganism is dependent upon the conditions under which the cell is grown. Observations by Hyder et al. (1977); Takama, (1980); Scott et al, (1981); Stanley et al, (1983); and Prior, (1985) showed this to be especially true of methylotrophic bacteria. The nature of the growth-limiting nutrient, levels of trace elements and specific growth rates are just some of the factors that go to determine the morphological and biochemical properties of the cell.

The overall objective of this project was to examine how growing cultures of M.capsulatus (Bath) responded to the introduction of various C_1 metabolites. Before this could be achieved, it was necessary to determine the physiological state of the cells prior to the introduction of such metabolites. This required cells to be grown under a series of defined conditions during which factors such as cell yield, pattern of gas metabolism and levels of enzyme activity could be examined. Once established, such properties could be used as references against which the effect of C_1 metabolite additions may be compared.

Another reason for performing such control continuous cultures was to assess the effectiveness of the analytical set-up described in the previous chapter.

4.2 Batch Culture of M.capsulatus (Bath)

Batch cultivation of cells is normally a prerequisite requirement before subjecting the organisms to continuous culture. To stimulate the batch growth of M.capsulatus (Bath), CO_2 was required along with air and methane in the gas mix entering the vessel. Stanley and Dalton, (1982) noted a similar requirement while examining the growth characteristics of the above organisms in shake flasks. During the initial stages of the project "biological" methane (supplied by B.O.C. Ltd, U.K.) was used along with

air to stimulate cell growth. Methane derived from biological sources normally contains between 1 and 3% CO_2. However within several weeks of commencing the project this supply of "biological" methane was no longer available and it proved difficult to initiate the growth of organisms using synthetically derived methane, the latter containing less than 0.01% CO_2. This apparent necessity for CO_2 during this period of growth was eliminated by using a 5% CO_2/air mix (supplied by B.O.C.), in addition to the synthetically-produced methane. Once cell growth could be detected in the fermenter and the cells were producing their own CO_2 as a result of methane metabolism, the air/CO_2 mixture was replaced with 100% air. After this switch growth continued uninhibited.

The choice of nitrogen source is important in standardizing any fermentation process since the metabolism of different N-containing compounds have different energy requirements. Methylotrophs are capable of utilizing a wide range of inorganic nitrogen-based compounds particularly ammonium-based salts, nitrate salts and even dinitrogen. Irrespective of the source of nitrogen, the N-containing compound must be reduced to the level of ammonia before entering cellular metabolism. The fixation of atmospheric nitrogen is the most energetically expensive of all the sources, requiring both ATP and NAD(P)H. Benemann and Valentine, (1972) reported that for every molecule of ammonia produced by way of the nitrogenase enzyme, the process required the input of 1.5moles of NAD(P)H and between 2 and 10 moles of ATP. Cells fixing dinitrogen also show an unusually high sensitivity to oxygen. Under such circumstances, problems were envisaged in maintaining sufficiently low concentrations of oxygen in the culture especially under nutrient-limitations other than oxygen.

The use of exogenously supplied ammonia or ammonium-based salts as a nitrogen-source for the growth of M.capsulatus (Bath) is complicated by the fact that ammonia may also act as a substrate for the MMO, which oxidises it to hydroxylamine, (O'Neill and Wilkinson, 1977; Dalton, 1977). Metabolism of ammonia by the MMO would therefore not only act as a drain on the availablity of nitrogen and reducing power for the cell but would also act as a competitive inhibitor of the MMO. Consequently nitrate was chosen as the N-source to be used in growth studies.

4.3 Continuous Culture of M.capsulatus (Bath) : Determination of Nutrient Limitation and Choice of Dilution Rate

The advantage of using continuous culture as a tool for the study of microbial metabolism is that it allows the operator to control the specific growth rate of the microbial population by adjusting the rate of nutrient supply to the culture, (the dilution rate). This is made possible when the medium is designed such that all but a single essential nutrient is available in excess of the amount required to synthesise a desired cell concentration. The single "growth-limiting" nutrient controls the size of the steady-state cell population . In practice both the nature of the limiting nutrient and whether it exists in the aqueous or gaseous phase of the culture will effect the relationship between cell concentration and dilution rate. Consequently to determine a set of defined growth conditions for the study of C_1 regulation in M.capsulatus (Bath), the operational nutrient limitation and dilution rate had to be established.

During the continuous culture experiments, the total gas flow entering the system was between 450 and 500cm^3 min^{-1}. 100cm^3 min^{-1} of the total gas flow was diverted into the mass spectrometer whilst the remainder was fed into the fermenter. These flow rates were measured routinely during each experiment. Under the above conditions, methane limitation occurred when the methane concentration entering the fermenter was less than 5%, (Stanley, 1977). If the methane concentration in the gas feed exceeded 15%, then oxygen-limitation prevailed. By altering individual gas flow rates during the continuous culture it was possible to check the nature of the limiting nutrient. Under conditions of methane-limitation, a reduction in the methane flow rate coupled with an increase in the air flow rate, (the latter being performed to maintain a constant total gas flow rate), resulted in a decrease in the steady-state cell density. Like-wise, under oxygen-limiting conditions, a decrease in the air flow rate, coupled with a similar increase in the methane flow rate also resulted in a decrease in the culture's cell density.

In a gas-limited continuous culture, increasing the

dilution rate does not increase the availability of the limiting nutrient, since the latter exists in the gas phase, consequently there is no increase in specific growth rate. Instead an increase dilution rate will effectively increase the rate at which the cells are washed out. Therefore, under gas-limiting conditions, lower cell densities are achieved at higher dilution rates.

Since one of the experimental objectives of this study was to examine any role that the MMO (Soluble or particulate) may play in determining the regulation of C_1 metabolism, it was essential to generate conditions that permitted the expression of either 100% soluble MMO or 100% particulate MMO. By using a dilution rate of $0.05h^{-1}$ and a copper-free medium, it was possible to obtain cell densities that were sufficiently high to generate the copper stressed conditions required for soluble MMO expression. Using the same dilution rate, and supplementing the medium with a specified amount of copper sulphate, it was possible to raise the copper : biomass ratio of the culture, such that it permitted the expression of the particulate form of the enzyme. The effect of altering the copper : biomass ratio on the type of MMO expressed and its effect on cell metabolism will be discussed in greater detail in the following sections. It is suffice to say that $0.05h^{-1}$ was assumed to be the most suitable dilution rate to be used during the study

4.4 Effect of Copper Levels and Nutrient Limitation on Growth Yield and Gas Metabolism in M.capsulatus (Bath)

The relationship between the type of MMO expressed by some methanotrophs and the level of copper ions in the cell's environment has been well documented (Stanley et al. 1983; Dalton et al, 1984 Prior, 1985). The observation by Leak and Dalton (1986a) that the growth yield of M.capsulatus (Bath) on methane was linked to the type of MMO expressed by the cell meant that this enzyme plays an important role in determining the overall physiological state of the cell. Therefore the type of MMO produced by the cell must be considered when examining any aspect of C_1 metabolism within the organism, including regulation. The aim of this section was to obtain conditions that permitted the expression of either 100% soluble MMO or

100% particulate MMO. Once achieved, it would then be possible to re-examine the effect of the MMO type on the energetics of methane metabolism, using on-line mass spectrometry. The results produced by Leak and Dalton (1986a) were based on gas exchange rates measured individually by gas chromatography. On-line mass spectrometry should represent a more efficient means of monitoring gas phase composition with rapid and automatic sampling of the inlet and exhaust gas phases, 24 hours a day.

Using gas-limited, continous cultivation techniques coupled with on-line mass spectrometry, a series of experiments were carried out using the conditions of growth described earlier. The carbon conversion efficiencies (C.C.E.) and gas stoichiometries measured during these experiments were calculated from data produced by the mass spectrometer and total carbon analysis of culture samples. These measurements were limited to periods of steady-state growth, which in turn were maintained over a period of three or more successive days. During such periods both the culture cell density and the gas exchange rates remained constant.

The results in table 4.1 show that under high copper conditions ($1.2mgl^{-1}$ $CuSO_4 \cdot 5H_2O$) cells grew more efficiently compared to low copper conditions ($0.2mgl^{-1}$ $CuSO_4 \cdot 5H_2O$) or in the absence of copper ions altogether. The observed increase in growth efficiency was reflected in not only a higher percentage of carbon being converted to biomass, but also a reduction in the amount of CO_2 produced per mole of methane consumed. The switch from high copper conditions to low copper conditions also corresponded to an observed increase in the amount of oxygen consumed per mole of methane utilized. This also reflected a reduction in the efficiency of cell growth since it implied that a higher proportion of carbon was being oxidised to CO_2. This pattern of results was obtained irrespective of whether the cells were grown under methane or oxygen-limiting conditions.

Cells grown in the absence of copper ions appeared more susceptible to culture washout than those grown in the presence of either 0.2 or 1.2mg l^{-1} copper sulphate. Consequently the values shown in table 4.1, concerning the efficiency of cell growth and gas exchange rates measured in copper-free conditions were only obtained after

Table 4.1 Effect of copper Concentration and nutrient limitations on cell yield and ratios of gas exchange in control continous cultures.

Key

1 C.C.E. = carbon conversion efficiency $\frac{\text{carbon incorporated into biomass}}{\text{total carbon consumed by cell}} \times 100$

2 Y_{CH_4} = cell yield on methane $\frac{\text{g cells produced dry weight}}{\text{g methane consumed}}$

3 $C_{recovery}$ = carbon recovery determination $\frac{\text{total carbon in}}{\text{total carbon out}} \times 100$

Nutrient Limitation	Dilution Rate (h^{-1})	Concentration of $CuSO_4 \cdot 5H_2O$ in medium (mgl^{-1})	C.C.E. (%)	Y_{CH_4}	Ratio of gas exchange: Moles O_2 Consumed : Moles CH_4 Consumed : Moles CO_2 Produced	Carbon recovery (%)
CH_4	0.05	1.2	37-39	0.61-0.63	1.54 : 1 : 0.61	99-98
O_2	0.05	1.2	36-38	0.58-0.61	1.54 : 1 : 0.61	99-97
CH_4	0.05	0.2	30-33	0.50-0.53	1.60 : 1 : 0.64	102-99
O_2	0.05	0.2	30-33	0.49-0.53	1.59 : 1 : 0.64	103-99
CH_4	0.05	0.0	28-31	0.47-0.50	1.63 : 1 : 0.67	99-97
O_2	0.05	0.0	26-29	0.47-0.50	1.60 : 1 : 0.67	99-97

repeated attempts at maintaining periods of steady-state growth. It was later realized that the frequency of culture wash-out could be reduced by increasing the level of Iron EDTA in the medium from 4mg l^{-1} to 8mg l^{-1}. Although this increase had no obvious effect on the efficiency of carbon conversion or on the stoichiometry of gas ultilization (table 4.2), it did appear to have a stabilizing effect on the culture. The significance of this effect will be discussed in greater detail in section 4.6.

4.5 Effect of Copper Levels and Nutrient Limitation on the Level of MMO activity in the Control Experiments

In the previous section an increase in the C.C.E. value of the culture was recorded when the conditions of growth were switched from low to high copper conditions. To confirm that this observed increase in growth efficiency correlated with a switch from soluble MMO expression to particulate MMO expression, as recorded by Leak and Dalton (1986a), samples of culture were removed during periods of steady-state growth and assayed for MMO activity.

Table 4.3.shows that in the presence of medium containing 1.2mg l^{-1} copper sulphate, cells possessed approximately 100% membrane-bound MMO activity. Under conditions of low copper, the MMO activity was mainly restricted to the soluble fraction of the cell extract. When this soluble fraction was run on an S.D.S. polyacrylamide gel, the characteristic protein bands associated with the polypeptide components of the protein A sub-unit of the soluble MMO could be clearly seen (fig4.1). Similar extracts prepared from the high copper continuous culture showed no such bands and likewise showed no soluble MMO activity.

4.6 Effect of Iron-EDTA Levels on MMO Activity

In the previous section, maintenance of a steady-state culture of M.capsulatus (Bath) in the absence of copper ions proved difficult. Under such conditions the culture appeared "unstable" with washout occurring at a dilution rate of 0.05h^{-1} for no obvious reason. MMO assays performed during the course of these experiments

Nutrient Limitation	Dilution Rate (h^{-1})	Level of $CuSO_4 \cdot 5H_2O$ in medium (mgl^{-1})	C.C.E. (%)	Gas exchange ratios O_2 : CH_4 : CO_2
CH_4	0.05	0	29	1.61 : 1 : 0.65
O_2	0.05	0	31	1.60 : 1 : 0.64

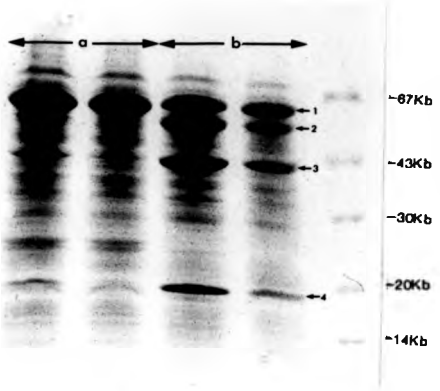
Table 4.2

C.C.E. values and gas stoichiometries obtained when the level of iron EDTA in the medium was raised from 0.4 to 0.8 mgl^{-1}

Nutrient Limitation	Dilution rate (h^{-1})	$CuSO_4 \cdot 5H_2O$ in medium (mgl^{-1})	soluble MMO ave	soluble MMO max	particulate MMO ave	particulate MMO max	% soluble MMO activity	% particulate MMO activity
CH_4	0.05	1.2	0.002	0.004	0.105	0.120	1.6	98.4
O_2	0.05	1.2	0.003	0.004	0.113	0.120	2.5	97.5
CH_4	0.05	0.2	0.035	0.045	0.01	0.015	78	22
O_2	0.05	0.2	0.037	0.048	0.008	0.015	82	18
CH_4	0.05	0	0.045	0.050	0.006	0.007	89	11
O_2	0.05	0	0.041	0.053	0.006	0.008	87	13

Table 4.3 Levels of MMO activity (Units mg^{-1}) in culture samples removed during control continuous cultures

Fig 4.1 SDS polyacrylamide gel of soluble cell extracts prepared from a) high copper continuous culture and b) low copper continuous culture



Key

Tracks a) soluble extract from culture expressing 100% particulate MMO
b) soluble extract from culture expressing 100% soluble MMO

Bands 1) Methanol dehydrogenase (MDH)

2) α Subunit of protein A (Soluble MMO)

3) β Subunit of protein A (Soluble MMO)

4) γ subunit of protein A (Soluble MMO)

M. wt. References

albumin	67,000
ovalbumin	43,000
carbonic anhydrase	30,000
trypsin inhibitor	20,000
α-lactalbumin	14,000

showed that the level of MMO activity in the culture dropped dramatically prior to washout. Activities obtained approximately 24 hours before washout were as low as 0.005 units mg^{-1}. When these experiments were repeated using medium containing 8mg l^{-1} Iron EDTA instead of 4mg l^{-1} Iron EDTA, the problem of culture wash-out at dilution rate $0.05h^{-1}$ did not occur. Examination of the level of MMO activity in these cultures showed that in the presence of raised Iron-EDTA levels the measured MMO activity was consistently higher than in the equivilent low-Iron EDTA cultures (table 4.4). In addition, the level of soluble MMO activity remained high throughout the duration of the experiment, implying that the raised levels of Iron EDTA had a stabilizing effect on the soluble MMO *in vivo*. This in turn resulted in an apparent increase in culture stability.

4.7 Discussion

The cell yield on methane was highest when the cells were expressing approximately 100% particulate MMO. The proposed reason for the observed difference in growth efficiency between cells expressing predominately soluble MMO and those expressing 100% particulate MMO is the latter's ability to obtain its required reducing power from sources other than NAD(P)H. This assumption was based on the observation by Leak and Dalton (1983) that NAD^+-independent methanol dehydrogenase was capable of driving particulate MMO activity in whole cells. In contrast soluble MMO activity has an obligate requirement for NAD(P)H and since Anthony (1978) previously proposed that methanotrophic growth was NAD(P)H-limited, $NAD(P)^+$-linked methane oxidation would prove a substantial drain on the availability of this co-factor.

The levels of C.C.E. calculated during these continuous cultivation experiments were between 2 and 4% lower than those obtained by Leak and Dalton (1986a). The observation that approximately 100% carbon recovery was obtained during each set of growth conditions suggested that any error in the estimation of either carbon consumption or production within the system was minimal. One possible reason why the efficiency of cell growth was lower than that recorded by Leak and Dalton may be in the fact that in the latter study the dilution rate used was

Nutrient Limitation	Dilution Rate (h^{-1})	Level of $CuSO_4 \cdot 5H_2O$ in medium	Soluble MMO activity	Particulate MMO activity
CH_4	0.05	0	0.055	0.005
O_2	0.05	0	0.052	0.007

Table 4.4

MMO activity levels (units mg^{-1}) recorded in cell extracts prepared from cultures grown in the presence of raised Iron EDTA levels (0.8 mgl^{-1})

$0.1h^{-1}$ and not $0.05h^{-1}$ as in this study. A recent report by Richards et al (1987) concerning the effect of dilution rate on the efficiency of growth in continuous cultures of M.capsulatus (Bath) concluded that cells grown at lower diltution rates produced increased levels of CO_2 for every mole of methane consumed. Therefore a reduction in dilution rate would effectively cause a drop in the efficiency of cell growth.

The in vitro soluble and particulate MMO activities generated during this study did not vary significantly with respect to whether the cells were grown under oxygen or methane-limited conditions. Such an observation may be explained by the fact that both oxygen and methane are required as substrates for the MMO-catalysed conversion of methane to methanol. Consequently the relative amounts of MMO expressed under either oxygen or methane-limited conditions will be determined by the relative affinity of the enzyme for these substrates. Green and Dalton (1986), reported that the purified soluble MMO had Km values of 3μM for methane and 16.8μM for oxygen. The equivolent Km values of the purified form of the particulate enzyme are not available since workers have been unable to purify this form of the enzyme. However, Joergensen (1985) using membrane-inlet mass spectrometry did estimate that the crude particulate MMO in the methanotroph OU-4-1 had a Km of 1μM and 0.14μM for oxygen and methane repectively. Therefore, assuming that the above particulate MMO is similar in nature to that expressed by M.capsulatus (Bath) it would appear that both the soluble and membrane bound forms of the enzyme possess a high affinity for both oxygen and methane. Consequently both forms of the enzyme will be very efficient systems for "scavenging" oxygen and methane when the extracellular concentration of either nutrient is low. Therefore the cell will not be required to express high levels of either enzyme in reponse to switching between oxygen and methane-limited growth.

One environmental parameter that did appear to influence cell physiology when M.capsulatus (Bath) was grown under soluble MMO conditions was the level of Iron-EDTA in the medium. In the absence of copper, increased levels of the above nutrient resulted in raised activity levels of the soluble MMO which appeared to increase the "stablility" of the culture. It has been known for some time that iron is an important structural component of the

soluble MMO. Colby and Dalton (1977) identified the presence of an acid-labile, iron-sulphur centre within the protein C sub-unit which is involved with the electron transfer between the FAD component and Protein A. Iron has also been identified as being an essential component of the protein A sub-unit (Woodland and Dalton 1984a, 1984b). It is now known that iron in the protein A is intimately involved in both the oxygen-carrying and oxygen-activating functions of the MMO (Dalton , Smith and Pilkington, in print). Recent work by Green and Dalton (1988) showed that the iron component of the protein A could be removed by repeated freeze/thaw cycles or by dialysing against 8-hydroxyquinoline. Subsequent incubation of the Iron-depleted protein with Iron-EDTA and dithiothreitol resulted in the re-assembly of the Iron centre within the protein. Green and Dalton (1988) also suggested that there may even be a "reconstitution" factor involved in this process, preliminary evidence implying that this factor is a protein. It is possible that the additional Iron-EDTA added to the medium, in the absence of copper sulphate helped stabilize the hydroxylase component of the MMO *in vivo*, the loss of which would compromise the cells capacity to metabolize methane. Exactly why the same culture instability was not observed when the cells were grown in medium supplemented with 0.2mg l^{-1} copper sulphate is difficult to explain since under these conditions the cells also expressed predominately soluble MMO. One possible explanation for the above observation may lie in the levels of membrane bound MMO activity detected in the presence of 0.2mg l^{-1} copper sulphate containing medium. Although the levels of particulate MMO activity would be limited by the availability of copper, the levels expressed (20% total MMO activity) may be sufficiently high to compensate for any loss of soluble MMO that might be incurred in the presence of lower Iron-EDTA levels. A more detailed analysis of the role of Iron-EDTA in the apparent stabilization of the soluble MMO may be forth-coming with the characterization of the *in vitro* properties of the so-called "reconstitution factor".

4.8 Effect of Copper Levels and Nutrient Limitation on other Methane Oxidation Pathway Enzymes.

To obtain a more complete picture concerning the flux of carbon along the methane oxidation pathway required the measurement of the relative activities of the enzymes responsible for this flux. Within this sequence of reactions there are several enzymes capable of fulfilling the same catalytic function; the oxidation of formaldehyde to formate. *In vitro* studies have identified three enzymes in *M.capsulatus* (Bath) capable of fulfilling such a catalytic role. These are the methanol dehydrogenase, $NAD(P)^+$-dependent formalde hyde dehydrogenase and an $NAD(P)^+$-independent formaldehyde dehdyrogenase. It is possible that all three enzymes are actively involved in the *in vivo* dissimilation of formaldehyde, but that one enzyme predominates under a specific set of growth conditions. Depending on which of these enzymes operates *in vivo* will effect the overall energetics of the cell since their catalytic action is coupled to the generation of reduced co-factors that not only differ in their capacity to be linked to ATP generation but also in their ability to act as electron donors for the MMO. The objective of this section was to monitor the activity levels of these different enzymes under the previously defined conditions of growth. The results of such measurements are discussed in the following sections.

4.8.1. Methanol Dehydrogenase

Of all the enzymes assayed, the ammonia-dependent methanol dehydrogenase showed the highest activity under all conditions of growth, (table 4.5). The activity was highest when it was measured using methanol as a substrate, although comparable activity levels were frequently obtained using formaldehyde in place of methanol. The methanol dehydrogenase utilizes PQQ as an electron acceptor *in vivo* and not $NAD(P)^+$. To measure the activity level *in vitro*, the non-physiological dye DCPIP is used as the electron acceptor. A similar arrangement is used to measure the $NAD(P)^+$ independent formaldehyde dehydrogenase. To ensure that the activity measured, using formaldehyde as a substrate was that of the methanol dehydrogenase and not the $NAD(P)^+$-independent formaldehyde

Nutrient Limitation	$CuSO_4 \cdot 5H_2O$ in medium (mgl^{-1})	Level of Methanol Dehydrogenase activity (Units mg^{-1})				
		a) methanol as substrate		b) formaldehyde as substrate		c) assays performed in the absence of ammonium ions
		ave	max	ave	max	
CH_4	1.2	0.421	0.445	0.343	0.370	0.008
O_2	1.2	0.452	0.473	0.392	0.41	0.003
CH_4	0.2	0.383	0.421	0.383	0.42	0.01
O_2	0.2	0.353	0.364	0.312	0.34	0.011
CH_4	0	0.388	0.371	0.302	0.343	0.009
O_2	0	0.384	0.420	0.347	0.368	0.009

Table 4.5 Levels of methanol dehydrogenase activity detected in culture samples.

dehydrogenase, a series of control assays were performed. These control assays involved repeating the methanol dehydrogenase assay with formaldehyde, but in the absence of ammonium ions. Unlike the $NAD(P)^+$-independent formaldehyde dehydrogenase, aerobically prepared methanol dehydrogenase has an obligate requirement for ammonium ions to act as an activator for the enzyme. When the methanol dehydrogenase assay was performed in the absence of ammonium ions, the level of activity detected was very low compared to the equivalent activity levels measured in the presence of ammonium ions. Therefore it was concluded that the high levels of methanol dehydrogenase measured in the presence of formaldehyde were not a consequence of $NAD(P)^+$-independent formaldehyde dehydrogenase activity but were due to the ability of the methanol dehydrogenase to utilize formaldehyde as a substrate.

4.8.2 $NAD(P)^+$-Independent Formaldehyde Dehydrogenase

The results in the previous section concerning the ammonium-independent, dye-linked oxidation of formaldehyde implied that the cells possessed relatively low levels of $NAD(P)^+$-independent formaldehyde dehydrogenase activity. To confirm this, the same cell extracts were assayed using the method described by Johnson and Quayle (1964). Although this method utilizes the same non-physiological dye to act as an electron acceptor, it is carried out in a phosphate buffer (pH7), instead of the glycine-based buffer (pH9), used in the methanol dehydrogenase assay. The levels of activity recorded are shown in table 4.6 and were between 4 and 10 fold higher than the activities obtained using the methanol dehydrogenase assay minus the ammonium ions. The level of $NAD(P)^+$-independent formaldehyde dehydrogenase activity in the culture was still considerably lower than the level of methanol dehydrogenase activity when the latter was measured in the presence of ammonium ions.

4.8.3.1 NAD^+-Dependent Formaldehyde Dehydrogenase

A formaldehyde dehydrogenase that requires $NAD(P)^+$ as an electron acceptor was isolated and purified from M.capsulatus (Bath) by Stirling and Dalton (1978). Stirling (1976) reported that the specific activity of

Nutrient Limitation	Concentration of $CuSO_4 \cdot 5H_2O$ (mgl^{-1}) in medium	NAD^+-Independent formaldehyde dehydrogenase activity (units mg^{-1})
CH_4	1.2	0.04
O_2	1.2	0.048
CH_4	0.2	0.035
O_2	0.2	0.04
CH_4	0	0.042
O_2	0	0.043

Table 4.6 NAD^+- independent formaldehyde dehydrogenase activity levels measured in culture samples

this enzyme, measured in cell extracts of the above organism were as high as 0.1 units mg^{-1}. Similar assays performed on cell extracts obtained during the previous continuous culture experiments routinely gave much lower levels of activity. Not only were these values low (0.004-0.01 units mg^{-1}), but they were also only obtained after a substantial period of time had elapsed between the initiation of the assay and the detection of activity. This period of time was frequently in excess of 20 minutes. Later studies showed that the length of this apparent lag period was indirectly related to the amount of crude extract used in the assay (fig 4.2). When the level of protein used in the assay was less than 2mg, the lag period was frequently greater than 30 minutes and in several instances no activity was detected after 45 minutes. The addition of an increased level of protein extract to the assay mix, effectively reduced this lag period, with immediate activity being recorded only after 8mg or more protein had been added. Increasing the level of protein also resulted in an increased level of specific activity.

The above obervations would appear to suggest that some essential co-factor was involved in the NAD^+-linked oxidation of formaldehyde. The use of low levels of protein in the assay may effectively dilute this co-factor to an extent where its ability to form a complex with the other enzyme component(s) is severly reduced. The observed decrease in specific activity with respect to the level of protein used may also be a consequence of the longer lag period generated under these conditions, since an increased proportion of the enzyme in the extract may be inactivated during this prolonged period of incubation at 45°c.

4.8.3.2 The Role of Heat-Stable Co-factor in the NAD^+-Linked Formaldehyde Dehydrogenase Activity

A heat-stable, low molecular weight co-factor was isolated by Stirling and Dalton (1978) and was shown to be a prerequisite for NAD^+-linked formaldehyde dehydrogenase activity in M.capsulatus (Bath). The authors showed this co-factor to be trypsin sensitive and it was therefore concluded that it was proteinaceous in origin. Following its method of preparation, the fraction of cell extract

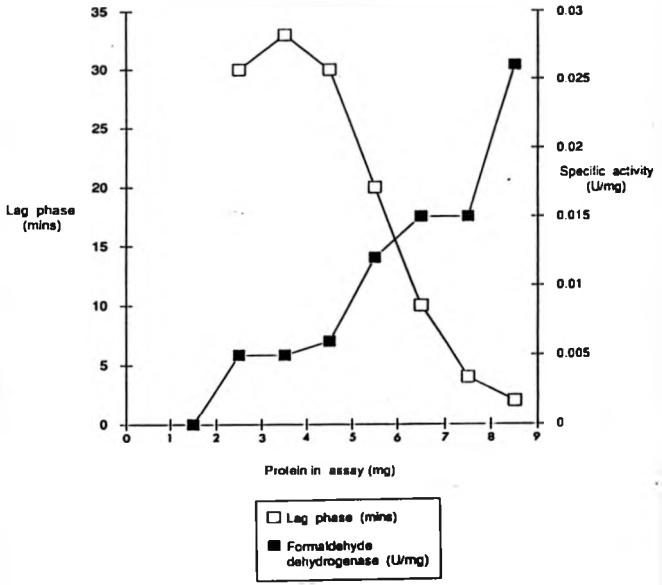


Fig 4.2 Level of NAD± linked formaldehyde dehydrogenase activity measured in crude extracts, while varying the level of protein used in the assay.

containing this co-factor was termed "heat-treated", soluble extract (HTSE).

To demonstrate the presence of HTSE in cell extracts and its role in the NAD^+-linked oxidation of formaldehyde, some concentrated, crude HTSE ($17mgml^{-1}$ protein) was prepared from a 100litre batch cultivation of cells. The large volume of culture harvested from the vessel permitted the preparation of a concentrated cell extract ($80mgml^{-1}$ protein). A sample of this extract was then used to prepare the HTSE according to the method described in chapter 2, while a similar amount was dialysed overnight at 4°c to prepare a HTSE-free fraction of cell extract.

To show that the crude extract contained NAD^+-linked formaldehyde dehydrogenase activity prior to any heat-treatment or dialysis, a sample (20mg protein) was added to a reaction mix containing excess NAD^+, formaldehyde and phosphate buffer. The subsequent disappearance of formaldehyde was monitored spectrophotometrically using Nash reagent, (fig 4.3). When an equivalent concentration of the dialysed fraction or HTSE fraction was added to the reaction mix instead of the un-treated crude extract, very little formaldehyde disappearance was recorded. By mixing the HTSE and dialysed fractions in the presence of NAD^+, it was possible to regain the capacity to utilize formaldehyde. A control was carried out involving the addition of formaldehyde to untreated crude extract in the absence of NAD^+ to ensure that the observed disappearance of formaldehyde was not the result of non NAD^+-linked enzyme activity. As expected, little formaldehyde disappearance was recorded in the absence of NAD^+.

In the earlier experiments low levels of NAD^+-linked formaldehyde dehydrogenase activity were obtained irrespective of the conditions of growth used (0.004-0.01 Units mg^{-1}). To determine whether these low levels of activity were a consequence of the presence of limiting levels of HTSE present in the crude cell extract, the assays were repeated in the presence of additional HTSE, prepared from the 100 litre culture. The amount of soluble extract used per assay was between 2 and 6mg protein, to which was added 1.7mg crude HTSE. The results (fig 4.4) showed that the previously recorded lag between initiating the assay and recording activity was effectively removed by the addition of excess HTSE. In

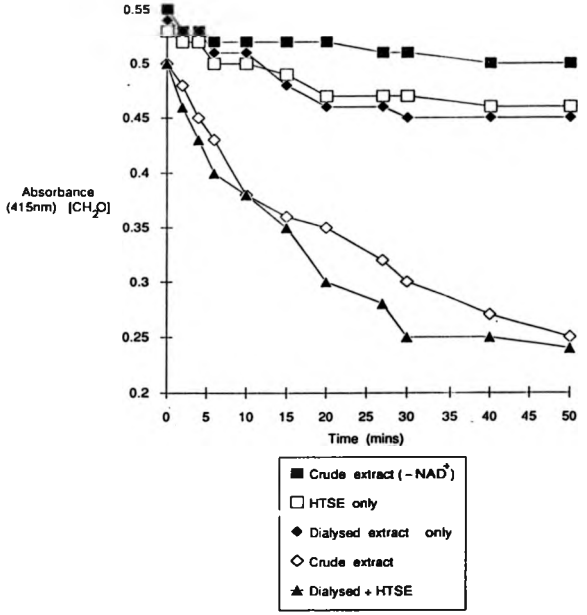


Fig 4.3 The role of the HTSE in the NAD^+- linked oxidation of formaldehyde, as determined by measuring the disappearance of formaldehyde

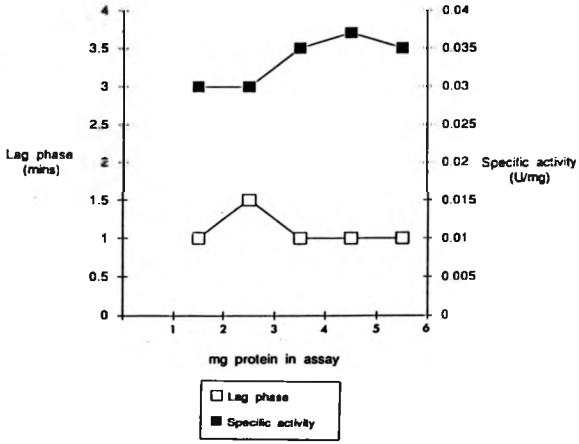


Fig 4.4 Pattern of NAD± linked formaldehyde dehydrogenase activity; measured in the presence of additional HTSE (1.7 mg).

addition, the calculated specific activities, based on the level of protein in the crude extract were between 40 and 100% higher. A control assay performed in the presence of HTSE and in the absence of the crude cell extract resulted in only a limited amount of activity (0.008 units mg^{-1}), implying that the HTSE on its own possessed only a limited amount of NAD^+-linked formaldehyde dehdyrogenase activity.

4.8.3.3. The Role of Heat-Stable Co-factor in the NAD^+-Linked Oxidation of Ethanal

To date the most recent work concerning the characterization of the NAD^+-linked formaldehyde dehydrogenase has been performed by Green, Millet and Dalton (unpublished). This work led to the purification of the HTSE component, shown to be a protein of molecular weight around 9,800. The authors subsequently named the purified HTSE component as "factor F" or "protein F".

Studies concerning the substrate specificity of the NAD^+-linked formaldehyde dehydrogenase using purified factor F and purified heat-sensitive formaldehyde dehydrogenase indicated that the latter required the presence of the co-factor to function as a formaldehdye dehydrogenase. In the absence of factor F, the heat-sensitive component lost its capacity to oxidise formaldehyde. Under the same circumstances, it aquired the capacity to utilize higher aldehydes such as ethanal, propanal and butanal in the presence of NAD^+, abeit with reduced levels of activity.

In the previous section it was shown that the addition of excess crude HTSE (soluble extract containing factor F) to an assay mix containing formaldehyde dehydrogenase activity caused a reduction in the lag period and an increase in the level of specific activity (fig 4.2). From this it was concluded that the factor F present in the crude extract was effectively limiting the NAD^+-linked formaldehyde dehydrogenase activity. According to the results generated by Green, Millet and Dalton, the above conditions should facilitate the NAD^+-oxidation of higher aldehydes by the heat-sensitive component of the formaldehyde dehydrogenase. To confirm this, the assays were repeated using similar levels of cell extract (1.5-9mg protein) added to a reaction mix containing NAD^+ and ethanal instead of formaldehyde. The

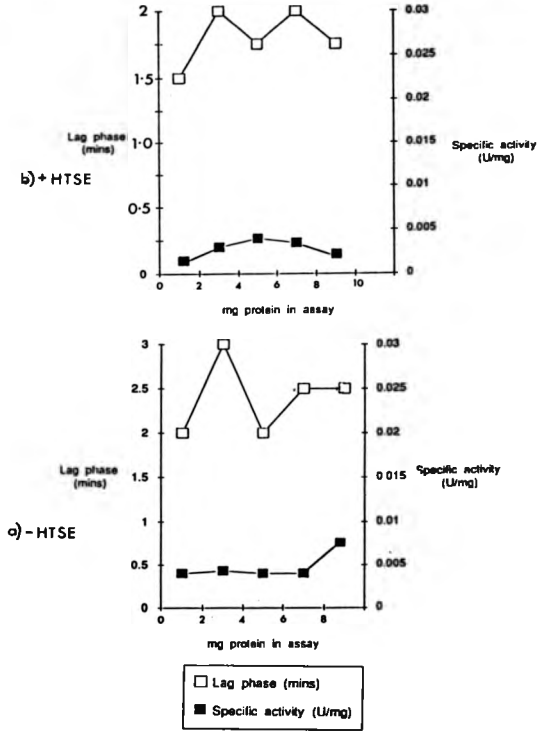


Fig 4.5 The results of the NAD^+- linked formaldehyde dehydrogenase assays using ethanal instead of formaldehyde as a substrate a) in the absence of added HTSE and b) in the presence of added HTSE

results (fig 4.5) showed that this did result in a reduced lag period compared with that observed when formaldehyde was used as a substrate , in the absence of HTSE. The level of activity produced under such conditions was however low. Addition of HTSE (1.7mg) to the ethanal-containing reaction had no effect on either the length of the lag period or on the level of activity. This was surprising since the work by Green et al implied that the addition of factor F (or HTSE) not only permitted the NAD^+-linked oxidation of formaldehyde but also restricted the same enzyme's capacity to utilize higher aldehydes. This effect was not observed during this study implying that the role of the F-factor in vivo may not simply be one of determining the substrate specificity of the NAD^+-linked formaldehyde dehydrogenase.

4.8.4 Formate Dehydrogenase

Formate dehydrogenase occupies the terminal position in the linear-oxidation pathway. Although it is not capable of utilizing formaldehyde as a substrate, the enzyme does play a key role in the metabolism of C_1 carbon since its activity is exclusively linked to the generation of NADH. High levels of this enzyme might be expected considering the high rate of CO_2 production recorded during earlier experiments, assuming that this CO_2 production occurs via the linear oxidation of carbon and not via the cyclic pathway of dissimilation.

Unlike the NAD^+-linked formaldehyde dehydrogenase, measuring the activity of the formate dehydrogenase in cell extracts was relatively straight forward. High levels of activity were recorded under all conditions of growth (table 4.7), the level of activity being proportional to the amount of protein used in the assay. The addition of KCN (3.75 μmol) to the assay caused the rapid and complete inactivation of the formate dehydrogenase. Since equivalent levels of KCN were used in the NAD^+-linked formaldehyde dehydrogenase assays, the activity levels recorded in the previous sections concerning the above enzyme were not the result of contaminating formate dehydrogenase activity.

Nutrient Limitation	Level of $CuSO_4 \cdot 5H_2O$ in medium (mgl^{-1})	Formate dehydrogenase activity (units mg^{-1})
CH_4	1.2	0.192
O_2	1.2	0.232
CH_4	0.2	0.210
O_2	0.2	0.201
CH_4	0	0.198
O_2	0	0.213

Table 4.7 Levels of formate dehydrogenase activity measured in cultures

4.9 Discussion

Unlike the MMO, the activities of the other methane oxidation pathway enzymes did not show any obvious response to changes in the level of copper ions in the medium. Throughout the growth conditions used, the methanol dehydrogenase consistently gave the highest levels of activity of all the enzymes monitored. The observation that the methanol dehydrogenase activity recorded with formaldehyde was higher than either the equivalent NAD^+-linked or NAD^+-independent formaldehyde dehydrogenase activites might imply that the former fulfils a major role in the dissimilation of formaldehyde under the conditions of growth used. Comparison of the kenetic properties of the purified forms of the methanol dehydrogenase and the NAD^+-linked formaldehyde dehydrogenase (table 4.8) would appear to underline this since the latter enzyme possesses both a lower affinity and lower Vmax for formaldehyde, compared to the methanol dehydrogenase.

The ability of the methanol dehydrogenase to oxidise methanol straight through to formate would have the advantage of reducing the availability of free formaldehyde, therefore avoiding any potential accumulation of the toxic metabolite. There are however a number of factors that would make formaldehyde dissimilation by the methanol dehydrogenase less favourable compared with the alternative NAD^+-linked step, particularly with respect to cell growth on methane. These include:-

1) Oxidation of formaldehyde by the PQQ-linked methanol dehydrogenase would be energetically less favourable than the equivalent NAD^+-linked oxidation. Dawson and Jones (1981a) showed that the PQQ-linked oxidation of methanol in Methylophilus methylotrophus resulted in a respiration-linked proton translocation of only $2H^+/O$, whereas the oxidation of NADH in the same organism produced a value of $6H^+/O$. Using these figures the authors estimated that in theory the PQQ-linked oxidation of one mole of substrate would generate only one mole of ATP, whilst the oxidation of one mole of NADH would produce 3 moles of ATP.
2) Unlike the $NAD(P)^+$-linked formaldehyde dehydrogenase, the PQQ-linked oxidation of formaldehyde would not

Enzyme	Km (CH_2O)	Vmax (CH_2O)	Reference
Methanol dehydrogenase	90.9μM	5.71μmol/min/mg	Green (Personal communication)
NAD^+-linked formaldehyde dehydrogenase	680μM	0.57μmol/min/mg	Stirling and Dalton (1978)

Table 4.8 Comparison of kinetic properties of purified methanol dehydrogenase and NAD^+-linked formaldehyde dehydrogenase; from M.capsulatus (Bath).

generate reducing equivalents [NAD(P)H] capable of being used directly in either assimilation reactions or in the supply of electrons to the soluble MMO.

3) In many methylotrophs the only source of NAD(P)H generated occurs at the final step in the linear oxidation sequence, the oxidation of formate to CO_2. Therefore any NAD(P)H-linked oxidation of formaldehyde would effectively reduce the percentage of carbon substrate required to be completely oxidised to CO_2 to generate the necessary NAD(P)H. This in turn would permit an increased level of carbon available for assimilation.

One other point concerning the potential disadvantage of formaldehyde dissimilation by the methanol dehydrogenase involves the envisaged problem of regulating the fate of carbon at the oxidation level of formaldehyde, since it is at this point that carbon may either be assimilated into cellular material or dissimilated to CO_2. If the methanol dehydrogenase is actively involved in the in vivo oxidation of formaldehyde, then the cell must possess some sort of regulatory mechanism that would effectively prevent the indiscriminate dissimilation of all the carbon reaching the oxidation level of formaldehyde. The cell must permit the assimilation of a proportion of this carbon in order to prevent a total loss of carbon to CO_2. Attwood and Quayle (1984) stated that it was uncommon for one enzyme such as the methanol dehydrogenase to catalyse two successive reactions, particularly when the product of the first reaction (formaldehyde) occupies such a key metabolic position.

A potential mechanism for the regulation of formaldehyde oxidation by the methanol dehydrogenase has been identified by Page and Anthony (1986). This involved the isolation of a modifier (M) protein from Methylophilus methylotrophus, which possessed the capacity to reduce the in vitro affinity of the enzyme for formaldehyde. Whether or not M.capsulatus (Bath) possesses a similar means of regulating the methanol dehydrogenase-catalysed oxidation of formaldehdye remains to be seen. The enzyme assays performed in this section indicated that the methanol dehdyrogenase retains the capacity to utilize formaldehyde under all the conditions of growth used. Therefore, according to the above theory, such an observation would imply that the M protein was not

present in the cell extracts assayed. One of the objectives of the following chapters will be to investigate whether or not it is possible to induce conditions of growth that would lead to a loss of the methanol dehydrogenase's capacity to utilize formaldehyde *in vitro*. Under such conditions one might expect to be able to demonstrate the presence of M protein in cell extract.

Although NAD^+-linked formaldehyde dehydrogenase activity was detected under the conditions of growth used, the levels of activity were low and difficult to measure in the absence of additional heat treated crude soluble extract (HTSE). From these observations it was concluded that the heat-stable co-factor was limiting in the assay mixtures. Arfman *et al* (1989) while examining the role of NAD^+-linked methanol dehydrogenase in a thermotolerant *Bacillus* strain reported that the activity of this enzyme was not proportional to the amount of cell extract added during its assay. Although no lag period was recorded between initiating the assay and detecting activity, increased specific activity levels were recorded when increased levels of protein were used in the assays. A more detailed study by these authors led them to conclude that the enzyme being studied, operated *in vivo* as part of a complex enzyme system and increased dilution of the protein effectively compromised the integrity of the complex. It is possible that a similar complex enzyme system operates in the NAD^+-linked oxidation of formaldehyde. Determination of the true role of this enzyme awaits a detailed study of the kinetics of the purified proteins associated with the "enzyme complex".

As with the NAD^+-linked formaldehyde dehydrogenase assays, the NAD^+-independent formaldehyde dehydrogenase assays consistently gave low levels of activity when measured in the presence of the non-physiological dyes, DCPIP and PMS. Similar observations were made by Marison and Attwood (1980) who examined the activity of the equivalent enzyme in *Hyphomicrobium* X, a facultative methylotroph. These authors reported that purified NAD^+-independent formaldehyde dehdyrogenase possessed both lower Km and higher Vmax values for aldehydes of carbon chain length C_4 and C_5 compared with the equivalent values for formaldehyde. Furthermore, the enzyme's activity was not induced during growth on C_1 compounds which contrasted

directly with the activity of the methanol dehydrogenase and the NAD^+-linked formaldehyde dehydrogenase in the same organism. Marison and Attwood therefore concluded that this enzyme did not play a major role in C_1 dissimilation but instead functioned primarily as a general aldehyde dehydrogenase.

Aldehydes occur widespread in nature and are often toxic to cells. In addition to the natural occurrence of such compounds, the combined substrate specificities of both the MMO and the methanol dehydrogenase may result in the frequent exposure of cells to aldehydes of varying chain length. Therefore, the possession of general aldehyde dehydrogenase under such circumstances would prove a selective advantage to M.capsulatus (Bath).

4.10 SUMMARY

The coupling of a gas-limited continuous culture to an on-line mass spectrometer permits the growth of M.capsulatus (Bath) to be studied under defined and reproducible conditions. Using such a system it was possible to generate a series of results concerning the effect of growth conditions on the efficiency of cell growth, the pattern of gas metabolism and the in vitro activities of enzymes associated with C_1 metabolism. The results generated under these conditions demonstrate the complexity of the situation, particularly with respect to the role of the MMO type in determining the physiological state of the cells and the potential fate of carbon at the oxidation level of formaldehyde. Although these results offer little information concerning the possible regulatory processes that operate in C_1 metabolism, they provide a starting point from which the effect of perturbing the above growth conditions can be examined.

The following chapters are concerned with the examination of the effect of deliberately perturbing the carbon flux within the C_1 oxidation pathway and monitoring the cells' physiological response to such changes.

CHAPTER 5

Regulation of Formate Metabolism

Chapter 5

Regulation of Formate Metabolism

5.1 Introduction

It was concluded in the previous chapter that cells expressing 100% particulate MMO grew more efficiently on methane than cells expressing the soluble form of the enzyme. The proposed reason for this was that while the soluble enzyme had an absolute requirement for NAD(P)H to act as a source of reducing power for methane oxidation, the particulate enzyme was able to utilize alternative sources of electrons for the same purpose. Since Anthony (1978) proposed that methylotrophic growth on methane was probably limited by the availability of NAD(P)H, the ability of the particulate MMO to utilize alternative sources of reducing power to NAD(P)H would effectively relieve this limitation, thereby permitting a higher cell yield on methane.

It should, in theory, be possible to demonstrate this limitation by stimulating metabolic reactions within the cell that result in the generation of surplus NAD(P)H. One such reaction is the oxidation of formate to CO_2 via the $NAD(P)^+$- linked formate dehydrogenase. One of the objectives of this section was to examine the effect of the addition of formate to an actively growing culture of M.capsulatus (Bath), especially with respect to its effect on the cell yield of the culture.

A second objective was to supplement the observation made by Eccleston and Kelly (1973), and Reed (1976) who reported that cultures of M.capsulatus (Bath) were capable of assimilating ^{14}C-labelled formate into cellular material. While both these studies were effective in demonstrating the accumulation of ^{14}C-label in amino acids such as serine and glycine, they did not give any indication as to the relative proportion of formate that was incorporated into biomass and that which was oxidised to CO_2. By using on-line gas analysis and total carbon analysis it should be possible to determine the extent by which M.capsulatus (Bath) is capable of assimilating

exogenously supplied formate and whether or not this isdependent upon the conditions of growth i.e. the nutrient limitation or the type of MMO expressed.

5.2 The effect of the Pulse Addition of Formate to Methane-Limited Cultures of M.capsulatus (Bath)

5.2.1. Experimental

Two different approaches were used in the study of the effect of formate addition to continuous cultures of M.capsulatus (Bath). Firstly a series of experiments were set up to examine the effect of the direct (pulsed) addition of the metabolite to the culture. A second approach, (to be discussed later in the section) involved the continuous addition of formate-supplemented medium to cells .

The initial study involving the pulse addition of formate to the culture was done for three reasons:-

1) To examine the cell's existing capacity to metabolize exogenously supplied formate. This approach avoided any physiological adaptation of the organism that might otherwise be induced by the continuous exposure of the cells to the metabolite.
2) To investigate the cells efficiency at removing excess formate from the environment. Unlike relatively non-polar compounds such as methanol or formaldehyde, formate exists predominately in its dissociated form in solution at neutral pH, since it has a pka value of 3.75. To date, no specific transport mechanism for the metabolite has been isolated in prokaryotic organisms. Therefore it is assumed that the extracellular formate traverses the cytoplasmic membrane by a process of diffusion when it is in its protonated form. If there is only a limited amount of protonated formate present at neutral pH, the factor limiting formate metabolism may not be an intracellular constraint but instead the inability of the cell to take up formate from its environment.
3) To determine the ability of the mass spectrometer to detect rapid changes in the gas phase composition of the culture, induced by the oxidation of additional formate.

Methane-limited continuous cultures were set up as

described previously using initially high copper conditions followed by low copper conditions. During periods of steady-state growth, controlled amounts of potassium formate were added directly into the culture. Under these conditions samples of culture were routinely removed to monitor the levels of extracellular metabolites, including formate in the culture.
The accumulation of metabolites such as methanol or formaldehyde would be indicative of a restriction in the flux of carbon through the methane oxidation pathway. Such a result might be expected if increased formate metabolism resulted in some sort of feed-back control of methane metabolism as was suggested by Reed (1976). The composition of the inlet and exhaust gases were also monitored throughout this period. Repeated measurement of CO_2, methane and oxygen levels in these gas streams made it possible to estimate the effect of additional formate metabolism on the rates of gas exchange within the system.

Prior to the addition of formate the cells were grown under carbon-limiting conditions. It was assumed that under such conditions, excess carbon added in the form of formate would be rapidly catabolized (Harder and Dijkuizen, 1983). This in turn would limit the possibility of any toxic effect caused by formate accumulation.

5.2.2. Results

The effect of the pulse addition of formate to cells growing firstly under high copper conditions and secondly low copper conditions are shown in fig. 5.1 and 5.2 respectively. According to these results the overall effect of the formate addition appeared to be independent of whether the cells expressed soluble MMO or particulate MMO, the response of cells being similar in both cases. The addition of increasing amounts of formate resulted in increased rates of CO_2 production; these rates reaching a maximum four or five minutes after the metabolite had been added, before dropping back to their original level. The observed pattern of extracellular formate consumption and raised CO_2 production implied that a certain proportion of the formate added was being oxidised to CO_2. However, since these studies were performed under non-steady state conditions it is difficult to equate the observed increase

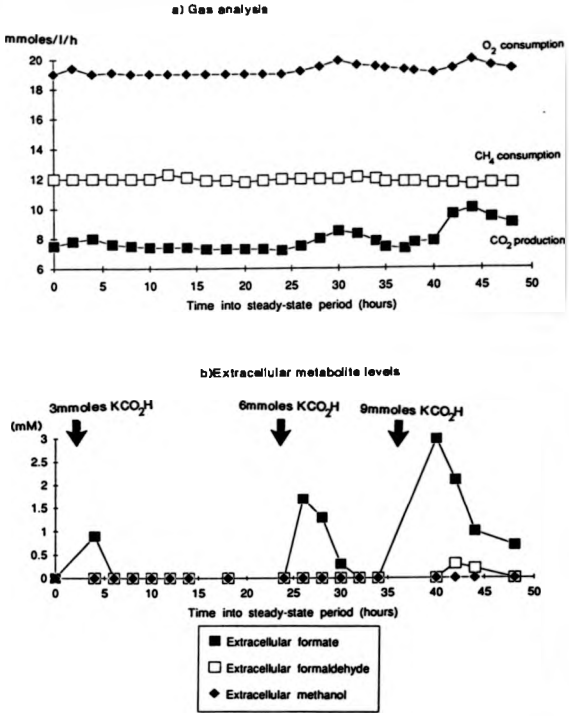


Fig 5.1 The effect of the pulse addition of formate to high copper continuous culture

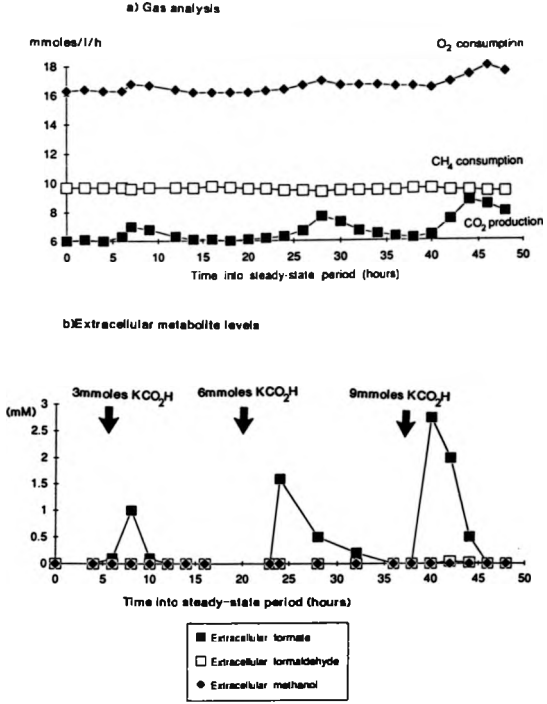


Fig 5.2 The effect of the pulse addition of formate to low copper continuous culture

in CO_2 production with the amount of formate added.

Analysis of the extracellular levels of methanol and formaldehyde showed that neither of these metabolites accumulated within the culture in response to the addition of formate. Therefore an increased rate of formate metabolism did not appear to cause any inhibition of the C_1 oxidation pathway prior to the oxidation level of formate. In addition, no change in the rate of methane consumption was observed throughout this study.

The increased levels of CO_2 production recorded after the periodic addition of formate occurred presumably via the oxidation of the metabolite by the NAD^+-linked formate dehydrogenase. Consequently, one might expect increased levels of NADH to be generated under such conditions. If the cells growing on methane are NADH-limited, as was suggested by Anthony (1978), then the generation of extra NADH should relieve this limitation and hence result in an increase in cell yield. Since these experiments were performed under non-steady state conditions it was very difficult to accurately determine the effect of increased formate metabolism on cell yield. To achieve the latter and to quantify the fate of exogenously supplied formate it was necessary to generate steady-state conditions that permitted the continuous metabolism of extracellular formate. This was achieved by growing M.capsulatus (Bath) in continuous culture using formate-supplemented NMS medium.

5.3 The Effect of Continuous Formate Addition to M.capsulatus (Bath)

5.3.1. Experimental

A second approach used in the study of the response of M.capsulatus (Bath) to exogenously supplied formate involved the continuous cultivation of cell in the presence of formate-supplemented medium. This method permitted formate-utilizing cells to attain periods of steady-state growth, during which it was possible to determine the effect of formate metabolism on cell yield. The on-line monitoring of gas exchange rates and total carbon analysis of culture samples during periods of steady-state growth also enabled the distribution of metabolized carbon between CO_2 and biomass to be examined

more closely, especially with respect to the fate of exogenously supplied formate.

The effect of continuous formate addition was examined firstly under methane-limited conditions followed by oxygen-limited conditions. This was done to determine whether or not the nutrient limitation affected the ability of the cells to consume formate or the eventual fate of the metabolized carbon. As with the studies involving the pulse addition of formate, the effect of the continuous addition of the metabolite was examined under high and low copper conditions. The results of these formate-fed continuous cultures are discussed in the following sections.

5.3.2. Results

5.3.2.1. Formate-Fed, Methane-Limited Continuous Culture

a) Low copper conditions

During the course of this experiment periods of methane-limited steady state growth were obtained in the presence of NMS medium containing 0,25,50 and 100mM formate. In each case the cells were grown under soluble MMO conditions since the medium also contained $0.2mgl^{-1}$ copper sulphate. The effect of formate addition on the distribution of carbon during each steady-state period is shown in fig 5.3.

Throughout the experiment no extracellular formate was detected in culture samples. The carbon recovery calculations indicated that virtually all the carbon in the added formate could be accounted for as CO_2. In addition to the increased level of CO_2 production, there was also a proportional increase in the rate of oxygen consumption. This would suggest that all the formate in the medium was oxidised to CO_2 and that additional oxygen was required as a terminal electron acceptor for the extra reducing power being generated.

Since all the formate added to the system was converted to CO_2 and not cellular material, the carbon conversion efficiency was reduced with increasing levels of formate addition. However, when the efficiency of growth was expressed in terms of cell yield on methane, the values obtained during the addition of 25 and 50mM

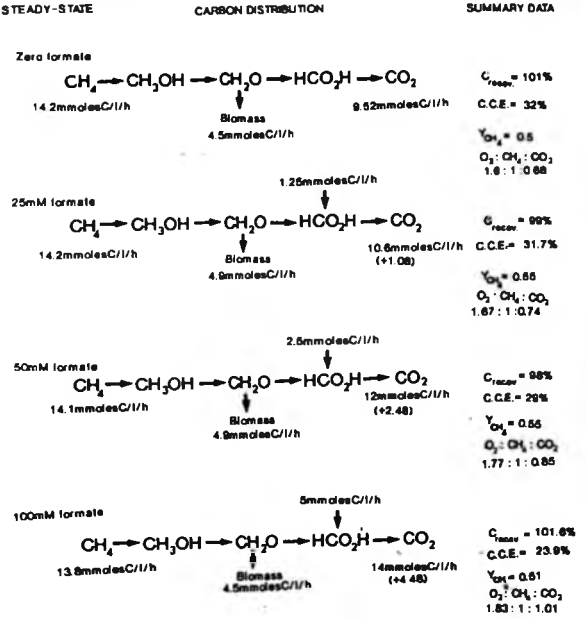


Fig 5.3 The distribution of carbon recorded during methane-limited, formate-fed continuous cultures: low copper conditions

formate were approximately 10% greater than the equivalent value produced in the absence of formate. Therefore, although the carbon conversion efficiency was reduced in the presence of additional formate metabolism, the efficiency of methane metabolism under the above conditions increased. Such a result might be expected if the extra NADH generated via additional formate metabolism was being used as a source of electrons for the soluble MMO, thereby effectively relieving the cells NADH-limitation.

The observation that a reduction in cell yield on methane was recorded when the formate concentration in the medium was raised from 50mM to 100mM implied that at this stage the cells were no longer limited for NADH. Instead growth was limited by some other factor and any formate added during this period was detrimental to the cells.

b) High Copper Conditions

The previously described continuous culture experiment was subsequently repeated using NMS medium supplemented with 1.2mg l^{-1} copper sulphate in order to achieve the expression of 100% particulate MMO within the cells. Under these conditions of growth, steady-states were achieved in the presence of 0,25,50,100 and 120mM formate-supplemented medium. The distribution of carbon within the system, under the above conditions is shown in fig 5.4.

As with the low copper, formate-fed continuous culture, no formate was detected in culture samples removed during periods of steady-state growth. Carbon balance results indicated that as in the previous experiment virtually all the formate added was oxidised to CO_2, resulting in a reduction in the C.C.E. valve of the culture. However, unlike the previous experiment the calculated yield on methane did not increase in response to the addition of formate. Consequently this would suggest that cells grown under particulate MMO conditions were not limited by the availability of NADH.

c) Formate - Fed, Methane-Limited Continuous Culture -

General Discussion

The results obtained in chapter 4 showed that soluble

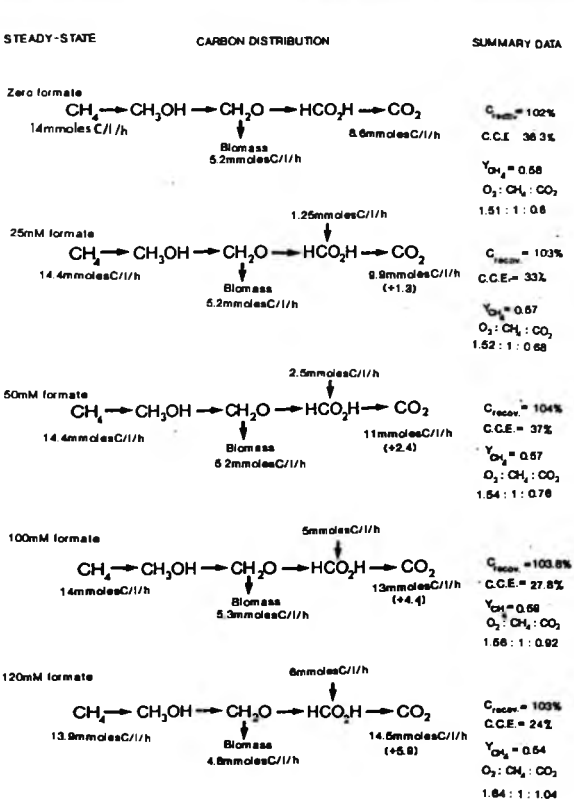


Fig 5.4 The distribution of carbon recorded during methane-limited, formate-fed continuous cultures: high copper conditions

MMO - containing cells grew less efficiently than those containing the particulate enzyme. It was concluded that this difference was due to the soluble enzyme's obligate requirement for NADH, coupled with the fact that methanotrophic growth was NADH-limited. Therefore it was suggested that it would be possible to eliminate this limitation by growing the cells in the presence of excess NADH, generated by the co-oxidation of additional formate. The results produced in this section showed this to be true, since increased formate metabolism by methane-grown, soluble MMO-containing cells resulted in an increased efficiency of methane metabolism. However, the maximum Y_{CH4} value obtained under these conditions was still lower than the equivalent value generated by particulate MMO-expressing cells grown in the absence of additional formate. Therefore either the metabolism of the additional formate did not completely relieve the apparent NADH-limitation or the uptake (and/or metabolism) of additional formate was energy dependent.

To date no active transport mechanism has been isolated for formate transport and it is generally assumed that formic acid enters the cell by means of passive diffusion in its protonated form, (Cobley and Cox, 1983; Baronofsky et al, 1984; Herrero et al, 1985). Dijkhuizen et al (1977) and van Verseveld and Stouthamer (1978), however postulated that while formate did enter the cell in its protonated form, it would do so at the expense of a pH gradient. Therefore when one molecule of formic acid crosses the cytoplasmic membrane, it is the equivalent of co-transport with one proton and according to van Verseveld and Stouthamer (1978), this would require between 0.33 and 0.5 mole ATP per mole of formate consumed. Therefore an increased rate of formate utilization would not only result in an increased rate of NADH production but also an increased level of energy expenditure linked to the uptake of the metabolite. This would explain why the Y_{CH4} value generated during the formate-fed, low copper continuous culture was lower than that obtained in the high copper formate-free continuous culture, since the latter would not require energy expenditure for the uptake of formate.

Energy-dependent formate uptake would also explain why the Y_{CH4} was reduced during both high and low copper continuous cultures when the formate concentration in the

medium was raised to 120 and 100mM respectively. At this concentration of formate the energy utilized in the metabolite's transport exceeded that which was produced as a result of the extra NADH generated during additional formate oxidation.

5.3.2.2. Formate-Fed, Oxygen-Limited Continuous Culture

a) Low Copper Conditions

In contrast to the methane-limited, formate-fed continuous culture, cells grown under oxygen-limited conditions on medium containing $0.2mgl^{-1}$ copper sulphate appeared more sensitive to increased levels of formate in the medium. An initial attempt at growing cells under oxygen-limited conditions in the presence of 25mM formate failed with the rapid accumulation of extracellular formate being detected in the culture. Formate accumulation was also accompanied by the accumulation of both formaldehyde (0.7mM) and nitrite (3mM) in the culture; the detection of these metabolites preceding the wash-out of the culture. Both formaldehyde and nitrite may have accumulated as a consequence of cell lysis, since at this stage the culture began to foam and there was an accompanying reduction in the pH of the culture, (pH7 to pH5)

This experiment was subsequently repeated under similar conditions except the initial formate concentration in the medium was reduced to 10mM in an attempt to avoid the previous problem of formate accumulation. Once a period of steady-state growth had been maintained for three consecutive days, the level of formate in the medium was increased, but by increments smaller than those used in the methane-limited experiments. Using this approach periods of steady-state growth were obtained with 10mM, 20mM, 30mM and finally 40mM; washout being recorded on the addition of 50mM formate-supplemented medium.

The pattern of carbon distribution obtained under the above set of conditions is shown in fig 5.5. At lower formate concentrations (10mM and 20mM), not all the carbon in the formate consumed could be accounted for in the increased rate of CO_2 production. Unlike the equivalent methane-limited experiments an increased rate of biomass

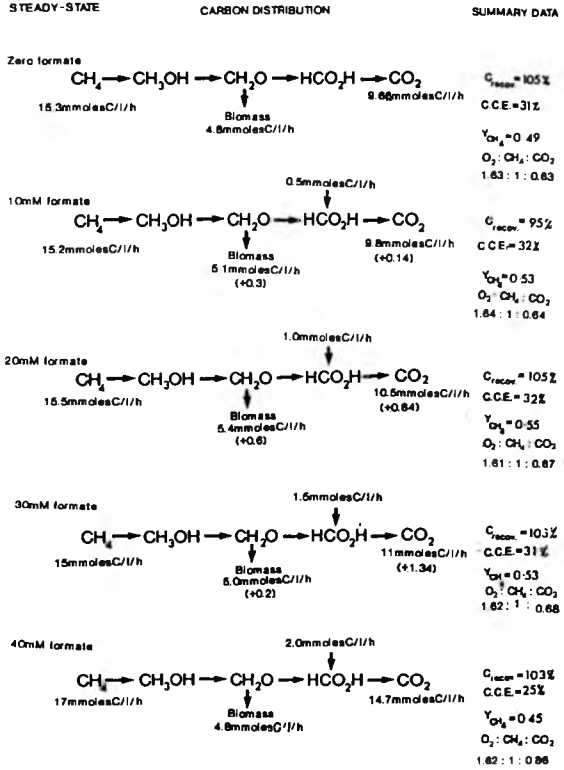


Fig 5.5 The distribution of carbon recorded during oxygen-limited, formate-fed continuous cultures: low copper conditions

accumulation was recorded while the rate of methane consumption remained constant. Therefore, under oxygen-limited conditions the cells appeared capable of assimilating carbon from the additional formate. As a consequence of the increased amount of carbon fixation, the C.C.E. value of the culture increased during the addition of 10mM and 20mM formate.

When the formate concentration in the medium was increased firstly to 30mM and then to 40mM, the proportion of carbon in the formate oxidised to CO_2 increased with respect to that which was assimilated. Consequently the C.C.E. value of the culture decreased under these conditions. When the medium was supplemented with 50mM formate , culture washout occurred with symptoms similar to those observed in the earlier oxygen-limited continuous culture, (excessive foam production, a drop in the culture pH and the accumulation of formate, formaldehyde and nitrite).

As in the low copper, methane-limited continuous culture, the addition of formate to the oxygen-limited culture resulted in an increase in the cell yield on methane. This was recorded on the addition of medium containing 10,20 and 30mM formate, and again suggests that cells grown under soluble MMO-expressing conditions were NADH-limited. The increased level of formate oxidation effectively increasing the level of available NADH.

b) High Copper Conditions

When the above experiment was repeated in the presence of NMS medium containing $1.2mgl^{-1}$ copper sulphate, periods of steady-state growth were achieved with 0,25 and 50mM formate added to the medium. The pattern of carbon distribution obtained under these conditions is shown in fig 5.6.

As with the low copper, oxygen-limited continuous culture, the addition of a low level of formate (25mM) resulted in an increased rate of biomass accumulation. The observed increase in CO_2 production was not sufficient to account for the amount of formate consumed. Therefore it was concluded that the cells were actively assimilating carbon from the exogenously supplied formate. The C.C.E. value of the culture under the above conditions did not alter significantly since the proportion of added formate

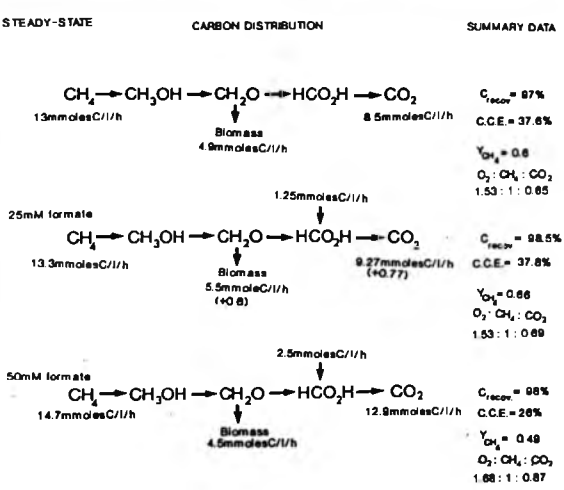


Fig 5.6 The distribution of carbon recorded during oxygen-limited, formate-fed continuous cultures: high copper conditions

being assimilated was similar to that being oxidised.

When the level of formate in the medium was increased to 50mM, an increase in the rate of both oxygen and methane consumption was recorded. In addition, a reduction in the steady-state biomass concentration was also detected indicating that the increased level of formate metabolism had caused a reduction in the efficiency of cell growth. It was possible that under such conditions the high levels of formate being consumed were to some degree uncoupling the process of oxidative phosphorylation. This would have caused a drop in cell yield, since although the methane consumption increased, the uncoupling would effectively reduce the rate at which utilizable energy (ATP) was formed.

During this period of growth one unexpected result was the observed increase in the rate of oxygen consumption. Throughout this experiment it had been assumed that the cells had been growing under oxygen-limited conditions and therefore any increase in oxygen availability would have been possible only through either an increase in the air flow rate or increased mixing. Such changes were not noted as having occurred during this steady-state period, nor were there any alterations in the medium constituents or dilution rate that might have resulted in an alternative nutrient limitation. Possible reasons for the observed increase in the rate of oxygen consumption included:

1) A problem with the calibration of the mass spectrometer resulting in the generation of abnormally high values for oxygen consumption.
2) Air leaking into the gas analysis sample lines.
3) An increase in the head-pressure within the fermenter due to a partially blocked exhaust filter, causing an increased rate of gas transfer into the culture.

Of the three possibilities, the last is the most probable since this problem had been previously recorded in an earlier experiment.

c) Formate-Fed, Oxygen-Limited Continuous Culture - General Discussion

The most obvious difference between the results

obtained during the continuous addition of formate to methane-limited and oxygen-limited continuous cultures was that under the latter conditions the cells were capable of assimilating low levels of formate carbon. The assimilation of formate carbon by M.capsulatus (Bath) was previously demonstrated by Eccleston and Kelly (1973), by the addition of ^{14}C-labelled formate to shake flask cultures. This label was shown to have been incorporated into several amino acids including serine and glycine; key intermediates in the serine pathway of carbon assimilation. Reed (1976) subsequently repeated this labelling experiment using an oxygen-limited continuous culture of M.capsulatus (Bath) instead of a shake flask. The pattern of metabolite labelling remained the same, underlining the ability of M.capsulatus (Bath) to assimilate formate carbon under oxygen-limited conditions.

Although the above ^{14}C-labelling experiments indicated that carbon in the formate was assimilated via the serine pathway, the means by which the formate is linked to the pathway is unclear. A pathway for formate assimilation in Hyphomicrobium X was outlined by Attwood and Harder (1978). This involved the conversion of formate to methylene tetrahydrofolate which is then capable of entering the serine pathway. The possibility of the methenyl THF pathway operating in M.capsulatus (Bath) will be discussed in the next section. Suffice to say at this stage whatever enzyme catalyses the initial step in formate carbon assimilation, it would have to compete with the NAD^+-linked formate dehydrogenase. It is possible that the formate dehydrogenase possesses a relatively high affinity for formate compared with the alternative assimilatory enzyme and hence the latter may only operate when formate is present at high intracellular concentrations. Such conditions being generated only when the cells are oxygen-limited. The accumulation of intracellular formate would explain the apparent sensitivity of oxygen-limited, formate-fed cultures to raised concentrations of formate in the medium. Under these conditions there would appear to exist a fine balance between formate carbon assimilation and the accumulation of toxic levels of formate.

The observed toxic effect of formate accumulation was probably a consequence of the uncoupling of oxidative phosphorylation. Compounds capable of functioning as

uncoupling agents are normally weak acids that are able to traverse the cytoplasmic membrane in their undissociated form. The intracellular pH of cells is normally alkaline with respect to their extracellular environment, in order to maintain a pH gradient. Therefore once inside the cell, the protonated acid dissociates; resulting in an increase in the local hydrogen ion content. This in turn causes the collapse of the proton motive force required to drive ATP synthesis. The role of formate as an uncoupler of oxidative phosphorylation was proposed by Wood and Kelly (1981) to explain the inhibitory effect of excess formate on the mixotrophic growth of Thiobacillus A2 on glucose and formate. Similarly, Drozd et al (1984) recorded that when excess formate was added to a culture of glucose-grown Beneckea natrigiens at pH 6.7, there was a marked increase in the rate of respiration, coupled with a drop in cell yield. This effect was similar to that observed when raised levels of formate were added to oxygen-limited cultures of M.capsulatus (Bath) and implies that formate effectively uncoupled oxidative phosphorylation in these cells.

5.3.3. Enzyme Levels in Formate-Fed Continuous Cultures

The previous observation that the assimilation of formate carbon occurred only under oxygen-limited conditions and not when the cells were grown under methane-limited conditions suggested that the enzymic capacity of the cells differed between the two nutrient limitations. To determine whether or not increased formate metabolism affected the relative activities of the C_1 oxidising enzymes, analysis of enzyme levels during the course of the formate-fed continuous cultures were performed.

The pattern of MMO activity throughout the previously described continuous cultures are shown in figs 5.7 and 5.8. Although the addition of formate to the cultures did result in a reduction in the level of MMO activity at no time during the experiments was the enzyme activity lost altogether. This was not surprising since the gas exchange rates measured indicated that high rates of methane consumption were maintained irrespective of the conditions of growth.

The activity levels of other C_1 oxidising enzymes

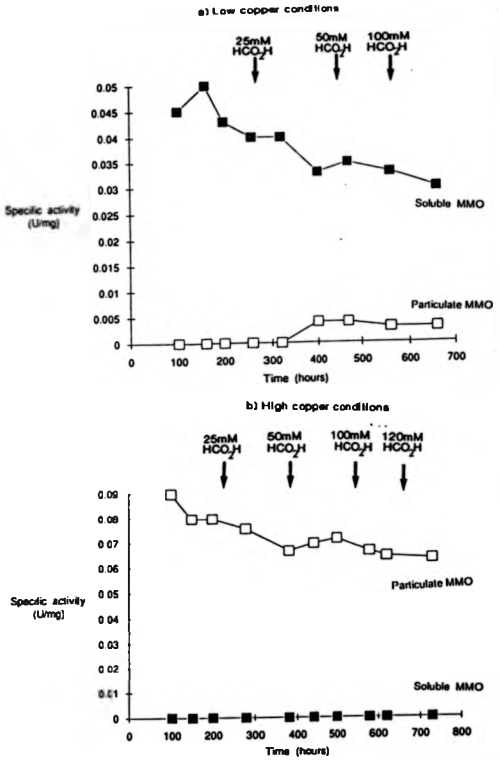


Fig 5.7 Levels of MMO activity recorded during methane-limited, formate-fed continuous cultures

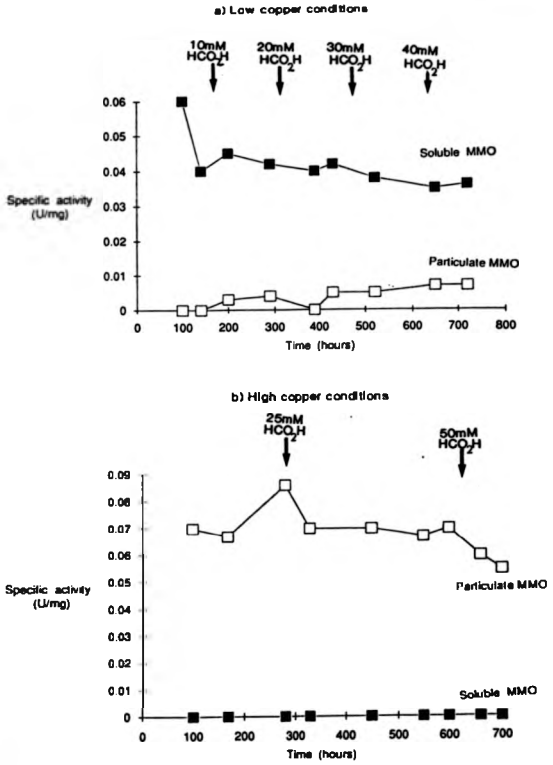


Fig 5.8 Levels of MMO activity recorded during oxygen-limited, formate-fed continuous cultures

Table 5.1 C_1 Oxidation enzyme levels measured during formate-fed, continuous cultures.

a) Methane-limited Growth

	Specific Activity Levels (Units mg^{-1})			
Level of formate in medium (mM)	MDH	FMDH (NAD^+)	FMDH (DYE)	FDH
0	0.41	0.03	0.056	0.15
25	0.38	0.03	0.06	0.154
50	0.42	0.035	0.07	0.15
100	0.39	0.03	0.06	0.13

b) Oxygen-Limited Growth

Level of formate in medium (mM)	MDH	FMDH (NAD^+)	FMDH (DYE)	FDH
0	0.36	0.04	0.05	0.136
20	0.35	0.032	0.05	0.15
40	0.33	0.035	0.043	0.123

Enzymes

MDH - Methanol dehydrogenase (Methanol)

FMDH (NAD^+) - Formaldehyde dehydrogenase (NAD^+-linked)

FMDH(DYE) - Formaldehyde dehydrogenase(NAD^+-independent)

FDH - Formate dehydrogenase

(table 5.1) changed little with respect to the addition of formate, indicating that these enzymes were neither induced nor repressed in response to the metabolism of the additional formate. The relative levels of activity were very similar to those obtained in the previous chapter.

In addition to the C_1 oxidation pathway enzymes, the activity levels of several other enzymes were monitored. Included in these were the hexulose phosphate synthase and the hydroxypyruvate reductase which have been previously used as indicators for the operation of the RuMP and serine pathways respectively (Lawrence and Quayle, 1970). The level of NADH oxidase activity within the culture was also measured since this enzyme has the potential to utilize additional NADH generated as a result of increased formate metabolism. The activities of the two enzymes involved in the methenyl - THF pathway were also examined since the operation of this cycle has been implicated in the assimilation of formate (Attwood and Harder, 1978). The result of these assays are shown in table 5.2.

The levels of hydroxypyruvate reductase, hexulose phosphate synthase and NADH oxidase activity changed little in response to the metabolism of additional formate. Anthony (1982) previously reported that NADH oxidase levels in methylotrophs were normally low. The results in this section implied that low levels of the latter enzyme were produced by the cells, even in the presence of additional NADH generation. The levels of methylene THF dehydrogenase and the formyl THF synthase measured in culture samples were also consistently low. Similar levels of activity were detected in formate-free continuous cultures and in cultures that appeared to be actively assimilating formate carbon. Low levels of methenyl THF pathway enzymes might have been expected in the absence of additional formate metabolism, since this pathway has been implicated in the maintenance of the cells C_1 pool for amino acid and purine biosynthesis. The individual reactions of this cycle are all reversible, with the exception of the non-enzymic synthesis of 5,10 methylene THF (fig 5.9); therefore it is feasible that carbon at the oxidation level of formate may be linked to assimilation by the serine pathway, as suggested by Large et al. (1961). The low level of methylene THF dehydrogenase measured under conditions of formate

Table 5.2 Activity levels of non-C_1 oxidising enzymes measured during formate-fed, oxygen-limited continuous culture.

Specific Activity Levels (Units mg^{-1})

1) High copper conditions ($1.2mgl^{-1}$ $CuSO_4.5H_2O$)

mM Formate in medium	HPS	HPR	NADH oxidase	Formyl-THF synthase	Methylene THF dehydrogenase
0	0.171	0.042	0.023	N.D	N.D
20	0.135	0.057	0.012	0.035	0
50	0.152	0.062	0.017	N.D	N.D

2) Low copper conditions ($0.2mgl^{-1}$ $CuSO_4.5H_2O$)

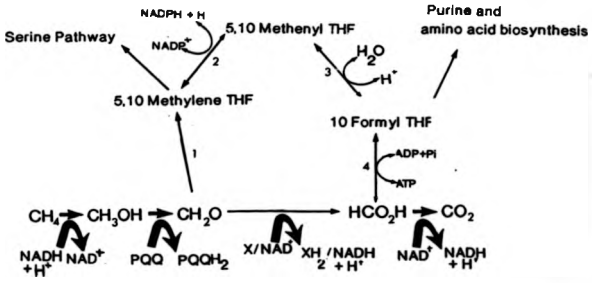
mM Formate in medium	HPS	HPR	NADH oxidase	Formyl-THF synthase	Methylene THF dehydrogenase
0	0.13	0.08	0.01	0.01	N.D.
20	0.143	0.095	0.015	0.045	0.005
30	0.140	0.13	0.016	0.049	0.002
40	0.162	0.08	0.08	0.02	0.002

Key

HPS - Hexulosephosphate synthase

HPR - Hydroxypyruvate reductase

N.D - Not Done



1) Non enzymic

2) Methylene THF dehydrogenase

3) Methenyl THF cyclohydrolase

4) Formyl THF synthase

Fig 5.9 Outline of Methenyl THF pathway

assimilation may reflect an unfavourable equilibrium of the enzyme for its operation in the direction measured. Under conditions of formate carbon fixation, the cell would be required to convert methenyl THF to methylene THF. However, the reverse activity was measured and under the above conditions of growth, may not reflect the cells true capacity to fix carbon via the methenyl THF pathway.

In theory carbon assimilation via the methenyl THF pathway would be energetically expensive, requiring one mole of ATP and NADPH per mole of carbon fixed, plus the energy demand of the serine pathway. In addition, carbon fixation via this route would also represent a loss of formate available for the NAD^+-linked oxidation of the metabolite by the formate dehydrogenase. If formate accumulation occurred via the above biochemical route, then its theoretically high energy expenditure might explain why only a maximum of 11% of the total carbon assimilated, originated from added formate.

5.4 Summary

According to the results generated in this chapter, the fate of formate added to methane-grown cultures of M.capsulatus (Bath) is dependent upon the conditions of growth. Under methane-limited conditions additional formate was rapidly oxidised to CO_2, presumably generating increased levels of NADH. Under oxygen-limited conditions the cells appeared capable of assimilating carbon in the added formate. The exact method by which the formate carbon enters the biosynthetic pathways has yet to be confirmed, although it was proposed that one possible mechanism may involve the coupling of the methenyl THF pathway with the serine pathway. During oxygen-limited growth the cells were also very susceptible to the toxic effects of formate accumulation and consequently the uncoupling of oxidative phosphorylation. Therefore under such conditions it was impossible to achieve high levels of formate assimilation without causing cell death.

CHAPTER 6

Regulation of Formaldehyde Metabolism

Chapter 6

Regulation of Formaldehyde Metabolism

6.1 Introduction

Formaldehyde is a key intermediary metabolite in methylotrophic growth since it occupies the metabolic branch-point between carbon assimilation and further oxidation to CO_2. Since the latter sequence of reactions are required to provide reductant and energy for the necessary assimilation reactions, the cells must effectively apportion the flux of carbon between these two reaction sequences. In M.capsulatus (Bath), this dilemma is further complicated by the fact that the organism possesses the enzymic capacity for carbon assimilation via either the RuMP pathway or the serine pathway. Likewise, the dissimilation of carbon may not be limited to the linear oxidation pathway, since the enzymes associated with the cyclic dissmilation of formaldehyde have also been identified in the above organisms (Stirling, 1978).

Attwood and Quayle (1978) pointed out that under conditions of methylotrophic growth, the relative flux between assimilation and dissimilation would be expected to vary between wide limits to accommodate changes in the cell's environment. Therefore one might expect methylotrophs to possess a means of regulating the fate of formaldehyde in response to changes in growth conditions. The need for efficient formaldehyde metabolism is made more important by the fact that the metabolite is a very toxic compound. Consequently any interruption in the intracellular disposal of formaldehyde may cause cell damage or even death as a result of its accumulation.

The toxic nature of formaldehyde also presents problems in the study of its metabolism by methylotrophic bacteria. Walker (1964) estimated that a formaldehyde solution containing 0.005% $^{w}/_{v}$ was sufficient to act as an antiseptic preventing bacterial growth. Therefore the study of formaldehyde metabolism by cells requires the use of continuous culture with formaldehyde-limited

conditions, where the concentration of free formaldehyde in the culture is effectively zero. Using this approach Goldberg et al (1976) showed that several strains of methylotrophic bacteria were capable of utilizing formaldehyde as their sole carbon and energy source. The ability of M.capsulatus (Bath) to grow under a similar set of conditions was reported by Whittenbury et al (1976) and Reed (1976).

More recently, Dijkhuizen and Levering (1987) reported the maintenance of steady-state cultures of the facultative methyltroph Arthrobacter P_1 on formaldehyde alone, at dilution rates as high as $0.2h^{-1}$. Such cultures were however only obtained after the smooth transition from growth under choline-limited conditions, via choline plus formaldehyde, to growth on formaldehyde alone. The subsequent analysis of enzyme activities within the culture during this period of transition showed the apparent induction of the RuMP cycle enzyme, hexulose phosphate synthase. Bussineau and Papoutsakis (1986) reported the growth of "formaldehyde-tolerant" cultures of Methylomonas L.3; these being obtained only after a prolonged exposure of the cells to a methanol/formaldehyde mixed substrate. The authors concluded that such formaldehyde-tolerant cells possessed an altered physiology to the same organisms grown on methanol alone, the physiological difference manifesting itself in the form of subtle differences in the activities of the dissimilation pathway enzymes.

One of the experimental objectives of this section was to examine whether or not it was possible to induce changes in the in vitro activity of formaldehyde-metabolizing enzymes in M.capsulatus (Bath), by exposing the cells to increasing levels of formaldehyde in the medium. According to the results generated by Reed (1976), is should be possible to adapt M.capsulatus (Bath) to growth on formaldehyde as its sole source of carbon. Further to Reed's work, there has been a considerable increase in our understanding of C_1 metabolism in this organism particularly with respect to the identification of enzymes capable of utilizing formaldehyde as a substrate. The results in chapter 4 did not offer any clear indication as to which enzyme fulfils the major role in formaldehyde dissimilation during growth on methane. Examination of the cellular response to metabolism of the

exogenously supplied formaldehyde, with or without methane may help resolve this dilemma.

Reed (1976), also reported that when a culture of M.capsulatus (Bath) was switched from growth on methane to growth on formaldehyde, there was a loss of specific protein bands when soluble cell extracts of the respective cultures were run on an SDS polyacrylamide gel. The bands that disappeared can now be ascribed as being those associated with the α, β and γ subunits of the protein A component of the soluble MMO. Therefore under conditions of growth on formaldehyde, the cells appeared to lose their capacity to synthesise soluble MMO. This led Dalton et al (1984) to conclude that the expression of this enzyme was probably induced by methane or methanol, both substrates of the MMO. A second objective of this study was to verify the observations made by Reed concerning the apparent repression of the soluble MMO during growth on formaldehyde.

6.2 The Effect of the Pulse Addition of Formaldehyde to Methane-Limited Cultures of M.capsulatus (Bath)

6.2.1 Experimental

The approach used to study the response of M.capsulatus (Bath) to exogenously supplied formaldehyde was similar to that used in study of formate regulation. An initial series of experiments involving the pulse addition of formaldehyde to a methane-limited continuous culture were performed to determine the existing capacity of the cells to metabolize additional formaldehyde. The toxic nature of this metabolite is such that any prolonged exposure of the culture to extracellular formaldehyde would be detrimental to the cells. Consequently methane was the chosen nutrient limitation as it was assumed that under conditions of carbon-stressed growth, formaldehyde uptake would be greatest.

Stock solutions of formaldehyde were prepared from 10% ($^{w}/_{v}$) suspensions of paraformaldehyde. Once methane-limited, steady-state conditions of growth had been achieved, a measured amount of the formaldehyde solution was added to the culture. The effect of the direct addition of formaldehyde was examined with respect to induced changes in the pattern of gas metabolism and the

possible accumulation of extracellular metabolites. As with the previous study the effect of the pulse addition of formaldehyde was examined under both high and low copper conditions, this being to determine whether or not the type of MMO expressed influenced the response of the cells to the metabolite addition.

6.2.2.. Results

6.2.2.1. High Copper Conditions

According to the results in fig 6.1 methane-limited cultures of M.capsulatus (Bath) grown under particulate MMO expressing conditions ($1.2mgl^{-1}$ copper sulphate) appeared efficient at metabolizing added formaldehyde. When exposed to extracellular formaldehyde levels of up to 2mM, the metabolite was rapidly removed from the culture by the cells. The disappearance of formaldehyde corresponded to an increase in the extracellular level of formate which itself was eventually metabolised, abeit at a lower rate than the formaldehyde. The amount of formate that accumulated was directly proportional to the level of formaldehyde added. The maximum level of formate detected in the culture was approximately 75% the molar equivalent of the formaldehyde added. The observed pattern of formaldehyde consumption and formate accumulation suggested that the increase in the latter occurred as a direct result of the metabolism of the additional formaldehyde. The disappearance of formate in the culture was also coupled to increased rates of CO_2 production and O_2 consumption, which would be predicted if the extra formate produced was subsequently oxidised to CO_2.

The exposure of M.capsulatus (Bath) to these conditions did not appear to have any long term, deleterious effect on the cells, since after the final addition of formaldehyde the culture regained its original steady-state. This assumption was based on the observation that cell yield and gas exchange rates, measured 24 hours after the final addition of formaldehyde were identical to the equivilent values obtained prior to the addition of formaldehyde.

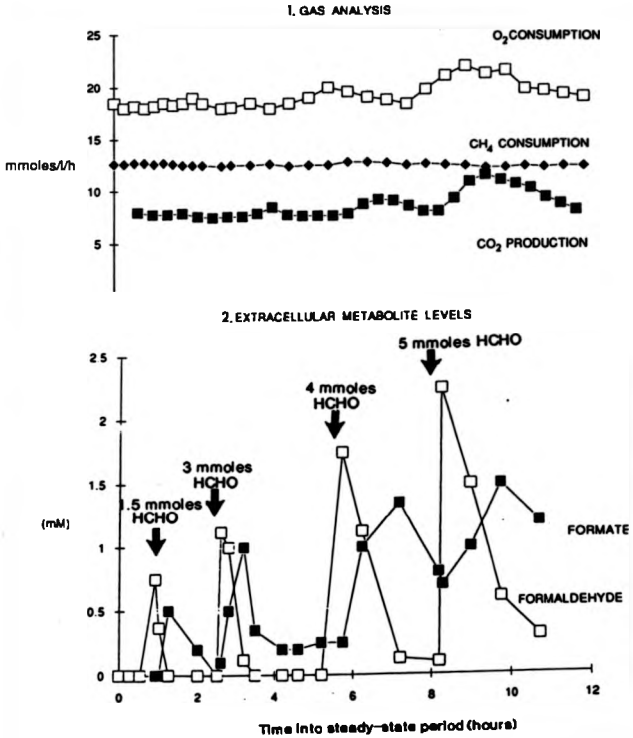


Fig 6.1 The effect of the pulse addition of formaldehyde: high copper conditions

6.2.2.2. Low Copper Conditions

When the previous study was repeated using NMS medium containing $0.2mgl^{-1}$ copper sulphate, a similar pattern of formaldehyde consumption and formate accumulation was obtained (fig 6.2). Further examination of the levels of formate produced indicated that the extracellular concentration of the latter was higher than the molar equivilent of formaldehyde added (table 6.1). Since the only other source of carbon fed into the culture during this period was methane, the pattern of metabolite consumption and production suggested that under low copper conditions, the metabolism of excess formaldehyde caused a restriction in the flux of carbon from methane to CO_2. The high levels of formate produced, indicated that this restriction occurred at the level of the formate dehydrogenase. This was especially evident after the addition of 5 mmoles of formaldehyde since several minutes after the addition, the culture required the addition of alkali to maintain its pH value of 7. The requirement of the culture for alkali addition coincided with formate accumulation; consequently it appeared that the observed pH reduction was a direct result of formate production by the cell.

The effect of additional formaldehyde metabolism on the pattern of gas exchange rates was similar to that observed in the previous section. Increased levels of formaldehyde addition corresponded to increased levels of CO_2 production and O_2 consumption, whilst methane consumption remained constant. Approximately 24 hours after the addition of 5mmoles of formaldehyde, the rates of O_2 and methane consumption, and CO_2 production all began to drop and the culture eventually washed-out. This observed reduction in the gas exchange rates was first noted several hours after both the extracellular formate and formaldehyde had been consumed, implying that the uptake and/or metabolism of these intermediary compounds had an irreversible, deliterious effect on the culture.

The results from the previous chapter concerning the direct addition of formate to steady-state cultures of M.capsulatus (Bath) indicated that although similar levels of extracellular formate could be periodically observed in the culture, no toxic effect was recorded. Therefore the accumulation of formate recorded in response to the

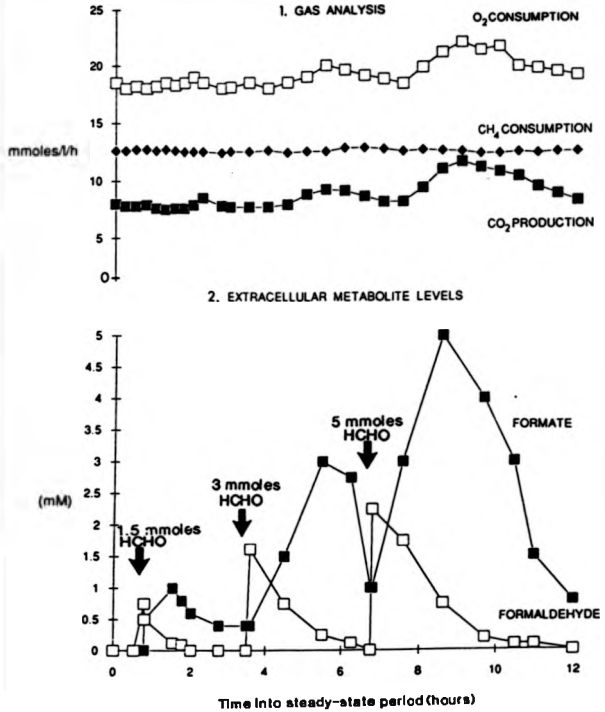


Fig 6.2 The effect of the pulse addition of formaldehyde: low copper conditions

m moles of HCHO added to 1.5 litres of culture	Estimated concentration of HCHO (mM), immediately after metabolite addition	Measured concentration of HCHO (mM), immediately after metabolite addition	Measured concentration of $HCOO^-$, maximum value
1.5	1.0	0.75	1.2
3	2.0	1.6	3
5	3.33	2.3	6

Table 6.1 Maximum concentrations of HCHO and $HCOO^-$ measured in the culture after the addition of HCHO, alongside the estimated theoretical maximum concentration of HCHO.

addition of formaldehyde was not the sole reason for washout, but that it was connected to the uptake and metabolism of the additional formaldehyde. It is feasible to suggest that the extracellular levels of metabolites measured during the course of these studies may not be representative of their respective intracellular concentrations. It is possible that highly localized concentrations of such metabolites are generated within the cells under these conditions, even when the extracellular concentration appear negligible. Consequently it is these that cause the observed toxic effect.

Overall, cells grown under soluble MMO-expressing conditions (low copper) were considerably more susceptible to formate accumulation after the addition of formaldehyde, than particulate MMO-expressing cells. Confirmation of any possible role that the MMO may play in determining the response of cells to increased formaldehyde metabolism will require a more detailed examination.

6.3 The Effect of Continuous Formaldehyde Addition to Methane-Limited Cultures of M.capsulatus (Bath)

6.3.1. Experimental

To examine in more detail the effect of increased formaldehyde metabolism on the physiological state of M.capsulatus (Bath), it was necessary to expose cells to additional formaldehyde over a longer period of time. Although the results obtained in the previous section implied that the cells were efficient at metabolizing excess formaldehyde, the continuous exposure of cells to extracellular levels of the metabolite would probably be detrimental to the culture. A previous study by Hirt et al (1978) reported that while methanol-grown continuous cultures of the methylo troph L3 were capable of oxidising excess formaldehyde when added directly to the culture, irreversible culture washout occurred when the extracellular concentration of the metabolite was allowed to accumulate. Therefore, to achieve the growth of M.capsulatus (Bath) on formaldehyde containing medium, it was essential that the available concentration of the metabolite in the culture remained zero.

Once achieved, steady-state growth on formaldehyde - containing medium would permit a more detailed examination of the role of the MMO in determining the cell's response to increased formaldehyde metabolism. In addition, this approach would also permit the examination of the activity levels of the methane oxidation pathway enzymes and these could be compared with the equivalent activity levels produced in the absence of added formaldehyde (Chapter 4). It was also hoped that at some stage it would be possible to maintain the cells on formaldehyde as their sole carbon source, as was reported by Reed (1976).

Prior to the addition of formaldehyde, cultures of M.capsulatus (Bath) were grown under methane-limited conditions and allowed to achieve steady-state growth. Once a 100% carbon recovery had been achieved, as determined by gas analysis and total carbon analysis data, a specified amount of formaldehyde solution was added to the medium reservoir. The final concentration of formaldehyde in the medium was estimated by Nash reagent, these assays being performed routinely throughout the experiment to ensure the level of metabolite in the medium remained constant. As with the formaldehyde pulse experiments, the effect of the continuous addition of formaldehyde was studied in both particulate MMO and soluble MMO - containing cells.

6.3.2. Results of Continuous Formaldehyde Addition to Particulate MMO - Containing Cells

6.3.2.1 Effect of Formaldehyde Addition on Carbon Distribution Within The System

In the presence of medium containing $1.2mgl^{-1}$ copper sulphate; methane-limited, steady-state growth was achieved with medium containing zero, 25,50, and 120 mM formaldehyde. In addition, it was also possible to remove the methane source altogether and maintain the culture on 120mM formaldehyde alone.

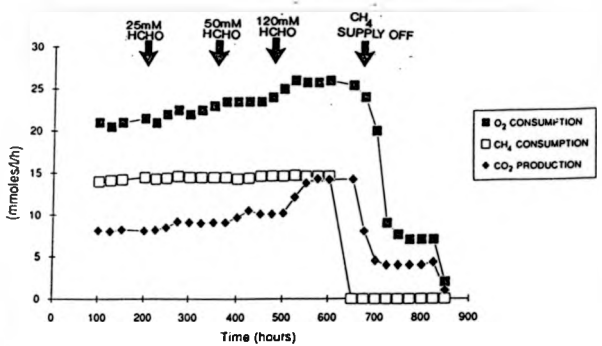
Fig 6.3a) shows the profile of the effect of the addition of formaldehyde-supplemented medium on cell density and extracellular metabolite levels. The dry weight measurements made during the experiment showed an increase in the cell density of the culture in response to the addition of increased levels of formaldehyde. In

theory the cells were growing under methane-limited conditions and therefore any observed increase in cell density was presumably due to either an increased efficiency of methane metabolism (excess formaldehyde oxidation providing additional NADH) or the cells were actively fixing carbon from the added formaldehyde. The latter assumption had to be true during the final stages of the experiment since the removal of the methane supply did not result in an immediate wash-out of the culture. The removal of the methane supply did result in an initial drop in the biomass level of the culture, however this reduction was not continuous and the cells appeared to attain a new steady-state, growing on formaldehyde in the absence of methane. A closer examination of extracellular metabolite levels during this period showed that in the absence of methane, formaldehyde began to accumulate in the culture. As the rate of formaldehyde accumulation increased, formate also began to accumulate. Therefore during the period of growth on formaldehyde alone, the culture did not attain a true steady-state since the cells' environment was continuously changing.

The measured changes in gas exchange rates (fig 6.3.b) were similar to those produced during the pulsed addition of formaldehyde to the culture. When the concentration of formaldehyde in the medium was raised, there was a corresponding increase in the rates of both CO_2 production and O_2 consumption, indicative of an increased proportion of carbon being oxidised to CO_2. The rate of methane consumption in the culture remained constant up until the methane supply was removed. Removal of the methane supply corresponded to a reduction in the rate of CO_2 production, this being a consequence of reduction in the total carbon level fed into the culture. The rate of oxygen consumption measured during this period was also considerably lower than that obtained in the presence of methane metabolism. The difference between the initial rate of oxygen consumption, prior to the addition of formaldehyde and that measured in the absence of methane reflected the oxygen requirement of the active MMO.

The effect of formaldehyde addition on the steady-state distribution of carbon in the culture is shown in fig 6.4. When the medium contained 25mM and 50mM formaldehyde, 80% of the additional carbon fed into the

b) gas exchange rates



a) cell density and extracellular metabolitc levels

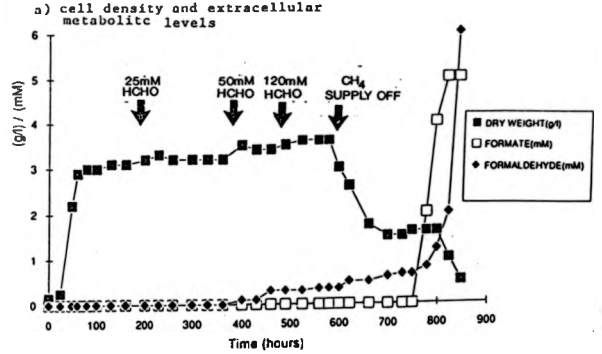


Fig 6.3 The effect of continuous formaldehyde addition (high copper conditions) on a) cell density and extracellular metabolite levels and b) gas exchange rates

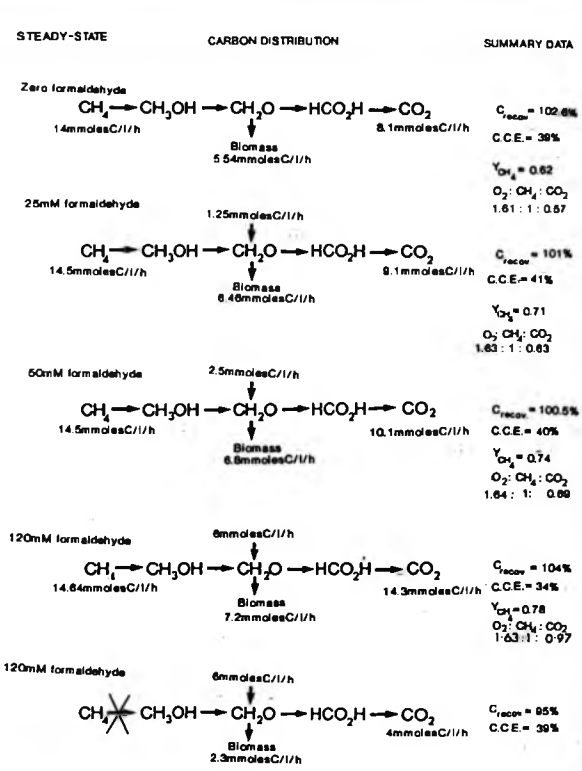


Fig 6.4 The distribution of carbon recorded during formaldehyde-fed, high copper continuous culture

system was oxidised to CO_2. Although an increase in the rate of methane consumption was recorded in response to the addition of 25mM formaldehyde, it was not sufficient to account for the observed increase in the steady-state biomass concentration. Consequently, it would appear that under these conditions not all the additional formaldehyde carbon was oxidised to CO_2, but rather a proportion was assimilated into biomass. Similarly when the formaldehyde concentration was raised to 50mM, there was an increase in both the rate of CO_2 production and biomass accumulation. This meant that the C.C.E. values calculated during these periods did not change significantly, since although the amount of carbon being fed into the culture had increased, the relative proportions of carbon being assimilated and dissimilated remained similar. When the formaldehyde concentration in the medium was raised to 120mM, the C.C.E. dropped from 40% to 34% as the majority of additional carbon added to the culture was oxidised to CO_2.

Although formaldehyde accumulation in the culture was recorded when the methane supply was removed, the rate at which it accumulated was relatively low (approximately 0.04mmoles C $l^{-1}h^{-1}$) compared with the rate of biomass accumulation (2.35 mmoles C $l^{-1}h^{-1}$) measured during the same period. Since this rate of carbon assimilation was maintained for a period of 150 hours, it was concluded that under these conditions M.capsulatus (Bath) was capable of utilizing formaldehyde as a sole carbon and energy source.

Tonge et al (1975) offered preliminary evidence to suggest that the particulate MMO in Methylosinus trichosporium was capable of obtaining its required reducing power from the direct recycling of electrons from the methanol dehydrogenase, during growth. Based on this theory and under similar conditions of particulate MMO expression, Leak et al (1985) estimated that such a process of electron recycling would make methane energetically equivalent to formaldehyde. The levels of copper sulphate used in the medium in this study should permit the expression of particulate MMO within the cells. Therefore the observation that the C.C.E. value obtained during growth on methane, prior to the addition of formaldehyde was similar to that obtained during growth on formaldehyde alone would add weight to the above theory.

6.3.2.2. Analysis of Enzyme Activity Levels in the Culture

Cell extracts were prepared from samples of culture removed throughout the course of the continuous culture. These extracts were subsequently assayed for the various methane oxidation pathway enzymes to identify any obvious changes in the enzymic capacity of the cells in response to the addition of formaldehyde. The results of these assays are shown in fig 6.5, 6.6 and 6.7.

a) Methane Oxidation Pathway Enzymes

The high copper conditions used in this experiment meant that the cells expressed 100% particulate MMO. When the medium was supplemented with 25mM formaldehyde, there was an initial reduction in the level of membrane-bound MMO activity (fig 6.5). However on continued exposure to the added formaldehyde, the level of MMO activity appeared to level off. A dramatic reduction in activity was recorded when the formaldehyde concentration in the medium was raised to 120mM and this pattern of activity continued when the methane supply was removed. Cell samples removed approximately 24 hours before the observed washout of the culture showed no MMO activity. The complete loss of MMO activity was probably a consequence of the removal of the methane supply since MMO expression may require the presence of an oxidisable substrate, such as methane. MMO inactivation may also have been a consequence of the accumulation of extracellular formaldehyde and this will be examined later in this chapter.

The measured levels of methanol dehydrogenase activity in the culture were high throughout the experiment, irrespective of whether the activity was measured using formaldehyde or methanol as substrates (fig 6.6). The activity levels of this enzyme showed an increase in response to the removal of the methane supply. Such an increase may reflect a direct response of the cells to increasing levels of formaldehyde in the culture, since this is a potential substrate for the enzyme. However under such conditions of formaldehyde accumulation, the observed increase in activity was not as fast as one might expect given the extreme toxic nature of the metabolite. Indeed, the raised levels of methanol

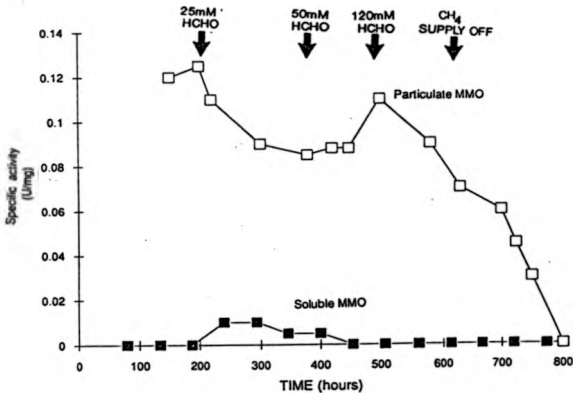


Fig 6.5 The levels of MMO activity measured during formaldehyde-fed, high copper continuous culture

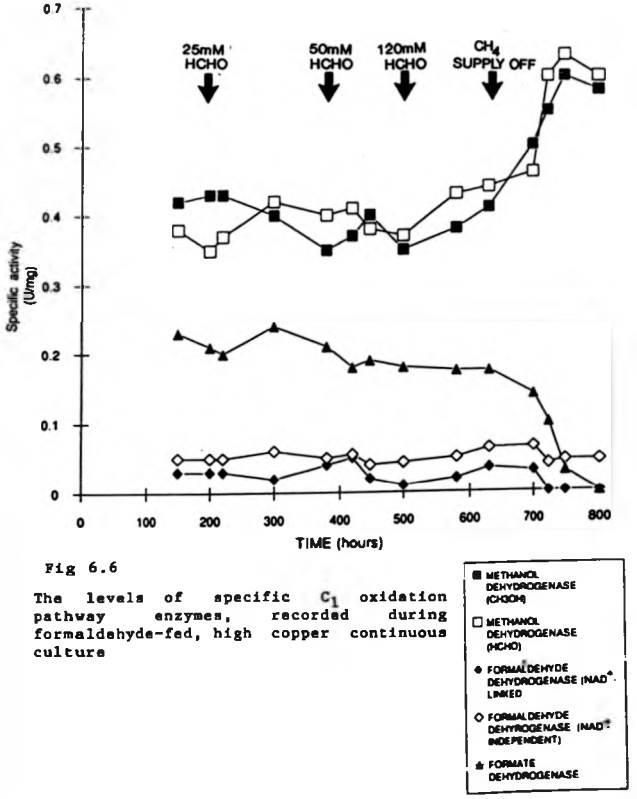


Fig 6.6

The levels of specific C_1 oxidation pathway enzymes, recorded during formaldehyde-fed, high copper continuous culture

dehydrogenase activity in the culture did not effectively reduce the rate at which formaldehyde accumulated.

The alternative formaldehyde dissimilating enzymes, the NAD^+-dependent and NAD^+-independent formaldehyde dehydrogenase did not show any apparent response to the addition of formaldehyde to the medium. In all the cell extracts prepared, the activity levels of these enzymes were low. When the NAD^+-linked formaldehyde dehydrogenase assays were performed in the presence of excess HTSE (as described in chapter 4), no great increase in activity was observed, indicating that the apparent lack of activity was not the consequence of a lack of factor F, but was due to a total lack of formaldehyde dehydrogenase activity.

The levels of formate dehydrogenase activity were consistently high throughout most of the continuous cultivation experiment, although a dramatic reduction in activity was recorded towards the end. The loss of formate dehydrogenase activity corresponded with the accumulation of extracellular formate within the culture. Therefore it would appear that during the latter stages of the culture, either the accumulation of formaldehyde or increased formaldehyde metabolism had a detrimental effect on the activity levels of the formate dehydrogenase.

b) Non-Methane Oxidation Pathway Enzymes

In addition to the levels of enzyme activity associated with the methane oxidation pathway, the activity levels of several other enzymes were measured during the experiment. The activity levels of the hexulose phosphate synthase (HPS) and the hydroxypyruvate reductase (HPR) were monitored as indicators for the RuMP and serine pathways of carbon assimilation. To determine whether the cyclic pathway of formaldehyde dissimilation functioned under conditions of increased formaldehyde availability the activity levels of the 6-phosphogluconate dehydrogenase (6-PGD) and the glucose-6-phoshate dehydrogenase (G-6-PD) were also measured. The effect of additional formaldehyde metabolism on these enzyme activity levels is shown in fig 6.7.

High levels of hexulose phosphate synthase activity were maintained throughout the experiment, irrespective of the carbon source used. In contrast, the hydroxypyruvate reductase activity level was reduced in response to the

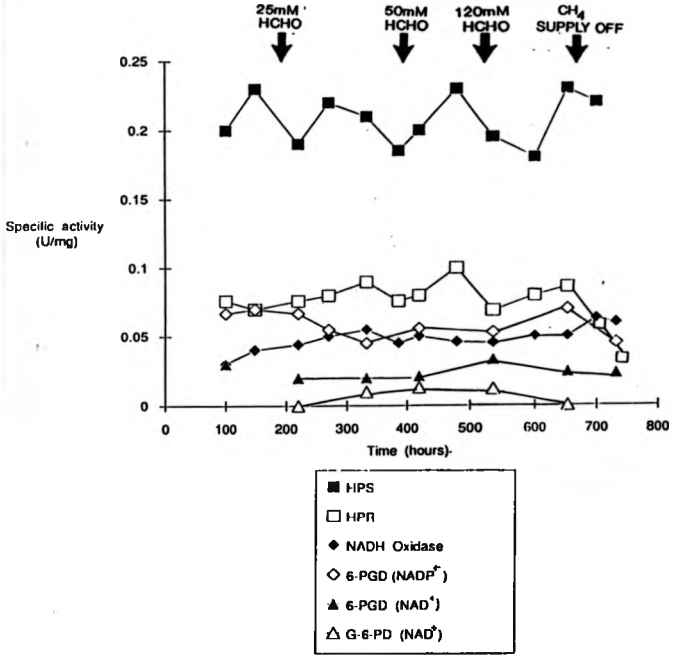


Fig 6.7 The effect of additional formaldehyde metabolism on specific non-C_1 oxidising enzymes

removal of the methane supply, implying that the assimilation of exogenously supplied formaldehyde occurred via the RuMP pathway.

Work by Stirling (1988) and Davey et al (1972) showed that M.capsulatus (Bath) possessed the necessary enzymes required to dissimilate formaldehyde via the cyclic route, outlined in section 1.3.3.3d. One would expect the effective operation of such a cycle, under conditions formaldehyde accumulation in the culture, to be advantageous to the cells, since it would not only reduce the levels of the toxic metabolite, but would also lead to the generation of additional $NAD(P)^+$. Unfortunately measurement of linked 6-PGD and G-6-PD activity levels indicated that the levels of the above enzymes were low throughout the experiment. Consequently the operation of the formaldehyde dissimilation cycle within M.capsulatus (Bath) did not appear to be regulated by the availability of formaldehyde.

NADH oxidase assays performed during the experiment showed that the activity levels of this enzyme were also low under the conditions of growth used (fig6.8). Anthony (1982) previously stated that the levels of this enzyme were normally low in methane-grown organisms. The results obtained in this study showed this to be true, even under conditions of growth that would presumably generate raised NADH levels: the oxidation of formaldehyde to CO_2.

6.3.3. Results of Continuous Formaldehyde Addition to Soluble MMO-Containing Cells

The results observed during the pulse addition of formaldehyde implied that cells containing particulate MMO responded differently compared with those containing the soluble form of the enzyme, the latter showing an increased sensitivity to formaldehyde accumulation. To examine in more detail the response of soluble MMO containing cells to formaldehyde addition, the formaldehyde-fed continuous cultivation experiment was repeated using medium containing $0.2mgl^{-1}$ copper sulphate.

Under conditions of low copper, periods of steady-state growth were achieved with medium supplemented with 25,50 and 100mM formaldehyde. Unfortunately, unlike the previous formaldehyde-fed continuous culture, growth was not achieved on formaldehyde alone. Instead rapid

formaldehyde accumulation in the culture was detected approximately 120 hours after the concentration of formaldehyde in the medium was raised to 100mM (fig6.8a) . The accumulation of formaldehyde was immediately followed by the washout of the culture.

6.3.3.1. Effect of Formaldehyde Addition on Carbon Distribution within the System

Unlike the high copper, formaldehyde-fed continuous culture, no increase in cell density was recorded in response to the addition of formaldehyde (fig6.8a). Gas analysis results (fig6.8b) showed that in response to the addition of formaldehyde, increased rates of CO_2 production were detected, along with an increase in the rate of oxygen consumption. Therefore it would appear that all the additional formaldehyde was immediately oxidised to CO_2.

The overall pattern of carbon distribution measured during the continuous addition of formaldehyde is shown in fig 6.9. When formate was fed into a culture growing under low copper conditions, an increase in cell yield on methane was recorded (chapter 5). It was assumed that such an increase was the direct result of extra NAD(P)H being generated via the oxidation of the formate. The pattern of gas analysis produced in response to formaldehyde addition indicated that this added metabolite was also oxidised completely to CO_2. Therefore one might have expected a similar increase in the cell yield on methane. However under the latter circumstances no increase in cell yield on methane was observed. One possible explanation for this may be linked to the means by which the cells consumed the extracellular formaldehyde. Active transport mechanisms have been implicated in formaldehyde uptake in Methylomonas L.3. (Diwan et al, 1983) and more recently in the methylotroph T15 (Bussineau and Papoutsakis, 1988). The operation of a similar system in M.capsulatus (Bath) would result in an effective drain on the cells available energy and consequently a reduction in cell yield.

6.3.3.2. Effect of Formaldehyde Addition on Extracellular Metabolite Levels

In common with previous attempts at introducing

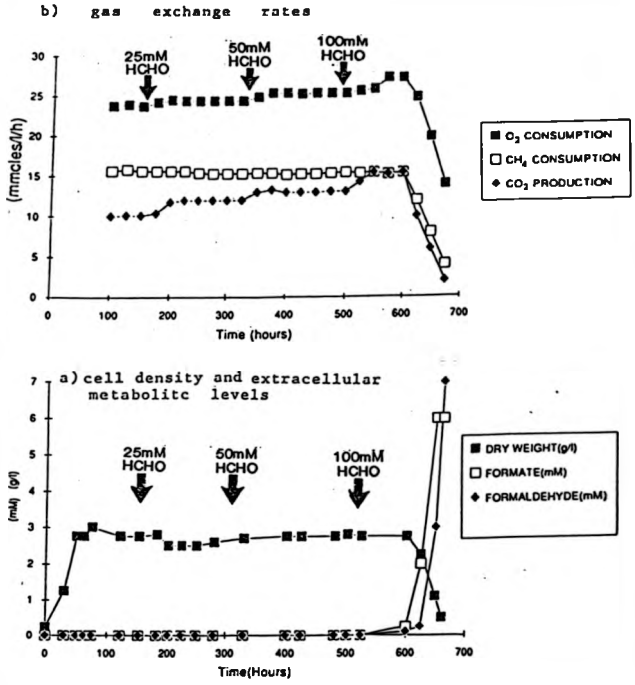


Fig 6.8 The effect of continuous formaldehyde addition (low copper conditions) on a) cell density and extracellular metabolitc levels and b) gas exchange rates

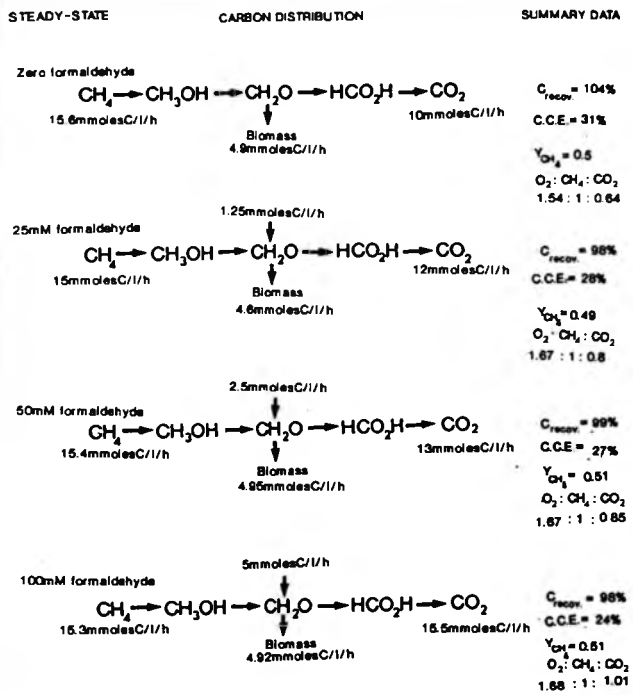


Fig 6.9 The distribution of carbon recorded during formaldehyde-fed, low copper continuous culture

formaldehyde into continuous cultures of M.capsulatus (Bath), culture washout was again accompanied by the accumulation of extracellular formate and later formaldehyde (fig6.8a). This observed pattern of metabolite accumulation might imply that the initial accumulation of formate effectively reduced the cell's capacity to metabolize the added formaldehyde and ultimately resulted in the death of the cells. As was concluded in the pulse addition of formaldehyde to soluble MMO-containing cells, the accumulation of formate suggests that there was a restriction in the carbon flux at the level of the formate dehydrogenase. Therefore the next step was to examine the in vitro activities of the C_1-oxidising enzymes in the culture, and in particularly that of the formate dehydrogenase.

6.3.3.3. Effect of formaldehyde Addition on MMO activity within the Culture

The activities levels of various enzymes were examined during the low copper, formaldehyde-fed continuous culture. Under such conditions the cells expressed predominately soluble MMO (fig6.10). The addition of 25mM formaldehyde resulted in an immediate reduction in the level of soluble MMO activity and the appearance of low levels of membrane-bound MMO activity. This level of particulate MMO activity was probably limited by the availability of copper. The level of soluble MMO activity continued to drop on the addition of formaldehyde and eventually reached zero. The loss of MMO activity corresponded to the washout of the culture.

During the initial stages of formaldehyde addition to the culture, the original soluble MMO activity level could be maintained by supplementing the assay mixture with purified protein B . Since this protein is one of the 3 sub-units of the soluble MMO essential for methane oxidation, it would appear that the level of protein B in cell extract was limiting the level of in vitro activity. The addition of equivalent levels of proteins A and C had no such effect indicating that these enzyme sub-units were not limiting. Previous studies by Green and Dalton (1985) showed that the protein B component of the MMO was essential to enable the enzyme to function as an oxygenase. The authors showed that the removal of protein

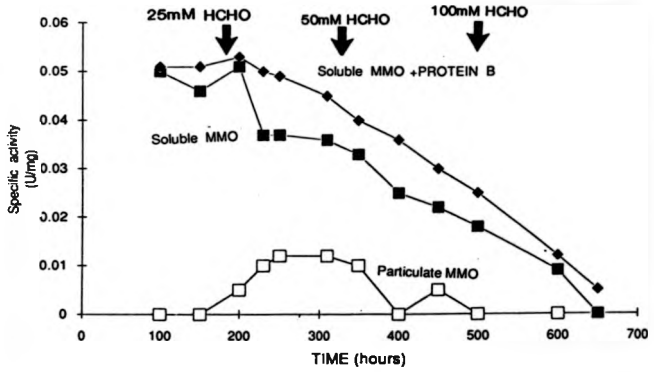


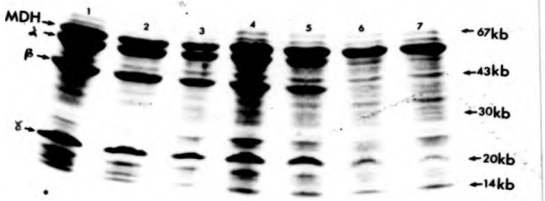
Fig 6.10 The levels of MMO activity measured during formaldehyde-fed, low copper continuous culture

B from the enzyme complex caused the activity of the enzyme to be uncoupled, Proteins A and C retaining an NADH oxidase capacity but losing their oxygenase function. The pattern of MMO activity generated during the initial addition of formaldehyde to the culture implied that although the enzyme's capacity to function as an oxygenase had been reduced, the enzyme still retained its capacity to function as an oxidase. Therefore the effect of formaldehyde addition on MMO activity during this period was one of uncoupling.

When the addition of formaldehyde to the culture was continued, it was accompanied by a continual decline in the culture's methane-oxidising capacity. At this stage, loss of soluble MMO activity in cell extracts was not alleviated by the addition of protein B, as in the initial period of formaldehyde addition. Under these circumstances, not only did the culture possess reduced oxygenase activity but also reduced oxidase activity. This observation contrasted with the effect of formaldehyde addition on particulate MMO-containing cells, the latter maintaining high levels of membrane-bound oxygenase activity in the presence of increased formaldehyde metabolism.

Comparison of the levels of MMO activity measured during the experiment (fig 6.10) with the rates of gas exchange measured during the same period (fig 6.8b) indicated that although a continuous reduction in the level of MMO activity was recorded with the addition of formaldehyde; the rate of methane consumption by the cells remained unaffected. A constant rate of methane consumption was maintained up until the total MMO activity in the culture had been reduced to approximately 20% its original value. Therefore up until the latter stages of the continuous culture, the reduction in MMO activity did not compromise the *in vivo* rate of methane oxidation, reflecting the enzyme's high affinity for methane.

SDS polyacrylamide gel analysis of soluble cell extracts, prepared during the cultivation period showed that the observed reduction in the rate of methane consumption towards the end of the experiment coincided with the loss of several major protein bands (fig 6.11). The bands that disappeared were those corresponding to the α, β and γ polypeptides of the protein A subunit of soluble MMO. Thus the observed loss of MMO



Track	Sample time (hours)	mM HCHO in medium
1.	160	0
2.	250	25
3.	280	25
4.	350	25
5.	500	50
6.	600	100
7.	700	100

Fig 6.11 SDS polyacrylamide gel containing soluble cell extracts prepared during the course of the formaldehyde-fed, low copper continuous culture

activity towards the end of the experiment and the coincidental reduction in the rate of methane consumption was a direct result of the loss of soluble MMO protein.

6.3.3.4 Methane Oxidation Pathway Enzyme Levels in Low Copper, Formaldehyde Fed Continuous Culture

The levels of methanol dehydrogenase, NAD^+-dependent and NAD^+-independent formaldehyde dehydrogenase activity in the culture were similar to those observed in previous studies. Increased formaldehyde metabolism by the cells had neither a positive nor a negative effect on the above enzyme levels (fig6.12). There was a considerable reduction in the level of formate dehydrogenase activity after the formaldehyde concentration in the medium was raised to 100mM. At the end of the experiment, the activity level of this enzyme was effectively zero which corresponded to the accumulation of formate in the culture.

Unfortunately, unlike the previous experiment, it was not possible to attain conditions of growth that permitted the accumulation of formaldehyde without inducing the washout of the culture. Therefore it was not possible to examine the response of soluble MMO - containing cells to the presence of non-toxic levels of extracellular formaldehyde.

6.4 Effect of the Addition of Formaldehyde on in vitro MMO Assays

According to the previous set of results, cells containing soluble MMO, lost their MMO activity on the introduction of formaldehyde to the culture. Loss of soluble MMO activity was observed immediately after formaldehyde was added. This was confirmed towards the end of the experiment by SDS gel profiles. In contrast, cells grown under high copper conditions maintained high levels of membrane bound activity in response to the addition of formaldehyde. A reduction in particulate MMO activity was only recorded once the formaldehyde had begun to accumulate, implying that under such circumstances formaldehyde had a direct, inactivating effect on the MMO. Further testing of the effect of formaldehyde on MMO activity *in vivo* was made difficult by the inability to

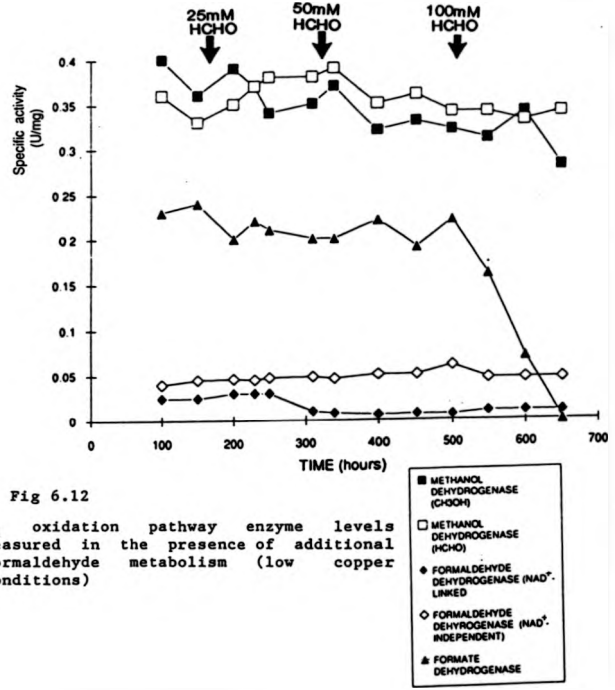


Fig 6.12

C_1 oxidation pathway enzyme levels measured in the presence of additional formaldehyde metabolism (low copper conditions)

maintain cultures of M.capsulatus (Bath) in the presence of non-toxic concentrations of formaldehyde, particularly if the cells contained soluble MMO. Therefore it was decided to examine the effect of formaldehyde on MMO activity in vitro rather than in vivo.

Varying levels of formaldehyde were added to in vitro MMO assays containing :-

1) Crude, soluble extracts, prepared from a methane-grown culture, expressing 100% soluble MMO (Fig 6.13).

2) Purified soluble MMO (proteins A, B and C having been purified individually and reconstituted), (fig 6.14).

3) Crude cell extract (membrane fraction), prepared from a methane-grown culture expressing 100% particulate MMO (fig. 6.15).

The results (fig 6.13, 6.14 and 6.15) showed that a degree of inactivation was recorded, but that the level of formaldehyde required to effect a reduction in activity of greater than 20% was high (>5mM formaldehyde). Such concentrations of formaldehyde would be unlikely to occur in vivo without incurring a general toxic effect. The degree of inactivation was dependent on the concentration of formaldehyde, the length of incubation in the presence of the metabolite and in the case of the soluble MMO, the level of protein B present. The addition of purified protein B (1.4mg) to crude cell extracts containing soluble MMO effectively reduced the level of inactivation by between 3 and 10% (fig 6.13).

6.5 DISCUSSION

The results generated during both the periodic and continuous addition of formaldehyde to cultures of M. capsulatus (Bath) indicated that the cellular response of these organisms to increased formaldehyde metabolism was in part dictated by the type of MMO expressed within the cells. Under conditions of low copper, cells containing the soluble form of the enzyme appeared less tolerant to exogenously supplied formaldehyde than cells possessing the particulate form the enzyme.

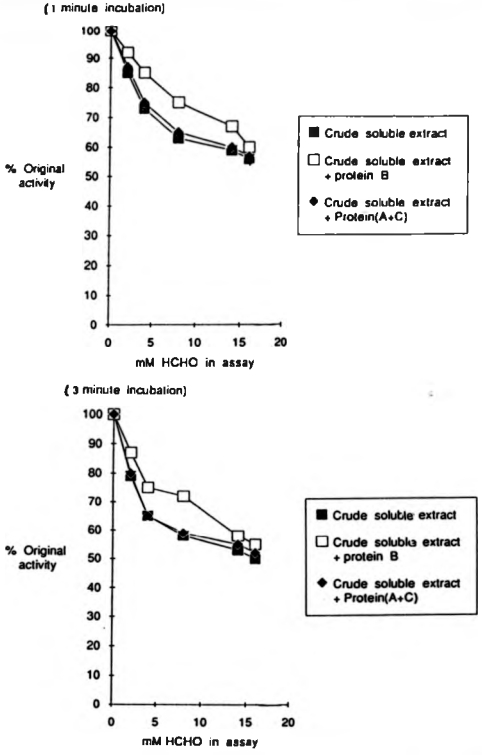


Fig 6.13 The effect of formaldehyde addition on in vitro MMO assays containing crude soluble cell extracts, prepared from a 100% soluble MMO expressing culture

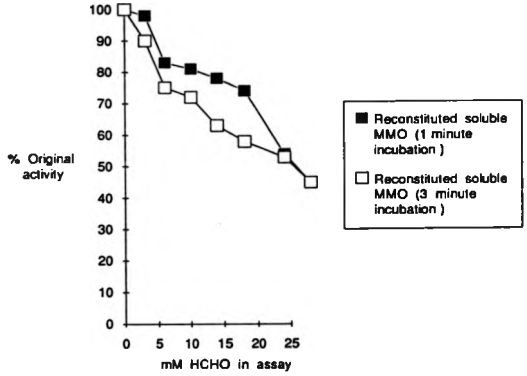


Fig 6.14 The effect of formaldehyde addition on in vitro MMO assays containing purified and reconstituted soluble MMO proteins

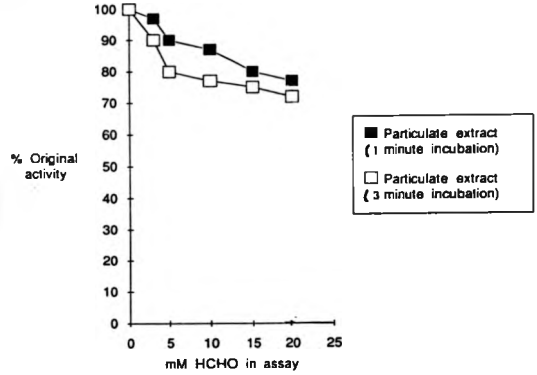


Fig 6.15 The effect of formaldehyde addition on *in vitro* MMO assays containing crude membrane fractions, prepared from cells expressing 100% particulate MMO

One of the most obvious effects of the addition of formaldehyde to low copper continuous cultures was the immediate reduction in the level of soluble MMO activity. The initial effect of formaldehyde addition appeared to be one of effectively uncoupling the enzyme's oxidase function from its oxygenase function, since during this period the total MMO activity could be regained by the addition of excess protein B. Continuous exposure to formaldehyde however resulted in the loss of both oxidase and oxygenase activities.

A possible explanation for the above observation may lie in the relative turnover rates of the individual protein components of the MMO. Green and Dalton (1989) offered some preliminary evidence to suggest that protein B present in crude cell extracts was more sensitive to protease action than either proteins A or C. More recent work by Green (unpublished) observed that once the soluble MMO has been inactivated by acetylene (a known inhibitor or the MMO), the turnover rate of protein A increased by approximately 60%. It is possible that uncoupled MMO proteins show increased sensitivity to protease action than when they exist in an active complex. Therefore while the initial effect of formaldehyde metabolism was one of uncoupling the MMO proteins, the overall loss of MMO activity (oxidase and oxygenase) was the result of an increased rate of turnover of the uncoupled proteins. Unfortunately this would not explain why the addition of formaldehyde to purified, reconstituted MMO caused a degree of inactivition that could not be recovered completely by the presence of additional protein B. Under such circumstances, the formaldehyde did appear to cause an overall loss of MMO activity and not just an uncoupling effect.

Another observed consequence of increased formaldehyde metabolism was the apparent impairment of formate metabolism within the cell. During the examination of the effect of continuous formaldehyde addition, extracellular formate accumulation was observed prior to both formaldehyde accumulation and culture washout. Therefore, under such conditions there appeared to be a restriction in the flux of carbon along the C_1 dissimilation pathway at the formate dehydrogenase catalysed step. Marison (1980), working with Hyphomicrobium X presented evidence to suggest that the

activity of formate dehydrogenase in this organism was regulated by the relative concentrations of $NAD(P)^+$ and NAD(P)H. Marison reported that an NAD^+: NADH ratio of 0.34 caused a 50% inhibition of purified formate dehydrogenase activity. A reduced level of inhibition was also recorded when NADPH was used instead of NADH. Jollie (personal communication) also reported tht formate dehydrogenase, purified from M. trichosporium OB3b was also subject to inhibition by NADH. This inhibition took the form of competitive inhibition even when NAD^+ was present in saturating concentratious, suggesting that the enzyme had a higher affinity for NADH compared with NAD^+.

Gas exchange rates measured during the addition of formaldehyde to methane-grown cultures indicated that a high proportion of the formaldehyde was oxidised to CO_2. The complete oxidation of formaldehyde, presumably via the linear oxidation pathway, would have led to increased levels of NAD(P)H being generated via the action of the formate dehydrogenase and possibly the formaldehyde dehydrogenase. Under normal conditions of growth, the MMO would act as a sink for NAD(P)H produced, the enzyme functioning either as an oxygenase or an oxidase., However towards the end of the formaldehyde-fed experiments, the level of in vitro MMO activity was virtually zero, and in the case of the soluble MMO, SDS polyacrylamide gel analysis indicated that the MMO protein had all but disappeared. Consequently the cell had effectively lost this meansofoxidising excess NAD(P)H; coupled or uncoupled to methane metabolism . Under such circumstances the NADH dehydrogenase enzyme may become the limiting enzyme involved in NADH oxidation (Harrison, 1976). Therefore in the absence of MMO activity, NADH accumulates to a level that inhibits the formate dehydrogenase and ultimately causes cell death. The subsequent estimation of the levels of NAD^+ and NADH, and the possible regulatory role of the MMO will be discussed in the following chapter.

In the introduction to this chapter it was stated that one of the objectives of this study was to determine which of the enzymes capable of oxidising formaldehyde, played the major role in the C_1 dissimilation pathway. It had been hoped that the cell's response to the addition of formaldehyde would have included either the synthesis of increased levels of one particular formaldehyde

metabolizing enzyme, or a change in the affinity of a specific enzyme through the removal or synthesis of a specific modifier protein. Either way the response of the cell would be reflected by a noticeable change in the specific activity of one the formaldehyde metabolizing enzymes.

The only observed change in the spectrum of formaldehyde metabolizing activity was the increase in methanol dehydrogenase activity. This was recorded in response to the accumulation of low, non toxic levels of formaldehyde in the culture. Such an observed change in activity was difficult to repeat since it was very difficult to reproduce the above conditions without causing the washout of the culture.

Under conditions of growth on either methane or methanol, the level of substrate availability for the formaldehyde metabolizing enzymes will be determined by the preceding rate of carbon flux. However, under conditions of exogenously supplied formaldehyde as in the previous set of experiments, the level of intracellular formaldehyde will be partially determined by the rate of formaldehyde uptake. During the continuous addition of formaldehyde to the culture; the accumulation of extracellular levels of the above metabolite might imply that the equivalent intracellular concentration had reached saturation levels. Under such circumstances, the methanol dehydrogenase would be more effective at metabolizing formaldehyde than the NAD^+-linked formaldehyde dehydrogenase, since the former has a higher Vmax for the substrate (table 4.8, chapter 4). Therefore in the event of intracellular formaldehyde accumulation, the methanol dehydrogenase may play as important role in the dissimilation of formaldehyde.

CHAPTER 7

Estimation of NAD^+/NADH Levels in *M.capsulatus* (Bath)

Chapter 7

7. Estimation of NAD^+/NADH Levels in M.capsulatus (Bath)

7.1 Introduction

If the sole function of the MMO is the hydroxylation of methane, then loss of MMO activity when the cells are growing on formaldehyde as their sole carbon source should not prove detrimental to the cells. However, in the previous set of experiments loss of MMO activity preceeded culture washout even when the cells were growing on formaldehyde alone. This might imply that in the absence of methane, the MMO still fulfils a critical metabolic function. Green and Dalton (1986) suggested that such a function might be the regulation of NAD^+/NADH levels within the cell.

Studies concerning aspects of metabolic regulation by $NAD(P)^+$/NAD(P)H in methylotrophic bacteria have been limited to the examination of the *in vitro* effect of these pyridine nucleotides on various pathway enzymes. For example Newaz and Hersh (1975) noted that PEP carboxylase purified from the serine pathway utilizing organism *Pseudomonas* MA showed complex allosteric kinetics, being activated by NADH and inhibited by ADP. Anthony (1982) subsequently pointed out that under conditions of high NADH and low ADP, the cell would possess sufficient energy to assimilate carbon via the serine pathway. Conversely if the NADH concentration were low and the ADP high then PEP carboxylase activity would be limited. Under the latter conditions the carbon flux in the serine pathway would be restricted, hence permitting increased carbon dissimilation and the generation of raised levels of NADH, (and ATP).

A similar form of metabolic control was envisaged by Beardsmore *et al* (1982) in the regulation of carbon flux between biosynthesis and carbon dissimilation in *Methylophilus methylotrophus*. In this case the carbon flow between the RuMP pathway and the cyclic dissimilation pathway could be effectively regulated by the relative ATP : AMP; and $NAD(P)^+$: NAD(P)H ratios. The authors showed that the *in vitro* activities of both the glucose-6-

phosphate dehydrogenase and the 6-phosphogluconate dehydrogenase (the latter enzyme occupying the branch point between the two cycles) were dependent upon the ratios of the coenzymes.

More recently a theory was proposed by Dalton and Higgins (1987) that linked the control of intracellular NAD^+ : NADH ratio with the type of MMO expressed by Methylosinus trichosporium OB3b. The basis of this theory came from the observation by Cornish et al (1984) that formaldehyde accumulation within the cell could only be detected by ^{13}C - NMR when the cells expressed particulate MMO. When the cells possessed soluble MMO no intracellular formaldehyde accumulation was recorded. Dalton and Higgins suggested that the reason for the above observation was that the particulate form of the enzyme was relatively ineffective at using NADH in vivo as a source of electrons. Therefore under particulate MMO generating conditions the NAD^+-linked dissimilation of formaldehyde would be effectively limited by the supply of NAD^+, causing formaldehyde to accumulate. Cells expressing the soluble form of the enzyme would not have the same problem since soluble MMO effectively converts NADH to NAD^+ thereby providing a source of co-factor for the NAD^+-linked dissimilation of formaldehyde. If the above theory is correct, then one might expect to see changes in the NAD^+ : NADH ratio within the culture when the growth of M.capsulatus (Bath) is switched from particulate MMO-expressing conditions to soluble MMO-expressing conditions.

The object of this chapter was to examine the levels of NAD^+/NADH in vivo, in a culture of M.capsulatus (Bath), grown under a series of defined conditions and to determine whether such levels may be altered with respect to the level and the type of MMO activity in the culture.

7.2 Extraction and Assay of Intracellular NAD^+ and NADH Levels

The accurate estimation of intracellular $NAD(P)^+$/NAD(P)H levels is difficult since not only are such metabolites present in the cell at very low levels, but are also subject to a very rapid rate of turnover; the latter being a consequence of the numerous enzymes requiring them as co-factors. Harrison (1976) estimated

that the rate of turnover might be as high as one second. In addition to these problems, the oxidised form of the pyridine nucleotide [NAD(P)$^+$] must be extracted in an acidic environment, whilst the reduced form [NAD(P)H] is most stable in an alkaline environment (Lowry et al, 1961). Therefore it was essential to obtain a suitable system for extraction and measurement of the cofactors. Such a system was required to be both accurate and reproducible.

In this study an LKB-luminometer was used to determine the levels of NAD$^+$/NADH in the culture. Unfortunately this method was discontinuous in its mode of operation and required both the removal of culture samples from the fermenter and the subsequent extraction of the coenzymes prior to their assay. To minimize any discrepancies between the NAD$^+$/NADH levels in the culture and those in the samples prepared for the assay, both sampling and extraction procedures had to be rapid and efficient.

To determine the efficiency of the extraction procedure to be used, stock solutions of predetermined concentrations of NAD$^+$ and NADH were prepared in NMS medium. These solutions were then subjected to an identical extraction and the degree of recovery estimated. The extraction procedure to be used was based on that described by Wimpenny and Firth (1972) who recorded levels of NAD$^+$ and NADH in a number of heterotrophic bacteria. In the above study the authors reported that the total NAD$^+$/NADH concentration varied between 4.0 and 11.7 μmoles g^{-1} dry weight of cells, the actual value being dependent upon both the organisms studied and the conditions of growth used. The standard concentrations of NAD$^+$ and NADH used to test the efficiency of the extraction procedure were based on the values obtained by Wimpenny and Firth. Under conditions of methane-limited growth on NMS medium the culture cell density normally achieved was approximately $3gl^{-1}$ dry weight (chapter 4). Consequently standard solutions of NAD$^+$ and NADH were prepared consisting of between 1 and 8nmoles ml^{-1}. After these solutions had been passed through the complete extraction and assay procedure, the level of recovery was determined, (table 7.1).

The results showed that little loss in NAD$^+$ or NADH was recorded at the highest concentration used. As the

Pyridine Nucleotide being measured	Standard Solution (nmoles ml^{-1})	% Recovery
NAD^+	8	94
	4	88
	1	79
NADH	8	98
	4	95
	1	90

Table 7.1 Recovery of NAD^+ and NADH during the standard extraction and assay procedure.

concentration of the cofactors was reduced there was a corresponding decrease in the level of recovery, especially with respect to samples containing NAD^+. When Wimpenny and Firth (1972) carried out a similar study, they too observed an increased loss of NAD^+ and NADH at lower concentrations, obtaining a minimum recovery of between 83 and 86% for NAD^+ and NADH respectively. The results in table 7.1 showed that while a maximum loss of 10% was obtained when subjecting standard solutions of NADH to the extraction process, the recorded loss in NAD^+-containing samples exceeded 20% when the samples contained concentrations of 1nmoles ml^{-1} NAD^+. The decreased level of recovery in samples containing NAD^+ was probably a consequence of the extended process of sample preparation, since the measurement of NAD^+ levels required the conversion of the oxidised cofactor to NADH, before assaying in the luminometer. Given time, it should be possible to optimize the conditions used in this conversion step to reduce the level of observed loss.

7.3 Effect of MMO Type and Formaldehyde Addition on NAD^+/NADH Levels in M.capsulatus (Bath)

7.3.1. Experimental

The initial objective of this section was to examine whether the MMO type expressed by the cells had any influence on the levels of NAD^+ and NADH within the culture as suggested by Dalton and Higgins (1987). To achieve this it was necessary to set up a continuous cultivation experiment that permitted the expression of firstly 100% particulate MMO followed by the expression of 100% soluble MMO. Therefore the culture conditions used were based on those decribed in chapter 4, the cells growing under methane-limited conditions and at a dilution rate of $0.05h^{-1}$. An initial period of steady-state growth was achieved using NMS medium supplemented with $1.2mgl^{-1}$ $CuSO_4 \cdot 5H_2O$, to encourage the expression of the particulate MMO, before switching to a copper free medium which encouraged the expression of the soluble form of the enzyme.

During the latter stages of the experiment, increasing levels of formaldehyde were added to the medium. The results in the previous chapter had implied

that in the presence of methane, extracellular formaldehyde was oxidised to CO_2. The increased level of formaldehyde oxidation was also accompanied by a decrease in the level of soluble MMO activity, which eventually disappeared altogether. Both increased formaldehyde oxidation and loss of MMO activity should influence the NAD^+/NADH levels within the cell.

7.3.2 Results

7.3.2.1 Cell density and MMO activity.

A profile of the cell density and the levels of MMO activity within the culture are shown in fig 7.1. As expected, the switch from medium containing $1.2mgl^{-1}$ copper sulphate to that free of copper caused a dramatic reduction in the level of particulate MMO activity and a corresponding increase in soluble MMO activity. The increase in soluble MMO activity corresponded with the expression of the enzyme, fig 7.2 showing the appearance of the polypeptides characteristic of protein A on an SDS polyacrylamide gel. The subsequent disappearance of the above protein bands coincided with the addition of formaldehyde. An attempt at maintaining the cells on formaldehyde alone failed, washout of the culture being detected immediately after the methane supply had been removed. As with the previous formaldehyde-fed cultures, no detectable MMO activity (soluble or particulate) could be detected at this stage.

7.3.2.2 Intracellular levels of NAD^+ and NADH

The levels of NAD^+ and NADH measured in culture samples removed during the experiment are shown in fig 7.3. These results are expressed in terms of the individual concentrations of NAD^+ and NADH, the total (NAD^+ + NADH) pool size and the ratio of NAD^+:NADH.

For the majority of the experiment, the total NAD(H) pool size remained between 1.0 and 0.8 μmoles g^{-1} dry weight. This observation corresponded with reports by London and Knight (1966), and Harrison and Chance (1970) that the total nucleotide pool in actively growing bacteria did not vary significantly from one growth condition to another, but only the ratio of oxidised to

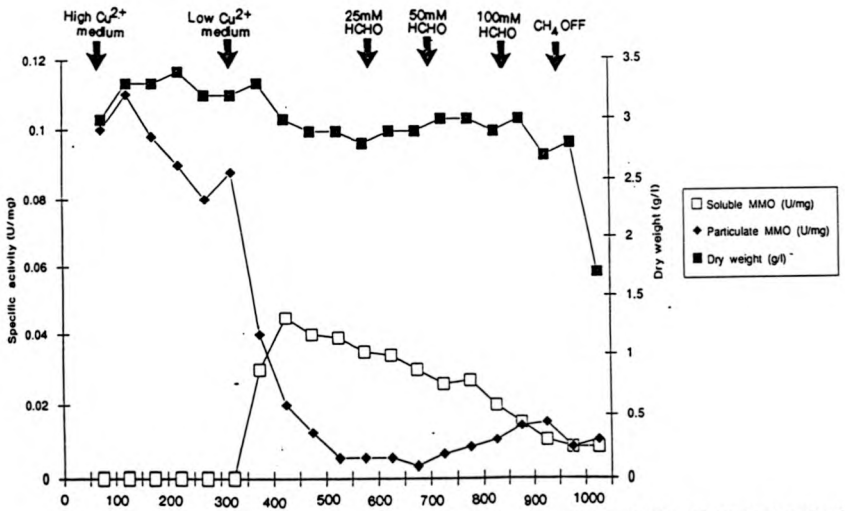
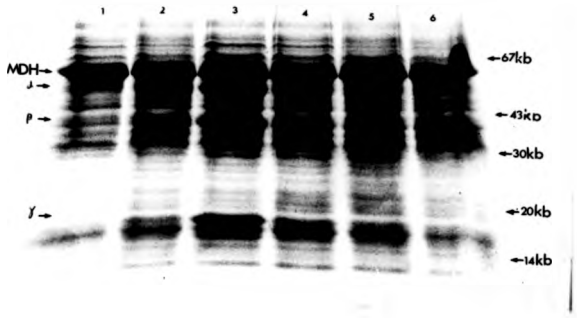


Fig 7.1 Profile of MMO activity levels and culture cell density recorded during the switch from high to low copper conditions and the addition of formaldehyde



Track	Sample time (hours)	Growth conditions
1.	150	high copper
2.	300	high copper
3.	550	low copper
4.	775	low copper + 50mM HCHO
5.	900	low copper + 100mM HCHO
6.	950	low copper + 100mM HCHO ($-CH_4$)

Fig 7.2 SDS polyacrylamide gel prepared from soluble cell extracts, showing the effect of the change in copper levels and the addition of formaldehyde

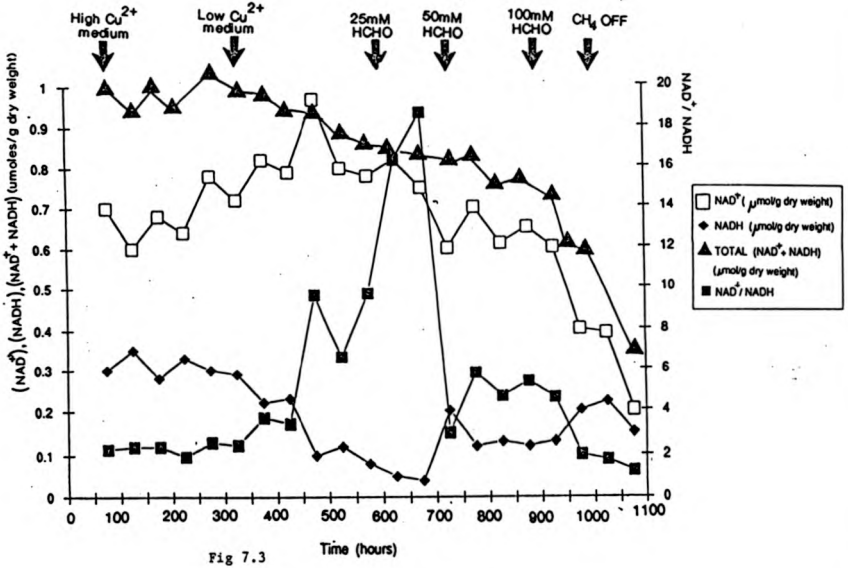


Fig 7.3

Intracellular concentrations of NAD^+ and NADH recorded during the continuous cultivation experiment

reduced species. A reduction in the total NAD(H) pool size was recorded towards the end of the experiment. This probably represented a loss of cellular activity within the culture since Wimpenny and Firth (1972) previously estimated that under non-growing conditions both NAD^+ and NADH would be destroyed.

The ratio of NAD^+ : NADH was highest when the cells were growing under soluble MMO conditions. Such a result might be predicted if the soluble MMO was actively utilizing NADH during its oxidation of methane, thereby effectively increasing the availability of NAD^+. Under conditions of particulate MMO expression the ratio of NAD^+ : NADH was lower compared with that generated in the presence of soluble MMO. In cells possessing high levels of particulate MMO activity, higher levels of NADH might be expected, assuming the particulate enzyme obtains reducing power from sources other that NADH. Under such circumstances, reduced levels of NAD^+ would be generated, effectively lowering the NAD^+:NADH ratio.

The addition of formaldehyde to the medium corresponded to an increase in the level of NADH, particularly during the latter stages of the experiment. The pattern of gas exchange was produced in response to increased formaldehyde metabolism (chapter 6), implying that formaldehyde added in the presence of methane was rapidly oxidised to CO_2. This presumably occurred by way of the NAD^+-linked formate dehydrogenase and possibly the NAD^+-linked formaldehyde dehydrogenase. Therefore additional formaldehyde oxidation would predictably lead to the production of increased levels of NADH and a corresponding decrease in NAD^+ levels.

The levels of NADH measured in culture samples during the addition of 25 and 50mM formaldehyde were not significantly higher than those measured in the absence of exogenously supplied formaldehyde. This observation could be explained by the fact that cells grown under these conditions still possessed a means of oxidising additional NADH via the soluble MMO. It has already been shown that during the initial stages of additional formaldehyde oxidation, the soluble MMO still retains an NADH oxidase capacity.

When the formaldehyde concentration in the medium was raised from 50mM to 100mM, the NADH concentration in the culture effectively doubled. This coincided with the loss

of MMO activity within the culture; washout being recorded approximately 100 hours later. As with previous formaldehyde-fed cultures, washout was also preceeded by a reduction in in the culture's capacity to metabolize the added formaldehyde and the accumulation of extracellular formate (5mM). Up until this point neither metabolite had been detected in the culture. According to the observation made by Marison (1980) and Jollie (personal communication), the *in vitro* activity of the formate dehydrogenase is restricted in the presence of NADH. Therefore the increased NADH levels detected in the culture during the latter stages would effectively compromise the formate dehydrogenase *in vivo*, thereby resulting in the observed accumulation of formate and ultimately cell death.

7.4 Final Comments

The results generated in this chapter implied that the MMO type expressed by *M.capsulatus* (Bath) played a major role in determining the intracellular concentration of NAD^+ and NADH. Dalton and Higgins (1987) suggested that the reason Cornish *et al* (1984) observed the intracellular accumulation of formaldehyde in the particulate MMO-expressing *Methylosinus trichosporium* OB3b was that formaldehyde oxidation was limited by the availability of NAD^+. The results in this study indicated that NAD^+ (abeit at reduced levels) was still detectable in the culture when *M.capsulatus* (Bath) was grown under particulate - MMO generating conditions. It is feasible to suggest that using the discontinuous technique to sample and extract the coenzymes from cells means that there will always be some NAD^+ present in the prepared sample; this being a consequence of the rapid turnover of the coenzymes.

Despite this, the overall pattern concerning the effect of switching between particulate MMO-expressing conditions and soluble MMO conditions on the NAD^+: NADH ratio was as predicted; the ratio being higher under the latter set of conditions. Similarly the results obtained, concerning the effect of formaldehyde additions to soluble MMO-expressing cells was similar to that predicted, given that the oxidation of added formaldehyde to CO_2 would result in the increased generation of NADH.

CHAPTER 8

Regulation of Methanol Metabolism

Regulation of Methanol Metabolism

8.1 Effect of Methanol Addition to Methane-grown Continuous Cultures of M.capsulatus (Bath)

8.1.1. Introduction

Over the years there have been numerous reports published concerning the ability of methanotrophs to switch from growth on methane to growth on methanol as their sole source of carbon and energy. The results in the literature however suggested that the ease with which such a transfer can occur is questionable. Leadbetter and Foster (1958), Stocks and McCleskey (1964) and Whittenbury et al (1970) reported that methane utilizing bacteria grew poorly when transferred to medium containing methanol as a sole source of carbon. The exact reason for this is not known although it has been attributed to the accumulation of formaldehyde (Ribbons et al 1970). Linton and Vokes (1978) also reported formaldehyde accumulation in methanol-grown shake flasks cultures of the methanotroph Methylococcus NICB 11083.

To achieve the transfer of methane-grown cultures of Methylocystis parvus OBBP to growth on methanol, Hou et al (1978) reported that a period of adaptation was required, during which the cells were gradually exposed to increased levels of methanol over a period of several days. A similar observation was made by Best and Higgins (1981) during the study of the growth of Methylosinus trichosporium OB3b on methanol. This led the authors to conclude that such an adaptation process reflected some form of physiological response of the cells to methanol or the selection of a mutant tolerant to either methanol or formaldehyde.

One of the experimental objectives of the present work was to examine whether or not a similar form of physiological adaptation was required when M.capsulatus (Bath) was switched from growth on methane to growth on methanol. An earlier study by Prior and Dalton (1985) showed that M.capsulatus (Bath) could be adapted to growth on methanol in continuous culture over a period of 4 days. During this period the level of methane entering the

vessel was gradually reduced while the level of methanol in the medium was increased. In that study, the effect of the switch in carbon sources was examined with respect to the location and activity of the MMO enzyme. In this section it was decided to examine the effect of a similar switch in carbon sources in more detail, particularly with respect to its effect on the activities of all the methane oxidation pathway enzymes. One would expect any physiological adaptation of the cells during the switch from growth on methane to methanol, would be reflected in a change in the levels of specific enzymes associated with C_1 metabolism.

8.1.2 Experimental

A continuous culture of M.capsulatus (Bath) was set up, growing on methane as its sole carbon and energy source. The culture was initially grown on a methane/air (1:4 v/v), as used by Prior and Dalton (1985). The total gas flow rate was 0·4 litres min^{-1}, which with a dilution rate of 0·05h^{-1} meant the culture was oxygen-limited.

Unlike the method used by Prior and Dalton to adapt methane-grown cells to growth on methanol, the switch in carbon sources was not limited to a period of 4 days. Instead, a series of steady-states were generated with different combinations of methane/methanol mixed carbon sources, the overall object being to decrease the level of methane and correspondingly increase the level of methanol in the medium. Ultimately, the methane supply was removed and the cells maintained on methanol. During each period of steady-state growth, samples of culture were routinely removed to monitor the levels of enzyme activity. Initially the effect of switching from methane to methanol was studied using cells containing particulate MMO. This was then repeated using cells containing soluble MMO.

8.1.3. Carbon distribution in Methanol-fed Continuous Culture

A description of the conditions under which the different steady-states were generated are shown in table 8.1 for the high copper continuous culture and table 8.2 for the low copper continuous culture. In both cases the

Steady-state --------->		<---------- Measurements ---------->						<- Summary ->	
Period	% methane in total gas in	methanol in medium (% v/v)	culture dry wt. ($g\ l^{-1}$)	rate of biomass accumulation ($mmoles\ Cl^{-1}h^{-1}$)	rate of CH_3OH consumption ($mmoles\ Cl^{-1}h^{-1}$)	rate of CH_4 consumption ($mmoles\ Cl^{-1}h^{-1}$)	rate of O_2 consumption ($mmoles\ Cl^{-1}h^{-1}$)	rate of CO_2 production ($mmoles\ Cl^{-1}h^{-1}$)	C.C.E. (%)	Qrecov (%)
1	20	0	4	7.8	0	21	33	12.8	37	102
2	5	0.5	4.1	8.4	6.2	18	34	15	35	102
3	5	1	3.9	7.6	12.4	8	26	13.3	37	103
4	0	1	2.8	5.4	12.4	0	11	7	42	100

Table 8.1 Effect of methanol addition to methane-grown, high copper continuous culture

Steady-state ---------> <---------- Measurements ----------> <- Summary ->

Period	% methane in total gas in	methanol in medium (% v/v)	culture dry wt. ($g\ l^{-1}$)	rate of biomass accumulation ($mmoles\ Cl^{-1}h^{-1}$)	rate of CH_3OH consumption ($mmoles\ Cl^{-1}h^{-1}$)	rate of CH_4 consumption ($mmoles\ Cl^{-1}h^{-1}$)	rate of O_2 consumption ($mmoles\ Cl^{-1}h^{-1}$)	rate of CO_2 production ($mmoles\ Cl^{-1}h^{-1}$)	C.C.E. (%)	Crecov (%)
1	20	0	3.6	7	0	21	36	13.8	33	101
2	5	0.5	3.8	7.4	6.2	17.6	37	15.4	32	104
3	5	1	3.9	7.6	12.4	9.6	27	13.2	34.5	105
4	0	1	2.8	5.5	12.4	0	11	6.4	44	104

Table 8.2 Effect of methanol addition to methane-grown, low copper continuous culture

overall pattern of carbon distribution was similar.

Throughout the experiment the rate of methanol consumption by cells was equivalent to the rate at which methanol was added, since at no time was any free methanol detected in the culture. In addition, neither formate nor formaldehyde accumulation was observed in the culture and consequently there appeared to be no deleterious effects induced by switching the carbon source from methane to methanol.

The carbon recovery calculations performed routinely gave values of greater than 100%. This can be explained by the fact that the level of methanol consumption was probably overestimated, since this value did not take into account any loss of methanol that might have been incurred as a result of evaporation of the metabolite as it passed through the culture head-space. As the level of methane entering the culture was reduced, there was a corresponding drop in the level of methane consumption. This reduction was off-set by the increased rate of methanol consumption, the metabolism of which was split between oxidation to CO_2 and assimilation to biomass. Consequently under both low and high copper conditions, the C.C.E. value obtained during mixed methane/methanol growth did not change significantly.

As expected the C.C.E. value obtained prior to the addition of methanol was higher in high copper experiment than the equivalent value generated in the low copper environment. This presumably was a consequence of the type of MMO being expressed. When the methane source was finally removed, there was in both cases a significant increase in the C.C.E. value. In theory the cell yield on methanol should always be higher than on methane, since the methane oxidation step represents drain on available reducing power and consequently is energy-requiring. Under conditions of growth on methanol the C.C.E. values for both the low copper and high copper cultures were similar. The reason for this was that unlike growth on methane, the different energetics of the particulate and soluble MMO-catalysed reactions would not influence cell yield, although this conclusion is based on the assumption that the MMO does not play a major role in the oxidation of methanol.

The measured cell yield on methanol under both low and high copper conditions was between 0.33 and 0.35 g

cells g CH_3OH^{-1}. This value was lower than that predicted by Anthony (1978) and by Harder and van Dijken (1976), (0.45-0.67gcells gCH_3OH^{-1}). Anthony (1982) stated that such theoretical predictions did not take into account the efficiency of ATP generation and utilization. Consequently such theoretical predictions were liable to be an overestimate of the true yield, since normally some degree of ATP wastage will occur through "uncoupled growth" or "slip reactions" (Forrest and Walker 1971; Neijssel and Tempest, 1976).

8.1.4 Enzyme Levels in Methanol-fed Continuous Culture

Methane oxidation pathway enzyme activities were measured during each period of steady-state growth. This was done to determine whether or not any of these enzymes were subject to induction or repression during the changes in the culture conditions.

The results in fig 8.1 and fig 8.2 showed that with the exception of soluble MMO activity under low copper conditions, the pattern of relative enzyme activities remained constant. The individual activity levels of methanol dehydrogenase, formaldehyde dehydrogenase (NAD^+-linked and NAD^+- independent) and the formate dehydrogenase were not affected by the switch in carbon source. Under conditions of high copper, relatively high levels of particulate MMO activity were maintained, even in the absence of methane. In contrast, under low copper conditions; the level of soluble MMO activity in the culture dropped continuously in response to the addition of methanol. This was similar to the response observed during the addition of formaldehyde to methane-grown cells indicating that soluble MMO activity is lost in the presence of a carbon substrate other than methane. The lack of soluble MMO activity during growth on methanol corresponded to the observation by Prior and Dalton (1985), concluding that the MMO activity in methanol-grown cells was always associated with the particulate fraction. The fact that no changes in the methanol dehydrogenase or formaldehyde dehydrogenase activity levels were observed was unexpected. One might have expected any physiological adaptation of the cells to growth on methanol to have been coupled to the production of raised levels of the above enzymes, especially since the toxic effect recorded by

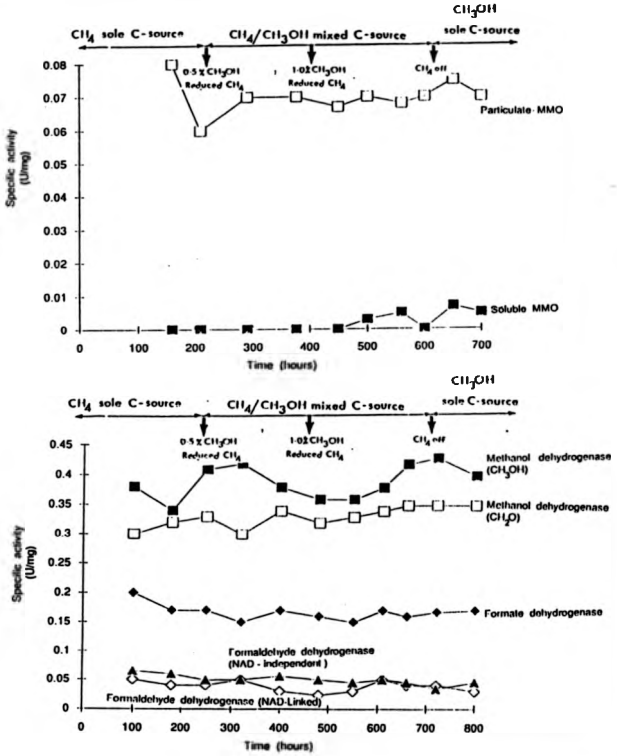


Fig 8.1 Methane oxidation pathway enzyme levels measured during high copper, methanol fed continuous culture

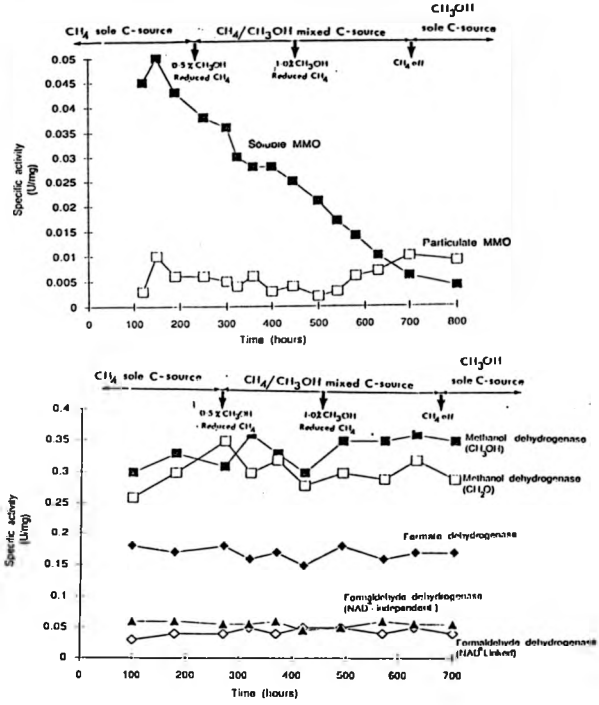


Fig 8.2 Methane oxidation pathway enzyme levels measured during low copper, methanol fed continuous culture

earlier workers in the absence of any "adaptation" period concerned the accumulation of formaldehyde. The results in this section implied that any physiological adaptation that occurred in reponse to the addition of methanol was minimal.

8.2 The Role of MMO in Transfering Cultures From Growth on Methane to Growth on Methanol

8.2.1. Introduction

The results generated by Ribbons et al (1970) and Linton and Vokes (1978) implied that the major reason for the observed toxic effect during the transfer of cells from a methane-supplemented environment to one containing methanol, was the accumulation of formaldehyde. The results in the previous section however indicated that the achievement of such a transfer did not require any substantial change in the levels of either the methanol or formaldehyde dehydrogenase enzymes. One other enzyme that may affect the rate of intracellular formaldehyde accumulation during the switch in carbon sources is the MMO, since it has been shown to have the capacity to oxidise methanol (Colby et al, 1977; Stirling et al. 1979). Indirect evidence for the in vivo oxidation of methanol by the MMO came from the observation that measured growth yields of methanotrophs on methanol were lower than the equivalent values obtained for typical methanol-utilizers using the same assimilatory pathway.

It was suggested that the reason for such discrepancies lay in the fact that some of the methanol was oxidised by the MMO, at the expense of the energetically more efficient methanol dehydrogenase. In vivo methanol oxidation was demonstrated by ^{13}C NMR in whole cells of Methylosinus trichosporium OB3b, using a pulsed methanol feed by Cornish et al (1984). These authors reported that inhibition of MMO activity coupled with the simultaneous addition of ^{13}C-labelled methanol caused a reduction in both the rate of methanol oxidation and formaldehyde formation. From this it was concluded that the MMO was directly involved in the conversion of methanol to formaldehyde. The inhibitor used in the above study was acetylene which has been reported as being an inhibitor of both soluble and particulate MMO in a number

of methylotrophs (Dalton and Whittenbury, 1976; Stirling and Dalton, 1977; Stanley et al 1983).

The purpose of this section was to demonstrate that formaldehyde accumulation could be detected when cultures of M.capsulatus (Bath) were switched directly from methane-limited to methanol-limited growth. Thereafter, to reduce the rate of formaldehyde accumulation during this period by temporarily inactivating the MMO with the addition of acetylene. This should facilitate the direct transfer of cells from methane to methanol growth without any period of physiological adaptation.

8.2.2. Effect of Changing Carbon Substrates in the Absence of Acetylene

A continuous culture of M.capsulatus (Bath) was established under methane-limited, low copper conditions. Once steady-state had been achieved, the supply of methane was removed and growth was switched to medium containing 1.0%(v/v) methanol.

The result of this experiment (fig 8.3) indicated that when the carbon source was switched from methane to methanol, both methanol (0.2mM) and formaldehyde (0.13mM) were detected in the culture. The accumulation of these metabolites did not however result in the wash-out of the culture, although a drop in cell density was recorded. Therefore the direct transfer of M.capsulatus (Bath) from growth on methane to methanol was possible under the above conditions.

8.2.3 Effect of Changing the Carbon Substrate in the Presence of Acetylene

A continuous culture was set up under indentical conditions to that described previously. When the carbon source of the culture was switched from methane to methanol, 1.5mll^{-1} acetylene was simultaneously added to the culture via the gas inlet. The level of acetylene used was based on that described by Suzuki (1989), who previously recorded that using a similar level resulted in the complete inactivation of the MMO within the culture for a limited period of time. The process of inactivation was too rapid to permit the routine preparation and assay of MMO activity in cell extracts. Consequently the

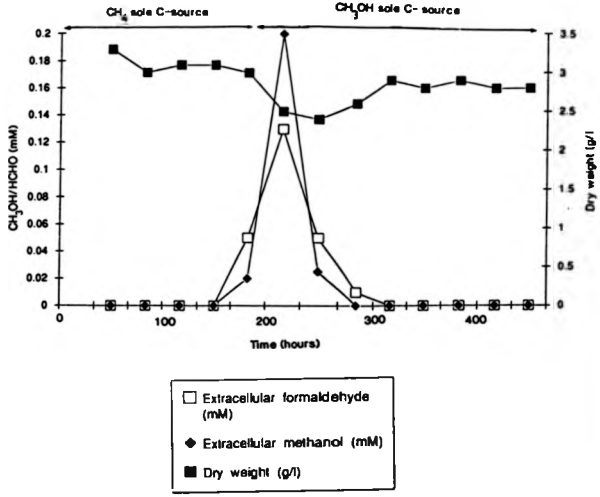


Fig 8.3 Effect of switching growth from methane-limited conditions to growth on methanol as a sole carbon source, without any period of adaptation

methane-oxidising capacity of whole cells was measured during this period. The results of such measurements are shown in fig 8.4 and show that up to 95% of the methane-oxidising capacity of the culture was temporarily inactivated within 30 minutes of the acetylene addition. The acetylene was readily removed by flushing the culture with air plus methane. This resulted in the reactivation of the MMO. Approximately 70% of the orginal methane-oxidising capacity was re-activated within 5 hours of acetylene addition.

The extracellular levels of metabolites detected in the culture during the period of acetylene addition are shown in fig 8.5. The level of methanol accumulation recorded in this experiment, in response to the change in carbon sources was approximately 2.5 times greater than the equivalent value generated in the absence of the addition of acetylene. At no time during the course of the experiment was extracellular formaldehyde detected. This result implied that the removal of the methane oxidising capacity of the culture effectively reduced the rate of methanol metabolism and consequently the rate of formaldehyde formation.

8.2.4 Conclusion

It can be concluded from the above observations that the MMO is actively involved in the oxidation of methanol when cells are transfered from methane-limited to methanol-limited conditions of growth. Under methanol-limited conditions, the relative amount of methanol oxidised by the MMO would be minimal. This assumption was based on the observation that Colby et al (1977) estimated the Km of the MMO from M.capsulatus (Bath) for methanol was 950μM, whilst Green (unpublished) showed the Km of methanol dehydrogenase for methanol was 6μM in the same organism. However under conditions that permitted the direct switch from methane-limited growth to methanol-limited growth, methanol was shown to temporarily accumulate within the culture. Under conditions of methanol saturation it is highly likely that the MMO is involved in the oxidation of methanol.

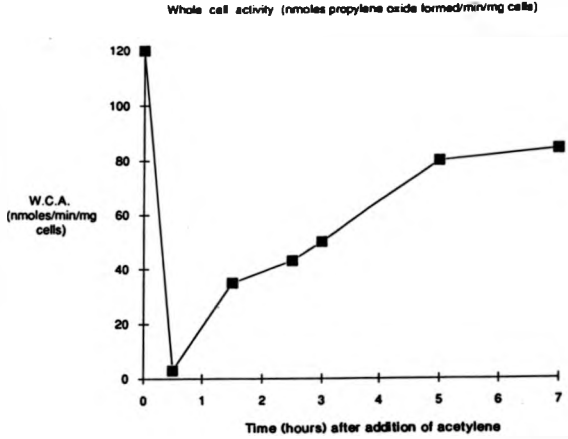


Fig 8.4 The use of acetylene in the temporary inactivation of the methane oxidising capacity of the culture

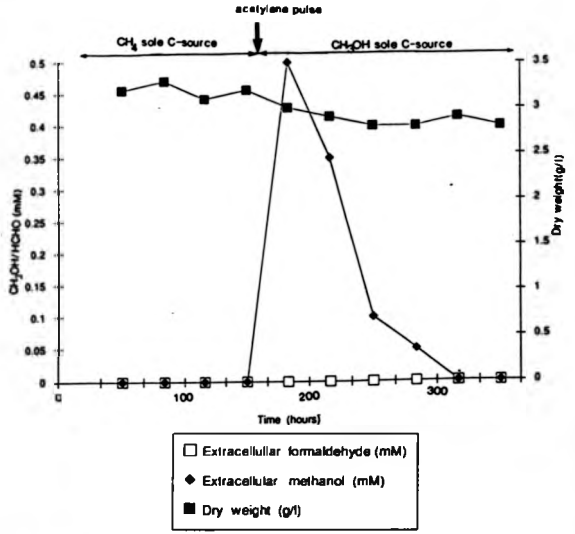


Fig 8.5 Effect of switching from methane-limited growth to methanol as a sole source of carbon, in the presence of the acetylene

8.3 Environmental Regulation of Methanol Metabolism by M.capsulatus (Bath)

8.3.1 Introduction

The successful transfer of M.capsulatus (Bath) from methane-limited conditions to growth on methanol as a sole source of carbon and energy meant that it was possible to examine aspects of the environmental regulation of methanol metabolism within the cells. Studies concerning the effect of growth rate and nutrient limitation on key metabolic pathway enzymes in methylotrophic bacteria have been routinely carried out using continuous culture techniques (Rokem and Goldberg, 1984). Bussineau and Papoutsakis (1986) observed that the levels of methanol dehydrogenase, formaldehyde dehydrogenase (NAD^+-linked) and the formate dehydrogenase in Methylomonas L3 were all subject to derepression at lower dilution rates. Similarly Greenwood and Jones (1986), and Jones et al (1987) reported that the levels of specific enzymes including the methanol dehydrogenase in Methylophilus methylotrophus were intimately linked to enviromental factors such as dilution rate and nutrient limitation. The purpose of this study was to examine whether or not C_1 oxidising enzymes in M.capsulatus (Bath)were subject to environmental regulation in a manner similar to that described above. This required the examination of the effect of dilution rate and substrate composition (particularly the level of methanol in the medium) on the levels of enzyme activity within the culture.

8.3.2 Effect of Raised Methanol Concentration during the Continuous Culture of M.capsulatus (Bath)

8.3.2.1 Experimental

A continuous culture was established to examine the response of M.capsulatus (Bath) to increased levels of methanol in the medium, at a constant dilution rate. Periods of Steady-state growth were obtained using NMS medium supplemented with 1.0%,1.5% and 2.0% $^{v}/_{v}$ methanol, as a sole source of carbon. The results generated in the previous experiments had shown that the particulate MMO

was active in the presence of methanol and that it was capable of oxidising methanol *in vivo*. Consequently high copper conditions were used to determine whether the levels of MMO activity in the cells might be regulated by the level of methanol in the culture.

8.3.2.2 Effect of Raised Methanol Levels on Cell density and Extracellular Metabolite Levels.

When the methanol concentration in the medium was increased from 1.0%($^{v}/_{v}$) to 1.5%($^{v}/_{v}$), an increase in cell density was observed (fig8.6). Up until this point no methanol had been detected in the culture and it was assumed that under such conditions the cells were methanol-limited. Immediately after the methanol concentration in the medium was raised, low levels of methanol (0.15mM) were detected in the culture, although this rapidly disappeared on continuous exposure to medium containing 1.5% ($^{v}/_{v}$) methanol. A similar effect was recorded when the methanol concentration was raised from 1.5% to 2.0%($^{v}/_{v}$) although the subsequent accumulation of extracellular methanol did not disappear. Instead a steady-state was generated in the presence of low levels of methanol (0.03-0.05mM) in the culture. Under these conditions, the cells appeared to be methanol-saturated and limited by some other nutrient. When the methanol concentration in the medium was finally raised to 2.5% ($^{v}/_{v}$), a rapid accumulation of methanol was recorded and there was no increase in cell density. The rapid accumulation of formate and formaldehyde in the culture was also detected and this coincided with the washout of the culture.

8.3.2.3 Effect of Raised Methanol Levels on Enzyme Activity Within the Culture.

The effect of increased methanol availability on the pattern of enzyme activity in the culture is shown in fig 8.7. The most dramatic effect concerned the observed reduction in methanol dehydrogenase activity in response to the accumulation of methanol in the culture. This occurred irrespective of whether the enzyme's activity was measured in the presence of methanol or formaldehyde as a substrate. When samples of soluble extract were run on an

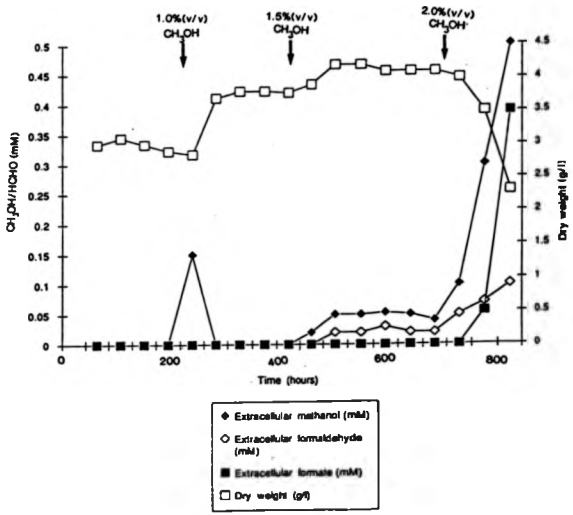


Fig 8.6 The effect of increased methanol levels in the medium on cell density and extracellular metabolite levels, using methanol as a sole source of carbon

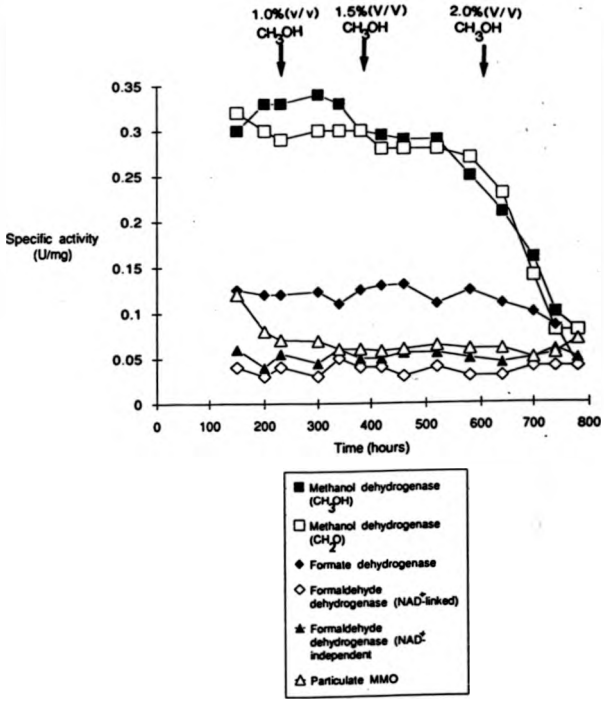


Fig 8.7 Changes in the levels of enzyme activity observed in response to increased levels of methanol in the culture

SDS polyacrylamide gel with a sample of "purified" methanol dehydrogenase (fig 8.8), the protein band corresponding to the methanol dehydrogenase was less intense in cell extracts prepared towards the end of the experiment. Therefore the observed loss in methanol dehydrogenase activity during the experiment was not the result of inactivation but a consequence of an overall reduction in the level of enzyme expressed. This result corresponded with the observation by Greenwood and Jones (1986) that the concentration of methanol dehydrogenase in Methylophilus methylotrophus was inversely proportional to the standing concentration of methanol in the culture.

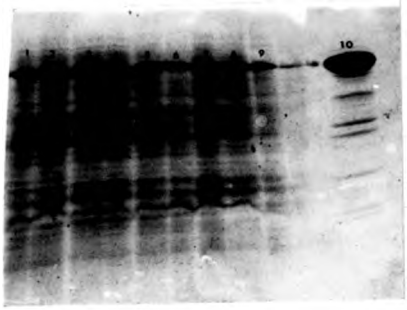
The initial accumulation of formaldehyde in the culture also coincided with the loss of methanol dehydrogenase activity, suggesting that the above enzyme is capable of oxidising formaldehyde in vivo. The corresponding levels of the formaldehyde dehydrogenase (NAD^+-linked and NAD^+-independent) were consistently low and showed no response to the accumulation of either methanol or formaldehyde. Similarly the level of particulate MMO activity did not change with respect to the increased levels of methanol. This corresponded to the report by Prior and Dalton (1985) that the particulate MMO was synthesised constitutively when the cells were grown on methanol and the level of activity was dependent on the level of copper ions available.

The pattern of formate dehydrogenase activity in the culture indicated that the enzyme was not affected by the initial accumulation of methanol in the culture. However, when the methanol concentration in the medium was raised to 2.5%($^v/_v$) a dramatic reduction in the level of activity was observed. This coincided with the rapid accumulation of formate, formaldehyde and methanol. Under these conditions there was a total loss of cellular activity and the culture washed out.

8.3.3 Influence of Dilution Rate on Enzyme Activities in Methanol-Grown M.capsulatus (Bath)

8.3.3.1 Introduction

The purpose of this work was to examine whether or not the enzymic capacity of methanol-grown M.capsulatus (Bath) was influenced by the specific growth rate of the



Track	Time of sample (hours)
1	100
2	200
3	250
4	300
5	400
6	550
7	600
8	650
9	700
10	Purified Methanol dehydrogenase

protein tracks 1 - 9 were loaded with 50 μg crude soluble extract.

Fig 8.8 Effect of increasing methanol availability on on the levels of methanol dehydrogenase protein in cell extracts, as shown by SDS polyacrylamide gel electrophoresis

cells. The results in the previous section indicated that the concentration of methanol dehydrogenase in the cell was linked to the level of methanol in the culture, the highest levels of activity being expressed under methanol-limited conditions. In a carbon-limited chemostat culture, the residual concentration of carbon substrate in the growth medium increases with increasing dilution rate. Consequently if the level of methanol dehydrogenase activity is inversely related to the residual concentration of methanol in the culture then it should be possible to demonstrate a reduction in enzyme activity with respect to an increasing dilution rate.

8.3.3.2 Experimental

Carbon limited, steady-state conditions of growth were obtained over a range of dilution rates (0.05 to $0.2h^{-1}$) using 1.0% ($^{v}/_{v}$) methanol-supplemented medium. Repeated culture samples were removed during each steady-state, to monitor the levels of enzyme activity within the culture.

8.3.3.3 Effect of Dilution Rate on In vitro Enzyme Activities

The activities of the methanol dehydrogenase and formate dehydrogenase in culture samples showed a marked decrease as the dilution rate was increased from 0.075 to $0.2h^{-1}$(fig 8.9). In both cases the degree of activity reduction was approximately 40% the maximum specific activity achieved. In contrast the specific activities of the formaldehyde dehydrogenase(NAD^{+} or dye-linked), and the hexulose phosphate synthase showed no change in activity when the dilution rate was increased. (fig 8.10)

A study by Bussineau and Papoutsakis (1986) concluded that the activities of all the early catabolic enzymes (methanol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase) were closely linked to the specific growth rate in Methylomonas L.3. With each enzyme, the authors recorded a reduction in activity levels with increasing dilution rate. The results obtained with M.capsulatus (Bath) showed a similar pattern with respect to the methanol dehydrogenase and formate dehydrogenase activities. The levels of formaldehyde

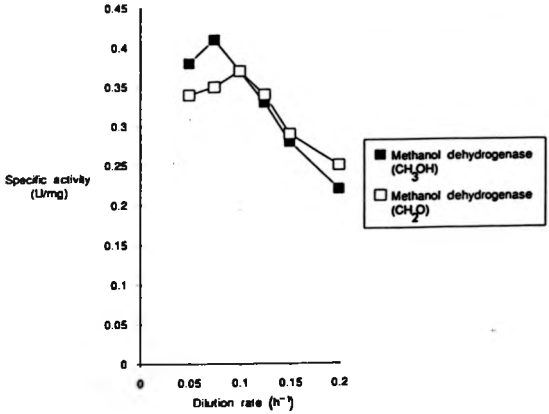


Fig 8.9 Effect of increasing dilution rate on the level of methanol dehydrogenase activity

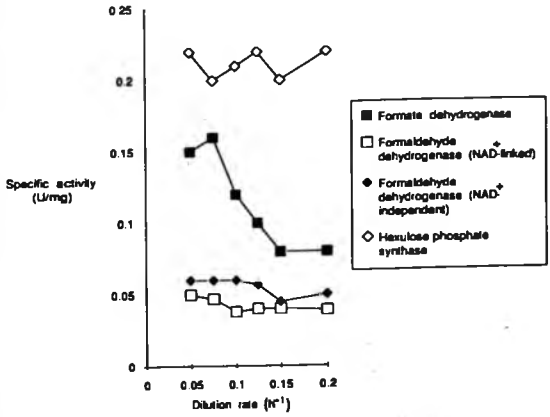


Fig 8.10 Effect of increasing dilution rate on the levels of specific C_1 metabolizing enzymes

dehydrogenase activity however did not change with respect to dilution rate, implying that the NAD^+-linked and the NAD^+-independent formaldehyde dehydrogenase's were expressed constitutively and not subject to regulation by the specific growth rate. Bussineau and Papoutsakis also reported that the hexulose phosphate synthase activity was greatest at an intermediate dilution rate. The results in this study however indicated that the activity levels of this enzyme were consistently high, irrespective of the dilution rate.

Studies by Greenwood and Jones (1986) and Jones et al (1987) concluded that both methanol dehydrogenase activity and formate oxidation were repressed at high dilution rates in methanol-limited Methylophilus methylotrophus. In addition, the level of hexulose phosphate synthase activity remained high irrespective of the dilution rate and whether the cells were methanol or oxygen-limited. This pattern of enzyme activity with respect to dilution rate corresponded to that generated during the methanol-limited growth of M.capsulatus (Bath). Jones et al (1987) also concluded that the activity ratio of the methanol dehydrogenase to the hexulose phosphate synthase, (the first two enzymes of methanol metabolism in M.methylotrophus) determined the carbon flux entering the linear oxidation pathway. This was subsequently demonstrated by monitoring the pattern of metabolite over-production with respect to increased rates of methanol metabolism, using both chemical analysis and ^{13}C-NMR.

No extracellular accumulation of metabolites was detected during the growth of M.capsulatus (Bath) on methanol, irrespective of the levels of methanol dehydrogenase and formate dehydrogenase activity generated at high dilution rates. This implied that the reduced levels of the above enzymes did not cause any permanent "bottle-neck" in the flow of carbon down the C_1 oxidation route. It is possible that low, non-toxic levels of formaldehyde or formate may have temporarily accumulated within the cell during changes in growth conditions. However to effectively demonstrate such dynamic changes in the cells intracellular environment would require the use of in situ analysis techniques such as ^{13}C-NMR.

3.3.3.4 DISCUSSION

Dilution rate (specific growth rate) can markedly

influence the enzymic composition of bacteria growing in continuous culture (Dean,1972). In a chemostat, the specific growth rate is determined by the level of growth-limiting nutrient in the medium. As the dilution rate is increased, the concentration of metabolites in the cell derived from the growth-limiting nutrient would also be expected to increase. Matin et al (1976) suggested that enzymes that showed reduced activity with increasing dilution rate did so as a result of catabolite repression. As the dilution rate is increased, the intracellular concentration of the metabolites capable of acting as catabolite repressors would also increase. Alternatively when the dilution rate is decreased, the intracellular pools of such metabolites would also decrease, relieving any catabolite repression.

Regulation by catabolite repression would involve a loss of enzyme activity via a reduction in the rate of enzyme synthesis. It was concluded in section 8.3.2 that the observed loss in methanol dehydrogenase activity was the result of a reduced concentration of methanol dehydrogenase protein in the cell. Harder and Dijkhuizen (1983) also pointed out that cells grown under carbon-limited conditions, tended to derepress the synthesis of their catabolic enzymes. In addition, under the same conditions, the synthesis of enzymes associated with anabolic functions were adjusted to levels in keeping with the growth rate of cells. This corresponds to the pattern of methanol dehydrogenase and formate dehydrogenase activity obtained in response to the increased dilution rates in section 8.3.3. The results generated concerning the level of hexulose phosphate synthase activity implied that the synthesis of this enzyme was constitutive and was not linked to dilution rate.

Low specific growth rates due to nutrient limitation are probably the rule rather than the exception when methylotrophic bacteria are growing in their natural environment. High levels of methanol dehydrogenase and formate dehydrogenase, observed at low dilution rates would probably be representative of the cell's physiological state in its natural environment. The raised levels of these enzymes would reflect a means of survival, since they would effectively enhance the cell's capacity to metabolize nutrients present at sub-saturating

concentrations. This would prove to be a selective advantage in a nutritionally poor environment.

8.4 Summary

It has long been established that M.capsulatus (Bath) is capable of utilizing methanol as its sole source of carbon and energy. The results generated in this chapter showed that methanol utilization did not require the physiological adaptation of methane-grown cells. Instead, cells could be transferred directly from methane-limited growth to methanol-limited growth. Although non-toxic levels of formaldehyde accumulation were detected during the change in carbon source, these could be removed by temporarily inactivating the MMO. Under conditions of methanol-saturation, the MMO appeared capable of oxidising methanol *in vivo*. Consequently loss of MMO activity reduced the rates of both methanol oxidation and formaldehyde formation.

Having achieved growth of M.capsulatus (Bath) on methanol, the effects of dilution rate, and the level of methanol in the medium on the pattern of enzyme activity in the cells, were examined. It was concluded that the activities of the methanol dehydrogenase and to a lesser extent the formate dehydrogenase were inversely proportional to the concentration of methanol in the culture. At increased dilution rates, the activities of the above enzymes were also reduced, under methanol limiting conditions. In contrast the level of hexulose phosphate synthase activity remained high, irrespective of the dilution rate. Therefore it was concluded that carbon-limited cells growing at a low specific growth rate effectively derepressed the synthesis of the methanol dehydrogenase and the formate dehydrogenase. This ability would represent a selective advantage for cells, since it would enable them to grow more efficiently in a nutritionally poor environment.

CHAPTER 9

Coda

Chapter 9

Coda

Continuous culture has long been established as an effective means of investigating the physiology and biochemistry of cells grown under defined and reproducible conditions. The technique not only allows the study of steady-state growth but also permits the examination of the response of cells to perturbations in the conditions of growth.

Throughout this study the response of M.capsulatus (Bath) to the addition of intermediate C_1 metabolites was monitored directly using on-line mass spectrometry. Among the points noted during these experiments were:

1) When the cells were grown under methane-limited conditions, additional carbon in the form of formate, formaldehyde or methanol was for the most part oxidised to CO_2. Formaldehyde carbon was assimilated in the absence of methane, as was formate carbon under oxygen-limited conditions. In both cases however, there was a fine balance between assimilation and metabolite accumulation. The toxic nature of these compounds made the generation of such conditions difficult.

2) The results generated during the continuous addition of formaldehyde appeared to imply that the successful growth of M.capsulatus (Bath) on formaldehyde required the cells to possess an active form of MMO. Under both high and low copper conditions rapid formaldehyde (and formate) accumulation was observed immediately after the loss of MMO activity in the culture. Under low copper conditions, the addition of formaldehyde (in the presence of methane) effectively repressed the synthesis of the soluble MMO proteins. Exactly why such repression should occur is difficult to explain as subsequent results implied that the MMO was actively involved in the regulation of NAD^+/NADH levels within the cell, in addition to its role in the oxidation of methane. A complete explanation as to why such repression occurs in the presence of formaldehyde

will require a detailed analysis of the regulation of enzyme synthesis at the molecular level.

3) Analysis of the activity levels of enzymes capable of oxidising formaldehyde did not give any clear indicaton as to which enzyme(s) are involved in the *in vivo* dissimilation of formaldehyde. The high levels of methanol dehdyrogenase activity recorded during this study, coupled with its low Km and high Vmax values for formaldehyde implied that this enzyme would be efficient at fulfilling the above role. However the cells energetic requirements would still favour the NAD^+-linked dissimilation of formaldehyde, either via the formaldehyde dehydrogenase or via the cyclic dissimilatory cycle. Unfortunately the levels of these NAD^+-linked enzymes were low, even under conditions of additional formaldehyde metabolism.

In addition, there was no evidence to suggest that regulatory proteins such as the M protein described by Page and Anthony (1986) or the F protein described by Green, Millet and Dalton (unpublished) had any involvement in the regulation of methane oxidation in *M.capsulatus* (Bath). Page and Anthony (1986) reported that the ratio of M protein to methanol dehydrogenase in *Methylophilus methylotrophus* was higher in cells grown under carbon-limited conditions compared with oxygen-limited conditions. They concluded that under the former set of conditions, the M protein effectively reduced the affinity of the methanol dehydrogenase for formaldehyde. The results obtained in this project showed that the level of methanol dehydrogenase activity measured with formaldehyde was high irrespective of whether the cells were methane-limited or oxygen-limited, implying that M protein was not present under either set of conditions. If *M.capsulatus* (Bath) does possess the capacity to synthesise M-protein, then demonstration of its existence will require the growth of cells under conditions that would effect a reduction in the level of formaldehyde-catalysed methanol dehdyrogenase activity without compromising the specific activity of the enzyme with methanol. Ideally, conclusive proof will require the purification and characterization of the protein in *M.capsulatus* (Bath).

4) One regulatory reponse that was noted concerning methanol dehydrogenase activity, was the apparent repression of the enzyme's synthesis in the presence of high levels of methanol. It was concluded that under conditions of high specific growth rate, the enzyme was subject to catabolic repression. The next step in the examination of this reponse would involve the determination of the nature of the metabolite acting as the repressor. This would require repeating the experiment under different nutrient limitations such that it should be possible to manipulate the concentration of different types of intracellular metabolite. For example, Matin et al (1976) reported that the levels of several catabolic enzymes in a Pseudomonas Sp increased with decreasing dilution rate, not only under carbon-limitation, but also under nitrogen and phosphate-limitation. From this it was concluded that the catabolite repressor for these enzymes contained carbon, nitrogen and phosphorous. A similar approach could be used to examine in more detail regulation of methanol metabolism in M.capsulatus (Bath).

Although the results discussed in this study clarify several points concerning the metabolism of C_1 compounds in M.capsulatus (Bath), they also raise several new questions. Such questions will need to be answered before it is possible to obtain a more complete picture concerning the regulation of C_1 metabolism in M.capsulatus (Bath).

REFERENCES

ANTHONY, C. (1975). The microbial metabolism of C_1 compounds: The cytochromes of Pseudomonas AMI. Biochem J. 146, 289-298.

ANTHONY, C. (1978). The prediction of growth yields in methylotrophs J. Gen. Microbiol 104 91-104.

ANTHONY, C. (1982). "The Biochemistry of Methylotrophs," Academic Press, London.

ANTHONY, C (1989). Quinoproteins in C_1 dissimilation by bacteria. Anton. van Leeuwenhoek 56 13-23.

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

ANTHONY, C. and ZATMAN L.J. (1964b). The microbial oxidation of methanol. II. The methanol-oxidising enzyme of Pseudomonas M27 Biochem J. 92 614-621.

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

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

ANTRANIKIAN, G., HERZBERG, C. and GOTTSCHALK, G. (1985). In vivo phosphorylation of proteins in Clostridium spenoides. FEM Microbiology Letters 27 135-138.

ARFMAN, N., WATLING, E.M., CLEMENT, W., VAN OOSTERWIJK, DE VRIES, G.E., HARDER, W. ATTWOOD, M.M. and DIJKHUIZEN, L. (1989) Methanol Metabolism in thermotolerant methylotrophic Bacillus strains involving a novel catabolic NAD^+- dependent methanol dehydrogenase as a key enzyme. Arch. Microbiol 152 280-288.

ATTWOOD, M.M. and HARDER, W. (1978). Formate assimilation by Hyphomicrobium X. FEMS Microbiol. Lett. 3 111-114.

ATTWOOD, M. M. and QUAYLE, J.R. (1984). Formaldehyde as a central intermediary metabolite of methylotrophic metabolism. In "Microbial Growth on C, compounds. Proceedings of the 4th International Symposium" pp 315-325. ASM Washington, U.S.A.

BAMFORTH, C. W and QUAYLE, J.R. (1977). Hydroxypyruvate reductase activity in Paracoccus denitrificans. J. Gen. Microbiol. 101 259-267.

BAMFORTH, C.W. and QUAYLE, J.R. (1978). Aerobic and Anaerobic growth of Paracoccus denitrificans on methanol. Arch. Microbiol. 119 91-97.

BARNES, L.J. DROZD, J.W., HARRISON, D.E.F. and HAMER, G. (1976). Process considerations and techniques specific to protein production from natural gas. In: "Microbial Production and Utilization of Gases (H_2, CH_4, CO). Schlegel, H.G., Gottschalk, G. and Pfennig, N. eds. pp 389-402.

BARONOFSKY, J.J., SCHREURS, W.J.A. and KASHKET, E.R. (1984). Uncoupling by Acetic Acid Limits Growth and Acetogenesis by Clostridium thermoaceticum. App. Environ. Microbiol 48, 1134 - 1139.

BEARDSMORE, A. APERGHIS P.N.G. and QUAYLE, J.R. (1983). Characterization of the assimilatory and dissimilatory pathways of carbon metabolism during growth of Methylophilus methylotrophus J. Gen Microbiol. 128 1423-1439.

BELLION, E., KENT, M.E., AUD, J.C., ALIKHAN, M.Y. and BOLBOT, J.A. (1983). Uptake of methylamine and methanol by Pseudomonas strain AMI. J. Bacteriol 154 1168-1173.

BENEMANN, J.R. and VALENTINE, R.C. (1972) The pathways of nitrogen fixation. Adv Microbial Physiol. 8 59-104.

BEST, D.J. and HIGGINS, I.J. (1981). Methane - oxidising activity and membrane morphology in a methanol-grown obligate methanotroph. Methylosinus trichosporium OB3b.J. Gen. Microbiol 125 73-84.

BOLBOT, J.A. and ANTHONY, C. (1980). The metabolism of 1, 2 propanediol by the facultative methylotroph Pseudomonas AMI. J. Gen. Microbiol 120 245-254.

BONNER. W, (1955) Succinate Dehydrogenase. Methods in Enzymology 1 722-729.

BUCKLAND, B. BRIX T., FASERT, H., GBEWONYO, K. HUNT, G. and JAIN, D. (1985). Fermentation exhaust gas and analysis using mass spectometry. Bio/technology 3 982-988.

BUSSINEAU, C. M. and PAPOUTSAKIS, E.T. (1986). Intracellular reaction rates, enzyme activities and biomass yields in Methylomonas L3: growth rate and substrate composition effects. Appl. Microbiol Biotechnol. 24 435-442.

BUSSINEAU, C. M. and PAPOUTSAKIS, E.T. (1988). Evidence suggesting energy-dependent formaldehyde transport in an RuMP-type methylotroph (T15). Arch. Microbiol 149 214-219.

COBLEY, J.G. and COX, J.C. (1983). Energy Conservation in Acidophilic Bacteria. Microbiological Review 47 579-595.

COLBY, J and ZATMAN, L.J. (1972). Hexulose Phosphate Synthase and T.C.A. Cycle enzymes in bacterium 486. Biochem. J. 128 1373-1376.

COLBY, J and ZATMAN, L.J. (1975). Enzymological aspects of the pathways of trimethylamine oxidation and C, assimilation in obligate methylotrophs and restricted facultative methylotrophs. Biochem. J. 148 513-520.

COLBY, J and DALTON, H. (1976). Some properties of a soluble methane monooxygenase from Methylococcus capsulatus strain Bath. Biochem. J. 157 495-497.

COLBY, J and DALTON, H. (1978). Resolution of the methane monooxygenase of Methylococcus capsulatus (Bath) into three components. Purification and properties of component C, a flavoprotein. Biochem J. 171 461-468.

COLBY, J and DALTON, H. (1979). Characterization of a second prosthetic group of the flavoenzyme NADH - acceptor reductase (component C) of the methane monooxygenase from Methylococcus capsulatus (Bath). Biochem J. 177 903-908.

COLBY, J., STIRLING D.I. and DALTON, H. (1977). The soluble methane monooxygenase of Methylococcus capsulatus (Bath). Its ability to oxygenate n-alkanes, n-alkenes, ethers and alicyclic aromatics and heterocyclic compounds. Biochem. J. 395-402.

COLBY, J., DALTON, H and WHITTENBURY, R. (1979). Biological and biochemical aspects of microbial growth on C, compounds. Ann. Rev. Microbiol 33 481-517.

CORNISH, A., NICHOLLS, K. M., SCOTT, D. HUNTER, B.K., ASTON, W.J., HIGGINS, I.J. and SANDERS, J.K.M. (1984). In Vivo ^{13}C NMR Investigations of methanol oxidation by the obligate methanotroph Methylosinus trichosphorium OB3b. J. Gen. Microbiol 130 2565-2575.

DALTON, H. (1977). Ammonia oxidation by the methane oxidising bacterium Methylococcus capsulatus strain (Bath). Arch Microbiol. 114 273-279.

DALTON, H. (1980). Oxidation of hydrocarbons by methane monooxygenase from a variety of microbes. Adv. Appl. Microbiol 26 71-87.

DALTON, H and WHITTENBURY, R. (1976). The acetylene reduction technique as an assay for the nitrogenase activity in the methane-oxidising bacterium Methylococcus capsulatus. Arch Microbiol 109 147-151.

DALTON, H and LEAK, D.J. (1985). In "Microbial Gas Metabolism : Mechanistic, Metabolic and Biotechnological Aspects" 173-200. Eds. Poole, R.K. and Dow, C.S., SGM. Pub.

DALTON, H and HIGGINS, I.J. (1987). Physiology and biochemistry of methylotrophic bacteria. Anton. van Leeuwenhoek 53 23-28.

DALTON, H., PRIOR, S.D., LEAK, D.J. and STANLEY, S.H. (1984). Regulation and control of methane monooxygenase. In : "Microbial Growth on C-1 compounds. Proceedings for the 4th International Symposium" pp 75-82 Eds. Crawford, R.L. and Hanson, R.S. ASM Washington U.S.A.

DAVEY, J.F., WHITTENBURY, R. and WILKINSON, J.F. (1972). The distribution in methylotrophs of some key enzymes of intermediate metabolism. Arch. Microbiol 87 359-366.

DAWSON, M.J. and JONES, C.W. (1981a). Chemiosmotic aspects of respiratory chain energy conservation in Methylophilus methylotrophus in "Microbial Growth on C. compounds" 251-257. Ed. Dalton, H. Heyden and Sons Ltd., London.

DAWSON, M.J. and JONES, C.W. (1981b). Respiration-linked proton translocation in the obligate methylotroph Methylophilus methylotrophus Biochem. J. 194 915-924.

DEAN, A.C.R. (1972). Influence of environment on the control of enzyme synthesis J. App. Chemistry and Biotechnology 22 245-259.

VAN DIJKEN, J.P. and HARDER, W. (1975). Growth yield of microorganisms on methanol and methane: a theoretical study. Biotechnol. Bioeng. 17 15-30.

DIJKHUIZEN, L., WIERSMA, M and HARDER, W. (1977). Energy production and growth of Pseudomonas oxalaticus OXI on oxalate and formate. Arch Microbiol 115 229-236.

DIJKHUIZEN, L., TIMMERMAN, J.W.C. and HARDER, W. (1978) A pyridine nucleotide-independent membrane bound formate dehydrogenase in Pseudomonas oxalaticus OXI. FEMS Microbiol. Letts 6 53-56.

DIJKHUIZEN, L. KNIGHT, M. and HARDER, W. (1978). Metabolic regulation in Pseudomonas oxalaticus OXI. Autotrophic and heterotrophic growth on mixed substrates. Arch. Microbiol. 116 77-83.

DIJKHUIZEN, L and LEVERING, P.R. (1987). Metabolic regulation in facultative methylotrophs. In "Microbial Growth on C, compounds". 95-104, Eds van Verseveld, H.W. and Duine, J.A. Martinus Nijhoff, Dordecht.

DIWAN, A.R., CHU, I-M and PAPOUSAKIS, E.I. (1983). Substrate transport and its effect on the dynamics of methylotrophic growth. Biotech. Letts. 5 579-584.

DROZD, S.W., LINTON, J.D., PORTER, N. and RATCLIFFE, H.D. (1984). Continuous culture as a tool in the study of growth energetics of aerobic bacteria. In "Continuous Culture 8; Biotechnology, Medicine and the Environment" pp 239-250 Eds. Dean A.C.R., Ellwood, D. C. Evans, C.G.T. Pub. ellis Horwood Ltd.

DUINE, J.A. FRANK, J and De RUITER, L.G. (1979). Isolation of a methanol dehydrogenase with a functional coupling to cytochrome c. J. Gen. Microbiol 115 523-526.

DUINE, J.A. FRANK, J. and De RUITER, L.G. (1980). The prosthetic group of methanol dehydrogenase. Purification and some of its properties. Eur. J. Biochem 108 187-192.

DUINE, J.A., FRANK, J. and BERKHOUT. M.P.J. (1984). NAD^+ -dependant, PQQ-containing methanol dehydrogenase in a multienzyme complex. FEBS Letts. 168 217-221.

DUINE, J.A., FRANK, J and DIJKSTRA, M. (1987). Quinoproteins in the dissimilation of C, compounds. in: "Microbial Growth on C, compounds. Proceedings of 5th International Symposium" 105-112. Van Verseveld, H.W. and Duine, J.A. eds. Martinus Dortrecht.

DUINE, J.A. (1989). PQQ and quinoproteins: an important novel field in enzymology. Ant. Van Leeuwenhoek 56 3-12.

DUNSTAN, P.M., ANTHONY, C and DRABBLE, W.T. (1972). Microbial metabolism of C_1 and C_2 compounds. The role of glyoxylate, glycollate and acetate in the growth of Pseudomonas AMI on ethanol and on C_1 compounds. Biochem J. 128 107-115.

DWORKIN, M and FOSTER, J.W. (1956). Studies on Pseudomonas methanica (Söhngen). Nov. Comb. J. Bacteriol. 72 646-659.

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

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

EGGELING, L. and SAHM, H. (1985). The formaldehyde dehydrogenase of Rhodococcus erythropolis, a trimeric enzyme requiring a cofactor and active with alcohols. Eur. J. Biochem. 150 129-134.

EHHALT, D.H. (1976). In: "Microbial Production and Utilization of Gases." pp 13 Ed. Schegel H. et al, Goltze, Gottingen, W. Germany.

EGOROV, A.M. AVILOV, T.V. DIKOV, M.M., POPOV., Y.V. and BEREZIN, I.V. (1979). NAD^+ - dependent formate dehydrogenase from Methylotrophic bacterium strain I. Eur. J. Biochem 99 569-576.

FERENCI, T. (1974). Carbon monoxide stimulated respiration in methane utilizing bacterium. FEBS Letts 41 94-98.

FERENCI, T. STROM, T. and QUAYLE, J.R. (1974). Purification and properties of 3-hexuloisomerase from Methylococcus capsulatus (Bath). Biochem J. 144 477-486.

FERENCI, T., STROM, T. and QUAYLE, J.R. (1975). Oxidation of carbon monoxide and methane by Pseudomonas methanica. J. Gen. Microbiol 91 79-91.

FORD, S., PAGE, D.M. and ANTHONY, C. (1985). The role of a methanol dehydrogenase modifier protein and aldehyde dehydrogenase in the growth of Pseudomonas AMI on 1, 2 - propandiol. J. Gen. Microbiol 131 2173-2182.

FORREST, W.W. and WALKER, D.J. (1971). The generation and utilization of energy during growth. Adv. Microbiol Physiol 5 213-274.

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

GALCHENKO, V.F. and ANDREEV, L.V. (1984). Taxonomy of obligate methanotrophs. In: "Microbial Symposium" pp 269-275. American Society for Microbiology Washington, U.S.A.

GREEN, J. and DALTON, H. (1985). Protein B of soluble methane monooxygenase from Methylococcus capsulatus (Bath). J. Biol. Chem. 260 15795-15801.

GREEN, J. and DALTON, H. (1986). Steady-state kinetic analysis of soluble methane monooxygenase from Methylococcus capsulatus (Bath). Biochem J. 236 155-162.

GREEN, J. and DALTON, H. (1988). The biosynthesis and assembly of protein A of soluble methane monooxygenase of Methylococcus capsulatus (Bath). J. Biol Chem 263 17561-17565.

GREEN, J. and DALTON, H. (1989). A stopped-flow kinetic study of soluble methane monooxygenase from Methylococcus capsulatus (Bath). Biochem J. 259 167-172.

GREEN, J., PRIOR, S.D. and DALTON, H. (1985). Copper ions as inhibitors of protein C of soluble methane monooxygenase of Methylococcus capsulatus (Bath). Eur. J. Biochem 153 137-144.

GREENWOOD, J.A. and JONES, C.W. (1986). Environmental regulation of the methanol oxidase system of Methylophilus methylotrophus. J. Gen. Microbiol 132 1247-1256,.

GOLD, T. (1979). Terrestrial sources of carbon and earthquake outgassing. J. Petroleum Geology 1 3-19.

GOLDBERG, I., ROCK, J.S., BEN-BASSAT, A and MATELES, R. I. (1976). Bacterial yields on methanol, methylamine, formaldehyde and formate. Biotechnol. Bioeng. 18 1657-1668.

HARDER, W. and DIJKHUIZEN, L. (1983). Physiological responses to nutrient limitation. Ann. Rev. Microbiol 37 1-23.

HARDER, W. and VAN DIJKEN, P.J. (1976). Theoretical considerations of the relation between energy production and growth of methane utilizing bacteria. In: "Microbial Production and Utilization of Gases" pp 403-418 eds. Schlegel, H.G. Gottschalt, G and Pjennig, N. Akademie der Wissenschaften, Gottingen.

HARDER, W., VAN DIJKEN, J. P. and ROELS, J.A. (1981). Utilization of energy in methylotrophs. In: "Microbial Growth on C, compounds" pp258-269 ed. Dalton, H.

HARRISON, D.E.F., TOPIWALA, H.H. and HAMER, G. (1972). Yield and productivity in single cell protein production from methane and methanol. In: "Fermentation Technology Today." pp491-495, ed. G. Terui, Soc. of Fermentation Technology Japan.

HARRISON, D.E.F. Physiological effects of dissolved oxygen tension and redox potential on growing populations of microorganisms. J. Appl. Chem. Biotecnol. 22 417-440.

HARRISON, D.E.F. (1976). The regulation of respiration rate in growing bacteria. In: "Advances in Microbial Physiology" 14 243-313.

HARRISON, D.E.F. and CHANCE, B. (1970). Fluorimetric technique for monitoring changes in the level of reduced nicotinamide nucleotides in continuous cultures of microorganisms. App. Microbiology 19 446-450.

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

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

HERRERO, A.A., GOMEZ, R.F., SNEDECOR, B. TOLMAN, C.J. and ROBERTS, M.F. (1985). Growth inhibition of Clostridium thermocellum by carboxylic acids: a mechanism based on uncoupling by weak acids. App. Microbiol Biotechnol 22 53-62.

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

HIGGINS, I.J., BEST, D.J., HAMMOND, R.C. and SCOTT, D. (1981). Methane-oxidising microorganisms. Microbiological Reviews. 45 556-590.

HIRT, W., PAPOUTSAKIS, E., KRUG, E., LIM, H.C. and TSAO, G.T. (1978). Formaldehyde incorporation by a new methylotroph (L3). App. Environ. Microbiology 36 56-62.

HOOPER, A.B., TERRY, K.R. and MAXWELL, P.C. (1977) Hydrooxylamine oxidoreductase of Nitrosomonas. Oxidation of diethyldithiocarmanate concomitant with the stimulation of nitrite synthesis. Biochem. Biophys. Acta 462 141-152.

HORECKER, B.L. and SMYRNIOTIS, P.Z. (1955). 6-Phosphogluconate dehydrogenase. Methods in Enzymology 1 323-327.

HOU, C.T., LASKIN, A.I. and PATEL, R. (1978). Growth and polysaccaride production by Methylocystis parvus OBBP on methanol. App. Environ. Microbiol 37 800-804.

HYDER, S.L., MEYERS, A and CAYER, M.L. (1979). Membrane modulation in a methylotrophic bacterium. Methylococcus capsulatus (Texas) as a function of growth substrate. Tissue and Cell 11 597-610.

JOERGENSEN, L. (1985). The methane mono-oxygenase reaction system studied in vivo by membrane-inlet mass spectrometry. Biochem J. 225 441-448.

JOHNSTON, P.A. and QUAYLE, J.R. (1964). Oxidation of C, compounds by Pseudomonas AMI. Biochem J. 93 281-290.

JONES, C.W., GREENWOOD, J.A., BURTON, S.M., SANTOS, H. and TURNER, D.L. (1987) Environmental regulation of methanol and formaldehyde metabolism by Methylophilus methylotrophus. J. Gen. Microbiol 133 1511-1519.

JORDAN, P.M. and AKHTAR, M. (1970). The mechanism of action of serine transhydroxmethylase. Biochem. J. 116 277-286.

KAUFMAN, S. (1962). Phenylalanine hydroxylase. Methods in Enzymology 5 809-816.

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

KOMANO, J., BROWN, N., INOUYE, S. and INOUYE, M. (1982). Phosphorylation and methylation of proteins during Myxococcus xanthus spore formation. J. Bacteriol. 151 114-118.

KORNBERG, A and HORECKER, B.L. (1955). Glucose-6-phosphate dehydrogenase. Methods in Enzymology 1 323-327.

LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. T4 Nature. 227 680-689.

LANG, E. and LANG, H. (1972). Spezifche Farbreaktion Zum direkten Nachweis der Ameisensaure. Zeitschrift fur analytische Chemie. 260 8-10.

LARGE, P.J. and BAMFORTH, C.W. (1988). In: "Methylotrophy and Biotechnology". Longman Scientific and Technical.

LARGE, P.J. and QUAYLE, J.R. (1963). Microbial growth on C. compounds. 5. Enzyme activities in extracts of Pseudomonas AMI. Biochem. J. 87 386-396.

LARGE, P.J., PEEL, D. and QUAYLE, J.R. (1961). Microbial growth on methanol and formate. Biochem. J. 81 470-479.

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

LEADBETTER, E.R. and FOSTER, J.W. (1958). Studies on some methane utilizing bacteria. Arch fur Mikrobiologie. 30 91-118.

LEAK, D.J. and DALTON, H. (1983) In Vivo studies of primary alcohols, aldehydes and carboxylic acids as electron donors for the methane monooxygenase in a variety of methanotrophs. J. Gen. Microbiol 129 3487-3497.

LEAK, D.J. and DALTON, H. (1986a). Growth yields on methanotrophs. I. Effect of copper on the energetics of methane oxidation. App. Microbiol. Biotechnol 23 470-476.

LEAK, D.J. and DALTON, H. (1986b). Growth yields on methanotrophs. 2. A theoretical Study. App. Microbiol. Biotechnol. 23 477-481.

LEAK, D.J. STANLEY, S.H. and DALTON, H. (1985). Implications of the nature of MMO on carbon assimilation in methanotrophs, In: "Microbial Gas Metabolism-Mechanistic Metabolic and Biological Aspects". pp201-208. Eds. Poole, R.K. and Dow, C.S.

LINTON, J.D. and VOKES, J. (1978). Growth of methane utilizing bacterium Methylococcus NCIB 11083 in mineral salts medium with methanol as the sole source of carbon. FEMS Microbiol Letts 4 125-128.

LLOYD, D. and SCOTT, R.I. (1985). Mass spectrometric monitoring of dissolved gases. In: "Microbial Gas Metabolism, Mechanistic, Metabolic and Biotechnological Aspects" pp 239-262 eds. Poole, R.K. and Dow, C.S.

LONDON, J. and KNIGHT, M. (1966). Concentrations of nicotinamide nucleotide coenzymes in microorganisms. J. Gen. Microbiol 44 241-254.

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

LOWRY, O.H., PASSONNEAU, J.V. and ROCK, M.K. (1961). The stability of pyridine nucleotides, J. Biol. Chem. 236 2756-2759.

LUND, J and DALTON, H. (1985). Further characteristics of the FAD and Fe_2S_2 redox centres of component C, the NADH: acceptor reductase of soluble methane monooxygenase of Methylococcus capsulatus (Bath). Eur. J. Biochem 147 291-296.

LUND, J. WOODLAND, M.P. and DALTON, H. (1985). Electron transfer reations in the soluble methane monoxygenase of Methylococcus capsulatus (Bath). Eur. J. Biochem 147 297-305.

MARISON, I.W. (1980). Possible Mechanism(s) of formaldehyde oxidation and regulation of metabolism in Hyphomicrobium X PhD Thesis, University of Sheffield.

MARISON, I.W. and ATTWOOD, M.M. (1980). Partial purification and characterization of a dye-linked formaldehyde dehydrogenase from Hyphomicrobium X. J. Gen. Microbiol. 17 305-313.

MARISON, I.W. and ATTWOOD, M.M. (1982). A possible alternative mechamism for the oxidation of formaldehyde to formate J. Gen. Microbiol 128 1441-1446.

MARTIN, B.R. (1987). In: "Metabolic Regulation: A Molecular Approach", Blackwell Scientific Publications.

MATIN, A. GROOJANS, A. and HOGENHUIS, H. (1976). Influence of dilution rate on enzymes of intermediary metabolism in two freshwater bacteria grown in continuous culture. J. Gen. Microbiol. 94 323-332.

MULLER, U., WILLNOW, P., RUSCHIG, U. and HOPFNER, T. (1978). Formate dehydrogenase from Pseudomonas oxalaticus Eur. J. Biochem 83 485-498.

MURRELL, J.C. and DALTON, H. (1983). Nitrogen fixation in obligate methanotrophs. J. Gen. Microbiol 129 3481-3486.

NASH (1953). The colourimetric estimation of formaldehyde by means of the Hanzsch reaction. Biochem. J. 55 416-421.

NEIJSSEL, O.M. and TEMPEST, D.W. (1976). The role of energy-spilling reactions in the growth of Klebsiella aerogenes NCTC in aerobic chemostat cultures. Arch. Microbiol 110 305-311.

NEWAZ, S.S. and HERSH, L.R. (1975). Reduced nicotinamide adenine dinucleotide - activated phosphoenolpyruvate carboxylase in Pseudomonas MA: Potential regulation between carbon assimilation and energy production. J. Bact. 124 825-833.

NIMMO, H.G. (1984). Control of Escherichia coli Isocitrate dehydrogenase: an example of protein phosphorylation in the prokaryote. TIBS (November, 1984) 475-478.

O'CONNOR, M.L. (1981). Regulation and genetics in facultative methylotrophic bacteria. In: "Microbial Growth on C, - compounds". pp 294-300. Ed Dalton, H., Heyden and Sons Ltd.

O'KEEFFE, D.T. and ANTHONY, C. (1978). The microbial metabolism of C, compounds. The stoichiometry of respiration driven proton translocation in Pseudomonas AMI and in mutant lacking cytochrome C. Biochem. J. 170 561-567.

O'KEEFFE, D.T. and ANTHONY, C. (1980). The interaction between methanol dehydrogenase and the autoreducible cytochromes c of the facultative methylotroph Pseudomonas AMI. Biochem. J. 190 481-484.

O'NEIL, J.G. and WILKINSON, J.F. (1977). Oxidation of ammonia by methane-oxidising bacteria and the effects of ammonia on methane oxidation. J. Gen. Microbial 100 407-412.

VAN OPHEM, P.W. and DUINE, J.A. (1989). Three different formaldehyde dehydrogenase activities in Nocardia sp 239. Poster presented at 6th International Symposium on Microbial Growth on C, compounds, Gottingen, FRG.

OTHOMO, T., IIZUKA, H. and TAKEDA, K. (1977). Effect of copper sulfate on the morphogenic transition of a strain of Methonomonas margaritae. Eur. J. Microbiol 4 267-272.

PAGE, D.M. and ANTHONY, C. (1986). Regulation of formaldehyde oxidation by methanol dehydrogenase, modifier proteins of Methylophilus methylotrophus and Pseudomonas AMI. J. Gen. Microbiol 132 1553-1563.

PATEL, R.N. HOU, C.T. LASKIN, A.I. and FELIX, A. (1982). Microbial oxidation of hydrocarbons: properties of a soluble methane monooxygenase from a facultative methane-utilizing organism Methylobacterium Sp. Strain CRL-26. Appl. Environ. Microbiol. 44 1130-1137.

PEARCE, F. (1989). Methane: the hidden greenhouse gas. New Scientist 6th May, 1989 pp37 - 41.

PETERSON, G.L. (1977). a simplification of the protein assay method of Lowry et al, which is more generally applicable. Analytical Biochemistry 83 346-356.

PIRT, S.J. (1975). In "Principles of Microbe and Cell Cultivation" Blackwell Scientific Publications, Oxford, pp 63-80.

PRIOR, S.D. (1985). Effect of copper ions on methane oxidation by the obligate methylotroph Methylococcus capsulatus (Bath). PhD. Thesis, University of Warwick.

PRIOR, S.D. and DALTON, H. (1985). The effect of copper ions on membrane content and methane monooxygenase activity in methanol-grown cells of Methylococcus capsulatus (Bath). J. Gen. Microbiol 131 155-163.

RATNER, S. (1955). Glycine oxidase. Methods in Enzymology 2 225-227.

RABINOWITZ, J.C. and PRICER, W.E. (1963). Formyl-tetrahydrofolate synthetase. Methods in Enzymology 6 375-376.

REED, H.L. (1976). A study of certain unusual biochemical and physiological properties of obligate methane-utilizing bacteria. PhD. Thesis, University of Warwick.

RIBBONS, D.W. (1975). Oxidation of C_1 compounds by particulate factions from Methylococcus capsulatus: distribution and properties of methane dependent reduced nicotinamide adenine dinucleotide oxidase (methane hydroxylase) J. Bacteriol 122 1351-1363.

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

RICHARDS, A., STANLEY, S.H. and DALTON, H. (1987) On-line mass spectrometry in the analysis of eight gases during the microbial oxidation of gaseous hydrocarbons. In: "Mass Spectrometry in Biotechnology Process Analysis and Control", pp 163-177. Eds. Heinzle, E. and Reuss, M.; Phelum Press J.S.

RODINOV, Y.V. and ZAKHAROVA, E.U. (1980). Two pathways of formate oxidation in methylotrophic bacteria. Biochem (USSR) 45 654-661.

ROKEM, J.S. and GOLDBERG, I. (1984). The application of continuous culture in the study of the biological utilization of C, compounds. In: "Continuous Culture, Biotechnology, Medicine and the Environment" pp 251-265 Eds. Dean, A.C.R. and Evans, C.G.I. Pub. Ellis Horwood Ltd.

SCRIMGEOUR, K.G. and HUENNEKENS, F.M. (1963). 5, 10 - Methylene tetrahydrafolic dehydrogenase. Methods in Enzymology 6, 368-372.

SCOTT, D., BRANNAN, J and HIGGINS, I.J. (1981). The effect of growth conditions on intracytoplasmic membranes and methane monooxygenase activities in Methylosinus trichosporium OB3b. J. Gen. Microbiol. 125 63-72.

SMITH, D.D.S. and DALTON, H. (1989). Solubilisation of methane monooxygenase from Methylococcus capsulatus (Bath). Eur. J. Biochem. 182 667-671.

SMITH, A.J., LONDON, J. and STANIER, R.Y. (1967). Biochemical basis of obligate autotrophy in blue-green algae and Thiobacilli J. Bacteriol 94 972-983.

SPERL, G.T., FORREST, H.S. and GIBSON, D.T. (1974). Substrate specificity of the purified primary alcohol dehydrogenases from methanol-oxidising bacteria J. Bacteriol. 118 541-550.

SÖHNGEN, N.L. (1906). Ueber bakterion, welchmethan als kohlenstaff nahrung und energiequelle gebrauchen. Zentl. Bakt. Parikade. Abte II, 15 513-517.

STANLEY, S.H. (1977). A study of the physiology and gas-limited growth of Methylococcus capsulatus PhD. Thesis, University of Warwick.

STANLEY, S.H. and DALTON, H. (1982). Role of ribulose 1, 5, bisphosphate carboxylase/oxygenase in Methylococcus capsulatus (Bath). J. Gen. Microbiol. 128 2927-2935.

STANLEY, S, H., PRIOR, S.D., LEAK, D.J. and DALTON, H. (1983). Copper stress underlies the fundamental change in intracellular location of methane monooxygenase in methane oxidising organisms. Studies in batch and continuous culture. Biotechnology Lett. 5 487-492.

STIRLING, D.I. (1978). Oxidation of carbon compounds by Methylococcus capsulatus. PhD Thesis, University of Warwick.

STIRLING, D. I. and DALTON, H. (1978). Purification and properties of an NAD(P)+- linked formaldehyde dehydrogenase from Methylococcus capsulatus (Bath). J. Gen. Microbiol 107 19-29.

STIRLING, D.I. and DALTON, H. (1977). Effect of metal binding agents and other compounds on methane oxidation by two strains of Methylococcus capsulatus. Arch. Microbiol 114 71-76.

STOCKS, P.K. and McCLESKEY, C.S. (1964). Morphology and physiology of Methylomonas methanooxidans. J. Bacteriol 88 1071-1077.

STROM, T, FERENCI, T. and QUAYLE, J.R. (1974). The carbon assimilation pathways of Methylococcus capsulatus, Pseudomonas methanica and Methylosinus trichosporium during growth on methane. Biochem. J. 144 465-476.

SUZUKI, M. (1988). Development of a process for the production of propylene oxide in methane-oxidising bacteria. PhD. Thesis, University of Warwick.

TAKEDA, K. and TAKANA, K. (1980). Ultrastructure of intracytoplasmic membranes of Methanomonas margaritae cells grown under different conditions. Anton. van Leeuwenhoek 46 15-25.

TAYLOR, S.C. (1977). Evidence for the presence of ribulose 1, 5 bisphosphate carboxylase and phosphoribulokinase in Methylococcus capsulatus (Bath). FEMS Microbiol Letts. 2 305-307.

TAYLOR, S.C. DALTON, H and DOW, C.S. (1981). Ribulose, 1,5 bisphosphate carboxylase/oxygenase and carbon assimilation in Methylococcus capsulatus (Bath). J. Gen. Microbiol. 122 89-94.

TONGE, G.M., HARRISON, D.E.F., KNOWLES, C.J. HIGGINS, I.J. (1975). Properties and partial purification of the methane oxidising enzyme system from Methylosinus trichosporium FEBS Letts. 58 293-299.

TONGE, G.M., HARRISON, D.E.F. and HIGGINS, I.J. (1977). Purification and properties of the methane monooxygenase enzyme system from Methylosinus trichosporium OB3b. Biochem. J. 161 333-344.

TROTSENKO, Y.A. (1976). Isolation and characterization of obligate methanotrophic bacteria. In: "Microbial Production and Utilization of Gases" pp 3 29-336 ed. Schlegel, H. G., Gottschalk, G. and Pfennig, N. Gottingen; Goltze.

TURNER, A.M. and MANN, N.H. (1986). Protein phosphorylation in Rhodomicrobium vannielli. J. Gen. Microbiol. 132 3433-3440.

TURNER, A.M. and MANN, N.H. (1989). Protein kinase activities in cell-free extracts of Rhodomicrobium vanielli. FEMS Microbiol Letts 57 301-305.

VAN VERSEVELD, H.W. and STOUTHAMER, A.H. (1978). Growth yield and efficiency of growth during autotrophic growth of Paracoccus denitrificans on methanol and formate. Arch. Microbiol. 118 21-26.

WADZINSKI, A.M. and RIBBONS, D.W. (1975). Oxidation of C, compounds by particulate fractions of Methylococcus capsulatus: properties of methanol oxidase and methanol dehydrogenase. J. Bacteriol 122 1364 -1374.

WALKER, J.F. (1964). "Formaldehyde" 3rd ed. Reinhold Pub. Corp., New York.

WANG, H.Y., COONEY, C.L. and WANG, D.I.C. (1979). On-line gas analysis for material balances and control. In: Biotechnology Bioengineering Symposium No. 9". J. Wiley and Sons Inc.

WHITTENBURY, R. COLBY, J. DALTON, H and REED, H.L. (1975). Biology and ecology of methane oxidisers. In: "Microbial Production and Utilization of Gases" pp 281-292, eds Schlegel, H.G., Gottschalt, G and Pfennig, N.; Gottingen: Goltze.

WHITENBURY, R and DALTON, H. (1981). The methylotrophic bacteria. In: "The Prokaryotes" pp 894-902 eds. Starr, M.P. et al. Springer Verlag, Heidelberg.

WHITTENBURY, R., PHILLIPS, K.C. and WILKINSON, J.F. (1970a). Enrichment, isolation and some properties of methane-utilizing bacteria. J. Gen. Microbiol. 61 205-218.

WHITTENBURY, R. DAVIES, S.L. and DAVEY, J.F. (1970b). Exospores and cysts of methane utilizing bacteria J. Gen. Microbiol 61 214-226.

WIMPENNY, J.W.T. and FIRTH, A. (1972). Levels of nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide in facultative bacteria and the effect of oxygen. J. Bacteriol. 111 24-32.

WINTER, M.J. (1987). The application of single detector magnetic sector mass-spectrometer systems in fermentation off-gas and liquid analysis. In: "Mass spectrometry in Biotechnological Process Analysis and Control" pp 17-38 eds Heinzle, E.H. and Reuss, M.

WOOD, A.P. and KELLY, D.P. (1981). Mixotrophic growth of Thiobacillus A2 in chemostat culture on formate and glucose. J. Gen. Micro 125 55-62.

WOODLAND, M.P. and DALTON, H. (1984a). Purification and properties of component A of the methane monooxygenase from Methylococcus capsulatus (Bath). J. Biol. Chem. 259 53-59.

WOODLAND, M.P. and DALTON, H. (1984b). Purification of component A of the methane monooxygenase of Methylococcus capsulatus (Bath). by high-pressure gel permeation chromatography. Anal. Biochem. 139 459-462.

ZATMAN, L.J. (1981). A Search for Patterns in Methylotrophic Pathways. In: "Microbial Growth on C, compounds" pp 42-45 ed Dalton, H. Heyden and Sons Ltd., London.

ZENDER, A.J.B. and BROCK, T.D. (1979). Methane formation and Methane oxidation by Methanogenic bacteria. J. Bacteriol. 137 420-432.