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THE BACTERIAL METABOLISM OF PROPANE

BY

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This thesis is presented for the Degree of Doctor of Philosophy, in the Department of Biological Sciences, University of Warwick.

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Dedication

To Mum and Dad, who made it all possible;
Kate, who showed me the path, and Julie,
who kept me on it.

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My special thanks go to my excellent supervisor Dr. J. Colin Murrell without whose continual harassment, cajouling and faith this would not have been; to Bill, Don and Chris for their combined lunacy; and to Simon, Jeff and Gaz for all their help and advice.

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Declaration

The work contained in this thesis was the result of original research conducted by myself under the supervision of Dr. J. C. Murrell. All sources of information have been specifically acknowledged by means of reference.

None of the work contained in this thesis has been used in any previous application for a degree.

N R Wood.

Summary

The bacterial metabolism of propane

A range of enrichment/isolation procedures yielded over 80 strains of Gram-positive propane-utilizing bacteria from a variety of environments. All appeared to be members of the Corynebacterium-Mycobacterium-Nocardia complex. No Gram-negative organisms were isolated and screening of Pseudomonas spp. culture collections failed to isolate any gaseous alkane-utilizing strains. Three of the isolated strains, identified as Rhodococcus rhodochrous, R. erythropolis and a Mycobacterium sp., were subjected to further analyses. They showed differing specificities towards gaseous alkane substrates, R. rhodochrous growing only on propane, the Mycobacterium sp. on ethane and propane, and R. erythropolis on all three. None could grow on alkenes but all could epoxidate propene to 1,2-epoxypropane after growth on propane.

R. rhodochrous (designated strain PNKb1) was selected for detailed study. Its potential to epoxidate alkenes was investigated further. Attempts to grow the organism in steady-state, continuous culture on propane were unsuccessful. It grew batchwise on most of the putative intermediates of propane metabolism. Simultaneous adaptation studies using whole cells suggested that the organism had the potential to use either the terminal or subterminal pathways of propane metabolism.

SDS-polyacrylamide gel electrophoresis revealed proteins of molecular weight 67, 59, 57 kDal specific to cells grown on propane, which may be components of the propane-oxidizing system. Oxygenase activity induced by propane, was studied in whole cell and cell-free systems and results suggest that it may be different in nature to those previously described alkane oxygenase systems.

The enzyme complement of propane-grown cells suggested that propane could be assimilated by either terminal or subterminal oxidation pathways and the relative importance of each remains unclear.

Abbreviations

ABER	Mineral salts medium of Stephens and Dalton (1987b)
AMS	Ammonium mineral salts medium
AMMSV	Ammonium/nitrate mineral salts medium plus vitamins
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CoA	Coenzyme A
CoASH	Coenzyme A, reduced form
CMN-complex	*Corynebacterium-Mycobacterium-Nocardia* complex
DCPIP	Dichlorophenolindophenol
DEAE-cellulose	Diethylaminoethylcellulose
EDTA	Ethylene diamine tetraacetic acid
E_{540nm}	Optical density at 540nm
FAD	Flavin adenine dinucleotide
FID	Flame ionisation detector
GC	Gas chromatography
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
id	Internal diameter
kDal	Kilodaltons
K_m	Michaelis constant
MOPS	3-(N-morpholino) propane sulphonic acid
MS	Mineral salts medium of Whittenbury *et al.* (1970)
$NAD(P)^+$	Nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) reduced form
NCIMB	National Collection of Industrial and Marine Bacteria
NCPPB	National Collection of Plant Pathogenic Bacteria

NMR	Nuclear magnetic resonance
NM$_3$	Nitrate mineral salts medium
Pa	Pascals
PAGE	Polyacrylamide gel electrophoresis
PI	Pirt's mineral salts medium, Pirt (1985)
PMS	Phenazine methosulphate
PMSF	Phenyl methyl sulphonyl fluoride
ppm	Parts per million
psi	Pounds per square inch
rpm	Revolutions per minute
RMP pathway	Ribulose monophosphate pathway
SDS	Sodium dodecyl sulphate
TCA cycle	Tricarboxylic acid cycle
TES	N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid
TRIS	Tris (hydroxymethyl)-methylamine
$T_{\frac{1}{2}}$	Half life
UV	Ultra violet
v/v	Concentration, volume by volume
w/v	Concentration, weight by volume
w/w	Concentration, weight by weight
λ_{max}	Wavelength of maximum absorption

A man would do nothing,
if he waited until he could do it so well
that no one would find fault
with what he has done.

Cardinal Newman

CHAPTER 1

INTRODUCTION

1.1 Historical perspective

From the initial observations of Miyoshi (1895) and Sohngen (1906), which mark the birth of hydrocarbon microbiology, has grown a wide and varied field of investigation which is probably receiving as much attention now as it ever has.

It was in the late 1950's that a rapid rise in interest began. Dworkin and Foster (1956) reisolated the lost Sohngen strain (renaming it Pseudomonas methanica) and began investigating the metabolism of methane in this organism. At about the same time, Davis et al (1956) began investigating the metabolism of ethane by Mycobacterium paraffinicum and similar work was being carried out in the Soviet Union (e.g. Kuznetsov and Telegina, 1957).

The 1960's with its relatively low cost petroleum hydrocarbons saw interest split between the fundamental aspects of understanding hydrocarbon metabolism and the more applied aspects particularly with regard to the production of single cell protein (e.g. Champagnat et al., 1963).

This applied interest waned somewhat in the 1970's with the increasing cost of petroleum feedstocks. Fundamental studies began investigating the biochemistry of oxygenase enzymes (e.g. Cardini and Jurtshuk, 1970; Colby and Dalton, 1976) and their potential use in biotransformation processes became recognised.

The Torrey Canyon disaster in 1967 was seen by some authors (e.g. Atlas, 1984, Wyatt, 1984) as the start of more ecological studies on how microorganisms are affected by hydrocarbons in the environment as well

as how they might be applied to "clean-up" after such events.

The 1980's has seen the arrival of molecular biology into hydrocarbon microbiology (e.g. Owen et al., 1984). Interest is now directed at understanding the genetics and regulation of hydrocarbon degradation. It is no coincidence that the first genetically engineered organism to be patented was a petroleum degrading strain. The interaction of microorganisms with hydrocarbons is also of considerable interest in the 1980's as thoughts turn to the increasingly difficult extraction of fossil fuels and the use of microbially enhanced oil recovery (e.g. Finnerty and Singer, 1983).

As our understanding of the molecular biology of these hydrocarbon-utilizing organisms increases no doubt new applications will be found. Of particular interest in the immediate future will be roles in the syntheses of pharmaceuticals, enzyme electrodes and environmental pollution monitoring and control (see May and Padgette, 1983).

Throughout this development of hydrocarbon microbiology interest in n-alkane metabolism has always focused on methane oxidation and liquid alkane oxidation, probably due to their abundance as potential feedstocks. The higher gaseous alkanes (ethane, propane and butane) seem to have received scant attention giving rise to such comments as; "gaseous alkanes are not readily utilized by hydrocarbonoclastic organisms" (Ratledge, 1978) and; "Relatively few bacteria have the ability to grow on alkanes shorter than n-octane" (Wyatt, 1984). Perry (1980) points out that although this may be true in part, "there are some misconceptions as to the relative number of hydrocarbon utilizers that can grow on gaseous alkanes based on substrate specificity tests in

which the gaseous alkanes often were not included".

Any study of higher gaseous alkane metabolism cannot, therefore, be considered in isolation from methane metabolism or liquid alkane metabolism about which relatively more is known. On that account, although this introduction is directed primarily at the state of knowledge regarding ethane, propane and butane metabolism, examples will be drawn from and references made to methane and higher alkane metabolism where this serves to illustrate a point or highlight gaps in our knowledge of gaseous alkane metabolism. For this reason a brief summary of the salient features of each subject is here included.

1.1.1 Methane oxidation by bacteria

1.1.1.1 Historical overview

In 1906 Sohngen reported isolating a bacterium that could convert methane to carbon dioxide. He named this isolate Bacillus methanica. References to this organism all but disappeared until Dworkin and Foster (1956) reisolated what appeared to be the same organism, renaming it Pseudomonas methanica. This organism was the subject of much of the early investigations into methane metabolism. Leadbetter and Foster (1958) showed that $^{14}CO_2$ was fixed in a heterotrophic manner and not autotrophically indicating that methane was not oxidized to carbon dioxide and then fixed. They then showed that $^{18}O_2$ was incorporated into cellular material to a much greater extent by methane-grown cells than methanol-grown cells, thus indicating that molecular oxygen was required for methane metabolism (Leadbetter and Foster, 1959). Then in the 1960's Quayle and co-workers began studying the enzymology of carbon

metabolism in P. methanica which led eventually to them postulating the ribulose monophosphate (RMP) pathway of carbon assimilation. The 1970's saw the number of methane-oxidizers isolated increase to over 100 with the microcolony selection procedures of Whittenbury et al., 1970. The initial enzyme of the pathway, methane monoxygenase, was characterized (Colby and Dalton, 1976) and the serine pathway of carbon assimilation was proposed (some details of which are still unknown). Now, into the 1980's, molecular biology is being applied to methane-oxidizers to elucidate regulatory mechanisms and complement fine biochemical analyses (Mullens and Dalton, 1987; Oakley and Murrell, 1988).

1.1.1.2 Taxonomy

The relationship of methane oxidizers to other bacteria remains somewhat unclear. The eighth edition of Bergey's Manual places them in the Family Methylomonodaceae along with the Family Pseudomonadaceae and other Gram-negative aerobic rods and cocci.

As a group they can be divided into two major subgroups denoted Type I and II. Division is based on the type of intracytoplasmic membrane present and the major carbon assilimation pathway. Each type has recognised sub-groups (see Dalton and Leak, 1985). Type I organisms have membranes as bundles of vesicular disks and use the ribulose monophosphate pathway for carbon assimilation, whilst Type II organisms have paired membranes around the periphery of the cell and use the serine pathway for carbon assimilation.

1.1.1.3 Pathways of methane metabolism

Methane is sequentially oxidized by methanotrophs as shown in Fig. 1.1. The enzymes of this series (methane to carbon dioxide) have been characterized and are discussed in a later section (1.7). Assimilation of carbon occurs at the level of formaldehyde by two different pathways in Type I and II organisms (see Fig.'s 1.2 and 1.3).

Type I organisms, using the ribulose monophosphate pathway, fix three molecules of formaldehyde with three molecules of ribulose-5-phosphate to form three molecules of hexulose-6-phosphate which can isomerize to three molecules of fructose-6-phosphate. The enzymes involved are 3-hexulose phosphate synthetase and phospho-3-hexuloisomerase which are unique to this pathway. Fructose-6-phosphate then undergoes cleavage and rearrangement via the hexose monophosphate cycle (see Quayle and Ferenci, 1978). The cycle has three distinct phases (i) the fixation phase, (ii) the cleavage phase, in which one fructose-6-phosphate molecule is cleaved to two 3 carbon units (usually glyceraldehyde-3-phosphate or dihydroxyacetone phosphate), one of which is assimilated to cell material, (iii) the rearrangment phase in which the two remaining 6 carbon sugars and the 3 carbon unit rearrange to give three ribulose-5-phosphate molecules that continue the cycle. Variations on this basic pathway exist and are discussed by Quayle and Ferenci (1978).

Type II organisms assimilate carbon via the serine pathway (Fig. 1.3). Formaldehyde reacts with a C_1 carrier (tetrahydrofolate), and two of these molecules then hydroxymethylate two glycine molecules to form two serine molecules using the enzyme serine transhydroxymethylase. The serines are then deaminated by serine glyoxylate aminotransferase to

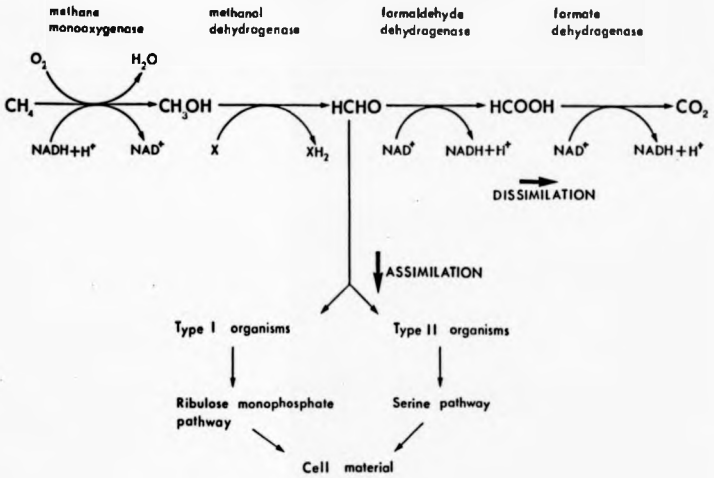


Figure 1.1 Pathways of methane metabolism in methane-oxidizing bacteria

Figure 1.2 RMP pathway and its variations

Ru5P, Ribulose-5-phosphate
Hu6P, D-erythro-L-glycero-3-hexulose-6-phosphate
F6P, fructose-6-phosphate
FDP, fructose-1,6-diphosphate
G3P, glyceraldehyde-3-phosphate
DHAP, dihydroxyacetone phosphate
E4P, erythrose-4-phosphate
Xu5P, xylulose-5-phosphate
S7P, sedoneptulose-7-phosphate
R5P, ribulose-5-phosphate
G6P, glucose-6-phosphate
6PG, 6-phosphogluconate
pyr, pyruvate

1 3-hexulosephosphate synthase
2 phospho-3-hexuloisomerase
3 6-phosphofructokinase (EC 2.7.1.11)
4 fructose diphosphate aldolase (EC 4.1.2.13)
5 transketolase (EC 2.2.1.1)
6 transaldolase (EC 2.2.1.2)
7 ribulose phosphate epimerase (EC 5.1.3.1)
8 ribulose phosphate isomerase (EC 5.3.1.6)
9 fructose diphosphatase (EC 3.1.3.11)
10 triosephosphate isomerase (EC 5.3.1.1)
11 glucose phosphate isomerase (EC 5.3.1.9)
12 glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
13 6-phosphogluconate dehydratase (EC 4.2.1.12)+ phospho-2-keto-3-deoxygluconate aldolase (EC 4.1.2.14)
14 transketolase + triokinase (EC 2.7.1.30)

Taken from Colby, Dalton and Whittenbury (1979).

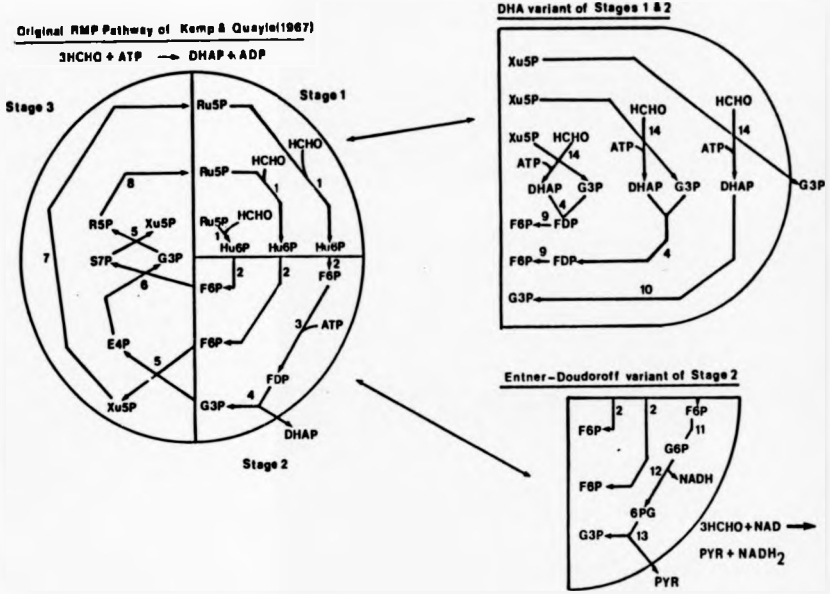

Original RMP Pathway of Kemp & Quayle(1967)
3HCHO + ATP → DHAP + ADP
Stage 3
Stage 1
Stage 2
Ru5P
HCHO
R5P
Xu5P
S7P
G3P
E4P
Hu6P
F6P
FDP
ATP
DHAP
DHA variant of Stages 1 & 2
Entner-Doudoroff variant of Stage 2
6PG
NADH
3HCHO+NAD
PYR + NADH2
PYR

Figure 1.3 Serine pathway

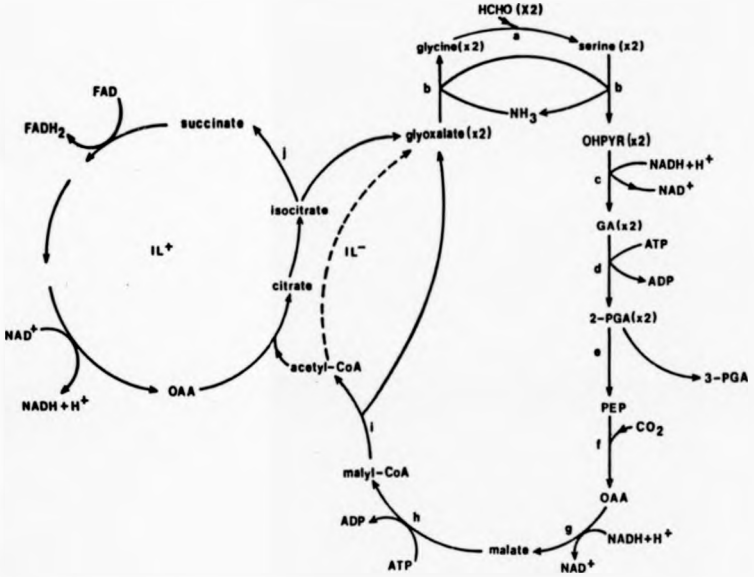
a Serine transhydroxymethylase (EC 2.1.2.1)
b serine glyoxylate amino-transferase
c hydroxypyruvate reductase (EC 1.1.1.29)
d glycerate kinase (EC 2.7.1.31)
e phosphopyruvate hydratase (EC 4.2.1.11)
f phosphoenol-pyruvate carboxylase (EC 41.1.31)
g malate dehydrogenase (EC 1.1.1.37)
h malate thiokinase (EC 6.2.1.-)
i malyl-CoA lyase (EC 4.1.3.24)
j isocitrate lyase (EC 4.1.3.1)

- - - - unknown reactions

CHPYR. hydroxypyruvate
GA. glycerate
PGA. phosphoglycerate
PEP. phosphoenolpyruvate
OAA. oxaloacetate

Net reaction (IL$_e$): $2HCHO + CO_2 + FAD + 2NADH_2 + 3ATP \longrightarrow$
3-PGA + 2ADP + 2NAD + $FADH_2$

Taken from Colby, Dalton and Whittenbury (1979).


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

hydroxypyruvate which is then reduced to glycerate by hydroxypyruvate reductase. It is the presence of these three enzymes that is used to indicate the operation of the serine pathway. The glycerates are then converted to 2-phosphoglycerates and one is converted to 3-phosphoglycerate which is used to produce cell material. The other is converted via phosphoenolpyruvate and carboxylated to oxaloacetate and then to malate. This is converted to malyl CoA which is cleaved to acetyl CoA and regenerates a glyoxylate molecule that can be aminated to glycine to restart the cycle. The acetyl CoA is then condensed with oxaloacetate to form citrate and enters the TCA cycle. If the organism posessess isocitrate lyase the enzyme is induced and the glyoxylate formed is used to make another glycine for formaldehyde fixation whilst the succinate replenishes oxaloacetate to allow the cycle to continue. The pathway in organisms lacking isocitrate lyase is less clear but acetyl CoA is probably oxidized to glyoxylate via glycollate (Colby *et al.*, 1979; Bellion *et al.*, 1981). In total then, two molecules of formaldehyde and one of carbon dioxide are used to make one molecule of 3-phosphoglycerate for cell synthesis.

Catabolism of formaldehyde occurs either directly via formate (Fig. 1.1) or via a cyclic route using the enzymes of the ribulose monophosphate pathway, which can obviously only occur in Type I organisms.

1.1.1.4 Facultative methane oxidizers?

Stephens (1983) surveys much of the evidence against the existence of facultative methane-oxidizers and suggests reasons why obligate methanotrophy exists. The evidence against many reports of facultative methane-oxidizers rests on two points, namely the purity of the

substrates employed for growth studies and the purity of the culture itself.

An example of the first case would be Mycobacterium vaccae strain JOB5 (Ooyama and Foster, 1965), reported to grow on n-alkanes from C_1 to C_{22}. Hubley (1975) using the same strain could not get it to grow on or oxidize methane. In the original studies the methane used was only 99% pure and the Mycobacterium could have been growing on impurities in the methane. Davis et al. (1956) had already shown that methane containing as little as 0.3% ethane could support the growth of Mycobacterium paraffinicum.

The problem of culture purity is highlighted by "Methylobacterium ethanolicum" a facultative methane-oxidizer described by Lynch et al. (1980) which was shown to be a syntrophic association of a Methylocystis sp. and a Xanthobacter sp. by Lidstrom-O'Connor et al. (1983).

Shishkena and Trotsenko (1982) noted that methanotrophs lacked many of the enzymes of heterotrophic metabolism (e.g. Type I and II organisms lacked pyruvate kinase, Type II organisms lacked 2-oxoglutarate dehydrogenase) which would explain their inability to grow on many organic substrates and which may contribute to the current view by those studying methanotrophy that all methanotrophs have an obligate requirement for C_1 substrates. Clearly, at present there is some doubt as to the existence of a true facultative methanotroph (Murrell, pers. comm.).

1.1.2 Liquid n-alkane metabolism

1.1.2.1 Introduction

Liquid n-alkanes (C_5 to C_{16}) are widely dispersed in the environment, the most obvious source being crude oil. Much of this enters the environment as a result of human activities (12 million tons of crude oil per year are discharged into the sea from oil tankers alone (Gutnick and Rosenberg, 1977)). Other sources, such as from plant and bacterial metabolites are also recognised (Tornabene, 1976; Hunt et al., 1980).

Bacteria capable of growth on liquid n-alkanes have been isolated from terrestrial, freshwater and marine environments using simple enrichment techniques (Rosenberg and Gutnick, 1981), these authors also enumerating the hydrocarbonoclastic bacteria found in several different environments. Jones and Edington (1968) reported that 1-10% of bacterial isolates from non-contaminated soils could grow on hydrocarbons as sole source of carbon and energy. Zobell (1969) discussed many of the factors that affect the growth of hydrocarbon-oxidizing bacteria in the environment such as temperature, pH, salinity and oxygen levels. Nutritional factors such as the availability of nitrogen, phosphorus, and trace elements will also limit their growth as will the nature of the hydrocarbon, its effective concentration and the presence of other toxic products.

Rosenberg and Gutnick (1981) list the following genera of organisms as being the most frequently isolated in hydrocarbon enrichments: Pseudomonas, Acinetobacter, Flavobacterium, Brevibacterium, Corynebacterium, Arthrobacter, Mycobacterium and Nocardia. Other

genera listed as showing growth on hydrocarbons are Achromobacter, Alcaligenes and Xanthomonas. This list is by no means exhaustive and earlier reviews (e.g. Fuhs, 1961) give more comprehensive listings.

In 1950, Zobell formulated four rules describing the specificity shown by microorganisms for hydrocarbon substrates. In the light of more recent work they were modified by Shennan and Levi (1974) and are listed by Ratledge (1978).

1) Aliphatic hydrocarbons are assimilated by a wide variety of microorganisms. Other classes of compound, including aromatics, may be oxidized but are assimilated by only a few bacteria.
2) n-alkanes of chain length shorter than n-nonane are not usually assimilated but may be oxidized. Only some bacteria have the ability to grow on alkanes shorter than n-octane. As the chain length of the alkane increases beyond C_9 the yield factor increases but the rate of oxidation decreases.
3) Saturated compounds are degraded more readily than unsaturated ones.
4) Branched-chain compounds are degraded less readily than straight-chain compounds.

These rules appear to have general applicability to all microorganisms and state the preference for liquid n-alkanes (at least those $\geq C_9$).

A problem encountered with liquid n-alkanes (but not gaseous alkanes) as growth substrates is that of solubility. Whereas the gaseous alkanes form truly soluble aqueous concentrations of 2-0.2mM which, according to Watkinson (1980), are enough to support observed growth rates, solubility of the liquid n-alkanes from hexane to hexadecane falls from

0.1mM to 0.03μM (Bell, 1973). Watkinson (1980) defines soluble as meaning that individual hydrocarbon molecules are solvated and are transported into the cell as individual molecules. To handle liquid n-alkanes of low solubility two mechanisms have been proposed. One involves direct contact between microorganism and hydrocarbon and the other involves the production of specific agents that cause the dispersion of hydrocarbons into micelles which are subsequently taken up. Both these mechanisms are discussed in reviews by Watkinson (1980), Ratledge (1978) and Britton (1984).

1.1.2.2 Pathways of liquid n-alkane utilization

a) Terminal oxidation

It appears generally accepted by most authors that monoterminal oxidation of short and long chain n-alkanes is the main route of assimilation. The alkane is converted to the corresponding primary alcohol, then aldehyde, then fatty acid (Fig. 1.4). Fatty acids are then oxidized via β-oxidation to the level of acetate (or propionate for odd chain length n-alkanes). Much of the evidence for these pathways remains circumstantial being based on such techniques as simultaneous adaptation and analyses of excreted metabolites. Evidence of an enzymological nature is still in the minority.

Bearing this in mind, the early evidence that suggested monoterminal oxidation as occuring included the identification of primary alcohols (Finnerty and Kallio, 1964) and fatty acids (Senez and Konovalschikoff-Mazoyer, 1956) in culture supernatants of alkane-grown bacteria and the simultaneous adaptation of hexane grown Pseudomonas aeruginosa to the

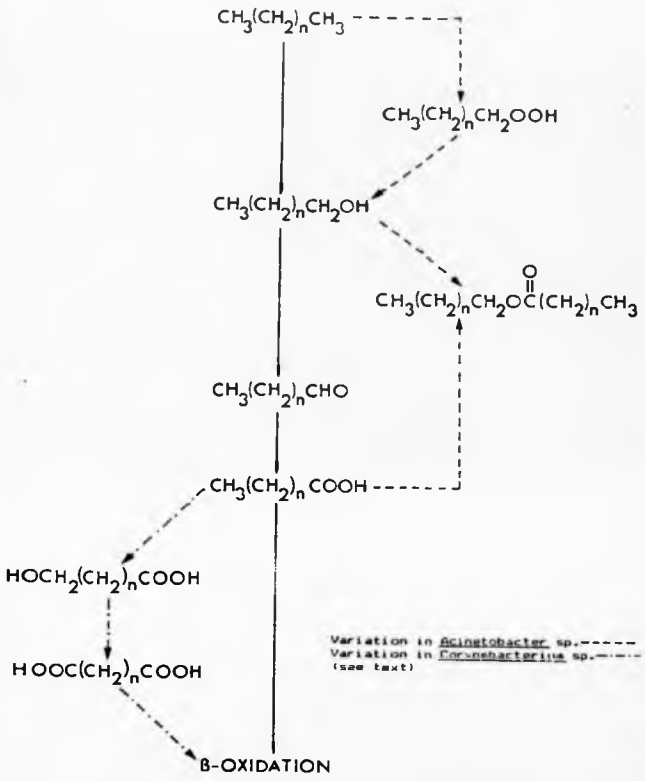


Figure 1.4 Terminal oxidation of n-alkanes

oxidation of hexan-1-ol, hexanal and hexanoate (Azoulay and Senez, 1960).

The first direct evidence of terminal oxidation occurred when Baptist et al. (1963) showed that cell-free extracts of Pseudomonas oleovorans produced octan-1-ol, octanal and octanoate from octane. Similar results were obtained by Cardini and Jurtshuk (1968) working with cell-free extracts of Corynebacterium sp. 7E1C.

The isolation of dicarboxylic acids from a Corynebacterium sp. grown on n-alkanes (Kester and Foster, 1963) is not inconsistent with terminal oxidation. It is possible that first one end of the molecule and then the other may undergo a sequence of oxidations (Fig. 1.4).

The isolation of esters from culture supernatants of Acinetobacter sp. HO1-N grown on alkanes (Stewart et al., 1959) and the fact that growth on alkyl hydroperoxides produces the same esters has led to suggestions that such hydroperoxides are intermediates of n-alkane metabolism and the postulated pathway is as shown in Fig. 1.4.

b) Subterminal oxidation

That subterminal oxidation of liquid n-alkanes occurs is beyond doubt. What is still very open to speculation is the relative importance of this pathway. Fredricks (1967) showed that Pseudomonas aeruginosa growing on decane excreted subterminal alcohols and the corresponding ketones. Lukins and Foster (1963) reported a similar phenomenon in Mycobacterium smegmatis grown on propane, butane, pentane and hexane. These studies showed alkane grown organisms producing ketones. Other

studies, (Klein et al., 1968; Klein and Henning, 1969) involved an Arthrobacter sp. that grew only poorly on hexadecane but which, when grown on yeast extract, could oxidize hexadecane to a number of internal hexadecones. This indicated that subterminal oxidation was occurring, but to assign such a role to alkane metabolism would be wrong when the organism grew so poorly on hexadecane.

Markovetz and co-workers studied tridecan-2-one metabolism in various Pseudomonas strains and found when they were grown on the ketone they produced a number of products including tridecan-2-ol, undecan-1-ol, undecanoate and undecyl acetate (Forney et al., 1967). They proposed that tridecan-2-one was cleaved at the keto group in a Baeyer-Villiger type reaction whereby oxygen was inserted into the molecule by an oxygenase to form undecyl acetate which was then cleaved to form undecan-1-ol and acetate. Markovetz (1971) later showed that Pseudomonas aeruginosa grown on n-tridecane produced the same products and suggested that the same pathway was operating (Fig. 1.5).

1.1.2.3 Anaerobic oxidation of n-alkanes

A number of reports of anaerobic oxidation of n-alkanes exist in which the postulated pathway involves dehydrogenation of the alkane to the alkene which is then hydroxylated to the primary alcohol. Perhaps the most credible of these reports is Parekh et al. (1977) who isolated from a Pseudomonas sp., growing anaerobically on hexadecane with nitrate as terminal electron acceptor, an NAD^+-linked alkane dehydrogenase and an NADPH-linked alkene hydroxylase. Both enzymes were partially purified and the dehydrogenase produced dec-1-ene from decane and the hydroxylase decan-1-ol from decene. This organism may be a special case since it

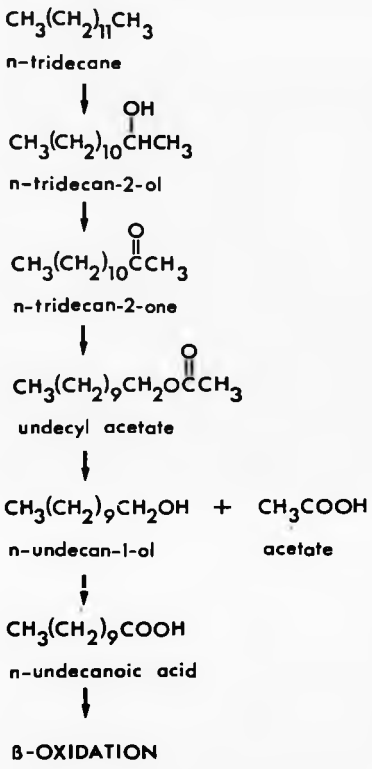


Figure 1.5 Pathway of subterminal oxidation of n-tridecane in Pseudomonas spp (adapted from Klug and Markovetz, 1971)

grew poorly on decane aerobically. Other reports of alkene production from alkanes have occurred (e.g. Abbot and Casida, 1968) but there have been a number of arguments raised against alkenes as intermediates in alkane metabolism (see McKenna and Kallio, 1965; Klug and Markovetz, 1971; Ratledge, 1978, and see below).

1.1.2.4 Mechanisms of n-alkane oxidation

Three mechanisms for the oxidation of n-alkanes to alcohols have been postulated and they are summarized in Fig. 1.6.

The formation of alkyl hydroperoxides (equation 1) is based on indirect evidence particularly from Acinetobacter sp. HO1-N which when grown on alkanes and alkyl hydroperoxides produced the same excreted products (Stewart et al., 1959). Cell-free extracts could also degrade alkyl hydroperoxides and there was some evidence that an NADH-dependant alkyl hydroperoxide reductase was present. Also of note is that rubredoxin from Pseudomonas oleovorans can act as an electron carrier in the reduction of alkyl hydroperoxides (Lode and Coon, 1971). There have been no reports of these compounds having been isolated as free intermediates.

The dehydrogenation mechanism (equation 2) was proposed by Senez and Azoulay (1961) to explain their detection of hept-1-ene in cultures of Pseudomonas aeruginosa grown on heptane. The hydroxylation of alkene to alcohol is shown involving water but it could possibly involve molecular oxygen thus forming an epoxide which could be reduced to the alcohol. As mentioned previously, a number of arguments have been used against this mechanism (see Klug and Markovetz, 1971) particularly the

1. $RCH_2CH_3 + O_2 \rightarrow RCH_2CH_2OOH + NAD(P)H + H^+ \rightarrow RCH_2OH + NAD(P)^+ + H_2O$

2. $RCH_2CH_3 + NAD(P)^+ \rightarrow RCH{=}CH_2 + NAD(P)H + H^+ + H_2O \rightarrow RCH_2CH_2OH$

3. $RCH_2CH_3 + O_2 + NAD(P)H + H^+ \rightarrow RCH_2CH_2OH + NAD(P)^+ + H_2O$

Figure 1.6 Mechanisms of n-alkane oxidation (adapted from Singer and Finnerty, 1974)

thermodynamic unfeasability of converting an alkane to an alkene (McKenna and Kallio, 1965).

Equation 3 describes the direct incorporation of one atom of molecular oxygen into the alkane molecule using a mixed-function oxygenase (monooxygenase). This is the mechanism that has most support. Although equations 1 and 3 both have the same overall stoichiometry and would both give the expected results from $^{18}O_2$ labelling studies, Klug and Markovetz (1971) suggest that in the hydroperoxidation mechanism NAD(P)H would have the additional function of donating electrons to form an active oxygen species for formation of the alkylhydroperoxide. This would then be contrary to the 1:1:1 ratio of O_2:NADH:octane observed with octane monooxygenase by Peterson et al. (1969).

The initial reaction mechanisms of alkane oxidation remain obscure. However it does seem likely that molecular oxygen is an obligatory reactant. Whether or not oxygen is activated to a more reactive species prior to incorporation into the alkane molecule or if the alkane molecule itself is activated to receive the oxygen molecule is not known. Britton (1984) and Singer and Finnerty (1984) have reviewed the subject.

1.2 Species involved in gaseous alkane oxidation

1.2.1 Filamentous fungi

The observation by the Japanese botanist Miyoshi (1895) that the mould Botrytis cinerea could grow on paraffin is generally recognized as the first report of microbial hydrocarbon utilization. Since then many

species of fungi have been found to grow at the expense of n-alkanes. The apparently greater propensity for oxidation of n-alkanes by bacteria in relation to fungi is probably due simply to the lack of investigation using fungi.

Lowery et al. (1968) screened 29 species of filamentous fungi on a series of n-alkanes from methane to hexadecane. 18 were capable of growth on one or more of these n-alkanes. Of these 18, all but 3 grew well on n-alkanes of chain length 10 or greater. Those listed as growing on the gaseous n-alkanes were Epicoccum sp. (C_2-C_4), Fusarium sp. (C_1-C_4), Gliocladium sp. (C_4), Paecilomyces sp. (C_4) and Penicillium sp. (C_1-C_4).

Alkanes were supplied as a 50% (v/v) atmosphere in air and the investigators point out that due to the extensive nature of the survey no attempt was made to determine whether of not those hydrocarbons described as unsuitable for growth could in fact support growth when supplied under different conditions or at different concentrations. The purity of the gaseous alkanes employed is not reported and neither is the extent of growth so it cannot be ruled out that, for instance, what is recorded as growth on methane could be growth on low levels of a contaminant (e.g. ethane) in the methane.

Davies et al. (1973) identified several ethane-utilizing Hyphomycetes isolated from sewage as being Graphium sp., Phialophora sp. and Acremonium sp. They considered that as the ability to utilize ethane was present in such diverse genera, it was not inconceivable that the ability could be widespread in nature. Klug and Markovetz (1971) considered the ability of fungi to utilize n-alkanes as a property that

had no taxonomic value.

Very little biochemical data exists regarding the pathways of n-alkane metabolism in filamentous fungi. Lowery et al. (1968) mention that methyl ketones are often produced as a result of n-alkane oxidation by fungi. Klug and Markovetz (1971) suggest that the presence of methyl ketones in some systems and terminal acids in others indicates that subterminal or terminal oxidation of alkanes could be occurring.

Zajic et al. (1969) described the growth of a Graphium sp. on natural gas composed of (v/v) 90.5% methane, 6.0% ethane, 3% nitrogen, 0.25% carbon dioxide and 0.21% propane with trace amounts of higher alkanes. It was isolated from sewage and grown in stable co-culture with a Trichoderma sp. and an acid-tolerant bacterium (sic). The Graphium sp. could grow in pure culture on natural gas as could the bacterium. The Trichoderma sp. was apparently being cross-fed by metabolites from the other two organisms. One and two carbon alcohols, aldehydes and acids were detected in the culture.

Volesky and Zajic (1971) describe the same Graphium sp. as growing on the ethane component of the natural gas in a later paper that investigates protein production from ethane or natural gas. They used a submerged batch culture in a stirred-tank fermentor and investigated the physico-chemical parameters involved in the production of fungal biomass. The production of biomass was considered to be 30% efficient on a weight to weight conversion basis and the tissue was approximately 50% protein of a quality that compares favourably with the standards set by the Food and Agricultural Organisation of the United Nations.

Davies et al. (1974) performed respirometric studies on an Acremonium sp. that grew on ethane. Resting cells were tested in a Warburg respirometer and found to oxidize ethane, propane and butane in the presence of oxygen. Methane was not oxidized alone or in the presence of ethane. Two, three and four carbon alcohols, aldehydes and acids which were regarded as potential intermediates of gaseous alkane metabolism were also oxidized and the patterns of oxidation were said to point to a common pathway of alkane oxidation to the level of fatty acid. The authors do add the proviso that the possibility of other minor pathways cannot be eliminated and concede that the oxidation of secondary alcohols may indicate some subterminal oxidation.

In a subsequent paper (Davies et al., 1976) the same authors present further evidence using ^{14}C-labelled intermediates to support a pathway of ethane oxidation via ethanol and acetaldehyde to acetate. They also succeeded in obtaining cell-free activity and showed ethane oxidation to be NADPH dependant and inhibited by CO. However, no cytochrome P_{450} could be demonstrated by a CO difference spectrum. Activity could be located within the microsomal fraction and electron micrographs revealed stacked membrane structures in ethane-grown cells.

1.2.2 Yeasts

Lowery et al. (1968) screened 66 strains of yeast from 16 genera and found that only 11 strains from the genera Candida, Rhodotorula and Debaryomyces could grow on n-alkanes in the range decane to hexadecane. None could grow on gaseous n-alkanes. Shennan and Levi (1974) reviewed the growth of yeasts on hydrocarbons and listed the many genera that have exhibited the phenomenon. They too did not report any strains

growing on gaseous alkanes.

Wolf and Hanson (1979) isolated 5 strains of yeast capable of growth on methane. All showed at least slight growth on ethane and butane. Propane was not tested. The purity of the gases used was not recorded but growth was recorded as being equivalent to that on methane controls so it would appear to be a genuine observation (although nothing further has appeared in the literature).

Kormendy and Wayman (1974) reported a strain of Candida utilis which could grow on butane and butan-1-ol and this appears to be the only report of a yeast growing on a higher gaseous alkane. They did not investigate the physiology of the organism but did report on its morphology (see Section 1.5).

1.2.3 Bacteria

The ability of microorganisms to grow with propane as sole source of carbon and energy was originally suggested (according to Perry (1980)) by Tausz and Donath in 1930. They described a "Methanbakterium" that utilized the n-alkanes from methane to hexane. Since that report, numerous lists have appeared of organisms capable of growth on hydrocarbons and many include reports of bacteria growing on ethane, propane and butane.

The ability to oxidize the C_2-C_4 gaseous alkanes appears to be confined mainly to the Gram-positive genera and particularly to the Coryneform and Actinomycete groups. Genera that appear most often in reports are Corynebacterium, Mycobacterium, Nocardia, Arthrobacter, Brevibacterium and

Rhodococcus (Perry, 1980; Hou, 1984). Other genera that have been mentioned are Pseudomonas, Alcaligenes, Flavobacterium and Streptomyces (Dworkin and Foster, 1958; Hou, 1984; Davis et al., 1956).

The Mycobacteria are particularly common in enrichments on gaseous alkanes and screening stock cultures also yields a high percentage of gaseous alkane-utilizers (Perry, 1980).

Actinomycetes and coryneforms also occur regularly in enrichments for liquid alkane-utilizers as do pseudomonads. In fact it is probably the Pseudomonas species that have been most studied as far as liquid alkane utilization is concerned. Despite their well documented ability to grow on liquid alkanes there are considerably fewer reports of their occurrence in gaseous alkane enrichments. Hou (1984) lists 6 species of pseudomonads that are "known" gaseous alkane utilizers but cites no primary source for this information. Smirnova (1962) lists three named species and three unidentified species of Pseudomonas capable of growth on propane, and Kuznetsov and Telegina (1957) isolated an organism, growing on propane, that was named as a Pseudomonas species. Unfortunately, in all these reports, no details of identification are given. Takahashi et al. (1980) described in some detail a bacterial strain capable of growth on n-alkanes from C_2 to C_9. There seems little doubt that this organism is a Pseudomonas species. It is listed in the current addition of "Bergey's Manual of Determinative Bacteriology" (9th Edition) as "Pseudomonas butanovora" but it's name has not yet been validly published.

That the same groups of organisms should be responsible for the degradation of both liquid and gaseous alkanes is perhaps not surprising

in view of the similarity of the two substrates. The methane oxidizing bacteria isolated to date appear to be a taxonomically distinct group of organisms, particularly with regard to their carbon metabolism. They all appear to have an obligate requirement for methane or methanol as carbon/energy sources. A possible explanation for this, based on enzymic lesions found in these organisms, has been put forward (Shishkina and Trotsenko, 1982). Reports of facultative methane oxidizers do occur, but can often be criticised and explained as mixed cultures or growth on contaminants in the methane (Lidstrom-O'Connor et al., 1983). The C_2-C_4 gaseous alkane-utilizers do not generally show any such specialised features. They are capable of growing on substrates such as sugars, alcohols, and organic acids in addition to n-alkanes. An interesting exception to this would appear to be Mycobacterium paraffinicum (Davis et al., 1956). This organism, isolated from garden soil, could grow only on ethane, ethanol, acetate and n-alkanes from C_2 to C_{10}.

The range of n-alkanes utilized by a particular organism is often the subject of some discrepancy between different authors, probably due to a failure to observe the same growth regimes, not checking culture purity or using impure substrates (Stephens, 1983).

Why the Corynebacterium-Mycobacterium-Nocardia (CMN) group of organisms should be so common in enrichments in n-alkanes was speculated on by Stephens and Dalton (1987a). Although their arguments were aimed primarily at liquid alkane utilizers, some points may well be applicable to gaseous alkane utilizers too. Firstly there is the presence of a highly hydrophobic cell surface on these organisms which would facilitate diffusion of a hydrophobic substrate into the organism.

Secondly these organisms are known to produce surfactants to aid in the dispersion of hydrophobic substrates. This is probably of less significance with gaseous alkane substrates as they are more soluble in water than liquid alkanes. Finally the cell wall biochemistry of these organisms may endow them with an enzyme complement particularly suited to handling n-alkane substrates, for example the possession of oxygenase enzymes. These points may explain, in part, the predominance of these CMN organisms in n-alkane enrichments, but no systematic study has been done on this phenomenon and other unforseen factors may be involved.

One final point that should be made under this heading is about the apparently confused state of the taxonomy of these Coryneform-Actinomycete groups. This manifests itself in the literature by the appearance of synonyms, or the renaming of organisms in papers. For example, de Bont and Pack (1980) describe their organism as a Nocardia and a Rhodococcus in the same paper! They also point out it's earlier description as a Mycobacterium species. Nocardia paraffinicum has also been named as Rhodococcus rhodochrous (MacMichael and Brown, 1987) and probably Mycobacterium rhodochrous too (Perry, 1980). Corynebacterium sp. 7E1C has also been described as Mycobacterium sp. 7E1C (Perry, 1980). This inconsistency in nomenclature only serves to confuse much of the work and particularly to cloud any comparative studies in this field.

1.3 Occurrence and distribution in nature

1.3.1 Sources of gas and occurrence of organisms

The most obvious source of hydrocarbons in the environment are petroleum

deposits and their associated natural gases. These so called fossil fuels represent a "leak" in the organic carbon cycle. They actually constitute a small fraction of the buried organic matter that has escaped oxidation, most of which lies distributed in a dilute form in sedimentary rocks (Ourisson et al., 1984). Only under precise geological conditions do petroleum deposits occur. Gas deposits can occur with oil deposits as a result of thermal cracking at elevated temperatures and pressures or they may be associated with coal deposits (Anonymous, 1981). As with oil, gas deposits do not exist in pockets or hollows but are held within porous rocks usually at depths between 800m and 4000m.

In addition to this "geological" source of hydrocarbons a large amount of "biological" hydrocarbons are produced continually. For example, the action of bacteria on plant terpenes can yield n-alkanes (Hunt et al 1980) and fatty acids from membranes can also be a source of microbial n-alkane production (Tornabene, 1976)

The production of methane by methanogenic bacteria is well established (Winfrey,1984) but the microbial production of ethane, propane and butane has only recently been reported. Previously it was believed that these gases could only be formed under conditions of high temperature and pressure. Oremland (1981) reported that ethane from anaerobic esturine sediments was of microbial origin and was produced optimally at 40^{o}C. Fukada et al. (1984a, 1984b) screened several strains of bacteria, yeasts and fungi and found many could produce ethane, propane and butane aerobically at 25-30^{o}C. Malik and Tauro (1986) speculated on the possibility of engineering conditions within anaerobic digestors in order to produce ethane and propane, in addition to methane, to increase

the thermal efficiency of the "biogas" produced. Belay and Daniels (1987) showed that several strains of methanogenic bacteria were capable of producing ethane when exposed to halogenated ethane. So it seems clear that ethane, propane and butane could all be produced biologically in the environment but to date no one has systematically studied the problem so no figures on estimated biological production are available. However, most authorities are still under the impression that these biological sources represent a fairly minor input to the total available to microorganisms, preferring seepage from geological sources as the m[illegible] supply.

The concentrations of gaseous-hydrocarbons recorded in soil vary widely. Kartsev et al. (1959) reported that in non-petroliferous regions the concentration of hydrocarbons in the subsoil atmosphere occurred in the range 2 to 4 ppm by volume. Values for soils overlying petroleum deposits vary from 0.2 to 800 ppm depending on the soil type, reservoir depth and sampling technique (Brisbane and Ladd, 1965). Analyses of hydrocarbons adsorbed to soil particles gave figures of 0.025-0.1 ppm (by weight of dried soil sample) for non-petroliferous areas and 0.1-2 ppm for soils above petroleum deposits (Brisbane and Ladd, 1965).

As far as aquatic environments are concerned, Ivshina et al. (1981) reported concentrations of dissolved ethane, propane and butane of 0.79 ppm in ground waters of a non-petroliferous region. In ground water overlying petroleum deposits the figure was 1.63 ppm. No reports exist on the concentration of ethane, propane and butane in marine environments but it is known that seepage of petroleum deposits from oil bearing strata on the ocean floor does occur (Floodgate, 1984) so it is likely that these compounds are present in the marine environment.

A final and fairly minor source of these alkanes in the environment is as a result of human activities particularly from leaks in submerged gas-carrying pipes. Adamse *et al*. (1972) investigated the effects of natural gas leakage from underground pipes on the surrounding microbial flora but gave no indication of the sorts of concentrations of gases that occurred in the soil around the leak. Presumably they were somewhat higher than those quoted for seepage from deep reservoirs.

As far as occurrence of gaseous alkane utilizing bacteria is concerned, they would appear to be ubiquitous in nature. Isolations are generally attempted from soils that have been exposed to gaseous or liquid alkanes (e.g. McLee *et al*., 1972; Linton *et al*., 1980) but such organisms have been isolated from sewage (Dworkin and Foster, 1958), garden soil (Davis *et al*., 1956) and pond and river samples (Stephens and Dalton, 1986).

No reports exist on isolations from marine environments but methane oxidizers and liquid alkane utilizers have been isolated from such environments (Hutton and Zobell, 1949; Rosenberg and Gutnick, 1981) so it is probable that no one has yet looked in such environments.

Gaseous alkane-oxidizers have only been isolated from aerobic environments and reports of anaerobic degradation of n-alkanes are scarce (see Section 1.1). However, James and Burns (1984) suggested that subsurface natural gas reservoirs were being microbially altered, in particular propane was being metabolized. Evidence of microbial activity is based on carbon isotope composition of the gaseous alkanes. They give no details of the environments in which these gas accumulations are held other than that they are at depths in excess of 2000m. Such environments were thought to be anoxic but these findings

and others (see Finnerty and Singer, 1983) perhaps suggest such environments can support the growth of aerobic bacteria.

If these organisms are to grow in the environment using gaseous alkanes as their sole carbon and energy source they must be able to grow on the low concentrations that are found. Mycobacterium paraffinicum the only species that appears to have an obligate requirement for n-alkanes or alkane metabolites (Davis et al., 1956), was shown to grow on 15 ppm ethane after a lag period of some 8 weeks (Brisbane and Ladd, 1972). Dostalek and Spurny (1962) showed that the growth of propane-utilizing bacteria in liquid culture suspended in sealed bore holes containing natural gas was proportional to the propane concentration in the range 0.2-10 ppm so it would appear that these bacteria are capable of utilizing the gaseous alkane substrate at the sort of concentrations that exist in the environment.

The presence and numbers of gaseous alkane-utilizing organisms in a soil containing gaseous alkanes will depend on other factors in the soil environment such as pH, oxygen, other nutrients (especially phosphorus and nitrogen) and may also be subject to seasonal variation. The actual numbers found in soil and their significance are discussed in the next section.

1.3.2 Use of microorganisms in oil and gas prospecting

Attempts have been made to correlate the number of gaseous alkane-utilizing bacteria found in soil with the presence of underlying deposits of gas and oil. According to Brisbane and Ladd (1965) this method was first suggested by Mogilevskii in 1938 who based his method

on an assay for methane-oxidizing bacteria. At the time the simplicity of the assay would have been attractive since alternative chemical methods would have been insensitive. The specificity of methane-oxidizing bacteria would make them ideal markers for the presence of methane, but unfortunately methane, being produced in large amounts biologically, is not a suitable marker of petroleum deposits. Ethane, propane and butane would make more suitable markers since they are not produced to any great extent in nature by biological processes. Unfortunately the bacteria that oxidize these substrates generally lack the growth substrate specificity shown by methane-oxidizers and therefore their presence in the soil does not necessarily indicate the presence of the alkane. This problem, coupled with other factors such as the length of time such assays take, the non-vertical seepage of gases and the sensitivity and reliability of modern geochemical techniques, has largely rendered this technique obsolete.

Data on the actual numbers of gaseous alkane utilizers in soil is lacking. Most authors prefer to measure the alkane-utilizing activity of the soil rather than actual numbers (e.g. Brisbane and Ladd, 1968). Davis *et al.* (1959) reported the relative abundance of ethane-oxidizing mycobacteria in soils from above known oil fields and from beyond the limits of such fields and noted a contrast between the two. Ivshina *et al.* (1981) did enumerate butane and propane-oxidizing bacteria from ground-waters within and beyond the limits of an oil field and recorded an order of magnitude difference between the two, 7×10^3 per ml within the field and 2.6×10^2 per ml beyond the field. Adamse *et al.* (1972) gave figures for the numbers of ethane-oxidizing bacteria in the soil around a natural gas pipe leak but this is obviously a somewhat artificial situation.

A brief synopsis of the subject of microbial oil prospecting by Vestal (1984) would suggest very little work has been carried out in this field recently.

1.4 Growth conditions

1.4.1 Isolation methods

Gaseous alkane-utilizing bacteria are easily isolated from the environment by enrichment on a mineral salts medium with the alkane as sole carbon source. Various different techniques have been employed with this approach.

In order to obtain a wide variety of organisms, Perry (1980) suggested that enrichment on agar plates is the best approach. Perry and Scheld (1968) used diluted soil samples spread directly onto mineral salts agar and incubated with alkane as sole carbon source to obtain a variety of organisms and attempt their enumeration. Dworkin and Foster (1958) used the same method to isolate ethane-utilizing bacteria and also pointed out what could be considered as the drawback of the technique, that is that it allows selection of the slower growing organisms that may have been over grown in liquid cultures.

Vestal (1984) described a similar method by which methane-oxidizers were isolated. Suspensions of samples were drawn through filters of pore size 0.2-0.4μm. Filters were then incubated on a pad soaked with mineral salts media below an atmosphere of methane in air.

Enrichment in liquid media seems to be the most widely used method (e.g.

Stephens and Dalton, 1986; Hou, 1984; McLee et al., 1972). This method should be employed in studies that require further physiological/biochemical studies of the organism as it allows direct selection of organisms capable of rapid, non-flocculent growth. Stephens and Dalton (1986) isolated a number of propane-utilizers with doubling times of approximately 5 hours and McLee et al. (1972) isolated a range of ethane, propane and butane-oxidizers with doubling times in the range of 4-15 hours.

Although enrichments are usually performed in batch culture, continuous enrichment in a chemostat has been used to isolate an ethane-utilizing fungus (Zajic et al., 1969) and this approach may prove useful in isolating bacteria that are adapted for chemostat growth.

Whilst most enrichment/isolation procedures for ethane, propane and butane-utilizing bacteria utilize the gaseous alkane as carbon source, Perry and Schold (1968) demonstrated that using other carbon sources that require the presence of an oxygenase for metabolism (e.g. phthalic acid) could result in isolation of bacteria capable of oxidizing propane.

1.4.2 Growth requirements

The requirements of gaseous alkane-utilizers for growth such as the gas/air atmosphere, nitrogen source, temperature etc. will tend to vary from strain to strain and probably reflects the conditions used during the isolation. There would appear to be few studies on the effect of these parameters on growth, most authors continuing to grow their isolates under the conditions used for isolation.

i) Gas atmosphere

The gas atmosphere should generally be in the range 30-50% v/v of alkane in air (Perry, 1980; Hou, 1984), but there is much variation in the actual figures used. Davis et al. (1956) used an atmosphere of 40:20:40 v/v ethane:oxygen:nitrogen to isolate Mycobacterium paraffinicum. Bokova (1954) and Kuznetsov and Telegina (1957) used a 1:3 v/v propane:air mixture and more recently Stephens and Dalton (1986) used a 1:1 v/v propane:air mixture to isolate propane-oxidizers. However, as mentioned previously (Section 1.3.1) gaseous alkane-utilizers are capable of growing on very low concentrations of alkane, in the order of a few ppm's.

McLee et al. (1972) investigated the effect of butane and oxygen partial pressures on the growth rate of several butane-utilizing bacteria. They concluded that altering the partial pressure of butane between 10-60% v/v (in air) did little to affect the generation time, but that high partial pressures of oxygen ($\geq$ 20% v/v) had an inhibitory affect on growth rates.

Blevins and Perry (1971) in a similar study investigated the effect of propane and oxygen partial pressures on the cell yield of Mycobacterium vaccae strain JOB 5. They found that optimal yields occurred with a gas:atmosphere of 50:40:10 v/v propane:oxygen:nitrogen. Unfortunately it is not possible to draw comparisons between these two studies as the authors chose to measure the affects of the gas atmosphere on different growth parameters.

The uptake of gaseous-alkanes is probably not a problem as they are

lipophilic and exist as an evenly dispersed dilute solution in water (unlike the longer chain alkanes which tend to form droplets when mixed with water and therefore require specialised forms of interaction with the bacteria that utilize them) (Watkinson, 1980). Their transfer from the gas phase to the liquid phase has been much studied as far as methane is concerned and no doubt parallels can be drawn with ethane, propane and butane. In their study, McLee et al. (1972) reported that increasing the transfer rate of butane into solution by using baffled flasks and more rapid agitation did not increase the growth rate suggesting that the rate of transfer of butane to solution was not limiting the rate of growth. Ichikawa et al. (1981) measured the effect of increasing the transfer rate of butane into solution by using pressurized culture of Pseudomonas butanovora. They concluded that the growth rate remained unchanged as the pressure increased from 1 to 4 atmospheres (provided that dissolved oxygen tension was kept below 15 ppm, suggesting an inhibitory role for high oxygen tensions). They could however increase the productivity of their system (measured in gl^{-1} hr) and suggested that pressurized culture could be used for efficient biomass production from butane.

ii) Nitrogen sources

The best source of nitrogen for gaseous-alkane utilizers will probably vary between strains. Perry (1980) suggested using both nitrate and ammonium salts in mineral salts media for enrichment and isolation. Smirnova (1962) showed that propane-oxidizing bacteria could utilize organic nitrogen in the form of asparagine, meat hydrolysate and gelatin as well as nitrate and ammonium salts. There exists only a single report of a nitrogen-fixing higher gaseous alkane-utilizer by Coty

(1967), but tests other than the ability to grow on nitrogen-free media were not performed.

iii) Temperature

Most ethane, propane and butane-utilizers grow within the range of 20-30°C (Perry, 1980). Linton et al. (1980) isolated an ethane-utilizing Mycobacterium which grew at 42°C, but no truely thermophilic gaseous alkane-utilizers have been reported. There are, however, instances of long chain alkane utilizers that are thermophilic with optimum growth temperatures of 60°C (Merkel et al., 1978b; Zarilla and Perry, 1984).

iv) Other requirements

Perry (1980) stated that a requirement for growth factors is not apparent in microorganisms that utilize aliphatic hydrocarbons but he did not preclude the possibility that such organisms exist in nature. Rosenberg and Gutnick (1981) discussed the nutritional requirements of hydrocarbon-oxidizing bacteria and, whilst their arguments are directed mainly towards liquid hydrocarbon-utilizers, they still omit to mention any requirement for growth factors. This apparent non-requirement may simply be a result of the enrichment/isolation procedures employed to obtain these organisms. Few authors included any such growth factors in the original enrichment so it is unlikely to enrich for organisms having an obligate requirement for such a factor. Takahashi et al. (1980) included 100mgl^{-1} yeast extract with their mineral salts medium that was used to isolate a butane-oxidizing Pseudomonas sp. It subsequently showed no requirement for any growth factor. Stirling et al. (1977) isolated a Norcardia sp. that grew on cyclohexane and had a requirement

for biotin, so hydrocarbonoclastic organisms showing auxotrophy do exist.

1.5 Morphological changes brought about by growth on gaseous alkanes

It is generally recognised that microorganisms growing on long chain alkanes may undergo changes in their cell ultrastructure (Ratledge, 1978). Reports of microorganisms growing on gaseous alkanes undergoing similar changes are much fewer but similar reports do exist. Typically these changes include the presence of intracellular cytoplasmic inclusions and intracytoplasmic membranes (Singer and Finnerty, 1984). The role played by these structures, which are absent when growth is on a non-hydrocarbon substrate, has been the subject of much speculation but their part in alkane oxidation remains unclear.

Kennedy and Finnerty (1975) and Kennedy et al. (1975) characterized the ultrastructure of Acinetobacter HO1-N when grown on hexadecane. They reported the presence of intracytoplasmic membranes extending across the length of the cell and continuous with the cytoplasmic membrane. They also appeared to be in contact with inclusion bodies. The role of the membranes was unclear, Singer and Finnerty (1984) suggesting four possibilities;

i) the enzymes responsible for the initial oxidation of the alkane and its subsequent oxidation may be associated with the membrane system,

ii) the membrane may provide a suitable lipid-rich hydrophobic environment necessary for alkane oxidation,

iii) the membrane may provide a channel from the cytoplasmic membrane to the intracellular site of alkane oxidation or to the hydrocarbon inclusions,

iv) the membrane may provide a site for additional respiratory activity and the increased cytochrome content of hexadecane-grown cells.

The inclusion bodies mentioned above appeared as electron transparent spheres of 0.2μm diameter bounded by a monolayer membrane and were shown to contain unmodified alkane. A survey by Scott and Finnerty (1976) revealed that similar hydrocarbon inclusions were present in bacteria from a number of different genera growing on hexadecane. A different type of inclusion was noted by Atlas and Heintz (1973) who observed electron-dense inclusions in Brevibacterium sp. and Flavobacterium sp. grown on crude-oil. Intracytoplasmic structures were observed in a cyclohexane-grown Nocardia sp. (Stirling et al., 1977).

This phenomenon is not restricted to prokaryotes and several reports exist of morphological changes occuring in yeasts and filamentous fungi growing on alkanes (see Singer and Finnerty, 1984).

Kormendy and Wayman (1974) reported that Arthrobacter spp. when grown on n-butane or butan-1-ol developed electron-dense inclusions. These inclusions were surrounded by electron transparent regions, but whether these were genuine or an artefact of the fixation and embedding techniques seemed to be open to conjecture. The inclusions appeared to lack any membrane structure and were linked to the cytoplasm via fine strands. The function of the inclusions remained unclear. Gas vacuoles do exist in microorganisms but they are usually associated with complex

internal membrane systems which were absent from these Arthrobacter spp. so it is unlikely that they represent sequestered butane (Griffin and Traxler, 1983). The authors termed the structures oxisomes. Iron appeared to be necessary for their organisation and there was some speculation that the structures may be involved in electron transport.

Ivshina et al. (1982) reported morphological changes in a Rhodococcus sp. growing on propane. Gross colony morphology was affected in addition to ultrastructural changes. Electron dense structures similar to the oxisomes described above were noted together with alterations in intracytoplasmic membranes. A well developed system of membranes located primarily along the periphery of the cell were observed, and the similarity of these structures to those found in certain methanotrophs led to speculation that they may be functionally related to the metabolism of gases.

1.6 Pathways of C_2-C_4 gaseous n-alkane metabolism

1.6.1 Introduction

As mentioned previously it would appear that the liquid n-alkanes can be metabolized by both terminal and subterminal pathways. Methane-oxidizing bacteria when co-oxidizing higher n-alkanes also produce a mixture of terminal and subterminal oxidation products (Dalton, 1980a). On this evidence it could be supposed that either (or both) terminal or subterminal pathways might operate in gaseous alkane-utilizers.

Fig. 1.7 shows a proposed pathway for the terminal oxidation of propane via propan-1-ol, propanal and propanoate. Propanoate could be oxidized

to CO_2 by a number of enzymes involved in heterotrophic metabolism. Figs. 1.8 and 1.9 show two possible variations of a subterminal pathway. In both cases propane is oxidized to propan-2-ol and then to acetone. Acetone can then undergo a Baeyer-Villiger type reaction to form methyl-acetate which could then be hydrolysed to methanol and acetate (Fig. 1.8). Alternatively acetone could undergo further terminal oxidation to form acetol (Fig. 1.9). The subsequent fate of acetol is shown in Fig. 1.10. It could be oxidized by a dehydrogenase to pyruvate via pyruvic aldehyde (see Taylor et al., 1980) or it could undergo a Baeyer-Villiger type oxidation to form acetate and formaldehyde via an unstable intermediate hydroxy methyl acetate (see Hartmans and de Bont, 1986).

The question of major interest is therefore; is the initial oxidation of the propane molecule terminal or subterminal or perhaps indiscriminate? The trend of opinion as will be discussed below, favours subterminal oxidation as the major route of propane oxidation in those organisms studied. Much of the evidence for this opinion is based on simultaneous adaptation studies and the analysis of products in culture supernatants. Neither of these techniques offer very firm evidence for the existence of a proposed metabolic sequence.

Dagley and Chapman (1971) discuss the limitations and pitfalls of such methods. They make the point on product excretion studies that the easier it is to isolate a compound from a culture supernatant, the greater the caution to be exercised before assigning it a role as an intermediate because such a role implies rapid removal as well as rapid formation. On the technique of simultaneous adaptation they point out that a compound may be a reaction intermediate and yet may not be oxidized by intact cells because it is excluded from the relevant

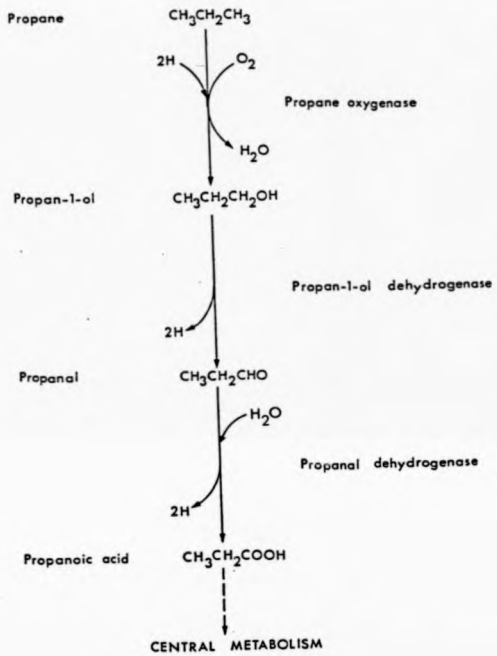


Figure 1.7 Terminal oxidation of propane

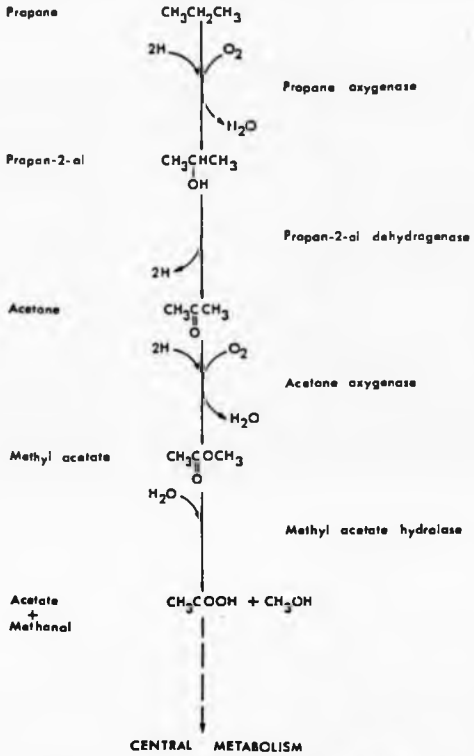


Figure 1.8 Subterminal oxidation of propane (via methylacetate)

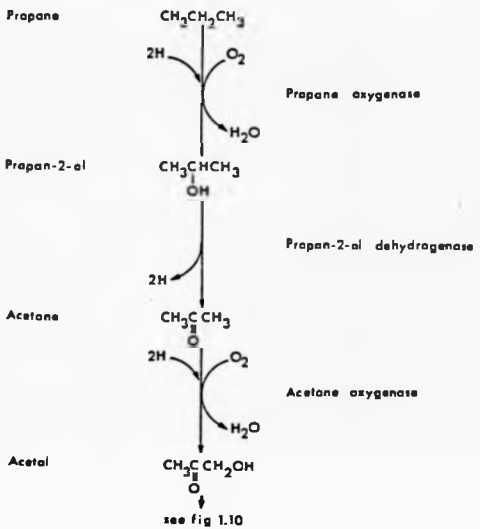


Figure 1.9 Subterminal oxidation of propane (via acetol)

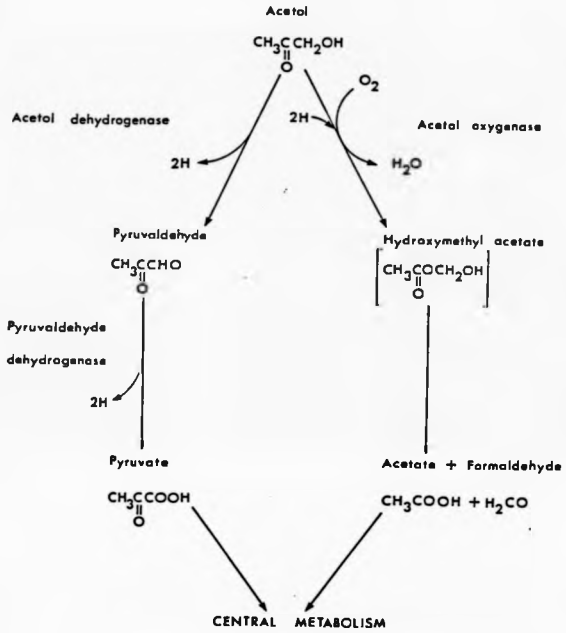


Figure 1.10 Pathways of acetol metabolism

enzymes by a permeability barrier. Also that, conversely, a compound may be rapidly oxidized when it is not an intermediate in the postulated sequence because the enzymes for its metabolism may be constitutive. They also state that the best criteria to support the assigned status of a compound as an intermediate in degradation are those supplied by a study of enzymes isolated from the organisms. As will be seen in the subsequent discussions such evidence is lacking for gaseous alkane metabolism.

Perhaps the greatest single contribution to understanding propane metabolism has come from the work of Jerome J. Perry and his co-workers who have done most of their work with the propane-oxidizing mycobacteria, M. vaccae JOB5, M. smegmatis and M. convolutum R-22. It is due more to their work than any other that the notion of subterminal oxidation has been applied to propane metabolism, and it is their work that is discussed first.

1.6.2 The work of J. J. Perry et al

Perry (1968) investigated the substrate specificity of three bacteria isolated from soil and capable of growth on propane. The one studied in most detail was designated Brevibacterium sp. JOB5 (later to become Mycobacterium vaccae JOB5).

Respirometric studies were performed using a Warburg apparatus to measure oxygen uptake by resting cell suspensions. The fact that propan-2-ol and acetone-grown cells were adapted for propane oxidation was taken to implicate these two compounds as intermediates in propane oxidation. This need not necessarily be so since these compounds may

require an oxygenase in their metabolism (see Figs. 1.8 and 1.9) which could also oxidize propane fortuitously. Also JOB5 grown on propane was capable of oxidizing propan-1-ol and propanoate at rates of 0.27 and 0.099μl O_2 min^{-1} mg dry $weight^{-1}$ respectively, whereas acetone was only oxidized at a rate of 0.023μl O_2 min^{-1} mg dry $weight^{-1}$, which must also be compared with the rate of acetone oxidation by propan-2-ol-grown cells of 0.4μl O_2 min^{-1} mg dry $weight^{-1}$. These figures would appear to suggest that acetone was not a potential intermediate.

More substantial evidence in favour of subterminal oxidation was presented by Vestal and Perry (1969). Using the same organism (Brevibacterium JOB5) they performed ^{14}C radiotracer experiments that apparently showed subterminal oxidation of propane was occurring.

In the presence of the pyruvate carboxylase inhibitor sodium arsenite, propanoate and propan-1-ol-grown cells could accumulate ^{14}C-pyruvate upon incubation with $^{14}CO_2$. Upon incubation of propan-1-ol and propanoate-grown cells with ^{14}C-propanoate labelled in the 1, 2 or 3 position the patterns of $^{14}CO_2$ evolution suggested that propanoate was being metabolized via the methylmalonate pathway (Fig. 1.11). Cells grown on propane, propan-2-ol and propanoate were then exposed to the corresponding 2-^{14}C substrate with and without sodium arsenite. Propanoate metabolism resulted in a significant accumulation of label in pyruvate, whereas propane and propan-2-ol metabolism resulted in relatively little label in pyruvate. These results suggested that propane and propan-2-ol were not being metabolized via pyruvate. Isocitrate lyase was induced by growth on propane and propan-2-ol but not on propanoate or propan-1-ol suggesting that propane was metabolized via acetate. By the addition of unlabelled potential intermediates

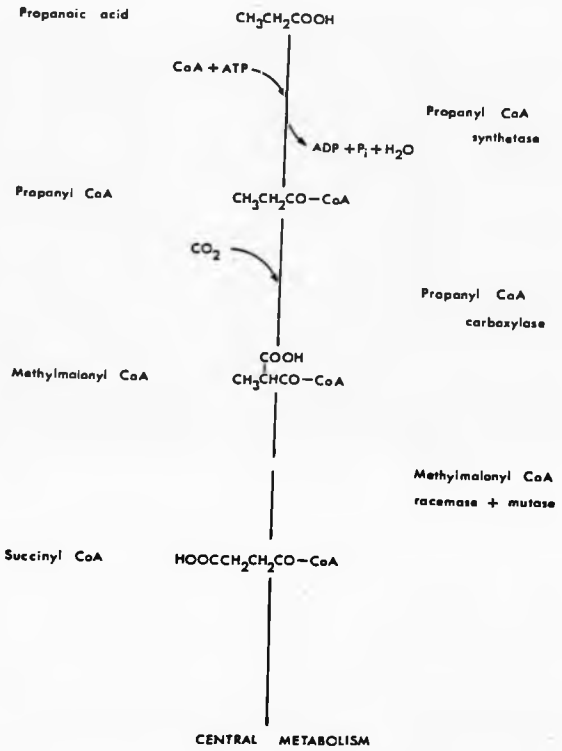


Figure 1.11 Methylmalonate pathway of propanoate oxidation

during the oxidation of 2-^{14}C-propane and 2-^{14}C-propan-2-ol and looking for accumulation of label in metabolic products they were able to show that acetone and perhaps acetol (hydroxyacetone) were intermediates of propan-2-ol metabolism which led them to propose the pathway in Fig. 1.12 to be operating in propane metabolism. Markovetz (1971) pointed out that acetone being converted to methyl acetate followed by cleavage to acetate and a C_1 fragment (methanol?) could also explain the induction of isocitrate lyase in propane-grown cells of Brevibacterium JOB5 but Taylor et al. (1980) could find no evidence for the methyl acetate pathway in this organism.

Stephens (1983) raised some doubts as to the interpretation of the data from this experiment. Firstly in the absence of arsenite apparently significant amounts of 2-^{14}C-propan-2-ol were being converted to pyruvate suggesting that arsenite may have been inhibiting the intitial stages of propan-2-ol metabolism. Subsequent experiments by Taylor et al. (1980) to show that acetone could be converted to pyruvate by Brevibacterium JOB5 were unsuccessful but assays (using cell-free extracts) were only attempted under one condition which may have been suboptimal for the enzymes required. Even the presence of isocitrate lyase during growth on propane and propan-2-ol does not preclude oxidation via pyruvate since Kornberg (1966) showed that pyruvate does not repress isocitrate lyase activity in all species. The presence of isocitrate lyase does not rule out the possibility of oxidation via propanoate since other pathways of propanoate oxidation exist that require the presence of isocitrate lyase (e.g. via malonic semialdehyde). These pathways may be induced by growth on propane but not on propanoate, when the methylmalonate pathway is operative.

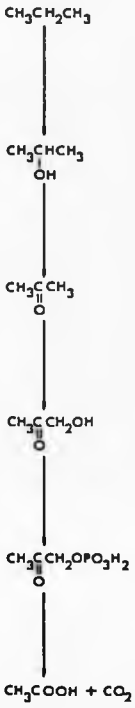


Figure 1.12 Perry's proposed pathway of propane metabolism in Mycobacterium vaccae JOB5 (from Perry, 1980)

Vestal and Perry (1971) analysed the fatty acid content of Mycobacterium vaccae JOB5 (formerly Brevibacterium JOB5) after growth on propane, propan-2-ol, propanoate and acetate. It was suggested that if propane was oxidized to propanoate, then propane-grown cells should contain high levels of odd chain fatty acids, whereas if it was oxidized via propan-2-ol to acetate then predominantly even chain fatty acids should result. The results indicated that considerable amounts of odd chain fatty acids were present in propane-grown cells indicating that terminal oxidation of propane to propanoate could have been occurring.

Blevins and Perry (1972) used a similar approach to establish the propanoate oxidation pathways in M. convolutum R-22. They also found that isocitrate lyase activity in propane-grown cells was inconsistent but offered no explanation for this. $^{14}CO_2$ recovery from 1-^{14}C-propanoate exceeded recovery from 2 and 3-^{14}C-propanoate over a 30 minute period thus indicating propanoate oxidation via the methylmalonate pathway. The authors did not note that propanoate-grown cells not only accumulate more label in pyruvate when fed $^{14}CO_2$ and propanoate (in the presence of sodium arsenite) than do propane and acetone-grown cells fed their respective substrates under the same conditions, but propanoate-grown cells also incorporate more label into pyruvate when incubated with malate than do acetone and propane-grown cells. Also an identical experiment using M. vaccae JOB5 showed overall much higher counts and the amount of $^{14}CO_2$ incorporation by M. convolutum R-22 appears insignificant. A simultaneous adaptation experiment did show that propane-grown cells could rapidly oxidize propan-2-ol (1.83μl O_2 min^{-1} mg dry weight^{-1} of cells) and acetone (2.47μl O_2 min^{-1} mg dry weight^{-1}). Propan-1-ol oxidation was also significant (1.53μl O_2 min^{-1} mg dry weight^{-1}) but the low levels of

propanoate oxidation (0.1µl O_2 min^{-1} mg dry $weight^{-1}$) could be due to lack of an uptake system and cannot rule out propanoate as a possible intermediate in propane metabolism. Overall then, this work produced little firm evidence on the route of propane oxidation in this organism.

Phillips and Perry (1974) investigated butane metabolism by M. vaccae JOB5 and came to the conclusion that it was metabolized terminally. Fatty acid profiles of propanoate and butan-2-one (an intermediate of subterminal oxidation of butane)-grown cells were similar in containing significant levels of odd chain fatty acids. n-butane, butanoate and acetate-grown cells contained less than 1% odd chain fatty acids. This suggested that butan-2-one could be metabolized via propanoate and that butane was not, thereby indicating that butane was not oxidized subterminally. Cells grown on n-butane, butanoate, acetate and β-hydroxy-butyrate were induced for isocitrate lyase whereas after growth on butan-2-one and propanoate, cells were isocitrate lyase negative. This again suggested that butan-2-one was not an intermediate in butane metabolism.

Incorporation of labelled $^{14}CO_2$ into pyruvate by butan-2-one and propionate-grown cells but not by n-butane, butanoate and acetate-grown cells again suggests that butan-2-one was not an intermediate of butane metabolism. Strangely the roles of butan-1-ol and butan-2-ol were not investigated, as analysis of cells after growth on these substrates and comparison with n-butane-grown cells would have shown which was the more likely intermediate in n-butane metabolism. It also seems odd bearing in mind that propane-grown cells are adapted for butane, butan-1-ol and butanoate oxidation (Perry, 1968) that the pathways of n-butane and propane metabolism are so dissimilar. Why, if they are indeed

metabolized by a common oxygenase, should propane be oxidized subterminally and butane terminally?

Between 1976 and 1984 Perry's interest seemed to shift to isolation and characterization of thermophilic hydrocarbon oxidizers (e.g. Phillips and Perry, 1976; Merkel et al., 1978a; Merkel et al., 1978b, Merkel et al., 1980; Zarilla and Perry, 1984). Then Coleman and Perry (1984) returned to the problem of the fate of the C_1 moeity resulting from the supposed C_2 and C_1 cleavage that occurred in propane metabolism. This was originally suggested to be released as CO_2 (Fig. 1.12). Upon exposing cell suspensions to 2-^{14}C-acetone and 1, 3-^{14}C-acetone and measuring patterns of CO_2 evolution it was seen that $^{14}CO_2$ was produced at the same rate from each substrate. This would not be the case if CO_2 was released from the cleavage of acetol when there would be a higher rate of $^{14}CO_2$ evolution from 1, 3-^{14}C-acetone than from 2-^{14}C-acetone. It was then postulated therefore that the C_1 moeity could enter the reduced C_1 metabolite pool. To demonstrate if this was happening cells were grown on propan-2-ol in the presence of 1-^{14}C-acetate or ^{14}C-bicarbonate or ^{14}C-formaldehyde or 1-^{14}C-1,2- propandiol (this latter compound being used instead of 1-^{14}C-acetol but supposedly being metabolized to acetol). After incubation, patterns of label incorporation into lipid, nucleic acid and protein were determined. Cells exposed to labelled diol (= acetol) showed the same pattern as those exposed to formaldehyde suggesting that metabolism of acetol results in the formation of a reduced C_1 moeity. The nucleic acid fraction was also hydrolysed to release guanine and cytosine. 40% of the carbon in guanine is derived from the reduced C_1 pool whereas none of the cytosine is so derived. Cells exposed to labelled 1,2-propandiol and formaldehyde showed a higher percentage of label in their

guanine than did cells exposed to acetate or bicarbonate, again suggesting that acetol is metabolized via a reduced C_1 moeity.

Of course this experiment is using the *a priori* assumption that propane is metabolized to acetol in the first place in proposing a reduced C_1 moeity as an intermediate in propane metabolism. All the experiment actually shows is that 1, 2 propandiol (or acetol?) is metabolized to produce a reduced C_1 moeity.

Subsequently Hartmans and de Bont (1986) have shown an acetol monooxygenase from Mycobacterium Pyl which is capable of converting acetol to acetate and formaldehyde via hydroxymethylacetate in a Baeyer-Villiger type reaction (see Section 1.7.4).

1.6.3 Other work

In 1956, Davis *et al* isolated an unusual organism Mycobacterium paraffinicum. It appeared to have an obligate requirement for n-alkanes or their potential metabolic intermediates. It grew well on ethane and poorly on ethanol and acetate. It was capable of oxidizing ethane, ethene, ethanol, acetaldehyde and acetate and the authors postulated this as the pathway of ethane oxidation. Rates of ethene oxidation were, however, about half that of the other intermediates and in hindsight ethene is probably not an intermediate of ethane oxidation. Ethane-grown cells were able to oxidize propane and butane but no mention is made of any products formed as a result of this.

Dworkin and Foster (1958) isolated a number of ethane-oxidising mycobacteria. They attempted, unsuccessfully, to form cell-free

extracts of these organisms and the constitutive nature of ethane oxidation precluded simultaneous adaptation studies. All the strains oxidized ethanol and ethandiol. Acetaldehyde and acetate were only oxidized slowly and only one strain oxidized ethene. The authors were careful to point out that these studies neither established nor eliminated the involvement of any of these compounds in ethane metabolism.

Lukins and Foster (1963) showed that Mycobacterium smegmatis 422 produced methylketones when oxidizing n-alkanes. No, or very little, terminal oxidation products were detected in culture supernatants. Ketone production occurred under rather artificial conditions. Washed cell suspensions of n-alkane-grown cells were resuspended in mineral salts medium lacking a nitrogen source and incubated with n-alkane for 15 hours. Whilst it was clear that subterminal oxidation was occurring, whether this was so under growth conditions was not established. M. smegmatis 422 was also able to grow on a range of methylketones but this did not correlate with its ability to grow on the corresponding n-alkane. This observation was subject to the reservation that the ketones were supplied as growth substrates at 0.2% (v/v), a concentration at which they may have been toxic. Simultaneous adaptation studies showed that growth on propane greatly enhanced the oxidation rate of subterminal oxidation intermediates (propan-2-ol and acetone). However, cells grown on propane or on non-hydrocarbon substrates rapidly oxidized the primary alcohol, aldehyde and acid. Cells grown on propane, propan-1-ol and acetone were tested for the ability to oxidize these three substrates. Propane and acetone-grown cells oxidized propane and acetone whereas propan-1-ol-grown cells did not thus indicating that acetone-grown cells were "back-adapted" to propane

Three possible hypotheses were put forward to explain this phenomenon i) acetone was acting as an analogue inducer of propane oxygenase, ii) propane was being oxidized by an acetone oxygenase, iii) propane produced from acetone was acting as an inducer of propane oxygenase.

If i) were true then propan-2-ol-grown cells should also be adapted to propane oxidation. This was found for M. vaccae JOB5 (Perry, 1968) but was not reported in this study. If ii) were true it could account for the build up of ketones reported when M. smegmatis 422 was growing on n-alkanes. If propane and acetone were competing for the same active site and the K_m for acetone was higher, then acetone might be excreted. No evidence for the third explanation has been reported.

Lukins and Foster (1963) also showed that propylene could not be an intermediate in propane metabolism by using medium enriched in D_2O and analysing the mass spectra of the acetone formed.

O'Brien and Brown (1967) isolated a strain of Mycobacterium phlei that was capable of growth on isobutane. The configuration of the isobutane molecule precludes the involvement of a ketone in isobutane metabolism and the authors concluded, on the basis of simultaneous adaptation studies, that the molecule was oxidized terminally via isobutanol, isobutanal and isobutanoate. Resting cells grown on propane, butane and 2-methylbutane were adapted for oxidizing one or more of the saturated gaseous alkanes, but cells grown on liquid hydrocarbons (with the exception of 2-methylbutane) were not so adapted. This would suggest that a separate oxidation system exists for liquid ($>C_5$) and gaseous n-alkanes.

Taylor et al. (1980) carried out an extensive study on acetone metabolism by four Gram-positive organisms isolated from soil. They compared patterns of enzyme activities in cell-free extracts and also used ^{14}C tracer studies with whole cells to conclude that acetone was metabolized via acetol and pyruvaldehyde (methylglyoxal) to pyruvate (see Fig. 1.10). The authors included Perry's M. vaccae JOB5 in this study and concluded that since no acetol or methylglyoxal dehydrogenase activity could be measured in acetone-grown cells then metabolism via pyruvate was unlikely. This was backed up by the presence of isocitrate lyase activity suggesting metabolism via acetate. The authors also assayed for methylacetate hydrolase to see if a Baeyer-Villiger type oxidation was occurring to convert acetone to methylacetate but no hydrolase activity could be measured. It should be noted that the conditions used for the enzyme assays were rather conservative and it could be that no activity was recorded because conditions in the assay were deleterious. A range of conditions (buffers, pH., cofactors, etc.) may have revealed some activity.

Hou et al. (1983a) showed that methylketones were produced by resting cell suspensions of gaseous alkane-grown bacteria when incubated with secondary alcohols or n-alkanes. This clearly indicated that subterminal oxidation was occurring but the authors did not rule out the possibility that terminal oxidation was also occurring. Propan-1-ol was metabolized more rapidly than propan-2-ol or acetone and propanoate could be detected following acidification of the reaction and extraction with benzene. Using a cell-free extract Hou et al were able to directly demonstrate that propane was oxidized to equimolar amounts of propan-1-ol and propan-2-ol. Propane-grown cells contained both primary and secondary alcohol dehydrogenase activity (NAD^+-linked). It does,

however, seem extremely wasteful for these organisms to convert half of their available substrate to propan-2-ol which is then dehydrogenated to acetone, a substrate that is excreted and hardly oxidized at all.

Babu and Brown (1984) investigated the metabolism of propane and isobutane by "Nocardia paraffinicum" (Rhodococcus rhodochrous). The only product they detected (in more than trace amounts) was propan-1-ol. $^{18}O_2$ was incorporated into the propan-1-ol whereas $H_2{}^{18}O$ was not, thus indicating an oxygenase system was present. The stoichiometry of the oxidation appeared to be two propane molecules to one oxygen molecule and the authors suggested a novel intermolecular dioxygenase system might be operating.

MacMichael and Brown (1987) showed that the rate of propane oxidation in N. paraffinicum was directly related to the intitial CO_2 concentrations. The organism would not grow on either propane or propan-1-ol in the absence of CO_2. Respirometric studies suggested that CO_2 was required for propan-1-ol, propanal and propanoate, but not for propan-2-ol, catabolism. Significantly higher amounts of $^{14}CO_2$ were fixed when propane was the substrate compared to isobutane or n-butane. In fact $^{14}CO_2$ was fixed in considerable amounts when all the potential intermediates of a terminal oxidation pathway were metabolized. Addition of sodium arsenite increased the amount of ^{14}C trapped in pyruvate. Similarly 2-^{14}C-propane could also lead to ^{14}C trapped in pyruvate. This data led the authors to conclude that propane was metabolized via the methylmalonoate pathway (see Figs. 1.7 and 1.11).

Stephens and Dalton (1986) isolated several strains of Arthrobacter sp. that could utilize propane. They were divided into three groups on the

basis of their ability to utilize acetone. One group could not grow on acetone and could not oxidize acetone after growth on propane. Propan-2-ol was converted stoichiometrically to acetone which was excreted. These organisms were considered to oxidize propane terminally. The second group could grow slowly on acetone but could not oxidize acetone after growth on propane. Acetone oxidation was rapid after growth on acetone or propan-2-ol. Acetone was excreted during growth on propane and was not considered a likely intermediate in propane metabolism. The third group grew rapidly on acetone and were induced for acetone oxidation after growth on propane. No products could be detected in culture supernatants. Simultaneous adaption studies suggested that both propan-1-ol and propan-2-ol were metabolized completely.

Van Ginkel et al. (1987) elucidated the pathway of butane oxidation in Nocardia TB1. The presence of isocitrate lyase in butane-grown cells and the excretion of butanoate when cell suspensions were incubated with butane and sodium arsenite led the authors to suggest that butane was oxidized terminally. For some unknown reason no butan-1-ol dehydrogenase activity was measured in cell-free extracts but a butanol reductase activity was measured along with butanal dehydrogenase, and butanoate CoA synthetase which were used to infer the presence of terminal oxidation.

1.6.4 Conclusions

It can be seen from the surveyed literature that the pathways of ethane, propane and butane metabolism are far from clear. Ethane, being a small symmetrical molecule, has received scant attention and the idea that it is metabolized in an analogous fashion to methane (i.e. via alcohol,

aldehyde and acid) seems to have been accepted. Propane and butane have received considerably more attention but still their metabolism remains unclear.

The work of Perry and his coworkers when viewed as a whole would appear to suggest subterminal oxidation via acetol and acetate. But the evidence is not unambiguous and too few investigations of the enzymes of the pathway have yet been carried out. Perhaps, as a rule, the mycobacteria do oxidize propane subterminally. If that is the case then from the work of MacMichael and Brown (1987) and Babu and Brown (1984) it would appear that the nocardiae oxidize propane terminally. However, given the confused state of the actinomycete-complex taxonomy, such comparative statements should be viewed with extreme caution.

It is also intriguing why in the same organism propane should be oxidized subterminally and butane terminally, (Vestal and Perry, 1969; Phillips and Perry, 1974) and from the work of Hou et al. (1983a) why a propane oxygenase should produce terminal and subterminal oxidation products when only the terminal products appear capable of being oxidized further.

These apparent anomalies will only be resolved by extensive biochemical and physiological studies on the enzymes involved to build up a more coherent, integrated picture of the pathways. This will be the approach used in this particular study. Other techniques (genetics, molecular biology, NMR spectroscopy) will no doubt also be used in the future to provide answers to the questions of gaseous alkane metabolism.

1.7 Enzymes involved in n-alkane metabolism

1.7.1 Oxygenases

Oxygenases were originally defined by Hayaishi (1975) as enzymes that incorporate molecular oxygen into substrates. They are widespread, occurring in plant and animal tissues as well as in microorganisms. Hayaishi further divides this group of enzymes into di- and monooxygenases depending on whether both or just one atom of molecular oxygen is incorporated into the substrate.

The monooxygenases appear to be the most important class of oxygenases in n-alkane metabolism, being implicated in both methane and liquid alkane metabolism. No firm evidence yet exists of their role in ethane, propane or butane metabolism. Patel et al. (1983b) showed that cell-free extracts of an Arthrobacter sp. catalysed the oxygen and NADH-dependant hydroxylation of ethane, propane and butane which would suggest the involvement of a monoxygenase type enzyme.

Three different n-alkane monoxygenase systems have been characterized in some detail and these are summarised below.

1.7.1.1 Methane monooxygenases

These enzymes catalyse the first step in methane metabolism, its oxidation to methanol. A number of enzymes have been characterized to greater or lesser degrees (see Dalton, 1980b; Dalton and Leak, 1985) but it is the soluble methane monooxygenase from Methylococcus capsulatus (Bath) that is the best characterized.

This enzyme is composed of three components A, B and C. Component C is an NADH-dependant oxidoreductase. It is a single polypeptide chain of molecular weight 39 to 44 kilodaltons and contains one mole of FAD, one mole of non-haem iron and one mole of acid-labile sulphide per mole of protein (Dalton and Leak, 1985). It catalyses the transfer of electrons from NADH (or NADPH) to a range of acceptors including protein A. Protein A is the hydroxylase component and the site of oxygen addition to methane. It has a molecular weight of 220 kilodaltons and is comprised of three subunits of molecular weights 54, 42 and 17 kilodaltons which suggests an α_2, β_2, γ_2 arrangement. It contains 2 to 3 moles of non-haem iron, 0.2 to 0.5 moles of zinc and no acid labile sulphide per mole of protein (Dalton and Leak, 1985). Protein B is a low molecular weight (20 kilodaltons) protein with a single polypeptide chain and appears to lack any metals or prosthetic groups.

Based on reconstitution studies a tentative scheme of electron transfer has been postulated (Fig. 1.13). Protein B appears to act as a regulator, controlling the flow of electrons from protein C to protein A. In the absence of protein B, proteins A and C will catalyse the NADH-driven reduction of oxygen to water. Even if methane is present it is not oxidized. If B is added back (in the absence of methane) then electron flow falls to zero. If methane is then added, rapid oxidation of the substrate occurs (Green and Dalton, 1985).

Green and Dalton (1986) established the mechanism of methane monooxygenase activity with propylene as a substrate. They suggested that the enzyme reacted first with propylene and NADH to form a reduced form of the enzyme which then reacted with oxygen in a separate step.

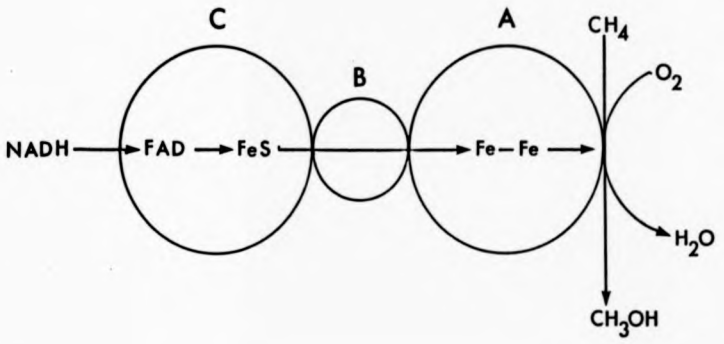


Figure 1.13 Postulated pathway of electron transfer in soluble methane monooxygenase from M. capsulatus (Bath) (from Dalton and Leak, 1985)

All the methane monooxygenases studied so far have wide substrate specificities (see Dalton, 1980a). There is some disagreement over whether this is an adaptation to give the organisms a survival advantage (Higgins et al., 1980; 1981) or whether it is a dictate of the enzyme mechanism (Stirling and Dalton, 1981). Since liquid alkane monooxygenases are capable of effecting similar oxidations (alkanes to alcohols, alkenes to epoxides) it could be that this is due to some mechanistic similarity between alkane monooxygenases but this is just speculation and requires further investigation.

1.7.1.2 Octane monooxygenase from Pseudomonas oleovorans

The octane monooxygenase from P. oleovorans is a three component system first described by Baptist et al. (1963). It oxidizes octane to octan-1-ol in an oxygen and NADH-dependant reaction. The three components are i) an NADH-rubredoxin reductase which is a single polypeptide of molecular weight 5 5 kilodaltons containing one mole of FAD per mole of protein (Ueda et al., 1972). It appears to contain no metals and is similar in size and amino acid composition to the reductase in the Corynebacterium P_{450} system described by Cardini and Jurtshuk (1970), ii) rubredoxin, which is a single polypeptide chain of 19 kilodaltons molecular weight. It contains two moles of iron per mole of protein and no prosthetic groups (Lode and Coon, 1971), iii) ω-hydroxylase, which is a non-haem iron protein containing one iron atom and one cysteine residue per polypeptide chain of molecular weight 40.8 kilodaltons (Ruettinger et al., 1977).

The FAD group of the reductase can be reduced by NADH and in turn pass the electrons to the non-haem iron prosthetic group of rubredoxin. The

reductase and rubredoxin interact on a 1:1 basis to form a complex and the redox reaction occurs as described in Fig. 1.14 (Peterson et al., 1967). Both reductase and rubredoxin are soluble enzymes but the hydroxylase is membrane bound (Benson et al., 1979).

The possibility of the involvement of hydroperoxides as intermediates in octane oxidation has been raised, because the reductase, in the presence of rubredoxin and NADH, will catalyse the reduction of octylhydroperoxide to octan-1-ol. Ratledge (1978) considers this possibility unlikely as hydroperoxides have never been isolated as intermediates and the reactivity of the enzyme towards hydroperoxides is probably due to its broad specificity.

McKenna and Coon (1970) tested the substrate range of the octane monooxygenase and found it oxidized n-alkanes from C_6 to C_{16} (no others were tested), maximum rates occurring with octane and nonane. It was also active against a range of long chain fatty acids and cyclic alcohols. It showed no activity on aromatics or highly branched aliphatic molecules. May and Abbott (1973) attempted to show that the hydroxylase could also epoxidate alkenes but were unable to unequivocally state this due to being unable to purify the hydroxylase component to homogeneity. However, it seemed likely and was later shown by May et al. (1976) that the hydroxylase was capable of epoxidating alkenes. It was suggested that the two reactions were mechanistically similar and may involve the same species of activated oxygen. May and Padgette (1983) report unpublished results suggesting that the mechanism of oxygen activation is not concerted and probably proceeds through a carbocation intermediate after initial oxygen attack on the terminal carbon atom.

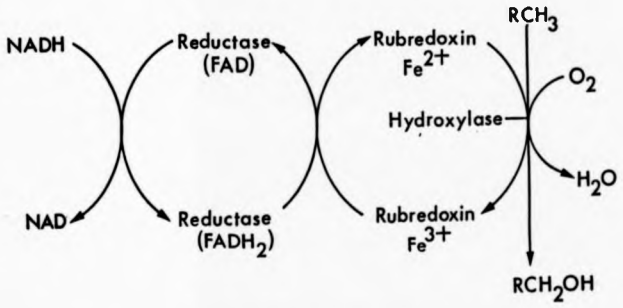


Figure 1.14 Postulated pathway of electron transfer in octane monooxygenase from P. oleovorans (from Ratledge, 1978)

1.7.1.3 Octane monooxygenase from Corynebacterium sp. 7E1C

Cardini and Jurtshuk (1968; 1970) described an octane monooxygenase enzyme isolated from octane-grown Corynebacterium sp. 7E1C. They found that soluble cell-free extracts could catalyse the oxygen and NADH-dependant oxidation of octane to a mixture of octan-1-ol and octanoate. In contrast to the system in Pseudomonas oleovorans this reaction was inhibited by carbon monoxide. Ammonium sulphate precipitation resulted in two fractions both of which were required for activity. One fraction gave a characteristic cytochrome P_{450} spectrum when reduced in the presence of carbon monoxide. The other fraction contained flavin and catalysed the transfer of electrons from NADH to a variety of acceptors including the cytochrome P_{450} component. Both components were induced by growth on octane. The authors suggested that a third non-haem iron component may be involved since other cytochrome P_{450} systems from bacteria and eukaryotes consist of three proteins. They suggested the flow of electrons from NADH to oxygen would be as shown in Fig. 1.15.

Unfortunately, although Corynebacterium sp. 7E1C grows on n-alkanes from C_3 to C_{18} the authors did not investigate the substrate specificity of the monooxygenase.

1.7.2 Alcohol dehydrogenases

Methanol dehydrogenase is the enzyme that oxidizes methanol to formaldehyde. It was first characterised by Anthony and Zatman (1964) in Pseudomonas M27. Since then many such enzymes have been described in both methylotrophs and methanotrophs (see Dalton and Leak, 1985). They all appear to be similar, utilizing phenazine methosulphate (PMS) as an

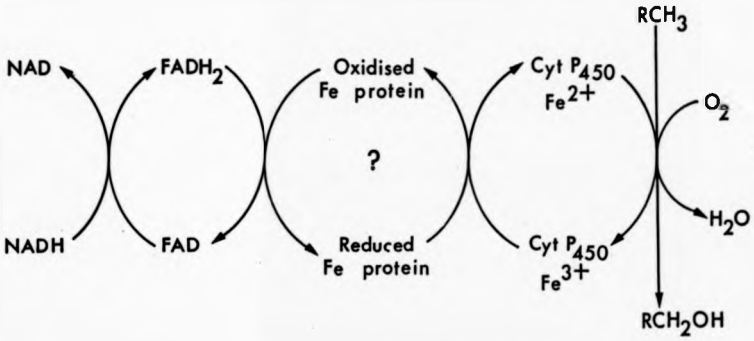


Figure 1.15 Postulated pathways of electron transfer in octane monooxygenase from Corynebacterium sp. 7E1C (from Ratledge, 1978)

electron acceptor *in vitro*. The *in vivo* electron acceptor is probably cytochrome C (O'Keefe and Anthony, 1980). This coupling can occur *in vitro* only if the enzyme is purified anaerobically. Methanol dehydrogenase enzymes contain the prosthetic group pyrroloquinoline quinone (PQQ) which has been extracted from the enzyme in one and two-electron reduced forms (Duine *et al*., 1980). All are optimal at high pH and require ammonia or methylamine for activity. A wide range of primary alcohols will serve as substrates (Anthony and Zatman, 1967).

Long chain alcohol dehydrogenases have been studied in most detail in *Pseudomonas* sp. Singer and Finnerty (1984) list a selection of work in this field. Tassin and Vandacasteele (1972) showed that *P. aeruginosa* 196Aa grown on glucose or hexadecane contained several soluble, NAD^+-linked alcohol dehydrogenase activities capable of using long chain primary alcohols as substrates. They then went on to show that a membrane bound NAD^+-independent alcohol dehydrogenase was induced after growth on hexadecane. Only PMS was used as an acceptor *in vitro* (the *in vivo* acceptor being unknown). Its' affinity for long chain primary alcohols was twenty times greater than that of the NAD^+-linked enzymes, this further implicating its role in alkane metabolism. The utilization of PMS instead of NAD^+ was suggested as having physiological significance since an acceptor of higher redox potential (PMS E_o' - 0.08V, NAD^+ E_o' - 0.31V) will shift the equilibrium of the reaction in favour of aldehyde formation. The enzyme produced aldehydes from a range of alcohols from C_4 to C_{16}. The authors drew parallels between this enzyme and the methanol dehydrogenases.

P. putida PpG1 (formally *P. oleovorans*). the plasmid-less strain, grows on primary aliphatic alcohols from C_3 to C_{12} (Grund *et al*., 1975).

Mutants designated Alc A^- grew on short chain alkanes but not on octanol, nonanol or dodeconol. This inferred the presence of at least two chromosomally encoded alcohol dehydrogenases. Introduction of the OCT plasmid restores octanol, nonanol and dodecanol utilization when the alk C locus on the plasmid is induced. This suggests that the Alc A^- mutants had lost an alcohol dehydrogenase activity that could be replaced by the gene product of alk C. However, Benson and Shapiro (1976) showed that Alc A^- mutants still possess constitutive NAD^+-dependant alcohol dehydrogenase activity and the Alc A^- gene product remains unclear. On an Alc A^- background alk C^- mutants will not grow on octanol, nonanol or dodecanol (or alkanes), thus demonstrating the obligatory role of alcohols as intermediates in alkane catabolism by this organism.

Hou et al. (1983b) isolated and purified a secondary alcohol dehydrogenase from propane-grown Pseudomonas fluorescens. Two NAD^+-linked alcohol dehydrogenase activities were present in cell extracts, one showing preference for primary alcohols and the other for secondary. The secondary alcohol dehydrogenase was purified to homogeniety and its properties investigated. It had a molecular weight of 144.5 kilodaltons and consisted of four subunits. The pH and temperature optima were 8 to 9 and 60 to 70°C respectively. The thermal stability was unusual for this type of enzyme, it retained 75% of its activity after 80 minutes at 85°C. Activity was inhibited by strong thiol reagents and certain metal chelating agents. Secondary alcohols were oxidized at much higher rates than primary alcohols, pentanol being the longest alcohol oxidized. The K_m for propan-2-ol was 8.5×10^{-5}M.

Coleman and Perry (1985) purified an inducible secondary alcohol

dehydrogenase from propane-grown Mycobacterium vaccae JOB5. It had a molecular weight of 136 kilodaltons and consisted of four subunits. The pH optimum was 10 to 10.5. NAD^+ could act as an electron acceptor but $NADP^+$ could not. The K_m for propan-2-ol was 4.9×10^{-5}M and the activity was inhibited by thiol reagents and metal chelators. Although it had (low) activity against primary alcohols it was only induced by growth on propane, propan-2-ol and acetone, not by growth on propan-1-ol.

The alcohol dehydrogenase from M. vaccae JOB5 was similar in many respects to that isolated from P. fluorescens by Hou et al. (1983). They were of similar size and subunit structure and oxidized a similar narrow range of substrates. Both were NAD^+-linked making them fundamentally different from the methanol and hexadecanol dehydrogenases described above. The enzyme from M. vaccae JOB5 could not oxidize methanol or ethanol but was capable, in the presence of NADH, of catalysing the reverse reaction of acetone to propan-2-ol. In these respects it differed from that described by Hou et al. Neither Coleman and Perry (1985) or Hou et al. (1983b) described any attempts to measure NAD^+-independant propanol dehydrogenase activity. The affinity of these latter two enzymes for their substrate were of the same order as that of hexadecan-1-ol dehydrogenase so it is possible that they do indeed play a role in gaseous alkane metabolism.

1.7.3 Aldehyde dehydrogenases

A range of formaldehyde dehydrogenase enzymes exist in methanotrophs and methylotrophs (see Dalton and Leak, 1985). There are both NAD^+-dependant and NAD^+-independant variaties of these enzymes. Some require

glutathione for activity and Duine et al. (1987) in a summary of the subject mention NAD^+-dependant formaldehyde dehydrogenases that do not require glutathione but some other, as yet unidentified, factor for activity.

Pseudomonas aeruginosa grown on heptane contained both soluble and particulate NAD^+-dependant aldehyde dehydrogenases which could oxidize aliphatic C_4 to C_{13} aldehydes (Bertrand et al., 1973).

Gaurrillot and Vandercasteele (1977) showed that glucose-grown P. aeruginosa contained two aldehyde dehydrogenases. An NAD^+-specific enzyme had a high affinity for short and medium (C_2 to C_{10}) chain length aldehydes and an $NADP^+$-specific enzyme from longer chain aldehydes. Hexadecane-grown cells contained a third, membrane bound, NAD^+-dependant aldehyde dehydrogenase which had high specific activities against long chain aldehydes perhaps implying a role in alkane metabolism. The role of the constitutive enzymes was unclear.

Acinetobacter sp. HO1-N was also shown to have inducible (by hexadecane, long chain alcohols and aldehydes) $NAD(P)^+$-dependant aldehyde dehydrogenase (Singer and Finnerty, 1984).

In P. putida the isolation of aldehyde negative (ald A^-), alkane-negative mutants in plasmid carrying strains (alkane hydroxylase positive, alcohol dehydrogenase positive) would suggest that the chromosomally encoded aldehyde dehydrogenase is required for alkane metabolism (Grund et al., 1975). Other studies, as mentioned above, would imply such a role. The role of constitutive aldehyde dehydrogenases is less clear but Duine et al. (1987) suggest they may

have a detoxification role.

1.7.4 Ketone monooxygenases

Forney and Markovetz (1969) demonstrated the presence of an NADPH-requiring tridecanone monooxygenase in tridecanone-grown P. aeruginosa. Unfortunately they did not try the assay with tridecane-grown cells but simply inferred that the ketone monooxygenase may play a role in alkane metabolism. Hasegawa et al. (1983) were able to demonstrate NAD(P)H-dependant cycloketone oxygenase activity in a cyclohexanone-grown Nocardia sp. Taylor et al. (1980) could not measure acetone monooxygenase activity in cell-free extracts of acetone-grown coryneforms, but inferred its presence from other whole cell studies. They suggested that the acetone monooxygenase was a labile system that was disrupted during preparation of the extracts. Hartmans and de Bont (1986) demonstrated acetol monooxygenase activity in cell-free extracts of Mycobacterium Pyl after growth on acetol or propan-1, 2-diol. The activity converted acetol to acetate and formaldehyde (see Fig. 1.10) and was inhibited by carbon monoxide, suggesting the involvement of a cytochrome P_{450} type system. The authors pointed out that such an acetol monooxygenase could play a role in propane metabolism by M. vaccae JOB5 since it would give rise to the acetate necessary to induce isocitrate lyase and a reduced C_1 moeity as described by Coleman and Perry (1984).

1.7.5 Summary

From the above survey, it can be seen that similarities exist between the enzymes involved in methane oxidation and those involved in liquid

alkane metabolism. These parallels will probably extend to the enzymes of ethane, propane and butane metabolism as they are investigated.

Methane oxidation enzymes are obviously constitutive because methanotrophs are obligately so. Growth on liquid alkanes tends to induce a series of enzymes specifically to metabolize the substrate to a heterotrophic intermediate. Ratledge (1978) suggests the switch to an alkane substrate has more far reaching affects switching the whole of cell metabolism from a glycolytic, lipogenic mode to a gluconeogenic lipolytic one. Both methane and octane monooxygenases appear to oxidize similar substrates to similar products perhaps suggesting like mechanisms. Similarities have also been pointed out between methanol dehydrogenases and other long chain alcohol dehydrogenases. The enzymology of ethane, propane and butane metabolism lags behind that of longer chain alkanes and methane but it is likely to turn out to be similar in many respects.

1.8 Genetics and regulation of n-alkane metabolism

The genetics and regulation of n-alkane metabolism has been most intensively studied in the liquid alkane-utilizing Pseudomonas sp. (see Williams, 1981; Singer and Finnerty, 1984). Elegant models describing the relationships between the genes involved in alkane oxidation have been proposed. Conversely the genetics of organisms such as mycobacteria, nocardioforms and coryneforms appears to be at a much less advanced stage generally. Consequently knowledge of the genetics and regulation of hydrocarbon metabolism in these organisms is virtually non-existent.

Van Eyk and Bartels (1968) working on Pseudomonas aeruginosa showed that whole cell oxidation of hexane was induced by C_4 to C_8 n-alkanes. n-butane was unable to support growth but was still capable of inducing the hexane oxidation system. Ethane and propane were ineffective as inducers. Several non-metabolizable substrates could also act as inducers such as diethoxymethane, 1,2-dimethoxyethane, and cyclopropane. Alkane analogues containing a sulphur or nitrogen atom could not act as inducers whereas an oxygen atom did not affect induction. Repressors of whole cell hexane oxidation included glucose, C_4 dicarboxylic acids, acetate glycerol and yeast-peptone extract. Certain amino acids, α ketoglutarate and malonate did not repress hexane oxidation.

The genes for the inducible octane hydroxylase system of P. putida are carried on the OCT plasmid (Chakrabarty et al., 1973). Shapiro and co-workers have elucidated the structure and regulation of the octane-oxidizing system in P. putida PpG6 (OCT) and this is summarised in Figs. 1.16 and 1.17. The OCT plasmid is a large plasmid of the P2 incompatability class. It has been shown to carry the alk A, B, C, D, E, and R loci.

alk A and alk B both determine components of the ω-hydroxylase. alk A gene product is a soluble protein presumed to be the rubredoxin component and the alk B product has been identified as a 40k cytoplasmic membrane component (Benson et al., 1979). alk E specifies a membrane bound $NAD(P)^+$-independant alcohol dehydrogenase. alk A, B and E are in a single operon under the control of a regulatory protein coded by alk R.

Two other genes are plasmid borne, alk C which is associated with

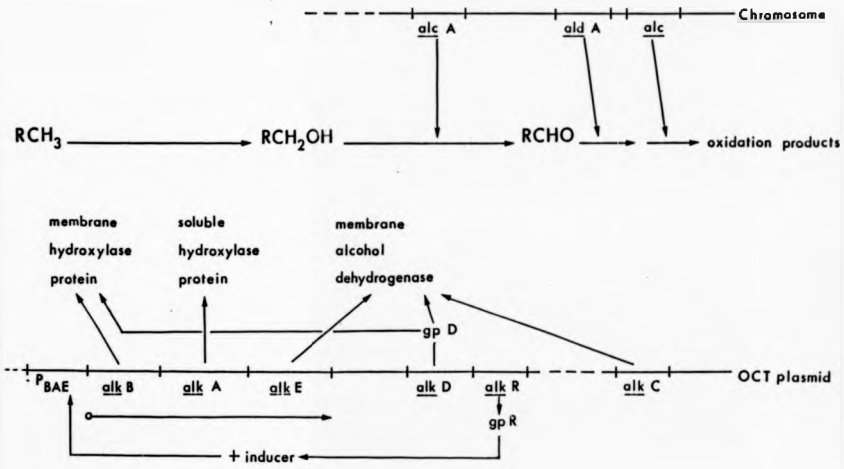


Figure 1.16 Genetic control of n-alkane oxidation in P. putida PpG6 (OCT) (from Williams, 1981)

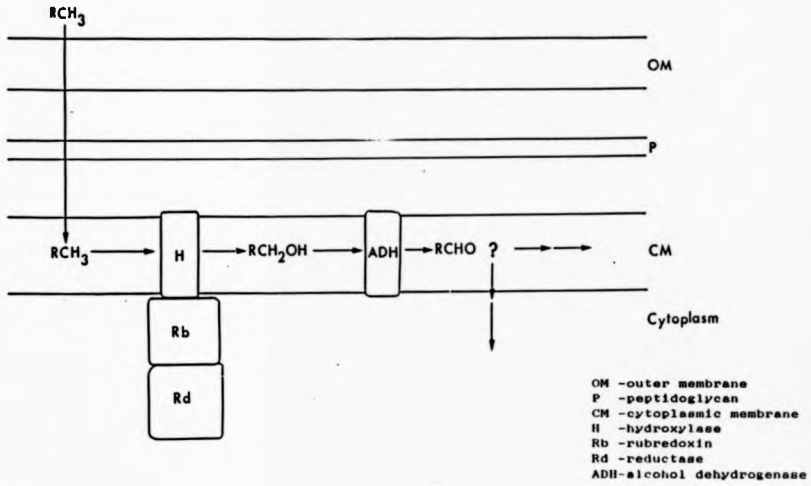


Figure 1.17 Membrane model of n-alkane oxidation in *P. putida* PpG6 (OCT) (from Benson *et al.*, 1979)

alcohol dehydrogenase activity and alk D. alk D mutants are pleiotropic and lack both the membrane bound hydroxylase activity and the membrane bound alcohol dehydrogenase activity. The alk D gene product is presumed to play some role in the membrane association of these two activities. The interaction of these loci and their products is shown in Fig. 1.16. This probably does not constitute a full picture since Benson et al. (1979) describe two other plasmid determined components in the cytoplasmic membrane, the genetic loci of which have not yet been identified. The location of the gene products led Benson et al. (1979) to propose the model of alkane oxidation shown in Fig. 1.17.

The other organism on which some work has been done in this field is Acinetobacter sp. HO1-N. This bacterium grows on n-alkanes from C_{10} to C_{22}. Despite extensive searches no plasmids have ever been found in this particular strain (Singer and Finnerty, 1984). The Alk^+ phenotype could not be removed by introduction of plasmids of several incompatability groups. Chemically induced Alk^- mutants could not be complemented by mating with Alk^+ phenotypes but the Alk^+ phenotype can be transferred by transformation of Alk^- mutants with highly purified chromosomal DNA. All this suggests that the alkane-oxidizing genes are chromosomally encoded in this organism.

The chemically induced point mutants of Acinetobacter with an Alk^- phenotype do not grown on any n-alkanes but are still capable of using C_{10} to C_{22} alcohols and aldehydes. This skewed distribution of mutants has been interpreted as meaning that either alcohols and aldehydes do not play a role in alkane oxidation or that there exist separate pathways for alcohol and aldehyde metabolism not related to alkane metabolism (Cruze et al. 1979).

None of the organisms subjected to physiological investigations of gaseous alkane metabolism have been subjected to genetic analyses. Jenkins et al. (1972) generated mutants of Mycobacterium rhodochrous that had altered patterns of n-alkane utilization but only n-alkanes from C_8 to C_{28} were investigated. The CMN group of bacteria are known to harbour plasmids (Sandoval et al., 1985) but to date the only catabolic activities assigned to such plasmids are nicotine degradation by Arthrobacter oxidans (Brandsch et al., 1982) and aniline degradation by Rhodococcus An1 (Atkins and Cain, 1985).

1.9 Potential uses of gaseous n-alkane oxidizing microorganisms

Much literature has appeared on the potential applications of methane and liquid alkane utilization (see Shennan, 1984). Comparatively little exists on the potential uses of ethane, propane and butane-oxidizing microorganisms. No doubt this reflects the state of knowledge of the more fundamental biochemistry and physiology of these organisms, it being difficult to speculate upon their usefulness without a knowledge of their functioning. However it is possible to consider some of the advantages and disadvantages of using these microorganisms in processes and the sort of processes they might be applied to.

Ethane, propane and butane are clearly not as abundant as methane, forming usually between 4 and 10% v/v of natural gas. Their occurrence is nonetheless widespread and they can be considered as quite abundant (Perry, 1980). They are usually available at a relatively low cost and tend to be free of objectionable side products not utilized during the fermentation. For this latter reason they are considered "high purity"

substrates in comparison to gas-oil fractions (C_{12}-C_{18} alkanes) and carbohydrates (soyabean meal) (Perry, 1980). Obviously their cost as feedstock must be considered in relation to other potential feedstocks such as methanol, gas-oil and carbohydrates. Their relative attractiveness will vary as economic and political variation alters the prices of these other feedstocks (see Linton and Niekus, 1987).

In addition to economic factors there are process considerations to take account of. Propane is the most water soluble of all the n-alkanes and has a mass transfer rate some 1.5-2 times that of methane. This means that the load on the fermenter for supplying the same mass of gaseous substrate is reduced to one-half or one-third when propane rather than methane is used (Takahashi, 1980). One disadvantage with all alkane substrates is the relatively large amount of oxygen required for their complete oxidation. This not only increases the cost of the fermentation but adds the additional design problem when using gaseous alkanes of creating explosive gas-air mixtures.

Other points to consider in any process employing ethane, propane or butane as a feedstock is the long doubling time most organisms have on these substrates. This coupled with a low growth yield (Perry, 1980) will lead to low productivity thus resulting in a dilute product which in turn increases down stream processing costs.

So allowing for these process considerations, what sort of products could be obtained from ethane, propane and butane-oxidizing bacteria? The answer is probably much the same as those obtained from methane and liquid alkane-utilizers.

Single cell protein production from ethane, propane and butane-oxidizing microorganisms is unlikely to be any more successful than it was from methane, methanol or gas-oil. The fluctuations in the market and the gradual realization that the world protein gap is a political and not a technological problem will almost certainly put an end to this sort of process. Perry (1980) gives some figures for the protein content of propane-grown bacteria and Takahashi (1980) discusses protein production from n-butane using Pseudomonas butanovora.

Overproduction and excretion of metabolites such as amino acids, organic acids, alcohols, fatty acids and vitamins have been considered by some authors (Perry, 1980; Shennan, 1984) and Hou (1984) mentions the production of polymers by a butane-grown Nocardia sp.

Biotransformations are likely to be a worthwhile field of investigation with this group of organisms. These fall into two categories. Firstly the bulk production of low value chemicals such as epoxypropane from propene (Hou et al., 1983c) and secondly more restricted production of high value chemicals, particularly drugs and hormones, for example specific modifications to complex sterols (Srivastava et al., 1985).

In all cases gaseous alkane-utilizers will be competing against the already exploited methane-oxidizers and liquid alkane-oxidizers, but until they are understood more fully it is impossible to state their possible usefulness or impact.

1.10 Synopsis

The study of the metabolism of n-alkanes by microorganisms has grown to a vast field since its start some 90 years ago. The methane-oxidizing bacteria have been intensively studied over the last 20 years as their potential usefulness became apparent. Much of their biochemistry and physiology is now understood but questions such as "is there such a thing as a facultative methane-oxidizer?" still stand unanswered. The metabolism of liquid n-alkanes aroused much interest from an environmental point of view. A variety of pathways appear to be used by different bacteria to assimilate these substrates. Questions still arise concerning the mechanisms of n-alkane oxidation and the feasibility of anaerobic metabolism on these substrates.

Filamentous fungi and yeasts are capable of growth on gaseous alkanes but comparatively little work has been done on them in comparison to bacteria. Ethane, propane and butane metabolism appears to be facultative (unlike methane oxidation). The Corynebacterium-Mycobacterium-Nocardia group of organism appear particularly prevalent in gaseous alkane metabolism.

The higher gaseous alkanes are widespread in nature, occurring in natural gas and petroleum deposits. They are not produced biologically to any great extent (unlike methane). Reports exist of increased abundance of gaseous alkane- metabolizing bacteria in soils overlying petroliferous regions.

Ethane, propane and butane-oxidizers are easily isolated by simple enrichment procedures. They do not appear to require any particular

supplements in their growth medium.

Growth on the higher gaseous alkanes leads to morphological changes within the cell as a result of the special nature of these substrates.

The pathways of gaseous alkane metabolism are far from certain. Much of the work is equivocal and the relative importance of terminal versus subterminal oxidation of propane is unclear. Firm evidence of the enzymes involved in propane metabolism is lacking.

Methane monooxygenase and two types of octane monooxygenase have been well characterized. Alcohol and aldehyde dehydrogenases and ketone monooxygenases have been described in methane and liquid alkane-utilizers. There appear to be common features which gaseous alkane oxidizers may share with these other two groups.

The genetics of alkane metabolism in Gram-positive bacteria is only poorly understood. The OCT plasmid system in Pseudomonas putida is the only well documented system in terms of its genes and regulation.

The potential usefulness of higher gaseous alkane-utilizers is difficult to assess given the present limited knowledge about them. They may find uses in processes similar to those involving methane and liquid alkane-utilizers.

CHAPTER 2

MATERIALS AND METHODS

2.1 Media and materials

Propane-utilizing bacteria were routinely grown in the mineral salts medium (MS) of Whittenbury et al. (1970) which was supplemented with $1g.l^{-1}$ NH_4Cl to give ammonium mineral salts (AMS). Solid medium was prepared by the addition of $15g.l^{-1}$ Difco Noble Agar to AMS medium. Medium was sterilized by autoclaving at 121°C for 15 minutes. Sterile phosphates were subsequently added to the cool medium. The medium was used at pH 6.8. Flammable carbon sources (e.g. alkanes) were added to the medium after inoculation. Non-flammable carbon sources (e.g. acetate, pyruvate) were added prior to inoculation. Concentrations of carbon sources used are given in Table 2.1.

During the initial enrichment and isolation of propane-utilizing bacteria, MS medium was supplemented with a dual nitrogen source of $0.5g.l^{-1}$ NH_4Cl and $0.5g.l^{-1}$ KNO_3 plus a vitamin supplement (Gest et al., 1983) (5ml of a X200 stock solution per litre of medium) and called ANMSV medium. Other mineral salts media employed during enrichment and isolation procedures were FI medium (minus glucose) (Pirt, 1985) and ABER medium (Stephens and Dalton, 1987b). Both were supplemented with the above mixture of vitamins. Subsequent demonstration of the non-requirement of isolates for these vitamins and the preference for ammonium as nitrogen source led to the use of AMS medum for routine growth studies.

Oxoid nutrient agar was prepared according to the manufacturers instructions.

Table 2.1 Substrate concentrations for routine growth studies

Substrate		concentration (% v/v except * % w/v)
Propane		50% in air
Propan-1-ol		0.1
Propan-2-ol		0.1
Propanal		0.05
Propanone (Acetone)		0.05
Propanoate	*	0.1
Hydroxypropanone (Acetol)		0.05
Acetate	*	0.1
Pyruvate	*	0.1
Succinate	*	0.1
Methane		50% in air
Ethane		50% in air
Butane		50% in air
Propene		50% in air
Methanol		0.1, 0.05
Methylacetate		0.1, 0.05
Methylglyoxal		0.1, 0.05

2.2 Enrichment and isolation methods

2.2.1 Samples

Soil and water samples were collected in sterile universals from a number of sites that had been exposed to gaseous or liquid alkanes (e.g. soil from an oil depot, scrapyard, gas works) and from sites showing no obvious exposure to alkanes (e.g. garden soil, running freshwater).

A few grams of soil were suspended in 10ml of sterile medium and the supernatant was allowed to clear slightly before being used as an inoculum. Water samples were used directly as inocula.

2.2.2 Enrichment of gaseous alkane-utilising bacteria in shake flasks and on agar plates

250ml "quickfit" flasks containing 50ml of mineral salts medium (with vitamin supplement) were inoculated with 100µl of soil suspension or water sample. The carbon source was then added to the specified concentration (Table 2.1). In the case of gaseous and volatile carbon sources the flask was closed with a "suba-seal". Air was replaced with gaseous alkane to give a 50% (v/v) alkane:air mixture. Flasks were incubated with shaking at 30°C until turbidity developed, usually after 3-10 days. Samples of the cultures were serially diluted to 1 in 10^8 and 20µl aliquots of the 10^{-4} and 10^{-8} dilutions were spread onto mineral salts agar plates. These were incubated at 30°C in "Gaspak" anaerobic jars containing a 50% (v/v) gaseous alkane:air mixture. When individual colonies appeared, these were streaked onto fresh ANMSV plates and incubated as before. Subsequently, the resulting colonies were used

to inoculate flask cultures with propane as carbon/energy source. Serial dilution of these cultures onto both mineral salts agar and nutrient agar plus detailed microscopic examination confirmed the presence of pure cultures.

In addition to the above method, 200ml of water samples were filtered through filters of 0.2μm pore size and these filters were then incubated on mineral salts agar plates in a propane:air atmosphere. Direct plating of 20μl of soil suspensions was also used as a method of enrichment for propane-utilizing bacteria. Purity checks were as for the flask enrichment method.

2.2.3 Continuous enrichment

An LH Fermentation (Stoke Poges, Bucks., U.K.) 100 series fermentor with a working volume of 800ml was inoculated with 5-10ml of soil or water samples. Stirring was at 600rpm and air was supplied at 60ml.min^{-1}. The minimal media and carbon sources employed are given in Table 2.2. The culture was grown batchwise until an optical density of $E_{540nm} = 1.0$ was attained, whereupon the fermentor was switched to continuous operation at the dilution rates specified in Table 2.2, with propane, supplied at 60ml.min^{-1}, as the carbon source. Samples were removed daily until a steady population appeared to be present or the culture washed out. Samples were serially diluted and plated onto ANMSV agar and incubated in a propane:air atmosphere as described previously.

2.3 Maintenance of isolates

Stock cultures of propane-utilizing bacteria were maintained by

Table 2.2 Continuous enrichment for propane-utilizing organisms

Experiment No.	Minimal medium	Batch growth carbon source (% w/v or % v/v *)	Dilution rate (h^{-1})
1.	ANMSV	Sodium benzoate 0.1	0.04
2.	PIV	Propan-1-ol 0.2 * and Propan-2-ol 0.2 *	0.02
3.	PIV	Sodium salicylate 0.1	0.014
4.	ABER	Sodium benzoate 0.1	0.03
5.	ABER	Propane (50% in air)	0.03

subculturing monthly onto AMS agar plates. The inoculated plates were placed in "Gaspak" anaerobic jars and the jars were gassed with propane from an inflated football bladder to give an approximate atmosphere of 50% propane in air (v/v). The containers were sealed and incubated at 30°C.

2.4 Biochemical tests to identify isolates

Preliminary identification of the isolates involved observation of cell morphology and motility with biochemical tests for catalase, spores, oxidative/fermentative metabolism and Gram stains. These tests were carried out using the methods described by Doetsch et al. (1981).

2.5 Routine growth and cell dry weight measurements

Flask cultures on propane were grown in 250ml "quickfit" flasks containing 50ml of AMS medium. Cultures were inoculated with a loopful of cells from a plate or with 0.5ml of an exponential growth phase liquid culture. Flasks were sealed with a "suba-seal" and 100ml of air withdrawn using a sterile needle and syringe. 100ml of propane was injected to give a 50% (v/v) propane:air mixture. In experiments to determine the effects of propane concentration on growth, nitrogen was used to replace portions of the propane. "Suba-seals" were swabbed with 70% (v/v) ethanol prior to injection of the gases but it was not found necessary to filter sterilize the gases. Flasks were incubated on an orbital shaker at 200rpm and 30°C.

Propane-grown batch cultures of gaseous alkane-utilizers were established using a 2 litre LH 500 series fermentor or a 100 litre LH

100 series fermentor. Inocula for the 2 litre fermentor were 2 x 50ml shake flask cultures in mid-exponential phase of growth. Inocula for the 100 litre fermentor were 10 litres of culture generated from the 2 litre fermentor operating in a fed-batch mode. Propane and air were supplied to the 2 litre fermentor at 60ml.min^{-1} each and to the 100 litre fermentor at 500ml.min^{-1} each. AMS medium was used at a constant pH of 6.8 and a temperature of 30^{o}C.

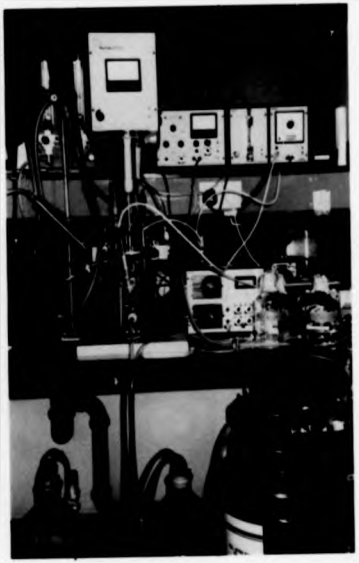
Continuous cultivation of the cells was attempted using a 2 litre LH 500 series fermentor (see Plate 2.1). Conditions used were as for the above growth experiments. In some experiments, dissolved oxygen tension was controlled using a polarographic oxygen electrode coupled to an oxygen controller which altered the stirring speed of the fermentor. pH was kept constant by addition of 1M HCl or 1M KOH using an automatic controller. Dilution rates were varied from 0.01 to 0.05h^{-1} and the AMS medium was sometimes supplemented with vitamin solution (see Section 2.1), casamino acids (Difco) 0.01% (w/v) or yeast extract (Difco) 0.01% (w/v).

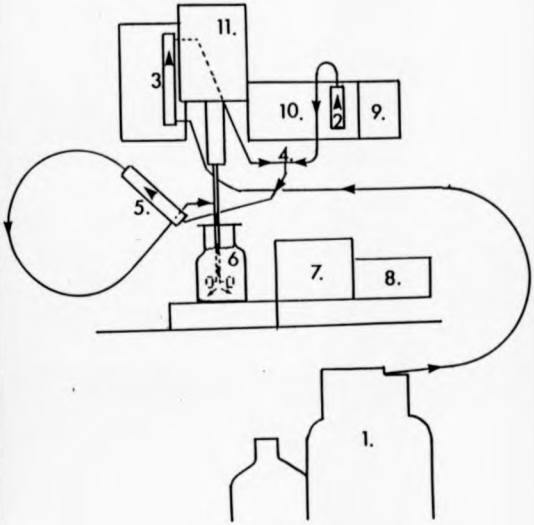
Cell density was routinely measured as optical density at 540nm (E_{540nm}) against a medium blank.

Growth rates were determined from flask cultures. 1ml samples were drawn aseptically from the culture at suitable intervals and their E_{540nm} measured. Results were plotted on semi-logarithmic graph paper and linear regression analysis performed on the data lying on the linear part of the resulting graph.

Plate 7.1 Fermentor system used for attempted continuous culture of R.rhodochrous PNKb1 on propane.

1. Propane supply
2. Air supply and flowmeter
3. Propane flowmeter
4. Gas mixing manifold
5. Sterile filter
6. Gases enter fermentor via stirrer shaft
7. pH control
8. Media pump
9. Temperature control
10. pO_2 control
11. Stirrer drive control




11.
3
10.
A2
9.
4
5.
6
7.
8.
1.

2.5.1 Cell dry weight measurement

Dry weights were estimated by constructing a standard curve of dry weights of cells versus optical density. Dry weights were measured by a filtration method (Gerhardt, 1981). Washed samples of cultures at different phases of growth and known optical densities were filtered under vacuum through preweighed, dried filters of pore size 0.2μm. Filters and cells were then dried in an oven at 90^{o}C to constant weight.

2.5.2 Total cell protein

Total cell protein was determined using a whole cell Biuret protein assay (Herbert et al., 1971). 2ml of a washed cell suspension was placed in a test tube and 1ml of 2M NaOH added. The suspension was then boiled in a water bath for 5 minutes. 1ml of a 2.5% (w/v) copper sulphate solution was then added, the tube was inverted once and allowed to stand for 5 minutes. The contents of the tube were centrifuged for 2 minutes in a bench microcentrifuge at 5000xg and the resulting clear blue/violet supernatant was transferred to a 1.5ml plastic cuvette and its' optical density measured at 555nm in a Pye Unicam SP1800 spectrophotometer (Pye Unicam, Cambridge, U.K.). A standard curve was prepared using bovine serum albumin at a range of concentrations from zero to 5mg.ml^{-1}.

2.6 Light microscopy

Slides were prepared by allowing a drop of 1% (w/v) noble agar to solidify on a slide under a cover slip. Once the agar had set the cover slip was lifted and a drop of culture placed on the agar. After

replacing the cover slip and applying immersion oil, photographs were taken using the fitted camera unit containing Kodak Panatomic X film. The microscope (Leitz, Wetzlar, W. Germany) was set in phase-contrast mode at a magnification of 1250X.

2.7 Studies using whole cells

2.7.1 Preparation of cell suspensions

Cultures from flasks or fermentors were centrifuged at 20,000xg for 10 minutes at $4^{o}C$. Cell pellets were washed in 20-30ml of ice-cold buffer (20mM TRIS HCl pH 6.8) and centrifuged as before. The cell pellets were then resuspended in the same buffer to an E_{540nm} of between 20 and 50 and the suspension used for whole cell studies.

2.7.2 Oxygen electrode assays

The ability of propane-utilizing bacteria to oxidize a variety of substrates was tested by measuring the stimulation of oxygen uptake on addition of the substrate to cell suspensions contained in a Clark-type oxygen electrode. (Rank Brothers, Bottisham, Cambridge, U.K.). Assays were done at $30^{o}C$ in 2.9ml of 20mM phosphate buffer pH 6.8. Air-saturated buffer was placed in the reaction chamber, the plunger inserted and the system allowed to equilibrate. 50µl of cell suspension was then added by syringe and the endogenous rate of oxygen uptake measured. 50µl of substrate was then injected and any stimulation in oxygen uptake recorded. Rates of oxygen uptake were corrected for endogenous rate and expressed as n moles O_2 min^{-1} mg dry $weight^{-1}$ of cells.

The dissolved oxygen concentration of air-saturated buffer was calculated using the method of Robinson and Cooper (1970).

Gaseous alkane substrates were prepared by degassing 5ml of distilled water under vacuum then passing the contents of a football bladder inflated with the gas through the water. Liquid alkanes and other relatively insoluble substrates were prepared as saturated solutions. The final concentrations of these substrates in the assay are given in Table 2.3 and were calculated from the data of McAuliffe (1966). Substrates readily soluble in water were prepared as 2mM solutions giving a final concentration in the assay of 33.3μM.

2.7.3 Gas chromatographic assays for product formation

2.7.3.1 Product formation from alkanes

Assays were performed in 2ml GC vials (Owens Polyscience, Macclesfield, U.K.) in a total volume of 0.25ml. The buffer used was either 20mM TRIS HCl pH 6.8 or 20mM phosphate pH 6.8. 12μl of a cell suspension was added to 238μl of buffer in a sealed vial. The vial was preincubated to 30°C in a shaking water bath for 30 seconds then 0.9ml of air was removed from the vial and replaced with 0.9ml of substrate (gaseous alkane). The vial was returned to the shaking water bath and incubated for 15 minutes. 5μl samples were removed at 5 minute intervals and injected into a gas chromatograph. The column used was glass 1.5m x 2.3mm i.d. packed with 50% (w/w) Porapak Q and Porapak N. It was run isothermally at 160°C with nitrogen as a carrier gas at 30ml.min^{-1}. Flame ionisation detection (FID) was used, output being logged on a chart recorder.

Table 2.3 Final concentration of substrates in oxygen electrode studies

Substrate	Solubility in water (mM)	Concentration in assay (μM)
Methane	1.53	25.50
Ethane	2.01	33.50
Propane	1.42	23.67
n-Butane	1.06	17.67
iso-Butane	0.84	14.00
n-Pentane	0.54	9.00
n-Hexane	0.11	1.8
n-Octane	0.0058	0.0965
Propene	4.76	79.33

2.7.3.2 Product formation from alkanes

The formation of epoxyalkanes from alkanes was assayed as above. The column used was packed with Porapak Q run at 180°C with 30ml.min^{-1} nitrogen as carrier. Output from the FID was logged on a reporting integrator (Hewlett Packard, Avondale, Pa., U.S.A.) that had been calibrated using standard epoxyalkane solutions. Rates were expressed as n moles epoxyalkane formed min^{-1} mg dry weight^{-1} of cells.

2.7.4 Inhibitor studies

Potential inhibitors were made up as 100mM solutions (except CO and C_2H_2 which were used as saturated solutions in water having concentrations of 1mM and 44mM respectively (Stirling, 1978)). They were added to GC vials to give final concentrations of 1, 5 or 10mM before addition of the substrate. Control vials lacked inhibitor. In oxygen electrode assays, substrate stimulated oxygen uptake was measured before and after addition of inhibitor.

2.7.5 Acetylene hydrase assay

Acetylene hydrase activity was measured as the rate of acetylene utilization by resting cell suspensions. Conditions for the assay were identical to those used for assaying product formation from alkanes (2.7.3.1) except that 0.9ml of acetylene was substituted for 0.9ml of gaseous alkane.

The gas chromatograph contained a 1.5m x 2.3mm i.d. glass column packed with Porapak Q run at 180°C with 30ml.min^{-1} nitrogen as carrier gas.

Output from the FID was logged on a reporting integrator. The integrator was calibrated using known gas mixtures of acetylene in argon. Rates were expressed as n moles acetylene-utilized min^{-1} mg dry $weight^{-1}$ of cells. Assays using boiled cells were used as controls.

2.8 Studies using cell-free extracts

2.8.1 Cell breakage methods

A variety of breakage techniques were used in an attempt to obtain satisfactory cell-free extracts. Cell suspensions were prepared as previously mentioned (Section 2.7.1) and subjected to physical, chemical and enzymatic breakage techniques.

i) French pressing: Cell suspensions were passed through a French pressure cell (Aminco, Silver Spring, Maryland, U.S.A.) operated at a pressure of 138MPa. Three passages were necessary to obtain sufficient protein for assays. Pressure cells were cooled to $4^{o}C$ and extracts collected on ice in precooled universals unless stated otherwise.

ii) Sonication: Cell suspensions were subjected to bursts of ultrasound from an MSE sonicator (MSE, Crawley, Sussex, U.K.). Cells were kept on ice during the treatment and were subjected to seven bursts at maximum amplitude (24 microns) of 30 seconds duration each, with 30 seconds cooling time between bursts.

iii) Tissue disintegrator: 10ml of cell suspension were placed in a small breakage vessel of a Braun tissue disintegrator (FT Scientific, Tewkesbury, U.K. Suppliers). Glass beads of diameter 0.1 - 0.11mm were added according to the manufacturers

instructions. The disintegrator was then operated on maximum setting (4000rpm) for five 1 minute bursts, allowing 30 seconds cooling between each burst. The vessel was cooled using CO_2 gas.

iv) Enzymatic lysis: Cell suspensions were centrifuged and resuspended in 20mM TRIS HCl pH 8.0 containing 100mM EDTA and 150mM NaCl. This was left on ice for 30 minutes. Cells were then centrifuged and resuspended in 250mM TRIS HCl pH 8.0 containing 5mM EDTA. A solution of lysozyme ($10mg.ml^{-1}$ in 250mM TRIS HCl pH 8.0, 5mM $MgCl_2$) was then added to a final concentration of $1mg.ml^{-1}$. The suspension was incubated at 37°C for 10 minutes, 20 minutes or 30 minutes.

2.8.2 Preparation of cell-free extracts

After breakage, unbroken cells were removed by centrifugation at 10,000Xg for 2 minutes. Occasionally a second spin was required to clear all unbroken cells. Crude extracts were viewed under a microscope to ensure they were free of whole cells. Before loading extracts onto purification columns, they were further cleared by passage through a 0.2μm pore size filter. The presence of both soluble and membrane bound proteins in crude extracts was shown by assaying for NADH-oxidase (a membrane bound protein) in an oxygen electrode. To separate soluble and membrane bound proteins, crude extracts were subjected to centrifugation at 38,000Xg for 30 minutes. The supernatant was then taken as the soluble fraction and the pellet, resuspended in 20mM TRIS HCl pH 6.8, as the particulate fraction.

The protein content of extracts was determined using the "BioRad" standard protein assay according to the manufacturers instructions. A

standard curve was prepared using bovine serum albumin.

2.8.3 Assay of cell-free propane oxygenase activity in the oxygen electrode

Sufficient 20mM TRIS HCl pH 6.8 (or other buffer as indicated in the results section), to give a final assay volume of 3ml, was equilibrated at 30°C in the oxygen electrode. Cell-free extract was added (2-10mg protein). Endogenous oxygen uptake was measured. 50µl of 100mM NADH and/or 50µl of 100mM NADPH were added and the change in rate recorded (other electron donors used are mentioned in the results and discussion). 100µl of a saturated propane solution (1.42mM) were then added and any change in rate recorded. A crude cell-free extract from Methylosinus trichosporium OB3b which is known to oxidize propane (Dalton, 1980a) was used as a positive control.

2.8.4 Assay of 1,2-epoxypropane formation from propane by cell-free extracts

Sufficient 20mM TRIS HCl pH 6.8 (or other buffers as mentioned in the results section) to give a final assay volume of 0.25ml was placed in a GC vial along with 1-5mg of protein from a cell-free extract. The vial was sealed and preincubated at 30°C for 30 seconds in a shaking water bath (250rpm). 10µl of 100mM NADH were added and 0.9ml of air removed and replaced with 0.9ml of propane. The vial was returned to the shaking water bath and 5µl samples removed every 5 minutes. These were assayed as described in Section 2.7.3.2. Rates were reported as n moles 1,2-epoxypropane formed min^{-1} mg $protein^{-1}$.

2.8.5 Stabilization of propane oxygenase activity

Attempts to stabilize propane oxygenase activity involved breaking the cells in different buffer solutions containing different putative stabilizing agents. Buffers were made up with stabilizing agents and cells were resuspended in these buffers prior to breakage. Cell suspensions were broken in a French pressure cell as described in Section 2.8.1. Agents used during the stabilization studies are listed in Table 2.4. Preparation of cell-free extracts under anaerobic conditions was also attempted. All solutions were degassed and sparged with oxygen-free nitrogen prior to use. All manipulations (e.g. filling the pressure cell, collecting the extract) were performed under a blanket of oxygen-free nitrogen gas.

2.8.6 Purification of propane oxygenase activity

Three different techniques were used in an attempt to isolate the propane oxygenase activity from cell-free extracts.

i) Gel filtration: 10mg of protein from a cell-free extract was loaded onto a 21.5 i.d. x 600mm preparative HPLC column packed with TSK G3000. The column had been equilibrated for 2 hours with 10mM phosphate buffer pH 7.0. The column was operated at a flow rate of 3ml.min^{-1} and fractions of 3ml volume were collected. Fractions were assayed for the ability to convert propene to 1,2-epoxypropane (see Section 2.7.3.2).

ii) Ion-exchange chromatography: 10mg of protein from a cell-free extract was loaded onto a 7.7 id x 250mm HPLC column packed with TSK DEAE 25W. The column had been equilibrated for 2 hours with

Table 2.4 Putative stabilizing agents

Stabilizing agent	Concentration (mM)
HEPES	20
Imidazole	20
MOPS	20
TES	20
Dithiothreotol	5, 10
Sodium thioglycollate	1, 5, 10
2-mercaptoethanol	1, 5
PMSF	2, 5
Procaine	2, 5
Mg^{2+}	5, 10
Mn^{2+}	5, 10
Trace elements (Vishniac and Santer, 1957)	$1ml.l^{-1}$ buffer

10mM phosphate buffer pH 7.0. The column was operated at a flow rate of 1ml.min^{-1}. A sodium chloride gradient from zero to 0.5M was run through the column to elute bound protein. Protein eluting from the column was monitored by its UV absorbance at 206nm. Fractions were assayed for the ability to convert propene to 1,2-epoxypropane (see Section 2.7.3.2).

iii) Affinity column: 25mg of protein from a cell-free extract was loaded onto a 10 x 60mm column packed with "Affi-gel Blue" (Activated blue sepharose - BioRad, Watford, Herts., U.K.). The column had been equilibrated for 2 hours at 4^{o}C with 20mM TRIS HCl pH 6.8. Protein was eluted from the column in 6 fractions as below. The flow rate was 12ml.hr^{-1}.

Fraction	Volume (ml)	Elutant
1	10	TRIS 20mM
2	6	NADH 1mM
3	6	NADH 5mM
4	8	KCl 0.2M
5	8	KCl 1M
6	15	KCl 3M

All elutants were made up in 20mM TRIS HCl pH 6.8. All fractions were assayed for the ability to convert propene to 1,2-epoxypropane (see Section 2.7.3.2).

2.8.7 Polyacrylamide gel electrophoresis (PAGE)

The method was based on that of O'Farrell (1975) and used a discontinuous buffer system as described by Laemmli (1970). The resolving gel was prepared in 3.0M TRIS HCl pH 8.8 and the stacking gel in 0.5M TRIS HCl pH 6.8. The running buffer was TRIS-glycine (0.025M TRIS base, 0.192M glycine). Gels were formed from either 10 to 30% (w/v) exponential gradients of acrylamide or single percentage acrylamide (7.5% (w/v)). Electrophoresis was done at a constant current of 15mA. For sodium dodecyl sulphate (SDS) electrophoresis the stacking gel, resolving gel and running buffer contained SDS at 0.1% (w/v) and 2-mercaptoethanol at 1mM.

Cell-free extracts for SDS-PAGE were boiled for 5 minutes in sample buffer (0.125M TRIS HCl pH 6.8, 10% (w/v) sucrose and 4% (w/v) SDS). Extracts for non-denaturing gels were prepared in 10% (w/v) sucrose. Prior to loading onto the gel, samples were mixed with bromophenol blue tracking buffer (10µl 0.1% (w/v) bromophenol blue per 0.5ml of sample).

Gels were stained for 5 hours in 0.1% (w/v) Coomassie Blue R in 10% (v/v) acetic acid and 40% (v/v) methanol. Gels were destained in the same solvent for 2-4 hours.

2.8.8 Alcohol dehydrogenase activity stain

The method used was based on that of Stirling and Dalton (1978). Cell-free extracts were run on 7.5% (w/v) polyacrylamide gels then incubated for 30 minutes in the dark at 30°C in the assay mixture (170ml) containing 20mM TRIS-NaCL pH 10.0, nitroblue tetrazolium 4mM, phenazine

methosulphate 1.5mM, NAD^+ 15mM and propan-1-ol or propan-2-ol 200mM. A control gel with no substrate present was used.

2.9 Enzyme assays

2.9.1 Alcohol/Aldehyde dehydrogenase

i) $NAD(P)^+$-linked activity: This was measured spectrophotometrically using a Pye Unicam SP1800 spectrophotometer by monitoring the change in absorbance at 340nm due to the formation of NAD(P)H. Assays were done in a total volume of 1ml. The cuvette contained, in 20mM TRIS NaOH pH 10.0 (unless specified otherwise), 0.2μmol of $NAD(P)^+$ and 0.1-1.0mg of protein extract (sufficient to give a linear rate for more than 5 minutes). Cuvettes were allowed to equilibrate at 30°C for 1 minute before the reaction was commenced by addition of 10μmol of alcohol or aldehyde. The change in absorbance at 340nm was followed for 5-10 minutes.

ii) Phenazine methosulphate (PMS) linked activity: This was measured spectrophotometrically by the change in absorbance at 600nm due to the reduction of dichlorophenol indophenol (DCPIP) by reduced PMS. Assays were done in a total volume of 1.5ml. The cuvette contained, in 20mM TRIS NaOH pH 9.0 (which had been sparged with oxygen-free nitrogen for 1 minute), 0.11μmol of PMS, 0.13μmol DCPIP, 45μmol NH_4Cl and 0.1-1.0mg of protein extract. Cuvettes were allowed to equilibrate at 30°C for 1 minute before the reaction was initiated by addition of 10μmol of alcohol or aldehyde. The change in absorbance at 600nm was followed for 5-10 minutes.

2.9.2 Ketone monooxygenase

Acetone and acetol monooxygenase activity was measured as substrate-dependant stimulation of oxygen uptake in a Clark-type oxygen electrode, based on a method by Hartmans and de Bont (1986).

Sufficient 20mM TRIS HCl pH 6.8 (unless stated otherwise) to give a final volume of 3ml was equilibrated at 30°C in the oxygen electrode. Cell-free extract was added to give 0.1-1.0mg of protein. Endogenous oxygen uptake was recorded. 1μmol of NAD(P)H was added and any change in rate measured. 8μmol of substrate (acetone or acetol) were subsequently added and any further change in rate recorded.

2.9.3 Acetol dehydrogenase

This was measured spectrophotometrically using an $NAD(P)^+$-linked assay based on that of Taylor et al. (1980). Assays were done in a total volume of 1ml. The cuvette contained, in 20mM TRIS HCl pH 6.8 (unless stated otherwise), 0.5μmol of $NAD(P)^+$ and 0.1-1.0mg of protein. Cuvettes were allowed to equilibrate at 30°C for 1 minute before the reaction was initiated by the addition of 2μmol of acetol. The change in absorbance at 340nm was followed for 5-10 minutes.

2.9.4 Propionyl CoA synthetase

This was measured by determination of propanoate dependant sulphydryl-CoA disappearance. It was based on the method of Grunert and Phillips (1951).

Assays were performed in small glass test tubes in a total volume of 0.2ml. These contained, in 50mM TRIS HCl pH 7.4, 2.5μmol $MgCl_2$, 1 μmol KBH_4 (from a freshly prepared solution), 0.4μmol ATP, 0.2μmol propanoate pH 8.0 and 0.1-0.5mg of protein. Tubes were incubated at 30°C for 15 minutes under a nitrogen atmosphere. A developing reagent was prepared in a second tube which consisted of 2ml saturated NaCl, 0.4ml NaCN/Na_2CO_3 solution (0.44g NaCN and 21.2g Na_2CO_3 per 100ml of water) and 0.4ml of nitroprusside reagent (2.7g nitroprusside per 100ml of water). The resulting mixture was poured into the assay tube, mixed and the absorbance read at 520nm immediately against a blank lacking propanoate. Under these conditions a change in CoASH concentration of 0.1μmol is equivalent to a change in absorbance at 520nm of 0.2 units.

2.9.5 Isocitrate lyase

This was measured in a spectrophotometric assay measuring the formation of glyoxylate which was converted to its phenylhydrazone derivative with a λ_{max} at 324nm. The method is based on that of Dixon and Kornberg (1951).

Assays were done in quartz cuvettes, in a total volume of 1.5ml. These contained, in 20mM TRIS HCl pH 7.5, 10μmol phenylhydrazine HCl, 0.05μmol cysteine HCl, 5μmol $MgCl_2$ and 0.1-0.5mg of protein. The reaction was initiated by the addition of 1μmol of (threo) potassium isocitrate. Formation of the glyoxylate derivative was followed for 5-10 minutes.

One unit of activity was defined as 1μmole of glyoxylate phenylhydrazone formed per minute. One unit in the described system catalyses a change in absorbance at 324nm of 11.55 min^{-1}. Therefore a change in absorbance

of 0.1 min^{-1} is equivalent to 8.7m units.

2.9.6 Cytochrome P_{450} spectrum

Washed cell suspensions were resuspended in 20mM TRIS HCl pH 6.8 containing 10% (v/v) glycerol. 1ml of the suspension was placed in each of two 1ml glass cuvettes. Air was bubbled through each for 1 minute. They were then placed in a Pye-Unicam SP1800 scanning spectrophotometer and a spectrum measured from 380nm to 600nm. This gave a difference spectrum base line. A few crystals of dithionite were added to the sample cuvette, mixed and a second scan taken to give an oxidized/reduced difference spectrum. Gaseous CO was then bubbled through the sample cuvette for 1 minute and another scan taken to give an oxidized/reduced/CO-treated difference spectrum.

2.10 Gas chromatographic analysis of excreted products

Supernatants from batch cultures of organisms were analysed by gas chromatography for the presence of any excreted compounds. The gas chromatograph contained a 1.5m x 2.3mm i.d. glass column packed with Porapak Q and operated isothermally at 160°C. Nitrogen was used as a carrier gas at 30ml.min^{-1}. Under these conditions it was possible to resolve all the suspected products. Products were identified by the use of authentic external standards. Output from the FID was logged by a reporting integrator.

2.11 Photography

Polyacrylamide gels were photographed from above using a Pentax SP500 camera fitted with a yellow filter and using Kodak Panatomic X film. Fermentors and plates were photographed with the same camera containing suitable Kodak colour film. Slide cultures were photographed using the microscope mounted camera supplied by the manufacturer containing Kodak Panatomic X film.

2.12 Chemicals

Most compounds, inhibitors, enzymes, substrates, media components of the highest purity available etc., were obtained from the following manufacturers:

Sigma (London) Chemical Co. Ltd., Poole, Dorset, U.K.; Aldrich Chemical Co. Ltd., Gilingham, Dorset, U.K.; BDH Chemicals Ltd., Poole, Dorset, U.K.; Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K.; Fisons Scientific Apparatus, Loughborough, Leics., U.K.; British Oxygen Co. Ltd., London, U.K.; Cambrian Chemicals, Croydon, Surrey, U.K.; Matheson Gases, Stoke-On-Trent, Staffs., U.K.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Enrichment and isolation of ethane, propane and butane-utilizing bacteria

Introduction

It would appear from the literature that Gram-positive bacteria and filamentous fungi are the main groups of organisms concerned with the oxidation of the higher gaseous alkanes. What reports there are on Gram-negative gaseous alkane-utilizers are few and give little information on the identification of such isolates, the one exception being Takahashi et al. (1980).

Of the Gram-positive organisms isolated on gaseous alkanes, few have been subjected to detailed biochemical analysis. This is probably due, in part, to the difficulty encountered in forming cell-free extracts of these organisms. Blevins and Parry (1972) reported that a propane-utilizing Mycobacterium sp. required sonicating for 20 minutes to form a cell-free extract. Dworkin and Foster (1958) encountered similar problems. Such harsh extraction procedures will not favour the study of labile enzyme systems.

For this reason it was considered worthwhile to try and isolate new gaseous alkane-utilizing organisms that would be more amenable to biochemical characterization. Special emphasis was placed on trying to isolate Gram-negative gaseous alkane-utilizers, particularly pseudomonad type organisms, since these have proved amenable to study in the case of liquid alkane utilization.

For the same reason it was decided to screen a range of Pseudomonas sp.,

particularly those having shown growth on hydrocarbon substrates, for growth on ethane, propane and butane.

Results

Samples were obtained from a variety of different environments in order to obtain a variety of different gaseous alkane-utilizers (Table 3.1). All environmental samples screened yielded ethane, propane and butane-utilizing organisms irrespective of whether or not the environment appeared to have been exposed to gaseous alkanes. No attempt was made to enumerate the organisms isolated from each environment so it is not possible to compare the results between the different environments. It was noted however, that all the environments yielded similar types of organisms. Brightly pigmented Gram-positive organisms exhibiting cell morphologies consistent with CMN-group bacteria appeared to dominate the enrichments. Whether these organisms are the most abundant in the environment or whether they are just the most adapted for these substrates is impossible to say from this study. Other work (e.g. Foster, 1962) would seem to suggest that these organisms are numerically the most significant in alkane degradation in the enviroment. They are certainly ubiquitous in their distribution.

Of the different methods of enrichment and isolation employed, the shake-flask enrichment on gaseous alkane was the most successful in that it yielded all three organisms considered worthy of further investigation. Enrichment on other carbon sources such as benzoate or propan-1-ol gave rise to mixed cultures in which the predominant organisms were motile Gram-negative rods. However, these organisms appeared unable to grow on gaseous alkanes and were soon replaced by the

Table 3.1 Environments from which ethane, propane and butane-utilizing bacteria were isolated

Environment	Source	Description
Soil	Leamington Spa, Warks., UK.	Site of old gas works. Well drained light soil. Abundant plant growth.
	Yellowstone Nat. Park, WY., USA.	Wet, clay mud from edge of hot-spring.
	Slochteren, Groningen, The Netherlands.	Dark, wet, clay mud from a ditch above the Groningen gas field.
	Kingsbury, Warks., UK.	Dark, wet, clay mud from below oil storage drums.
Water	Leamington Spa, Warks., UK.	River Leam. Free flowing freshwater. Little evidence of pollution.
	Leamington Spa, Warks., UK.	Grand Union Canal. Standing freshwater. Dark water with oily scum on surface.
Effluent	Knottingly, West Yorks., UK.	"Synthol" Chemical Works Waste Treatment Plant. Liquid waste containing hydrocarbons.

large non-motile Gram-positive organism (characteristic of the CMN-complex) on transferring to gaseous alkane substrates.

Enrichment on gaseous alkane susbtrates in the presence of compounds supposedly inhibiting the growth of Gram-positive organisms (malachite green, brilliant green and crystal violet, see Krieg, 1981) were also unsuccessful in obtaining Gram-negative organisms. High concentrations ($\geq 1 mg.ml^{-1}$) inhibited all growth whereas lower concentrations ($\leq 0.5 mg.ml^{-1}$) allowed abundant growth of Gram-positive organisms. Intermediate concentrations allowed growth of Gram-positive organisms that were presumably resistant to the inhibitory effects of these compounds.

Enrichment on agar plates allows isolation of slower growing organisms that would probably be outgrown in liquid culture. Again all the environments screened yielded gaseous alkane-utilizers by this method. As before it appeared that the brightly pigmented Gram-positive organisms were predominant. Slower growing, non-pigmented colonies appeared after 7-10 days incubation but in all cases they were apparently scavenging and never grew in liquid media on gaseous alkanes. Strains isolated in this manner often showed flocculent growth in liquid media and many had excessively long doubling times (20-25 hours). Samples drawn through filters and incubated on agar plates gave similar results.

Continuous enrichments performed in a fermentor gave similar results again. Enrichment on benzoate, salicylate and propanol (see Table 2.2) gave rise to dense cultures of predominantly motile Gram-negative rods. Upon switching to continuous operation with propane as sole carbon

source the density of the cultures dropped and Gram-positive organisms became the predominant forms. The use of different minimal media (see Table 2.2) appeared to have little affect on the type of organisms isolated.

Of the continuous enrichments attempted, experiment number 4 (Table 2.2) is worthy of particular mention. A fermentor containing 800ml of sterile ABER medium was inoculated with 20ml of resuspended sample from Slochteren (Table 3.1). After batch growth on benzoate a dense culture (E_{540nm}= 1.0) consisting of predominantly Gram-negative motile rods resulted. Upon switching to continuous operation (D = $0.03hr^{-1}$) with propane as carbon source, the optical density fell from 1.0 to 0.01 over a period of 7 days. The culture was then allowed to grow batchwise on propane and after a lag period of approximately 60 hours the culture began to grow again and consisted of just three organisms. Two Gram-positive rods giving pink and orange colonies and a Gram-negative rod giving cream coloured colonies. The Gram-positive organisms were both capable of growth on propane in pure culture but the Gram-negative organism was not. Cross-streaking of the three isolates on mineral salts agar under a propane atmosphere resulted in satellite growth of the Gram-negative organism around both Gram-positive organisms indicating that it was being cross-fed by the other two organisms. This co-culture was not investigated any further.

The organisms isolated from continuous enrichments generally grew well in liquid culture and had doubling times in the range of 8-20 hours.

In all, some 80 strains of ethane, propane and butane-oxidizing bacteria were isolated in pure culture from the environments listed using the

techniques mentioned. All were Gram-positive organisms showing CMN-group type morphologies. The majority were brightly pigmented falling into three major groups; pink/orange, orange/buff and yellow. All isolates had doubling times in the range 8-25 hours on propane. Some, particularly the yellow pigmented organisms, showed flocculent growth in liquid media, so following growth by measurements of optical density did not always give satisfactory results, but as these organisms were of no further interest and were rejected, this was not deemed significant.

There appeared to be no pattern in the utilization of gaseous (C_2-C_4) alkanes. Some isolates could utilize all three, others only two or one of the substrates. All were able to grow on nutrient agar thus showing the facultative nature of gaseous alkane metabolism.

Of the 42 strains of Pseudomonas sp. tested (Table 3.2) none showed growth on ethane, propane or butane even after subculturing on propan-1-ol or propan-2-ol.

None of the 5 strains of pink pigmented facultative methylotrophs were able to grow on gaseous alkane substrates. It should be mentioned that in all cases only one set of conditions were used when screening, namely ANMSV medium, 50% (v/v) alkane in air at 30°C. These conditions may not be optimal but none of the screened cultures showed even poor growth under these conditions whereas a Gram-positive control organism under the same conditions showed significant growth.

Table 3.2 Organisms screened for the ability to grow on ethane, propane and butane as a sole carbon/ energy source

Strain	known to grow on
Pseudomonas putida (7 strains)*	m-xylene, octane
P. aeruginosa PAO I*	m-xylene
P. aeruginosa (6 strains)	naphthalene
P. fragi ATCC 4973	
P. cichorii NCPPB 907	
P. marginelis NCPPB 667	
P. chlororaphis ATCC 9446	
P. ausrofaciens ATCC 13985	kerosene
P. stutzeri ATCC 17592	
P. pseudoalcaligenes ATCC 17440	
P. oleovorans ATCC 8062	octane
P. fluorescens ATCC 13525	
P. acidovorans ATCC 15668	
P. multovorans ATCC 17759	octane
17 unnamed strains of the P. fluorescens-putida group	naphthalene
5 unnamed strains of pink pigmented facultative methylotrophs +	methanol

* Gift from M. Labens, University College of North Wales, Bangor, UK.
+ Gift from D. Hood, University of Warwick, Coventry, UK.
All other strains: Gift from M. Rhodes-Roberts, University College of Wales, Aberystwyth, UK.

Choice of organisms for further study

From the original 80 propane-utilizing isolates, 3 were eventually selected as being worthy of further study. Their characteristics have been summarised in Table 3.3. Apart from being fast growers they were considered representative of each of the three main colour groupings observed (pink/orange, orange/buff and yellow). They were selected chiefly for their ability to grow rapidly and without flocculating in liquid culture, characteristics considered desirable for future work.

3.2 Identification of selected isolates

Introduction

By far the most commonly reported organisms in gaseous alkane enrichments are "coryneform" bacteria belonging to the CMN-complex. As mentioned in Section 1.2.3 much confusion appears to exist in the field of this groups' taxonomy which probably accounts for the occasional renaming of such isolates.

Rogosa et al. (1974) state "The coryneform bacteria present a number of unresolved problems in taxonomy and classification. The rather widespread use of the term "coryneform" or "diptheroid" to describe any non-sporing Gram-positive rod of irregular outline has given rise to considerable confusion .....". The term is usually interpreted in a morphological sense and has consequently been applied to isolates of many different genera including Corynebacterium, Mycobacterium, Nocardia, Arthrobacter, Brevibacterium and Rhodococcus. Hence the term "Coryneform" is largely synonymous with what is now termed the CMN-

Table 3.3 Properties of selected propane-utilizing isolates

Isolate	Source	Colony Morphology[1]	Microscopic Appearance	Doubling Time (hrs)[2]	Growth on[3] Ethane	Propane	Butane
PNKb1	Soil sample Yellowstone Park, USA	Circular, 2-3mm dia. pink/orange waxy surface	Non-motile rods, often clumped 2-3 x 0.8-1.0μm Gram-positive	8	+	+++	+
GPYb1	Soil sample Ex-gas works site L/Spa, UK	Circular, 1-2mm dia. yellow shiney surface	Non-motile rods, 1.8-2.7 x 0.4-0.6μm Gram-positive	13	+++	+++	+
BPSd1	Soil sample from BP origin unknown	Circular, 2-3mm dia. waxy surface	Non-motile rods, 2.0-2.5 x 1.0-1.3μm Gram-positive	8	++	++	++

Notes

[1] Nutrient agar plates, 72 hours; [2] Mineral salts medium + propane; [3] Mineral salts agar + alkane, 14 days.

+++ good growth; ++ fair growth; + poor growth.

complex (Collins and Cummins, 1986). More recently Goodfellow and Minnikin (1981) have voiced much the same opinion "The taxonomy of the coryneform bacteria is widely acknowledged to be unsatisfactory".

The problem for the investigator of gaseous alkane physiology is that simple biochemical tests are not sufficient to distinguish between the different genera of the CMN-complex. The characteristic that appears to have been most usefully employed in this task is cell wall composition. Such characteristics are rarely reported in papers describing the isolation and characterization of gaseous alkane-utilizers. Phenotypes described usually allow the isolate to be placed in more than one of the CMN-complex genera, so selection of a genus is often arbitrary.

Results

The three isolates selected for further study were designated PNKb1, BPSd1 and GPYb1. On nutrient agar and AMS/propane plates they all gave brightly pigmented colonies (Plate 3.1). Upon examination of isolates under a low magnification binocular microscope it was apparent that isolates PNKb1 and BPSd1 were similar in possessing a mycelial-like structure to their colonies. GPYb1 showed no such morphology. Microscopic examination of individual cells revealed again similarities between PNKb1 and BPSd1. Samples removed at intervals from growing liquid cultures (AMS/propane) showed cells undergoing a morphological change from large, pleomorphic rods in the exponential phase to short rods/cocci upon entering the stationary phase of the growth cycle (Plate 3.2). Cells of GPYb1 showed no such rod/cocci transition and retained a consistent "brick-like" rod morphology through the growth cycle (Plate 3.2). The rod/coccus transition is generally attributed to members of

Plate 3.1 Gross colony morphology of isolates PNKb1,BPSd1 and GPYd1 grown on AMS/propane medium.

A.PNKb1,7 days growth

B.BPSd1,7 days growth

C.GPYb1,10 days growth

Plate 3.2 Micrographs of isolates PNKb1,BPSd1 and GPYb1 grown on AMS/propane medium.

A. PNKb1-exponential phase culture.

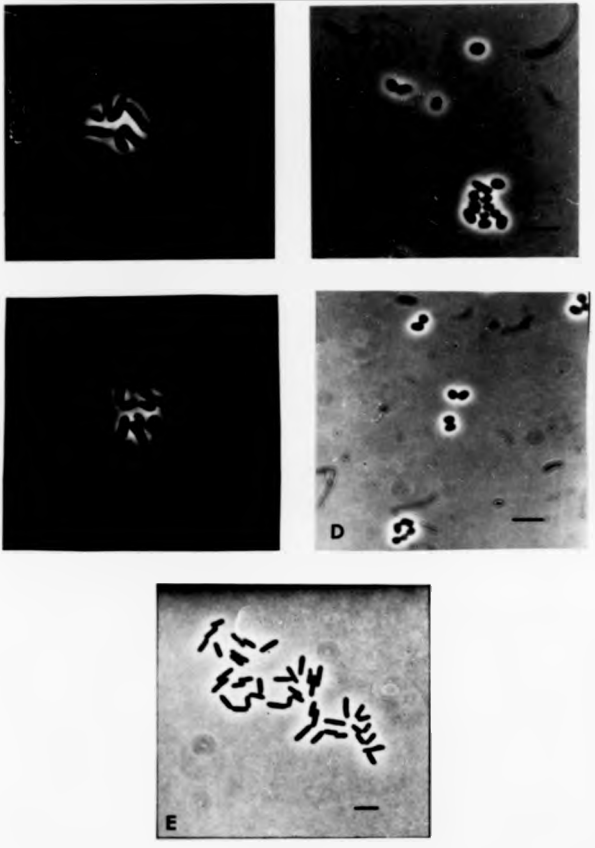
B. PNKb1-stationary phase culture.

C. BPSd1-exponential phase culture.

D. BPSd1-stationary phase culture.

E. GPYb1-exponential phase culture.

Bar=3µm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the genus Arthrobacter but members of Brevibacterium and Rhodococcus also exhibit such a phenomenon (Keddie et al., 1986).

Simple biochemical tests were performed on the isolates to assign them to the CMN-complex (Table 3.4). All stained Gram-positive though showing more decolouration than a Bacillus sp. used as a Gram-positive control organism. None showed the presence of spores even on older AMS/propane agar plates where nutritional conditions might be conducive to spore formation. This excluded GFYb1 from membership of the genus Bacillus which from its morphology was a possibility. None of the organisms exhibited motility in liquid media. Repeated observations of these isolates grown on a variety of media throughout this study failed to note any motile forms. Motility is rare in the CMN-complex. All isolates were catalase positive. This excludes GFYb1 from membership of the genus Lactobacillus. PNKb1 and BPSd1 both appeared to exhibit oxidative metabolism of glucose. The result for GFYb1 was consistently unclear, the indicator medium showing no change. CMN-complex bacteria are generally oxidative in their metabolism of glucose but some facultatively anaerobic members of the genus Corynebacterium exist. The acid-fast test (usually used to distinguish members of the genus Mycobacterium) was not performed as no positive control organism was available. Members of the other genera in the CMN-complex all show differing degrees of acid-fastness so the test is in fact of little diagnostic value in such a simple scheme of tests.

The results of the above tests when viewed as a whole would suggest that PNKb1 and BPSd1 probably belonged to either the genus Nocardia or the genus Rhodococcus. this being strongly suggested by their apparent mycelial growth form on solid media. GFYb1 was more difficult to assign

Table 3.4 Preliminary identification of isolates

Test	Isolate		
	PNKb1	BPSd1	GPYb1
Morphology	rod → coccus	rod → coccus	rod
Gram stain	+	+	+
Spore stain	-	-	-
Motility	-	-	-
Catalase	+	+	+
Oxid/Ferm (glucose)	O	O	?

Tests performed as described by Doetsch et al. (1981).

to a genus but the cell morphology, and the absence of any mycelial form or rod-coccus transition suggested that the genus Mycobacterium was most likely.

In order that the isolates could be more positively identified they were sent to the National Collection of Industrial and Marine Bacteria Ltd., Aberdeen, U.K., who kindly performed the necessary tests to allow more complete identification. Their results are shown in the Appendix. They concluded that PNKb1 was a strain of Rhodococcus rhodochrous. BPSd1 was R. erythropolis and GPYb1 a Mycobacterium sp.

3.3 Growth of propane-utilizing organisms

Introduction

Alkanes, due to their highly reduced nature, represent a special type of microbial substrate that requires a specialized metabolism to assimilate it. Such requirements may necessitate special growth conditions. Little systematic investigation has been done on the precise requirements of gaseous alkane-utilizing microorganisms. Blevins and Perry (1971) investigated the affect of various parameters on growth yield of Mycobacterium vaccae JOB5 and McLee et al. (1972) carried out similar studies on Arthrobacter spp. Ichikawa (1981) investigated factors affecting biomass production from Pseudomonas butanovora grown on n-butane. All these studies are discussed in Section 1.4.2.

In this study the growth conditions for propane-utilizing bacteria were not fully optimized but observations were made on the ability of Rhodococcus rhodochrous PNKb1 to grow on aliphatic hydrocarbon

substrates and of the effect of certain parameters on the growth rate of this organism.

Results

Propane-utilizing organisms were grown routinely in shake flasks and fermentors as described in Section 2.5. A typical growth curve for R. rhodochrous PNKb1 is shown in Fig. 3.1. It was obtained by inoculating a loopful of cells from a nutrient agar plate into 50ml of AMS medium below a 50% (v/v) propane:air atmosphere at 30°C and shaken on an orbital shaker at 200rpm. A long lag phase was observed which has been noted before. Blevins and Perry (1971) attributed it to the length of time required to induce oxygenase activity. Stephens (1983) suggested it may be due to oxygen toxicity. However, in this study, using inocula from AMS/propane plates or reducing the oxygen tension in the flask did not reduce the long lag phase and its cause remains obscure.

The effect of different nitrogen sources, oxygen, carbon dioxide and propane concentrations on the growth rate of R. rhodochrous PNKb1 in batch culture are summarised in Table 3.5. For the effect of different nitrogen sources MS medium was supplemented either with $1g.l^{-1}$ NH_4Cl or $1g.l^{-1}KNO_3$. The gas atmosphere was 50% (v/v) propane in air. Growth was at 30°C on a 200rpm shaker. The ammonium salt was the better nitrogen source, perhaps not surprisingly since presumably the nitrate had to be reduced to ammonia prior to being metabolized, an energy demanding process.

Decreasing the oxygen partial pressure appeared to have an adverse

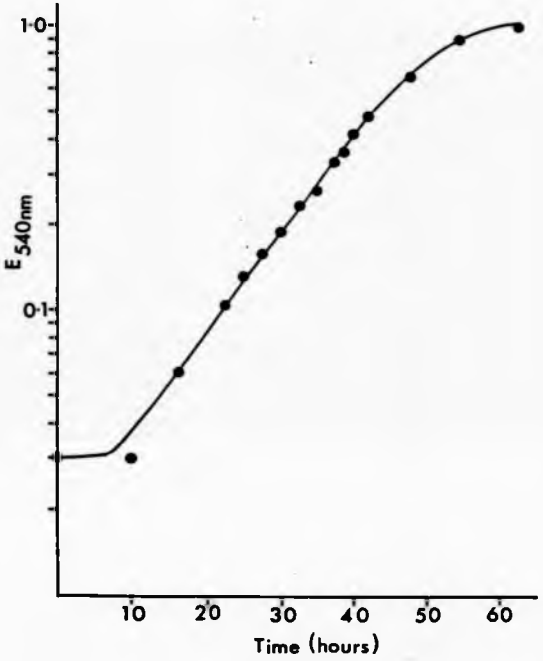


Figure 3.1 Growth curve of R. rhodochrous PNKb1 grown on AMS/propane medium

Table 3.5 The effect of nitrogen source, oxygen, carbon dioxide and propane concentration on the growth rate of R. rhodochrous PNKb1 grown on propane

			Specific growth rate (h^{-1})	Doubling time (h)
MS medium 50% v/v propane in air	+	NH_4Cl ($1g.l^{-1}$)	0.083	8.3
		KNO_3 ($1g.l^{-1}$)	0.044	15.7
AMS medium 50% v/v propane	+	O_2 10% (v/v)	0.083	8.4
		4% (v/v)	<0.023	>30
		2% (v/v)	NG	NG
AMS medium 50% v/v propane in air	+	CO_2 0.015% (v/v)	0.083	8.3
		5% (v/v)	0.083	8.3
		10% (v/v)	0.083	8.4
		20% (v/v)	0.083	8.4
AMS medium 50% v/v air	+	Propane 20% (v/v)	0.034	20.0
		30% (v/v)	0.058	12.0
		50% (v/v)	0.081	8.6

NG = no growth

All cultures were grown batchwise in 250ml quickfit flasks containing 50ml of medium. Head space made to 100% (v/v) using oxygen-free nitrogen where necessary. Incubation was at 30°C and 200rpm.

effect on growth rate. Shake flasks containing 50ml of AMS medium were degassed under vacuum and sparged with nitrogen gas. 50% (v/v) propane was then added to the head space along with the indicated amount of oxygen. No attempts were made to increase the partial pressure of oxygen above 10% (i.e. 50% propane:50% air) as McLee et al. (1972) reported that increased partial pressures of oxygen had deleterious effects on the growth rate of n-butane-grown organisms.

Normal atmospheric air contains approximately 0.03% (v/v) carbon dioxide. Therefore a 50% (v/v) propane:air mixture would contain 0.015% (v/v) carbon dioxide, assuming that the propane gas contained no carbon dioxide which according to the manufacturers specifications was the case. This amount is no more than a trace amount. Increasing the carbon dioxide concentration to 5, 10 or 20% (v/v) whilst keeping other parameters constant (i.e. 50% propane, 10% oxygen, 50ml AMS, 30^{o}C, 200rpm) had no effect on growth rate. Interestingly MacMichael and Brown (1987) using R. rhodochrous ATCC 21198 showed that growth on propane was dependant on the presence of carbon dioxide. They give no indication of the minimum concentration of carbon dioxide required for growth but their organism was grown routinely in an atmosphere containing 5% (v/v) carbon dioxide and would not grow in an atmosphere stripped of carbon dioxide by sodium hydroxide. No attempt was made in this study to grow the organisms in the total absence of carbon dioxide.

Decreasing the propane concentration below 50% v/v in air had an adverse effect on growth rate. This is contrary to the findings of McLee et al. (1972) who measured no such effect when n-butane concentrations were altered from 10-60% (v/v) with their organism.

Care should be taken with all these studies on interpreting the effects of altering atmospheric gas concentrations on growth rates. The organism only interacts with the gases in the aqueous phase and consideration should therefore be given to the relative solubilities of the gases (Pirt, 1985). However, for the purposes of this study these parameters were altered more from an empirical viewpoint to try and increase the growth rate of the organism rather than to make any statements about their required levels. A more comprehensive study could have been undertaken if it had proved possible to use continuous culture techniques.

The terminal or subterminal oxidation of propane leads to metabolic pathways containing different sequences of intermediates. The ability of an organism to grow on these supposed intermediates may give some indication of which pathway it can utilize. Table 3.6 summarises the ability of R. rhodochrous PNKb1 to grow on supposed intermediates of propane metabolism. Propanal, propanone (acetone) and hydroxypropanone (acetol) appeared to be toxic when given at 0.1% (v/v) and would not allow growth. Reducing their concentration to 0.05% (v/v) allowed growth to occur. Methylacetate, methylglyoxal and methanol did not support growth at 0.1 or 0.05% (v/v). They did not inhibit growth on propane when present at 0.05% (v/v) suggesting that they are not toxic. Either they are not taken up by the organism or it has no ability to oxidize them (see Section 3.4.5).

It is interesting to note that the growth rates of R. rhodochrous PNKb1 with terminally oxidized intermediates as growth substrates, are greater than with subterminally oxidized intermediates. The significance of this observation remains unclear, although their solubility may play a

Table 3.6 Growth rates of R. rhodochrous PNKb1 on various potential intermediates of propane metabolism

Intermediate	Specific growth rate (h^{-1})	Doubling time (h)
(Propane)	0.083	8.3
Propan-1-ol	0.289	2.4
Propanal	0.124	5.6
Propanoate	0.289	2.4
Propan-2-ol	0.151	4.6
Propanone (acetone)	0.096	7.2
Hydroxypropanone (acetol)	0.144	4.8
Methylacetate	NG	NG
Methanol	NG	NG
Methylglyoxal	NG	NG
Pyruvate	0.239	2.9
Acetate	0.257	2.7
Succinate	0.224	3.1

NG = no growth

Growth conditions were as described in Section 2.5.

role.

Table 3.7 shows the range of aliphatic hydrocarbons tested as growth substrates. The specificity for propane amongst all the n-alkane substrates tested is unusual but not unique (see Perry, 1968). The other n-alkane substrates do not appear to be toxic and did not inhibit growth on propane when present at 20% (v/v) for methane, ethane and butane or 0.05% (v/v) for pentane, hexane, and octane. Subsequent work (see Section 3.4.1) suggested that this specificity may be due to the specificity of the oxygenase system.

There is little evidence to suggest a role for alkenes in propane metabolism (McKenna and Kallio, 1965) and attempts to grow R. rhodochrous PNKb1 on alkenes were unsuccessful. Addition of 5% (v/v) propene to a propane culture totally inhibited growth. This was probably due to the ability of cells to convert propene to 1,2-epoxypropane (see Section 3.4.2) which is a powerful sterilizing agent.

The ability to grow on alkynes is an interesting phenomenon discussed more fully in Section 3.9.

No reports exist of propane-utilizing organisms being grown in steady-state continuous culture. Despite numerous attempts it was not possible to achieve such cultures of R. rhodochrous PNKb1 growing on propane. Steady-states were easily achieved with growth on propan-1-ol or propan-2-ol but upon switching to propane as sole carbon substrate, cultures slowly washed out over a period of days even at a dilution rate representing a doubling time of 15 hours (see Fig. 3.4).

Table 3.7 Ability of R. rhodochrous PNKb1 to grow on alkanes, alkenes and alkynes

Substrate	Growth
Methane	-
Ethane	-
Propane	+
n-Butane	-
iso-Butane	-
n-Pentane	-
n-Hexane	-
n-Octane	-
Ethene	-
Propene	-
Butene	-
Ethyne	+
Propyne	+
Butyne	ND

\+ Growth
\- No growth
ND Not determined

Growth conditions were as described in Section 2.5.

Batchwise growth on propane resulted in cultures of an optical density of E_{540nm} = 1.0 after 2-3 days, but upon switching to continuous operation, washout gradually occurred. The addition of 0.01% (w/v) yeast extract or doubling the trace element composition of the AMS medium did not prevent washout occurring. Analysis of culture supernatants showed no potentially toxic products (e.g. ketones or aldehydes) to be present. The reason for this inability to achieve a steady-state whilst growing on propane remains unclear. It was a serious handicap in this study and meant all work had to be carried out using batch-grown cells, grown as far as possible under identical conditions and harvested at identical points in their growth phase.

3.4 Whole cell studies

3.4.1 Inducible nature of the gaseous alkane oxygenase system

Introduction

Perhaps the most fundamental difference between methane metabolism and higher alkane metabolism is the inducible nature of higher alkane oxygenases. The ability to utilize methane appears to involve specialised pathways of metabolism and its obligate nature means that the enzymes of these pathways must be constitutively expressed. By contrast, organisms capable of growth on higher alkanes generally appear to have an inducible set of enzymes capable of metabolizing n-alkanes to intermediates of normal heterotrophic metabolism (such as acetate, pyruvate etc.). Examples of constitutively expressed gaseous alkane oxygenases have been reported (Davis et al., 1956; Dworkin and Foster, 1958) but these would appear to be in the minority.

Induction is usually shown to occur in the presence of the readily metabolized growth substrate but induction by non-metabolizable substrates has also been reported (van Eyk and Bartels, 1968, see Section 1.8). Perry (1968) showed that propane oxygenase in Mycobacterium vaccae JOB5 was induced after growth on either propan-2-ol or acetone, as well as by growth on propane. Perry and Scheld (1968) showed that structurally diverse compounds such as phthalic acid could induce propane oxygenase in an Arthrobacter sp. The exact structural requirement of the inducer molecule therefore remains obscure.

Once induced, organisms often show an ability to oxidize a range of n-alkane (and other) substrates. M. vaccae JOB5 after growth on propane could oxidize n-alkanes from C_2 to C_8 (Perry, 1968). This ability probably reflects a lack of specificity in the initial oxygenase enzyme but caution should be exercised in this type of judgement when dealing with whole cell systems as it is always possible that more than one oxygenase enzyme could be present. However, studies with purified methane monooxygenase and octane monooxygenase would suggest that such enzymes can oxidize a range of n-alkanes (see Dalton, 1980a; McKenna and Coon, 1970).

Results

Table 3.8 shows the induction of a gaseous alkane oxygenase in Rhodococcus rhodochrous PNKb1 after growth on propane. Pyruvate-grown cells did not exhibit any activity against n-alkanes. A similar effect is seen with Mycobacterium sp. GPYb1 and R. erythropolis BPSd1 in Table 3.9. None of the isolates exhibited any activity towards methane. This is probably the case with most gaseous alkane oxygenases and early

Table 3.8 Ability of R. rhodochrous PNKb1 to oxidize n-alkanes after growth on propane or pyruvate

Assay substrate	Growth substrate	
	Propane	Pyruvate
Methane	0	0
Ethane	3.8	0
Propane	18.1	0
Butane	9.2	0
(iso-Butane)	0	ND
Pentane	0	0
Hexane	0	0
Octane	0	0
(Pyruvate)	ND	18.9

ND = Not determined

Rates quoted as n moles oxygen consumed min^{-1} mg dry weight $cells^{-1}$

Table 3.9 Ability of Mycobacterium sp. GPYb1 and R. erythropolis BPSd1 to oxidize n-alkanes after growth on propane or pyruvate

Mycobacterium sp. GPYb1

Assay substrate	Growth substrate	
	Propane	Pyruvate
Methane	0	0
Ethane	7.2	0
Propane	10.6	0
Butane	3.4	0
(Pyruvate)	ND	21.8

R. erythropolis BPSd1

Assay substrate	Growth substrate	
	Propane	Pyruvate
Methane	0	0
Ethane	5.7	0
Propane	13.2	0
Butane	9.0	0
(Pyruvate)	ND	17.6

ND = Not determined

Rates quoted as n moles oxygen consumed min^{-1} mg dry weight $cells^{-1}$.

reports of their ability to oxidize methane may have been due to impurities in the methane. In this study only the highest quality gases (instrument grade, > 99.5% pure) were used and supplied at low concentrations (< 30μM) so that any impurities would be present at negligable levels (< 0.15μM) and would not interfere with the assay.

All three isolates could oxidize ethane, propane and butane but only R. erythropolis BPSd1 was capable of growth on all three compounds. Mycobacterium sp. GPYb1 showed poor oxidation of butane and was incapable of growth on that substrate. R. rhodochrous PNKb1 showed rates of oxidation of butane equivalent to R. erythropolis BPSd1 but is unable to grow on butane. Analysis of the supernatant from metabolically active resting cell suspensions exposed to butane failed to identify any possible oxidation products of butane so the fate of the oxidized butane molecule remains unclear. R. rhodochrous PNKb1 exhibited low activity towards ethane which might account for its inability to grow on ethane.

The inability of R. rhodochrous PNKb1 to oxidize higher n-alkanes (pentane, hexane and octane) is interesting in that it suggests that the oxygenase has a specific requirement for short chain alkanes. Liquid alkanes were supplied at very low concentrations due to the presence of impurities but analysis of supernatants from resting suspensions exposed to these substrates showed no appreciable disappearance of the substrate over 30 minutes.

The report of Babu and Brown (1984) suggested that R. rhodochrous ATCC 21198 could grow on isobutane. The R. rhodochrous strain isolated in this study could neither grow on isobutane nor oxidize it after growth

on propane, indicating that significant differences exist between the two strains.

In addition to pyruvate-grown cells, propan-1-ol, propan-2-ol or acetone-grown cells showed no activity towards propane. This indicates that the system is different from that in M. vaccae JOB5, which was adapted for propane oxidation after growth on propan-2-ol or acetone (Perry, 1968).

3.4.2 Epoxidation of alkenes by resting cell suspensions of propane-grown bacteria

Introduction

The ability of alkane oxygenase systems to epoxidate alkenes is well documented. The three best characterized systems, methane monooxygenase from Methylococcus capsulatus (Bath), octane hydroxylase from Pseudomonas oleovorans and octane hydroxylase from Corynebacterium sp. 7E1C are all capable of epoxidating alkenes as well as hydroxylating alkanes (see Section 1.7). In all three cases the epoxide is not metabolized further and its accumulation can be monitored. The latter two organisms are unable to grow on alkenes as sole carbon sources and the inability to metabolize the epoxide probably explains this.

Patel et al. (1983a) demonstrated that the epoxidation of propene by resting cell suspensions of butane-grown Arthrobacter sp. was inhibited by the presence of propane in the assay, thus suggesting that propane and propene were competing for the same active site on the oxygenase enzyme.

The range of alkanes epoxidated is usually equivalent to the range of alkanes hydroxylated, again suggesting that the same active site is involved in both processes. The availability of optically active alkanes to act as substrates has led to their use as active site probes to help elucidate mechanisms of hydroxylation by monooxygenases (Leak and Dalton, 1987). The ability of these organisms to produce stereospecific epoxides has also lead to commercial interest (see Habets-Crützen et al., 1985).

Results

All three isolates were able to epoxidate propene to 1,2-epoxypropane. No other products were observed during the assays. Table 3.10 lists the rates of 1,2-epoxypropane formation in five minute assays (the rate being linear over this period). The two Rhodococcus spp. isolates appear to oxidize propene rapidly whereas the Mycobacterium sp. seems less able to deal with propene as a substrate. Epoxidating activity, like alkane hydroxylating activity, was only present after growth on propane. R. rhodochrous PNKb1 was able to epoxidate ethene and but-1-ene to the corresponding epoxides (Table 3.11) but exhibited no activity against either isomer of the subterminal but-2-ene. This may be due to the but-2-enes not permeating into the cells but in view of the activity against but-1-ene this seems unlikely. Perhaps more likely is that moving the double bond away from a terminal methyl group has rendered it less susceptible to attack by the oxygenase enzyme. Longer chain alkenes were not investigated due to an inability to identify potential products using the GC columns available. However, given the specificity of n-alkane substrates shown by R. rhodochrous PNKb1 it would seem unlikely that long chain alkanes would serve as substrates.

Table 3.10 Ability of propane grown organisms to convert propene to 1,2-epoxypropane

Isolate	Growth substrate	
	Propane	Propan-1-ol
R. rhodochrous PNKb1	22.7	0
R. erythropolis BPSd1	18.2	0
Mycobacterium sp. GPYb1	2.5	0

Rates quoted as n moles 1,2-epoxypropane formed min^{-1} mg dry weight $cells^{-1}$.

Table 3.11 Ability of propane-grown R. rhodochrous PNKb1 to epoxidate alkenes

Substrate	Product	Rate
Ethene	1,2-epoxyethane	11.6
Propene	1,2-epoxypropane	26.2
But-1-ene	1,2-epoxybutane	13.7
cis but-2-ene	none observed	0
trans but-2-ene	none observed	0

Rates quoted as n moles product formed min^{-1} mg dry weight $cells^{-1}$.

The ability of R. rhodochrous PNKb1 to epoxidate propene was investigated further as it was considered of use in later studies to isolate the oxygenase system in cell-free extracts. The temperature optimum of the activity was found to be 30°C and the amount of product formed was proportional to the dry weight of cells used in the assay (up to 0.5mg) and the reaction was linear for over 30 minutes. This last result is interesting in that the organism was capable of sustaining the biotransformation of a non-growth substrate for such a long period in the absence of any exogenous supply of energy. The addition of 2mM propan-1-ol to "drive" the reaction had no affect on the rate over a 30 minute period. This would suggest that the organism possessed readily metabolizable reserves from which it could regenerate cofactors to sustain the biotransformation. Such a situation is known to exist in methane-oxidizers where Type II organisms, which are capable of synthesizing the storage polymer polyhydroxybutyrate, can carry out such biotransformations in the absence of exogenous donors, whereas Type I organisms usually require exogenous donors (Leak and Dalton, 1983). No attempt was made to reduce the level of reserve materials as this was not considered pertinant to this study.

That propene was being oxidized by a propane oxygenase was suggested by performing epoxidation assays in the presence of increasing amounts of propane (Fig. 3.2). Increasing the amount of propane in the assay decreased the amount of 1,2-epoxypropane formed suggesting that propane and propene were competing for the same active site on the enzyme. Patel et al. (1983) described a similar phenomenon with cell suspensions of a butane-grown Arthrobacter sp. whereby addition of 50% (v/v) propane to the assay reduced the activity to 65% of the control activity. Similar results were obtained with this study, addition of 50% (v/v)

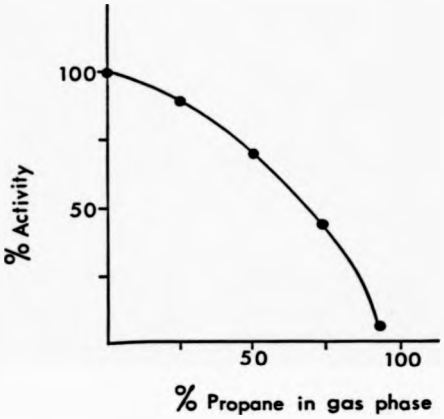


Figure 3.2 The effect of adding propane to the gas phase during assaying the conversion of propene to 1,2-epoxypropane by resting cells of propane-grown R. rhodochrous PNKb1

Activity in n moles 1,2-epoxypropane formed min^{-1} mg dry weight $cells^{-1}$

propane to the assay giving 68% of the control activity.

3.4.3 Inhibition of oxygenase activity

Introduction

The effect of inhibition on oxygenase activity has been employed in a number of studies to elucidate the nature of the enzyme. Such studies are performed on purified enzymes or cell-free extracts (see Section 3.5) but provided that inhibitors can enter cells they can provide some information when used with whole cell systems. Table 3.12 summarises the findings of six studies involving inhibition of hydrocarbon oxygenase systems. The systems under study were; Corynebacterium sp. 7E1C (Cardini and Jurtshuk, 1970) using partially purified enzyme (octane hydroxylase); Pseudomonas oleovorans (McKenna and Coon, 1970) using purified enzyme (octane hydroxylase); Methylococcus capsulatus (Bath) (Stirling and Dalton, 1977) using whole cell suspensions (methane monooxygenase); Xanthobacter Py2 (van Ginkel and deBont, 1986) using cell-free extracts (propene monooxygenase); Arthrobacter sp. CRL-60 (Patel et al., 1983) using whole cell suspensions (propane monooxygenase); Rhodococcus rhodochrous PNKb1., this study using whole cell suspensions (propane monooxygenase).

It is difficult to draw exact parallels from the data presented since different assay conditions were employed in the various studies but it does appear that each system is distinct. Corynebacterium sp. 7E1C octane hydroxylase is highly susceptible to carbon monoxide due to its P_{450} cytochrome component. The octane hydroxylase of P. oleovorans is particularly susceptible to 8-hydroxyquinoline indicating an easily

Table 3.12 Summary of the effects of various inhibitors on some hydrocarbon oxygenase systems

Inhibitor	Concentration employed (mM)	System	% inhibition of activity
Azide	1	*Corynebacterium* sp. 7E1C	20
	1	*Methylococcus capsulatus* (Bath)	0
	1	*Pseudomonas oleovorans*	0
	1	+ *Rhodococcus rhodochrous* PNKb1	34
KCN	1	*Corynebacterium* sp. 7E1C	13
	1	*M. capsulatus* (Bath)	0
	1	*P. oleovorans*	32
	1	*Xanthobacter* Py2	33
	1	*Arthrobacter* sp. CRL-60	60
	1	+ *R. rhodochrous* PNKb1	50
$HgCL_2$	0.1	*Corynebacterium* sp. 7E1C	98
	1	+ *R. rhodochrous* PNKb1	100
p-hydroxy-mercuribenzoate	0.1	*Corynebacterium* sp. 7E1C	51
	1	*Arthrobacter* sp. CRL-60	35
	10	+ *R. rhodochrous* PNKb1	0
2-mercapto ethanol	0.1	*Xanthobacter* Py2	0
	5	+ *R. rhodochrous* PNKb1	12
EDTA	1	*Corynebacterium* sp. 7E1C	3
	1	*P. oleovorans*	14
	5	+ *R. rhodochrous* PNKb1	20
8-hydroxy-quinoline	1	*Xanthobacter* Py2	0
	1	*P. oleovorans*	96
	1	*M. capsulatus* (Bath)	0
	10	+ *R. rhodochrous* PNKb1	0
Carbon monoxide	* 10%	*Xanthobacter* Py2	0
	0.2	*M. capsulatus* (Bath)	0
	* 10%	*Corynebacterium* sp. 7E1C	91
	0.5	+ *R. rhodochrous* PNKb1	0
Acetylene	* 5%	*Xanthobacter* Py2	0
	7.5	*M. capsulatus* (Bath)	100
	7.5	+ *R. rhodochrous* PNKb1	0

* As % v/v in gas phase of assay.
\+ This study.

chelated metal centre. M. capsulatus (Bath) is totally inhibited by acetylene which appears to bind irreversibly to the protein A component, the site of methane oxidation (Prior and Dalton, 1985). These studies show then that the common problem of inserting an oxygen molecule into a hydrocarbon molecule has been overcome in a number of different ways.

Results

Figures on the effects of inhibitors on R. rhodochrous PNKb1 were included in Table 3.12 for ease of comparison. The full results of this study are shown in Table 3.13 (results for cell-free assays are given in Section 3.5).

The results show that both propane and propene oxidation have similar inhibitor profiles which further supports the notion that they are metabolized by the same enzyme system. Effects on propan-1-ol oxidation were included to show when an inhibitor had an effect on general metabolism rather than specifically on hydrocarbon oxidation. For example, 1mM KCN is totally inhibitory to all oxidations when measured in the oxygen electrode but appears to have less serious effects on the formation of 1,2-epoxypropane from propane. Longer preincubation with KCN may have had more deleterious effects on epoxide formation as cells became depleted of reduced cofactors. This was not tested. Of the systems mentioned in Table 3.12 only M. capsulatus (Bath) is not inhibited by KCN suggesting that the system under study here is not akin to M. capsulatus (Bath) methane monooxygenase in this respect.

The effect of azide on the propane oxygenase of R. rhodochrous PNKb1 is difficult to assess. It appears to have a general effect by also

Table 3.13 Effects of inhibitors on oxygenase activity of resting cell suspensions of R. rhodochrous PNKb1

Inhibitor	Concentration (mM)	Type of assay			
		GC	Oxygen electrode		
		Assay substrate			
		Propene	Propane	Propene	Propan-1-ol
None	-	0	0	0	0
Azide	1	34	39	37	59
	5	55	65	45	62
KCN	1	47	100	100	100
	5	59	ND	ND	ND
	10	67	ND	ND	ND
$HgCl_2$	1	100	100	100	100
EDTA	1	0	ND	ND	ND
	5	20	32	38	31
	10	25	ND	ND	ND
2-mercapto-ethanol	1	0	ND	ND	ND
	5	12	ND	ND	ND
	10	26	ND	ND	ND
8-hydroxy-quinoline	1	0	ND	ND	ND
	5	0	ND	ND	ND
	10	0	ND	ND	ND
p-hydroxy-mercuribenzoate	1	0	ND	ND	ND
	5	0	ND	ND	ND
	10	0	ND	ND	ND
CO	0.1	0	0	0	0
	0.5	0	0	0	0
Acetylene	7.5	0	0	0	0

Figures are % inhibition, 0% inhibition = 100% activity.

Uninhibited rates were:

28 n moles 1,2-epoxypropane formed min^{-1} mg dry weight $cells^{-1}$ for the GC assay.

For the oxygen electrode assays:

With propane as substrate: 8 n moles min^{-1} mg dry weight $cells^{-1}$.
With propene as substrate: 15 n moles min^{-1} mg dry weight $cells^{-1}$.
With propan-1-ol as substrate: 24 n moles min^{-1} mg dry weight $cells^{-1}$.

ND = Not determined.

inhibiting propan-1-ol oxidation. The inhibition of Corynebacterium sp. 7E1C octane hydroxylase by azide is probably due to its binding to the haem moeity. This also explains the susceptibility of this enzyme to inhibition by carbon monoxide. Clearly the R. rhodochrous PNKb1 enzyme system is different in this respect since it shows no inhibition in the presence of carbon monoxide and therefore does not appear to contain a haem group (see also Section 3.4.5).

EDTA appeared to have non-specific inhibitory effects but 8-hydroxyquinoline exhibited no such inhibitory effects. This suggested that any metal centres present in the enzymes of propane metabolism were tightly bound or inaccessible to chelation by 8-hydroxyquinoline. P. oleovorans octane hydroxylase is particularly susceptible to this inhibitor thus indicating differences between itself and the enzyme in this study.

Mercuric chloride showed a non-specific inhibitory effect but the other thiol inhibitor p-hydroxymercuribenzoate exhibited no effect. 2-mercaptoethanol inhibited epoxide formation but its effects on propan-1-ol oxidation were not tested, so whether this was a specific effect on the oxygenase or was a result of a more general effect is unclear.

Acetylene had no inhibitory effect on hydrocarbon oxidation by R. rhodochrous PNKb1 suggesting that the hydrocarbon binding site is dissimilar to that of the methane monooxygenase of M. capsulatus (Bath).

In summary, the inhibitor profile of the propane oxygenase of R. rhodochrous PNKb1 appears to suggest that the enzyme system may be fundamentally different from those systems characterized to date.

3.4.4 Time course of propane oxygenase induction

Introduction

Little work appears to have been done on studying the events following exposure of microorganisms to alkanes. van Eyk and Bartels (1968) were able to measure the induction of hexane oxidation within 30 minutes of adding n-hexane to an exponentially growing culture (with malonate as substrate) of *Pseudomonas aeruginosa* 473.

In stark contrast, Blevins and Perry (1971) stated that it took 12-15 hours to induce propane oxygenase activity in resting cells of *Mycobacterium vaccae* JOB5 grown on acetate.

Results

A chemostat culture ($D = 0.046h^{-1}$) of *Rhodococcus rhodochrous* PNKb1 growing on propan-1-ol was assumed to be carbon limited when substrate could no longer be detected in the culture. At this point the medium was switched to minimal salts minus propan-1-ol and then propane was passed through the fermentor at $60ml.min^{-1}$ (see Fig. 3.4). Samples were removed every 2 hours and assayed for the appearance of oxygenase activity (using the 1,2-epoxypropane formation assay; Section 2.7.3). Oxygenase activity was first detected 6 hours after the addition of propane. Activity continued to increase for a further 6 hours (Fig. 3.3).

The reason why this system takes so long to induce is unclear. It could be that cells have accumulated storage materials and are metabolizing

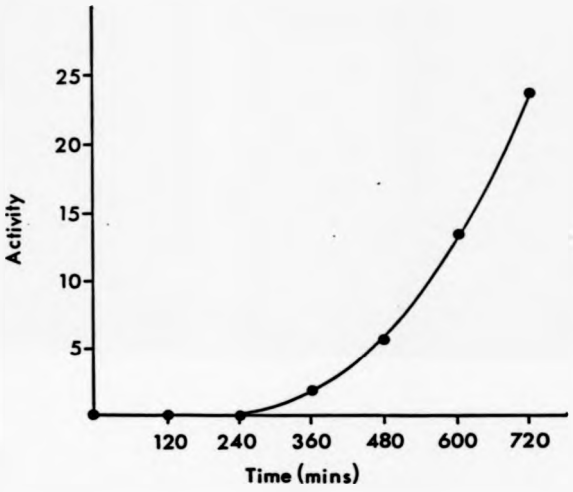


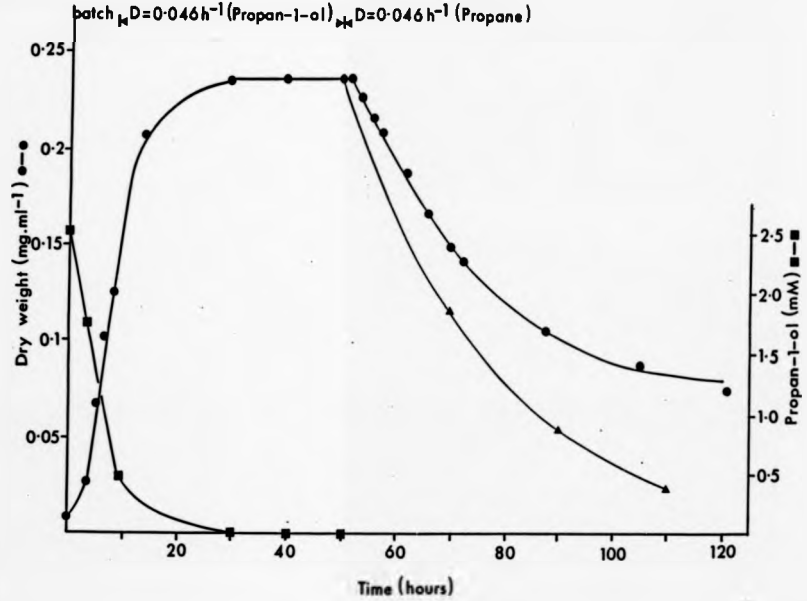
Figure 3.3 Time course of oxygenase activity induction in R. rhodochrous PNKb1

Activity in n moles 1,2-epoxypropane formed min^{-1} mg dry weight $cells^{-1}$

Figure 3.4 Induction experiment.

R.rhodochrous PN1b1 was grown batchwise, in a fermentor, on AMS/propane medium with propan-1-ol as sole carbon source. The fermentor was then switched to continuous operation until it appeared to be carbon limited. The culture was then switched to propane as sole carbon source at the same dilution rate and assayed for the appearence of oxygenase activity. (see Fig.3.3)

▲—▲ indicates a theoretical washout curve for the system described at D=0.046h^{-1}


batch
D=0·046 h⁻¹ (Propan-1-ol)
D=0·046 h⁻¹ (Propane)
Dry weight (mg.ml⁻¹) ●—●
Propan-1-ol (mM) ■—■
0·25
0·2
0·15
0·1
0·05
2·5
2·0
1·5
1·0
0·5
20
40
60
80
100
120
Time (hours)

these first, but in view of the fact that the culture was probably carbon-limited to start with this is unlikely. The availability of propane is not likely to be a problem since propane is readily soluble and mixing within the fermentor was efficient.

Fig. 3.4 also shows the gradual washing out of a propane chemostat (mentioned in Section 3.3) even though the dilution rate ($0.046h^{-1}$) would allow a doubling time of 15 hours and the doubling time on propane was 8 hours in batch culture.

3.4.3 Pathways of propane metabolism in R. rhodochrous PNKb1

Introduction

Much of the work done on propane metabolism has employed the technique of simultaneous adaptation. The advantages and limitations of this technique were described in Section 1.6.1 and need not be reiterated here. Bearing in mind these limitations and in view of the results of growth studies, propane, propan-1-ol, propan-2-ol and pyruvate-grown cells of R. rhodochrous PNKb1 were tested for their ability to oxidize potential intermediates of propane metabolism.

Results

Table 3.14 summarises the results of these simultaneous adaptation studies. It is clear that propane oxidation is inducible only by growth on propane (of the four substrates tested). The ability of pyruvate-grown cells to oxidize all the intermediates tested suggests that the enzymes required for their metabolism are constitutively expressed.

Table 3.14 The ability of R. rhodochrous PNKb1 to oxidize potential intermediates of propane metabolism after growth on propane, propan-1-ol, propan-2-ol or pyruvate

Assay substrate	Growth substrate			
	Propane	Propan-1-ol	Propan-2-ol	Pyruvate
Propane	21.0	0	0	0
Propan-1-ol	24.6	31.3	27.1	9.2
Propanal	26.3	45.8	33.1	10.2
Propanoate	6.9	43.8	8.5	2.7
Propan-2-ol	35.2	45.8	44.8	4.6
Propanone	41.9	50.0	20.6	5.6
Acetol	12.1	50.0	38.8	33.3
Methylacetate	0	0	0	ND
Methanol	0	0	0	ND
Methylglyoxal	0	ND	ND	ND
Acetate	27.7	22.9	15.8	ND
Pyruvate	6.0	4.8	0	22.9
Succinate	5.8	4.4	0	ND

ND = Not determined

Rates quoted as n moles oxygen consumed min^{-1} mg dry weight $cells^{-1}$.

However, given the low levels, these activities could be the result of non-specific alcohol/aldehyde dehydrogenase etc. which play no primary role in alkane metabolism but can fortuitously oxidize intermediates. Propane, propan-1-ol and propan-2-ol-grown cells possess increased oxidation activities against all the intermediates, with the exception of propane-grown cells oxidizing acetol. This result is somewhat perplexing (but consistent) and whether it represents a high level of constitutive acetol oxidation in pyruvate-grown cells or a genuinely depressed level in propane-grown cells remains unclear.

No clear pattern exists to suggest that oxidation of propane is primarily terminal or subterminal. The low level of propanoate oxidation by propane-grown cells could be due to a permeability barrier preventing propanoate from entering the cells rather than from a lack of the enzymes required for propanoate oxidation. Although why propan-1-ol-grown cells should have high levels of propanoate oxidation under similar conditions is puzzling.

The inability of propane-grown cells to oxidize methylacetate and methylglyoxal could again be due to uptake problems and on this evidence alone it is not possible to discount a possible role for these compounds as intermediates in propane metabolism. It is unlikely that a small polar molecule such as methanol would have any problems permeating the cell wall and membrane, and it does therefore seem likely that methanol is not an intermediate of propane metabolism.

All in all these results provide little, if any, real clues to the pathway of propane metabolism in this organism. For this reason studies were instigated to look at levels of specific enzyme activities in cell-

free systems (Sections 3.5, 3.6 and 3.7).

The involvement of cytochrome P_{450} systems in alkane metabolism (Cardini and Jurtshuk, 1970; Hartmans and deBont, 1986) prompted an investigation into the presence of these proteins in R. rhodochrous PNKb1.

Reduced and reduced/CO treated absorption spectra of propane and pyruvate-grown cells showed no characteristic absorption peaks around the 450nm wavelength that are indicative of the presence of these proteins. From this (in conjunction with the results of inhibitor studies) it was concluded that P_{450} cytochromes were not involved in alkane metabolism in R. rhodochrous PNKb1.

3.5 Studies on cell-free extracts of R. rhodochrous PNKb1

3.5.1 Preparation of cell-free extracts

Introduction

Few studies of ethane, propane and butane metabolism have been conducted on cell-free systems. This is surprising in view of the amount of effort directed at the field. Possible reasons were hinted at in Section 3.1 in that most of the organisms isolated in these studies have been highly resistant to breakage, making the formation of active cell-free extracts difficult. The members of the CMN-complex are renowned for their ability to survive extreme environments (particularly dessication) and this has been explained, in part, by their thick cell walls. These walls are much cross-linked endowing them with great mechanical strength as well as rendering them resistant to wall

degrading enzymes such as lysozyme.

The limitations of whole cell studies have been discussed and a clearer view of propane metabolism would be obtained from working with cell-free systems. Of particular interest would be the isolation of the oxygenase enzyme from R. rhodochrous PNKb1 since in vitro assaying of the enzyme would allow identification and quantification of the products thus allowing a direct measure of the relative importance of terminal and subterminal oxidation. Much emphasis was placed in this study on achieving this.

Results

Initially cell suspensions of several strains of propane-utilizing bacteria were mechanically disrupted by three cycles through a French pressure cell operated at 138MPa. It was apparent that these organisms were highly resistant to breakage and yielded very little total protein. A series of studies were then begun on R. rhodochrous PNKb1 to increase the protein yield from disrupted cell suspensions. It was apparent from earlier studies (Stephens, 1983) that excessively harsh treatments would render the cell-free extract inactive. So different techniques and combinations of techniques were applied to release protein whilst maintaining activity.

Mechanical disruption of R. rhodochrous PNKb1 in a French pressure cell proved to be the most effective method and was eventually used routinely to produce extracts. Four cycles of treatment in a precooled pressure cell were required to form cell-suspensions containing 5-10mg.ml^{-1} protein from cell suspensions containing 60-80mg.ml^{-1} dry weight of

cells. More than four cycles yielded little more protein. Using a larger pressure cell capable of a greater pressure drop (276MPa) slightly more protein was made available, but this larger pressure cell contained much "dead-space" in which cell suspensions became lost and so was not used for practical reasons.

Sonication was less effective at producing cell-free extracts. Bursts of maximum amplitude (24 microns) for periods of 30 seconds, with 30 seconds cooling intervals in an ice bath, for a total time of 3.5 minutes yielded only $4mg.ml^{-1}$ protein from a cell suspension containing $62mg.ml^{-1}$ dry weight of cells. Sonication over longer periods gave only marginal improvements in the protein yields.

Violent agitation of cell suspensions with fine glass beads is often the method of choice for breaking the tough cell walls of Gram-positive bacteria and yeasts. Such a tissue disintegrator apparatus was loaded with a cell suspension and glass beads and operated according to the manufacturers instructions. After 5 minutes of operation the protein yield was no better than that from French pressing.

Combinations of sonicating and freeze thawing in ethanol/dry ice followed by French pressing did not enhance the protein yield.

Attempts were made to soften the cell walls prior to French pressing by treatment with salt/EDTA and lysozyme (Section 2.7.1). The progress of the lysozyme treatment was followed under the microscope but there appeared to be no morphological change in the cells during the period of their treatment. Subsequent French pressing of the cell suspension failed to release any more protein than from untreated cells. It was

possible that other wall degrading enzymes such as mutanolysin (from Streptomyces globisporus) and zymolyase (from Arthrobacter luteus) would have weakened the cell walls. However, these enzymes are prohibitively expensive for routine use and may have interfered with subsequent purification steps so they were not employed.

Attempts were also made to soften the cell walls of actively growing cells by the addition of glycine to cultures prior to harvesting them. Glycine becomes incorporated into the murein matrix and interferes with its cross linking, thus weakening the cell wall. Addition of 1 or 5% (v/v) glycine to mid-exponential phase cultures of R. rhodochrous PNKb1 caused the cells to clump and flocculate. Harvested cells when French pressed yielded no more protein than untreated cells.

Total cell protein was determined as described in Section 2.5.2 after cells were boiled in 3M NaOH. By dry weight, propane-grown cells were determined to be 52% protein. Therefore a 60mg.ml^{-1} dry weight suspension of cells should yield approximately 30mg.ml^{-1} protein. Even assuming that some protein remains associated with cell debris and is centrifuged from the extract it is clear that a yield of only 5mg.ml^{-1} is poor. In fact it should be pointed out that the whole cell protein determination uses a different assay to that applied to extracts so figures may not be directly comparable. Perry (1980) states that propane-grown cells of a Mycobacterium sp. were 56.3% protein by weight and an Arthrobacter sp. grown on propane was 75-87% protein by weight. So the estimate of 52% in this study may err on the low side which would mean the measured yields may be even poorer.

It is difficult to compare these results with other studies on

Rhodochrous spp. since most authors do not mention the density of cell suspensions from which subsequent extracts were made. By way of comparison though, Hommel et al. (1987) obtained an extract of a Rhodococcus sp. containing 11mg.ml^{-1} protein after disrupting a suspension of at least 400mg.ml^{-1} wet weight by agitation with glass beads for 15 minutes. Colby et al. (1975) obtained an extract of Methylomonas methanica containing 15-20mg protein after a single passage through a French pressure cell at 138MPa of a suspension containing about 200mg.ml^{-1} wet weight of cells. It would appear from these figures that the Rhodococcus sp. was certainly more difficult to disrupt than the Methylomonas sp.

The resistance to disruption was not limited to propane-grown cells either. Growth on intermediates of propane metabolism or heterotrophic substrates still gave rise to resilient cell suspensions.

3.5.2 Assays for oxygenase activity

Introduction

In previous studies of oxygenase systems it was possible to measure the build-up of products from the oxygenase reaction in cell-free systems. Colby and Dalton (1976) were able to measure methanol formation from methane by crude extracts of Methylococcus capsulatus (Bath). Addition of KCN enhanced methanol formation by inhibiting methanol oxidation. Cardini and Jurtshuk (1970) reported that extracts of Corynebacterium sp. 7E1C could accumulate octan-1-ol and octanoate when offered octane, and Peterson et al. (1966) showed a similar effect with cell-extracts of Pseudomonas oleovorans.

Normally a metabolic sequence of enzymes will be regulated to prevent build-up of a particular intermediate, therefore the above studies rely on the fact that forming the cell-free extract in some way disrupts the pathway and allows a product to accumulate. Alternatively if a specific inhibitor of one of the enzymes of the sequence is known this may be added to effect the build-up of an intermediate.

Other assays of methane monooxygenase activity in cell-free systems have been reported. Indirect assays of methane-stimulated NADH disappearance and methane-stimulated oxygen uptake have been used (Ribbons, 1975; Feranci, 1974). Bromomethane, a soluble derivative of methane was utilized by crude extracts of Methylomonas methanica (Colby et al., 1975) and propane was epoxidated to 1,2-epoxypropane (Colby et al., 1977).

It was based on these earlier studies that an assay for propane oxygenase in cell-free extracts was sought.

Results

Using whole cells, it was possible to measure propane-stimulated oxygen uptake in an oxygen electrode. Therefore it was this assay that was attempted first using cell-free extracts consisting of total cell protein (soluble and particulate, see Section 2.8.3). Addition of 2-10mg of protein extract to the oxygen electrode resulted in a measurable endogenous rate. Addition of NADH or NADPH gave an increase in oxygen uptake rate but subsequent addition of saturated propane solution gave no increase in rate. Using an extract of Methylosinus trichosporium OB3b under identical conditions it was possible to measure a propane-

stimulated oxygen uptake rate. The use of other potential electron donors (ascorbate, malate, succinate, acetate) still resulted in no propane- stimulated oxygen uptake with extracts of R. rhodochrous PNKb1. A range of different buffers were tried in the assay (see the next section) in case TRIS was inhibitory to oxygenase activity but to no avail. The reasons for this failure to detect propane-stimulated oxygen uptake in cell-free extracts were unclear. It could be that high rates of endogenous NADH-oxidase activity were masking propane-stimulated oxygen uptake. In the absence of NADH or other electron donors no stimulation could be achieved which is surprising since earlier results suggested that production of reducing power in whole cell assays was not a problem. Perhaps disrupting the cell interfered with this ability. No satisfactory explanation of the failure of this assay system was found but another assay system that was successful was developed.

Analysing cell-free extracts exposed to propane for products of propane metabolism was also unsuccessful. No products of propane metabolism could be detected even after prolonged incubation. It was assumed that the metabolic sequence from propane to some intermediate of heterotrophic metabolism was still intact and that intermediates were not accumulating at any step on the pathway. Attempts to remove the propanol dehydrogenase activity by centrifugation or by the addition of potentially specific inhibitors (formamide and propionamide, see Sokolov et al., 1984) were also unsuccessful.

The ability of cell-free extracts exposed to propene to accumulate 1,2-epoxypropane was shown and this was adopted as the assay for oxygenase activity in cell-free extracts. The activity was characterized and the results are shown in Section 3.5.4.

3.5.3 Stability of oxygenase activity in cell-free extracts

Introduction

Release of enzymes from the cell environment to the in vitro assay environment almost inevitably leads to loss of activity. This may be as a result of denaturation caused by pH or temperature, catalytic site inactivation caused by loss of cofactors, metal centres or a covalent modification (e.g. oxidation of sulfhydryl residues) or protein degradation by proteases.

These effects can be minimised by careful choice of buffer, temperature and the addition of stabilizing agents to the extract.

Cardini and Jurtshuk (1968) made no mention of the stability of the octane hydroxylase from Corynebacterium sp. 7E1C apart from to say it could be stored at -25°C for 2 months without appreciable loss of activity. They did not need to add stabilizers to the breakage buffer so presumably the extract was relatively stable.

Baptist et al. (1963) state that octane hydroxylase activity in crude extracts of Pseudomonas oleovorans was stable for prolonged periods when frozen but that ascorbate was required to stabilize the partially purified enzyme.

Colby et al. (1975) reported that methane monooxygenase activity in crude extracts of Methylomonas methanica was unstable at 2°C losing 30-75% of activity after 24 hours. Extracts frozen to -70°C were stable for several weeks. No stabilizing compounds were present with the

extract.

Soluble extracts of Methylococcus capsulatus (Bath) containing methane monooxygenase activity were stable at 0°C retaining 75-100% of their activity for 24 hours. In extracts frozen at -70°C activity was retained for several months (Colby and Dalton, 1976).

The methane monooxygenase of Methylosinus trichosporium OB3b in crude extracts was more unstable, losing all activity overnight at 4°C. Storage at -80°C resulted in it being stable for several weeks (Lund, 1983). Soluble fractions containing methane monooxygenase activity lost all activity in 2 hours at 4°C. Addition of the protease inhibitor PMSF (phenylmethylsulphonyl fluoride) or preparing and keeping extracts anaerobically allowed 40-45% of activity to be retained at 0-4°C for 24 hours.

Hou et al. (1983c) reported that the epoxidating activity in soluble extracts of propane-grown Brevibacterium sp. CRL-56 was very stable losing only 10% of its activity at 4°C for 1 week. No stabilizing agents were present in the extract.

So it would appear that alkane oxygenase enzymes exhibit a range of stabilities from the propane oxygenase of Brevibacterium sp. CRL-56 losing only 10% of its activity after 1 week at 4°C to the soluble methane monooxygenase of Methylosinus trichosporium OB3b which loses 100% of its activity after 2 hours at 4°C.

Results

From the early attempts in this study to measure propane oxygenase activity in cell-free extracts it was suspected that the system was very labile. Extracts were always formed on ice using precooled apparatus. Extracts were centrifuged at 4°C to remove unbroken cells and held on ice whilst assaying. The decay of oxygenase activity is shown in Fig. 3.5. The half life at 0°C is 150 minutes. To check that the enzyme was not cold labile (a situation that arises when a decrease in temperature weakens the hydrophobic forces holding the molecule together) activity was assayed whilst the extract was left at room temperature (18°C). All activity was lost after 300 minutes, the half life being 60 minutes. Clearly the enzyme was not cold labile.

The buffer into which the extract is made can also affect the activity of the extract. As well as the constituent molecules of the buffer system having inhibitory affects on enzyme activity (e.g. phosphates), buffers have metal chelating properties which may remove vital metal centres from enzyme systems. In addition to phosphate and TRIS buffers, HEPES, MOPS, TES and imidazole buffers were also used at 20mM pH 7.0. None appeared to improve the stability of the oxygenase activity. Those other alkane oxygenase systems described to date have all been investigated in phosphate or TRIS buffers so it would seem unlikely that these buffers are detrimental to such systems.

One of the major problems with forming extracts is the fact that proteins are released from a relatively reduced oxygen-free environment to a relatively oxidized one which can lead to covalent modification of sulphydryl residues and loss of enzyme activity. Addition of compounds

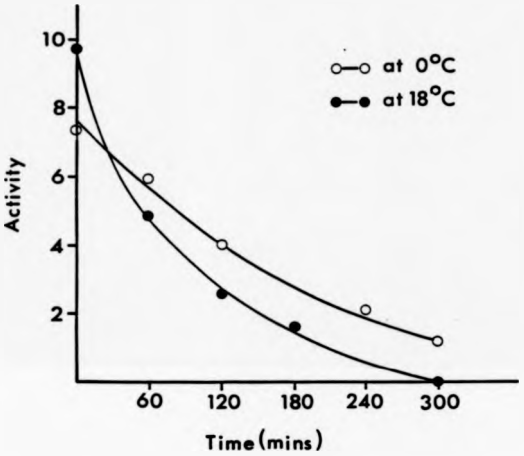


Figure 3.5 Stability of oxygenase activity in crude cell-free extracts of propane-grown R. rhodochrous PNKb1

Activity in n moles 1,2-epoxypropane formed min^{-1} mg $protein^{-1}$

such as dithiothreotol, sodium thioglycollate and 2-mercaptoethanol can prevent this from happening. These compounds have been used to stabilize both the soluble methane monooxygenase from Methylococcus capsulatus (Bath) and that from Methylosinus trichosporium OB3b. Addition of these compounds at 1, 5 or 10mM had no affect on the stability of the propane oxygenase activity in this study.

Denaturation by protease degradation has been implicated in the instability of methane monooxygenases and the addition of the protease inhibitor phenylmethylsulphonyl fluoride remedied this. PMSF inhibits a whole range of protease enzymes and those it does not affect, the acid proteases, can be effectively inhibited by procaine. PMSF and procaine were included in breakage buffers at 2 and 5mM but had no affect on the instability of oxygenase activity in this study.

It was noted that the breakage buffers employed in methane monooxygenase studies contained 5mM $MgCl_2$. The presence of cations such as Mg^{2+} and Mn^{2+} can have a stabilizing affect on extracts, so addition of $MgCl_2$ and $MnCl_2$ at 5 and 10mM was attempted. Also additions of small volumes of a trace element solution (Vishniac and Santer, 1957) containing Cu^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+} and Ni^{2+} to the breakage buffer were tried. The presence of these cations did not make the enzyme any more stable.

Loss of cofactors has also been implicated in loss of activity upon cell breakage so NADH, NADPH and FAD at concentrations of 5 and 10mM were included in breakage buffers but to no avail.

The preparation of extracts anaerobically was also attempted since certain enzymes are known to be oxygen sensitive. All buffers used were

thoroughly degassed under vacuum and sparged with oxygen-free nitrogen. All operations were carried out under a blanket of nitrogen gas. Cell suspensions were degassed prior to breakage and collected under nitrogen. Only upon assaying was the extract brought into contact with air. This procedure did not improve the half life of the enzyme.

The presence of glycerol is known to stabilize many enzymes by lowering the water activity in the in vitro environment. The use of glycerol in this study was precluded for two reasons. Firstly, in oxygen electrode assays glycerol is rapidly oxidized giving a rate which masks any subsequent propane stimulated oxygen uptake. Secondly, to be effective glycerol has to be present at about 5-10% (v/v) in the extract. When assaying for products using a GC this then involves injecting high concentrations of glycerol onto the column which tend to mask all other peaks.

The inability to stabilize propane oxygenase activity was a serious hindrance to this particular study. All subsequent work had to be performed on freshly formed extracts. Extracts drop-frozen in liquid nitrogen and stored at -70°C or at -20°C were inactive when thawed so there was no means of storing extracts. The reasons for the inability to stabilize the activity are hard to understand. Approaches that had proved successful in the past with similar systems were unrewarding in this case. Numerous combinations of the agents listed above were tried but never with any success. The most likely explanation is that the system is very labile and requires a precise set of conditions in which to remain active. Despite all the conditions tried in this study the right criteria were obviously not met. If this oxygenase system is entirely different from those described to date then it may prove

necessary to approach its stabilization from an entirely different angle since all that has worked on similar systems before has failed with this system.

3.5.4 Characteristics of cell-free oxygenase activity

Introduction

Methane monooxygenase from Methylococcus capsulatus (Bath) and octane hydroxylases from Pseudomonas oleovorans and Corynebacterium sp. 7E1C have been well characterized (see Section 1.7.1 and 3.4.3). Their structure and function is well understood and, in the case of methane monooxygenase work is now directed at understanding the finer points of the mechanisms of methane oxidation.

In stark contrast, studies on ethane, propane and butane oxygenases have only just entered the realm of cell-free studies. Mention has already been made as to why this might be. The only work published to date is that of Patel et al. (1983a, 1983b) and Hou et al. (1983c).

Patel et al. (1983a) reported results from propane-grown Arthrobacter sp. CRL60, Brevibacterium sp. CRL56 and Pseudomonas sp. NRRL B1244. Extracts were formed by single passage of the cell suspension through a French pressure cell at 20,000psi. Extracts were fractionated by centrifugation at 40,000xg for 60 minutes to form a particulate (P(40)) and soluble (S(40)) fraction. The S(40) fraction was then centrifuged for 60 minutes at 80,000xg to yield P(80) and S(80) fractions. For the Arthrobacter sp. CRL60 all the hydroxylating activity was located in the P(40) fraction and when offered propane produced propan-1-ol and propan-

2-ol at 10 and 5 n moles min^{-1} mg $protein^{-1}$ respectively. The activity required oxygen and NADH. The Brevibacterium sp. CRL56 and Pseudomonas sp. NRRL B1244 contained hydroxylating activity in the S(80) fractions. The rates of product formation were 0.42 and 0.50 n moles min^{-1} mg $protein^{-1}$ for propan-1-ol and propan-2-ol respectively by the Pseudomonas sp. NRRL B1244 and 0.54 and 0.60 n moles min^{-1} mg $protein^{-1}$ for propan-1-ol and propan-2-ol respectively by the Brevibacterium sp. CRL56.

Patel et al. (1983b) reported that a butane-grown Arthrobacter sp. CRL60 P(40) fraction contained epoxidating activity and formed 1,2-epoxypropane from propene at a rate of 6 n moles min^{-1} mg $protein^{-1}$. In the same report they showed that propane and propene apparently compete for the same active site suggesting that this epoxidating activity was the alkane hydroxylase activity.

Hou et al. (1983c) located an epoxidating activity in Brevibacterium sp. CRL56 grown on propane. Activity located in the S(80) fraction epoxidated propene to 1,2-epoxypropane at a rate of 52 n moles min^{-1} mg^{-1}, had a pH optimum of between 6.0 and 7.0 and a reduced level of activity (50%) when NADPH replaced NADH in the assay.

These results summarise the known facts on cell-free propane oxygenase systems. The activity identified in this study was investigated in a similar manner to compare and contrast it with other known systems.

Results

The temperature optimum of the oxygenase activity was shown to be 30°C using whole cells and is unlikely to be significantly different for cell-free extracts. The pH optimum was found to be about 6.8 (Fig. 3.6).

The absolute requirement for oxygen and NAD(P)H was difficult to assess. Extracts prepared anaerobically and offered propane in an atmosphere of nitrogen rather than air produced no product so it would seem oxygen is a requirement. Dialysis to remove endogenous amounts of NAD(P)H resulted in inactive extracts even when NAD(P)H were added back. This was probably due to loss during dialysis of another cofactor, or perhaps cation, required for activity. It was never possible to measure activity in dialysed extracts. However, extracts left on ice for an hour showed only low levels of activity in the absence of added NAD(P)H (1.1 n mole min^{-1} mg^{-1}) but addition of 2μmoles of NADH resulted in an activity of 2.3 n moles min^{-1} mg^{-1} whereas addition of 2μmoles of NADPH gave no increase in activity. This would suggest that endogenous levels of cofactor had become depleted and that addition of NADH but not NADPH could restore activity.

Centrifugation at 38,000xg for 1 hour resulted in a soluble and a particulate fraction. Activity was located in the soluble fraction but not the particulate fraction. This suggests that the enzyme is soluble but further fractionation of the supernatant by longer, faster centrifugation would be required before this can be categorically stated.

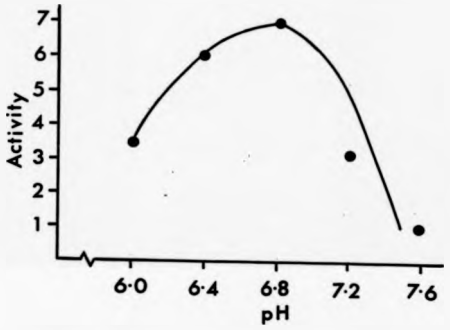


Figure 3.6 pH profile of oxygenase activity in crude cell-free extracts of propane-grown R. rhodochrous PNKb1

Activity in n moles 1,2-epoxypropane formed min^{-1} mg $protein^{-1}$

The effect of various inhibitors on cell-free oxygenase activity is shown in Table 3.15. Results are similar to those shown with whole cells, activity being inhibited by azide, KCN, $HgCl_2$, EDTA and 2-mercaptoethanol. No inhibition was seen with 8-hydroxyquinoline, p-hydroxymercuribenzoate, carbon monoxide or acetylene.

When cell-free extracts of propane-grown cells and cells grown on other intermediates of propane metabolism were analysed using SDS-PAGE (Plate 3.3), it was apparent that propane-grown cells possessed three major protein bands of molecular weights 67, 59 and 57 kilodaltons (as judged from standard marker proteins). These bands were only present on the track containing extract from propane-grown cells and may represent components of a propane oxygenase system.

The temperature optimum of the enzyme probably reflects the growth temperature of this organism. Having been enriched for and selected at 30°C it is likely that this will represent the optimum temperature. Both octane hydroxylase systems (from Corynebacterium sp. 7E1C and P. oleovorans) had temperature optima of 30°C, again this being the growth temperature of the organism. The soluble methane monooxygenase of M. capsulatus (Bath) is most active at 45°C which is the growth temperature for this organism. None of the oxygenase systems appear to be very thermostable, all losing activity rapidly when assayed at temperatures higher than the organisms' growth temperature.

The pH requirements of all the systems studied, including this one, also appear to be similar having pH optima in the range 6.5-7.5.

Cofactor requirements are a little more diverse. In this study it was

Table 3.15 Effect of inhibitors on oxygenase activity of cell-free extracts of R. rhodochrous PNKb1

Inhibitor	Concentration (mM)	% inhibition
None	-	0
Azide	1	30
	5	48
KCN	1	49
	5	61
	10	71
$HgCl_2$	1	100
EDTA	1	0
	5	25
	10	30
2-mercaptoethanol	1	0
	5	10
	10	30
8-hydroxyquinoline	1	0
	5	0
	10	0
p-hydroxymercuribenzoate	1	0
	5	0
	10	0
CO	0.1	0
	0.5	0
Acetylene	7.5	0

Figures are % inhibition, 0% inhibition = 100% activity.

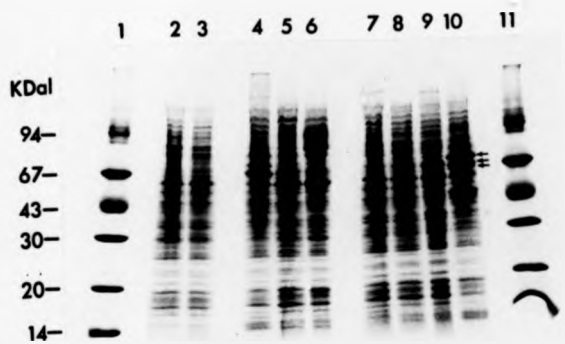
100% activity = 10.5 n moles 1,2-epoxypropane formed min^{-1} mg $protein^{-1}$.

Plate 3.3. SDS-polyacrylamide gel electrophoresis of cell-free extracts of *R.rhodochrous* PNKb1 grown on various potential intermediates of propane metabolism.

Track	Growth substrate
1	(Molecular weight markers)
2	Pyruvate
3	Succinate
4	Acetol
5	Acetone
6	Propan-2-ol
7	Propanoate
8	Propanal
9	Propan-1-ol
10	Propane
11	as 1

50µg of protein per track

Arrows indicate proteins specific to propane grown cells.


1
2 3
4 5 6
7 8 9 10
11
KDal
94–
67–
43–
30–
20–
14–

not possible to measure activity with NADPH as cofactor. This was the case with the octane hydroxylase from Corynebacterium sp. 7E1C which showed very little activity with NADPH compared to NADH (Cardini and Jurtshuk, 1970). The octane hydroxylase from P. oleovorans by contrast had very little difference, the activities being 26.6 and 23.6 n moles min^{-1} mg $protein^{-1}$ for NADH and NADPH respectively (McKenna and Coon, 1970). The propane oxygenase system in Brevibacterium sp. CRL56 (Hou et al. 1983c) exhibited 50% of the activity measured with NADH when NADPH was used. Similarly the methane monooxygenase of Methylosinus methanica exhibited a likewise effect (Colby et al., 1975).

The fact that the propane oxygenase activity in this study was located in the soluble fraction after a 38,000xg centrifugation for 1 hour suggests that the enzyme is soluble. However a more rigorous demonstration would be necessary before this could be categorically stated. The propane oxygenase activity of Brevibacterium sp. CRL56 would appear to be soluble since it was located in the soluble fraction after a 40,000xg and an 80,000xg centrifugation (Hou et al., 1983c). The octane hydroxylase of P. oleovorans has two soluble components (reductase and rubredoxin) whilst the hydroxylase is particulate in nature. Similarly the octane hydroxylase of Corynebacterium sp. 7E1C, whilst located in the soluble fraction after a 144,000xg spin for 2 hours was still described as having a particulate component (the P_{450} cytochrome component). The situation with methane monooxygenase in M. capsulatus (Bath) is more complex since there are two distinct enzymes, one soluble and the other particulate in nature whose presence or absence is determined by the copper:biomass ratio of the culture (Stanley et al., 1983).

The effect of inhibitors on cell-free extracts of R. rhodochrous PNKb1 was not dissimilar to that seen on whole cell suspensions. Particularly striking is the lack of inhibition by carbon monoxide which apparently rules out the involvement of a P_{450} cytochrome type system such as that found in Corynebacterium sp. 7E1C (Cardini and Jurtshuk, 1970). This is backed up by the absorbance spectra mentioned in Section 3.4.5 which also showed the absence of P_{450} cytochromes. Given that carbon monoxide has no effect on oxygenase activity the inhibition caused by azide is somewhat harder to explain since azide should inhibit iron-porphyrin containing enzymes as does carbon monoxide. Cyanide is also known to inhibit enzymic activity by binding to iron-porphyrin groups but it must also show other inhibitory mechanisms since the octane hydroxylase of P. oleovorans was not found to contain an iron-porphyrin moeity and yet was still inhibited by cyanide (McKenna and Coon, 1970). Cyanide inhibition of R. rhodochrous PNKb1 propane oxygenase activity could be as a result of the ability of cyanide to complex with other metal ion cofactors. In magnitude it is more akin to that shown by cell-free extracts of Arthrobacter sp. CRL60 (Patel et al., 1983b).

Any metal centres within the propane oxygenase of R. rhodochrous PNKb1 would appear to be tightly complexed with the enzyme judging by its resistance to inhibition by 10mM 8-hydroxyquinoline and only 30% inhibition by 10mM EDTA. Such concentrations severely inhibited the octane hydroxylase of P. oleovorans showing that its metal centre was susceptible to chelation by these compounds (McKenna and Coon, 1970).

Conflicting results appear with the thiol inhibitors mercuric chloride and p-hydroxymercuribenzoate. Both should attack the same -SH groups and yet mercuric chloride causes a total loss of activity whilst p-

hydroxymercuribenzoate has no apparent affect. It is difficult to state, therefore whether or not sulphydryl groups are significant in the propane oxygenase of this study.

The lack of inhibition by acetylene, which is thought to bind irreversibly to the methane binding component of methane monooxygenase in M. capsulatus (Bath) (Prior and Dalton, 1985), suggests that the hydrocarbon binding site or mechanism of the enzyme in this study is different from that in M. capsulatus (Bath).

The presence of three distinct protein bands in electrophoresed cell extracts of propane-grown cells suggests parallels with two earlier studies. The soluble methane monooxygenase of M. capsulatus (Bath) gives a similar banding pattern on SDS-polyacrylamide gels. The α, β and γ components of protein A give distinct bands at 54, 42 and 17 kilodaltons (Woodland and Dalton, 1984). P. oleovorans treated in the same manner after growth on octane yields three distinct bands at 50, 41 and 19 kilodaltons corresponding to the reductase, hydroxylase and rubredoxin components respectively. If the three propane specific proteins present in R. rhodochrous PNKb1 are components of an alkane oxygenase system then it could be a large multicomponent enzyme which may also account for its instability.

In summary then, although parallels have been drawn between the possible component structure of the propane oxygenase in R. rhodochrous PNKb1 and other systems, the inhibitor profile is dissimilar in many respects to these systems. It is possible, therefore, that the propane oxygenase of R. rhodochrous PNKb1 may be a novel enzyme system.

3.3.5 Purification of oxygenase activity

Introduction

The three alkane monooxygenase systems mentioned in Section 1.7.1 as being the most studied have been purified to varying degrees. The octane monooxygenase from Corynebacterium sp. 7E1C has been the least purified, being separated into two ammonium sulphate precipitable fractions (Cardini and Jurtshuk, 1970). One fraction contained a P_{450} cytochrome and the other a flavoprotein reductase. Both fractions were required for hydroxylating activity.

The octane monooxygenase from Pseudomonas oleovorans has been resolved into three components. Cell-free extracts could be centrifuged to yield a supernatant containing the reductase and rubredoxin components and a pellet containing the hydroxylase component. The reductase and rubredoxin could be resolved by passage through a DEAE-cellulose ion exchange column which bound the rubredoxin but not the reductase. The hydroxylase was recovered from the pellet fraction by resuspension, sonication and precipitation (with ammonium sulphate) followed by gel filtration chromatography (Peterson et al., 1966). All three components have been subjected to further purification and characterization (see Peterson and Coon, 1968; McKenna and Coon, 1970).

The soluble methane monooxygenase from Methylococcus capsulatus (Bath) has also been resolved into its three components. Supernatant from centrifuged cell-free extract was loaded onto a DEAE-cellulose ion exchange column and the three components (A, B and C) could be eluted using an increasing sodium chloride gradient (0-0.5M). This and

subsequent purification steps are summarised by Dalton (1980b).

Although resolution into components and characterization of these components is ultimately desirable in a biochemical study of an enzyme, an initial consideration in this study was to attempt to separate the oxygenase activity from the alcohol dehydrogenase activity. If this could be achieved by a simple, one step process, then it would be possible to offer the partially purified oxygenase propane as a substrate and to measure a build up of the products of this activity in the absence of subsequent enzymes. This would then have demonstrated the relative importance of terminal and subterminal oxidation in R. rhodochrous PNKb1.

The methods employed are described in Section 2.8.6. Due to the unstable nature of the oxygenase ($T_{\frac{1}{2}}$ at 4°C = 150 mins) the separation techniques employed had to be rapid. High performance gel filtration chromatography met this criterion and the use of a suitable packing material allowed the rapid elution of the potentially large oxygenase system. High performance ion exchange chromatography was also a possibility since it might be possible to recombine collected fractions containing oxygenase components to give a functionally active complex without reconstituting alcohol dehydrogenase activity. Finally, affinity chromatography could be employed to selectively bind the oxygenase enzyme whilst removing alcohol dehydrogenase enzymes.

Results

Attempts to isolate partially purified propane oxygenase were wholly unsuccessful, despite the range of techniques employed. The reasons for

this failure are discussed below.

The time taken to remove fractions containing protein from the gel filtration and ion exchange columns was approximately 60 minutes and from the affinity column approximately 150 minutes. Fractions were placed on ice immediately upon collection and were assayed as quickly as possible. Throughout the assaying period crude extract, as loaded onto the columns, was kept on ice and this was checked for activity at the end of the experiment. Activity could be detected in this control thus indicating that the inability to measure oxygenase activity was not due entirely to a loss of activity with time. What is more likely is that dilution of the protein during its elution led to a more rapid denaturation of the oxygenase enzyme.

Gel filtration separates proteins on the basis of their size and a large multicomponent enzyme (such as oxygenases appear to be) would pass through the column rapidly. The column should be non-denaturing so a multicomponent enzyme might reasonably be expected to be isolated intact within a few fractions. The elution profile from the gel filtration column is shown in Figure 3.7. Under the conditions used and assuming the oxygenase enzyme constituted about 10% of the total cell protein (a not unreasonable assumption based on the degree of staining seen in polyacrylamide gels showing propane specific proteins - see Section 3.5.4) then the assay system employed should have been sensitive enough to detect activity if it was present. Dilution of protein solutions often leads to denaturation of the protein due to the increased water activity in these highly aqueous environments. The addition of large volumes of glycerol to buffers can reduce the water activity and thus slow down denaturation, but this unfortunately makes the solutions being

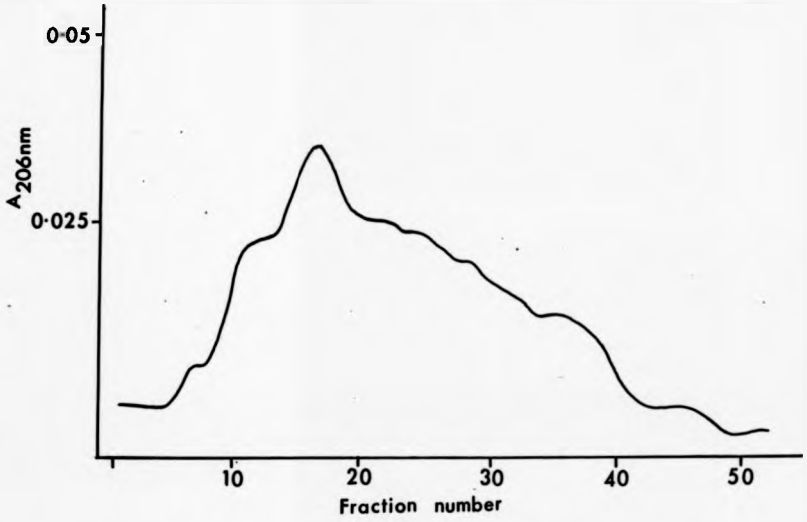


Figure 3.7 Elution profile of samples from gel filtration column (see Section 2.8.6)

used more viscous which can increase the time required for resolution in a gel filtration column. Addition of large amounts of "foreign" protein, such as bovine serum albumin, to mimic the *in vivo* situation of high protein concentration is obviously inappropriate during the resolution of protein solutions.

Ion exchange chromatography would almost certainly resolve a large multicomponent oxygenase into its constituent proteins. Elution of the column under the conditions stated using an increasing sodium chloride gradient gave rise to three major protein peaks (as determined by absorbance at 206nm - see Fig. 3.8). Recombining these major protein fractions did not result in measurement of oxygenase activity. Combining other minor fractions was also unsuccessful. When using ion exchange chromatography the choice of buffer, ionic strength and pH are critical and without a systematic evaluation of these parameters, successful purification using this technique is difficult.

A similar criticism can be levelled at affinity chromatography. Unless the buffer, ionic strength and pH are correct for the system of interest then the necessary binding of protein to ligand will not occur. The affinity column chosen in this study should have bound the oxygenase enzyme as it is designed to bind NADH requiring systems (see manufacturers specifications). However, no activity was measured in any of the eluted fractions and combination of fractions gave no activity either. There is always the possibility too, that the site of binding for the ligand is deep in a cleft and therefore inaccessible, thus resulting in the protein not binding. If this had been the case however then activity could still have been measured in fraction 1 which would contain material eluted straight through the column.

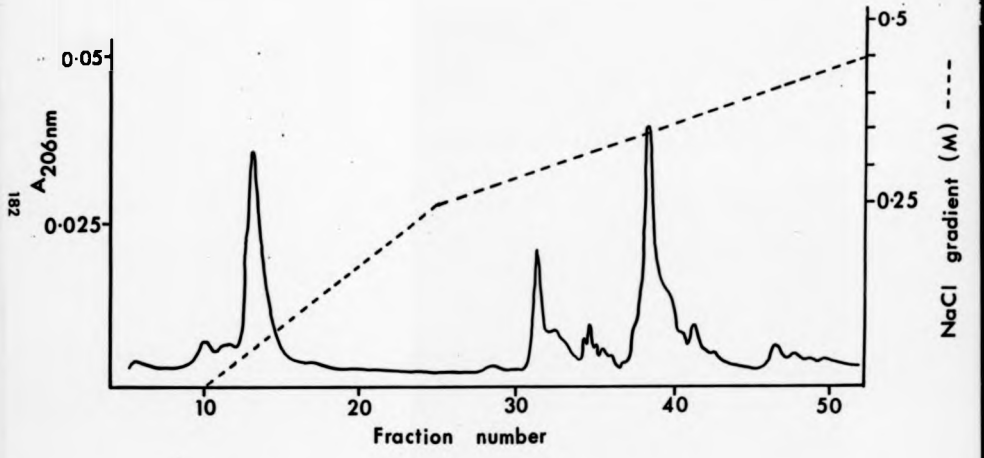


Figure 3.8 Elution profile of samples from ion exchange column (see Section 2.8.6)

The problem of protein solutions that were too dilute was probably the source of the disappointing results to these studies. Concentration by precipitation with ammonium sulphate could have overcome this problem when applying the protein solution to the gel-filtration column. However the time taken to concentrate the protein solution (with resulting loss in activity) must be weighed up against the stabilizing effect such a treatment might confer on the protein. Unfortunately such an approach would not have worked with the ion exchange or affinity chromatography techniques since the increased ionic strength of such solutions would interfere with the binding of the proteins to the column.

If the activity could have been stabilized more and loaded onto the gel filtration chromatography column at a higher concentration then this set of experiments may have achieved their desired aim in removing the alcohol dehydrogenase activity from any oxygenase activity and thus allowed the measurement of the build up of the products of oxygenase activity and answered the questions of the relative importance of terminal versus subterminal oxidation of propane.

3.6 Alcohol dehydrogenase activity

Introduction

Section 1.7.2 surveyed the current state of knowledge regarding the role of alcohol dehydrogenases in n-alkane metabolism. Parallels were drawn between the role in methane metabolism and liquid n-alkane metabolism of $NAD(P)^+$-independant alcohol dehydrogenases. This was in contrast to the two systems described in gaseous alkane-utilizing organisms both of

which were NAD^+-linked systems (Hou et al., 1983b; Coleman and Perry, 1985). The study of alcohol dehydrogenase activity in n-alkane-grown cells can be useful in inferring the pathway of n-alkane metabolism, but care should be exercised in interpreting results since some organisms appear to contain many alcohol dehydrogenase enzymes, the roles of some being unclear (see Singer and Finnerty, 1984).

Results

Cell-free extracts of propane-grown Rhodococcus rhodochrous PNKb1 (harvested at mid-late exponential phase E_{540nm} = 0.81) contained both primary and secondary NAD^+-linked propanol dehydrogenase activity. Low levels of $NADP^+$-linked activity could also be measured, but despite exhaustive measures using a wide range of assay buffers/pH's no dye linked activity (using either phenazine methosulphate or phenazine ethosulphate) could be measured. Control assays using cell-free extracts of Methylococcus capsulatus (Bath) were used to validate the assay systems. Dye linked methanol dehydrogenase activity could be measured in the M. capsulatus (Bath) extracts. The failure to detect dye linked alcohol dehydrogenase activity in propane (or propan-1-ol)-grown cells of R. rhodochrous PNKb1 may indicate that no such activity is present and that the $NAD(P)^+$-linked activities are responsible for further metabolism of the alcohols produced by the alkane oxygenase. Alternatively the possibility cannot be ruled out that an alcohol dehydrogenase using some other unknown physiological acceptor was present but that the artificial acceptor system used in the assay could not couple with the physiological system. Reasons for this suggestion will be apparent later.

The pH profile of the major activity present (NAD^+-linked) is shown for both primary and secondary substrates in Fig. 3.9. The pH optimum was approximately 10 which is comparable with that found by Coleman and Perry (1985). Fig. 3.10 shows the substrate range of the alcohol dehydrogenase activity. Interestingly activity is consistently greater against secondary alcohols (secondary propanol, butanol and hexanol) than the equivalent chain length primary alcohols. Methanol is not a substrate for this activity but ethanol is. Short chain diols could also act as substrates and again the presence of subterminal hydroxyl groups gave greater rates than terminal hydroxyl groups.

The fact that glycerol, tertiary butanol and cyclohexanol could also act as substrates may indicate a broad substrate range or, perhaps more likely, the presence of more than one alcohol dehydrogenase in the cell-free extract.

The propanol dehydrogenase activities appeared to be quite stable losing only 10% of their activity over a period of 13 hours at 0^oC. Centrifugation of cell-free extracts at 38,000xg for 1 hour gave supernatants containing all of the activity. Resuspended pellets were devoid of activity. This would suggest, as in the studies of Coleman and Perry (1985) and Hou et al. (1983b), that this NAD^+-linked propanol dehydrogenase activity is soluble and not associated with a membrane fraction (as is the dye linked hexadecanol dehydrogenase described by Tassin and Vandecasteele, 1972).

Table 3.16 shows the effects of various inhibitors on cell-free primary and secondary NAD^+-linked alcohol dehydrogenase activity. Activity was inhibited by both 1, 10 phenanthroline and cyanide but at quite high

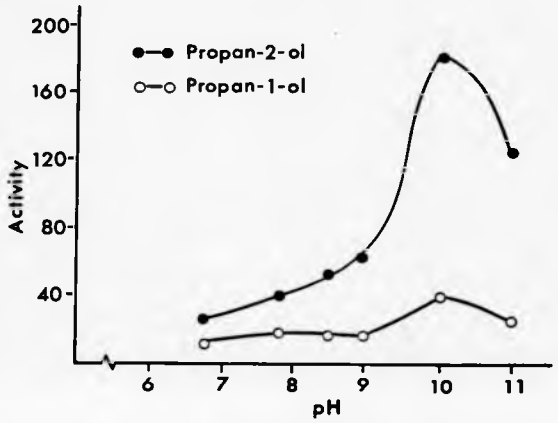


Figure 3.9 pH profile of NAD^+-linked propanol dehydrogenase activity in crude cell-free extracts of propane-grown R. rhodochrous PNKb1

Activity in n moles NAD^+-reduced min^{-1} mg $protein^{-1}$

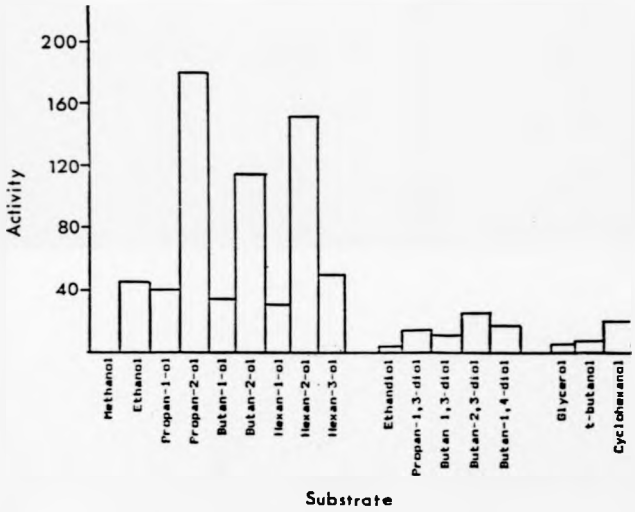


Figure 3.10 Substrate range of NAD^+-linked alcohol dehydrogenase activity in crude cell-free extracts of propane- grown R. rhodochrous PNKb1

Activity in n moles NAD^+-reduced min^{-1} mg $protein^{-1}$

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Table 3.16 Inhibition of NAD^+-linked alcohol dehydrogenase activity in cell-free extracts of propane-grown R. rhodochrous PNKb1

Substrate	Inhibitor	Concentration (mM)	% inhibition
Propan-2-ol	1,10-phenanthroline	5	38
	p-OH-mercuribenzoate	0.05	78
		0.01	0
	KCN	5	49
		1	34
	Formamide	25	8
		5	0
	Propionamide	50	33
Propan-1-ol	Formamide	50	0
	Propionamide	50	8

Propan-2-ol dehydrogenase activity 100% = 179 n moles NAD^+-reduced min^{-1} mg $protein^{-1}$.

Propan-1-ol dehydrogenase activity 100% = 39 n moles NAD^+-reduced min^{-1} mg $protein^{-1}$.

concentrations suggesting that a metal centre may be involved but it appears to be quite tightly bound to the enzyme protein. The thiol reducing reagent p-hydroxymercuribenzoate was highly inhibitory probably indicating the presence of a required thiol group. Similar results were reported by Coleman and Perry (1985) and Hou et al. (1983b), the inhibitory concentrations they reported for these compounds being slightly lower due to their using purified enzymes rather than crude cell-free extracts. This would suggest that the alcohol dehydrogenase activity present in Rhodococcus rhodochrous PNKb1 is similar to the enzymes described in Mycobacterium vaccae JOB 5 (Coleman and Perry, 1985) and Pseudomonas fluorescens NRRL B1244 (Hou et al., 1983b).

Formamide, at high concentrations (50mM) is known to be a specific inhibitor of methanol dehydrogenase (Sokolov et al., 1984). Therefore it was reasoned that formamide or perhaps propionamide might selectively inhibit propanol dehydrogenase. If this was the case, then by inhibiting the alcohol dehydrogenase it might be possible to measure the build up of the products of oxygenase activity in cell-free extracts offered propane. However, formamide appeared ineffective at inhibiting propanol dehydrogenase activity and although propionamide had some inhibitory effect it was deemed unsuitable for the experiment described above for two reasons. Firstly it appeared to inhibit secondary alcohol dehydrogenase activity more than the primary activity which would distort the measurements of possible build up products from oxygenase activity. Secondly it proved impossible to prevent the large concentrations of propionamide used in the assays from totally masking any product build up on the GC output.

In an attempt to elucidate if more than one enzyme was responsible for

the measured propanol dehydrogenase activities, cell-free extracts were electrophoresed into non-denaturing polyacrylamide gels and the gels subjected to activity stains with propan-1-ol or propan-2-ol as substrate. The results are shown in Plate 3.4. Extracts from propane, propan-1-ol, propan-2-ol and pyruvate-grown cells all appear to contain a common activity which appears to be more active (i.e. gives a darker stain) in the presence of the secondary alcohol than with the primary alcohol. In addition, propane and propan-2-ol-grown cells appear to contain an additional activity that also appears to be more active against the secondary alcohol. It is interesting to compare these observations with the results of Coleman and Perry (1985) who found that the alcohol dehydrogenase that appeared to be involved in propane metabolism was induced by growth not only on propane but on propan-2-ol too. It would appear then, that growth of Rhodococcus rhodochrous PNKb1 on propane (or propan-2-ol) may induce an additional secondary alcohol dehydrogenase not present in pyruvate or propan-1-ol-grown cells. This additional activity may therefore have a role in propane metabolism, (see also Section 3.7).

It is worth noting that continued incubation of the control gel (without substrate) beyond the 30 minutes prescribed in the original method (Stirling and Dalton, 1978) resulted in the appearance of a similar, but somewhat fainter, set of bands that had appeared on the gels incubated in the presence of substrate. Repeating the experiment using a different method (Grell et al., 1965) resulted in the same phenomenon. Whether this is due to the effects of trace amounts of alcohols present in the reagents or perhaps a non-specific interaction of the dyes involved with the alcohol dehydrogenases is uncertain.

Plate 3.4 Activity stain for propanol dehydrogenase in electrophoresed cell-free extracts of R.rhodochrous PNkb1 grown on propane,propan-1-ol,propan-2-ol and pyruvate.

Track	Growth substrate
1	(Yeast alcohol dehydrogenase control)
2	Pyruvate
3	Propan-2-ol
4	Propan-1-ol
5	Propane

Gel	Assay substrate
A	None(control)
B	Propan-1-ol
C	Propan-2-ol

15ug protein per track

a-indicates activity common to all extracts.
b-indicates activity only in propane and propan-2-ol grown cells.


1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
a
b
A
B
C

Studies of a Pseudomonas sp. grown batchwise on butane suggested that different alcohol dehydrogenase enzymes may be present through the growth cycle (Beers et al., 1988). It is thought that activities measured later in the growth cycle may have more relevance to the metabolism of storage materials, such as polyhydroxybutyrate, than with n-alkane metabolism. To investigate this effect, the affinity of the propanol dehydrogenase activity for the primary and secondary propanols was measured at three different points in the growth cycle. The results are shown in Table 3.17. The most striking features of these figures are the very low affinities for the two substrates. Even considering that these are being measured in crude cell-free extracts, which may give different results to measuring the same affinity with a purified enzyme, the apparent K_s values of 22mM for propan-2-ol and 3mM for propan-1-ol are orders of magnitude different from those measured by Coleman and Perry (1985) and Hou et al. (1983b). This must raise the question of whether these activities could be responsible for the metabolism of intermediates of propane metabolism or whether these are just incidental alcohol dehydrogenases responsible for other aspects of metabolism and the enzymes responsible for propane metabolism are evading measurement. It would be more likely that the enzymes of propane metabolism would exhibit much stronger affinities for their substrates, as this has been the case previously (e.g. Coleman and Perry, 1985, 49μM for propan-2-ol; Hou et al. 1983b, 85μM for propan-2-ol; Tassin et al., 1972, 4.5μM for tetradecan-1-ol). In the Pseudomonas sp. study mentioned above, there is a peak in NAD^+-independent alcohol dehydrogenase activity during mid exponential phase, falling off in late exponential phase. $NAD(P)^+$-linked activity increases at late exponential phase. This was interpreted as showing the role of NAD^+-independent enzymes in alkane metabolism whereas the $NAD(P)^+$-linked

Table 3.17 Variation in apparent K_m and V_{max} values of propanol dehydrogenase activity through the growth cycle of propane-grown R. rhodochrous PNKb1 (see Appendix 2).

Substrate	Parameter	Mid exponential phase (OD_{540} 0.51)	Mid late exponential phase (OD_{540} 0.75)	Late stationary phase (OD_{540} 0.99)
Propan-2-ol	K_m	20.7	23.3	22.8
	V_{max}	35.1	83.3	178.2
	V_{max}/K_m	1.69	3.58	7.82
	(r)	(0.98)	(0.9)	(0.99)
Propan-1-ol	K_m	2.5	3.9	2.9
	V_{max}	14.7	16.9	30.0
	V_{max}/K_m	5.86	4.33	10.10
	(r)	(0.98)	(0.9)	(0.95)

(r) = correlation coefficient of linear regression analysis yielding K_m and V_{max}.

K_m in mM.

V_{max} in n moles NAD^+-reduced min^{-1} mg $protein^{-1}$.

enzymes were considered to play no such role (Beers et al., 1988). However in this study with R. rhodochrous PNKb1 there is no evidence to suggest the presence of NAD^+-independant activity and the affinity for propan-1-ol and propan-2-ol appears almost constant through the growth cycle. There appears to be an increase in the specific activity of secondary alcohol dehydrogenase later in the growth cycle which may indicate the presence of additional enzymes but the consistently low affinities for propan-1-ol and propan-2-ol remain perplexing.

In summary, cell-free extracts of propane-grown R. rhodochrous PNKb1 contain NAD^+-linked alcohol dehydrogenase activity that is similar in many respects to that described by Coleman and Perry (1985) and Hou et al. (1983b) in propane-grown organisms. This activity may be limited to propane and propan-2-ol-grown cells and the specific activities measured in propane-grown cells appear to favour secondary alcohols. However, the affinity shown towards propan-1-ol and propan-2-ol appears very low and must place a question mark over the involvement of this activity in n-alkane metabolism.

3.7 Pathways of propane metabolism

Introduction

As discussed in Section 3.4.5 the evidence presented for the pathway of propane metabolism based on the measurement of enzyme activities in cell-free extracts is minimal. Perhaps the most complete studies of this kind are those of Taylor et al. (1980) looking at acetone metabolism in coryneform bacteria (but including work on Mycobacterium vaccae JOB5) and Van Ginkel et al. (1987) who looked at butane

metabolism in a Nocardia sp. In both cases, the level of enzyme activities in cell-free extracts of the organisms grown on different substrates was used to postulate the relevant metabolic pathway. The advantages of this method over the use of whole cell studies were discussed in Section 1.6. Dagley and Chapman (1971) add a caution to this method and state that it is not only the presence of an activity but its magnitude that must be considered too. Since different enzymes will be affected differently during the forming of the cell-free extract then the in vitro activities measured may differ from those present in vivo. Given a continuous culture at a fixed growth rate and steady state biomass, it is possible to calculate whether or not the measured enzyme activities are reasonable (i.e. whether they could support the observed growth rate/biomass concentration). Such calculations are impossible to perform on batch-grown systems.

This caution must be applied to this study where all growth was batchwise. To minimise differences between subsequent batches of cells, all cells were harvested at the same point in the growth cycle (mid to late exponential phase) and cell extracts were always prepared and assayed immediately to minimise the effects of denaturation. The levels of activity were compared in propane and pyruvate-grown cells and in cells grown on an intermediate that would require the activity of interest, for example propanal dehydrogenase activity was measured in propanal-grown cells.

Results

Table 3.18 summarises the results of enzyme assays on cell-free extracts of Rhodococcus rhodochrous PNKb1.

Table 3.18 Enzyme activities measured in cell-free extracts of R. rhodochrous PNKb1 grown on potential intermediates of propane metabolism

Enzyme activity	Growth substrate								
	Propane	Propan-1-ol	Propanal	Propanoate	Propan-2-ol	Propanone	Acetol	Pyruvate	Acetone
Propane oxygenase[1]	12	0	-	-	0	-	-	0	-
Propan-1-ol dehydrogenase[2]	40	34	-	-	47	-	-	33	-
Propan-2-ol dehydrogenase[2]	185	58	-	-	90	-	-	49	-
Propanal dehydrogenase[2]	14	-	32	-	-	-	-	3	-
Acetone oxygenase[3]	0	-	-	-	-	0	-	0	-
Propionyl CoA synthetase[4]	26	-	-	26	-	-	-	6	-
Acetol oxygenase[3]	27	-	-	-	-	-	19	1	-
Acetol dehydrogenase[2]	0	-	-	-	-	-	0	0	-
Isocitrate lyase[5]	12	-	-	-	-	-	-	0	78

Cont/...

1. Activity measured as n moles 1,2-epoxypropane formed min^{-1} $mg\ protein^{-1}$.
2. Activity measured as n moles NAD^+-reduced min^{-1} $mg\ protein^{-1}$.
3. Activity measured as n moles oxygen consumed min^{-1} $mg\ protein^{-1}$.
4. Activity measured as n moles -SH disappearing min^{-1} $mg\ protein^{-1}$.
5. Activity measured as units $mg\ protein^{-1}$ (see Section 2.9.5).

\- Not assayed.

Propane oxygenase activity (as measured by the formation of 1,2-epoxypropane - see Section 3.5.2) was only present in propane-grown cells. This would agree with earlier work in this study that suggested only alkane-grown cells possessed oxygenase activity. It would appear that only propane (of all compounds listed) could induce oxygenase activity. This is in contrast to the situation with M. vaccae JOB5 where oxygenase activity (albeit in whole cells) was present in propan-2-ol and acetone-grown cells.

The alcohol dehydrogenase activities were discussed in some detail in the previous Section (3.6). The activities presented in Table 3.18 are NAD^+-linked activities and represent the major activity present in the extracts, there being low levels of $NADP^+$-linked but no dye-linked activities present. Primary alcohol dehydrogenase activity appears to be fairly constant irrespective of growth substrate and may represent a constitutive activity. There are equivalent levels of secondary alcohol dehydrogenase activity in propan-1-ol and pyruvate-grown cells again suggesting the presence of a constitutive enzyme. Levels of activity in propan-2-ol-grown cells and propane-grown cells are 2 and 3 times greater respectively, indicating the possible presence of another enzyme induced by growth on these substrates. This may represent a similar situation to that found by Coleman and Perry (1985) in M. vaccae JOB5 in which a secondary alcohol dehydrogenase was induced by growth on propane or propan-2-ol.

Pyruvate-grown cells possessed low levels of aldehyde dehydrogenase activity. Propanal-grown cells show a ten fold increase in this NAD^+-linked activity. $NADP^+$-linked activity has been reported in a number of studies (see Section 1.7.3) but none was measured in this study. The

low level of NAD^+-linked activity found in pyruvate-grown cells may represent a constitutive enzyme having a detoxification role as suggested by Duine et al. (1987). Levels of activities in propane-grown cells were certainly increased five fold from those measured in pyruvate-grown cells but they were still only half that measured in propanal-grown cells, so the role of propanal dehydrogenase in propane metabolism remains unclear.

Acetone oxygenase activity was not measured in cell-free extracts of any of the cell suspensions tested. The assay employed was a polarographic one based on that of Hartmans and deBont (1986) (see Section 2.9.2). A range of buffers were employed at different pH's and the addition of both NADH and NADPH tried. Presumably a similar situation is arising as did with the alkane oxygenase, in that a very labile system is being disrupted by cell breakage or that a structural requirement (membrane system?) is being lost upon formation of the extract. A similar situation was reported by Taylor et al. (1980) who were also wholly unsuccessful at measuring cell-free acetone oxygenase activity. They had to resort to evidence from whole cell systems to place acetone oxygenase in the metabolic sequence for propane dissimilation. In this study the fact that cell suspensions of propane-grown cells were able to oxidize acetone at eight times the rate of cell suspensions of pyruvate-grown cells provides the only possible evidence of acetone as an intermediate of propane dissimilation.

By way of contrast, acetol oxygenase activity in cell-free extracts of acetol and propane-grown R. rhodochrous PNKb1 could be readily measured by the method of Hartmans and deBont (1986). Presumably this infers that acetone and acetol are not metabolized by the same enzyme and that

the acetol oxygenase is more stable and therefore easier to assay. Levels of activity in propane-grown cells were greatly enhanced compared to pyruvate-grown cells and slightly higher even than in acetol-grown cells. Activity was NADPH-dependant as was that described by Hartmans and deBont (1986) in cell-free extracts of Mycobacterium Py1 grown on acetol.

No acetol dehydrogenase activity could be measured using the method of Taylor et al. (1980). Again the proviso must be added that an exhaustive range of assay conditions were not attempted, but in view of the presence of acetol oxygenase activity in propane-grown cells and the inability of whole cell suspensions to oxidize methylglyoxal (the product of acetol dehydrogenase) it is probable that this activity is indeed absent. Indeed Taylor et al. (1980) found that acetol dehydrogenase activity was absent from acetone-grown M. vaccae JOB5.

The presence of acetol oxygenase, as mentioned above, would give rise to acetate. Assimilation of acetate requires the operation of the glyoxylate cycle and the presence of isocitrate lyase. Propane-grown cells did indeed contain isocitrate lyase albeit at a low level. Taylor et al. (1980) reported isocitrate lyase levels in acetone-grown M. vaccae JOB5 to be as high as in acetate-grown cells. Conversely Van Ginkel et al. (1987) postulated that butane was metabolized via acetate yet butane-grown cells had less than one third of the isocitrate lyase activity of acetate-grown cells.

Taken as a whole then, the presence of increased levels of propan-2-ol dehydrogenase, acetol oxygenase and isocitrate lyase in propane-grown cells would argue that oxidation of propane is occuring subterminally,

and assimilation is occurring via acetate (see Fig. 1.9). The lack of acetol dehydrogenase and presence of isocitrate lyase would argue against assimilation via pyruvate.

However mention has not yet been made of the high activity of propionyl CoA synthetase. Activities in propane-grown cells were greatly enhanced from those in pyruvate-grown cells, to a level equal to that found in propanoate-grown cells. This must surely argue for considerable propane oxidation via propanoate. Oxidation of propane via propanoate does not preclude the presence of isocitrate lyase either, since certain pathways of propanoate oxidation (e.g. via malonic semialdehyde - see Dagley and Nicholson, 1970) may be isocitrate lyase positive. Propionyl CoA is a central metabolite and it is conceivable that the levels of propionyl CoA synthetase may be enhanced for some other reason not related to propane metabolism. This however, is speculation and it must be concluded from the results in Table 3.18 that althogh subterminal oxidation via acetate is an attractive proposition on the basis of the levels of propan-2-ol dehydrogenase activity, acetol oxygenase activity and isocitrate lyase activity, the possibility of some terminal oxidation via propan-1-ol dehydrogenase, propanal dehydrogenase and propionyl CoA synthetase cannot be ruled out.

3.8 Product excretion studies

Introduction

Many studies on n-alkane-oxidizing bacteria report the presence of excreted products (usually alcohols and methylketones) in the culture medium during the growth on n-alkanes. Many authors have used the

presence of such compounds to infer what metabolic pathways are operative, a practice that should be viewed with some caution considering the words of Dagley and Chapman (1971) expressed in Section 1.6. For example, the presence of methylketones in culture supernatants after growth on n-alkanes may indicate that subterminal oxidation has occurred but it is doubtful if a complete subterminal pathway is operating if the organism is losing potential cell carbon in this manner. Why product excretion should occur is unclear and no systematic study of this phenomenon had occurred. One obvious possibility is that a non-specific oxygenase produces both terminal and subterminal oxidation products but the organism subsequently lacks the metabolic potential to use these products which are therefore excreted. Alternatively, product excretion may be a consequence of the culture conditions employed. Stephens (1983) suggested that the ammonium ion concentration of the medium may have some effect on product excretion.

To examine the effect of ammonium ion concentrations on Rhodococcus rhodochrous PNKb1 it was grown batchwise on propane in NMS medium with varying amounts of ammonium chloride and the culture supernatant analysed for the presence of products.

Results

Initially, supernatants from stationary phase cultures were analysed and found to contain no (or only trace amounts of) products. This was perhaps not unexpected since the organism appeared capable of oxidizing all the potential intermediates of propane metabolism. However, analysis of supernatants from growing cultures gave a different result entirely. Table 3.19 shows the results of culture supernatant analysis

from early, mid and late exponential phase and stationary phase cultures. Propan-2-ol was the only product detected on the GC column, which was capable of resolving propan-1-ol, propan-2-ol, propanal, acetone, acetol and propanoate. No other unidentified peaks were recordad. Product excretion appeared to occur early in exponential phase. The excreted product then appeared to be metabolized until the culture reached stationary phase when it could no longer be detected. Increasing the ammonium ion concentration did appear to increase the amount of product produced. Stephens (1983) suggested that this may be due to ammonium ion toxicity but since growth occurs more rapidly in AMS medium, containing $1.0g.l^{-1}$ ammonium chloride ($\mu = 0.083h^{-1}$), than in NMS medium, containing no ammonium chloride ($\mu = 0.044h^{-1}$) this may not be the case with this organism. If the ammonium ion was having some inhibitory affect on, for example, the alcohol dehydrogenase enzyme, which caused the build up of propan-2-ol, then the growth rate might be expected to decrease too. This was not the case.

The appearance of propan-2-ol may be a consequence of the poor affinity of the alcohol dehydrogenase activity for this substrate. However, one wonders how such an organism would survive in the environment if, when growing on such a poor substrate as propane it then proceeded to excrete propan-2-ol too, a substrate which is much more widely used by bacteria than propane. However, in the natural environment, concentrations of propane available are very low and perhaps this phenomenon would not occur under such conditions, i.e. it is only occurring as a result of the culture conditions employed.

Whatever the reason for the occurrence of product excretion, this experiment shows what care must be exercised in interpreting such a

Table 3.19 The effect of ammonium ion concentration on propan-2-ol excretion by R. rhodochrous PNKb1 growing on propane

Concentration of NH_4Cl ($g.l^{-1}$)	Propan-2-ol concentration (μm)			
	20 hrs	45 hrs	55 hrs	80 hrs
0	92	64	14	0
0.5	111	113	73	0
1.0	305	241	122	0

R. rhodochrous PNKb1 was grown on propane (50% v/v) in NMS medium to which various concentrations of NH_4Cl had been added. Samples were removed aseptically and analysed for the presence of propan-2-ol.

phenomenon since the amount of product measured in the culture supernatant was affected by the composition of the medium and the growth phase. A systematic survey of this effect may prove useful in determining optimal conditions for the production of compounds such as methylketones which are potentially useful for organic syntheses in the chemical industry.

3.9 Acetylene metabolism by Rhodococcus rhodochrous PNKb1

Introduction

The ability of bacteria to grow with acetylene (ethyne) as sole carbon and energy source was first reported by Birch-Hirschfeld (1932). She described a *Mycobacterium* sp. that exhibited growth in stationary cultures after 2 to 4 weeks incubation in a mineral salts-soil extract medium under an acetylene:air atmosphere.

Kanner and Bartha (1979) isolated a similar organism which they identified as a *Nocardia* sp. They concluded that their organism could have been the same as that of Birch-Hirschfeld. They investigated the optimal growth conditions for this organism and found maximal growth rates (doubling times 2.7-3.0 hours) on acetylene were obtained with 5 to 20% acetylene, 25 to 40% oxygen, pH 7.0 and 26 to 28°C. Acetylene utilization was not an obligate trait and a wide range of carbon sources were utilized.

Kanner and Bartha (1982) further characterized their isolate and showed that resting cell suspensions when exposed to acetylene excreted acetaldehyde. The organism could grow on acetylene, acetaldehyde,

acetate, ethanol and succinate. The levels of acetaldehyde dehydrogenase, acetothiokinase and isocitrate lyase were measured in cell-free extracts of cells grown on each of the aforementioned substrates. Their results suggested that in their organism acetylene may be metabolized as shown in Fig. 3.11. Repeated attempts to demonstrate acetylene hydrase activity in cell-free extracts were unsuccessful. Variations in the cell disruption techniques and the addition of stabilizers made no difference. Acetylene utilization was shown to be constitutive.

deBont and Peck (1980) isolated a Rhodococcus sp. capable of growth on acetylene and methylacetylene. In addition it could grow on acetaldehyde, propanal, acetate, propanoate, propan-1-ol, propan-2-ol, acetone, succinate and glucose. Acetylene hydrase activity appeared to be constitutive but with enhanced levels on acetylene and methylacetylene-grown cells. Resting cell suspensions cultured on acetylene excreted acetaldehyde when supplied with acetylene and excreted propanal when supplied with methylacetylene. Acetylene-grown cells had enhanced levels of acetaldehyde dehydrogenase and isocitrate lyase compared to succinate-grown cells. deBont and Peck (1980) were successful in measuring cell-free acetylene hydrase activity after assaying under nitrogen (with 160ppm acetylene) rather than air. Extracts dialysed for 5 hours retained all their activity and showed a stoichiometric conversion of acetylene to acetaldehyde. A spectrophotometric assay in which acetaldehyde formation was linked to NADH-oxidation via alcohol dehydrogenase allowed the K_m for acetylene to be estimated at 0.61mM. 95% of activity was lost upon storage at 4°C or -2°C for 24 hours.

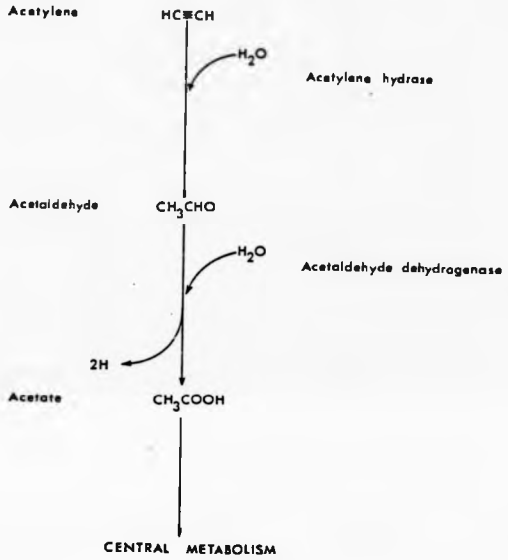


Figure 3.11 Pathway of acetylene metabolism in Rhodococcus spp. (from Kanner and Bartha, 1982)

The four reports mentioned above are the only examples known of the microbial metabolism of acetylene. That all should describe probably the same organism is interesting and suggests that this metabolic capability may be limited to a single group of microorganisms. Also interesting is the fact that acetylene is not a naturally occurring compound (Kanner and Bartha, 1979) but is released as a result of human activities such as in emissions from petrol engines and oil refineries (Kanner and Bartha, 1982). Acetylenic compounds do exist in nature and no doubt the Rhodococcus group of organisms must play a role in their breakdown.

This study strayed onto acetylene metabolism accidently whilst attempting to inhibit propane oxygenase activity using acetylene. It was apparent from the assays that not only was propane oxygenase activity not inhibited by acetylene but that acetylene was being removed from the assay system. Because of the novelty of this metabolism it was decided to investigate the phenomenon further.

Results

Rhodococcus rhodochrous PNKb1 was grown in shake flasks on 50ml AMS medium below an atmosphere of 50% (v/v) acetylene in air. Growth was slow, the culture taking 7 days to reach an E_{540nm} of 0.8. Dropping the acetylene concentration to 20 or 10% (v/v) did not improve the growth rate, neither did reducing the oxygen concentration from 10% (v/v) to 5 or 2.5% (v/v). Trace amounts of acetaldahyde were detected in late exponential phase cultures. Resting cell suspensions of acetylene-grown cells when exposed to acetylene did not produce acetaldahyde. However, it was possible to measure acetylene disappearance in the assay (Fig.

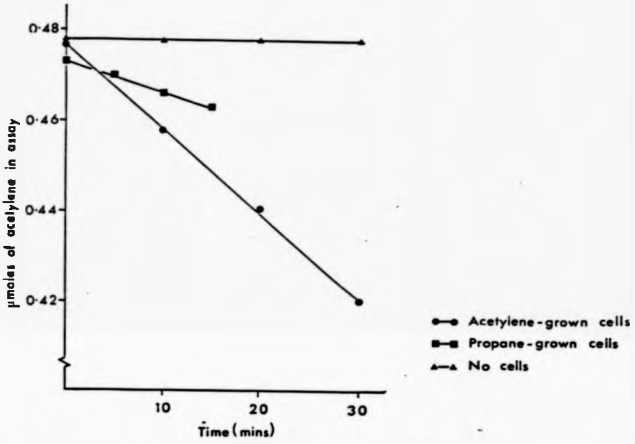


Figure 3.12 Metabolism of acetylene by resting cell suspensions of R. rhodochrous PNKb1

3.12). The rates were 6.3 and 1.6 n moles acetylene consumed min^{-1} mg dry weight $cells^{-1}$ for acetylene and propane-grown cells respectively. These rates are orders of magnitude less than those measured by deBont and Peck (1980). This would account for some of the differences seen between this organism and that of deBont and Peck. Resting cell suspensions of R. rhodochrous PNKb1 did not excrete acetaldehyde when exposed to acetylene and growth on acetylene was very slow. The organism(s) used in the other studies were obviously well adapted to acetylene metabolism having been selected and isolated on it as sole carbon source. The Rhodococcus sp. used in this study probably had a non-specific hydrase activity that was able to convert acetylene to acetaldehyde, which was then metabolized to acetate by a non-specific aldehyde dehydrogenase.

deBont and Peck (1980) speculated on the role of a monooxygenase in acetylene metabolism but decided that the intermediate so formed $-C(=O)-C-$ would be very unstable. Further recent work on R. rhodochrous PNKb1 suggests that propane oxygenase deficient mutants are still able to metabolize acetylene, thus eliminating the oxygenase from a role in acetylene metabolism (W. Ashraf pers. comm.).

The ability to utilize acetylenic compounds is another example of the extreme metabolic diversity of Rhodococcus spp. which will no doubt guarantee their exploitation in the future.

3.10 Synopsis

Using a variety of different techniques over eighty strains of ethane, propane and butane-utilizing bacteria were isolated from a range of

environments, showing the ubiquitous nature of these organisms. All appeared to be from the Corynebacterium-Mycobacterium-Nocardia group and no Gram-negative organisms were isolated. Screening of Pseudomonas spp. culture collections failed to isolate any gaseous alkane-utilizing strains.

Three of the isolates were selected as being suitable for further study and were identified as Rhodococcus rhodochrous, Rhodococcus erythropolis and a Mycobacterium sp.

All grew in liquid culture on propane with doubling times between 8 and 13 hours. Batch growth conditions of R. rhodochrous PNKb1 were partially optimised and its substrate range examined. It grew on most of the putative intermediates of propane metabolism and appeared to have the potential for using both terminal and subterminal pathways of propane metabolism. Of the n-alkanes from methane to octane it only grew on propane and it showed no growth on short chain alkenes. Despite growing in continuous culture with propan-1-ol a substrate, absolute steady-states of R. rhodochrous PNKb1 could not be established during continuous culture on propane.

The alkane oxygenase activity appeared to be inducible in the three isolates tested. All were capable of epoxidating alkenes to epoxyalkanes, and alkenes and alkanes appeared to compete for the same oxygenase. The oxygenase activity was investigated in terms of its inhibitor profile and it had features distinguishing it from the alkane oxygenase systems described to date. Oxygenase activity was slow to induce, and the results of simultaneous adaptation studies suggested that either terminal or subterminal pathways of propane oxidation could

be operative.

Cells of R. rhodochrous PNKb1 were highly resistant to disruption, making the formation of cell-free extracts difficult. It was not possible to measure a build up of the products of propane oxidation in cell-free extracts but the oxidation of propene to 1,2-epoxypropane could be readily measured. Oxygenase activity in cell-free extracts was very unstable and none of the established stabilizing factors could improve this instability. Proteins specific to propane-grown cells were identified on polyacrylamide gels and these may be components of an oxygenase system. The effect of inhibitors on cell-free oxygenase activity gave similar results to the whole cell studies suggesting that the propane oxygenase system of R. rhodochrous PNKb1 may be different to those systems described previously. Attempted partial purifications of the oxygenase system were hampered by its lability and earlier failures to stabilize it.

Alcohol dehydrogenase activity appeared to be largely NAD^+-linked and similar in nature to systems already described in gaseous alkane-utilizing bacteria. Activity towards secondary alcohols appeared predominant. However, the apparently low affinity of the measured activities to the alcohols left some doubt as to whether these activities could participate in the pathways of n-alkane metabolism.

The enzyme complement of propane and pyruvate-grown cells suggested that either terminal or subterminal oxidation of propane could occur and the realtive importance of each pathway remains unclear.

Propan-2-ol was excreted by R. rhodochrous PNKb1 growing on propane, the amount varying with the culture conditions and the phase of the growth cycle.

CHAPTER 4

GENERAL DISCUSSION

The isolation of ethane, propane and butane utilizing-bacteria proved relatively straightforward. They could be found in all of the environments tested regardless of whether or not the environment appeared to have been exposed to gaseous alkanes. The different techniques employed usually enriched and isolated the same types of organisms, members of the CMN-complex. The lack of Gram-negative organisms in these enrichments remains perplexing, although possible contributory factors were discussed in Section 1.2.3. The Pseudomonas spp. isolated by Takahashi et al. (1980) and Patel et al. (1983b) came from an activated sludge plant and soil samples both associated with oil refineries. Similar samples were used in this study but failed to yield any gaseous alkane oxidizing Pseudomonas spp. Why the above authors were successful in isolating these organisms when so many before and after have failed is not clear. They did not use any special media or isolation techniques. No mention is made of how many samples were screened to obtain these organisms but it would appear that an element of chance may have been involved.

The benefits of working with an organism such as a Pseudomonas sp. lie in the amount of work that has been published on these organisms. Techniques exist for the gentle disruption of these Gram-negative cells, thus allowing easy formation of cell-free extracts. Much data has been acquired on the continuous culture of these Pseudomonas spp. With this background knowledge to build upon, developing new techniques is more directed and has less of a trial and error approach.

The organisms isolated in this study and identified as strains of Rhodococcus rhodochrous, R. erythropolis and Mycobacterium sp. are typical of those organisms isolated in previous gaseous alkane

enrichments. Although these three strains were undoubtably the most amenable to work on, out of all those isolated, they were in retrospect prone to many of the same problems of previously studied isolates. Until the techniques exist for working on these organisms in a manner that will not disrupt the apparently delicate enzyme systems of alkane oxidation or until more amenable organisms become available our knowledge of gaseous alkane metabolism will remain limited.

The advantages of being able to culture an organism continuously are numerous. Optimisation of growth conditions to achieve higher growth rates or greater biomass concentrations is easier to implement on a continuous culture system than on a batch culture where alteration of the culture conditions by the growing culture cannot be controlled. Cells grown in chemostat cultures can be harvested to give cells of a constant metabolic condition. Batch-grown cultures contain a more heterogeneous population of cells and must be harvested at exactly the same point in their growth cycle to allow comparative studies to be performed.

Steady-state continuous cultures would have allowed the measurement of parameters such as growth yield, and carbon conversion efficiency. Such parameters are needed when considering practical applications of microorganisms and may have had an additional use in this study. The subterminal oxidation pathway contains three oxygenase reactions which makes it more energetically demanding than the terminal pathway which contains just one. This may be reflected in a lower theoretical growth yeild which it may prove possible to measure using a steady-state continuous culture.

Only chemostat continuous culture was attempted. Growth of the organism in turbidostat culture in which the biomass concentration is fixed and the dilution rate allowed to vary may have proved more successful. Technical difficulties can be encountered when attempting to monitor optical density continuously over a long period as organisms may adhere to the surfaces of the optical cell. Pirt (1985) suggests that any growth linked process (e.g. change in pH) can be utilized as a sensor for turbidostat control and this might prove easier to implement.

Failure to keep cultures of these organisms growing continuously will prove a problem in their exploitation since they will most likely be competing in processes similar to those using methane-oxidizers, whose growth in continuous culture has been well documented.

Perhaps the major obstacle in this study and one that was never satisfactorily overcome was that of cell breakage. The CMN-complex bacteria possess a much cross-linked cell wall structure endowing the cells with great mechanical strength. This was reflected by the lack of success using mechanical means to disrupt the cells. Their wall structure also makes the cells resistant to many wall degrading enzymes and the antibiotics that interfere with wall synthesis. No doubt new wall degrading enzymes will be discovered that many attack the peptidoglycan of these organisms and perhaps also compounds that can interfere with the synthesis of these wall structures. The best approach will no doubt be a combined one perhaps culturing cells in the presence of a wall synthesis inhibitor followed by incubation with a wall degrading enzyme followed by sonication and French pressing. However there is always the danger that at the end of such a procedure the extract formed will be lacking the relevant enzyme activity so the

development of a gentle lysis procedure is to be favoured.

The instability of the oxygenase activity is another problem that still requires much further work. Achieving the correct conditions for stabilization of propane oxygenase is very much a case of trial and error, at getting the right combination of factors together at the right concentration. In a study plagued by lack of protein to work on, the number of combinations of factors tried was limited. If the problem of releasing more protein from cell suspensions could be overcome then a thorough systematic search for the right combination of factors could be done. Until the problems of protein yield and stability are solved a successful purification of the propane oxygenase from R. rhodochrous PNKb1 remains unlikely.

Despite the problems mentioned above, much of value has emerged in this study. In particular, the whole cell and cell-free inhibitor studies indicate that the propane oxygenase system of R. rhodochrous PNKb1 may be dissimilar to those systems studied to date. In terms of its instability it may prove less easy to work on than the methane monooxygenases or octane hydroxylase, but it is potentially a novel enzyme system and deserves further study. The inability to assay its activity using its "natural" substrate (propane) prevented measurement of any kinetic parameters. Attempting such measurements on crude cell-free extracts is not the best way of obtaining such information as the early attempts of measuring these parameters for methane monooxygenase illustrates. A K_m of 160μM for methane was reported by Colby et al. (1977) but working with purified enzyme this was revised to 3μM by Green and Dalton (1986). The stoichiometry of the reaction of propane oxygenase also remains to be established to ascertain whether it is a

mono or dioxygenase.

Throughout the study crude cell-free extracts were used, containing both soluble and particulate (membrane associated) activities. The centrifugation of extracts at 38,000xg for 30 minutes would probably not be sufficient to sediment all membrane fragments so the location of activity in the supernatant cannot preclude the possibility that the enzyme may still be associated with a membrane. If one of the components does turn out to be particulate in nature this may further complicate subsequent purification as was the case with the hydroxylase component of the octane hydroxylase of Pseudomonas oleovorans (McKenna and Coon, 1970).

That the same oxygenase should be responsible for the hydroxylation of alkanes and the epoxidation of alkenes is well documented for methane and liquid alkane-utilizers (see Stirling and Dalton, 1981; May and Padgette, 1983). In common with these examples, R. rhodochrous PNKb1 can derive no benefit from epoxidating the alkene since it cannot metabolize the resulting epoxide any further. This fact has been used to argue that the epoxidating activity is a result of the enzyme mechanism rather than a particular adaptation (see Stirling and Dalton, 1981; Higgins et al., 1981). This must then suggest that although the enzyme in this study was dissimilar in nature to those previously studied it may possess mechanistic similarities, for example in the way the substrates are activated prior to reaction. It must be commented that the enzyme system in this study may not be typical of gaseous alkane-utilizers generally since Cerniglia et al. (1976) reported that propane-grown M. convolutum when offered propene produced acrylic acid rather than the epoxide, although it is conceivable that the epoxide was

formed and then further metabolized to acrylate.

The production of epoxides from alkenes is currently an area of much practical interest. The rate of epoxide formation by R. rhodochrous PNKb1 compares favourably with those rates published to date (see Habata-Crützen et al., 1984). Less encouraging from a practical viewpoint is the apparently limited range of alkenes used as substrates. If epoxidation can only occur with short chain terminal alkenes then the biotransformation of large compounds such as sterols may be beyond the capability of the propane oxygenase of R. rhodochrous PNKb1. It is interesting to speculate if this restriction to short chain terminal alkenes plus the limited range of alkanes oxidized indicates that there is restricted access to the hydrocarbon-binding site of the oxygenase. Investigation of the activity using a wider range of alkenes (e.g. aromatics and optically active compounds) may yield more information on the accessibility and reaction mechanism at the active site.

The pathways of propane metabolism were investigated using three different approaches; growth substrate specificity, simultaneous adaptation in whole cells and enzyme activities in cell-free extracts. None of these approaches gave an unequivocal answer in favour of terminal or subterminal oxidation. From all of these studies it must be concluded that R. rhodochrous PNKb1 appears to have the metabolic capability of utilizing either pathway. The poor affinity of the alcohol dehydrogenase activity for propanol is perplexing. It could be explained in terms of there being another pyridine nucleotide independant activity present that was not measured or perhaps, as mentioned above in the case of methane monooxygenase, the apparent K_m measured in crude cell-free extracts could be two orders of magnitude

too great. Figures of 200μM for propan-2-ol and 30μM for propan-1-ol would be more in line with those described by Coleman and Perry (1985) and Hou et al. (1983b).

Given that many of the oxygenase activities described can produce mixtures of both primary and secondary alcohols (Dalton, 1980a; Patel et al., 1983b) then an organism that had the capability to utilize both products further would obviously be at an advantage. There would be some metabolic cost in maintaining two parallel pathways but if, for example, non-specific primary alcohol dehydrogenase and aldehyde dehydrogenase activity was already present for some other purpose and these were able to metabolize further any primary alcohol resulting from the action of a non-specific oxygenase then the organism will out-compete, in carbon assimilation terms, the organism that must lose the primary alcohol, since it possesses only the subterminal oxidation pathway. In the environment, organisms relying on gaseous alkanes as sole carbon sources will probably be carbon limited due to the extremely low concentrations of these gaseous alkanes, therefore any means of assimilating all the carbon available will be advantageous.

The excretion of secondary alcohol by R. rhodochrous PNKb1 could be seen as the result of the "unnaturally" high concentration of propane present and the apparent lesser affinity of the alcohol dehydrogenase towards propan-2-ol. In the competitive environment where much lower concentrations of propane would be present then the poor affinity might be less significant and no product excretion might occur. This is obviously speculation and no data exist to support it, but perhaps the ability to utilize either pathway of propane metabolism is advantageous.

Even if the ability to produce both terminal and subterminal oxidation products is a consequence of some mechanistic dictate of the oxygenase enzyme, it would still be interesting to know the relative proportions of each product produced, but until the oxygenase has been purified, this may remain unknown. Alternatively non-destructive techniques such as NMR (Sanders, 1987) may be able to elucidate the relative proportions of products *in vivo* and succeed where all these "destructive" techniques have not.

By way of concluding comment then, this project has covered much ground on the subject of propane metabolism perhaps at the expense of not probing too deeply into any one aspect. This was considered a reasonable approach for such a preliminary study (one that started with just mud samples!) and the results, although a little disappointing from the view of the original aims, were satisfactory in many respects and open the way for numerous further studies.

APPENDIX

Summary of the results of the identification of isolates PNKb1, BPSd1 and GPYb1 by NCIMB Ltd.

On the basis of preliminary examinations, the results of which are shown in Table A.1 and A.2. NCIMB assigned PNKb1 and BPSd1 to the genus Rhodococcus. Further biochemical tests were proposed to allow identification to species level. The preliminary examination of GPYb1 was less revealing and four possible genera were suggested. Mycobacterium, Rhodococcus, Nocardia or Corynebacterium. To determine which of these genera GPYb1 belonged to, thin layer chromatography analysis of wall mycolic acids, gas-liquid chromatography analysis of wall fatty acid methyl esters, and the aryl sulfatase test were proposed.

Table A.3 shows the results of biochemical tests on PNKb1 and BPSd1. PNKb1 was identified as Rhodococcus rhodochrous. BPSd1 was identified as R. erythropolis despite giving atypical results for the benzoate and tyrosine utilization tests. It was distinguished from R. rhodochrous by the negative utilization of meta-hydroxybenzoate.

GPYb1 gave a negative arylsulfatase test, atypical of the genus Mycobacterium but some of the more rapidly growing species are arylsulfatase negative.

The cell wall diamino acid was identified as meso diamino pimelic acid. The major fatty acids were $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and tuberculostearic acids plus smaller amounts of $C_{14:0}$ and $C_{18:0}$. This suggested membership of either Rhodococcus or Mycobacterium but the presence of several mycolic acids led to the conclusion that GPYb1 was a Mycobacterium sp.

Table A.1 Preliminary identification of isolates PNKb1 and BPSd1

The National Collections of Industrial and Marine Bacteria Ltd., Torry Research Station, 135 Abbey Road, Aberdeen. AB9 8DG, Scotland, UK.

for

Source of isolates

FIRST STAGE

Morphological descriptions are from growth on LAB M Nutrient Agar except as stated

Isolate	PNK b1	BPS d1
°C incubation	25°	25°
Cell morphology 5 hours X630 48 hours	 Cells are in nutrient broth + 0.75% agar by phase contrast  Gram's stain of glucose agar	 
Gram	+	+
Spores	—	—
Motility	—	—
Colonial morphology Glucose agar 3 days	round, regular, entire smooth, opaque low convex orange-pink 2 mm diameter	round, regular, entire smooth, opaque, low convex pale pink, matt surface in confluent zone ≈ 2-2.5 mm diameter
°C, growth	37° + 41° +	37° + 41° —
Catalase Oxidase, Kovacs O-F glucose	+ (+) OXIDATIVE	+ — OXIDATIVE
First stage identification		

Table A.2 Preliminary identification of isolate GPYb1

The National Collections of Industrial and Marine Bacteria Ltd., Torry Research Station, 135 Abbey Road, Aberdeen. AB9 8DG, Scotland, UK.

for

Source of isolates

FIRST STAGE

Morphological descriptions are from growth on LAB M Nutrient Agar except as stated

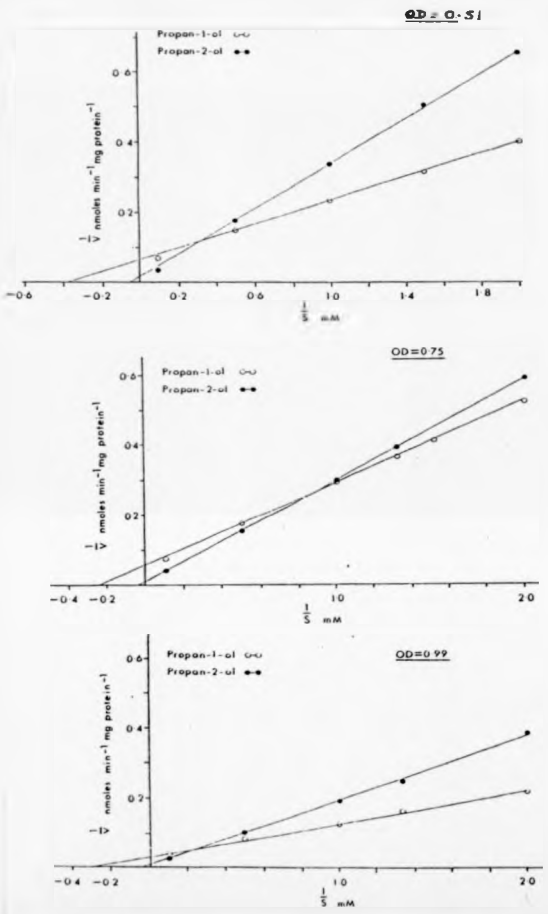
Isolate	GPY b1	
°C incubation	25°	
Cell morphology 6hrs ×630	 Cells are on nutrient broth + 0.75% agar, by phase contrast	
48hrs	Gram's Stain, ex glucose agar	
Gram	+	
Spores	—	
Motility	—	
Colonial morphology Glucose agar 7days.	round, regular, entire smooth, opaque, low convex yellow ± 1mm diameter	
°C. growth	37° — 44° —	
Catalase	+	
Oxidase, Kovacs	—	
O-F glucose	— No Change.	
First stage identification	223	

Table A.3 Biochemical characterization of isolates PNKb1 and BPSd1

Decomposition of:	PNKb1	BPSd1
Adenine	-	+
Tyrosine	+	+
Urea	-	+
Allantoin	-	+
Growth on sole C Sources:		
Glycerol	(+)	+
Sorbitol	2+	2+
Maltose	(+)	(+)
Trehalose	(+)	+
Benzoate	+	+
Citrate	(+)	(+)
Lactate	+	(+)
L-Tyrosine	+	(+)
ONPG	-	+
Tween 80	+	+
Phosphatase	+	+
growth at 10°C	(+)	+
growth at 40°C	-	-
growth at 5% NaCl	+	+
m-hydroxybenzoate	+	-
p-hydroxybenzoate	+	+
Acid from:		
Inositol	-	-
Trehalose	-	+
Mannitol	+	+
Sorbitol	+	+
Colour on		
glucose agar	orange pink	orange

APPENDIX 2

Lineweaver - Burk plots for data in Table 3.17.


OD = 0·51
Propan-1-ol
Propan-2-ol
0·6
0·4
0·2
−0·6
−0·2
0·2
0·6
1·0
1·4
1·8
1/V nmoles min⁻¹ mg protein⁻¹
1/S mM
OD=0·75
Propan-1-ol
Propan-2-ol
0·6
0·4
0·2
−0·4
−0·2
1·0
2·0
1/V nmoles min⁻¹ mg protein⁻¹
1/S mM
Propan-1-ol
Propan-2-ol
OD=0·99
0·6
0·4
0·2
−0·4
−0·2
1·0
2·0
1/V nmoles min⁻¹ mg protein⁻¹
1/S mM

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