THE GENETICS AND BIOCHEMISTRY OF A PROPANE-UTILIZING *RHODOCOCCUS RHODOCHROUS*

BY

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Dedication

For: Mam, Dad and Kate

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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.

M. Ashraf

Summary

The pathways of terminal and subterminal propane oxidation have been investigated in a propane-utilizing R.rhodochrous PNKb1. NTG-generated pleiotrophic mutants, characterized by their inability to utilize propane have been isolated. Several classes of mutants have been obtained which are unable to metabolize potential propane oxidation intermediates, e.g. propanol (alcA$^-$ or alcB$^-$), propanal (ald$^-$), acetone (ket$^-$), propanoate (oate$^-$) and acetate (ace$^-$). Only ket$^-$ mutants retained the ability to metabolize propane. Mutants defective in the first step of propane metabolism (alk$^-$), were also unable to metabolize acetol (a potential subterminal intermediate). Mutant analysis suggests that propane is oxidized via terminal and subterminal pathways in R.rhodochrous PNKb1. However, acetone (a potential subterminal intermediate) does not appear to have a role in propane metabolism.

A propane-specific 86 kDa NAD^+-dependent secondary alcohol dehydrogenase has been purified to homogeneity. This enzyme oxidizes a range of primary and secondary aliphatic alochols (C_2 to C_8). It is also responsible for both propan-1-ol and propan-2-ol dehydrogenase activities measured in cell-free extracts of propane-grown cells. Western-blot analysis has shown that it is induced during growth on propane, propan-2-ol, acetol and acetate (subterminal intermediates); but not propan-1-ol, propanal propanoate (terminal intermediates) or acetone. This technique has also demonstrated that a conserved NAD^+-dependent alcohol dehydrogenase was induced in Rhodococcus - Nocardia bacteria after growth on propane.

SDS-PAGE revealed proteins specific to cells grown on propane and acetol, which may be components of a novel propane/acetol oxygenase system. Oxygenase activity, as demonstrated by the epoxidation of propene, was induced after growth on propane and acetol. NADPH-dependent acetol oxygenase activity was also detected. These results suggest a relationship between the metabolism of propane and acetol.

Mutants unable to utilize propan-1-ol or propan-2-ol (alcA$^-$ and alcB$^-$ respectively) were examined by assaying for NAD^+-dependent propan-1-ol and propan-2-ol dehydrogenase activities, by using SDS-PAGE analysis of cell-free extracts and comparing the pattern and distribution of polypeptides with the wild-type, and by Western-blot analysis of the NAD^+-dependent secondary alcohol dehydrogenase synthesized by alc$^-$ mutants. Results demonstrated the alc$^-$ mutants had generally lower NAD^+-dependent alcohol dehydrogenase activities, altered polypeptide patterns and that alcB$^-$ mutants synthesized NAD^+-dependent secondary alcohol dehydrogenase which had altered electrophoretic mobility after non-denaturing PAGE. The latter result may explain the inability of these mutants to utilize propan-2-ol as a growth substrate.

The development of a plasmid transformation and gene transfer system for R.rhodochrous PNKb1 based on previously published methods has also been assessed.

Finally, a model for the pathway of propane oxidation in R.rhodochrous PNKb1 is also presented showing oxidation via terminal and subterminal carbon atoms.

Publications

Ashraf, W. & Murrell, J.C. (1990). Purification and characterization of a NAD^+-dependent secondary alcohol dehydrogenase from propane-grown Rhodococcus rhodochrous PNKb1. Arch. Microbiol., 153, 163-168.

Ashraf, W. & Murrell, J.C. (1990). Propane-specific alcohol dehydrogenase from Rhodococcus rhodochrous PNKb1. Methods in Enzymology (In Press).

Murrell, J.C. & Ashraf, W. (1990). Cell-free assay methods for enzymes of propane utilization. Methods in Enzymology (In Press).

Abbreviations

AMS	Ammonium mineral salts medium
ATP	Adenosine triphosphate
CoA	Coenzyme A
CMN-complex	*Corynebacterium*-*Mycobacterium*-*Nocardia* complex
DCPIP	Dichlorophenolindophenol
DEAE-cellulose	Diethylaminoethylcellulose
E'_o	Standard redox potential
FID	Flame ionization detector
GC	Gas chromatogrphy
HPLC	High performance liquid chromatography
id	Internal diameter
IEF	Isoelectric focusing
kb	Kilobase
kDa	Kilodaltons
K_m	Michaelis constant
$NAD(P)^+$	Nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) reduced form
NMR	Nuclear magnetic resonance
NTG	N-methyl-N-nitro-N-nitrosoguanidine
OD_{540nm}	Optical density at 540 nm
Pa	Pascals
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PMS	Phenazine methosulphate
PQQ	Pyrrolo quinoline quinone
rpm	Revolutions per minute
RMP pathway	Ribulose monophosphate pathway

SDS	Sodium dodecyl sulphate
TRIS	Tris (hydroxymethyl)-methylamine
UV	Ultra-violet
V_{max}	Maximum possible reaction velocity
v/v	Concentration, volume by volume
w/v	Concentration, weight by volume

Chapter 1: Introduction

INTRODUCTION

1.1 The Rhodococcus genus

In the current edition of Bergey's Manual of Systematic Bacteriology (Goodfellow, 1986) fourteen species of Rhodococcus are recognized and five taxa listed as species incertae sedis. The genus provides a niche for actinomycetes previously assigned to genera such as Arthrobacter, Brevibacterium, Corynebacterium, Mycobacterium and Nocardia (Goodfellow & Cross, 1984). The taxonomy of the Coryneform-Actinomycete group appears in a confused state. This is apparent by the appearance of synonyms, or the renaming of organisms in papers. For example, de Bont & Peck (1980) describe the same organism as both Nocardia and Rhodococcus; and also Nocardia paraffinium has been named as Rhodococcus rhodochrous (MacMichael & Brown, 1987). These inconsistencies in nomenclature makes comparative studies between these organisms difficult.

Rhodococci are soil bacteria noted for their ability to degrade toxic compounds such as acrylamide (Arai et al., 1981), aromatic compounds (Cain, 1981), insecticides (Larkin, 1988) and terpenes (Williams et al., 1989). Nocardioforms, including Rhodococcus, are one of the few groups of prokaryotes which can degrade lignin (Eggeling & Sahm 1980). They also have the ability to interconvert steroid compounds, e.g. converting cholesterol to substances which are precursors of steroid hormones or oral contraceptives (Ferreira et al., 1984). Although, perhaps more relevant to this study is the ability to degrade hydrocarbons (Jenkins et al., 1972; MacMichael & Brown, 1987 and German & Knowles, 1988). A review on the transformation of xenobiotics by actinomycetes, including Nocardia and Rhodococcus, is given by Peczynska-Czoch & Mordarski

(1984). In the diversity of their catabolic abilities they resemble pseudomonads, but they are less well studied, particularly their genetic systems. A general review on the genetics of the Nocardioform bacteria is given by Brownell & Denniston (1984). One report describes the development of a Rhodococcus - Actinophage gene cloning system which involved protoplasting and transfecting the Rhodococcus host (Brownell et al., 1982). Another report described the isolation of a bacteriophage from soil which mediated the transduction of a number of unlinked markers in Rhodococcus erythropolis. Under optimal conditions transduction to prototrophy of auxotrophic markers was over 50 times the spontaneous reversion rate (Dabbs, 1987). Plasmids have also been reported in Rhododoccus, a genetic approach was used to isolate plasmids in Rhodococcus erythropolis ATCC 12674 which carried genes for increased resistance to arsenate, cadmium and chloramphenicol (Dabbs & Sole, 1988). A system for the conjugative transfer of a 138 kb plasmid conferring cadmium resistance has been developed for Rhodococcus fascians (Desomer et al., 1988). A plasmid transformation system for Rhodococcus sp. strain H13-A has been developed using an Escherichia coli - Rhodococcus shuttle vector and heterologus gene expression was demonstrated (Singer & Finnerty, 1988). Finally the cloning and expression of Rhodococcus genes encoding pigment production in E.coli has been reported by Hill et al. (1989).

1.2 Alkane oxidation

Throughout the development of hydrocarbon microbiology, interest in n-alkane metabolism has always concentrated on methane oxidation and liquid n-alkane oxidation, probably due to their abundance and potential as feedstocks. By comparison 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). However, Perry (1980) points out that, "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".

Therefore, any study of higher gaseous alkane metabolism cannot be considered in isolation from methane or liquid alkane metabolism about which relatively more is known. However, it is not the intention here to give a comprehensive review of the physiology and biochemistry of C_1 and liquid alkane-utilizing organisms; but the reader will be referred to a number of relevant reviews and references.

1.2.1 Methane-oxidizing bacteria

Bacteria which oxidize methane belong to a group of organisms known as the C_1-utilizers, which are recognised by their ability to use compounds which are more reduced than carbon dioxide and contain no carbon-carbon bonds as sole carbon source for growth e.g. methane, methanol, N-methyl and S-methyl compounds. The term methylotroph was defined by Colby & Zatman (1973), and Quayle & Ferenci (1978), as those organisms that obtain their energy from the oxidation of C_1-compounds and assimilate carbon as formaldehyde or a mixture of formaldehyde and carbon dioxide. The term methanotroph has been applied to bacteria which obilgately utilized methane (and methanol) as sole carbon sources. The first well characterized methane utilizing bacterium was isolated in pure culture by Sohngen (Sohngen, 1906) and named Bacillus methanica. In 1956, Dworkin & Foster re-isolated Bacillus methanica and renamed it

Pseudomonas methanica (Dworkin & Foster, 1956). In the succeeding decade only three other new species of methanotroph were isolated and described in any detail (see Anthony, 1982).

There have been reports of facultative methanotrophs but Stephens (1983) surveys much of the evidence against their existence 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.

One of the main consequences of the isolation of over 100 strains of methanotroph by Whittenbury and his colleagues was that, a simple classification scheme was proposed which divided methanotrophs into Type I and Type II, based largely on internal membrane arrangements and cell shape (Davies & Whittenbury, 1970). Type I bacteria have bundles of disc-shaped vesicles which appear to be formed by invagination of the cytoplasmic membrane, while Type II bacteria have a system of paired membranes situated around the periphery of the cell. Methylococcus capsulatus (Bath) is an organism which represents a third major type of methanotroph which has been placed in a group known as Type X. A broad division into Types I, II and X is now generally accepted.

The fundamental understanding of microbial methane oxidation has improved drastically, according to Dalton & Leak (1985), in the last decade or so due to three important reports. Firstly, Whittenbury et al. (1970) demonstrated that it was possible to isolate, in pure culture, a wide range of methanotrophs from many habitats. The second report was by Ribbons & Michalover (1970), who were the first to demonstrate that extracts of Methylococcus capsulatus (Texas) could be

prepared which retained methane-oxidizing activity and that such extracts required NADH, oxygen and methane for activity. The third finding was reported by Colby et al. (1977), that the enzyme responsible for the conversion of methane to methanol, methane monooxygenase (MMO), was able to insert an atom of oxygen into a wide range of organic compounds.

The oxidation of methane to carbon dioxide appears to proceed via a series of two electron oxidation steps; resulting in the oxidation of methane to methanol, formaldehyde, formate and then carbon dioxide. The evidence for this pathway has been extensively reviewed by several workers (Anthony, 1982; Dalton & Leak, 1985; Anthony, 1986).

Studies carried out on a number of methane and methanol oxidizing bacteria have shown the presence of a broad specificity NAD(P)-independent methanol dehydrogenase (reviewed in Anthony, 1986). It has been established that the methanol dehydrogenase is a quinoprotein with a pyrrolo quinoline quinone (PQQ) prosthetic group (Duine & Frank, 1980). PQQ has also been found in a number of other dehydrogenases e.g. glucose dehydrogenase (Duine et al., 1979).

Formaldehyde produced by oxidation of methanol can be either assimilated into biomass or dissimilated by complete oxidation to carbon dioxide. The latter involves the enzyme formaldehyde dehydrogenase of which two forms exist, an $NAD(P)^+$-dependent and a $NAD(P)^+$-independent form. An $NAD(P)^+$-dependent formaldehyde dehydrogenase was purified from M.capsulatus(Bath), the formate formed, is further oxidized by a NAD^+-dependent formate dehydrogenase to carbon dioxide (Stirling & Dalton, 1978).

Carbon for the biosynthesis of cellular material is diverted from the methane oxidation pathway at the oxidation stage of formaldehyde and is assimilated either via a ribulose monophosphate (RuMP) pathway or a serine pathway. The pathway used, can be correlated with the type of membrane system observed, i.e. Type I organisms used the RuMP pathway and Type II organisms use the serine pathway, facultative methylotrophs also use the serine pathway. The biochemistry of the methanotroph carbon assimilation pathways has been extensively reviewed (Quayle, 1980; Anthony, 1982).

Information on the genetic analysis and manipulation of a range of C_1-utilizing microorganisms has accumulated steadily (for reviews see Lidstrom, 1983; Listrom et al., 1987; Holloway et al., 1987). The genetics and molecular biology of the obligate methanotrophs has been reviewed by Cardy (1989).

Finally workers have been interested in the "applied" or biotechnological aspects of methylotrophy, the most notably being the production of SCP from petroleum feedstocks. For a review of potential applications see Large & Bamforth (1988).

1.2.2 Liquid n-alkane metabolism

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 & 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 & Gutnick, 1981), these authors also listed the following genera as being the most frequently isolated in hydrocarbon enrichments: Pseudomonas, Acinetobacter, Flavobacterium, Brevibacterium, Corynebacterium, Arthrobacter, Mycobacterium and Nocardia.

A problem encountered with liquid n-alkanes (but not gaseous alkanes) as growth substrates is that of solubility. Whereas gaseous alkanes form truly soluble aqueous concentrations of 0.2-2 mM which, according to Watkinson (1980), are enough to support observed growth rates, solubility of the liquid n-alkanes from hexane to hexadecane falls from 0.1 mM to 0.3 μM (Bell, 1973). Watkinson (1980) defines soluble as meaning that individual hydrocarbon molecules are solvated and are transported into the cell as individual molecules. To metabolize liquid n-alkanes of low solubility, two mechanisms have been proposed. One involves direct contact between microorganisms and the hydrocarbon and the other involves the production of specific agents that cause the dispersion of hydrocarbons into micelles which are subsequently taken up. Both mechanisms are discussed in reviews by Ratledge (1978), Watkinson (1980) and Britton (1984). For a review of the various aspects of petroleum microbiology see Atlas (1984).

1.2.2.1 Pathways of liquid n-alkane oxidation

(a) Terminal oxidation: It is generally accepted that mono-terminal oxidation of short and long chain n-alkanes is the main route of assimilation. The alkane is converted to the corresponding primary alcohol, aldehyde and then fatty acid (Fig 1.1). Fatty acids are then

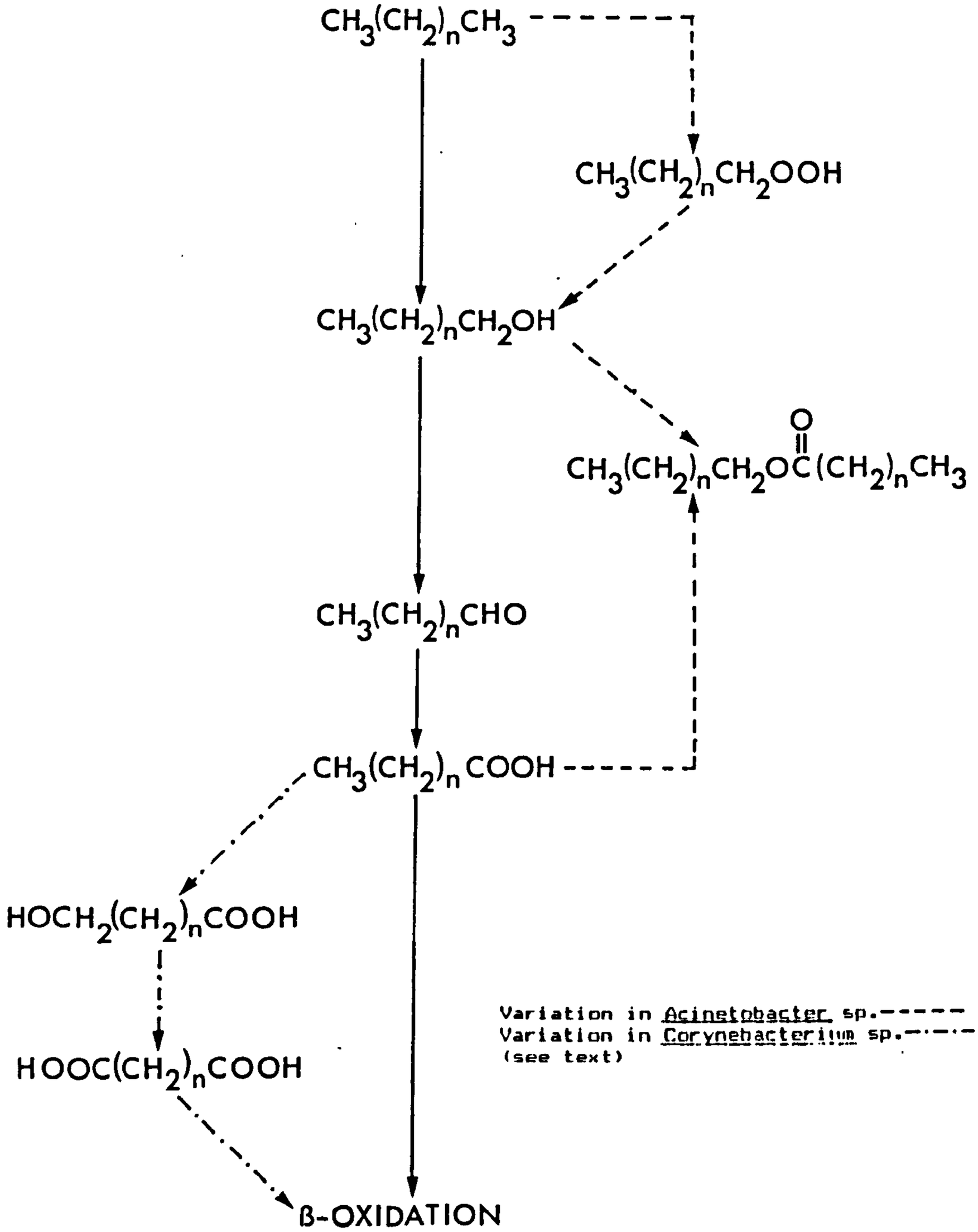


Figure 1.1 Terminal oxidation of n-alkanes

oxidized in a heterotrophic manner via β-oxidation to the level of acetate (or propanoate for odd chain length n-alkanes). Much of the evidence for terminal oxidation is circumstantial, being based on techniques such as simultaneous adaptation and analysis of excreted metabolites. Biochemical and genetic data is lacking in such studies.

The exception to the above is the work initiated in the early 1960's on hydrocarbon oxidation by Pseudomonas. The first direct evidence that terminal oxidation occurred was 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 & Jurtshuk (1968) working with cell-free extracts of Corynebacterium sp. 7E1C.

The isolation of dicarobxylic acids from a Corynebacterium sp. grown C_{10}-C_{14} n-alkanes was thought to be consistent with diterminal oxidation (Kester & Foster, 1963), see Fig. 1.1.

The isolation of esters from culture supernatants of Acinetobacter sp. H01-N grown on alkanes and the fact that growth on alkyl hydroperoxides produced the same esters has lead to the suggestion that hydroperoxides are intermediates of n-alkane oxidation and the postulated pathway is shown in Fig. 1.1 (Stewart et al., 1959).

For a recent review of the monoterminal and diterminal oxidation pathways of alkanes to fatty acids see Buhler & Schindler (1984).

(b) Subterminal oxidation: The fact that subterminal oxidation of liquid n-alkanes occurs is beyond doubt. However, the relative importance of this pathway biologically is open to speculation.

Studies with Pseudomonas aeruginosa growing on decane (Fredricks, 1967) and Mycobacterium smegmatis grown on propane, butane, pentane and hexane (Lukins & Foster, 1963) showed the production of excreted secondary alcohols and their corresponding ketones.

Markovertz (1971) has reviewed the subject of subterminal oxidation of aliphatic hydrocarbon by microorganisms. He showed that Pseudomonas aeruginosa grown on n-tridecane produced tridecan-2-ol, tridecan-2-one, undecyl acetate, undean-1-ol and acetate. It was suggested that tridecan-2-one was cleaved at the keto group by a biological 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 (Fig. 1.2).

For a recent review of subterminal oxidation pathways see Buhler & Schindler (1984).

1.2.2.2 Anaerobic oxidation of n-alkanes

The postulated pathway involves the dehydrogenation of the alkane to alkene, which is then hydroxylated to the primary alcohol.

There have been a number of arguments raised against the metabolism of n-alkanes anaerobically via the formation of alkenes as intermediates (see McKenna & Kallio, 1965; Klug & Markovertz, 1971).

Abbott & Casida (1968) postulated that double bond insertion represented an early step in the pathway of n-alkane degradation. However, much of their evidence relied on suspensions of glucose-grown resting cells of Nocardia salmonicolor PSU-N-18 oxidizing hexadecane to a mixture of

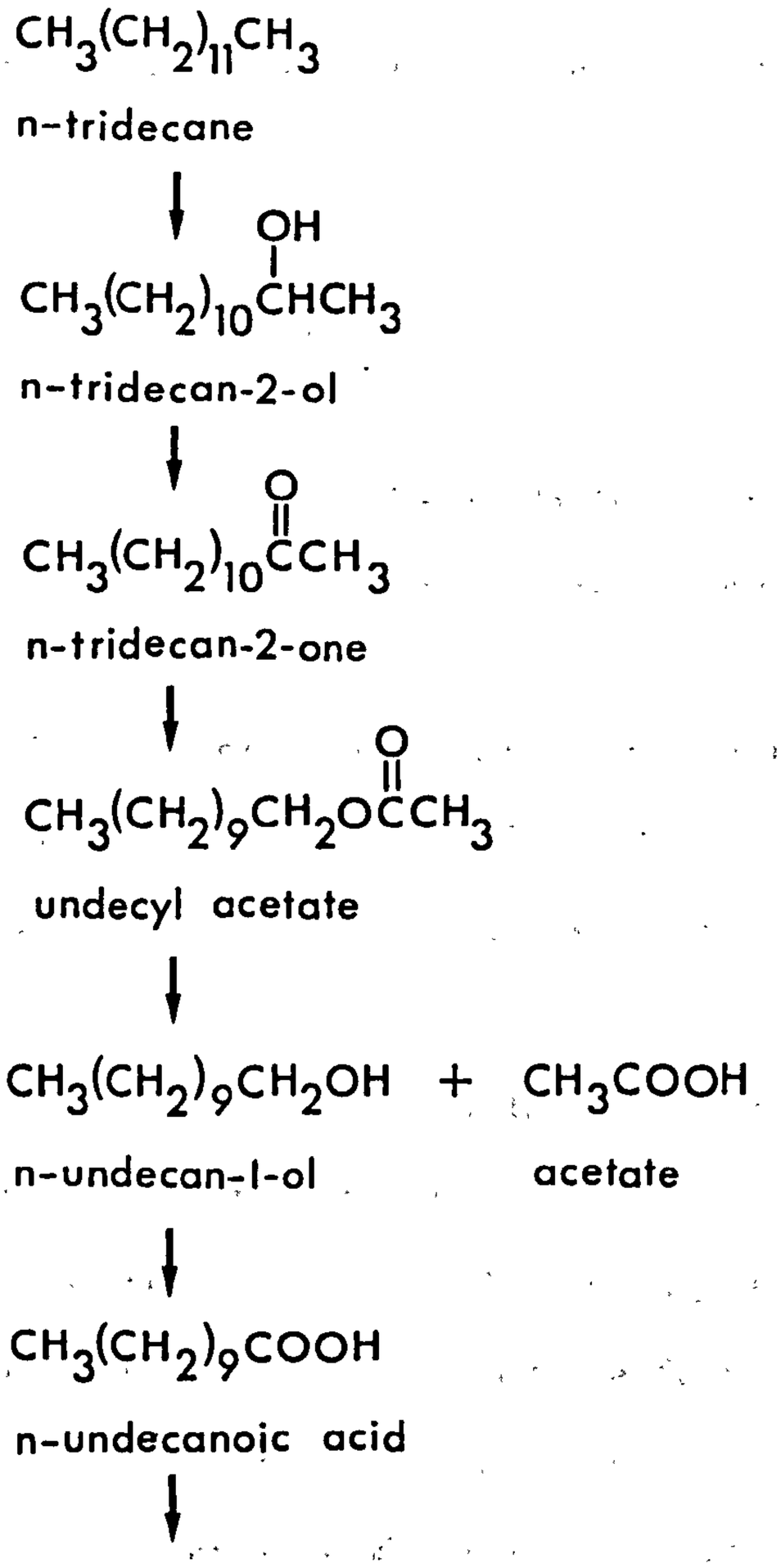


Figure 1.2 Pathway of subterminal oxidation of n-tridecane in Pseudomonas spp. (adapted from Klug & Markovetz, 1971).

internal monohexadecenes. A more credible report was given by Parekh et al. (1977) who isolated from a Pseudomonas sp., growing anaerobically on hexadecane with nitrate as terminal electron acceptor, an NAD^+-dependent alkane dehydrogenase and an NADPH-dependent alkene hydroxylase. Both enzymes were partially purified and the dehydrogenase produced dec-1-ene from decane and the hydroxylase decan-1-ol from decene. However, this organism may represent a special case since it grew poorly on decane aerobically.

1.2.2.3 Mechanisms of n-alkane oxidation

Three mechanisms have been proposed for the oxidation of n-alkanes to their corresponding alcohols and they are summarised below:

(1) $RCH_2CH_3 + O_2 \rightarrow RCH_2CH_2OOH + NAD(P)H + H^+ \rightarrow RCH_2CH_2OH + NAD(P)^+ + H_2O$

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

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

Equation 1 describes the formation of alkyl hydroperoxides as intermediates of n-alkane oxidation. Indirect evidence for this mechanism came from work by Stewart et al. (1959) on Acinetobacter sp. HO1-N grown on hexadecane. Their data indicated the formation of cetyl palmitate from the esterification of cetyl alcohol and palmitic acid produced from 1-hexadecyl-hydroperoxide which was suggested as the first intermediate in the oxidation of hexadecane. These compounds were detected as excreted products in culture supernatants. Cell-free extracts also degraded alkyl hydroperoxides and there was some evidence that NADH-dependent alkyl hydroperoxide reductase was present. In Pseudomonas, hydroxylation of hydrocarbons and fatty acids is catalyzed by three proteins, an alkane hydroxylase, rubredoxin and rubredoxin

reductase (see section 1.4.1.2). The latter two components are an iron-sulphur redox protein and a flavoprotein reductase, which in the absence of the hydroxylase can promote NADH-dependent reduction of a wide range of alkyl hydroperoxides and it was speculated that these may be intermediates in the conversion of alkane to alcohol (Peterson et al., 1966; Boyer et al., 1971). Recently an alkyl hydroperoxide reductase from Salmonella typhimurium has been purified (Jacobson et al., 1989) and the gene, ahp, cloned and characterized (Storz et al., 1989). The enzyme was shown to play a role in protecting against alkyl hydroperoxide mutagenesis and the ahp genes encoded two proteins (F52a and C22) that make up the alkyl hydroperoxide reductase. It is interesting to speculate whether the oxidation of n-alkanes, at least in some cases, is a fortutious consequence of an organism's ability to resist the deleterious effects of oxidative damage to DNA.

The second mechanism (equation 2) was a dehydrogenation proposed by Senez & 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. However, as already stated there have been a number of arguments raised against this mechanism (see Klug & Markovetz, 1971) particularly the thermodynamic unfeasibility of converting an alkane to an alkene (McKenna & Kallio, 1965).

The direct incorporation of one atom of molecular oxygen into the alkane molecule catalyzed by a mixed-function oxygenase (monooxygenase) is described by equation 3. This is the most accepted mechanism for alkane oxidation. Two systems are known which involve either cytochrome P-450 or rubredoxin, but in neither case has the reaction mechanism been

worked out unambiguously (for review see Buhler & Schindler, 1984). Equations 1 and 3 have the same overall stoichiometry and would both give the expected results from $^{18}O_2$ labelling studies. Klug & Markovetz (1971) suggested that in the hydroperoxidation mechanism NAD(P)H would have the addition function of donating electrons to form an active oxygen species for the formation of the alkyl hydroperoxide. However, they stated that this would be contrary to the 1:1:1: ratio of O_2:NADH:octane observed with octane monooxygenase by Peterson et al. (1969) since; NADH would be required to donate electrons for the formation of an active oxygen species and to reduce the hydroperoxide to the alcohol.

The initial reaction mechanisms of alkane oxidation are unclear. However, most evidence points to molecular oxygen as an obligatory reactant. It is not known whether oxygen or the alkane is activated to allow the formation of alcohol. Britton (1984), Singer & Finnerty (1984) and Buhler & Schindler (1984) have reviewed the subject.

1.3 Pathways of C_2-C_4 gaseous n-alkane metabolism

Woods (1988) surveys much of the literature concerned with the species involved in gaseous alkane oxidation including filamentous fungi, yeasts and bacteria. The sources of gas, occurrence of organisms in nature and possible uses of these organisms were also discussed. Of particular note with regard to the species of bacteria involved in n-alkane oxidation is the frequent isolation of the Corynebacterium-Mycobacterium-Nocardia (CMN) group of organisms in n-alkane enrichments. The reasons for this was speculated upon by Stephens & Dalton (1987), although their arguments were aimed primarily at liquid alkane utilizers some points may be applicable to gaseous alkane utilizers too. Firstly there is the presence of a highly hydrophobic cell surface on these organisms which would facilitate the diffusion of a hydrophobic substrate into the organism. Secondly, these organisms are known to produce surfactants to aid the dispersion of hydrophobic substrates, although this is probably less significant with gaseous alkane substrates more soluble in water than liquid alkanes. Finally, the cell wall biochemistry of these organisms may endow them with an enzyme complement well suited to handling n-alkanes substrates, for example the possession of oxygenase enzymes.

Only one well documented case exists for the isolation of a Gram-negative propane-utilizer, Takahasi et al. (1980) described in some detail the isolation of a Pseudomonas butanovora capable of growth on n-alkanes from C_2-C_9. However, Stephens (1983) and Woods (1988) only observed Gram-positive organisms when isolating propane-utilizers.

1.3.1 Pathways

When compared with the oxidation of methane and liquid n-alkanes the pathways of ethane, propane and butane oxidation have received scant attention, and what reports there are on the subject are not unambiguous.

As previously discussed it would appear that liquid n-alkanes can be metabolised by both terminal and subterminal pathways. Methane-oxidizing bacteria when co-oxidising higher n-alkanes produce a mixture of terminal and subterminal oxidation products (Dalton, 1980). Therefore, it might be reasonable to suppose that either (or both) terminal or subterminal pathways operate in the metabolism of gaseous alkane-utilizers.

Much of the evidence for the routes of oxidation of gaseous alkanes is based on simultaneous adaptation studies (as described by Stanier, 1947) and the analysis of products in culture supernatants. Neither of these techniques have offered firm evidence for the existence of proposed pathways. The limitations of such techniques have been discussed by Dagley & Chapman (1971). When using the technique of simultaneous adaptation they point out that a compound may be a reaction intermediate and yet may not be oxidized by whole cells because of the permeability barrier. Also, a compound may be rapidly oxidized when it is not an intermediate in the proposed sequence because the enzymes involved in its metabolism may be constitutive. 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 given before assigning it a role as a possible intermediate, because of its rapid removal as well as formation. They also stipulate that the best

criteria to support the role of a compound as an intermediate in degradation are those gained by a study of enzymes isolated from the organism. The following discussion shows a lack of a biochemical and genetic information when dealing with gaseous n-alkane metabolism.

1.3.2 Ethane

The metabolism of ethane has not been studied in great detail, primarily because it is such a small symmetrical molecule it has been assumed that it is oxidized by a monooxygenase to ethanol, then to acetaldehyde and then to acetate, for review see Vestal (1984).

Davis et al. (1956) isolated a Mycobacterium paraffinicum which appeared to have an obligate requirement for n-alkanes or their potential intermediates. It grew well on ethane and poorly on ethanol and acetate, as well as C_3-C_{10} n-alkanes. Ethane-grown cells were also able to oxidize propane and butane. They also oxidized ethene, ethanol and acetaldehyde and acetate and this was postulated to be the pathway for ethane oxidation. Although the authors suggested that ethene was an intermediate of ethane oxidation, the relatively slow oxidation rate observed makes this unlikely.

Dworkin & Foster (1958) isolated a number of ethane-oxidizing mycobacteria. Preliminary attempts were made to study the intermediate steps in the oxidation of ethane. However, these were unsuccessful due to the lack of obtaining enzymatically active cell-free extracts. Also the constitutive nature of ethane oxidation precluded the use of simultaneous techniques. The problems of obtaining active and/or stable cell-free extracts are not unique, both Stephens (1983) and Woods (1988) encountered such problems with their work on propane-utilizing bacteria.

However, all of Dworkin & Foster's strains oxidized ethanol and ethandiol. Acetaldehyde and acetate were oxidized slowly and only one strain oxidized ethene. They were careful to stress that these studies neither established nor precluded the involvement of any of these compounds in ethane metabolism.

The metabolism of two-carbon gaseous hydrocarbons also includes ethylene and acetylene and their bacterial oxidation has been reported by members of the CMN-complex. However, details will not be discussed here, instead the reader is referred to Vestal (1984).

1.3.3 Propane

As the hydrocarbon chain length increases to three, there is the possibility of two oxidation mechanisms. The possible site(s) of oxidation on the molecule can be in either the terminal (w-) and/or the central or subterminal carbon atom. The question of whether propane oxidation proceeds via the terminal, subterminal or indiscriminate oxidation has not been resolved. The majority of opinion in the literature favours subterminal oxidation as the route of propane oxidation. This opinion stems mainly from the work by J.J. Perry *et al.*, although much of their evidence is circumstantial being based on simultaneous adaptation studies and analysis of products in culture supernatants (see 1.3.1).

Fig. 1.3 shows a proposed pathway for the terminal oxidation of propane *via* propan-1-ol, propanal and propanoate. Propanoate could be then oxidized to CO_2 by a number of enzymes involved in heterotrophic metabolism as shown in Fig. 1.4. Two possible variations on the subterminal pathway are shown in Figs 1.5 and 1.6, both envisage the

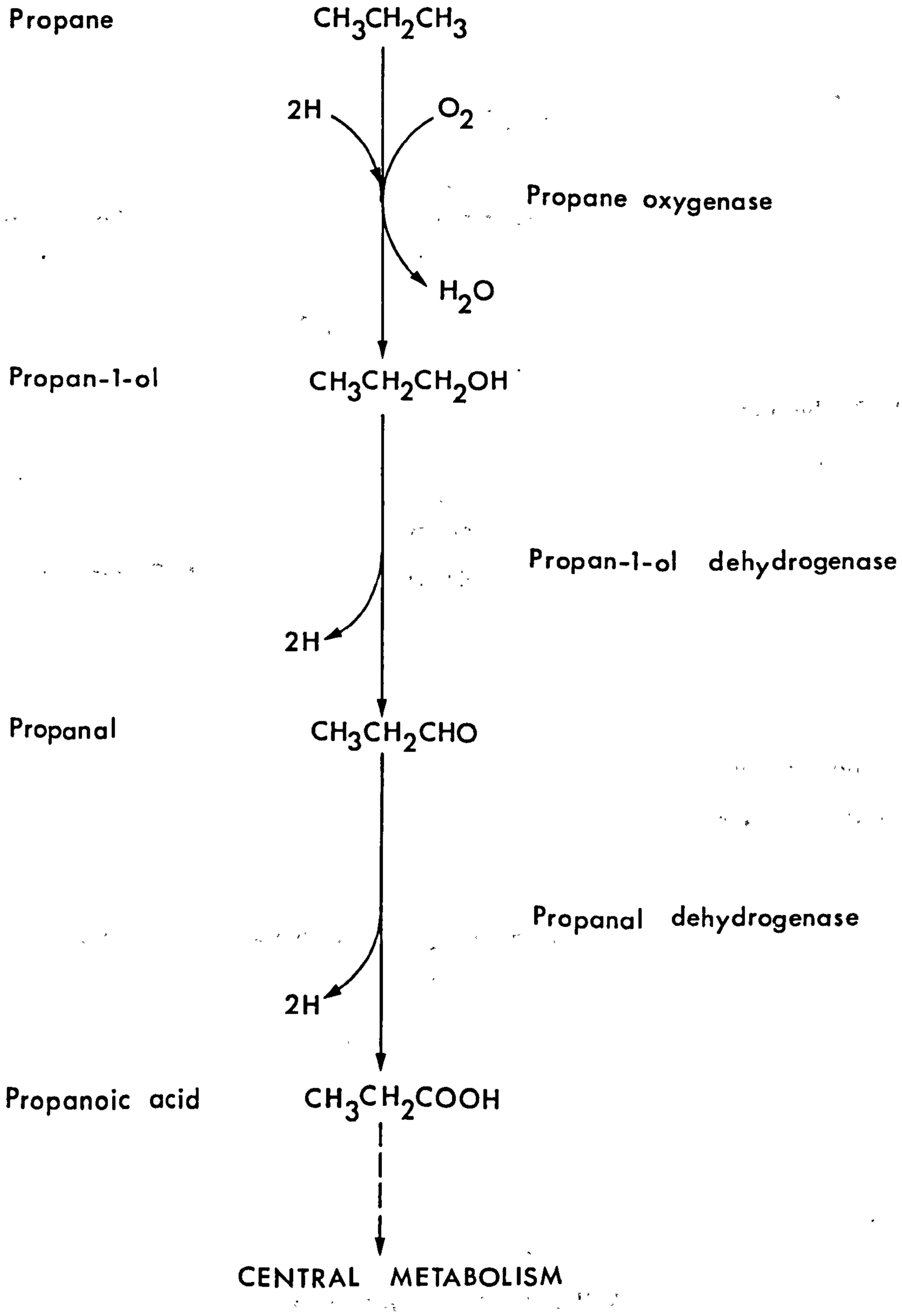


Figure 1.3 Terminal oxidation of propane

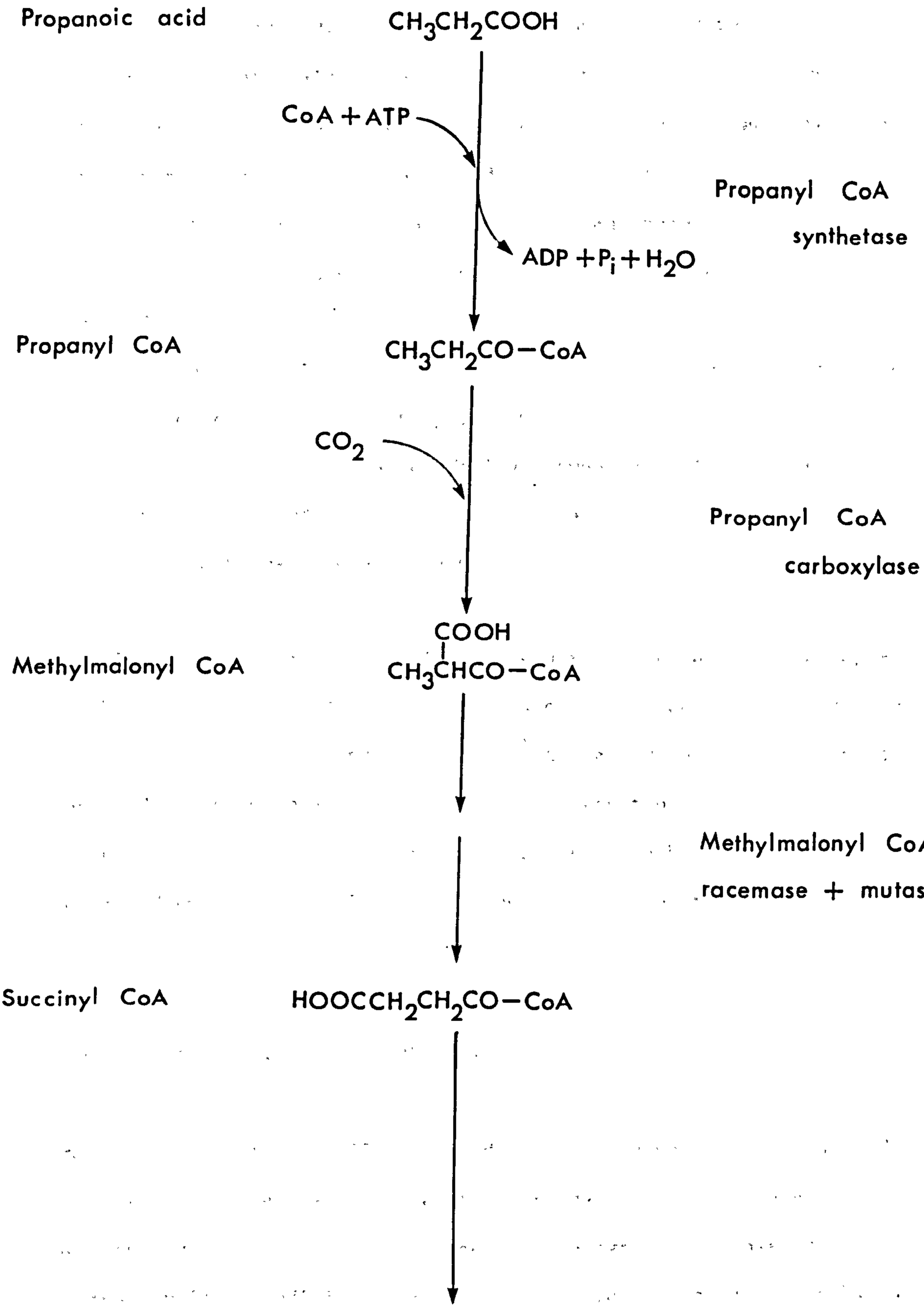


Figure 1.4 Methylmalonate pathway of propanoate metabolism

oxidation of propane *via* propan-2-ol and then to acetone. Acetone may then undergo a biological Baeyer-Villiger type reaction to form methylacetate which could be hydrolyzed to methanol and acetate (Fig. 1.5); or alternatively acetone could undergo further terminal oxidation to form acetol (Fig. 1.6). Both situations may require the participation of an oxygenase enzyme. The fate of acetol is shown in Fig. 1.7:

(i) it could be oxidized by a dehydrogenase to pyruvate via pyruvic aldehyde (see Taylor *et al*., 1980)

(ii) it could undergo a Baeyer-Villiger type oxidation to form acetate and formaldehyde *via* an unstable intermediate hydroxymethylacetate (see Hartmans & deBont, 1986).

In relation to propane metabolism it is worth considering some reports on the metabolism of propan-1,2-diol, acetone and acetol which will be discussed in the following sections. Although propan-1,2-diol is not reported to be an intermediate of propane oxidation, inclusion in this discussion is justified because it is reported to be metabolized *via* acetol (Goepfert, 1941; Levine & Krampitz, 1952; Hartmans & deBont, 1986).

1.3.3.1 Propan-1,2-diol metabolism

In *Flavobacterium* sp. NCIB 11171 under aerobic conditions propan-1,2-diol was exclusively metabolised to lactaldehyde by a propandiol oxidase, subsequently it was metabolised to pyruvate. However, under microaerophilic conditions some propan-1,2-diol was catabolized by an inducible diol dehydrase to propanal and then reduced to propan-1-ol (Willetts, 1979). Later work showed the presence of an inducible catalase from diol-grown cells (Willetts, 1983). Bolbot & Anthony

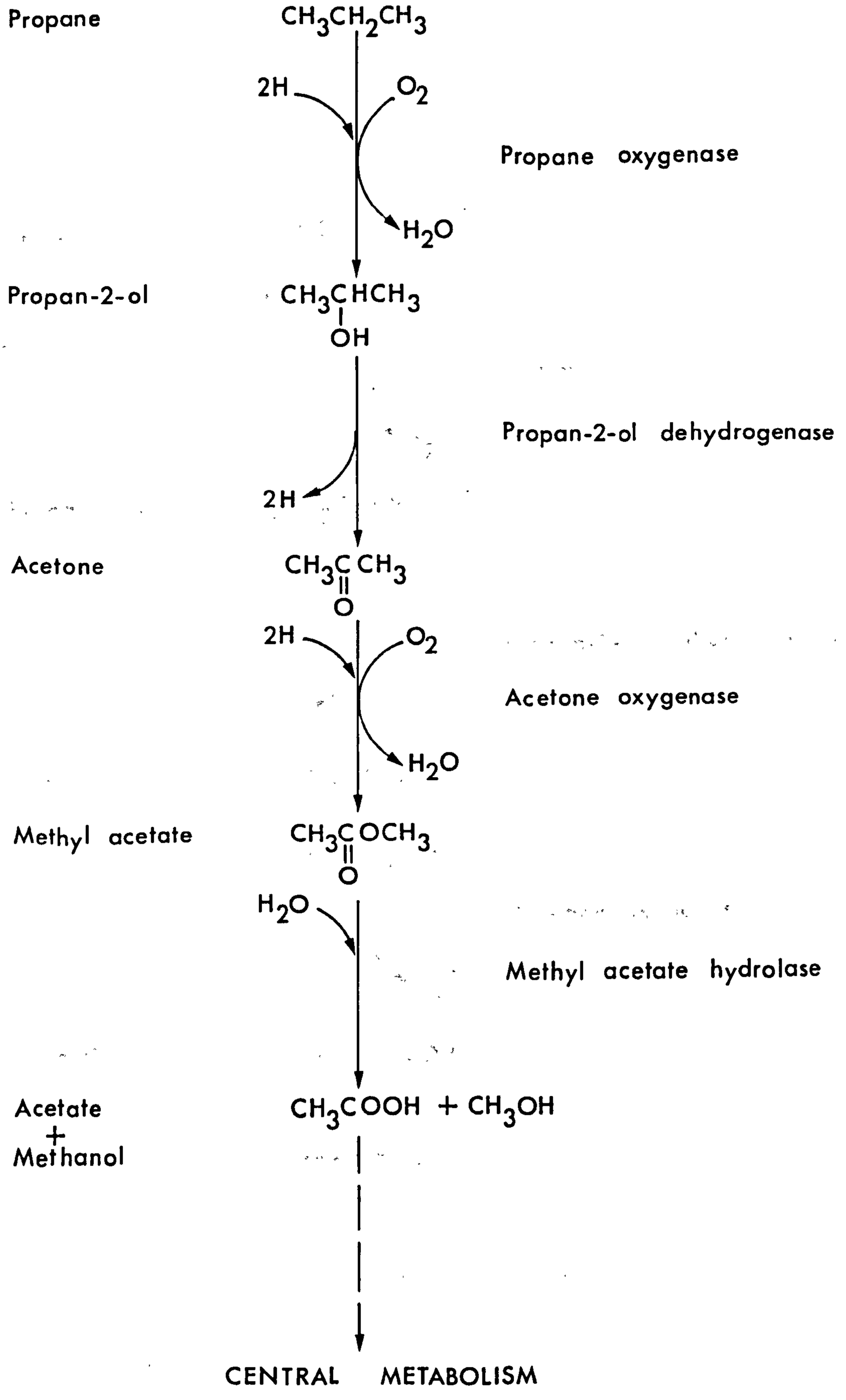


Figure 1.5 Subterminal oxidation of propane (via methylacetate)

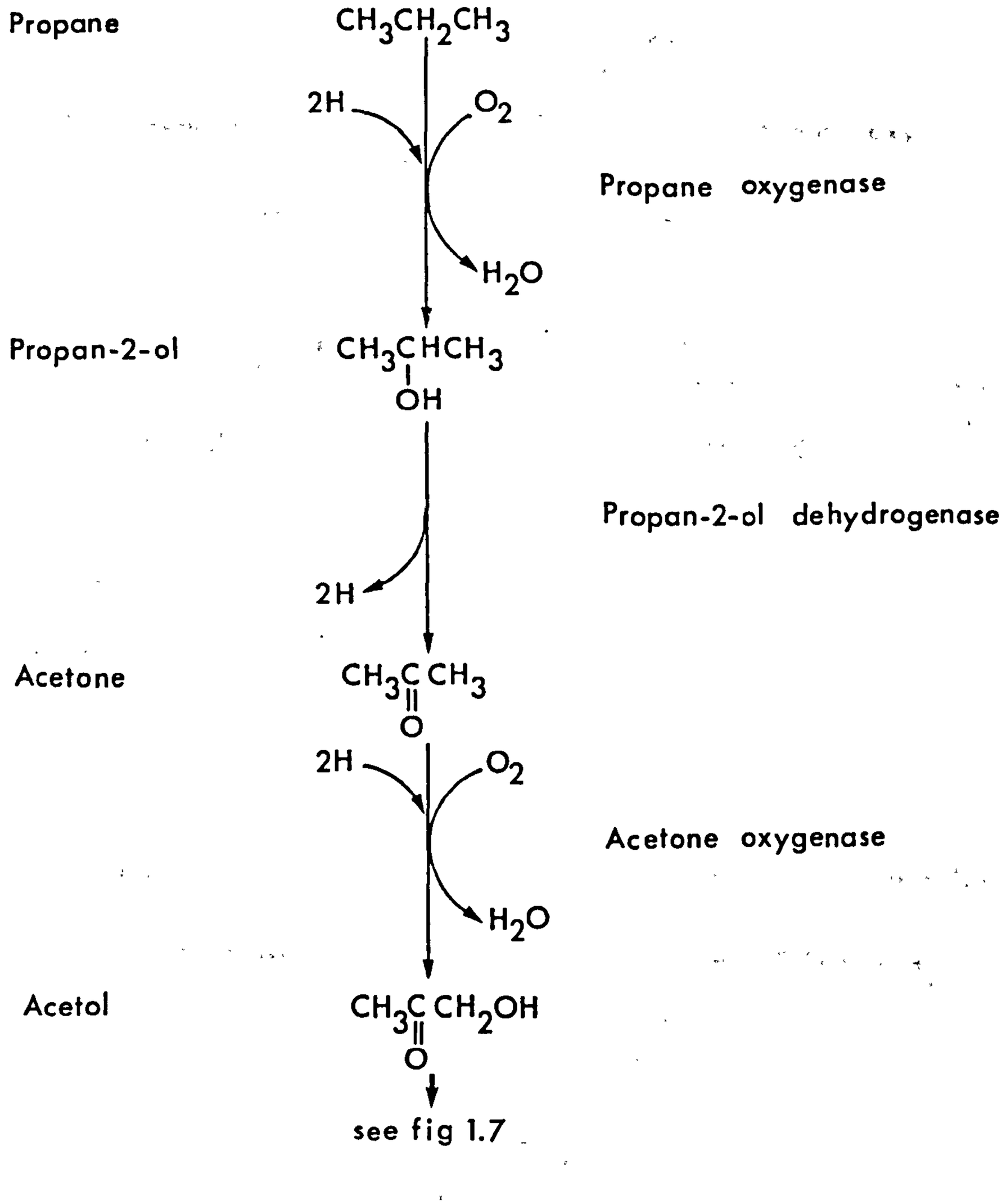


Figure 1.6 Subterminal oxidation of propane (via acetol)

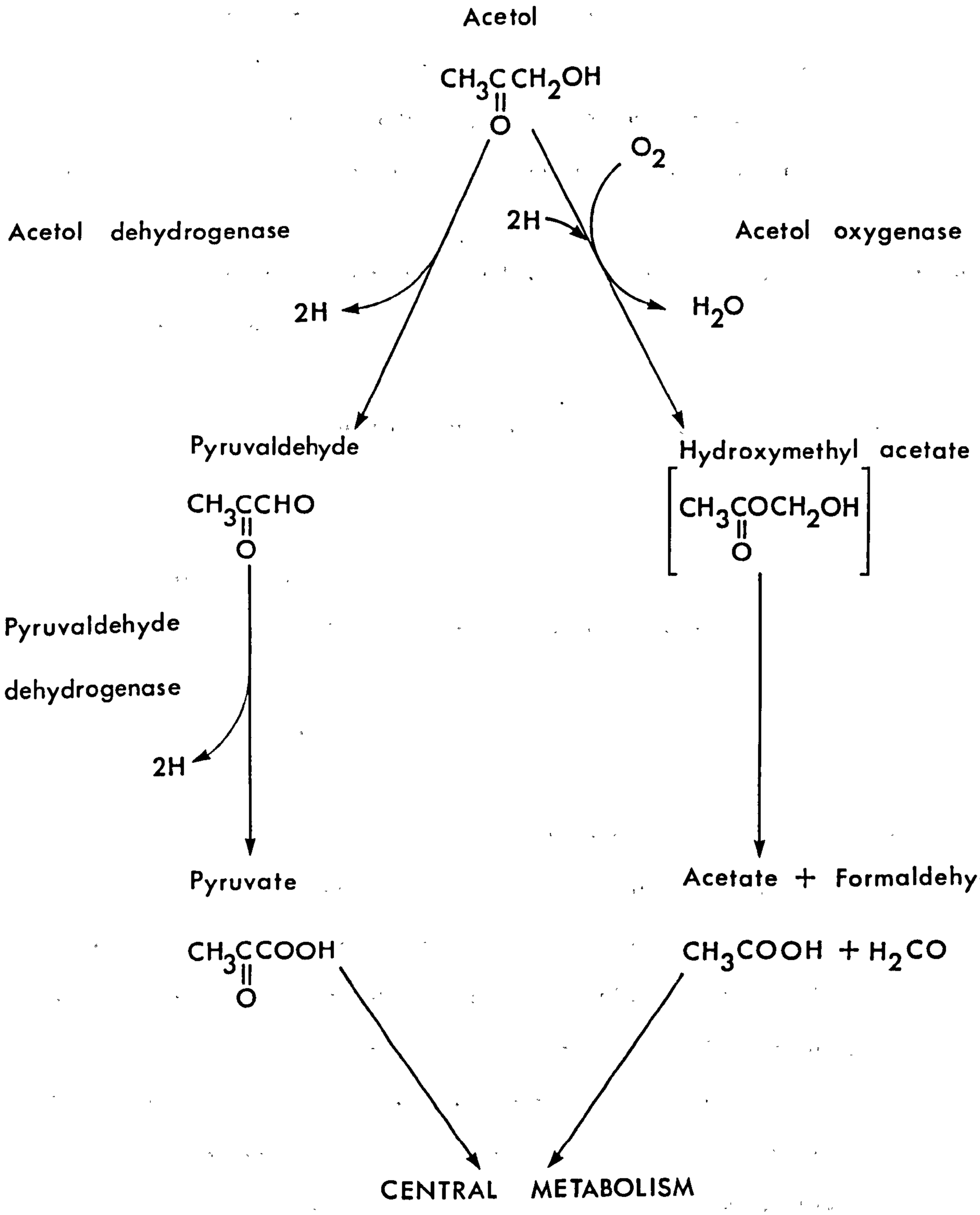


Figure 1.7 Pathways of acetol metabolism

(1980) reported that propan-1,2-diol was oxidised by methanol dehydrogenase in the presence of a large molecular weight protein. This "stimulatory factor" seemed to alter the substrate specificity of the enzyme, the oxidation product of propan-1,2-diol was lactate. This "stimulatory factor" or modifer protein (M-protein) was later purified by Ford et al. (1985) and shown to consist of two proteins:

(i) the M-protein which increased affinity of methanol dehydrogenase for propan-1,2-diol

(ii) a dye-linked aldehyde dehydrogenase which oxidized lactaldehyde to lactate.

These reports serve to highlight the other possibilities for the metabolism on propan-1,2-diol other than via acetol.

1.3.3.2 Acetone metabolism

Levine & Krampitz (1952) suggested that acetol was an intermediate in the oxidation of acetone. Results showed that acetol was further metabolized via a C_2-C_1 cleavage in which acetaldehyde was formed. They also proposed that acetone and propan-1,2-diol was metabolized through acetol. Mycobacterium smegmatis 422 produced homologous ketones during the oxidation of propane, butane, pentane and hexane (Lukins & Foster, 1963). An oxygenase reaction was postulated for the attack of methyl ketones, acetol being isolated as the oxidation product from acetone. They also proposed a possible diterminal oxidation analogous to that reported by Kester & Foster (1963) for the further metabolism of acetol to produce the glycolytic intermediate dihydroxyacetone. Studies on the metabolism of acetone, propan-2-ol and propane have indicated that the

C_3 skeleton was cleaved to yield organic C_1 and C_2 fragments before the entry of carbon into central metabolism (Goepfert, 1941; Levine & Krampitz, 1952; Vestal & Perry, 1969). However, while acetol has been implicated as an intermediate in the degradation of acetone, the mechanism of formation and further metabolism remained unclear.

However, Taylor et al. (1980) outline another route of metabolism by which acetone is converted to pyruvate. The reaction sequence was proposed as follows: propan-2-ol → acetone → acetol → methylglyoxal → pyruvate, see Figs 1.6 and 1.7. The latter two reactions were catalyzed by inducible acetol and methylglyoxal dehydrogenases respectively.

Contrary to nearly all acetone-degrading isolates described so far (Levine & Krampitz, 1952; Lukins & Foster, 1963; Vestal & Perry, 1969; Taylor et al., 1980; Coleman & Perry, 1984) the following reports concern Gram-negative acetone-utilizers. These reports describe the anaerobic metabolism of acetone, which occurs by carboxylation to acetoacetate, which was then polymerized to poly-β-hydroxybutyrate (PHB) (Platen & Schink, 1987; Bonnet-Smits et al., 1988; Platen & Schink 1989a).

Recently, Platen & Schink (1989b) reported the presence of an ATP- and $MgCl_2$-dependent acetone carboxylating enzyme in cell-free extracts of isolate BunN.

1.3.3.3 Acetol metabolism

Goepfert (1941) reported that after Fusarium sp. was grown on propan-2-ol, propan-1,2-diol and acetone; formaldehyde and acetol were detected. During growth of Mycobacterium vaccae JOB5 on propane, acetol was formed

and then cleaved to acetate and CO_2 (Vestal & Perry, 1969). This was later modifed by Coleman & Perry (1984) when they proposed that the C_1 moiety was metabolized *via* the reduced C_1 pool. Much of the work on acetol metabolism lacked any biochemical analysis. However; Hartmans & deBont (1986) reported a cytochrome P-450 type acetol monooxygenase in *Mycobacterium* Py1. The enzyme activity was only detected in propan-1,2-diol and acetol-grown cells; acetate and formaldehyde were the reaction products. The formaldehyde formed could possibly be metabolized by a formaldehyde dehydrogenase as described by Eggeling & Sahm (1985), see 1.4.3.2.

1.3.3.4 Propane metabolism

Much of our understanding of propane metabolism has come from the work of J.J. Perry *et al*. with propane-utilizing mycobacteria, *M.vaccae* JOB5, *M.smegmatis* and *M.convolutum* R-22. The notion that subterminal oxidation of propane is the major route of assimilation has come from conclusions arising out of their work, however, their conclusions have been criticized (Stephens, 1983; Woods, 1988). Perry (1980) has reviewed the subject of propane- utilization by microorganisms.

1.3.3.4.1 The work of Perry et al.

Perry (1968) investigated the substrate specificity of three propane-utilizing soil bacteria, *M.vaccae* JOB wás studied in the most detail. Respirometric studies were performed using a Warburg apparatus to measure oxygen uptake by resting cell suspensions. Propan-2-ol and acetone were adapted for propane oxidation and this was taken to implicate these two compounds as intermediates in propane oxidation. However, the possibility exists that these compounds may require an

oxygenase for their metabolism (as previously shown) which could oxidize the propane molecule fortuitously. Propane-grown M.vaccae JOB5 was also capable of oxidizing propan-1-ol and propanoate at rates of 0.27 and 0.099 μl O_2 min^{-1} mg dry $weight^{-1}$ respectively, whereas acetone was only oxidized at a rate of 0.023 μl O_2 min^{-1} mg dry $weight^{-1}$. However, the rate of acetone oxidation by propan-2-ol-grown cells was 0.4 μl O_2 min^{-1} mg dry $weight^{-1}$. As already pointed out by Woods (1988) these results are inconsistent with acetone as a potential intermediate of propane oxidation.

Vestal & Perry (1969) using the same organism performed ^{14}C radiotracer experiments which they suggested showed subterminal oxidation of propane. The presence of isocitrate lyase in cells grown on propan-2-ol, acetate or propane and the absence of this enzyme in propan-1-ol and propanoate-grown cells suggested to them that propane was not metabolized *via* terminal oxidation. They stated that metabolism does not involve pyruvate as an intermediate, however, Kornberg (1966) showed that pyruvate does not repress isocitrate lyase activity in all species. Also 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 this enzyme, for example *via* malonic semialdehyde. These pathways may be induced by growth on propane but not on propanoate, when the methylmalonate pathway is in operation (Fig. 1.4).

Further experiments using the pyruvate carboxylase inhibition sodium arsenite gave results which they suggested showed propane and propan-2-ol were not being metabolised *via* pyruvate. This in combination with the isocitrate lyase results suggested that propane was being metabolised *via* acetate (Fig. 1.8). However, Stephens (1983) raised

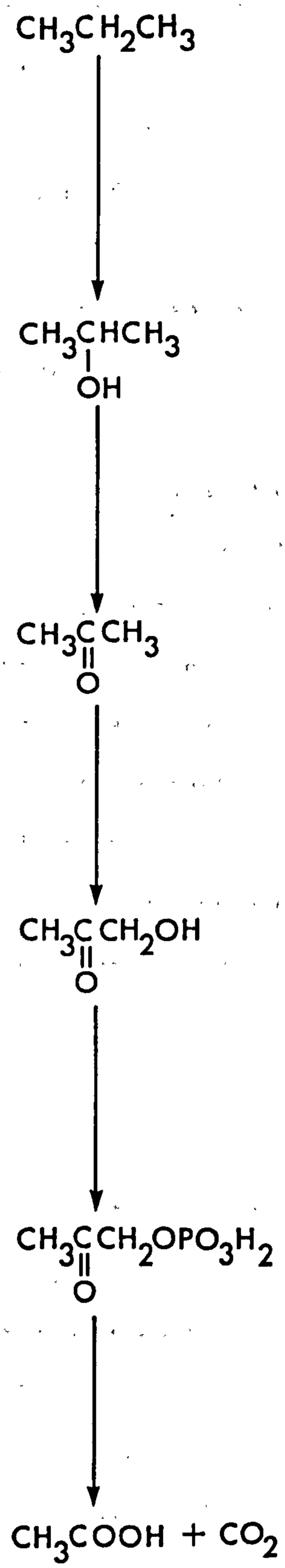


Figure 1.8 Perry's proposed pathway of propane metabolism in Mycobacterium vaccae JOB5 (from Perry, 1980)

doubts about the interpretation of experimental data from this work. For example, in the absence of arsenite significant amounts of 2-^{14}C-propan-2-ol were being converted to pyruvate suggesting that arsenite may have been inhibiting the initial steps of propan-2-ol metabolism. Dagley & Chapman (1971) have pointed out the limitations of using inhibitors for the elucidation of biochemical pathways, namely that it is rarely possible to use an inhibitor so specific in its action that it only inhibits one type of enzyme.

Vestal & Perry (1971) analyzed the fatty acid content of M.vaccae JOB5 after growth on propane, propan-2-ol, propanoate and acetate. They proposed that if propane was oxidized to propanoate, then propane-grown cells should contain high levels of odd chain length fatty acids, converse if it is oxidized via acetate then predominantly even chain fatty acids should be present. In contrast to the above discussion, which favoured subterminal oxidation, considerable amounts of odd chain length fatty acids were observed in propane-grown cells, thus implicating terminal oxidation of propane via propanoate.

Blevins & Perry (1972) carried out similar studies on a propane-, propanoate- and n-propylamine-utilizing M.convolutum R-22. They proposed that propane was metabolized via acetone, propanoate and n-propylamine were metabolized via the methylmalonate pathway. A simultaneous adaptation experiment showed that propane-grown cells could rapidly oxidize propan-2-ol (1.83 μl O_2 min^{-1} mg dry $weight^{-1}$) and acetone (2.47 μl O_2 min^{-1} mg dry $weight^{-1}$). Propan-1-ol oxidation was also relatively significant (1.53 μl O_2 min^{-1} mg dry $weight^{-1}$), the low levels of propanoate oxidation (0.1 μl O_2 min^{-1} mg dry $weight^{-1}$) could be due to a lack of an uptake system and cannot rule out propanoate as a possible intermediate in the oxidation of propane, according to Woods

(1988). Interestingly, propan-1,2-diol also had a significant level of oxidation (1.75 μl O_2 min^{-1} mg dry $weight^{-1}$) although the reason for this was not discussed.

Coleman & Perry (1984) worked on the fate of the C_1 moeity resulting from the proposed C_2-C_1 cleavage for the metabolism of propane by M.vaccae JOB5. This was originally suggested to be released as CO_2 (Vestal & Perry, 1969); see Fig. 1.8. When propane-grown cell suspensions were incubated with 2-^{14}C-acetone and 1,3-^{14}C-acetone and the patterns of CO_2 evolution measured, it was observed that $^{14}CO_2$ was produced at the same rate by both substrates. If CO_2 was released from the cleavage of acetol then there would be a higher rate of $^{14}CO_2$ evolution from 1,3-^{14}C-acetone than from 2-^{14}C-acetone. They suggested that "The C_1 unit enters stable cellular constituents". Further evidence was provided by growing cells on propan-2-ol in the presence of 1-^{14}C-acetate, ^{14}C-bicarbonate, ^{14}C-formaldehyde or 1-^{14}C-1,2-propandiol. The latter was used in place of acetol, as it is supposedly metabolized *via* acetol; although an earlier discussion on the metabolism of propan-1,2-diol shows there are other possibilities (see section 1.3.3.1). After incubation, incorporation of labelled ^{14}C into lipid, nucleic acid and protein was determined. Cells exposed to the diols showed the same level of incorporation as those exposed to formaldehyde suggesting the metabolism results in the formation of a reduced C_1 moiety. This may involve the participation of enzymes involved in C_1-group transfers using tetrahydrofolate or S-adenosylmethionine, although this is not speculated upon (for review on C_1-group transfers, see Walsh, 1977). Cells exposed to labelled diol and formaldehyde showed a higher percentage label in their guanine than did cells exposed to radiolabelled acetate or bicarbonate. This suggested that acetol is metabolized *via* a reduced C_1 moiety, as 40% of the carbon in guanine is

derived from the "reduced C_1 pool". However, Woods (1988) commented that all this experiment showed was that propan-1,2-diol was metabolised to produce a reduced C_1 moiety.

1.3.3.4.2 Other work on propane metabolism

Other studies have suggested a role for terminal and/or subterminal oxidation of propane. Lukins & Foster (1963) showed that Mycobacterium smegmatis 422 produced methylketones when oxidizing n-alkanes. However, ketone production occurred under 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. It is clear that subterminal oxidation was occurring, although the relative biological importance cannot be ascertained from this type of experiment. Propane and acetone-grown cells oxidized propane and acetone whereas propan-1-ol cells did not, which indicated that acetone-grown cells were simultaneously adapted to propane.

Pabst & Brown (1967) isolated a Mycobacterium sp. which was capable of growth on ethane and propane, but not n-butane or isobutane. It also grew on propan-1-ol and propan-2-ol, but not acetone, although this was oxidized by propane-grown cells. Propane and propan-2-ol-grown cells were shown to oxidize propane, while propan-1-ol and glucose-grown cells could not. They proposed that propane was metabolized via propan-2-ol which can then be converted to acetone or propan-1-ol, although the evidence for this is lacking.

Patel et al. (1983) using cell-free extracts of Arthrobacter sp. 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. This suggests that propane could be oxidized *via* terminal and subterminal oxidation. In another report Hou *et al*. (1983a) reported the ability of resting-cell suspensions of propane-grown cells to epoxidate a range of alkenes (the reader is referred to Weijers *et al*. 1988; for a discussion on the microbial production and metabolism of epoxides). They reported that the propane monooxygenase was relatively stable.

The oxygenase involved in the metabolism of propane and isobutane by *Nocardia paraffinicum* (*Rhodococcus rhodochrous*) was investigated by Babu & Brown (1984). Analysis of culture supernatants from propane-grown cultures showed the presence of propan-1-ol, propan-2-ol and acetone were only found in trace quantitites. Experiments using $^{18}O_2$ and $H_2{}^{18}O$ demonstrated that molecular O_2 was incorporated into propan-1-ol, thus indicating the action of an oxygenase in the terminal oxidation of the propane molecule. They observed 2:1 ratio of hydrocarbon to oxygen consumption and suggested the participation of a novel intermolecular dioxygenase, which catalyzed the oxidation of two molecules of propane with one molecule of O_2 to yield two molecules of a C_3 alcohol.

MacMichael & Brown (1987) showed the metabolism of propane involved CO_2 fixation after its oxidation to propanoate. The rate of propane utilization was directly related to the initial CO_2 concentration, and Warburg respirometry suggested that CO_2 was an obligate requirement for the metabolism of propan-1-ol, propanal and propanoate but not for propan-2-ol. This led them to propose that propane was metabolized *via* terminal oxidation involving the methylmalonate pathway (Figs 1.3 and 1.4 respectively).

Stephens and Dalton (1986) isolated several strains of propane-utilizing bacteria, they could be classified into three groups depending on their ability to grow on acetone. The three strains selected for detailed study belonged to the genus Arthrobacter. Strain B3aP could not grow on acetone and could not oxidize acetone after growth on propane. Strain PrIO3 grew slowly on acetone but could not oxidize acetone after growth on propane. However, acetone was excreted during growth on propane. They suggested that B3aP and $PrIO_3$ oxidized propane to both propan-1-ol and propan-2-ol and concluded that the propane oxygenase must be non-specific with respect to the site of insertion of oxygen into the propane molecule. The third strain, B2, grew rapidly on acetone and was induced for acetone oxidation after growth on propane, which suggested that both propan-1-ol and propan-2-ol were produced and metabolized completely as no products were detected in culture supernatants. It was suggested that B2 employed both terminal and subterminal oxidation pathways in the metabolism. Attempts to measure the production of propan-1-ol and/or propan-2-ol by a propane monooxygenase were unsuccessful because of the organisms resistance to breakage by physical and chemical methods.

Woods & Murrell (1989) isolated a strain of Rhodococcus rhodochrous strain PNKb1. Despite testing for growth on n-alkanes (C_1-C_8) it grew only on propane. The organism also grew on most of the potential intermediates of propane metabolism, except methylacetate, methanol and methylglyoxal (pyruvaldehyde). Simultaneous adaptation studies demonstrated that it oxidized both terminal and subterminal intermediates. The propane-specific oxygenase activity was measured by its ability to co-oxidize propene to epoxypropane. Specific activities of "key enzymes" of propane oxidation in cell-free extracts of R.rhodochrous PNKb1 were measured after growth on propane and pyruvate.

NAD^+-dependent propan-1-ol dehydrogenase activity showed little increase in propane-grown cells compared to pyruvate grown cells. The other enzymes of the terminal oxidation pathway, propanal dehydrogenase and propionyl-CoA synthetase, showed a five fold increase in levels of activity in propane-grown cells compared to pyruvate-grown cells. NAD^+-dependent propan-2-ol dehydrogenase activity showed a four fold increase in propane-grown cells, and acetol oxygenase activity, which was absent from pyruvate-grown cells, was present in propane-grown cells. They postulated that the absence of acetol dehydrogenase activity and the presence of acetol oxygenase activity may rule out the conversion of acetol to pyruvate. However, acetol dehydrogenase activity was only measured under one set of conditions, at pH 10 and using NAD^+ as the electron acceptor; which may not be optimal for enzyme activity. Propane oxygenase activity was shown to be induced only during growth on propane. SDS-PAGE of cell-free extracts of cells grown on various intermediates of propane metabolism showed the presence of three major polypeptides, of 69, 59 and 57 kilodaltons (kDa) which according to them were only specific to propane-grown cells. However, closer examination reveals similar polypeptides which are also specific to acetol-grown cells. They proposed that these polypeptides were likely to be components of the propane oxygenase system.

They were unable to measure the propane-stimulated oxygen uptake or the build-up of propan-1-ol or propan-2-ol in cell-free extracts from propane-grown cells, however, it was possible to measure the formation of 1,2-epoxypropane from propene. They were unable to purify oxygenase activity because of the organisms resistance to breakage and the extreme liability of the oxygenase activity (half-life of 150 min). Inhibition studies on the oxygenase showed that it was not cytochrome P-450 linked

in contrast to the only well characterized oxygenase system from a Gram-positive bacterium (Cardini & Jurtshuk, 1970).

They concluded that the metabolism of propane by R.rhodochrous PNKb1 may proceed via terminal or subterminal oxidation, although the relative importance of each remained unclear. They also stated that the establishment of growth on potential intermediates of propane metabolism would enable a genetic approach to the analysis of the pathways of propane metabolism.

1.3.4 Butane

O'Brien & Brown (1967) isolated a strain of Mycobacterium phlei that was capable on growth on isobutane, it also utilized propane and n-butane; but not methane or ethane as growth substrates. The spacial arrangement of the isobutane molecule is such that the involvement of a ketone intermediate in its metabolism is precluded. On the basis of simultaneous adaptation studies the molecule undergoes terminal oxidation via isobutanol, isobutanal and isobutanoate. Isobutane-grown resting cells also oxidized propan-1-ol and propanal, however; acetone and propanoate were oxidized relatively slowly. Resting cells grown on propane, butane and 2-methylbutane were adapted for oxidizing one or more saturated gaseous alkanes, but cells grown on liquid hydrocarbons (with the exception of 2-methylbutane) were not adapted. A possible explanation for this is that a separate oxidation system exists for liquid ($>C_5$) and gaseous n-alkanes.

Phillips & Perry (1974) investigated the metabolism of n-butane and butan-2-one by M.vaccae JOB. They proposed that n-butane was metabolized via terminal oxidation and butan-2-one through propanoate,

and stated that subterminal oxidation in M.vaccae JOB5 was limited to propane. The similarity of fatty acid composition and induction of isocitrate lysase activity after growth on n-butane, butyrate, PHB and acetate was their main evidence for their terminal oxidation proposal. Incorporation of labelled $^{14}CO_2$ into pyruvate by butan-2-one and propanoate but not by n-butane, butanoate and acetate-grown cells suggested that butan-2-one was not an intermediate of butane metabolism. Their experimental evidence for the pathway would have been improved if the roles of butan-1-ol and butan-2-ol had been investigated as growth substrates. Another problem with this report stems from earlier work (Perry, 1968) which demonstrated that propane-grown cells are adapted for butane, butan-1-ol and butanoate oxidation. If these two reports are to believed, why should a common oxygenase oxidize propane subterminally and butane-terminally?

A more comprehensive study into butane oxidation was carried out on Nocardia TB1, isolated from soil on trans-2-butene (Van Ginkel et al., 1987). The presence of isocitrate lysase in butane-grown cells and excretion of butanoate when cell suspensions were incubated butane and sodium arsenite suggested that butane was oxidized terminally. The activities of butanol reductase, butanal dehydrogenase and butanoacyl CoA synthetase were detected in cell-free extracts which inferred terminal oxidation, however, no butan-1-ol dehydrogenase activity was reported.

1.3.5 Summary

From a survey of the literature the pathways of ethane, propane and butane are not well characterized compared to those of methane or liquid n-alkanes. This is mainly due to the lack of biochemical and genetical

data, and the dependence on simultaneous adaptation and product excretion studies.

Reports suggest that propane may be metabolized subterminally from the work of Perry et al. (1.3.3.4.1), terminally (Babu & Brown, 1984; Stephens & Dalton, 1986; MacMichael & Brown, 1987), and both terminally and sub-terminally (Stephens & Dalton, 1986; Woods & Murrell, 1989).

1.4 Emzymology of n-alkane metabolism

Very little is known about the enzymology of ethane, propane and butane metabolism. The following discussion serves to highlight points which may have applicability to higher gaseous n-alkane metabolism.

1.4.1 Oxygenases

Oxygenases have been defined by Hayaishi (1975) as enzymes which incorporate molecular oxygen into substrates. This group of enzymes is further divided into di- and monooxygenases depending on whether both or just one atom of molecular oxygen is incorporated into the substrate.

Methane monooxygenase from M.capsulatus (Bath) has been purified (Colby & Dalton, 1978) and well characterized when compared to other oxygenases, however, it will not be discussed in this work, the reader is referred to Dalton & Leak (1985) and Dalton et al. (1990). Work has also been undertaken on aromatic hydrocarbon oxygenases, but again will not be discussed here (see Gibson & Subramanian, 1984).

1.4.1.1 Propane "oxygenase" activity

There are no reports of a purified propane oxygenase system and there is no firm evidence as to a possible role in propane oxidation.

However, Patel et al. (1983) demonstrated that cell-free particulate fractions from Arthrobacter sp. CRL-60 and soluble extracts derived from Pseudomonas fluorescens NRRL B-1244 and Brevibacterium sp. NRRL B-11319 catalyzed an NADH and oxygen-dependent hydroxylation of ethane, propane

and butane. Both primary and secondary alcohols were detected as the products of n-alkane oxidation.

Babu & Brown (1984) proposed the presence of a novel propane intermolecular dioxygenase in N.paraffinicum which had two hydrocarbon-binding sites. Although no attempt was made to measure cell-free activity.

More recently, Woods & Murrell (1989) reported a propane-specific oxygenase from R.rhodochrous PNKb1. The activity was measured by its ability to co-oxidize propene to 1,2-epoxypropane. The formation of propan-1-ol or propan-2-ol from propane could not be demonstrated in cell-free extracts. It was investigated in terms of its inhibitor profile. Cytochrome P-450 was not involved as demonstrated by a lack of inhibition by carbon monoxide. Attempts to purify propane-oxygenase activity was unsuccessful due to the difficulty in obtaining high-protein concentrations from cell-free extracts and the labile nature of the activity.

1.4.1.2 Octane monooxygenase from Pseudomonas putida

The three component octane monooxygenase from Pseudomonas oleovorans(putida) has been studied in great detail and similar enzyme systems have been reported for Pseudomonas aeruginosa (Van Eyk & Bartels, 1970; Matsuyama et al., 1981).

The system was first described by Baptist et al. (1963). Cell-free extracts oxidized octane to octanoic acid, n-octanol and octadldehyde were also identified as reaction products. NAD^+-dependent dehydrogenases acting on octanol and octaldehyde were also demonstrated.

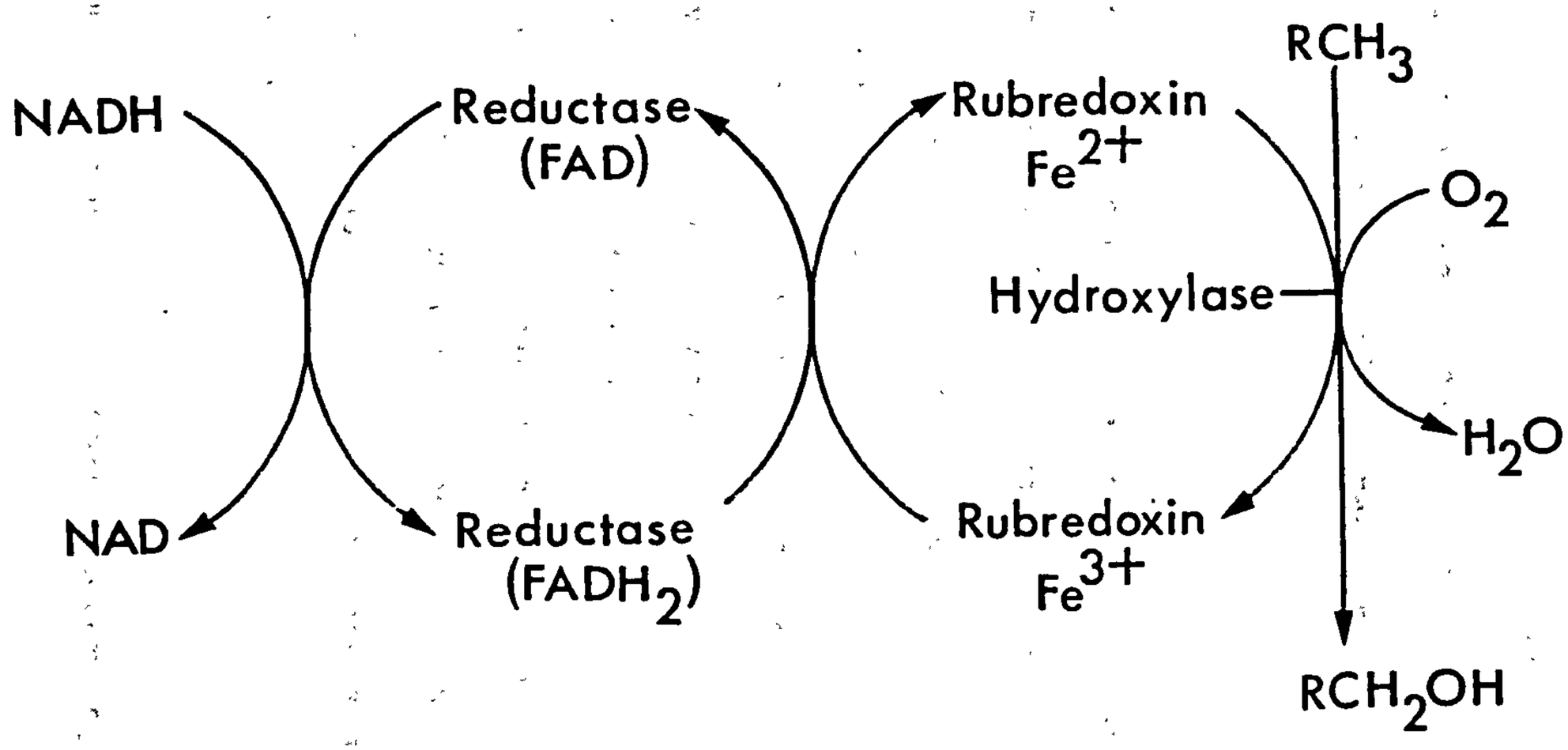


Figure 1.9 Postulated pathway of electron transfer in octane monooxygenase from P.oleovorans (from Ratledge, 1978)

Peterson et al. (1966) described the separation of the ω-hydroxylation system into three components, which were identified as a rubredoxin, NADH-rubredoxin reductase and a ω-hydroxylase. The conversion of octane to octanol was shown to be oxygen and NADH-dependent. Evidence was presented by Peterson et al. (1967) that rubredoxin and the reductase function as electron carriers during the hydroxylation of octane in the presence of the ω-hydroxylase. The reductase and rubredoxin interact on a 1:1 basis to form a complex and the redox reaction occurs as described in Fig.1.9.

Further studies on the three components showed that (a) the NADH-rubredoxin reductase was a single polypeptide of 55 kDa containing one mole of FAD per mole of protein (Ueda et al., 1972); (b) the rubredoxin was a single polypeptide chain of 19 kDa, which contained two moles of iron per mole of protein and no prosthetic groups (Lode & Coon, 1971); (c) the ω-hydroxylase functions as a methyl hydroxylase in attacking fatty acids and n-alkanes (McKenna & Coon, 1970) and Ruettinger et al. (1977) demonstrated it to be a non-haem iron protein containing one iron atom and one cysteine residue per polypeptide chain of 40.8 kDa. The ω-hydroxylase is membrane bound but the reductase and rubredoxin are soluble (Benson et al. 1979).

1.4.1.3 Octane monooxygenase from Corynebacterium sp 7E1C

Corynebacterium sp. 7E1C was isolated from a propane enrichment by Kester & Foster (1963) and has the ability to grow on C_3-C_{18} n-alkanes. Cell-free extracts from sonically disrupted cells oxidized n-octane to octan-1-ol and octanoate in an oxygen and NADH-dependent reaction (Cardini & Jurtshuk, 1968; 1970). In contrast to the system in Pseudomonas oleovorans the reaction was sensitive to carbon monoxide.

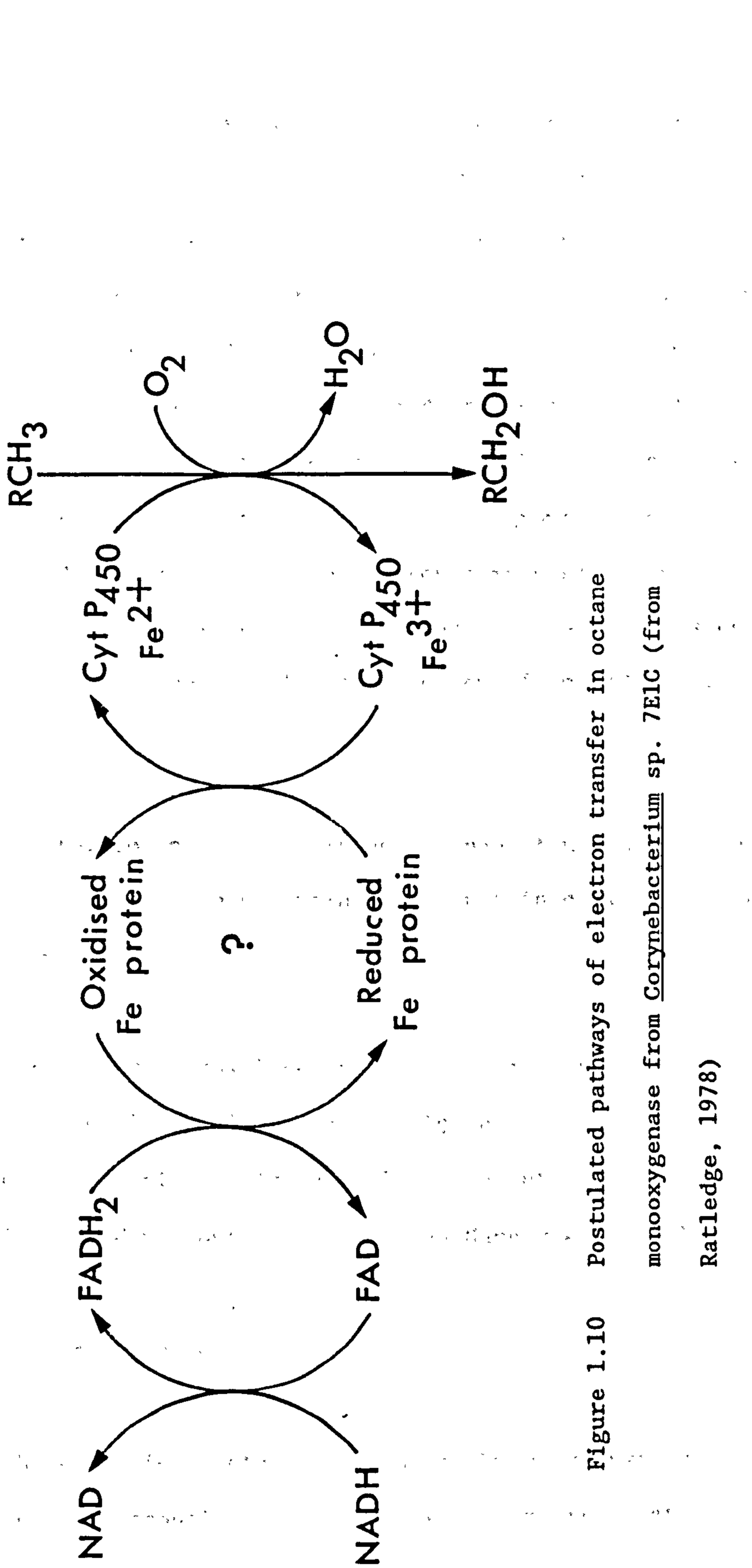


Figure 1.10 Postulated pathways of electron transfer in octane monooxygenase from Corynebacterium sp. 7E1C (from Ratledge, 1978)

The hydroxylating system was shown by ammonium sulphate fractionation, to consist of at least two protein fractions. The S_3 (25-40)D, which contains cytochrome P-450, appears particulate or at least associated with lipids. The flavoprotein fraction, S_3(60-100)D, was soluble and they proposed that it was similar to NADH-cytochrome P-450 reductase. Both fractions were required for activity and it was proposed that the flavin catalyzed the transfer of electrons from NADH to the cytochrome P-450 component. Both components were induced by growth on octane. It was also postulated that a third non-haem iron component may be involved, since other P-450 systems from bacteria and eukaryotes consist of three proteins. The proposed flow of electrons from NADH to oxygen is shown in Fig. 1.10.

Although Corynebacterium sp. 7E1C grows on a range of n-alkanes the substrate specificity of the monooxygenase was not investigated. For a review see Jurtshuk & Cardini (1971).

The involvement of P-450 hydroxylases have also been reported for Candida tropicalis (Gallo et al., 1973) and Acinetobacter sp. (Asperger et al., 1981) when utilizing n-alkanes. For a recent review on cytochrome P-450 dependent monooxygenases, see Mansuy et al. (1989).

1.4.1.4 Ketone monooxygenases

Several groups have studied acetone degradation by microorganisms, however, the precise metabolic routes involved and their relationship to central metabolism remains unclear (see 1.3.2.2).

Taylor et al. (1980) tried unsuccessfully to measure acetone monooxygenase activity in cell-free extracts of Corynebacterium sp.

grown on acetone, but its presence was inferred. They proposed the hydroxylation of acetone to form acetol and suggested the reason for the absence of monooxygenase activity was due to the extreme liability of the enzyme, or a requirement for a structural organization that was lost upon cell rupture. Woods & Murrell (1989) were also unable to detect acetone monooxygenase activity in propane-grown R.rhodochrous PNKb1, however an oxygen and NADPH-dependent acetol monooxygenase was detected in cell-free extracts. However, microsomal P-450 dependent acetone and acetol monooxygenase activities have been reported for acetone and propan-1,2-diol treated rats (Casazza et al., 1984; Casazza & Veech, 1985).

Britton & Markovetz (1977) described the purification and characterization of a ketone monooxygenase from Pseudomonas cepacia grown on tridecan-2-one as sole carbon source. They reported the formation of an ester, undecyl acetate, from tridecan-2-one in an oxygen and NADPH-dependent reaction. They proposed this was equivalent to the chemical Baeyer-Villiger oxidation of carbonyl compounds by peracids (see Sykes, 1975). The substrate specificity of the tridecan-2-one monooxygenase for other methyl ketones C_3-C_{14} was tested; all were oxidized except acetone.

Hasegawa et al. (1983) were able to demonstrate NAD(P)H-dependent cycloketone oxygenase activity in a cyclohexanone-grown Nocardia sp.

Trudgill (1984) has reviewed the microbial degradation of alicyclic compounds and reports the properties of cycloalkaneone monooxygenases cyclohexanol-grown Acinetobacter NCIB 9871 and cyclopentanol-grown Pseudomonas NCIB 9872. The reaction proceeds via the lactonization of the alicylic ring which ultimately leads to ring-fisson.

Hartmans & deBont (1986) demonstrated acetol monooxygenase activity in cell-free extracts of Mycobacterium Py1 after growth on acetol and propan-1,2-diol. They proposed the reaction involves the formation of a transient ester intermediate which spontaneously forms acetate and formaldehyde. The enzyme was shown to be cytochrome P-450 dependent as it was inhibited by carbon monoxide. A role for this type of enzyme in the metabolism of propane was postulated on the basis of the work described by Coleman & Perry (1984).

An inducible NADPH-dependent 6-oxocineole oxygenase was demonstrated in a Rhodococcus sp. after growth on 1,8-cineole (Williams et al., 1989). An investigation of the substrate specificity of partially purified oxygenase, in which a wide range of cyclic and acyclic ketones was used, failed to reveal a single alternative substrate to 6-oxocineole. The authors proposed that the oxidation involved a Baeyer-Villiger reaction. The ability of this Rhodococcus sp. to grow on acetone was also noted.

Trower et al. (1989) have reported the purification and characterization of an FMN-containing cyclohexanone monooxygenase from a cyclohexane-grown Xanthobacter sp. The monooxygenase catalyzed the transformation of cyclohexanone to the lactone 1-oxa-2-oxocyclohexanone in an oxygen ring insertion reaction. The substrate specificity of the monooxygenase was investigated with a range of cyclic and straight-chain ketones substrates, none of the latter stimulated monooxygenase activity. The enzyme was also compared and contrasted with other reported ketone monooxygenases in terms structure and cofactor characteristics.

It can be concluded that ketone monooxygenases are lactone and ester-forming enzymes which are flavoproteins. A repeated theme is that the reactions are catalyzed by a biological manifestation of a Baeyer-

Villiger type reaction mechanism. Recently, Walsh & Chen (1988) have reviewed the enzymatic Baeyer-Villiger oxidations by flavin-dependent monooxygenases.

1.4.2 Alcohol dehydrogenases

Methanol dehydrogenase is a well characterized enzyme which oxidizes methanol to formaldehyde. Many such enzymes have been described in both methylotrophs and methanotrophs (see Dalton & Leak, 1985). Methanol dehydrogenases contain the prosthetic group pyrroloquinoline quinone (PQQ); the role of quinoproteins in C_1-dissimilation by bacteria has been reviewed by Anthony (1989). However, an NAD^+-dependent PQQ-containing methanol dehydrogenase multienzyme complex has been reported for methanol-grown Nocardia sp. 239 (Duine et al., 1984). Recently isolated thermotolerant methylotrophic Bacillus sp. (Dijkhuizen et al., 1988) have been shown to contain NAD-dependent (non-PQQ containing) methanol dehydrogenases (Dijkhuizen, 1989; Arfman et al., 1989).

Alcohol dehydrogenases and their role in n-alkane metabolism have been studied in most detail in Pseudomonas sp. Singer & Finnerty (1984a) list a variety of alcohol dehydrogenases found in alkane-grown bacteria. Most of these enzymes are constitutive, soluble and require NAD(P) as cofactors.

1.4.2.1 Alcohol dehydrogenases from Pseudomonas sp.

Tassin & Vandecasteele (1972) studied the long-chain alcohol dehydrogenases from P.aeruginosa in relation to their involvement in hydrocarbon metabolism. Three soluble $NADP^+$-dependent alcohol dehydrogenases were present when cells were grown on glucose or n-

hexadecane. However, in a latter report Tassin et al. (1973) reported a particulate and phenazine methosulphate (PMS)-dependent alcohol dehydrogenase which was induced by growth on hydrocarbons. The purified enzyme did not use $NAD(P)^+$ as cofactors. The product of alcohol oxidation was the corresponding aldehyde. The apparent K_m values for this PMS-dependent dehydrogenase were lower for the longer chain n-alkan-1-ols than for the $NADP^+$-dependent dehydrogenase. They also postulated that the utilization of an electron acceptor with a higher reduction potential such as PMS (PMS E'_o = 0.08V; NAD^+ E'_o = 0.31V) in preference to NAD^+, may have a physiological value as it may facilitate the oxidation of long-chain alcohols by displacing the thermodynamic equilibrium towards the formation of aldehyde. Only PMS was used as an acceptor in vitro, the in vivo acceptor being unknown.

Chakrabarty et al. (1973) reported the presence of an NAD^+-dependent octanol dehydrogenase in P.oleovorans. The gene for this enzyme was chromosomally encoded, and the enzyme was induced by octanol but not octane. The enzymes responsible for the oxidation of octane where shown to be octane inducible, and were coded by genes carried on a transmissible plasmid (OCT). Studies on the regulation of OCT-plasmid expression demonstrated that after growth on octane, but not octanol, under conditions where octanol dehydrogenase induction was inhibited by the camphor (CAM) plasmid; suggested that the OCT-plasmid must also carry an octanol dehydrogenase gene under octane regulation.

Grund et al. (1975) working on the regulation of alkane oxidation in the wild-type P.putida PpG1 (OCT^-) demonstrated the existence of inducible plasmid-encoded primary alcohol and constitutive chromosomally coded alcohol dehydrogenase activity. This was confirmed by the isolation of alcO (later changed to alk C^-; Benson et al., 1977) and alcA^- mutants,

respectively. Mutant analysis lead them to the following conclusions with respect to alcohol dehydrogenases:

(i) the chromosome coded for at least two alcohol dehydrogenase activities, mutants of strain PpG1 with no plasmid selected for the inability to grow on octanol or nonanol can still grow on shorter-chain alcohols;

(ii) the plasmid coded for an alcohol dehydrogenase;

(iii) the specificity of the plasmid-coded alcohol dehydrogenase differed from that of the corresponding chromosomal enzyme;

(iv) strain PpG6 (OCT^+) grown on alkanes contained an NAD^+-dependent octanol dehydrogenase in cell-free extracts, but this same enzyme activity is present in an alcA$^-$ strain without an OCT plasmid, therefore, this activity is not responsible for the metabolism of octanol in vivo.

Benson & Shapiro (1976) identified an alcohol dehydrogenase activity in P.putida strains carrying the OCT-plasmid, the activity was NAD^+-independent, particulate and showed 20-fold greater activity with octanol rather than butanol as a substrate. The enzyme was induced by unoxidized alkane and was only present in strains that have the OCT plasmid with the wild-type alkC locus. Alcohol dehydrogenase activity in vitro was measured using PMS as the electron acceptor. It was also proposed that this particulate alcohol dehydrogenase activity was similar to that reported by Tassin et al. (1973) for alkane-utilizing P.aeruginosa. Wild-type and alcA$^-$ mutants without plasmids both contained constitutive NAD^+-dependent soluble alcohol dehydrogenase activity. They suggested that alcA$^-$ mutants were cryptic for octanol oxidation and also that the particulate plasmid-coded alcohol dehydrogenase activity was active on surface- or membrane-bound substrate.

The obligatory role of alcohols as intermediates in alkane metabolism by this organism is demonstrated by the fact that on an alcA$^-$ background alkC$^-$ mutants will not grow on octanol, nonanol or dodecanol or the corresponding alkanes.

1.4.2.2 Alcohol dehydrogenases from Acinetobacter

The alcohol dehydrogenases in Acinetobacter calcoaceticus have also been investigated in relation to alkane metabolism (for review see Singer & Finnerty, 1984a). A.calcoaceticus 69V contains a constitutive, soluble, $NADP^+$-dependent alcohol dehydrogenase; alkane-grown cells also contain an $NAD(P)^+$-independent alcohol oxidizing activity.

The pattern that emerges from the discussion is the presence of alkane inducible, particulate $NAD(P)^+$-independent alcohol dehydrogenase activity in alkane-grown cells. These enzymes have a higher affinity for long-chain alcohols than $NAD(P)^+$-dependent activities. Whether this is a general trait associated with the metabolism of higher alkanes is open to debate owing to the limited number of genera examined.

1.4.2.3 Alcohol dehydrogenases from gaseous n-alkane utilizers

The first indirect report came from studies on the microbial metabolism of acetone (Taylor et al., 1980). A comparison of enzyme activities in cell-free extracts of Arthrobacter A1 grown on acetone, succinate or acetate demonstrated the inducible nature of NAD^+-dependent dehydrogenase activity towards propan-2-ol, acetol and methylglyoxal.

In other studies an NAD^+-dependent propane-specific secondary alcohol dehydrogenase was purified from Pseudomonas fluorescens NRRL-B-1244 (Hou

et al., 1983b). Propane-grown cells of this organism possessed two NAD^+-dependent alcohol dehydrogenases, one which showed a preference for primary alcohols (alcohol dehydrogenase I), the other showing a preference for secondary alcohols (alcohol dehydrogenase II). The secondary alcohol dehydrogenase was purified to homogenity and was found to have a molecular weight of 144.5 kDa 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. Enzyme activity was inhibited by strong thiol reagents and certain metal chelators. A very broad substrate range was observed, substrates oxidised included n-alkan-2-ols, n-alkan-1-ols, aldehydes, diols, cyclic and aromatic alcohols. The k_m for propan-2-ol was 85 μM.

An NAD^+-dependent propan-2-ol dehydrogenase has been purified from M.vaccae JOB-5 (Coleman & Perry, 1985). This enzyme was implicated in the subterminal oxidation of propane since high activities of this enzyme were induced during growth on propane, propan-2-ol and acetone but not during growth on propan-1-ol. It is worthwhile noting that this enzyme was purified from cells grown on propan-2-ol with succinate and not propane-grown cells. The reason for this was not given. The enzyme was purified to homogeneity and had a molecular weight of 136 kDa and consisted of four subunits. The pH optimum was 10 to 10.5. The K_m for propan-2-ol was 49 μM. The activity was inhibited by thiol reagents and metal chelators. This alcohol dehydrogenase catalyzed the conversion of propan-2-ol to acetone, and also catalyzed the reverse reaction using NADH as an electron donor. It exhibited a narrower substrate specificity range than the Pseudomonas dehydrogenase of Hou et al. (1983b).

It is apparent that the alcohol dehydrogenase from M.vaccae JOB-5 was similar in many respects to that isolated from P.fluorescens. However, neither Coleman & Perry (1985) or Hou et al. (1983b) described any attempts to measure $NAD(P)^+$-independent alcohol dehydrogenase activity which was previously discussed.

Woods (1988) reported the presence of soluble NAD^+-dependent propan-1-ol and propan-2-ol dehydrogenase activities in cell-free extracts from propane-grown R.rhodochrous PNKb1. However, no dye-linked activity (using either PMS or phenazine ethosulphate) could be detected, despite attempts using a wide range of assay buffers and pH's. Enzyme activity was inhibited by thiol and chelating reagents. The apparent K_m values of 22 mM for propan-2-ol and 3 mM for propan-1-ol were orders of magnitude greater than those determined by Hou et al. (1983b) and Coleman & Perry (1985). He questioned whether these activities could be responsible for the metabolism of intermediates in propane metabolism, or whether they are just incidental activities responsible for other aspects of metabolism.

The distribution and characteristics of alcohol dehydrogenases expressed by butane-grown Pseudomonas butanovora has been examined by Beers (1988). Growth on butane resulted in the expression of four alcohol dehydrogenases. Three were found to be soluble and NAD^+-dependent, one being a primary alcohol dehydrogenase and the others being secondary alcohol dehydrogenases. The fourth enzyme was a membrane-bound $NAD(P)^+$-independent primary alcohol dehydrogenase. The molecular weights for each enzyme were as follows; $NAD(P)^+$-independent dehydrogenase consisted of a single subunit of 63 kDa; NAD^+-dependent primary alcohol dehydrogenase was 80 kDa consisting of two subunits; NAD^+-dependent secondary alcohol dehydrogenase A and B consisted of single subunits of

35 and 29 kDa respectively. The $NAD(P)^+$-independent dehydrogenase had a comparatively lower k_m for butan-1-ol (1.2 μM) than the soluble NAD^+-dependent dehydrogenases, a similar result was reported by Tassin et al. (1983). The particulate enzyme also catalyzed the oxidation of a wide range of substrates including methanol and ethanol, but not secondary alcohols. The soluble dehydrogenases showed a restricted substrate specificity. The expression of the various alcohol dehydrogenases varied during the growth cycle of P.butanovora. He postulated that the particulate $NAD(P)^+$-independent dehydrogenase was involved in the metabolism of PHB.

1.4.3 Aldehyde dehydrogenase

A range of formaldehyde dehydrogenase enzymes exist in methanotrophs and methylotrophs (see Dalton & Leak, 1985). There are both NAD^+-dependent and NAD^+-independent varieties of these enzymes.

Singer & Finnerty (1984a) list a selection of aldehyde dehydrogenases found in alkane-grown bacteria. As with alcohol dehydrogenases, most studies involved Pseudomonas sp. which contain $NAD(P)^+$-dependent and $NAD(P)^+$-independent aldehyde dehydrogenase activity.

1.4.3.1 Aldehyde dehydrogenases from Pseudomonas sp.

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

Guerrillot & Vandecasteele (1977) demonstrated two soluble, constitutive, aldehyde dehydrogenases in glucose-grown P.aeruginosa. An

NAD^+-specific enzyme had a high affinity for short- and medium-chain length aldehydes (C_2 to C_{10}) and an $NADP^+$-specific enzyme for longer chain aldehydes. Hexadecane-grown cells contained a third, particulate, NAD^+-dependent aldehyde dehydrogenase which had high specific activities for long chain aldehydes which implied a role in alkane metabolism. The role of the constitutive enzymes remained unclear.

Poels et al. (1987) described the purification of an $NAD(P)^+$-independent aldehyde dehydrogenase from butan-1-ol-grown Pseudomonas testosteroni. The enzyme appeared to oxidize a wide range of aldehydes, including C_1 to C_4 aldehydes. This novel enzyme contained neither PQQ or cytochrome c, but did contain molybdopterin, FAD, iron and sulphur. Their results were compared to other listed bacterial cofactor-containing aldehyde dehydrogenases.

In P.putida the isolation of aldehyde negative (aldA$^-$), alkane-negative mutants in plasmid carrying strains (alkane hydroxylase positive and alcohol dehydrogenase positive) would suggest that a chromosomally encoded aldehyde is required for alkane metabolism (Grund et al., 1975). However, recent work on the P.oleovorans plasmid alkBAC operon showed the alkA region contains three coding sequences, and one, alkH codes for a 52 kDa aldehyde dehydrogenase. The product of the alkH cistron restored, albeit weakly, the growth of a P.oleovorans aldA$^-$ mutant on aliphatic alcohols and aldehydes. Its amino-acid sequence also showed considerable homology to previously characterized aldehyde dehydrogenases from mammalian and fungal origin (Kok et al., 1989a).

1.4.3.2 Aldehyde dehy. drogenases from other bacteria

Acinetobacter sp. HO1-N was shown to have $NAD(P)^+$-dependent aldehyde dehydrogenase activity which was induced by hexadecane, long-chain alcohols and aldehydes (Singer & Finnerty, 1984a).

The involvement of an NAD^+-dependent aldehyde dehydrogenase in the metabolism of 1,2-dichloroethanol and 2-chloroethanol in Xanthobacter autotrophicus GJ10 was shown by Janssen et al. (1987). The enzyme was induced by the latter two compounds and ethanol. Its involvement in the metabolism of 2-chloroethanol metabolism was confirmed by the observation that the enzyme was lacking in a mutant which could not grow on 2-chloroethanol or 1,2-dichloroethane.

When the methylotrophic bacterium Nocardia sp. 239 is grown on methanol, a novel type of methanol dehydrogenase is present (Duine et al., 1984). This was found to be NAD^+-dependent and forms part of a multi-enzyme complex together with NAD^+-dependent aldehyde dehydrogenase. Recently three different formaldehyde dehydrogenase activities have been semi-purified from Nocardia sp. 239 (Van Ophem & Duine, 1989).

A trimeric formaldehyde dehydrogenase requiring an unknown cofactor has been purified from Rhodococcus erythropolis. (Eggeling & Sahm, 1985). The enzyme is only present when the organism is utilizing compounds which result in the generation of formaldehyde (e.g. methylamines or methoxylated benzoic acids). A mutant was also isolated which lacked this enzyme and could not grow on the above substrates. It is interesting to speculate whether this type of enzyme would have a role in the subterminal oxidation of propane by R.erythropolis BPSd1 isolated by Woods (1988). Such an enzyme may be involved in the metabolism of

the reduced C_1 moiety produced by the proposed C_2-C_1, cleavage of acetol (Coleman & Perry, 1984; Hartmans & deBont, 1986), see 1.3.2.2.

1.5 Genetics of n-alkane-utilizing microorganisms

Cardy (1989) has reviewed the genetics and molecular biology of the obligate methanotrophs, and as such will not be discussed here. The study of the genetics of hydrocarbon-utilizing microorganisms has mainly developed within the last decade. However, a greater understanding of the genetics of aromatic hydrocarbon-utilizing microorganisms has been achieved compared to the genetics of n-alkane-utilizing microorganisms, see Singer & Finnerty (1984b). A wide diversity of metabolic activities are coded for by catabolic plasmids. The two best studied plasmids are the alkane (alk) genes on a recombinant of two catabolic plasmids, CAM-OCT (for camphor and octane) and the TOL plasmid pWWO (for the toluene-xylene pathway) from P.putida ; see Williams (1981).

Conversely studies into the genetic systems of hydrocarbon-utilizing bacteria such as Corynebacterium, Mycobacterium, Nocardia and Rhodococcus are virtually non-existent.

1.5.1 Pseudomonas sp

Studies on Pseudomonas aeruginosa showed that whole-cell oxidation of hexane was induced by C_4 to C_8 n-alkanes (Van Eyk & Bartels, 1968). Butane induces hexane oxidation but does not support growth, although butan-1-ol does. Ethane and propane were ineffective as inducers. Malonate, a non-repressive carbon source, cyclopropane and diethoxymethane allowed gratuitous enzyme synthesis. Macham & Heydeman (1974) isolated NTG-generated mutants of P.aeruginosa defective in the metabolism of heptane. They showed by mutant complementation studies that the heptane-oxidizing system possessed at least two components, a

membrane-bound and labile component. They postulated the existence of a third component, probably rubredoxin.

Chakrabarty et al. (1973) demonstrated that the genes for the inducible octane hydroxylase were carried on the OCT plasmid in P.putida PpG6. Their conclusions were based on three pieces of evidence, each strongly suggesting plasmid involvement:

(i) Growth in the presence of mitomycin C resulted in the appearance of Oct^- strains;

(ii) Introduction of the CAM plasmid by conjugation resulted in CAM^+ exconjugants which were Oct^-;

(iii) The Oct^+ phenotype could be transferred from P.putida PpG6 by conjugation to isogenic Oct^- cured strains and to other Oct^- strains of P.putida at frequencies of 10^{-9} and 10^{-7}/donor, respectively.

Phenotypic examination of Oct^- strains revealed that all were capable of growth on octanol (Oct^+), suggesting a chromosomal locus for octanol dehydrogenase. They also proposed that the OCT plasmid must code for an octane-regulated octanol dehydrogenase. Shapiro et al. have elucidated the structure and regulation of the OCT plasmid, which is a large plasmid of the Inc P2 incompatibility class. It has been shown to carry alkA, B, C, E and R loci. Details of the genetic control and membrane model for octane oxidation are summarized in Figs 1.11 and 1.12.

The ability of P.putida PpG6 to utilize C_6-C_{10} n-alkanes was demonstrated by Neider & Shapiro (1975). NTG-generated revertible point mutants were isolated which had simultaneously lost the ability to grow on all five n-alkanes, but still grew on octanol or nonanol. An acetate-negative mutant (ace$^-$) defective in isocitrate lyase activity

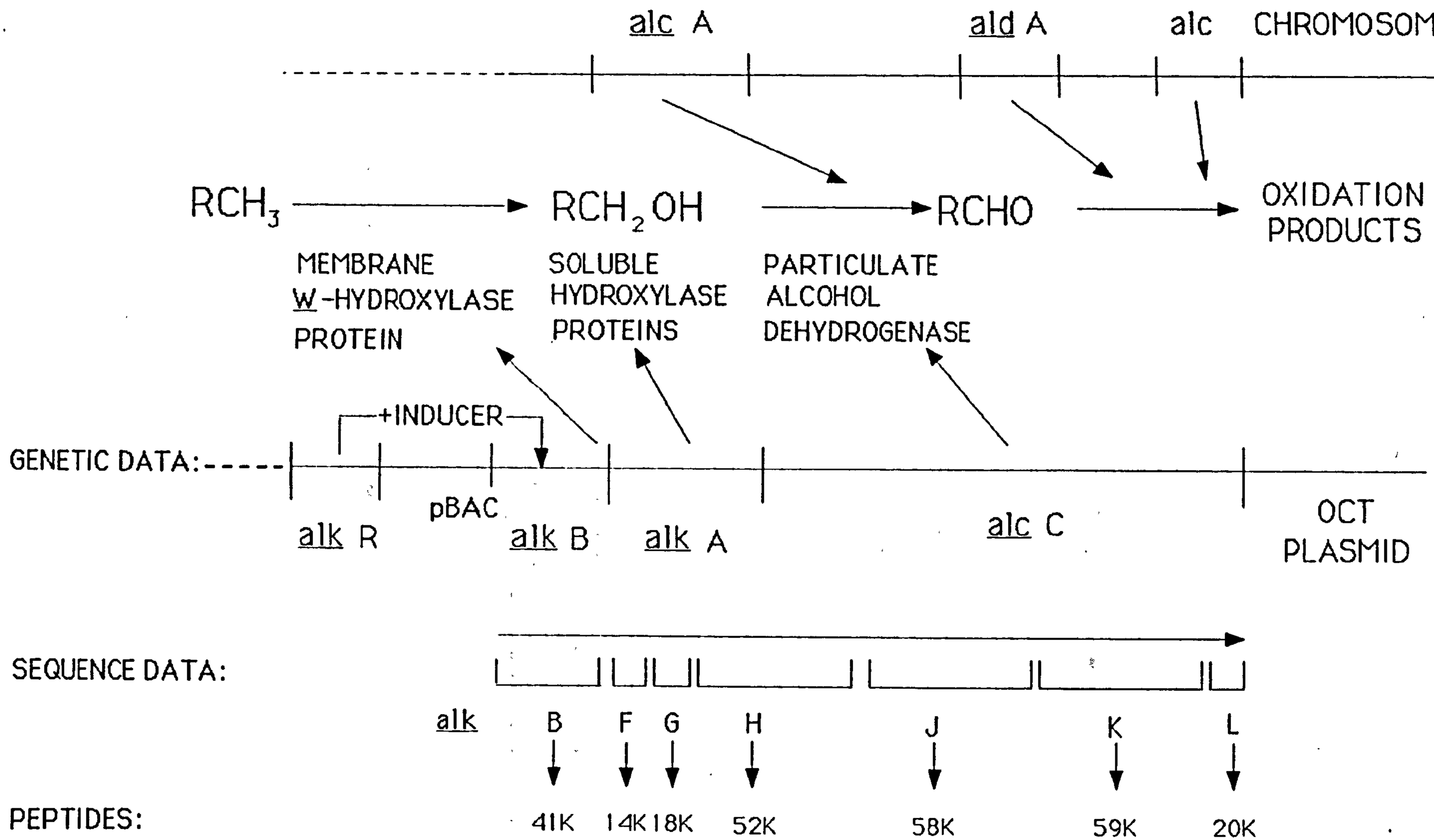


Figure 1.11 Genetic control of the n-alkane oxidation system in Pseudomonas (adapted from Williams (1981) and Kok et al (1989a))

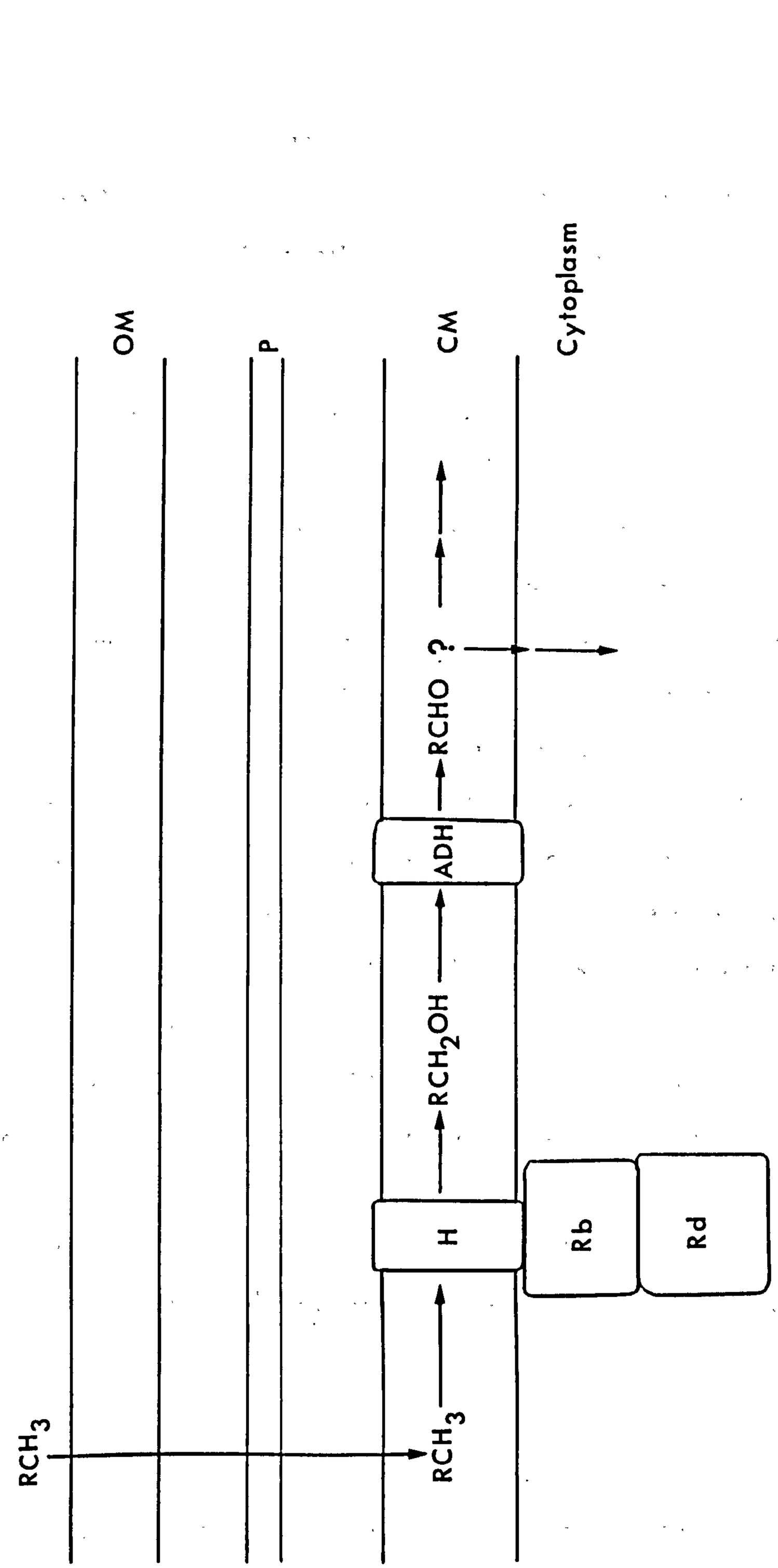


Figure 1.12 Membrane model of n-alkane oxidation in P.putida PpG6 (OCT) (from Benson et al., 1979).

was unable to grow on even chain-length alkanes and fatty acids. Analysis of double mutants defective in acetate and propanoate (ace$^-$ prp$^-$) metabolism showed the n-alkane was only assimilated *via* acetyl-CoA and propionyl-CoA.

Grund *et al*. (1975) demonstrated that the OCT plasmid codes for inducible alkane-hydroxylating and primary alcohol-dehydrogenase activities, and that the chromosome codes for constitutive oxidizing activities for primary alcohols, aliphatic aldehydes, and fatty acids. Mutant isolation confirmed the presence of an alcohol dehydrogenase locus on the OCT plasmid and indicated the presence of multiple alcohol and aldehyde dehydrogenase loci on the *P.putida* chromosome. Studies by Benson & Shapiro (1975) and Grund *et al*. (1975) also demonstrated that the unoxidized alkane molecules, as well as alcohols (pentan-1-ol, 1-, 2-, hexan-3-ol), ketones (heptan-2-one, octan-2-one), and terminal dienes (1,7-octadiene); are physiological inducers of both plasmid activities. Non-metabolizable compounds, such as dicyclopropyl ketone (DCPK), dicyclopropyl methane (DCPM) and diethyoxyethane, also served as effective gratuitous inducers of alkane-oxidizing activity.

Benson & Shapiro (1976) reported the identification of a plasmid-determined particulate NAD^+-independent alcohol dehydrogenase in octane-grown cells, see 1.4.2.1.

The plasmid-determined inducible alkane hydroxylase of *P.putida* was resolved into particulate and soluble fractions by Benson *et al*. (1977). The plasmid-determined activities were defined as a soluble alkane hydroxylase (*alk*A$^+$), particulate alkane hydroxylase (*alk*B$^+$) and particulate alcohol dehydrogenase (alkC$^+$). Growth tests and *in vitro* complementation revealed plasmid mutations which blocked expression of

alkane hydroxylase activity, alk$^-$, alkB$^-$ and a pleiotropic-negative class; which included nine mutations that lead to the loss of alkA$^+$, alkB$^+$ and alkC$^+$ activities. They suggested that the alk$^+$ gene cluster contained at least four cistrons.

Fennewald & Shapiro (1977) presented results which showed that the plasmid alk gene cluster constituted an operon. They also identified one cistron (later to become alkD) whose gene product participates in inducer recognition. The evidence for a single regulon was two-fold:
(i) alkA, alkB and alkC loci all responded to the same regulatory gene products;
(ii) mutations that blocked induction, altered inducer specificity, or lead to constitutive synthesis affected all three activities in the same way.

Studies by Fennewald & Shapiro (1979) and Fennewald et al. (1979) allowed the mapping of alk loci on the OCT plasmid; an excellent review of this work is given by Singer & Finnerty (1984b). Although this has been modified by the advent of molecular biological techniques.

The regulation of membrane peptides by the Pseudomonas plasmid alk regulon, and a membrane model for alkane oxidation has been discussed by Benson et al. (1979), Fig. 12. Induction of the alk regulon resulted in the appearance of three plasmid-determined cytoplasmic membrane peptides of about 59, 47 and 40 kDa molecular weight. The 40 kDa peptide was the membrane component of the alkane hydroxylase and product of the alkB gene. This was determined by the analysis of an alkB$^-$ mutant which had altered properties of alkane hydroxylase in whole cells, reduced thermal stability in cell-free extracts, and led to increase electrophoretic mobility of the inducible "40 kDa" peptide.

Local anaesthetics blocked the induction of the Pseudomonas alk operon (Benson, 1979). The results supported the hypothesis that induction of the plasmid-determined alkane oxidizing system in P.putida involves a membrane component(s).

Recently advances in molecular biology, including gene cloning and DNA-sequencing techniques, have allowed an even greater analysis of the alk operon in Pseudomonas, see Fig 1.11. Owen et al. (1984) cloned sequences of the alk operon and characterized them physically and genetically. These sequences were used to construct a DNA restriction map of the alkBAC region. Physical mapping of alkC::Tn7 insertions and complementation of alkC point mutations by cloned sequences from the alkBA region showed that previous studies by Fennewald & Shaprio (1979) were mistaken in inferring the existence of a separate unlinked alkC cluster. Studies with an alkB-lacZ transcription fusion construct established that the operon is transcribed in the order alkBAC and is under positive regulation by alkR. Results also demonstrated that the alkR gene was located upstream of alkB.

Eggink et al. (1987) constructed plasmids containing fusions of cloned alkBAC and alkR DNA to study the functional expression of the alkBAC operon and regulation in P.putida and E.*coli*. Both grew on octane and as the sole source of carbon and energy when they were supplied with the complete alk system cloned in the broad host range plasmid pLAFRI. However, while the system is active in P.putida, it is only active in fadR mutants of E.coli in which fatty acid degradation enzymes are expressed constitutively. The alkR locus was strictly required for the expression of the alkBAC operon.

Kok *et al*. (1989b) have sequenced a 2 kb segment of DNA which includes the *alk* promoter and *alk*B gene. This gene encodes a 401-amino acid polypeptide, the translation product contained nine hydrophobic sequences of which eight are sufficiently long to be membrane-spanning segments.

The most recent study showed that the *alk*BAC operon encoded seven proteins (Kok *et al*., 1989a). The *alk*A region contained three coding sequences, encoding two related rubredoxins (*alk*F and *alk*G) of 14 and 18 kDa and 52 kDa aldehyde dehydrogenase (*alk*H). The *alk*C region codes for another three polypeptides of 58, 59 and 20 kDa; *alk*J, *alk*K and *alk*L respectively, see Fig. 11.

The nucleotide composition of the *alk* genes (47% G + C) differed considerably from the G + C content of *Pseuomdonas* genome suggesting that the *alk* regulon may have originated from an unrelated organism.

1.5.2 *Acinetobacter*

In contrast to the alkane-oxidizing system of *P.putida* PpG6, that of *Acinetobacter* displays several significant differences. Unlike *P.putida*, *Acinetobacter* sp. HO1-N and *A.calcoaceticus* do not grow on alkanes shorter than decane (Singer & Finnerty, 1984b). Despite extensive studies no plasmids have been found in these strains. The Alk^+ phenotype could not be removed by introduction of plasmids of several incompatibility groups. Chemically induced Alk^- mutants could not be complemented by mating with the Alk^+ phenotype. All the evidence suggests chromosomal loci for alkane-oxidizing genes in *Acinetobacter*.

Singer & Finnerty (1984b) isolated several classes of Alk$^-$ mutants and using reciprocal transformation crosses (adapted from Sawula & Crawford (1972) and Ginther (1978)) postulated the existence of two separate alkX and alkY loci necessary for n-alkane oxidation.

The chemically induced point mutants of Acinetobacter with an Alk$^-$ phenotype do not grow on any n-alkanes but are still capable of using C_{10} to C_{22} alcohols and aldehydes. It was proposed that either alcohols and aldehydes do not have a role in alkane oxidation or that there exists separate pathways for alcohol and aldehyde metabolism not related to alkane metabolism (Cruze et al., 1979).

1.5.3 Saccharomycopis lipolytica

The only other organism which has been reasonably characterized with regard to the genetics of alkane metabolism is Saccharomycopsis lipolytica; for a review see Bassel & Ogrydziak (1979). The genes responsible for alkane metabolism seem to be chromosomally encoded. UV-generated mutants have been isolated which are blocked in the metabolism of alkane and the subsequent terminal oxidation products.

1.5.4 CMN-complex

In comparison to the work done on the genetics of liquid n-alkane oxidation, none has been done of the genetics of higher gaseous n-alkane oxidation. Mutants of Mycobacterium rhodochrous with modified patterns of n-alkane utilization were isolated after NTG-mutagenesis by Jenkins et al. (1972). All the mutants were defective in the first step of alkane oxidation, the activation of alkane to corresponding alcohol. This was shown by the fact mutants failed to grow on alkanes but still

utilized all oxidation products, e.g. alcohols, aldehydes and fatty acids. A possible reason for this skewed distribution is the method of mutant isolation, which was a penicillin enrichment with octane after mutagenesis. This would select specifically for first step "oxygenase" mutants. Different classes of mutants might have been obtained after mutagenesis if a penicillin enrichment included octanol, octanal, or octanoate.

Studies with Rhodococcus (Corynebacterium) fascians, a plant pathogen showed the presence of a large plasmid in pathogenic strains (Murai, 1980). Results indicated that cytokinin synthase may be coded for by plasmid gene(s). Plasmid containing strains, but not strains without plasmid, were able to utilize C_{11} - C_{18} n-alkanes. It was suggested that plasmid was necessary for the oxidation of alkanes. The CMN group of bacteria are known to harbour plasmids (Sandoval et al., 1985). Other reports concerning the relationship between hydrocarbon degradation and catabolic plasmids have been described for nicotine degradation by Arthrobacter oxidans (Brandsch et al., 1982) and aniline degradation by Rhodococcus sp. An-1 (Atkins & Cain, 1985). There has recently been a report on the transformation on Rhodococcus sp. with an E.coli-Rhodococcus shuttle vector (Singer & Finnerty, 1988), which may open up hydrocarbon metabolism by rhodococci to the techniques of molecular biology. Finally, the report by Hill et al. (1989) has demonstrated that Rhodococcus sp. genes may be expressed in an E.coli.

1.6 Synopsis

The genus Rhodococcus represents a group of nutritionally diverse bacteria and as such resemble the pseudomonads. However, by comparison their characterization to a biochemical and genetic level is poorly understood.

The metabolism of liquid n-alkanes is well documented by microorganisms. The general opinion favours terminal oxidation of n-alkanes as the major route of metabolism, although subterminal oxidation also occurs. Much of the evidence for existence of certain pathways has relied on simultaneous adaptation and product excretion studies, which must be viewed with caution. The most favoured mechanism for n-alkane oxidation is the direct incorporation of one atom of molecular oxygen into the alkane molecule by a monooxygenase.

The metabolism of ethane, propane and butane and the pathways involved remain unresolved. The relative importance of terminal versus subterminal oxidation of propane is unclear, although reports suggest that it may be oxidized via terminal, subterminal, or a mixture of the two pathways. Evidence of an enzymological nature is lacking, and in the case of genetics non-existent, for propane metabolism.

There have been a few reports in the literature of propane oxygenase activity. However, this activity has not been purified and characterized, unlike the two types of octane monooxygenase thus far described. The roles of ketone monooxygenases, alcohol and aldehyde dehydrogenases have also been described in relation to alkane metabolism. Two similar alcohol dehydrogenases have been purified and characterized in propane-utilizing bacteria. Although the roles of

ketone monooxygenase and aldehyde dehydrogenase are not clear in relation to propane metabolism.

The genetic systems of the CMN-group of bacteria are only poorly understood. The OCT plasmid system in Pseudomonas putida is the only well understood system in terms of its genes and regulation of alkane oxidation. The understanding of the system has recently reached new levels of sophistication with the advent of molecular biological techniques. This work was made possible because the biochemistry of the system was well characterized. Recent developments may allow these molecular biology techniques to be applied to hydrocarbon metabolism by Rhodococcus sp.

Chapter 2: Materials and Methods

MATERIALS AND METHODS

2.1 Growth and media

Rhodococcus rhodochrous PNKb1 and other propane-utilizing bacteria were isolated and maintained as described by Woods & Murrell (1989). They were grown at 30°C in the presence of propane - air (50:50, v/v), in a mineral salts medium containing, per litre, the following: Na_2HPO_4, 0.72 g; KH_2PO_4, 0.26 g; NH_4Cl, 1 g; $MgSO_4.7H_2O$, 1 g; $CaCl_2$, 0.2 g; Fe EDTA, 4 mg; $Na_2MoO_4.2H_2O$, 2.5 mg; EDTA, 0.5 mg; $FeSO_4.7H_2O$, 0.2 mg; $ZnSO_4.7H_2O$, 10 μg; $MnCl_2.4H_2O$, 3 μg; H_3BO_3, 30 μg; $CaCl_2.6H_2O$, 0.2 mg; $CuCl_2.2H_2O$, 1 μg; $NiCl_2.6H_2O$, 2 μg; $Na_2MoO_4.2H_2O$, 3 μg. This was similar to the ammonium mineral salts (AMS) medium described by Whittenbury et al. (1970). Solid medium was prepared by the addition of 15 $g.l^{-1}$ Difco Agar to AMS medium. The medium was sterilized by autoclaving at 121°C for 15 minutes. The medium was adjusted to pH 6.8. Flammable carbon sources (e.g. alcohols) were filter sterilized and added to the medium after inoculation. Non-flammable carbon sources (e.g. acetate, succinate) were added prior to inoculation. Concentrations of carbon sources used are given in Table 2.1.

R.rhodochrous PNKb1 was also maintained on nutrient agar, prepared according to the manufacturers instructions (Difco). R.rhodochrous PNKb1 was also grown in tryptone (0.3 % w/v) and yeast extract (0.5% w/v) liquid media to provide overnight cultures for work the following day.

Table 2.1 Substrate concentrations for routine growth

Substrate	Concentration (% v/v except *% w/v)
Propane	50% in air
Propan-1-ol	0.1
Propan-2-ol	0.05
Propanal	0.05
Propanone (Acetone)	0.05
Propanoate	*0.1
Hydroxypropanone (Acetol)	0.05
Methanol	0.1, 0.05
Methylacetate	0.1, 0.05
Methylglyoxal	0.1, 0.05
Primary n-alkanols (C_2-C_8)	0.1
Secondary n-alkanols (C_3-C_8)	0.05
Acetate[a]	*0.1, 0.035
Succinate[a]	*0.1, 0.035
Pyruvate[a]	*0.1, 0.035

[a]Sodium salt

2.1.1 Maintenance of propane-utilizing bacteria

Stock cultures of propane-utilizing bacteria were maintained by subculturing monthly onto AMS 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.

Stock cultures were also stored by adding 0.5 ml liquid propane-grown culture into 0.5 ml glycerol and storing at -20°C. Mutants defective in propane metabolism were also stored in glycerol. The strains and mutants used in the course of this work are shown in Table 2.2 and Table 2.3 respectively.

2.1.2 Routine growth

Flask cultures on propane and volatile potential propane oxidation intermediates (e.g. alcohols) were grown in 250 ml "quick-fit" flasks containing 50 ml of AMS medium. Cultures were inoculated with a loopful of cells from a plate or with 0.5 ml of an exponential growth phase culture. Flasks were sealed with a "Suba-seal" and 100 ml of air withdrawn using a sterile needle and syringe. 100 ml of propane were injected to give a 50% (v/v) propane: air mixture. When using volatile intermediates, the substrate was pipetted directly into the 50 ml AMS and then the flask was sealed. "Suba-seals" were swabbed with 70% (v/v) ethanol prior to injection of gases. Flasks were incubated on an orbital shaker at 200 rpm and 30°C.

Table 2.3 Propane oxidation mutants

Mutant	Class	Phenotype
alk 3*		
alk 4*		
alk 7		
alk 10*	alk$^-$	Propane$^-$ & Acetol$^-$
alk 24		
alk 25		
alk 33		
alk 50		
alcA 9		
alcA 12	alcA$^-$	Propane$^-$ & Propan-1-ol$^-$
alcA 13		
alcA 14		
alcB 8		
alcB 12	alcB$^-$	Propane$^-$ & Propan-2-ol$^-$
alcB 14		
alcB 26		
alcAB 17*	alcAB$^-$	Propane$^-$, Propan-1-ol$^-$ &
alcAB 18*		Propan-2-ol$^-$
ald 14*	ald$^-$	Propane$^-$, Propan-1-ol$^-$ & propanal$^-$
ket 2	ket$^-$	Propane$^+$ & Acetone$^-$
ket 6		
oate 6	oate$^-$	Propane$^-$, Propan-1-ol$^-$, Propanal$^-$ and propanoate$^-$
oate 4		
ace 1	ace$^-$	Propane$^-$, Propan-2-ol$^-$, Acetone$^-$, Acetol$^-$ and Acetate$^-$

*derived from wild-type R.rhodochrous (streptomycin sensitive)

(minus phenotype denotes lack of growth on indicated substrate)

Table 2.2 Propane - Utilizers

Strain	Phenotype	Source
Wild-Type	Propane-utilizer	Woods (1988)
Rhodococcus rhodochrous PNKb1	Nal^r_{20}	
R.rhodochrous PNKb1-Str^r_{20}	Propane-utilizer Nal^r_{20}, Str^r_{20}	This work
Pseudomanas butanovora DSM 2080	Propane and butane utilizer	Beers (1988)
Nocardia sp. OU	Propane-utilizer	J. Hunt, University of Warwick
Nocardia caviae	Propane-utilizer	E. Wellington, University of Warwick
Rhodococcus sp. An-1	Propane-utilizer	L. Atkins, University of Kent
Corynebacterium sp. GPYb1	Propane-utilizer	Woods (1988)
Rhodococcus sp. 69	Propane-utilizer	Woods (1988)
Nocardia sp. 56	Propane-utilizer	Woods (1988)

Propane-grown batch cultures of R.rhodochrous PNKb1 were established using a 2 litre LH 500 Series fermentor or a 20 litre LH 100 Series fermentor (Stoke Poges, Bucks, UK). Inocula for the 2 litre fermentor were 2 x 50 ml shake flask cultures in mid-exponential phase of growth. Inocula for the 20 litre fermentor were 2 litres of culture generated from the 2 litre LH 500 Series fermentor. Propane and air were supplied to the 2 litre fermentor at 80 $ml.min^{-1}$ each and to the 20 litre fermentor at 500 $ml.min^{-1}$ each (to give 50:50 (v/v) propane - air mix). AMS medium was used at a constant pH of 6.8 and a temperature of 30°C.

The optical density was measured at 540 nm (OD_{540nm}) against a sterile medium blank. Samples were removed regularly and examined microscopically and the OD_{540nm} recorded. Samples were also streaked onto nutrient agar plates and incubated at 30 and 37°C to check the purity of the culture.

2.1.3 Growth of mutants under propane-inducing conditions with low concentrations of growth substrates

In studies with the wild-type organism and NTG-generated propane oxidation mutants, the propane oxidation system was induced by growing cells on low concentrations of growth substrates (succinate, pyruvate and citrate at 0.035% (w/v)) in the presence of 50% (v/v) propane in air. The formation of 1,2-epoxypropane from propene by whole-cells and the induction of propane-specific polypeptides (as described in the methods), were used to indicate the induction of the propane-oxidizing system; controls used low concentrations of growth substrates without propane. A similar rationale used by Weaver & Lidstrom (1987) in the

analysis of Xanthobacter H4-14 methanol mutants was followed in this study.

2.1.4 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 0.2 μm. Filters and cells were then dried in an oven at 90^{o}C to constant weight.

2.1.5 Light Microscopy

Cultures were examined regularly to check their purity. Slides were prepared by placing a drop of culture on a slide and then pressing down firmly onto it using a coverslip. The microscope (Kyoma Unilux 11, Japan) was set in phase-contrast mode at a magnification of 1600X.

2.2 Studies using whole cells

2.2.1 Preparation of cell suspensions

Cultures from flasks or fermentors were harvested by centrifuging at 20,000 xg for 10 min at 4^{o}C. Cell pellets were washed in 20-30 ml of ice-cold 20 mM TRIS-HCl buffer, pH 6.8 and centrifuged as before. The cell pellets were then resuspended in the same buffer to give 1/20th final volume and the suspension used for whole cell studies.

2.2.2 Oxygen electrode assays

The ability of R.rhodochrous PNKb1 to oxidize a variety of substrates was tested by measuring the stimulation of oxygen uptake on addition of the substrate to cell suspension contained in a Clarke-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK). Assays were done at 30°C in 2.9 ml of 20 mM phosphate buffer, pH 6.8. Air-saturated buffer was placed in the reaction chamber, the plunger inserted and the system was allowed to equilibrate. 50 μl of cell suspension was then added by syringe and the endogenous rate of oxygen uptake measured. 50 μl of substrate were then injected and any stimulation in oxygen uptake recorded on a chart recorder. Rates of oxygen uptake were corrected for endogenous rate and expressed as nmoles 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).

Saturated propane solutions were prepared by degassing 5 ml of distilled water under vacuum then passing the contents of a football bladder inflated with propane gas through the water. Substrates were prepared as 2 mM solutions giving a final concentration in the assay of 33.3 μM (Woods & Murrell, 1989). The final concentration of propane was calculated to be 23.7 μM using the data of McAuliffe (1966).

2.2.3 Formation of 1,2-epoxypropane from propene

The formation of 1,2-epoxypropane was determined by gas chromatography. Assays were performed in 5 ml "Suba-sealed" vials in a total volume of

1.0 ml. The buffer used was 20 mM TRIS-HCl, pH 6.8. 50 μl of a cell suspension were added to 950 μl of buffer in a sealed vial. The vial was preincubated to 30^{o}C in a shaking water bath for 30 seconds then 3 ml of air were removed from the vial and replaced with 3 ml of propene. At 5 minute intervals 5 μl samples were injected onto a glass column (1.5 m x 2.3 mm) packed with Porapak Q. It was run at 180^{o}C with 30 ml.min^{-1} nitrogen as carrier gas. Output from the FID was logged on a reporting integrator that had been calibrated with 1,2-epoxypropane standard. Rates were expressed as nmoles 1,2-epoxypropane formed min^{-1} mg dry weight^{-1} cells.

2.3 Studies using cell-free extracts

2.3.1 Preparation of cell-free extracts

Cells were harvested at culture densities (OD_{540nm}) of 0.8 i.e. during the late logarithmic phase of growth and centrifuged at 20,000 xg for 10 min at 4^{o}C. Cell pellets were washed in ice-cold 20 mM TRIS-HCl, pH 6.8 and centrifuged as before. Cells were resuspended in 1/20th volume of the same buffer and then disrupted by three passages through a French pressure cell (Aminco, Silver Spring, Maryland, U.S.A.) at a pressure of 138 MPa. The pressure cell was cooled to 4^{o}C and extracts collected on ice in precooled universals.

After breakage, unbroken cells were removed by centrifugation at 20,000 xg for 5 min at 4^{o}C. To separate soluble and membrane bound proteins, crude extracts were subject to centrifugation at 48,000 xg for 30 min at 4^{o}C. The supernatant was then taken as the soluble fraction and the pellet, resuspended in 20 mM TRIS-HCl, pH 6.8 as the particulate

fraction. The protein content of extracts was determined by the method of Bradford (1976) using the BioRad standard protein microassay according to manufacturers instructions. A standard curve was prepared using bovine serum albumin.

2.3.2 Enzyme Assays

2.3.2.1 Alcohol/Aldehyde dehydrogenase

(i) $NAD(P)^+$-dependent activity: This was measured spectrophotometrically using a Shimadazu UV-150-02 spectrophotometer by monitoring the alcohol/aldehyde dependent change in absorbance at 340 nm due to the formation of NADH(P). Assays were done in a total volume of 1 ml. The cuvette contained, in 20 mM TRIS-NaOH, pH 10.0, 0.2 μmol of NAD^+ and a sufficient amount of protein to give a linear rate for more than 5 min. Cuvettes were allowed to equilibrate at 30°C for 1 min before the reaction was started by the addition of 10 μmol of alcohol or aldehyde. The change in absorbance at 340 nm was followed for 5-10 min.

The ketone-dependent oxidation of NADH by purified secondary alcohol dehydrogenase was monitored as above except that the cuvette contained 20 mM TRIS-HCl, pH 6.5 and 0.2 μmol of NADH. The reaction was initiated by the addition of 10 μmol of ketone.

(ii) Phenazine methosulphate (PMS) linked activity: This was measured spectrophotomerically by the change in absorbance at 600 nm due to the reduction of dichlorophenol indophenol (DCPIP) by reduced PMS. Assays were done in a total volume of 1.5 ml. The cuvette contained, in 20 mM TRIS-NaOH, pH 9.0 (which had been sparged with oxygen-free nitrogen for

1 minute), 0.11 μmol of PMS, 0.13 μmol DCPIP, 45 μmol NH_4Cl and cell-free extract containing 0.1 - 1.0 mg of protein. Cuvettes were allowed to equilibrate at 30°C for 1 min before the reaction was initated by addition of 10 μmol of alcohol. The change in absorbance at 600 nm was followed for 5-10 min.

2.3.2.2 Assay of product formation from purified NAD^+-dependent Secondary Alcohol Dehydrogenase

The formation of products from the NAD^+/NADH-dependent oxidoreductase reaction was determined by gas chromatography; using a (2.0 m x 4 mm) glass column packed with Porapak R, operated isothermally at 160°C with nitrogen as carrier gas at 20 ml min^{-1}. Products were identified against freshly made 2 mM standards of propan-2-ol and acetone. Products were detected using a FID output of which was logged by a Hewlett-Packard 3390A Integrator.

The buffer used was 20 mM TRIS-NaOH/HCl, pH 10/6.5 and 0.2 μmol NAD^+/NADH (total volume 1.0 ml). The assay contained 10 μg of purified secondary alcohol dehydrogenase which was preincubated to 30°C in a shaking water bath for 30 seconds. The reaction was initiated by the addition of 10 μmol propan-2-ol or acetone. 10 μl samples were removed at 10 min intervals and injected onto the column.

2.3.2.3 Ketone monooxygenase

Acetol and acetone monooxygenase activity was measured as substrate-dependent stimulation of oxygen uptake in a Clarke-type oxygen electrode, based on a method by Hartmans & de Bont (1986).

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

2.4 Purification of NAD^+-dependent Secondary Alcohol Dehydrogenase

All steps were performed at 4°C. The buffer used throughout the purification was 20 mM TRIS-HCl, pH 6.8, containing 1 mM dithiothreitol (Buffer A). The soluble cell-free extract was applied to a DEAE-cellulose column (30 x 2.5 cm i.d.) equilibrated with Buffer A. After washing with two column volumes of Buffer A to remove unbound proteins, the enzyme was eluted with a step-gradient (each 0.1 M increment being one column volume) from 0 to 0.5 M NaCl in Buffer A. Fractions showing highest alcohol dehydrogenase activity were combined and dialysed overnight against 10 litres of Buffer A. The active fractions from the DEAE-cellulose column were applied to a type 3 NAD-agarose (Sigma) column (4 ml bed volume) [NAD is linked to agarose via the C8 of the purine ring (Barry & O'Carra, 1973)]. The column was washed with 100 ml Buffer A containing 0.5 M NaCl to remove any unbound proteins. Secondary alcohol dehydrogenase was selectively eluted with Buffer A containing 0.5 M NaCl and 1 mM NAD^+ and the active fractions dialysed overnight against 10 litres of Buffer A to remove NAD^+ and NaCl. The purified enzyme was stored at -70°C, after freezing by dropwise addition to liquid nitrogen.

2.5 M_r determination

The molecular weight of alcohol dehydrogenase was determined by HPLC-gel filtration using a 25.1 i.d. x 600 mm preparative TSK 3000 SW column (Tosoh corp., Toyko, Japan) fitted with 21.5 x 75 mm TSK SW guard column, equilibrated with Buffer A containing 100 mM NaCl and run at a flow rate of 3 ml min^{-1}. Bovine catalase (232,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic hydratase (29,000) and horse heart cytochrome C (12,400) were used as molecular weight standards.

The subunit size of alcohol dehydrogenase was determined by SDS-PAGE using the molecular weight markers phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (33,000), soybean trypsin inhibitor (20,100) and bovine α-lactalbumin (14,000). Relative mobilities were measured with respect to bromophenol blue.

2.6 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 3.0 M TRIS-HCl, pH 8.8 and the stacking gel in 0.5 M TRIS-HCl, pH 6.8. The running buffer was TRIS-glycine (0.025 M TRIS base, 0.192 M glycine). Gels were formed either 4.5 to 15% (w/v) exponential gradient of acrylamide or single percentage acrylamide (10.5% (w/v)). Electrophoresis was carried out at a constant current of 8mA overnight. For sodium dodecyl sulphate (SDS) electrophoresis, the stacking gel, resolving gel and running buffer contained SDS at 0.1% (w/v).

Cell-free extracts for SDS-PAGE were boiled for 5 minutes in sample buffer (0.125 M TRIS-HCl, pH 6.8, 10% (w/v) sucrose and 4% (w/v) SDS). Extracts for non-denaturing PAGE were prepared in the same buffer without SDS. Sample buffers contained 0.1% (w/v) bromophenol blue as a marker.

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.

Polyacrylamide gels were also silver stained according to the rapid and simple method of Wray et al. (1981). Gels were soaked overnight in 50% (v/v) methanol. After this a stain solution was prepared by dissolving 0.8 g of silver nitrate in 4 ml of distilled water to produce solution A. Then 21 ml of 0.36% (w/v) sodium hydroxide was mixed with 1.4 ml of 14.8 M ammonium hydroxide to produce solution B. Solution A was added dropwise to solution B with constant vortexing and finally made up to 100 ml with distilled water. The methanol washed gel was soaked in the stain solution for 15 min. Then the gel was washed with at least 1000 ml distilled water for 10 min. The gel was then soaked in developer solution until bands appeared in not more than 15 min. The developer solution was prepared by mixing 2.5 ml of 1% (w/v) citric acid and 0.15 ml of 37% (w/v) formaldehyde solution and making the volume up to 500 ml with distilled water. The gel was then washed with water and placed in 45% (v/v) methanol, 10% (v/v) acetic acid to stop stain development.

2.6.1 Alcohol dehydrogenase activity stain

The alcohol dehydrogenase activity stain was based on the method of Stirling & Dalton (1978). Samples were electrophoresed on non-denaturing 4.5-15% (w/v) gradient polyacrylamide gels at 4°C. The gel was then incubated for 15 min in the dark in the assay mixture containing 20 mM TRIS-NaOH, pH 10.0, 4 mM nitroblue tetrazolium; 1.5 mM phenazine methosulphate; 15 mM NAD^+ and 200 mM propan-1-ol or propan-2-ol. A gel incubated in the above mixture minus substrate was used as a control.

2.6.2 Isoelectric focusing (IEF)

This was carried out using preformed 3.0 -9.0 pH gradient polyacrylamide gels, electrophoresed on a Pharmacia Phast System in accordance with the manufacturers recommendations. Protein was visualized by silver staining using the automated system of the machine and staining kit supplied by the manufacturer (Pharmacia).

2.7 N-methyl-N-nitro-N-nitrosoguanidine (NTG) mutagenesis

CARE: NTG is a potent mutagen and carcinogen. It must be handled with caution. A stock solution was prepared to give a final concentration of 1000 μg ml^{-1} NTG in distilled water. This was then filter sterilized using a 0.22 μm filter and stored at -20°C until required.

Tryptone-yeast extract grown *R.rhodochrous* PNKb1-Str^r_{20} was incubated during the logarithmic phase of growth (OD_{540nm} 0.6) for varying times with NTG at 150 μg ml^{-1} at 30°C in a shaking waterbath. 5 ml samples

were taken at t = 0, 5, 10, 15, 20, 25 and 30 min, washed twice in AMS medium and then serially diluted and plated onto nutrient agar to give viable counts. The results were used to construct a 'kill-curve'. The time taken to give a 50% 'kill' was used to obtain mutants (12 min).

Individual colonies were replica-plated onto AMS succinate (master plate) and AMS plates for selection of propane defficient mutants, or AMS plates plus potential propane oxidation intermediates (for volatile intermediates e.g. propan-1-ol, propan-2-ol, etc plates were incubated in their vapor) to screen for mutants defective in particular steps of propane metabolism. A known volume of volatile intermediate was spotted onto filter papers in a petri-dish. The vapor was contained within a "Tupperware" box of known volume which was sealed with plastic tape. Plates were incubated from 2-3 weeks depending on the carbon source deficiency being screened for. Mutants gave a white streak of growth on the test plate. This was due to the utilization of the agar as a carbon source.

2.8 Immunological Techniques

2.8.1 Production of antibodies

100 μg of purified secondary alcohol dehydrogenase were mixed in 0.5 ml of distilled water 1:1 with Freund complete adjuvant, passed between two syringes to form an emulsion, and injected intra-muscularly into a New Zealand White rabbit. A blood sample (approx 5 ml) was taken prior to injection to yield the pre-immune serum. The rabbit was boosted after 14 days with another 100 μg of purified protein via the ear vein. Again

a blood sample was taken prior to injection to provide the immune serum. This process was repeated weekly for another 4 weeks.

Blood samples collected were left at room temperature for 1 hr in a glass universal, after which the clot was detached from the side of the universal. The sample was stored overnight at 4°C after which the clot was discarded and the serum centrifuged at 10,000 xg for 5 min to removed red blood cells and debris. The resultant supernatant was stored at -70°C until required.

2.8.2 Western-Blotting

Proteins separated by SDS-PAGE and non-denaturing PAGE were transferred to Hybond-C (Amersham) nitro-cellulose using a Biorad Trans-Blot system at 300 mA for 3 hr in buffer containing 25 mM TRIS-HCl, pH 8.3, 192 mM glycine, 20% (v/v) methanol as originally descirbed by Towbin et al. (1979). After this procedure, the filter was removed and washed in distilled water to remove excess salt. Ponceau S (0.5% (w/v) in 5 % (w/v) TCA) was used to visualize protein on the nitrocellulose filter. The filter was incubated in dye solution for 10 min, and unbound dye removed by washing with distilled water. Filters were transferred to TBS solution (50 mM TRIS-HCl, pH 8.0, 150 mM NaCl) to remove all remaining bound dye.

The method used for the immunological detection of secondary alcohol dehydrogenase was based on that first described by Burnette et al. (1981). Peroxidase conjugated goat anti-rabbit IgG (Sigma) was used as secondary antibody and 4-chloronaphthol (Sigma) was used as the colour reaction agent. After transfer, filters were blocked in 20 ml TBS

containing 2% (w/v) Marvel (dried skimmed milk) for 1-2 hrs. After this fresh 20 ml TBS Marvel were added containing primary antibody against the alcohol dehydrogenase at a dilution of 1:5000 and left shaking overnight. Primary antibody was removed by washing the filter three times for 10 min with 20 ml TBS containing 0.1% (v/v) Tween 20. Fresh TBS containing 0.1% (v/v) Tween-20 (20 ml) was added to the filter containing goat anti-rabbit peroxidase conjugate IgG at a dilution of 1:333 and left for 1-2 hrs shaking at room temperature. Secondary antibody was removed by washing the filter for 10 min each with two washes of TBS containing Tween-20 and then TBS.

Prior to staining solution A (1.5 g NaCl, 1 ml 1M TRIS-HCl, pH 7.5, in a total volume 50 ml distilled water) and solution B (30 mg chloronaphthol, 10 ml methanol in a total volume 50 μl distilled water) were made. 50 μl hydrogen peroxide was added to solution A. Then A and B mixed together, the 100 ml staining solution was sufficient for 1 or 2 full blots. The colour developed between 5-20 min after which time the filter was washed well in distilled water.

More details on the immunological techniques used are given by Harlow & Lane (1988).

2.9 Plasmid screening of Propane-utilizers

To obtain cells for plasmid screening, overnight tryptone-yeast extract grown cultures of propane-utilizers were inoculated (10% v/v) into fresh tryptone-yeast extract containing 3% (w/v) glycine. The cells were then grown for 24 hrs. Most strains of propane-utilizers grew as flocculent

aggregates in the presence of glycine, although these could be dispersed readily by vortexing briefly.

A plasmid isolation method was used from that of Hansen & Olsen (1978) (developed by L. Atkins, per. comm.) incorporating a phenol-chloroform extraction similar to that of Kado & Liu (1981). However, initial experiments used the plasmid isolating techniques of Eckhardt (1978), Hansen & Olsen (1978), Wheatcroft & Williams (1981) Kado & Liu (1981) and Maniatis et al. (1982).

2.9.1 Reagents

TBE buffer:	TRIS-Borate (0.89 M TRIS-base, 0.89 M BORIC acid) EDTA (0.002 M) (pH8)
Lysozyme solution:	Lysozyme (2.0 mg ml^{-1}) freshly dissolved in TBE buffer containing sucrose (20% w/v)
Lysing solution:	SDS (16% (w/v)), EDTA (200 mM) in TBE buffer
Phenol-Chloroform:	Phenol: Chloroform: isoamyl alcohol (24:25:1 by volume)
Loading buffer:	Bromophenol blue (0.1% (w/v)) in TBE: glycerol (1:1 by volume)

2.9.2 Cell lysis

Glycine treated cultures (5 ml) were harvested by centrifugation 3,000 xg for 10 min and the cells resuspended in 1.0 ml lysozyme solution. After incubation at 30°C for 40 min, cells were centrifuged as above and resuspended thoroughly in 0.5 ml TBE, followed by the addition of 125 µl lysing solution and mixed by inversion. The mixture

was maintained at room temperature for up to 15 min with occasional mixing until a substantially clear lysate was obtained.

2.9.3 Purification of plasmid DNA

The cell lysate was subjected to alkaline denaturation by addition of 55 μl 3 M NaOH. After 3 min 250 μl 3 M TRIS-HCl, pH 7.0 was added and mixed well by gentle inversion. Chromosomal DNA was precipitated by addition of 250 μl 5 M NaCl, followed by cooling to 4°C for 90 min, and centrifugation 14,000 xg for 10 min at 4°C. The supernatant was mixed with two volumes of phenol-chloroform by gentle inversion (x 20), followed by centrifugation 14,000 xg for 10 min at 4°C, after which the aqueous phase was carefully transfered to a fresh tube without disturbing the precipitate at the interface. Plasmid DNA was precipitated by addition of two volumes of ethanol (-20°C), followed by cooling to -70°C for 90 min, and centrifugation 14,000 xg for 10 min at 4°C. The pellet was washed with 1 ml of ice-cold ethanol, dried in a vacuum desiccator and dissolved in 50 μl TBE buffer.

2.9.4 Agarose gel electrophoresis

Horizontal agarose slab gels were used routinely. Slab gels were prepared by boiling Agarose (Type II medium EEO-Sigma) in TBE electrophoresis buffer and cooled to ca. 45°C before pouring. 0.6% (w/v) gels were used routinely. DNA samples were prepared by addition of 1/10th volume loading buffer. Electrophoresis was carried out in Cambridge Bio-Sciences mini-horizontal gel system with the gel completely submerged in TBE buffer at 40 mA for 1 hr.

The DNA was stained within the agarose gels with ethidium bromide (0.5 μg ml^{-1}) as described by Maniatis et al. (1982) and then visualized by transillumination with short-wave UV light and photographed using Polaroid Type 665 black and white film. The camera was fitted with a red filter.

2.10 Photography

Polyacrylamide gels and nitro-cellulose sheets were photographed from above using a Pentax SP500 camera fitted with a yellow filter and using Kodak Pantomic X film.

2.11 Chemicals

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

Sigma (London) Chemical Co Ltd, Poole, Dorest, UK; Aldrich Chemical Co Ltd, Gillingham, Kent, UK; BDH Chemicals Ltd, Poole, Dorest, UK; Koch-Light Laboratories Ltd, Colnbrook, Bucks, UK; Fisons Scientific Apparatus, Loughborough, Leics, UK; British Oxygen Co Ltd, London, UK.

Chapter 3: Results and Discussion

3.1 Isolation and characterization of NTG-generated propane oxidation mutants of R.rhodochrous PNKb1

3.1.1 Introduction

The aim of this study was to elucidate the route(s) of propane oxidation in R.rhodochrous PNKb1 using a genetical approach. A previous study by Woods (1988) used simultaneous adaptation techniques to determine the fate of propane, however, the route(s) of metabolism remained unclear. It was found that R.rhodochrous PNKb1 had the potential to metabolize propane by either terminal and/or subterminal pathways. An insight into the relative importance of terminal and subterminal oxidation would have been gained from purifying the inducible propane oxygenase, however, this was not possible because of the unstable nature of the system (see section 1.4.1.1).

The practical approach has been to isolate classes of NTG-generated mutants blocked at each step of the postulated propane oxidation pathway (see Woods & Murrell, 1989; for a summary of possible pathways). This work has been aided by the fact that the organism utilizes all potential propane oxidation intermediates, except methanol, pyruvaldehyde and methyl acetate.

It has been assumed that propane and potential oxidation intermediates are freely diffusible into the cell and do not require transport systems, however see 3.1.5. Most of these mutants are derived from a R.rhodochrous PNKb1-Str^r_{20} strain, which grew normally on all substrates. Incubation of cells with NTG (150 μg ml^{-1}) for 12 minutes gave a survival rate of 50%. This resulted in the generation of approximately 1% auxotrophs estimated by replica-plating individual

colonies after NTG-mutagenesis onto AMS plus succinate and nutrient agar plates and approximately 0.3% propane oxidation mutants; estimated by replica-plating individual colonies after NTG-mutagenesis onto AMS plus succinate and AMS plates incubated in an anaerobic jar with 50% (v/v) propane. Also notable after mutagenesis was the appearance (approx. 0.5%) of colourless or "white" mutant colonies, these were streptomycin resistant and still retained the ability to utilize propane as a growth substrate. No spontaneously occurring white colonies were ever observed. Pigment production in actinomycetes has been best studied in Streptomyces spp., for example the biosynthesis of actinorhodin and prodiginine (Gerber & Rechevalier, 1976; Rudd & Hopwood, 1980). Recently, Hill et al. (1989) have undertaken studies involving pigment genes from Rhodococcus, however the exact chemical nature of these pigments was not elucidated.

Mutants unable to grow on propane or its potential oxidation intermediates were isolated by using a lack of growth on a particular intermediate (e.g. propan-1-ol, propan-2-ol, etc), then the step at which they were blocked tested by growth in liquid culture. This was because initial experiments showed that when a propane deficiency trait was selected by replica-plating, a skewed population of first step mutants was obtained, i.e. they failed to utilize propane for growth but still retained the ability to oxidize all potential intermediates. However, when a particular metabolism deficient mutant was required it was necessary to directly select for it (e.g. alcA$^-$ mutants were selected for on propan-1-ol plates, etc). A similar procedure was adopted by Grund et al. (1975) when they isolated NTG-generated mutants of P.putida blocked in various steps of octane oxidation. Jenkins et al. (1972) working on the metabolism of n-alkanes by Mycobacterium rhodochrous also observed a skewed population of first step mutants,

i.e. they failed to utilize alkanes as growth substrates, but still retained the ability to utilize all potential oxidation intermediates.

The phenotypic symbols used to describe the mutants isolated are: alk, growth on propane and acetol; alcA, growth on propan-1-ol; ald, growth on propanal; oate, growth on propanoate; alcB, growth on propan-2-ol; ket, growth on acetone; ace, growth on acetate. The symbols alcAB$^-$ and alkR$^-$ designate propan-1-ol and propan-2-ol metabolism deficient double mutants, and theoretical propane oxidation regulatory mutants respectively. This nomenclature was based on that initially described by Grund et al. (1975).

There was the possibility that the deficiency trait could be assigned to the toxicity of a particular substrate being tested. However, this was not the case as all mutants could be grown on succinate in the presence of the intermediate they failed to metabolise (concentrations are described in Table 1.1). The NTG-generated mutants isolated can be regarded as revertable single point mutants as demonstrated by reversion frequencies of 2.4×10^{-7}-10^{-9}. It is probable that deletion or multiple point mutants would not yield any revertant phenotypes.

3.1.2 alk$^-$, alkR$^-$ mutants

Mutants which were alk$^-$ failed to utilize propane as a growth substrate, but retained the ability to utilize all potential terminal and subterminal intermediates except acetol (Table 3.1). It is proposed that alk$^-$ mutants are defective in the structural gene(s) (alk) or regulatory gene(s) (alkR - see section 3.4) of an acetol/propane oxygenase system. Indeed SDS-PAGE of wild-type R.rhodochrous PNKb1 cell-free extracts after growth on propane and acetol revealed proteins

Table 3.1 Characterization of alk$^-$ mutants

Class	No. of Mutants	Growth Substrates: Propane	Propan-1-ol	Propanal	Propanoate	Propan-2-ol	Acetone	Acetol	Acetate	Propan-1,2-Diol	Succinate
alk$^-$	8	-	+	+	+	+	+	-	+	+	+

- = no growth + = growth

N.B. Wild-type grows on all intermediates

of molecular weights 68, 64 and 48 kDa specific to cells grown on those substrates (Fig. 3.1). However polypeptides of 76 and 41 kDa were present which were specific to cell-free extracts from propane-grown cells. Such an enzyme system may metabolize propane by the reduction of an alkyl hydroperoxide to either propan-1-ol or propan-2-ol (Boyer et al., 1971); see section 1.2.2.4, and acetol by way of a Baeyer-Villiger reaction yielding acetate and a reduced C_1 unit (Hartmans & deBont, 1986), both reactions are mechanistically similar (see Sykes, 1975).

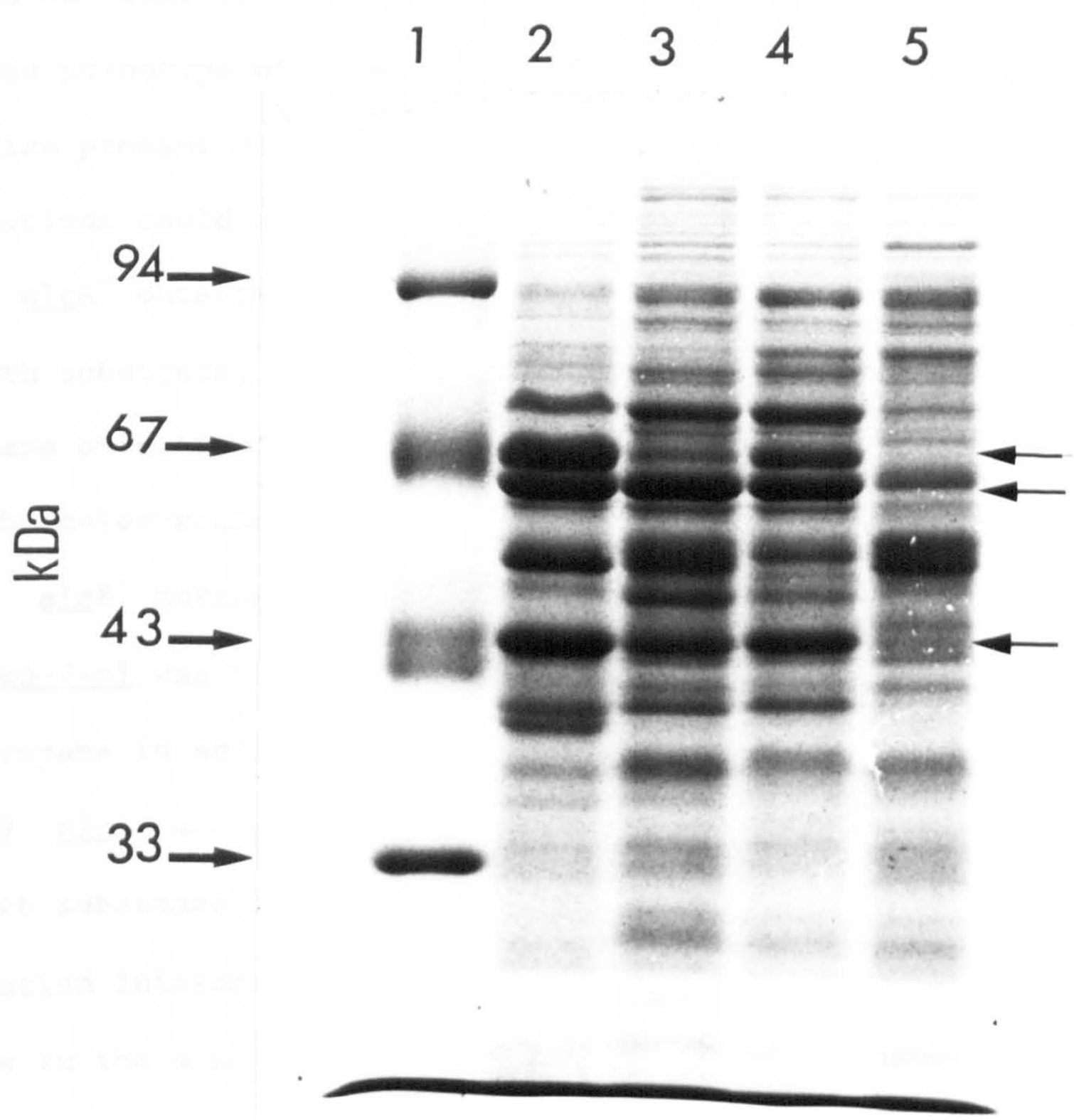
This is the first report to demonstrate a relationship between propane and acetol in terms of genetics and biochemistry (albeit at a preliminary level, see section 3.3 for further evidence). Studies by Perry (1968) showed that propan-2-ol and acetone were simultaneously adapted to oxidize propane, and it is these two compounds which have received most attention as potential oxidation intermediates, acetol has only received scant attention in the past (see section 1.3.3.2).

Alk$^-$ mutants, despite having the inability to utilize acetol (a proposed intermediate of propan-1,2-diol metabolism, see section 1.3.3.1) still had the ability to utilize propan-1,2-diol as a growth substrate. A possible explanation for this result is that the latter is not metabolized exclusively via acetol in R.rhodochrous PNKb1 (see section 1.3.2.2 for alternative pathways of propan-1,2-diol metabolism). Growth on the latter does not induce the synthesis of 68 kDa polypeptide, although the 64 and 48 kDa polypeptides were synthesized as was found in propane and acetol-grown cells (Fig. 3.1). A 72 kDa polypeptide was synthesized as in acetol-grown cells, and a 53 kDa polypeptide specific to propan-1,2-diol grown cells was also synthesized.

Figure 3.1 SDS-PAGE of cell-free extracts of R.rhodochrous PNKb1 grown on propane, propan-1,2-diol and acetol.

Track	Growth Substrate
1	(Molecular weight markers)
2	Propane
3	Propan-1,2-diol
4	Acetol
5	Citrate

100 μg protein in each track


1
2
3
4
5
94
67
43
33
kDa

3.1.3 alcA, alcB and alcAB mutants

Analysis of propan-1-ol and propan-2-ol growth negative mutants, alcA$^-$ and alcB$^-$ respectively; demonstrated that there are at least two genetic loci involved in the oxidation of these alcohols. Double mutants, alcAB, unable to utilize either propan-1-ol or propan-2-ol were also isolated after selecting for an alcohol deficiency phenotype. The common phenotype of these three classes of mutants is the inability to utilize propane as their sole carbon and energy source. Three possible situations could be envisaged before the mutagenesis experiments:

(i) alcA$^-$ mutants would still have the ability to utilize propane as a growth substrate, thus precluding propan-1-ol as an intermediate of propane oxidation; thus subterminal oxidation of propane would probably be the major route of assimilation;

(ii) alcB$^-$ mutants would give the opposite result, indicting that propan-2-ol was not an intermediate, thus implicating terminal oxidation of propane in assimilation.

(iii) alcA$^-$ and alcB$^-$ mutants both failed to utilize propane as a growth substrate, implicating both propan-1-ol and propan-2-ol as oxidation intermediates, thus terminal and subterminal would play major roles in the assimilation of propane.

The latter case represents the situation for the metabolism of propane by R.rhodochrous PNKb1 (Table 3.2). This suggests that the propane oxygenase inserts an atom of oxygen into the propane molecule indiscrimately producing a mixture of propan-1-ol and propan-2-ol (see section 1.4.2.1), which are then metabolized by distinct oxidation pathways. Woods & Murrell (1989) have already demonstrated that this organism has the metabolic capacity to operate terminal and subterminal oxidation pathways although they were unable to measure a build up of

Table 3.2 Characterization of alc⁻ mutants

		Growth Substrates:								
Class	No. of mutants	Propane	Propan-1-ol	Propanal	Propanoate	Propan-2-ol	Acetone	Acetol	Acetate	Succina
*alc*A⁻	4	-	-	+	+	+	+	+	+	+
*alc*B⁻	4	-	+	+	+	-	+	+	+	+
*alc*AB⁻	2	-	-	+	+	-	+	+	+	+

- = no growth + = growth

N.B. Wild-type grows on all intermediates

propan-1-ol or propan-2-ol in culture supernatants or cell-free extracts.

These mutations are located in the propan-1-ol or propan-2-ol dehydrogenase genes, which are probably located on the chromosome, as they still retain the ability to metabolize other intermediates further down their respective oxidation pathways, Table 3.2. These results reflect the dichotomy between the metabolism of primary and secondary alcohols.

3.1.4 ald⁻ and ket⁻ mutants

An ald⁻ mutant was isolated which had lost the ability to utilize propane, propan-1-ol and propanal as growth substrates. However, it still possessed the ability to grow on propanoate and all potential subterminal intermediates (Table 3.3). This means that the metabolic lesion is probably located in an aldehyde dehydrogenase gene. The isolation of this mutant demonstrates the oxidation sequence, propane → propan-1-ol → propanal; and also that terminal and subterminal pathways are distinct, as a lesion in terminal oxidation does not preclude growth on subterminal intermediates.

Two mutants defective in acetone metabolism (ket⁻) were isolated which still retained the ability to utilize propane, terminal and subterminal intermediates (including propan-2-ol) as growth substrates, Table 3.3. Therefore, it is proposed that acetone is not an intermediate of propane oxidation and that propan-2-ol is not metabolized via acetone. This also demonstrates that the enzymes necessary for the oxidation of acetone are not involved in the pathway of propane oxidation; or there is more than one pathway for the oxidation of acetone. This last point

Table 3.3 Characterization of ald⁻ and ket⁻ mutants

		Growth Substrates:								
Class	No. of mutants	Propane	Propan-1-ol	Propanal	Propanoate	Propan-2-ol	Acetone	Acetol	Acetate	Succina
ald⁻	1	-	-	-	+	+	+	+	+	+
ket⁻	2	+	+	+	+	+	-	+	+	+

- = no growth + = growth

N.B. Wild-type grows on all intermediates

is more difficult to assert due to the limited number of mutants isolated, if the latter were true a large sample size would show ket⁻ mutants which; (i) were defective in acetone, propan-2-ol and propane metabolism, or (ii) were defective in acetone metabolism alone. A number of pathways are known for the metabolism of acetone, these include terminal oxidation to form acetol, a Baeyer-Villiger type reaction yielding acetate and methanol and carboxylation to form acetoacetate. (see section 1.3.3.2 and Figs. 1.5, 1.6 and 1.7). Finally, it may be the case that these mutants are acetone-transport deficient.

3.1.5 Oate⁻ and ace⁻ mutants

Two classes of oate⁻ mutants were isolated (Table 3.4):

(i) $oate^-_1$ mutants failed to utilize propane, propan-1-ol, propanal and propanoate as growth substrates, but still retained the ability to metabolize subterminal intermediates;

(ii) $oate^-_2$ mutants failed to utilize propanoate as a growth substrate, but still utilize propane and all other intermediates. This sub-class probably represents a propanoate-transport deficient phenotype.

The isolation of $oate^-_1$ mutants demonstrates that propane and terminal intermediates are metabolized via propanoate. As these mutants utilize succinate as a growth substrate the metabolic lesion is probably between propanoate and succinyl CoA (Fig 1.4).

Only one ace⁻ mutant was isolated and this could not utilize propane or subterminal intermediates as growth substrates, but could still utilize terminal intermediates (propan-1-ol, propanal and propanoate). This

Table 3.4 Characterization of oate$^-$ and ace$^-$

		Growth Substrates:								
Class	No. of mutants	Propane	Propan-1-ol	Propanal	Propanoate	Propan-2-ol	Acetone	Acetol	Acetate	Succina
oate$^-_1$	2	-	-	-	-	+	+	+	+	+
oate$^-_2$	4	+	+	+	-	+	+	+	+	+
ace$^-$	1	-	+	+	+	-	-	-	-	+

- = no growth + = growth

N.B. Wild-type grows on all intermediates

suggests that propane is also metabolized *via* acetate. This is also true for subterminal intermediates.

These results are in agreement with those obtained for *alc*$^-$ mutants (3.2), which indicate that propane is metabolized *via* both terminal and subterminal oxidation pathways in *R.rhodochrous* PNKb1.

3.1.6 Summary

The isolation of the mutants discussed previously, represents the first report of propane oxidation mutants and one of the few reports describing mutant phenotypes blocked in more than the first step of n-alkane oxidation. Other reports include n-alkane mutants of *P.putida* (Neider & Shapiro, 1975; Grund *et al*., 1975) and *S.lipolytica* (Bassel & Ogrydziak, 1979).

Mutant analysis has indicated that for propane oxidation to proceed in *R.rhodochrous* PNKb1 it must possess the ability to oxidize both terminal and subterminal intermediates. However, *ket*$^-$ mutants are still able to utilize propane as a growth substrate, indicating that it is probably not an intermediate of propane oxidation.

Interestingly, *alk*$^-$ mutants have two phenotypic traits in that they are unable to metabolize either propane or acetol; suggesting a common enzyme or regulatory element for the metabolism of both substrates. SDS-PAGE of cell-free extracts revealed common inducible polypeptides.

The isolation of *oate*$^-_1$ and *ace*$^-$ phenotypes demonstrates the distinction between terminal and subterminal oxidation pathways in *R.rhodochrous*

PNKb1. The isolation of *oate*$^-_2$ mutants suggests that this organism also has a transport system for propanoate uptake.

One possibility was that a block in the terminal or subterminal pathway could still enable this organism to utilize propane, albeit less efficiently. However, this situation was not observed in this study. This may be due to the formation *in vivo* of high concentrations of toxic intermediates (e.g. propan-1-ol, propanal or propan-2-ol) directly from propane which cannot be further metabolized to the detriment of the cell.

The isolation of these classes of mutants provides a basis for future studies. These could include assaying the various enzyme activities in mutant classes with reference to wild-type *R.rhodochrous* PNKb1; *in vitro* enzyme complemention studies as carried out by Macham & Heydeman (1974) to determine the number of components involved in the propane oxygenase activity; cross-feeding experiments as described by Singer & Finnerty (1984b) using *Acinetobacter* defective in hexadecane metabolism; analysis of revertants (e.g. *alk*$^-$ mutants) for altered patterns of n-alkane utilization (Jenkins *et al*., 1972) and isolation of the gene(s) responsible for propane oxidation by complementation of mutants using recombinant DNA techniques.

In section 3.4 *alc*$^-$ mutants have been selected for further study by assaying for NAD^+-dependent alcohol dehydrogenase activity and comparing the results with the wild-type *R.rhodochrous* PNKb1. SDS-PAGE and Western-blot analysis have also been performed with *alc*$^-$ mutants.

3.2 Purification and characterization of a NAD^+-dependent secondary alcohol dehydrogenase from propane-grown R.rhodochrous PNKb1

3.2.1 Introduction

Section 1.4.3 surveyed the current literature with respect to the role of alcohol dehydrogenases in n-alkane metabolism.

It was not known how many types and locations of measurable alcohol dehydrogenase activities were being detected in cell-free extracts from propane-grown cells of R.rhodochrous PNkb1. Therefore, in order to gain a better understanding of the relative importance of terminal and subterminal oxidation in this organism, the purification and characterization of the alcohol dehydrogenase(s) from propane-grown cells was undertaken. A summary of this work is given by Ashraf & Murrell (1990).

3.2.2 Primary and secondary alcohol dehydrogenase activities

Cell-extracts of propane-grown R.rhodochrous PNKb1 possessed primary and secondary alcohol dehydrogenase activities. These activities were NAD^+-dependent and located in the soluble fraction of the cell lysate. Previous work by Woods (1988) and this study have shown there to be no $NAD(P)^+$-independent soluble or particulate alcohol dehydrogenase activity, despite exhaustive measures using a wide range of buffers, pH's (5-12) and electron acceptor dye (PMS and PES). However, in contrast to the work carried out by Woods no NAD^+-dependent propanal dehydrogenase could be detected in cell-free extracts, also no $NAD(P)^+$-independent soluble or particulate activities could be detected.

Table 3.5 shows the highest levels of NAD^+-dependent primary and secondary alcohol dehydrogenase activities were detected in propane-grown cells. The NAD^+-dependent primary alcohol dehydrogenase activity appears to remain at a basal constitutive level, except when the cells have been grown on propane or acetol. Elevated levels of NAD^+-dependent secondary alcohol dehydrogenase activity were observed in cell-free extracts after growth on the potential subterminal intermediates propan-2-ol, acetol and acetate, but not acetone. Cell-free extracts from citrate-grown cells possessed relatively high levels of NAD^+-linked primary and secondary alcohol dehydrogenase activity, and this may be due to the presence of non-specific alcohol dehydrogenases. From these results it was unclear whether primary and secondary alcohol oxidation was mediated by one or more soluble NAD^+-dependent alcohol dehydrogenases. For example, work undertaken by Beers (1988) showed the presence of four alcohol dehydrogenases involved in the metabolism of butane by Pseudomonas butanovora.

3.2.3 Enzyme Purification

A purification protocol was developed to clarify whether one or more alcohol dehydrogenases were responsible for the activities measured in cell-free extracts after growth on the various intermediates. Initial experiments using dye-ligand affinity chromatography supports such as Cibacron Blue, Procion Red, 5'AMP-Sepharose and the Amicon Dye-matrix kit failed to bind any alcohol dehydrogenase activity. Similar results were obtained by Coleman & Perry (1985) in that the secondary alcohol dehydrogenase from M.vaccae JOB5 failed to bind to Cibacron Blue F3GA in initial purifications, and Jaeger et al. (1981) obtained such results when purifying coniferyl alcohol dehydrogenase from Rhodococcus erythropolis. Previous work has postulated that this may be due to the

Table 3.5 Specific activities of alcohol dehydrogenase from propane and potential propane oxidation intermediate-grown cells of R.rhodochrous PNKb1

Enzyme Activity	Cells grown on: Propane	Propan-1-ol	Propanal	Propanoate	Propan-2-ol	Acetone	Acetol	Acetate	Citrate
	Specific Activity (units)[a]								
Propan-1-ol dehydrogenase	36	4.5	1.5	ND	3.0	2.0	19	1.0	12
Propan-2-ol dehydrogenase	176	34	27	ND	72	21	47	64	107

[a] 1 unit = 1 nmole NAD^+ reduced min^{-1} mg $protein^{-1}$

ND = Not Detected

absence of a dinucleotide fold in the enzyme (Wilson, 1976). A two-step purification scheme was developed which used DEAE-cellulose and NAD-agarose affinity chromatography; in which the NAD was linked to the agarose via the C8 of the purine ring (Barry & O'Carra, 1973).

During each purification step both primary (propan-1-ol) and secondary (propan-2-ol) dehydrogenase activities were determined, and both co-purified (Table 3.6). Purification factors and percentage recoveries showed a rough equivalence leading to the conclusion that both activities were due to the same protein. Most contaminating protein was removed after applying fractions from the DEAE-cellulose column to the NAD-agarose column. Experiments also demonstrated that enzyme activity was very stable in frozen whole-cells, cell-free extracts and even subsequent purification steps. Purified enzyme was stable for several months at -70°C with no loss of enzyme activity being observed.

The NAD-agarose purification step yielded only one major protein band after SDS-PAGE and Coomassie Blue staining. This protein corresponded to a subunit molecular weight of approximately 42 kDa (Fig. 3.2a). Only very minor traces of impurities were detected by careful visual examination of gels. Isoelectric focusing gels gave a single molecular species (pI = 5.2) after silver staining (Fig. 3.2b).

The molecular weight of the native secondary alcohol dehydrogenase enzyme, was determined by HPLC gel filtration and was estimated at 86 kDa. This suggests that the enzyme is composed of two identical subunits. The activity stain performed under non-denaturing conditions showed only one band when using propan-1-ol or propan-2-ol as substrates, Fig. 3.3a and b respectively. Fig. 3.3 shows the activity stains and corresponding Coomassie Blue stain (Fig. 3.4c) which relates

Table 3.6 Purification of NAD^+-dependent alcohol dehydrogenase from propane-grown Rhodococcus rhodochrous PNKb1

	Secondary Alcohol Dehydrogenase[1]						Primary Alcohol Dehydrogenase[2]			
Purification step	Total volume (ml)	Total protein (mg)	Total activity[a] (units)	Specific activity[a] (units [mg protein]$^{-1}$)	Yield (%)	Purification Factor	Total activity[a] (units)	Specific activity[a] (units [mg protein]$^{-1}$)	Yield (%)	Purification Factor
Cell-free extract	95.5	515.7	42,975	83.4	100	1	15,295	29.8	100	1
DEAE-cellulose	170	204	30,090	147.5	70	1.77	12,750	62.5	83	2.1
NAD-agarose	11.2	2.6	12,768	4,851	30	58.2	3,237	1,230	21	41.3

[a] 1 unit = 1 nmole of NAD^+ reduced min^{-1}

[1] Secondary alcohol dehydrogenase activity determined using propan-2-ol as a substrate

[2] Primary alcohol dehydrogenase activity determined using propan-1-ol as a substrate

Figure 3.2 (a) SDS-PAGE showing the steps for the purification of a NAD^+-dependent secondary alcohol dehydrogenase.

Track	Purification Step	
1	(Molecular weight markers)	
2	Cell-free extract	(100 μg)
3	DEAE-cellulose	(100 μg)
4	NAD-agarose	(20 μg)

(b) IEF-PAGE of purified NAD^+-dependent secondary alcohol dehydrogenase

Track		
1	Purified alcohol dehydrogenase	(0.25 μg)
2	Isoelectric point (pI) markers	

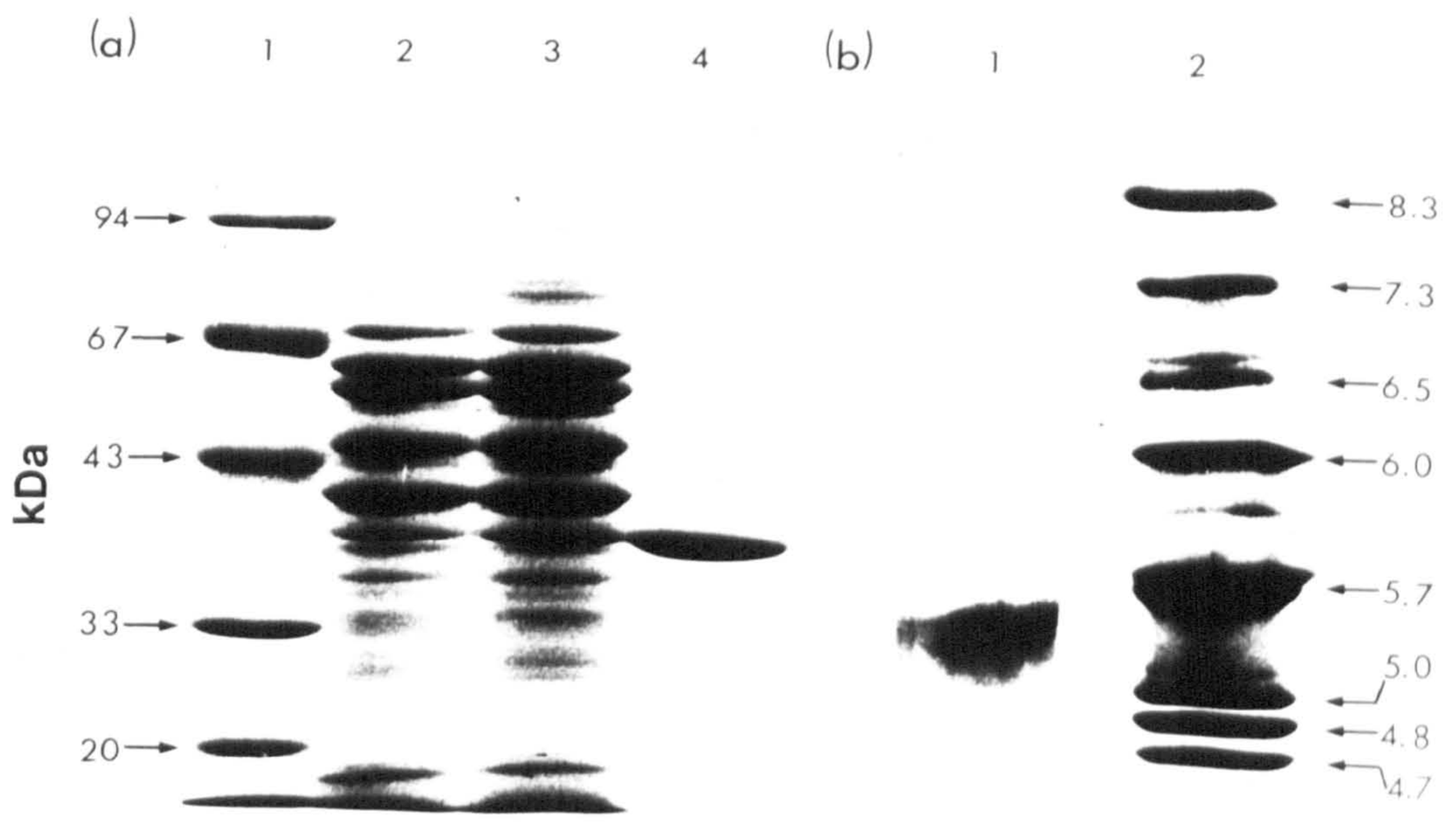

(a)
1
2
3
4
94
67
43
33
20
kDa
(b)
1
2
8.3
7.3
6.5
6.0
5.7
5.0
4.8
4.7
(Id)

Figure 3.3 Alcohol dehydrogenase activity stain

(a) using propan-1-ol as a substrate

(b) using propan-2-ol as a substrate

(c) Coomassie blue stain

Track	Purification Step	
1	Cell-free extract	(100 μg)
2	DEAE-cellulose	(100 μg)
3	NAD-agarose	(20 μg)

a.
1 2 3
b.
1 2 3
c.
1
2
3

to: (1) cell-free extract from propane-grown cells; (2) NAD^+-linked primary and secondary alcohol dehydrogenase activity from the DEAE-cellulose column and; (3) purified secondary alcohol dehydrogenase from the NAD-agarose column. The formazan bands produced in the activity stain reaction and the Coomassie Blue stained purified protein band migrated the same distance through non-denaturing gel. This indicates that the NAD^+-linked primary and secondary alcohol dehydrogenase activities are derived from one enzyme. This must be contrasted with numerous reports of multiple enzyme activities in other alkane-utilizing bacteria. Such bacteria may contain a multiplicity of constitutive and inducible $NAD(P)^+$-dependent and -independent alcohol dehydrogenases, (Singer & Finnerty, 1984a).

3.2.4 Enzyme characterization

The substrate specificity of the alcohol dehydrogenase was examined. Dehydrogenase activity was expressed relative to propan-1-ol for primary alcohols and other compounds, and propan-2-ol for secondary alcohols, Table 3.7. The enzyme showed a broad substrate specificity, although highest activities were observed with secondary alcohols. No activity was demonstrated with methanol, propan-1,3-diol or glycerol, which correlated well with the inability of R.rhodochrous PNKb1 to utilize these compounds as carbon and energy sources. The highest primary and secondary alcohol dehydrogenase activity was observed with pentan-1-ol and pentan-2-ol respectively. Representative, branched chain, cyclic and aromatic alcohols were also oxidized but at a much lower rate when compared with primary alcohols, except for cyclohexanol.

The enzyme could also reduce acetone (2,043 units), propanal (549 units), acetol (479 units), butanone (1,520 units) and cyclohexanone

Table 3.7 Relative specific activity of purified alcohol dehydrogenase from propane-grown R.rhodochrous PNKb1

Substrate	% Activity (relative to propan-1-ol or propan-2-ol)
Primary Alcohols	
Methanol	0
Ethanol	56
Propan-1-ol	100
Butan-1-ol	111
Pentan-1-ol	150
Hexan-1-ol	45
Octan-1-ol	33
Secondary Alcohols	
Propan-2-ol	100
Butan-2-ol	93
Pentan-2-ol	174
Hexan-2-ol	92
Octan-2-ol	37
Others	
Acetol	105
Propan-1,2-Diol	11
Propan-1,3-Diol	0
Glycerol	0
2-Methylpropan-1-ol	22
2-Methylpropan-2-ol	17
Cyclohexanol	111
Phenyl Ethyl Alcohol	73

100% activity = 1,230 units (mg protein)$^{-1}$ for propan-1-ol or 4,851 units (mg protein)$^{-1}$ for propan-2-ol

Others are measured relative to propan-1-ol

(5,263 units), in the reverse reaction using NADH as an electron donor at pH 6.5. This was determined by measuring the oxidation of NADH at 340 nm spectrophotometrically. Highest activity was observed with cyclohexanone as substrate. Production of acetone (forward reaction) and propan-2-ol (reverse reaction) was confirmed by gas chromatography.

The effect of pH on alcohol dehydrogenase activity for forward and reverse reactions were also investigated. The optimum pH for the conversion of propan-2-ol to acetone and for acetone to propan-2-ol were pH 10.5 and pH 6.5 respectively.

K_m values were determined for the substrates propan-1-ol, propan-2-ol, acetone and cofactors NAD^+ and NADH (Table 3.8). No activities were obtained when $NADP^+$ or NADPH were used as cofactors. The K_m values for propan-1-ol (12 mM) and propan-2-ol (18 mM) are notably higher than for other alkane associated alcohol dehydrogenases, although similar results were obtained by Woods (1988). However, K_m values for NAD^+ (57 μM) and NADH (100 μM) are of the same order. These data were obtained from Lineweaver-Burk plots, using duplicate assays at five sub-saturating concentrations for each substrate tested. Correlation coefficients from linear regression analyses for each K_m determination were all greater than 0.95.

Various potential inhibitors were tested against primary and secondary alcohol dehydrogenase activity of the purified enzyme (Table 3.9). Primary and secondary alcohol dehydrogenase activities were inhibited by metal-complexing agents 2,2'-bipyridyl and 1,10-phenanthroline and the thiol reagent iodoacetate. Other inhibitors or metal-binding agents such as potassium cyanide, EDTA and 8'-hydroxyquinoline and the carbonyl

Table 3.8 K_m determinations for purified alcohol dehydrogenase from propane-grown R.rhodochrous PNKb1

Substrate	K_m (mM)	V_{max} units[a] (mg protein)$^{-1}$
Propan-1-ol	12.0	1,053
Propan-2-ol	18.0	5,000
Acetone	57.0	2,222
NAD^+	0.057	3,448
NADH	0.10	1,269

[a] 1 unit = 1 nmole of NAD^+ reduced min^{-1}

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Propan-2-ol	18.0	5,000
Acetone	57.0	2,222
NAD^+	0.057	3,448
NADH	0.10	1,269

[a] 1 unit = 1 nmole of NAD^+ reduced min^{-1}

Table 3.9 Effect of various potential inhibitors on the activity of purified alcohol dehydrogenase from propane-grown R.rhodochrous PNKb1

Compound	Concentration (mM)	Activity[a] of alcohol dehydrogenase with propan-1-ol as substrate % Inhibition	Activity[b] of alcohol dehydrogenase with propan-2-ol as substrate % Inhibition
Iodoacetate	1	30	30
	2	70	50
2,2'-Bipyridyl	1	30	30
	5	80	50
1,10-Phenanthroline	1	10	0
	5	50	40

[a] 100% activity = 1,230 units (mg protein)$^{-1}$

[b] 100% activity = 4,851 units (mg protein)$^{-1}$

reagent semicarbazide (all tested at concentrations of 1-5 mM), failed to inhibit either activity.

3.2.5 Discussion

The presence of primary and secondary alcohol dehydrogenase activity in crude extracts of propane-grown R.rhodochrous PNKb1 supports the notion that the metabolism of propane may proceed via terminal and/or subterminal oxidation in this organism. The specific activity of the purified enzyme was consistently higher with secondary alcohols and can therefore be classed as a secondary alcohol dehydrogenase.

The purified secondary alcohol dehydrogenase from R.rhodochrous PNKb1 had a molecular weight of 86 kDa and was composed of two identical subunits of approximately 42 kDa. This is smaller than previously reported propane-associated alcohol dehydrogenase from both P.fluorescens NRRL B-1244 (144,500) (Hou et al., 1983b) and M.vaccae JOB5 (136,000) (Coleman & Perry, 1985). However, the purified enzyme from R.rhodochrous PNKb1 was similar in size and subunit composition to the NAD^+-dependent secondary alcohol dehydrogenases from methanol-grown Pseudomonas sp. (98,000) (Hou et al., 1979) and Pichia sp. (98,000) (Patel et al., 1979). Although similar in size to the Pseudomonas sp. ATCC 21489 and Pichia alcohol dehydrogenases, in terms of substrate specificity, alcohol dehydrogenase from R.rhodochrous PNKb1 was considerably different in that it does oxidize primary alcohols.

The propan-2-ol dehydrogenase from Candida boidinii is highly specific for short chain-length secondary alcohols, with no activity being detected with short chain-length primary alcohols as substrates (Schutte et al., 1982). However, the secondary alcohol dehydrogenase purified

from propan-2-ol grown M.vaccae JOB-5 exhibited a significant activity with short chain-length primary and secondary alcohols (C_3 to C_5) as substrates. The secondary alcohol dehydrogenase from R.rhodochrous PNKb1, by comparison, had broad substrate specificity, oxidizing a series of short chain-length primary and secondary alcohols (C_2- C_8) and representative cyclic and aromatic alcohols, and thus is similar to the secondary alcohol dehydrogenase from P.fluorescens NRRL B-1244 (Hou et al., 1983b) and the $NADP^+$-linked alcohol-aldehyde/ketone oxidoreductase from Thermonanerobium brockii (Lamed & Zeikus, 1981). The latter enzyme could also catalyze the reverse reaction, i.e. the conversion of ketone to alcohol, if the pH was lowered to pH 6.5 and NADH at 0.2 mM was provided. It is interesting to note the higher level of dehydrogenase activity when cyclohexanol was used as a substrate compared to propan-1-ol, and also in the reverse reaction cyclohexanone gave the highest activity in the reverse reaction. A NAD^+-linked cyclohexanol dehydrogenase which had broad specificity for straight-chain secondary alcohols (including propan-2-ol), cyclic and substituted cyclic alcohols and cyclohexane diols has been purified from a Nocardia sp. (Stirling & Perry, 1980). Reaction mixtures containing either butanone, acetol or cyclohexanone could stimulate NADH oxidation in vitro, it was postulated that these ketones are converted to their corresponding alcohols by the secondary alcohol dehydrogenase operating in reverse.

Studies on enzyme kinetics demonstrated relatively high K_m values for propan-1-ol (12 mM), propan-2-ol (18 mM) and acetone (57 mM). They appear two orders of magnitude higher than those previously reported for propane-associated alcohol dehydrogenases (Hou et al., 1983b; Coleman & Perry, 1985).

Woods (1988) raised doubts as to whether these activities could be responsible for the metabolism of intermediates of propane oxidation based on the comparatively high K_m values obtained in his work; however, these high values are not without precedent. Similar K_m values have been reported for purified primary and secondary alcohol dehydrogenase from Thermonanaerobacter ethanolicus; primary alcohol dehydrogenase had a K_m of 47.1 mM for butan-1-ol and the secondary alcohol dehydrogenase had a K_m of 16.4 mM for propan-2-ol (Bryant et al., 1988). An NAD^+-linked secondary alcohol dehydrogenase purified from Comamonas terrigena, exhibiting a preference for L-stereoisomers of secondary alcohols, had a K_m of 15.4 mM for propan-2-ol (Barrett et al., 1981). Pseudomonas BB1, grown on alcohols, contained a quinoprotein alcohol dehydrogenase whose apparent K_m values for methanol were approximately 31.5 mM for monomeric and dimeric forms of the enzyme, and 2.5 mM in the case of propan-2-ol (Dijkstra et al., 1985). Finally, the purification of long chain-length alcohol dehydrogenases isoenzymes from P.aeruginosa 196Aa demonstrated that lower K_m values were obtained with alcohols of increasing chain lengths. It was postulated that this was necessary because of the low water solubility of the enzyme substrate in question. Thus comparatively high K_m values were obtained from primary alcohols C_2-C_5 (Tassin & Vandecasteele, 1972). This may represent an explanation of the comparatively high K_m values for alcohols with secondary alcohol dehydrogenase purified from R.rhodochrous PNKb1, although specific activities are comparable with other work cited in this discussion.

Enzyme activity of purified alcohol dehdyrogenase was inhibited by 2,2'-biyridyl and 1,10-phenanthroline but at higher concentrations than were found necessary to inhibit other propane-associated secondary alcohol dehydrogenases (Hou et al., 1983b; Coleman & Perry, 1985). This suggests that a metal centre, possibly zinc, may be involved at the

active site or is necessary for stability of the enzyme. This metal centre is possibly tightly bound to the enzyme, as demonstrated by the comparatively higher concentrations of 2,2'-bipyridyl and 1,10-phenanthroline required to cause significant inhibition. The strong influence of these inhibitors on the activity of a NAD^+-linked secondary alcohol dehydrogenase purified from C.boidinii pointed towards a possible role for a metal ion, and zinc was found to have a profound effect on the production of that propan-2-ol dehydrogenase (Schutte et al., 1982). It has been suggested that the inhibitory effect of 1,10-phenanthroline on liver alcohol dehydrogenase and yeast alcohol dehydrogenase is due the chelation of zinc from the active site (see Branden et al., 1975). The alkylating reagent idoacetate was highly inhibitory to R.rhodochrous PNKb1 alcohol dehydrogenase, possibly indicating the presence of functional thiol groups. Similar results were obtained by Hou et al. (1983b) and Coleman & Perry (1985). The inhibition of both primary and secondary alcohol dehydrogenase activity was approximately the same for types and concentrations of inhibitors used, thus strengthening the evidence that both activities are due to the same enzyme.

In conclusion, propane-grown R.rhodochrous PNKb1 has a single NAD^+-dependent soluble secondary alcohol dehydrogenase that oxidizes both short-chain primary and secondary alcohols. However, the possibility cannot be ruled out that an alcohol dehydrogenase using some other unknown physiological acceptor was present, which could not be detected using in the artificial acceptor systems in this study and studies by Woods (1988). The purified alcohol dehydrogenase may best be described as a secondary alcohol dehydrogenase because of its consistently higher specific activity with secondary alcohols, the V_{max} for propan-2-ol was five-times greater than that of propan-1-ol. This novel secondary

alcohol dehydrogenase has characteristics and properties that are intermediate to those reported by Hou et al. (1983b) and Coleman & Perry (1985), the distinguishing features are a lower molecular weight and higher pI. The simple, quick and efficient purification method developed here may have general applicability to other NAD^+-linked dehydrogenases providing they have a high affinity for NAD which will enable the enzyme to bind tightly to NAD-agarose. It may be especially useful for those enzymes which fail to bind to the more conventional dye-ligand affinity chromatography supports.

The purification to homogeneity of this enzyme and its characterization has aided other studies to determine the potential role(s) of terminal and subterminal oxidation of propane. These have included the biochemical analysis of NTG-generated mutants blocked in the metabolism of propane, propan-1-ol and propan-2-ol (see section 3.4); production of antibodies for Western blot analysis of the induction of the secondary alcohol dehydrogenase after growth on propane and potential oxidation intermediates and the immunological relationship of NAD^+-linked alcohol dehydrogenases in other propane-utilizing bacteria (see section 3.3).

3.3 Growth on propane and potential oxidation intermediates

3.3.1 Introduction

R.rhodochrous PNKb1 has the ability to utilize all of the potential terminal and subterminal intermediates of propane oxidation; except methylglyoxal, methylacetate and methanol. Woods (1988) has demonstrated the inducible nature of the propane oxygenase activity and the synthesis of polypeptides of approximately 67, 59, and 57 kDa, which are specific to propane-grown cells. However, a closer examination of the work presented by Woods & Murrell (1989) revealed a similar distribution of polypeptides after cells had been grown on acetol. However, the ability to oxidize propane and co-oxidize propylene was not tested. If the above polypeptides form a propane oxygenase complex, as they suggest, it is reasonable to assume that acetol-grown cells would be adapted to oxidize propane.

To answer this question simultaneous adaptation studies using propane, propan-1,2-diol and acetol-grown cells has been undertaken. The inclusion of propan-1,2-diol, although not a postulated intermediate in the literature, is justified because of its reported metabolism via acetol (see section 1.3.3.1). Cell-free acetol oxygenase and whole-cell propane oxygenase activity has also been determined.

The purification to homogenity of an NAD^+-linked secondary alcohol dehydrogenase from propane-grown cells (see section 3.2) has enabled the production of antibodies, which allows Western-blot analysis of enzyme induction after growth on various intermediates, and a range of primary and secondary alcohols (C_2-C_8). This technique has also been used to examine if a conserved NAD^+-dependent alcohol dehydrogenase was present

after growth on propane for other propane-utilizing bacteria (see Table 2.2).

3.3.2 SDS-PAGE of extracts from propane and oxidation intermediate-grown cells

Soluble cell-free extracts from R.rhodochrous PNKb1 after growth on propane and potential oxidation intermediates are shown in Fig 3.4a. Propane and acetol-grown cells contained similar polypeptides of approximately 68, 64 and 48 kDa which are specific to those substrates, and are not present in for example propan-1-ol or acetone-grown cells. Woods (1988) suggested that these were components of a large oxygenase complex. The soluble methane monooxygenase of M.capsulatus (Bath) gives similar patterns after SDS-PAGE; α, β and γ components of protein A give distinct protein bands at 54, 42 and 17 kDa (Woodland & Dalton, 1984). Also the octane monooxygenase from P.oleovorans gives three distinct protein bands at 50, 41 and 19 kDa corresponding to the reductase, hydroxylase and rubredoxin components respectively (see section 1.4.2.2).

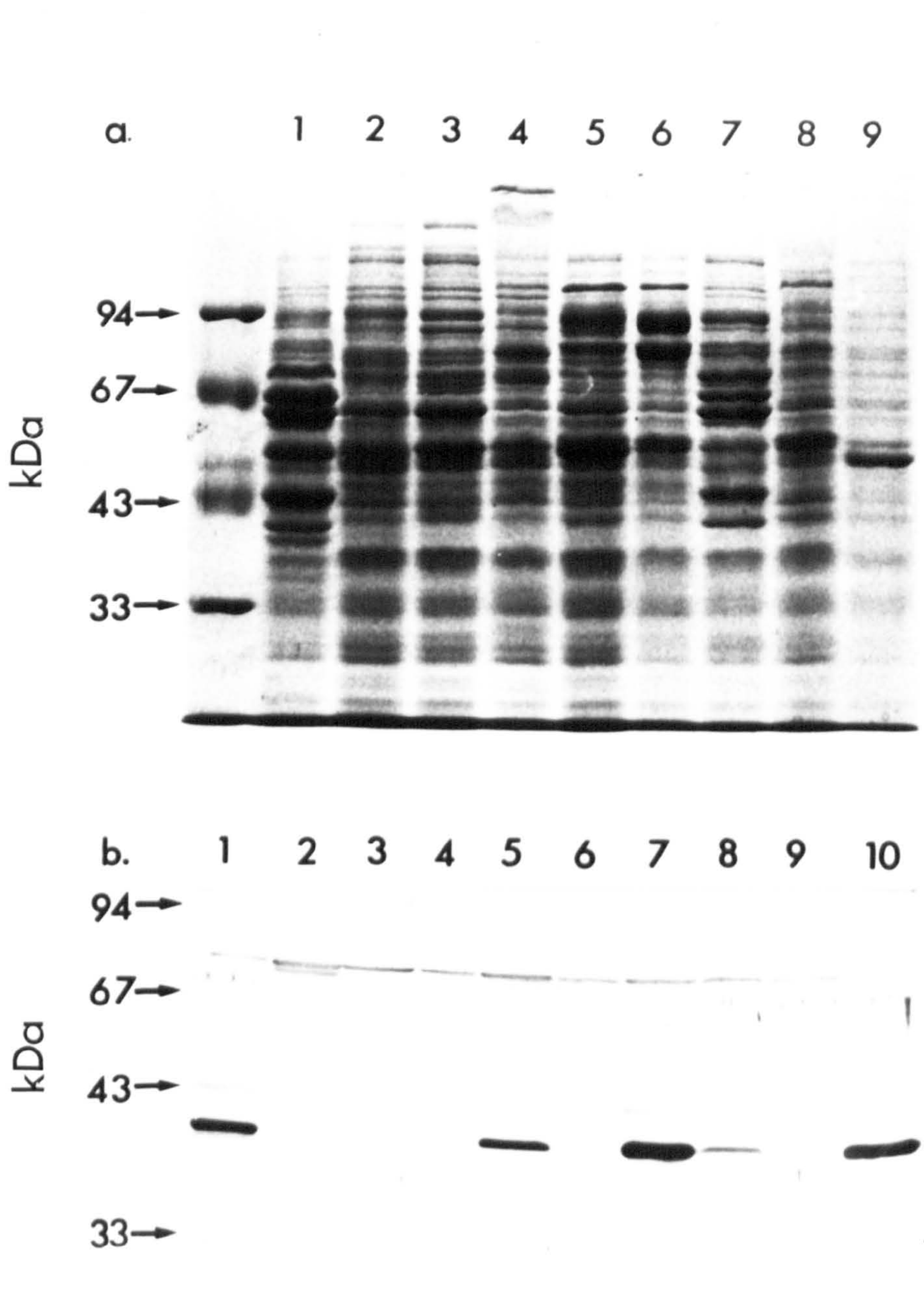
Cell-extracts from propan-2-ol and acetone-grown cells have a similar specific polypeptide of approximately 100 kDa. However, acetone-grown cells also contained specific polypeptides of approximately 92 and 84 kDa. These unique polypeptides may represent an enzyme complex required specifically for the metabolism of acetone. Mutant characterization of the ket$^-$ mutants demonstrated the ability to utilize propane and all other potential oxidation intermediates. Therefore, it is necessary to propose that a different and separate pathway for the metabolism of acetone exists (see section 3.1.4), as initially proposed by Woods (1988).

Figure 3.4 (a) SDS-PAGE of cell-free extracts of R.rhodochrous PNKb1 grown on propane and various potential oxidation intermediates

(b) Corresponding Western-blot analysis using antibodies against purified NAD^+-dependent secondary alcohol dehydrogenase.

Track	Growth Substrate
1	Propane
2	Propan-1-ol
3	Propanal
4	Propanoate
5	Propan-2-ol
6	Acetone
7	Acetol
8	Acetate
9	Citrate
10	Purified alcohol dehydrogenase (1 μg)

100 μg protein in each track for (a) and (b), except for propane and acetol in (b) (10 μg).


a.
1 2 3 4 5 6 7 8 9
94→
67→
43→
33→
kDa
b.
1 2 3 4 5 6 7 8 9 10
94→
67→
43→
33→
kDa

The synthesis of polypeptides after growth on propan-1,2-diol is shown in Fig 3.5a, which allows direct comparison between propane and acetol-grown cells. There are similarities and differences in terms of the induction of specific polypeptides after growth on propan-1,2-diol compared with growth on propane or acetol, however, a specific 53 kDa was synthesized (see section 3.1.1).

After growth on the other intermediates there were no distinct patterns of major polypeptide synthesis, although synthesis of comparatively low amounts of specific polypeptides could be observed for propanal and propan-2-ol-grown cells. This may, to some extent, reflect the fact that the other intermediates do not require metabolism <u>via</u> 'specialized' biochemical pathways and rely on the synthesis of constitutive enzymes for their metabolism.

3.3.3 <u>Synthesis of the NAD^+ linked secondary alcohol dehydrogenase</u>

Specific activities for NAD^+-linked alcohol dehydrogenase activity after growth on propane and potential oxidation intermediates have been previously discussed (section 3.2 and Table 3.5). Elevated levels of primary and secondary alcohol dehydrogenase activity were observed after growth on propane and acetol, and higher levels of the latter after growth on propan-2-ol, acetate and citrate.

As stated previously it was not known how dehydrogenases were involved in the metabolism of propane, although after the isolation of <u>alc</u>A$^-$ and <u>alc</u>B$^-$ mutants at least two enzymes must be involved. This hypothesis has been confirmed by the Western-blot analysis shown in Fig 3.4b.

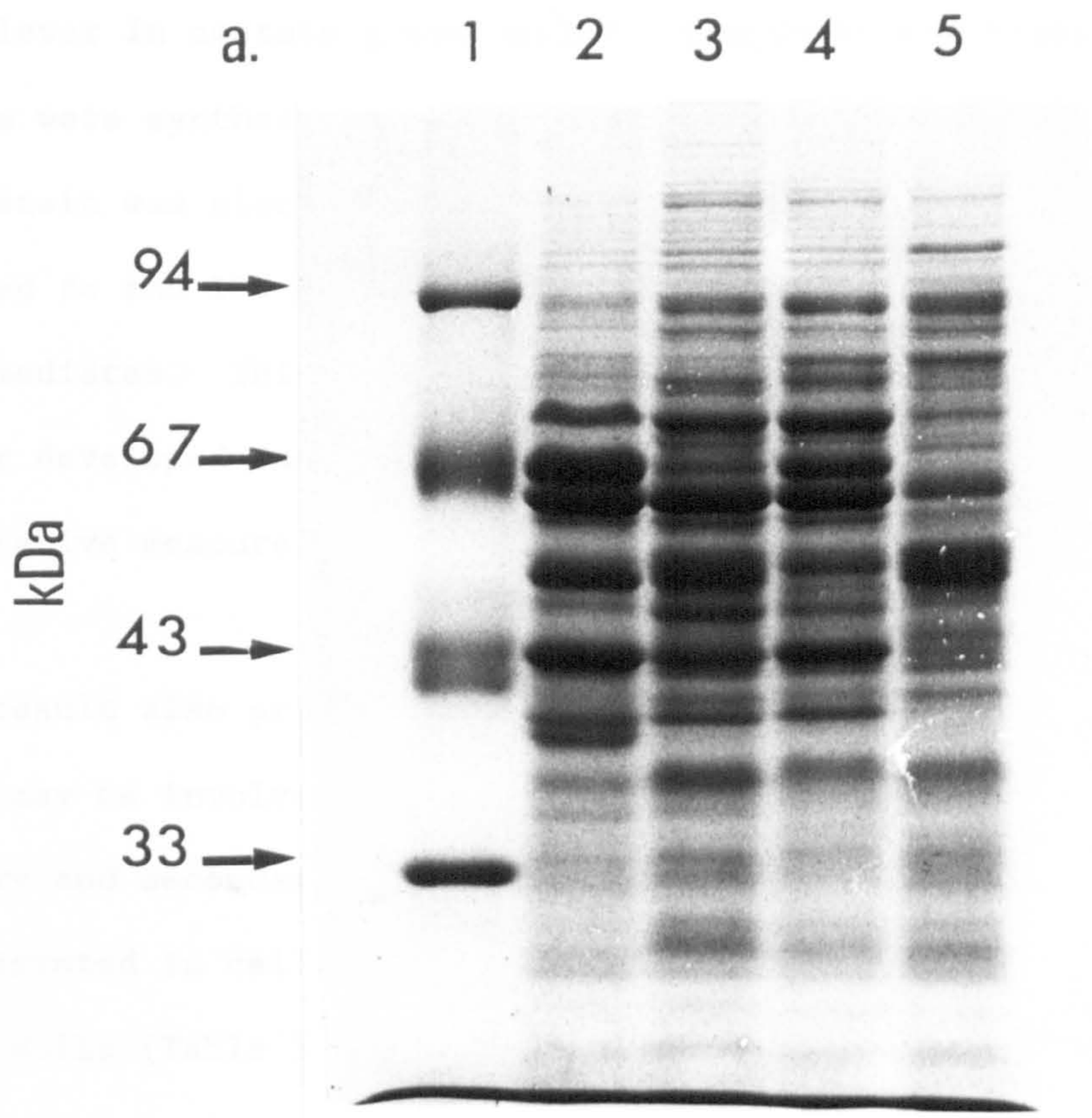
Figure 3.5 (a) SDS-PAGE of cell-free extracts of R.rhodochrous PNKb1 grown on propane, propan-1,2-diol and acetol.

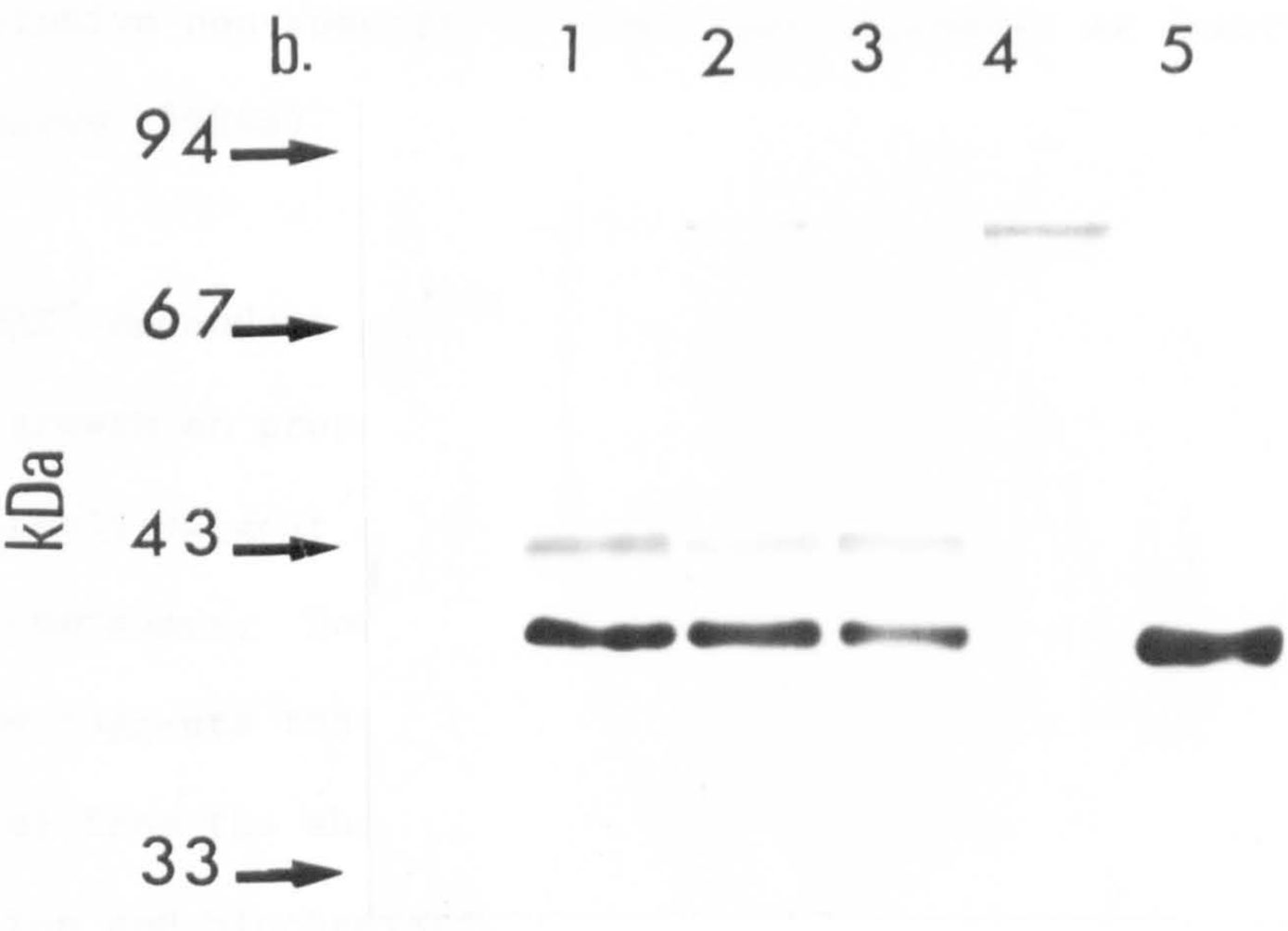
Track	Growth Substrate
1	(Molecular weight markers)
2	Propane
3	Propan-1,2-diol
4	Acetol
5	Citrate

100 μg of protein per track

(b) Corresponding Western-blot analysis using antibodies against purified NAD^+-dependent secondary alcohol dehydrogenase.

Track	Growth Substrate	
1	Propane	(10 μg)
2	Propane-1,2-diol	(10 μg)
3	Acetol	(10 μg)
4	Citrate	(100 μg)
5	Purified alcohol dehydrogenase	(1 μg)


a.
1
2
3
4
5
94
67
43
33
kDa


b.
1
2
3
4
5
94
67
43
33
kDa

The NAD^+-linked secondary alcohol dehydrogenase was induced after growth on propane, propan-2-ol, acetol and acetate (although synthesis appears much lower in acetate-grown cells). Comparatively higher amounts of enzyme were synthesized during growth on propane and acetol, as only 10 μg protein was electro-blotted after growth with the former compounds as opposed to the 100 μg protein used after growth on the other intermediates. This was based on a visual observation of intensity of colour developed during the staining reaction and is therefore only a qualitative measure.

This result also proves the existence of other alcohol dehydrogenase(s) which may be involved in the metabolism of propan-1-ol. NAD^+-dependent primary and secondary alcohol dehydrogenase activity has been demonstrated in cell-extracts from propan-1-ol, propanal and acetone-grown cells (Table 3.5), but this activity does not arise from the alcohol dehydrogenase which has been purified and characterized in this study. Interestingly, citrate-grown cells also contained high levels of alcohol dehydrogenase activity and this must reflect the synthesis of constitutive non-specific alcohol dehydrogenases as described by Singer & Finnerty (1984a).

This NAD^+-dependent secondary alcohol dehydrogenase was synthesized after growth on propane and subterminal intermediates, which might tentatively suggest a common regulatory system for the metabolism of the above compounds. However, the absence of this enzyme after growth on acetone suggests that this compound is metabolized *via* different route(s) from the above, which share common features in terms of mutant isolation and biochemistry.

Figure 3.5b shows that this secondary alcohol dehydrogenase is also synthesized after growth on propan-1,2-diol to the same extent as growth on propane and acetol. This may suggest a relationship between a metabolism of the above three compounds. Indeed studies by Ashraf & Murrell (1990) showed that the purified enzyme could oxidize acetol and propan-1,2-diol although at comparatively lower rates when compared with propan-1-ol and propan-2-ol.

The synthesis of this secondary alcohol dehydrogenase was also examined after growth on primary (C_2-C_8) and secondary (C_3-C_8) alcohols, Figs 3.6 (a) and (b) respectively which show the Western-blot analysis of soluble proteins obtained after growth on these alcohols. The enzyme was not synthesized after growth on primary alcohols, except for ethanol. This demonstrates again the necessity for other alcohol dehydrogenases which are required for the metabolism of primary alcohols. However, the enzyme was synthesized after growth on secondary alcohols; except butan-2-ol, propan-2-ol and pentan-2-ol being the most notable examples. The amount of enzyme synthesized after growth on hexan-2-ol, heptan-2-ol and octan-2-ol were comparatively low. This suggests the presence of other alcohol dehydrogenases required specifically for the metabolism of higher secondary alcohols. This could be tested by using *alc*B$^-$ mutants in growth tests on the above alcohols.

The reason for the lack of secondary alcohol dehydrogenase after growth on butan-2-ol is not immediately apparent, the same result was obtained after the experiment was repeated. Although the possibility cannot be discounted that the metabolism of butan-2-ol requires the presence of a specific NAD(P)$^+$-independent alcohol dehydrogenase which could not be detected.

Figure 3.6 SDS-PAGE Western-blot analysis of cell-free extracts of *R.rhodochrous* PNKb1 grown on primary and secondary alcohols.

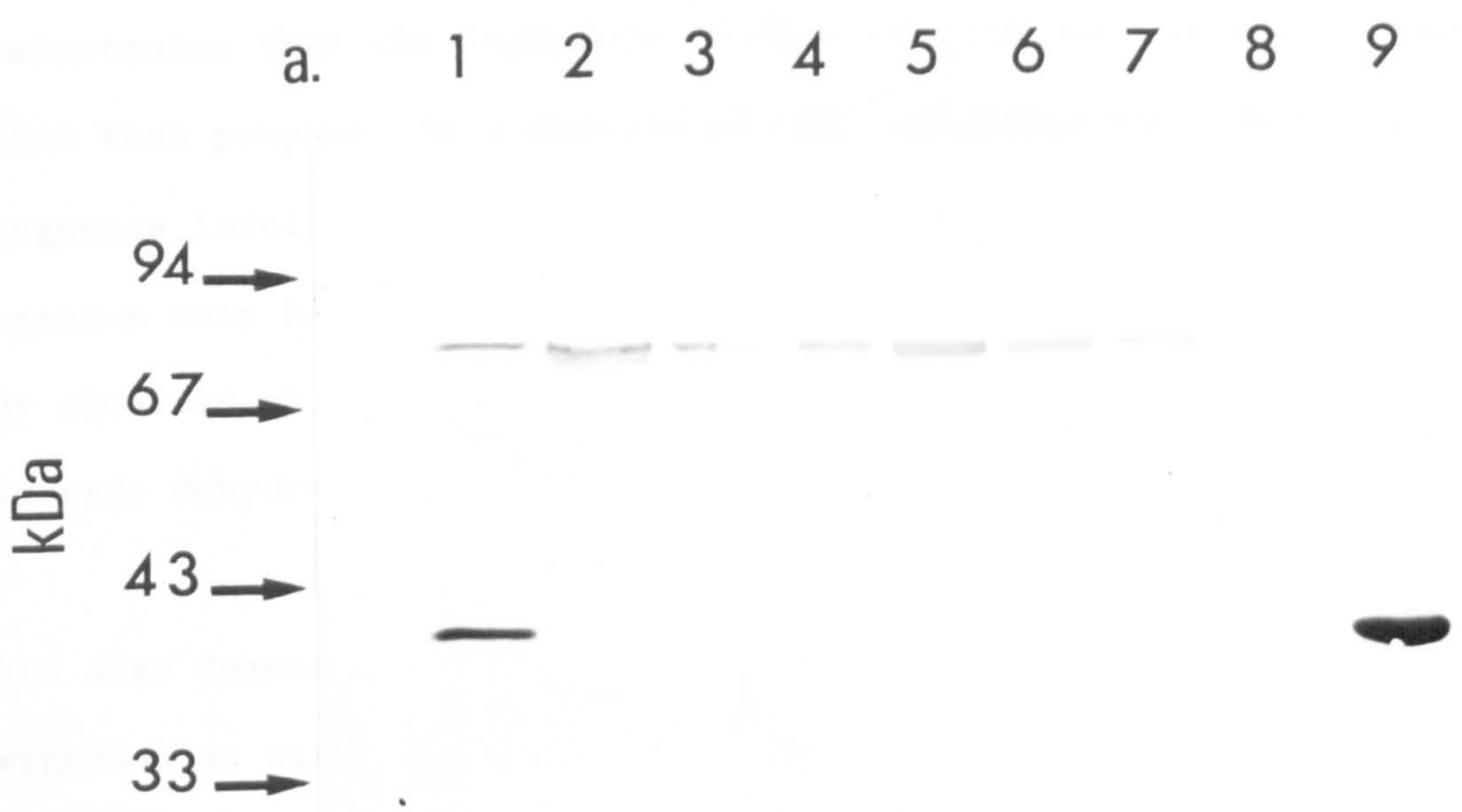
(a) Primary alcohols

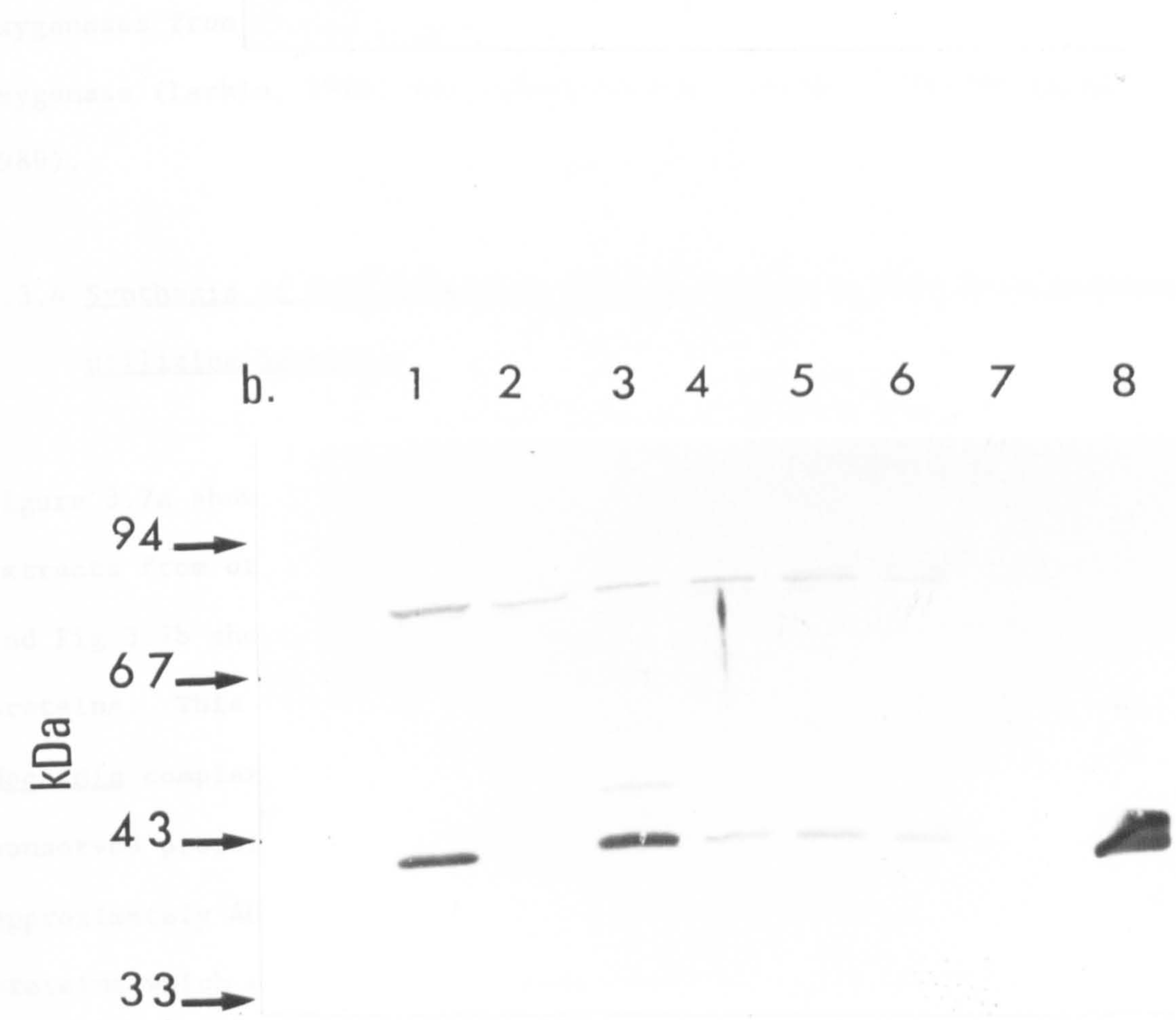
Track	Growth Substrate
1	Ethanol
2	Propan-1-ol
3	Butan-1-ol
4	Pentan-1-ol
5	Hexan-1-ol
6	Heptan-1-ol
7	Octan-1-ol
8	Citrate
9	(Purified enzyme)

(b) Secondary alcohols

Track	Growth Substrate
1	Propan-2-ol
2	Butan-2-ol
3	Pentan-2-ol
4	Hexan-2-ol
5	Heptan-2-ol
6	Octan-2-ol
7	Citrate
8	(Purified enzyme)

100 μg of protein per track, except purified enzyme (1 μg).


a.
1 2 3 4 5 6 7 8 9
94
67
43
33
kDa


b.
1 2 3 4 5 6 7 8
94
67
43
33
kDa

Growth of R.rhodochrous PNKb1 on C_2-C_8 primary and secondary alcohols demonstrates that the inability of this organism to utilize n-alkanes, other than propane, is a dictate of the hydroxylating capacity of the oxygenase involved in the biological activation of the n-alkane. This organism must have the ancillary enzymes available for the metabolism of any alcohols which might derive from C_2-C_8 n-alkanes, e.g. alcohol/aldehyde dehydrogenases, ketone monooxygenases, etc.

This also demonstrates the limited substrate specificity of the propane oxygenase as outlined by Woods (1988); the strict specificity of oxygenases from Rhodococcus sp. has been reported for 1-naphthol oxygenase (Larkin, 1988) and 1,8-cineole oxygenase (Williams et al., 1989).

3.3.4 Synthesis of NAD^+-dependent alcohol dehydrogenases from propane-utilizing bacteria

Figure 3.7a shows a silver stained gel after SDS-PAGE of soluble extracts from other propane-utilizing bacteria after growth on propane, and Fig 3.7b shows the corresponding Western-blot analysis of these proteins. This result demonstrated that members of the Rhodococcus - Nocardia complex had very similar protein profiles and also possessed a conserved protein, presumably an NAD^+-dependent alcohol dehydrogenase of approximately 40 kDa. The high degree of homology may be due to proteins which are components of a common propane oxygenase. It is interesting to speculate if these isolates would utilize acetol as a carbon and energy source, and if so whether similar polypeptide profiles are induced by the latter and propane, as is the case for R.rhodochrous PNKb1. Only two isolates, Pseudomonas butanovora and a Corynebacterium sp. showed major differences in polypeptide profiles and did not

Figure 3.7 (a) Silver-stained gel after SDS-PAGE of cell-free extracts of propane-utilizing bacteria grown on propane; (1) R.rhodochrous PNKb1, (2) Rhodococcus 69, (3) Nocardia 56, (4) Rhodococcus An-1, (5) Nocarida caviae, (6) Nocardia OU, (7) Corynebacterium GPYb1, (8) Pseudomonas butanovora. All 10 μg.

(b) Corresponding Western-blot analysis using antibodies against a purified NAD^+-dependent secondary alcohol dehydrogenase; (1) R.rhodochrous PNKb1, (2) Rhodococcus 69, (3) Nocardia 56, (4) Rhodococcus An-1, (5) Nocardia caviae, (6) Nocardia OU, (7) Corynebacterium GPYb1, (8) Pseudomonas butanovora, (all 10 μg); (9) R.rhodochrous PNKb1 after growth on citrate (100 μg) and (10) purified enzyme (1 μg).

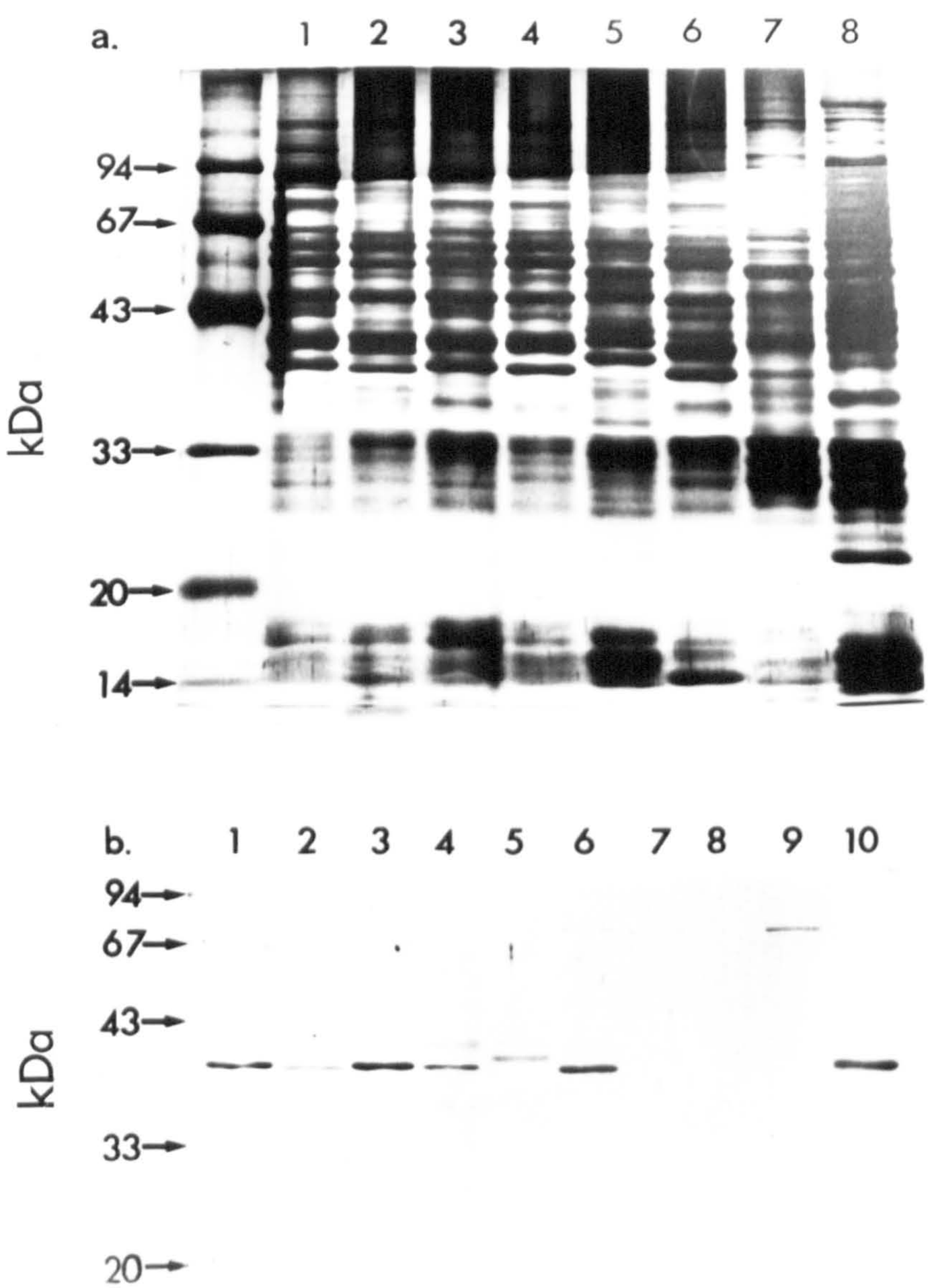

a.
1 2 3 4 5 6 7 8
94
67
43
33
20
14
kDa
b.
1 2 3 4 5 6 7 8 9 10
94
67
43
33
20
kDa

Table 3.10 Formation of 1,2-epoxypropane from propene by resting cell suspensions of R.rhodochrous PNKb1 after growth on propane, propan-1,2-diol and acetol

Growth Substrate	Formation of 1,2-epoxypropane from propene[a]
Propane	43
Propan-1,2-diol	0
Acetol	10
Pyruvate	0

[a]Rates quoted as nmoles 1,2-epoxypropane formed min^{-1} mg dry weight (determined by gas chromatography)

cells possess the same or similar oxygenase(s) activity. Further cell-free extract experiments involving studies carried out by Woods (1988) would address this problem in terms of substrate specificity, pH optimina, inhibitor profiles, etc.

The lack of epoxidating activity in propan-1,2-diol-grown cells might suggest that exogenously supplied propan-1,2-diol and acetol are metabolized *via* different oxidation pathways, this has already been suggested in section 3.1.1. Alternatively an oxygenase may be present which cannot perform epoxidation reactions, for example some strains of propane-utilizers converted propene to acrylic acid rather than 1,2-epoxypropane (Cerniglia *et al*., 1976). The lack of activity with pyruvate-grown cells demonstrates the inducible nature of the propane oxygenase system.

(b) Whole-cell oxidation of selected intermediates

Table 3.11 shows that propane, propan-1,2-diol and acetol grown cells are adapted to oxidize propane; the oxidation of various potential intermediates in the oxidation pathway are also shown.

Despite the inability of propan-1,2-diol-grown cells to epoxidate propene, they have the ability to oxidize propane. This gives further strength to the argument that another oxygenase may be present in *R.rhodochrous* PNKb1 other than the one initially described by Woods (1988); see above.

The rate of propane oxidation by propane-grown cells was comparable to that obtained by Woods (1988). Acetol and propan-1,2-diol-grown cells give similar, but lower rates of propane oxidation (Table 3.11).

Table 3.11 The ability of R.rhodochrous PNKb1 to oxidize potential intermediates of propane metabolism after growth on propane, propan-1,2-diol, acetol and pyruvate

Assay	Growth Substrate			
Substrate	Propane	Propan-1,2-diol	Acetol	Pyruvate
Propane	42	19	16	0
Propan-1-ol	202	71	42	10
Propan-2-ol	87	55	0	6
Propan-1,2-diol	20	139	57	40
Acetol	193	341	270	35

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

(determined by oxygen electrode studies)

Results from oxygen electrode studies and co-oxidation of propene (Table 3.10) by whole-cells suggests that propane and acetol-grown probably contain the same oxygenase, whereas propan-1,2-diol grown cells contain a different oxygenase. The inducible nature of the system was shown by a lack of activity with pyruvate-grown cells.

Propan-1-ol and propan-2-ol were oxidized by whole-cells; except acetol-grown cells which failed to oxidize propan-2-ol, although propane-grown cells gave the highest oxidation rates for those alcohols. The highest rate for propan-1,2-diol oxidation was obtained from propan-1,2-diol-grown cells, propane-grown cells gave the lowest rate. Propan-1,2-diol can be oxidized by constitutive enzymes as demonstrated by the result obtained for pyruvate-grown cells (Table 3.11).

The highest rate of acetol oxidation was obtained after cells were grown on propan-1,2-diol and comparatively high rates were obtained after growth on propane and acetol. These high rates of oxidation would by using simultaneous adaptation studies, suggest a role for acetol in the oxidation of both propan-1,2-diol and propane. The metabolism of acetol by constitutive enzymes was also demonstrated after growth on pyruvate.

(c) <u>NAD(P)H-dependent Acetol oxygenase activity</u>

Acetol oxygenase activity has been detected in <u>Mycobacterium</u> Py1 studies by Hartmans & deBont (1986) and in <u>R.rhodochrous</u> by Woods & Murrell (1989), both activities were inducible and NADPH-dependent.

NAD(P)H-dependent acetol oxygenase activity in soluble cell-free extracts of <u>R.rhodochrous</u> PNKb1 grown on propane, propan-1,2-diol and acetol has been demonstrated, (Table 3.12). This enzyme activity was

Table 3.12 Acetol oxygenase activity in cell-free extracts of R.rhodochrous PNKb1 grown on propane, propan-1,2-diol, acetol and pyruvate

Growth Substrate	Acetol oxygenase Specific Activities	
	NADPH[a]	NADH[b]
Propane	19	20
Propan-1,2-diol	18	1.5
Acetol	16	3
Pyruvate	0	0

[a]Specific activity measured as the NADPH-dependent oxygen consumption, expressed as nmoles oxygen consumed min^{-1} mg $protein^{-1}$

[b]Specific activity measured as the NADH-dependent oxygen consumption, expression as nmoles oxygen consumed min^{-1} mg $protein^{-1}$ (determined by oxygen electrode studies)

inducible as demonstrated by the lack of activity obtained from cell-free extracts of pyruvate-grown cells.

NADPH-dependent acetol oxygenase activity was approximately the same in all cell-free extracts, possibly indicating a common acetol oxygenase. However, NADH-dependent acetol oxygenase activity was only obtained at the same activity for cell-free extracts from propane-grown cells, propan-1,2-diol and acetol cell-free extracts had comparatively lower activities. This suggests the existence of two acetol oxygenase activities in cells grown on propane and a single NADPH-dependent activity cells grown on propan-1,2-diol and acetol. The low activities obtained for the latter two growth substrates with NADH may reflect the ability of the NADPH-dependent enzymes to utilize NADH as a cofactor, albeit poorly. However, the possibility cannot be discounted that the NADH-dependent activity in propane-grown cells was the result of some unrelated oxygenase oxidizing acetol fortuitously e.g. propane oxygenase is NADH-dependent (Woods, 1988).

The induction of NADPH-dependent acetol oxygenase activity after growth on propane and propan-1,2-diol suggests that these compounds may be metabolized <u>via</u> acetol. These results also demonstrated that propane and acetol oxygenase activities may be derived from separate enzymes which are NADH and NADPH-dependent respectively. Although this conflicts with the isolation of the <u>alk</u>$^-$ phenotype, which is propane and acetol deficient, hence, suggesting a single enzyme for the metabolism of both compounds.

3.3.6 Summary

Studies have shown that similar polypeptides are synthesized by R.rhodochrous PNKb1 after growth on propane and acetol, which may be components of a common oxygenase. Propan-1,2-diol grown cells synthesized polypeptides which were common to acetol-grown cells and could indicate a relationship between the metabolism of those compounds.

NAD^+-dependent secondary alcohol dehydrogenase was synthesized after growth on propane and subterminal intermediates; except acetone. This has demonstrated the existence of at least one other alcohol dehydrogenase which is required for the metabolism of propan-1-ol. Non-specific constitutive NAD^+-dependent alcohol dehydrogenase activity has also been demonstrated, albeit indirectly by Western-blot analysis. R.rhodochrous PNKb1 has the ability to utilize primary and secondary alcohols (C_2 - C_8), the NAD^+-dependent secondary alcohol dehydrogenase was only synthesized to any great extent after growth on ethanol, propan-2-ol and pentan-2-ol. This suggests the existence of alcohol dehydrogenases required for the metabolism of primary and higher secondary alcohols. The fact that this organism grows on a range of alcohols demonstrates that the inability to utilize n-alkanes, other than propane, is due to limitations of the first step oxygenase-dependent reaction. Studies by Woods (1988) have suggested this is due to a limited substrate specificity of the propane oxygenase.

Studies have also shown that propane, propan-1,2-diol and acetol-grown cells were simultaneously adapted to oxidize propane. Using precedents from the literature involving simultaneous adaptation this would suggest that propane is metabolized via propan-1,2-diol and acetol in R.rhodochrous PNKb1 (see section 1.3) although as outlined in section

1.3.2 such conclusions cannot be drawn without exercizing caution. However, although propan-1,2-diol-grown cells have the ability to oxidize propane, they cannot oxidize propene to 1,2-epoxypropane, unlike propane and acetol-grown cells. This might suggest the presence of a different non-specific oxygenase after growth on propan-1,2-diol which fortuitously oxidizes propane, studies on inhibitor profiles may prove this.

NADPH-dependent acetol oxygenase activity has been detected in cell-free extracts of the above grown cells. However, propane-grown cells possessed additional high levels of NADH-dependent activity. This result demonstrates the presence of two oxygenase activities after growth on propane. The induction of acetol oxygenase demonstrates that acetol may be an intermediate in the metabolism of propane and propan-1,2-diol.

SDS-PAGE and Western-blot analysis demonstrated that propane-utilizing bacteria belonging to the Rhodococcus - Nocardia complex synthesized homologous polypeptides and a conserved polypeptide of approximately 40 kDa, presumably an NAD^+-dependent alcohol dehydrogenase. Future studies could examine the enzymology of these organisms, for example the substrate specificity and stability of the propane oxygenases, as such systems may be more amenable to purification and characterization than the oxygenase activity reported by Woods (1988). Growth on acetol could also be investigated to examine if propane and acetol-grown cells are simultaneously adapted. The n-alkanes utilized by these organisms would also give an indication of the relationship between any n-alkane oxygenases which may be present in terms of substrate specificity. The results presented here tentatively indicate that the pathways for

propane oxidation may be common to members of the Rhodococcus - Nocardia complex.

A better understanding of the relationship between propane, propan-1,2-diol and acetol metabolism will only be gained when the inducible enzyme and activities are purified and characterized. Then direct and valid comparisons can be made for R.rhodochrous PNKb1 after growth on the above substrates.

3.4 Biochemical analysis of alc⁻ mutants

3.4.1 Introduction

AlcA⁻, alcB⁻ and alcAB⁻ mutants were isolated after NTG-mutagenesis, their phenotypes have already been discussed (see section 3.1). The following work describes the biochemical characterization of these mutants with respect to the soluble NAD^+-dependent alcohol dehydrogenase activity under 'growth supporting' and 'propane inducing' conditions (see below). The practical approach has been to assay for NAD^+-dependent alcohol dehydrogenase activity using propan-1-ol and propan-2-ol as substrates; undertake SDS-PAGE analysis of soluble cell-free extracts of mutants; to compare the pattern and distribution of polypeptides with the wild-type and to undertake Western-blot analysis of the NAD^+-dependent secondary alcohol dehydrogenase synthesized by alc⁻ mutants after non-denaturing PAGE. However, before this work was initiated experiments were done to find conditions which would support the growth of alc⁻ mutants in the presence of propane but which would also allow induction and expression of the propane oxidation system in R.rhodochrous PNKb1.

3.4.2 Growth of mutants under propane-inducing conditions with low concentrations of growth supporting substrates

Previous studies by Woods (1988) showed that the propane oxidizing system of R.rhodochrous PNKb1 was only induced after growth on propane. However, work contained within this study has demonstrated that probably the same system is also induced after growth on acetol (section 3.3).

Weaver & Lidstrom (1987) undertook phenotypic characterization of ethyl methanesulphonate generated methanol oxidation mutants of Xanthobacter H4-14. Methanol dehydrogenase was induced by growing the wild-type organism and mutants on growth supporting citrate (0.035% w/v) with methanol (0.15% v/v). A similar procedure has been developed for propane oxidation mutants of R.rhodochrous PNKb1 (see section 2.1.3). Conditions were found which allowed the growth of the wild-type on low concentrations of potential catabolite repressing growth supporting substrates but which still allowed expression of the propane oxidation system as demonstrated by the oxidation of propene to 1,2-epoxypropane, the distribution of 'propane specific' polypeptides after SDS-PAGE and the presence of NAD^+-dependent secondary alcohol dehydrogenase activities.

Table 3.13 shows the rates of formation of 1,2-epoxypropane from propene by whole cells of R.rhodochrous PNKb1 under growth conditions with low concentrations of growth supporting substrates in the presence and absence of 50% (v/v) propane. The propane oxygenase was repressed as demonstrated by the lack of epoxidating activity during growth on 0.035% (w/v) succinate, citrate and pyruvate. The maximum OD_{540nm} for cells after growth on the latter was approximately 0.3. The results also demonstrated that in the presence of propane, the oxygenase activity was expressed to the levels previously reported by Woods (1988). Succinate was chosen as the growth supporting substrate for alc$^-$ mutants in subsequent studies as this gave the highest rate of formation of 1,2-epoxypropane.

Figure 3.8a shows the soluble proteins after SDS-PAGE, from cells grown under conditions with low concentrations of supporting substrates in the absence (-) and presence (+) of propane. The results obtained from cells

Table 3.13 Formation of 1,2-epoxypropane from propene by whole cells of R.rhodochrous PNKb1 grown under propane-inducing conditions with low concentrations of growth supporting substrates

Growth Substrate	Propane oxygenase activity (units)[a]
Propane	43
Propane + Citrate*	17
Citrate*	0
Propane + Pyruvate*	36
Pyruvate*	0
Propane + Succinate*	38
Succinate*	0

[a] 1 unit = 1 nmole 1,2-epoxypropane produced min^{-1} mg dry $weight^{-1}$

*0.035% (w/v)

cells grown on succinate, citrate and pyruvate demonstrated that they do not synthesize propane specific polypeptides and this reflects their inability to epoxidate propene. However, in the presence (+) of propane these specific polypetides were synthesized, although qualitatively they appear to be in lower amounts when compared to the propane-grown cells. When cells were grown on citrate in the presence of propane the lowest rate of epoxidating activity was obtained (Table 3.13), and it can be seen that these cells contained the lowest amounts of specific propane polypeptides. Citrate-grown cells synthesized a large amount of specific polypeptide of approximately 57 kDa and this may be at the expense of the above polypeptides. The gel also showed that the NAD^+-dependent secondary alcohol dehydrogenase was also induced in the presence of propane with 0.035% (w/v) growth supporting substrates.

Figure 3.8b shows the corresponding Western-blot analysis. This result demonstrated that NAD^+-dependent secondary alcohol dehydrogenase was not synthesized after growth on 0.035% (w/v) citrate or pyruvate, however, synthesis was induced when propane was present. The result also showed that synthesis of this enzyme occurred after growth on 0.035% (w/v) succinate, albeit at lower amounts, as 100 μg of protein was used for the succinate (-) track of the gel whereas only 10 μg of protein was used for succinate (+) track. Table 3.14 shows that the NAD^+-dependent alcohol dehydrogenase activities were elevated after growth on the above substrates in the presence of propane. The highest propan-1-ol and propan-2-ol dehydrogenase activity was obtained after cells were grown on succinate in the presence of propane. After growth on citrate, pyruvate and succinate at 0.035% (w/v) both propan-1-ol and propan-2-ol dehydrogenase activities were approximately the same irrespective of carbon source, even through the NAD^+-dependent secondary alcohol dehydrogenase was not synthesized after growth on citrate or pyruvate

Figure 3.8 (a) SDS-PAGE of cell-free extracts of R.rhodochrous PNKb1 grown on propane with low concentrations of growth supporting substrates.

(b) Corresponding Western-blot analysis using antibodies against a purified NAD^+-dependent secondary alcohol dehydrogenase.

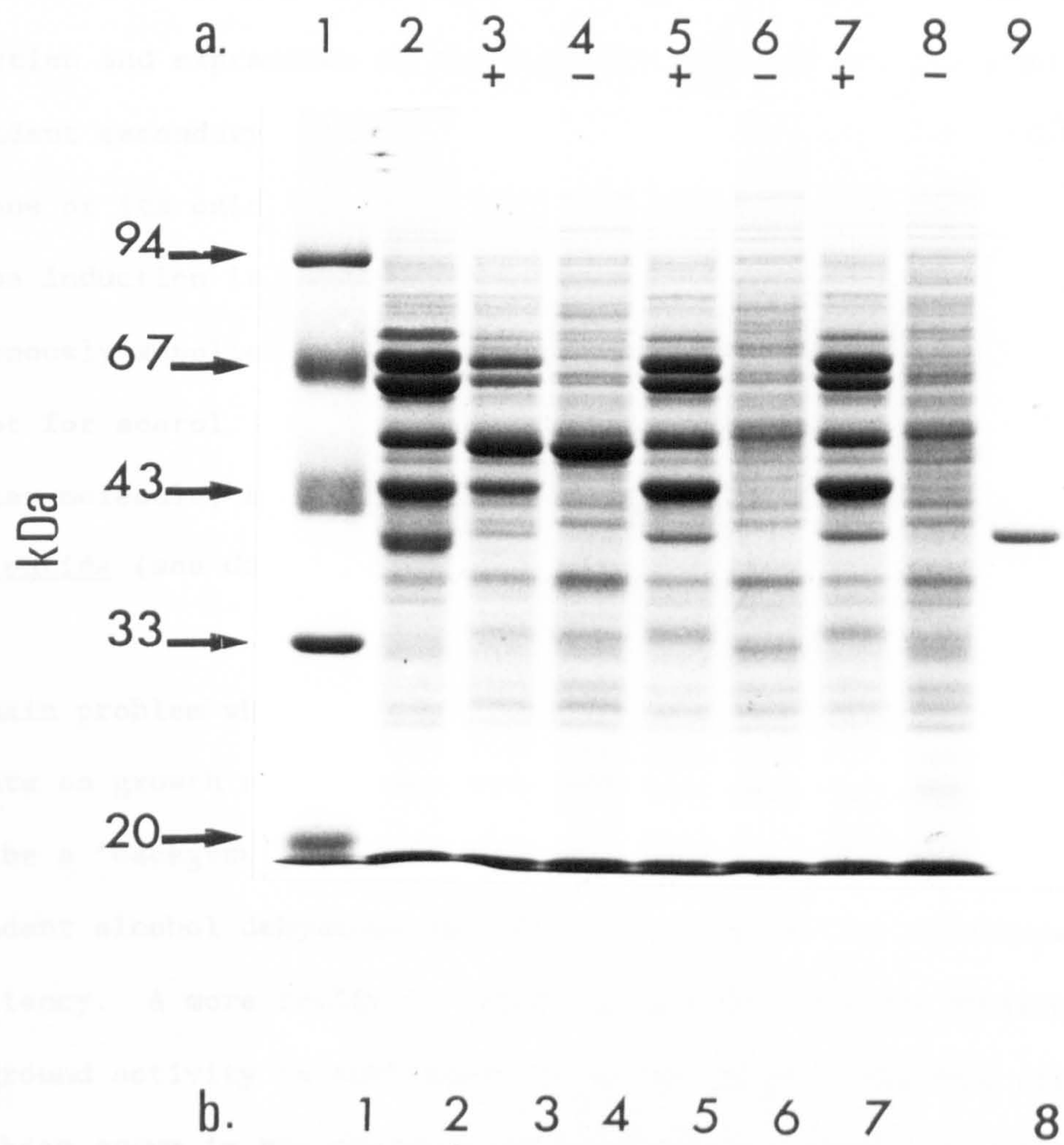
(a)

Track	Growth Substrate
1	(Molecular weight markers)
2	Propane
3	Propane + citrate
4	Citrate
5	Propane + pyruvate
6	Pyruvate
7	Propane + succinate
8	Succinate
9	Purified enzyme (1 μg)

(b)

Track	Growth Substrate	
1	Propane	(10 μg)
2	Propane + citrate	(10 μg)
3	Citrate	(100 μg)
4	Propane + pyruvate	(10 μg)
5	Pyruvate	(100 μg)
6	Propane + Succinate	(10 μg)
7	Succinate	(100 μg)
8	Purified enzyme	(1 μg)

All 100 μg protein per track, except b.


a.
1
2
3 +
4 −
5 +
6 −
7 +
8 −
9
94
67
43
33
20
kDa
b.
1
2 +
3 −
4 +
5 −
6 +
7 −
8
94
67
43
33
kDa

(see Fig 3.8b). This suggests the presence of constitutive NAD^+-dependent alcohol dehydrogenase activity as suggested in sections 3.2 and 3.3. The results from Tables 3.13 and 3.14 suggests that the induction and expression of the propane oxygenase and elevated NAD^+-dependent secondary alcohol dehydrogenase activities are dependent on propane or its oxidation products. If oxidation products are involved in the induction it must only arise if they are generated *in vivo*, as exogenously supplied intermediates do not induce the oxidation system, except for acetol. The possibility arises that propane may be the inducer molecule, as is the case of octane for the octane monooxygenase of *P.putida* (see Grund *et al*., 1975).

The main problem which can be foreseen is that after growth of *alc*$^-$ mutants on growth supporting substrates in the presence of propane there will be a 'background' activity from non-specific constitutive NAD^+-dependent alcohol dehydrogenase which may obscure any phenotype deficiency. A more realistic value for activity may be obtained if the background activity is subtracted from the results obtained after cells have been grown in the presence of propane plus growth supporting substrate. These figures are shown in brackets. After this adjustment cells grown on 0.035% (w/v) succinate in the presence of propane had the highest activity for NAD^+-dependent secondary alcohol dehydrogenase, the values were similar to those obtained from propane-grown cells (see Table 3.5, section 3.2). In common with the propane oxygenase activity cells grown on 0.035% (w/v) citrate in the presence of propane had the lowest specific activities for NAD^+-dependent secondary alcohol dehydrogenase.

From these results it was concluded that *alc*$^-$ mutants should grown on succinate, as the growth supporting substrate, as this yields cells with

Table 3.14 NAD^+-dependent alcohol dehydrogenase activities for wild-type R.rhodochrous PNKb1 grown under propane-inducing conditions with low concentrations of growth supporting substrates

Growth Substrate	Primary alcohol dehydrogenase[a] Specific Activity (units)[c]	Secondary alcohol dehydrogenase[b] Specific Activity (units)[c]
Propane	36	176
Propane + Citrate[d]	33 (30)	165 (93)
Citrate	3	72
Propane + Pyruvate[d]	54 (50)	171 (103)
Pyruvate	4	68
Propane + Succinate[d]	68 (63)	242 (169)
Succinate	5	73

[a]Primary alcohol dehydrogenase determined using propan-1-ol as a substrate.

[b]Secondary alcohol dehydrogenase determined using propan-2-ol as a substrate.

[c]1 unit = 1 nmole NAD^+ reduced min^{-1} mg $protein^{-1}$.

[d]citrate, pyruvate and succinate used at 0.035% (w/v).

( ) = NAD^+-dependent alcohol dehydrogenase activity obtained after growth on Propane + Growth supporting substrate minus that activity obtained after growth on that substrate alone.

the highest propane oxygenase and NAD^+-dependent secondary alcohol dehydrogenase activities.

3.4.3 alcA⁻ mutants

Table 3.15 shows the NAD^+-dependent alcohol dehydrogenase activities for alcA⁻ mutants. Propan-1-ol and propan-2-ol dehydrogenase activities were present after the mutants were grown on succinate and this represents the non-specific constitutive activity as discussed earlier. Mutants alcA9 and alcA13 had lower NAD^+-dependent alcohol dehydrogenase activities under propane-inducing conditions when compared with growth on succinate; except for alcA13 which had slightly higher propan-1-ol dehydrogenase activity. However, alcA 12 and alcA 14 had elevated NAD^+-dependent alcohol dehydrogenase activities when grown under propane-inducing conditions, but these were lower when compared with the wild-type values. Despite these lower dehydrogenase activities these mutants still grew on propan-2-ol, which suggests the presence of a functional NAD^+-dependent secondary alcohol dehydrogenase (alcA 12 and alcA 14) or another ancillary enzyme which enables growth on this alcohol but not propan-1-ol (alcA 9 and alcA 13).

Figure 3.9a shows the SDS-PAGE analysis of soluble proteins from alcA⁻ mutants after growth on succinate in the absence (-) and presence of propane (+). AlcA 9 and alcA 12 failed to synthesize any propane-specific polypeptides under propane-inducing conditions, this may suggest the involvement of a regulatory element (alkR, as discussed in section 3.2) which may be required for the induction and expression of these polypeptides. Thus SDS-PAGE has demonstrated that these mutants are alcA⁻ and alkR⁻. However, mutants alcA 13 and alcA 14 did synthesize propane-specific polypeptides, although lower amounts when compared with

Table 3.15 NAD^+-dependent alcohol dehydrogenase activities for alcA⁻ mutants

Mutant	Growth Substrate	Primary alcohol dehydrogenase[a] Specific Activity (units)[c]	Secondary alcohol dehydrogenase[b] Specific Activity (units)[c]
Wild-type	Propane + Succinate[d]	68 (63)	242 (169)
	Succinate	5	73
alcA 9	Propane + Succinate	9	123
	Succinate	11	147
alcA 12	Propane + Succinate	28 (25)	176 (92)
	Succinate	3	84
alcA 13	Propane + Succinate	6	54
	Succinate	4	80
alcA 14	Propane + Succinate	19 (15)	185 (80)
	Succinate	4	105

[a]Primary alcohol dehydrogenase determined using propan-1-ol as a substrate.

[b]Secondary alcohol dehydrogenase determined using propan-2-ol as a substrate.

[c]1 unit = 1 nmole NAD^+ reduced min^{-1} mg $protein^{-1}$.

[d]Succinate used at 0.035% (w/v).

( ) = NAD^+-dependent alcohol dehydrogenase activity obtained after growth on Propane + Succinate minus that activity obtained after growth on Succinate alone.

the wild-type. These latter mutants may represent a class of mutants where inducer recognition and subsequent synthesis of propane-specific polypeptides is inefficient. Thus alcA$^-$ mutants appear to be defective in the synthesis of all readily identifiable propane-specific polypeptides.

Western-blot analysis for the immunological detection of NAD^+-dependent secondary alcohol dehydrogenase and any mutant polypeptides of this enzyme has used non-denaturing PAGE as the separation medium. The rationale for using this system is as follows; the rate of migration of proteins in an electric field depends upon charge density (the ratio of charge to mass) of the protein concerned at a given pH. However, in the presence of SDS the intrinsic charges of the polypeptide are insignificant compared to the negative charges provided by the bound detergent, so that the SDS-polypeptide complexes have essentially identical charge densities and migrate in polyacrylamide gels according to polypeptide size. There may not be sufficient resolution of the separation of NTG-generated mutant polypeptides, in which only small changes in primary structure may be obtained. In contrast to the above technique non-denaturing PAGE of native proteins under non-dissociating buffer conditions fractionates a protein mixture in such a way that subunit interaction, native protein conformation and biological activity (see Fig. 3.3, section 3.2) are preserved. Thus the separation of native proteins occurs on the basis of both size and charge (see Hames, 1981). Changes in size or perhaps more importantly charge may occur after NTG-mutagenesis.

Figure 3.9b shows that the secondary alcohol dehydrogenase was synthesized by all of the alcA$^-$ mutants when grown on succinate in the presence and absence of propane. The electrophoretic mobility of the

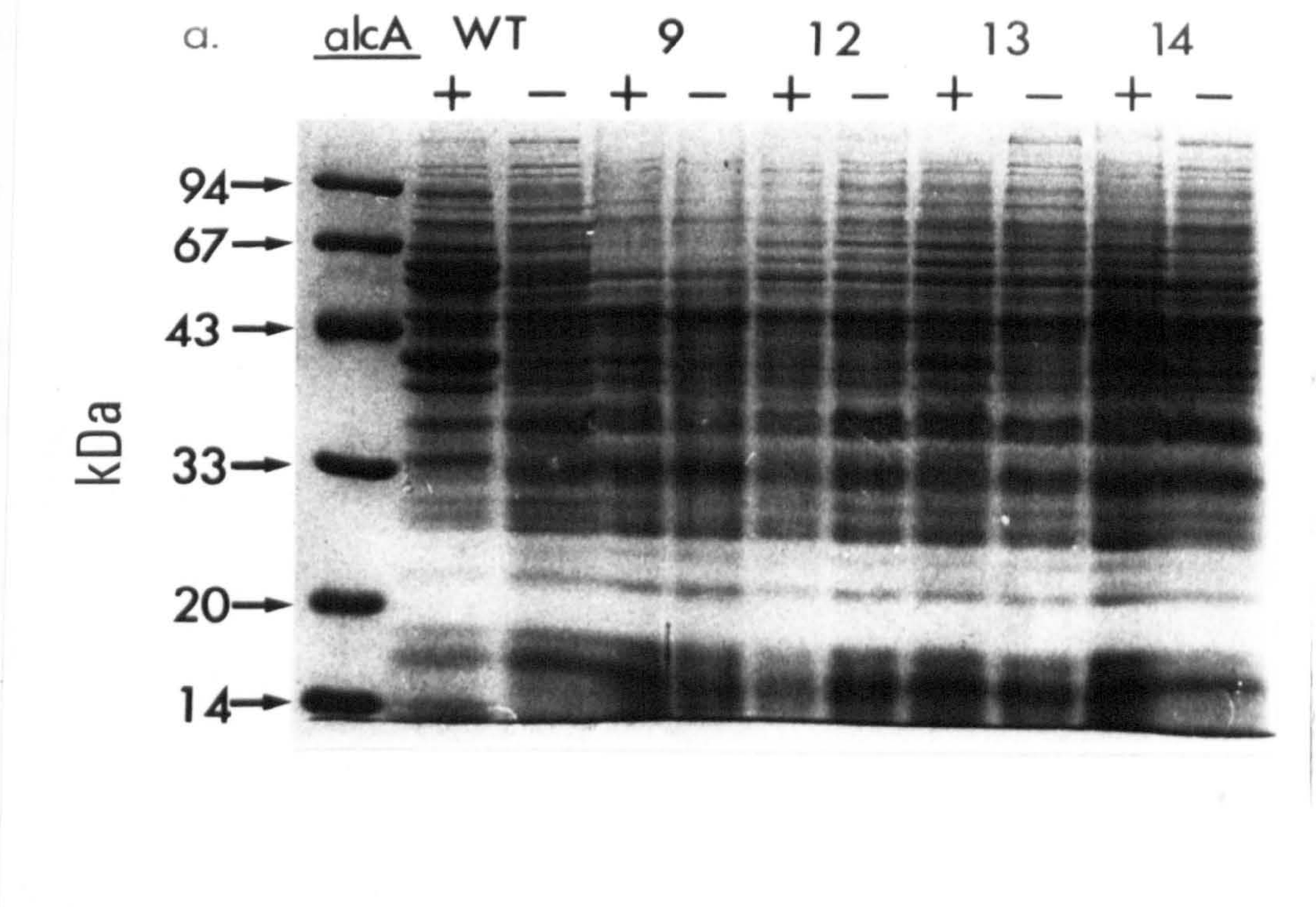
Figure 3.9 (a) SDS-PAGE of cell-free extracts of alcA$^-$ mutants, all 100 μg protein per track.

(b) Western-blot analysis of alcA$^-$ cell-free extracts after non-denaturing PAGE using antibodies against a purified NAD$^+$-dependent secondary alcohol dehydrogenase, 10 μg protein for + and 100 μg for - tracks. Tracks 1 and 2 represent 100 μg of cell-free extract from citrate-grown cells and 1 μg of purified enzyme respectively.

\+ = propane + succinate grown cells

\- = succinate grown cells

WT = wild-type


a.
alcA
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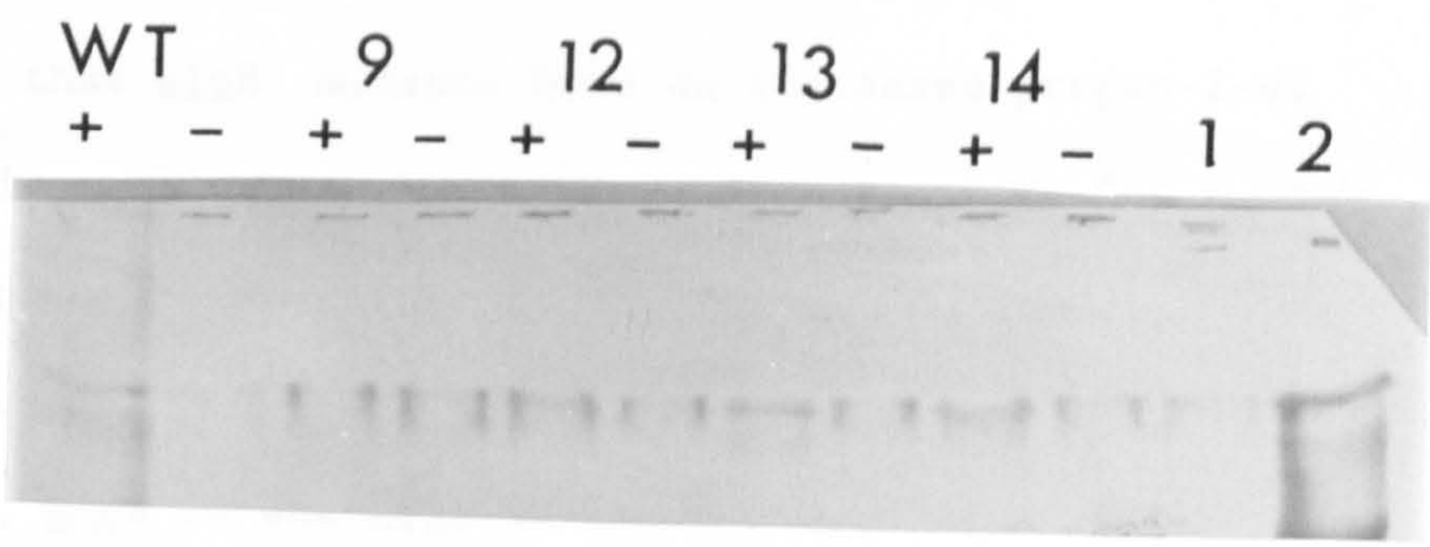
14
+ − + − + − + − + −
94
67
43
33
20
14
kDa


b. alc A
WT
9
12
13
14
+ − + − + − + − + −
1
2

native enzymes are comparable with the polypeptide synthesized by the wild-type and also the purified enzyme. This result suggests that alcA$^-$ mutants do not have a mutated secondary alcohol dehydrogenase, although SDS-PAGE showed that comparatively lower amounts of enzyme were synthesized. The retention of a wild-type NAD^+-dependent secondary alcohol dehydrogenase explains the ability of these mutants to utilize propan-2-ol as sole carbon and energy source.

3.4.4 alcB$^-$ mutants

NAD^+-dependent alcohol dehydrogenase activities for alcB$^-$ mutants are shown in Table 3.16. Propan-2-ol dehydrogenase activities were much lower for these mutants when compared with the wild-type dehydrogenase activities. All succinate grown cells had high propan-2-ol dehydrogenase activities when compared with the wild-type. AlcB 8 grown on succinate had higher propan-2-ol dehydrogenase activity than cells grown under propane-inducing conditions.

In contrast to propan-2-ol dehydrogenase activity, propan-1-ol dehydrogenase remained unchanged in succinate grown cells. These results demonstrate that alcB$^-$ mutants have an increased propan-2-ol dehydrogenase activity, this may be necessary to compensate for a lack of a functional propane-inducible secondary alcohol dehydrogenase. Although higher total propan-2-ol dehydrogenase activities were obtained for mutants alcB 12, alcB 26 and alcB 14 under propane inducing conditions compared with the wild-type they still could not be grown on propan-2-ol. This may mean that the non-specific dehydrogenase does not function efficiently *in vivo* to enable the metabolism of propan-2-ol. These background levels of alcohol dehydrogenase activity has made

Table 3.16 NAD^+-dependent alcohol dehydrogenase activities for alcB⁻ mutants

Mutant	Growth Substrate	Primary alcohol dehydrogenase[a] Specific Activity (units)[c]	Secondary alcohol dehydrogenase[b] Specific Activity (units)[c]
Wild-type	Propane + Succinate[a]	68 (63)	242 (169)
	Succinate	5	73
alcB 8	Propane + Succinate	6	69
	Succinate	8	98
alcB 12	Propane + Succinate	52 (45)	241 (89)
	Succinate	7	152
alcB 26	Propane + Succinate	54 (45)	271 (88)
	Succinate	9	183
alcB 14	Propane + Succinate	45 (38)	276 (78)
	Succinate	7	198

[a]Primary alcohol dehydrogenase determined using propan-1-ol as a substrate.

[b]Secondary alcohol dehydrogenase determined using propan-2-ol as a substrate.

[c]1 unit = 1 nmole NAD^+ reduced min^{-1} mg $protein^{-1}$.

[d]Succinate used at 0.035% (w/v).

( ) = NAD^+-dependent alcohol dehydrogenase activity obtained after growth on Propane + Succinate minus that activity obtained after growth on Succinate alone.

direct comparisons between the wild-type organism and alcB$^-$ mutants difficult.

The lower propan-2-ol dehydrogenase activity for alcB 8 may be due to a failure to respond to propane-inducing conditions and this is shown in Fig 3.10a. This demonstrates that this mutant cannot synthesize propane specific polypeptides as well as the NAD^+-dependent secondary alcohol dehydrogenase. This alcB 8, in common with the alcA$^-$ mutants discussed in 3.4.2, may represent an alkR$^-$ phenotype which cannot be induced by the propane molecule. Such a result might also suggest that the regulation of the propane specific polypeptides and secondary alcohol dehydrogenase are co-regulated to some extent. AlcB 12 synthesized only two major propane specific polypeptides, both of which had slightly altered molecular weights of approximately 62 and 45 kDa. AlcB 26 synthesized the specific polypeptides, but lower amounts were observed when compared with the wild-type. AlcB 14 also synthesized specific polypeptides but lower amounts were also observed, however, an additional polypeptide of approximately 41 kDa could also be observed. The results of SDS-PAGE for alcB$^-$ mutants have demonstrated that treatment with NTG has had a drastic affect upon the ability of this class of mutants to synthesize propane specific polypeptides; which Woods (1988) suggested may be components of a large propane oxygenase complex.

Western-blotting, Fig 3.10b, has also been used to examine the secondary alcohol dehydrogenase from the alcB$^-$ mutants. AlcB 8 failed to synthesize NAD^+-dependent secondary alcohol dehydrogenase and this may explain its inability to utilize propan-2-ol as a sole carbon and energy source. AlcB 12 and alcB 26 synthesized the secondary alcohol dehydrogenase, although slightly different electrophoretic mobilities

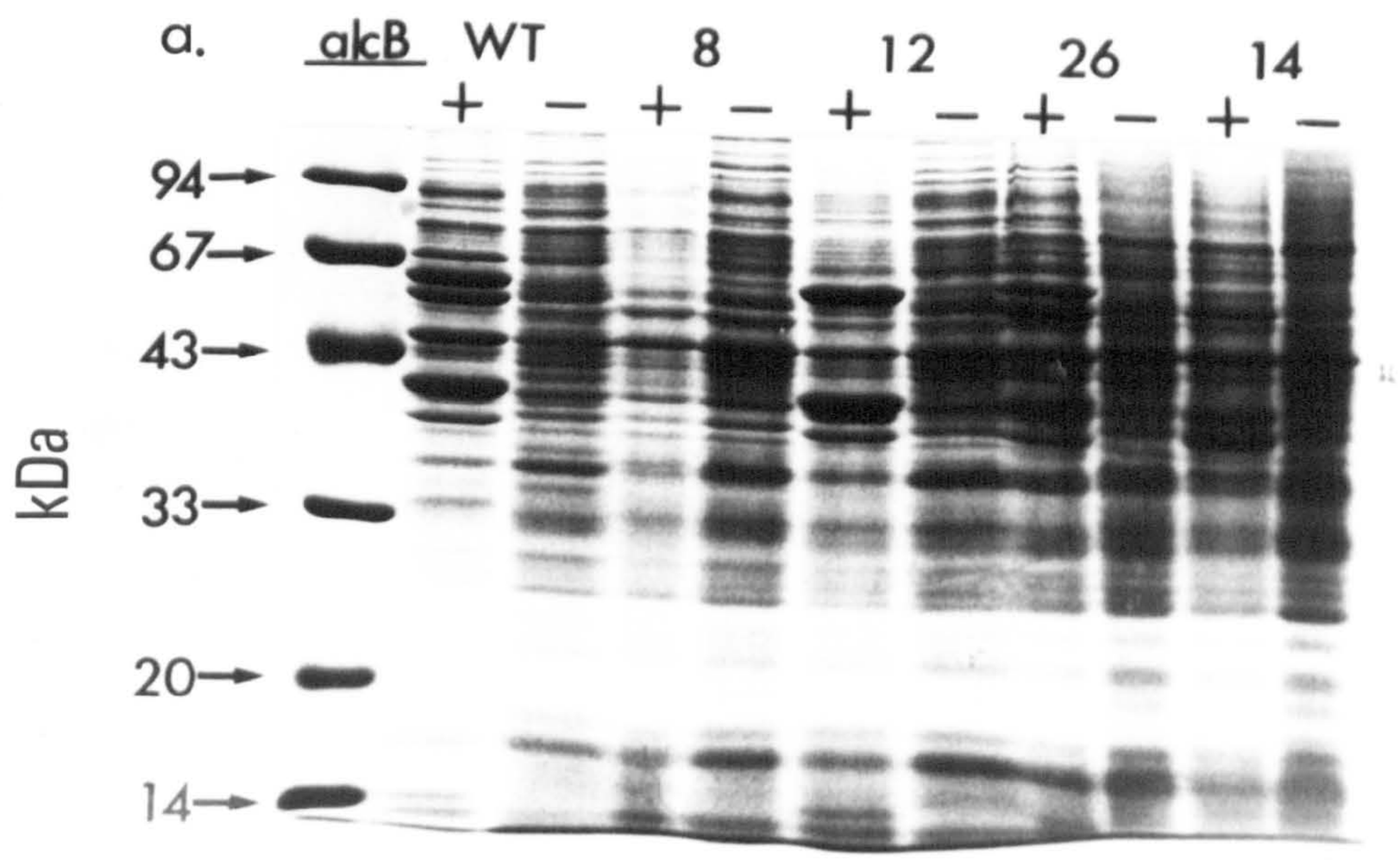
Figure 3.10 (a) SDS-PAGE of cell-free extracts of alcB$^-$ mutants, all 100 μg protein per track.

(b) Western-blot analysis of alcB$^-$ cell-free extracts after non-denaturing PAGE using antibodies against a purified NAD$^+$-dependent secondary alcohol dehydrogenase, 10 μg protein for + and 100 μg for - tracks. Tracks 1 and 2 represent 100 μg of cell-free extract from citrate-grown cells and 1 μg of purified enzyme respectively.

\+ = propane + succinate grown cells

\- = succinate grown cells

WT = wild-type


a.
alcB
WT
8
12
26
14
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94
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20
14
kDa

b. alcB
WT
8
12
26
14
+ − + − + − + − + − 1 2

were observed when compared to the purified enzyme. AlcB 14 synthesized comparatively large amounts of a polypeptide of approximately 41 kDa, however, this was probably defective enzyme (e.g. by premature translational termination of protein synthesis) as demonstrated by immuno-blotting. This sensitive technique detected significant amounts of the above enzyme, because polyclonal antibodies would have recognized antigenic determinants on the mutant 41 kDa NAD^+-dependent secondary alcohol dehydrogenase. These results confirm that these mutants synthesized mutant polypeptides or lower amounts of polypeptide corresponding to the above enzyme and thus may explain their phenotypic trait.

3.4.5 alcAB⁻ mutants

Two double mutants alcAB 17 and alcAB 18 have been examined with respect to their alcohol dehydrogenase activities, Table 3.17. The former had lower alcohol dehydrogenase activities under propane-inducing conditions when compared with cells grown on succinate. The non-specific propan-2-ol dehydrogenase activity was elevated three-fold when compared with the wild-type activity, this has also been demonstrated for some alcB⁻ mutants. The propan-1-ol dehydrogenase activity was also markedly lower when compared with the wild-type. AlcAB 18 had only slightly lower propan-1-ol and propan-2-ol dehydrogenase activity and this suggests that this mutant has reverted to the wild-type phenotype. This mutant had a reversion frequency of 2.5×10^{-7}, as demonstrated by the number of colonies growing on propane from a lawn of cells. Soluble proteins from these mutants have also been examined after SDS-PAGE, Fig 3.11a. The results show that the propane specific polypeptides were synthesized under propane-inducing conditions. However, alcAB 17 synthesized comparatively lower amounts of these polypeptides, except for the 53 kDa

Table 3.17 NAD^+-dependent alcohol dehydrogenase activities for alcAB$^-$ mutants

Mutant	Growth Substrate	Primary alcohol dehydrogenase[a] Specific Activity (units)[c]	Secondary alcohol dehydrogenase[b] Specific Activity (units)[c]
Wild-type	Propane + Succinate[d]	68 (63)	242 (169)
	Succinate	5	73
AlcAB 17	Propane + Succinate	30 (18)	169
	Succinate	12	229
AlcAB 18	Propane + Succinate	58 (58)	268 (115)
	Succinate	0	153

[a]Primary alcohol dehydrogenase determined using propan-1-ol as a substrate.

[b]Secondary alcohol dehydrogenase determined using propan-2-ol as a substrate.

[c]1 unit = 1 nmole NAD^+ reduced min^{-1} mg $protein^{-1}$.

[d]Succinate used at 0.035% (w/v).

Figure 3.11 (a) SDS-PAGE of cell-free extracts of alcAB⁻ mutants, all 100 μg protein per track.

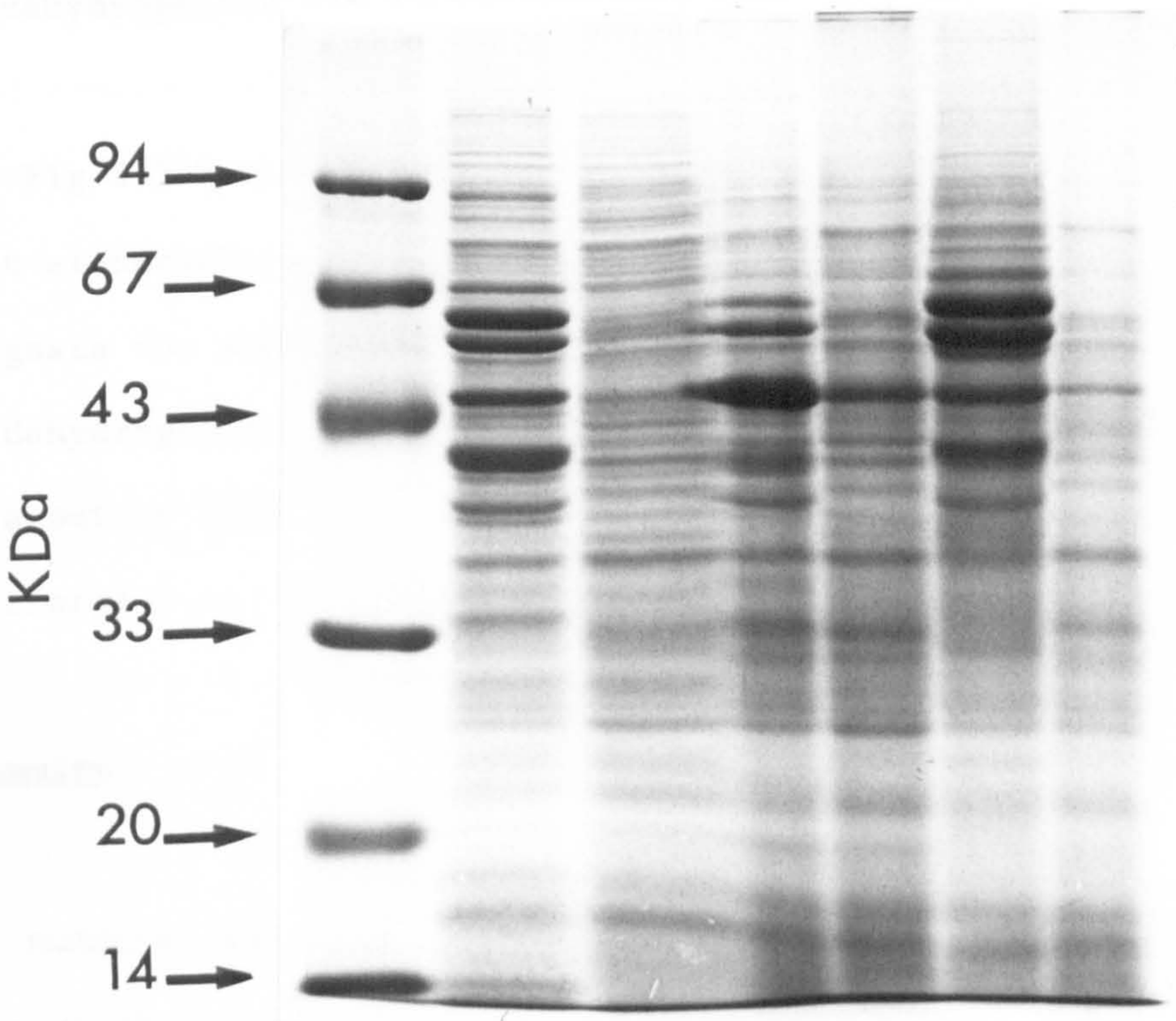
(b) Western-blot analysis of alcAB⁻ cell-free extracts after non-denaturing PAGE using antibodies against a purified NAD^+-dependent secondary alcohol dehydrogenase, 10 μg protein for + and 100 μg for tracks.

\+ = propane + succinate grown cells

\- = succinate grown cells

WT = wild-type

a. alcAB



b. alcAB

polypeptide which was over-expressed. AlcAB 18 synthesized comparatively more of these polypeptides than wild-type cells, leading to the conclusion that the revertant phenotype was under less stringent control for the induction and expression processes. The secondary alcohol dehydrogenase was synthesized by both mutants.

However, Fig 3.11b showed that the secondary alcohol dehydrogenase had different electrophoretic mobilities compared with the wild-type enzyme. This suggests the synthesis of a functional, but altered, secondary alcohol dehydrogenase for alcAB 18, the difference in mobility may be brought about by changes in the enzymes primary structure, affecting its overall charge.

3.4.6 Summary

The alc$^-$ mutants, with the exception of alcAB 18, had lower NAD^+-dependent alcohol dehydrogenase activities under propane-inducing conditions after the succinate associated alcohol dehydrogenase activity had been taken into account. Alcohol dehydrogenase activity was never absent and this 'background' activity made the interpretation of enzyme activities for alc$^-$ mutants more complicated and not entirely clear.

Western-blotting has shown that the alcA$^-$ mutants appear to synthesize secondary alcohol dehydrogenase which migrates through non-denaturing polyacrylamide gels at the same rate as the wild-type enzymes and may be classed therefore as wild-type dehydrogenase, which probably explains their ability to metabolize propan-2-ol. However, alcB$^-$ and alcAB$^-$ mutants synthesized a polypeptide which had altered electrophoretic properties as demonstrated by immuno-blotting after non-denaturing PAGE,

and this explains their inability to metabolize propan-2-ol as sole carbon and energy source.

SDS-PAGE demonstrated that alc$^-$ mutants;

(i) had altered patterns of propane-specific polypeptides,

(ii) had comparatively lower amounts of these polypeptides,

(iii) or that these polypeptides were not synthesized under propane-inducing conditions.

The first two observations may be due to mutations of structural genes required for propane metabolism and the latter is probably due to a mutation in regulatory gene(s) (alkR) which leads to a failure of the propane molecule or its subsequent oxidation products to induce the synthesis of these specific polypeptides. In addition these results also suggest that alc$^-$ mutants are also defective in some other aspects of propane metabolism. Such multiple mutations are known to occur after NTG-mutagenesis, especially for genes which are closely linked (Guerola et al., 1971). This may suggest that propane oxidation genes are clustered on the chromosome and may form an operon, the occurrence of such multiple effects is much less likely to occur if genes were located at different regions of the chromosomes.

The biological activity of these mutated secondary alcohol dehydrogenases is not known, although this could be assessed by performing activity stains after non-denaturing PAGE. A more efficient separation and detection of these mutated NAD^+-dependent secondary alcohol dehydrogenases, which probably only had a few changes to the enzyme, as demonstrated by only a slight change in electrophoretic mobility, would be achieved using isoelectric focusing or 2-D gels in combination with Western-blotting.

Different types of mutagens should be used in future studies to generate different types of mutants, for example deletion mutants of Nocardia sp. 239 have been generated after treatment with 1,2,7,8-diepoxyoctane (deBoer et al., 1988). Such mutants would be very stable and cause more drastic physical and chemical changes for any particular enzyme relating to a metabolic deficiency, e.g. inability to utilize propan-2-ol (alcB$^-$).

A better understanding of alcohol metabolism is required for alcA$^-$ mutants as a specific propan-1-ol/primary alcohol dehydrogenase activity has yet to be purified and characterized. The establishment of conditions which support the growth of mutants and allow induction of propane-specific polypeptides will allow a genetical analysis of the propane oxygenase system by in vitro complementation studies, similar to those discussed by Macham & Heydeman (1974).

3.5 Genetic studies on R.rhodochrous PNKb1

3.5.1 Introduction

Plasmid screening was undertaken to discover if there were any large catabolic type plasmids in propane-utilizing bacteria which might contain genes responsible for propane oxidation. The reasons for this assumption has come from other work carried out on hydrocarbon oxidation, which in some instances was coded for by information carried on plasmids (see section 1.5). There has been a report by Murai (1981) that Rhodococcus fascians harbours a 99 kb plasmid responsible for hydrocarbon utilization. It was also necessary to screen organisms for the presence of any small endogenous plasmids, which may be of use as replicons in the construction of shuttle vectors for the development of gene cloning (see Singer & Finnerty, 1988).

One of the reasons for the slow progress in the application of molecular genetics to the study of the biology of 'exotic' microorganisms is often that DNA cannot be introduced into them by any of the usual methods (transformation, transduction, conjugation or protoplast fusions). The possession of a method which enables efficient gene transfer in R.rhodochrous PNKb1 may be very useful in the study of propane oxidation by this organism. Therefore, these studies were undertaken in an attempt to develop a method of genetic transformation.

3.5.2 Plasmid screening of propane-utilizing bacteria

The most critical step in the isolation of plasmid DNA is cell lysis. Initial experiments demonstrated that this was extremely difficult to achieve without the incorporation of glycine into the growth media. The

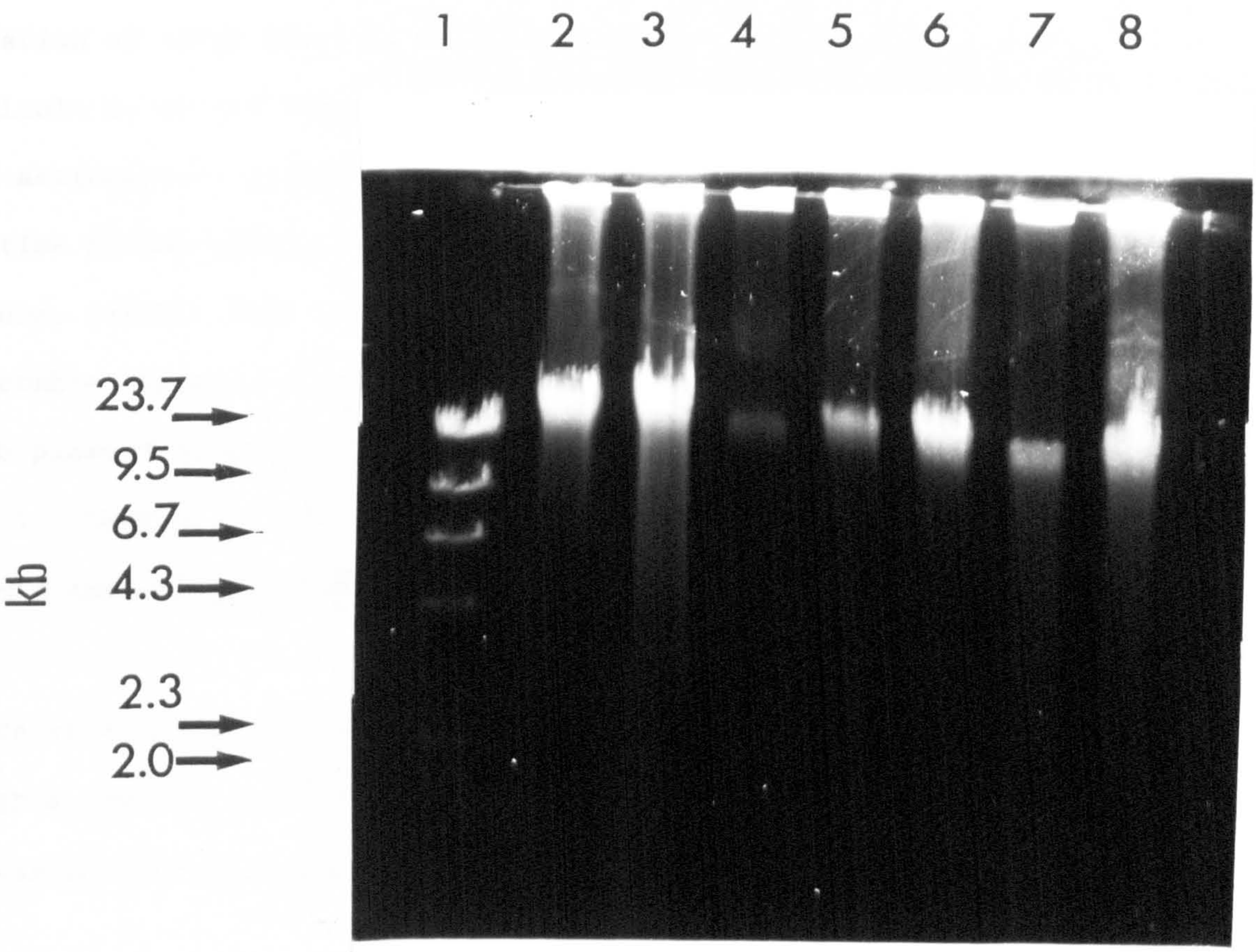
use of glycine is a well documented technique for obtaining protoplasts from Streptomycetes sp. (Hopwood et al., 1985). Heaton et al. (1988) in their work on mutants defective in the synthesis of D-alanine, discussed the effects of glycine on bacterial cells. They proposed a model in which glycine-induced lysis of Dal$^-$ mutant resulted from the effect of glycine on metabolic reactions related to D-alanine metabolism. At high glycine concentrations glycine can be incorporated into muramyl peptide, which reduces cross-linking of the peptidoglycan and weakens the cell wall (Hammes et al., 1973). Further uses for glycine-treated cultures may include the preparation of genomic DNA from Rhodococcus sp. which is difficult to lyse by conventional methods, and also the weakening of cell walls to enable the preparation of higher protein content cell-free extracts. Woods (1988) presented data which showed that the preparation of high protein content cell-free extracts was difficult, however, glycine treated propane-grown cells resulted in cell clumping, therefore, this technique may require more refinement to overcome this problem.

The results obtained (Fig 3.12) indicate that the gene(s) necessary for growth of a series of propane-utilizers appear to be coded by chromosomal genes. Two faint plasmid DNA bands can be observed above the chromosomal DNA from Rhodococcus 69. The phenotype coded by this plasmid and its size have not been determined. It is not known whether these bands represent one or two different cryptic plasmids in this strain. The two bands may be supercoiled and open circle forms of the same plasmid. No small endogenous plasmids were observed in any of the bacteria screened.

The lack of plasmids observed was despite the fact that a number of isolation techniques were employed including those of Eckhardt (1978),

Figure 3.12 Plasmid screening of propane-utilizing bacteria

Track	Propane-utilizer
1	(λ *Hind*III molecular weight markers)
2	*Rhodococcus* An-1
3	*R.rhodochrous* PNKb1
4	*Corynebacterium* GPYb1
5	*Nocardia* OU
6	*Nocardia caviae*
7	*Nocardia* 56
8	*Rhodococcus* 69


1 2 3 4 5 6 7 8
23.7
9.5
6.7
Kb 4.3
2.3
2.0

Hansen & Olsen (1978), Wheatcroft & Williams (1981), Kado & Liu (1981) and Maniatis et al. (1982). The possibility that extremely large plasmids were present, but refractory to the above methods of isolation, remained. For example, pulsed-field electrophoresis revealed several different linear mega-plasmids (180-510 kb) in Nocardia opaca strains which could not be detected by conventional isolation techniques (Kalkus et al., 1989), however, this method was not tried. A problem with the isolation of large plasmids is their low-copy number, which makes it difficult to detect them on agarose gels. Also it has been suggested that actinomycete plasmids may integrate into the chromosome and much of the time do not exist as autonomously replicating units (Charter & Hopwood, 1984). Such a situation may be responsible for the lack of detectable plasmid in Rhodococcus An-1, which is supposed to harbour a 90 kb plasmid responsible for aniline degradation (L. Atkins, per. comm.). Originally this organism was used as a positive control for plasmid screening of Rhodococcus-Nocardia type organisms.

Future studies could use the techniques of Chakrabarty et al. (1973) and Singer & Finnerty (1984b) which include conjugation and plasmid curing, to test whether plasmids are involved in hydrocarbon oxidation.

3.5.3 Antibiotic sensitivity of R.rhodochrous PNKb1

In order to determine potential vectors for use in subsequent transformation experiments, an antibiotic sensitivity spectrum of R.rhodochrous PNKb1 was determined.

Antibiotic multidisks (Oxoid and Mastring) were placed directly onto freshly prepared R.rhodochrous PNKb1 lawns on nutrient agar plates.

Table 3.18 Antibiotic sensitivity spectrum of R.rhodochrous PNKb1

Antibiotic	Concentration/disk	Sensitivity
Ampicillin	50 μg	+++
Naladixic Acid	20 μg	-
Rifampicin	20 μg	+++*
Gentamycin	20 μg	+++
Chloramphenicol	25 μg	+
Erythromycin	20 μg	+++
Kanamycin	30 μg	++
Tetracycline	20 μg	+++
Penicillin G	1 unit	+++
Streptomycin	20 μg	+++*
Thiostrepton	20 μg	+++

*Low frequency of spontaneous mutants obtained

+ - Degree of sensitivity determined by size of zone of inhibition around each disk, +++ being very sensitive, to + least sensitive.

- - No detectable sensitivity.

Table 3.18 shows the effect of each antibiotic on the R.rhodochrous lawn after 7 days exposure to the antibiotics.

Table 3.18 demonstrates that R.rhodochrous PNKb1 is sensitive to a broad range of antibiotics. The antibiotics which caused inhibition on plates, with the exception of choromphenicol, also caused inhibition of growth in liquid culture over a 2 week period. Therefore, any plasmid vectors conferring resistance to those antibiotics may be used in transformation experiments. Gram-positive plasmid vectors conferring resistance to thiostrepton were used in these experiments.

3.5.4 Development of a transformation system for R.rhodochrous PNKb1

Induction of competence of R.rhodochrous PNKb1 was initially attempted using the method of Maniatis et al. (1982), using CsCl-purified pIJ702 (see Hopwood et al., 1984) and pMVS302 (see Singer & Finnerty, 1988) purified from Streptomyces coelicolor and Rhodococcus sp. H13-A respectively. However, despite alteration of various parameters such as plasmid DNA concentration (0.25 → 5 $\mu g\ ml^{-1}$), heat shock (35 → 45°C) exposure time (2-10 min) and also length of expression time (2-18 hrs); no transformant colonies were observed.

Protoplast transformation was attempted using the method of Singer & Finnerty (1988). However, 3% (w/v) glycine had to be included into the growth medium if protoplasts were to be obtained.

Direct microscopic counts using a haemocytometer revealed that 25% of the bacteria population were in the protoplast form. However, < 1% of these protoplasts were regenerated. Control experiments using S.coelicolor and Rhodococcus sp. H13-A resulted in approximately 100%

protoplasts after treatment and gave > 50% regeneration. This demonstrates that methods for protoplast formation for R.rhodochrous PNKb1 require further development.

Despite incubation of R.rhodochrous PNKb1 protoplasts with pIJ702 and pMVS302 at concentrations 0.25 → 5 μg ml^{-1}, no transformant colonies were observed. However, control experiments with S.coelicolor yielded 10^3 transformants per μg of pIJ702 DNA. Singer & Finnerty (1988) reported that pMVS302 was transformed into several Rhodococcus sp. and coryneform bacteria. However, R.rhodochrous ATCC 13808 was not transformed and this may explain the lack of transformation in R.rhodochrous PNKb1, although this may be due to the very low regeneration frequency. These experiments involving protoplast transformation would be improved if other plasmid vectors were available which had a Rhodococcus origin of replication.

3.5.5 Deoxyribonuclease activity in R.rhodochrous PNKb1

A number of factors can strongly influence plasmid transformation of bacteria. For example, the cellular content of nucleases may impair transformation, as these enzymes cause the degradation of foreign plasmid DNA which can result in a decreased transformation efficiency; R.rhodochrous and Rhodococcus sp. are known to produce restriction endonucleases (see Old & Primrose, 1985). Therefore, due to the lack of transformation an analysis of the content of DNases of R.rhodochrous PNKb1 was carried out as described by Rama et al. (1987). Using this method, no DNase activity was detected in both whole cells or cell-free extracts.

3.5.6 Conjugation

Filter mating experiments between Rhodococcus An-1, Str^s_{20}, harbouring pLA1 and R.rhodochrous PNKb1-Str^r_{20} were performed using exponentially growing cultures (OD_{540nm} approx 0.6) in a ratio of 1:1, pLA1 is a conjugative plasmid which encodes the genes for aniline utilization (An^+). However, no R.rhodochrous PNKb1-Str^r_{20} An^+ transconjugants were isolated after 2 week incubation. This mating was repeated several times with the same result. Other work has demonstrated that pLA1 could be transferred to other Rhodococcus sp. by conjugation (L. Atkins, per. comm.). However, the result contained in Fig 3.12 demonstrated that Rhodococcus An-1 does not possess the 90 kb pLA1 plasmid. This plasmid may have integrated into the chromosome and this would be one explanation for the lack of transconjugants. Another possible reason for this result could have been the toxicity of aniline to R.rhodochrous PNKb1, however, this organism utilized propane in the presence of 0.2% (v/v) aniline.

Plasmid transfer by conjugation from E.coli to R.rhodochrous PNKb1-Str^r_{20} was also attempted using the filter-mating method of Trieu-Cuot et al. (1987). The broad-host range vector used was pAT187, which was reported to be mobilized into a range of Gram-positive bacteria by the self-transmisible IncP plasmid pRK212.1, co-resident in the E.coli donor. A number of attempts at filter mating, including varying the times of incubation (18-48 hrs) and the recipient:donor ratios, did not result in any kanamycin resistant (from pAT187) transconjugants of R.rhodochrous PNKb1-Str^r_{20}.

3.5.7 Transduction

Transduction of R.rhodochrous PNKb1 was attempted using the method and Q4 bacteriophage reported by Dabbs (1987). This phage mediated the transduction of a number of unlinked markers in Rhodococcus erythropolis. Control experiments using Q4 and R.erythropolis CW12 gave approximately 10^{10} pfu.ml^{-1} as reported by Dabbs (1987). However, no plaques were observed on lawns of R.rhodochrous PNKb1 despite a number of attempts. This result suggests that plaque formation by Q4 may be species, or even strain specific. To obtain transduction for R.rhodochrous PNKb1 may require phage isolation and characterization from environmental samples.

3.5.8 Electroporation

Recently, the technique of electro-transformation (electroporation) has revolutionized the transformation of bacteria. Electroporation works by the formation of transient holes or pores in the cell membrane by high voltage electric shock, and has been used successfully to give high transformation frequencies to a wide range of bacterial genera previously thought untransformable; for reviews see Potter (1988) and Chassy et al. (1988). There have been a number of recent reports of the transformation of Gram-positive bacteria (Chassy & Flickinger, 1987; Luchansky et al., 1988; Wolf, 1989).

Electroporation of R.rhodochrous PNKb1 was carried out using the method of Chassy & Flickinger (1987). Pulses of 5 kV cm^{-1} at 25 μF were generated using a Bio-Rad Laboratories Gene Pulser^{TM}. Although the conditions above were sufficient to transform Lactobacillus casei, no transformants of R.rhodochrous PNKb1 were isolated using pIJ702 and

pMV302 as plasmid vectors. Further experimentation is required in order to determine the usefulness of this method for transformation of this organism. Variables affecting transformation frequency with this method may include the shape and duration of the pulse, initial field strength, the number of discharges applied, the physiological condition of the cells and also the electroporation buffer used. For example, the field strength (5 kV cm^{-1}) used in these studies may not be great enough for transformation of R.rhodochrous, as Bordetella pertussis and Corynebacterium glutamicum required 25 and 40 $kVcm^{-1}$ respectively for optimal frequency of transformation, field strength was found to be the most important variable (see Zealey et al., 1988; Wolf et al., 1989). High field strengths may be particularly important in the transformation of R.rhodochrous PNKb1; as the cell wall is very resistant to mechanical, chemical and enzymatic breakage, and thus may also be resistant to the formation of pores by electroporation. Although in studies with Corynebacterium glutamicum showed that lysozyme treated cells (osmotically sensitive) required lower field strengths for efficient transformation, 25-30 $kVcm^{-1}$ (Wolf et al., 1989).

3.5.9 Summary

The main problem which will be encountered in any genetic transformation of R.rhodochrous PNKb1 is the lack of endogenous plasmids which would provide origin of replications for the construction of suitable plasmid vectors. Conventional systems for the introduction of DNA into R.rhodochrous PNKb1, transformation, conjugation and transduction, were unsuccessful in these preliminary studies. Future work should be spent on optiminizing conditions which may aid the introduction of DNA into cells. Even the relatively new electroporation technique proved unsuccessful in transforming this organism, however, this may be because

of the limited number of plasmid vectors tried and conditions used. However, electroporation is now the fastest and simplest method for transforming bacteria and its increasing success rate in transforming a wide range of organisms, has the greatest potential for use with R.rhodochrous PNKb1.

Although genetic transformation was not a success, these studies will provide a useful starting point for other researchers using this organism. Various classes of NTG-generated mutants have been isolated (alk$^-$, alc$^-$) in this study and now require a high frequency transformation system for their complementation by genomic DNA and subsequent genetic characterization.

Chapter 4: General Discussion

GENERAL DISCUSSION

4.1 Discussion

The pathways of propane metabolism in R.rhodochrous PNKb1 have been previously investigated using three different approaches; growth substrate specificity, simultaneous adaptation in whole cells and enzyme activities in cell-free extracts (Woods, 1988). However, none of these approaches gave an unequivocal answer as to the metabolism of propane via terminal or subterminal pathways. The aim of this study was to isolate NTG-generated mutants blocked in the metabolism of terminal and subterminal intermediates to determine whether or not propane was metabolized via the above pathways. During the course of the isolation and biochemical analysis of alc$^-$ mutants it became necessary to purify the NAD^+-dependent alcohol dehydrogenase activity. The induction of the NAD^+-dependent secondary alcohol dehydrogenase was examined after growth on the above intermediates. Genetic transformation of R.rhodochrous PNKb1 was also attempted with the eventual aim of complementing alk$^-$ mutants.

Growth studies with various classes of mutants have demonstrated that propane oxidation probably proceeds via terminal and subterminal oxidation, see Fig. 4.1. This suggests that the first step in metabolism, catalyzed by a propane oxygenase, is non-specific in its insertion of an atom of oxygen into the propane. Studies by Patel et al. (1983) have demonstrated the production of propan-1-ol and propan-2-ol from propane using cell-free extracts of propane-grown Arthrobacter sp. and Brevibacterium sp. However, the actual stoichiometric amounts of these alcohols produced by R.rhodochrous PNKb1 is not known and must await the purification of the oxygenase for this to be elucidated.

Fig. 4.1 **Proposed propane oxidation pathway for Rhodococcus rhodochrous PNKb1.**

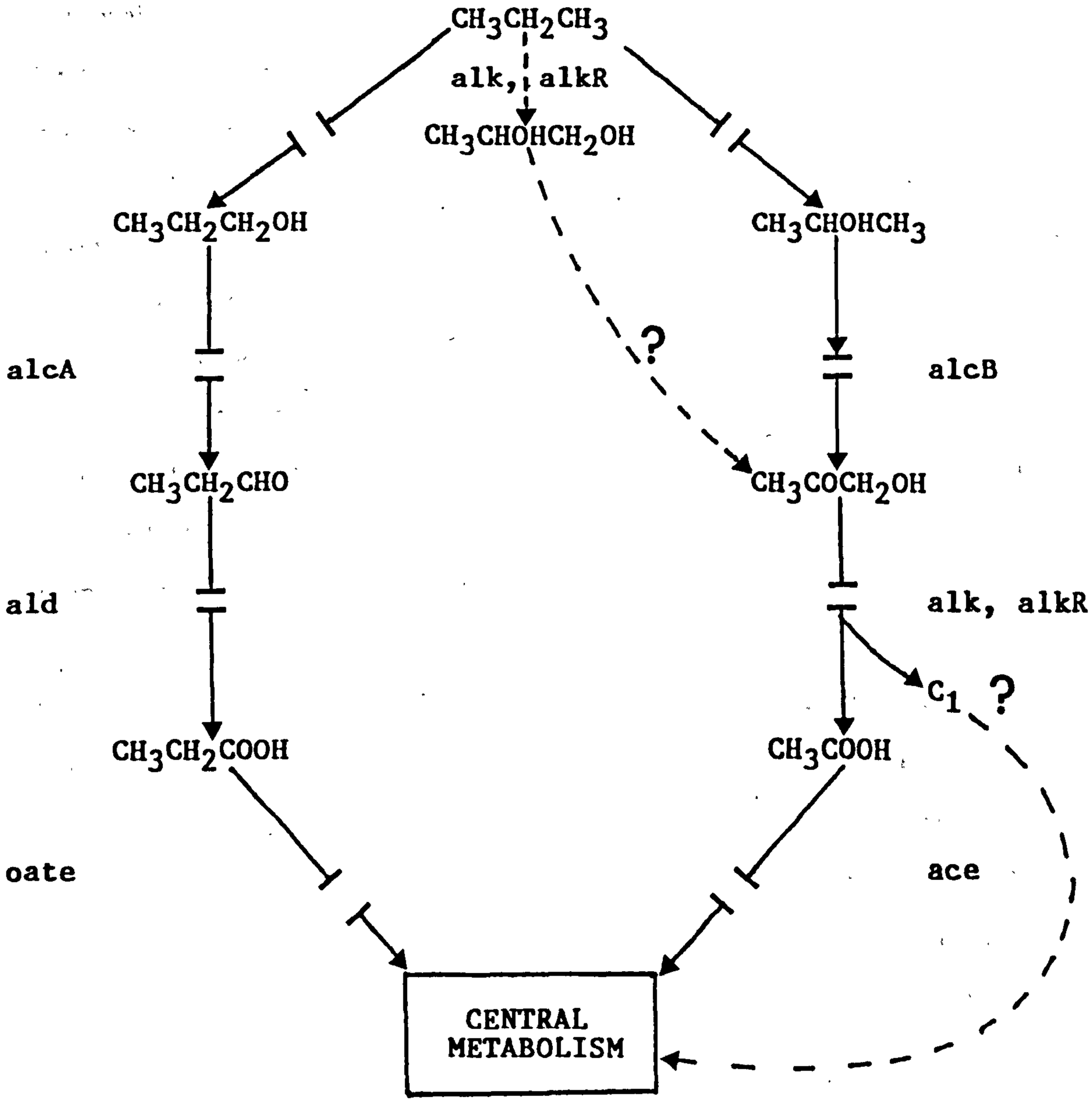


Figure 4.1 shows the proposed propane oxidation pathway for R.rhodochrous PNKb1 on the basis of phenotypic characterization of mutants using growth tests. The breaks in the metabolic pathway arrows (- →) are particular metabolic lesions obtained after NTG-mutagenesis; dashed lines (---) represent theoretical alternatives to this scheme, although there is no direct evidence from this study for their operation by this organism (?). The formation of propan-1,2-diol from propane may provide a direct route to acetol, this hypothesis could be tested by gas chromatography if the propane mon(di)-oxygenase were purified, however, see Woods (1988) for problems on purification. The formation of the C_1 moiety (?) has been proposed by Coleman & Perry (1984) and Hartmans & deBont (1986) as a product of the metabolism of propane and acetol respectively. The most notable feature of this pathway is the absence of acetone as a subterminal oxidation intermediate, this has been deduced from the ket$^-$ phenotype which still allows growth on propane and propan-2-ol.

The roles for propan-1-ol and propan-2-ol in the metabolism of propane has been confirmed by the isolation of alcA$^-$ and alcB$^-$ mutants respectively and this result also confirms the existence of two genetic loci for two alcohol dehydrogenases. The isolation of mutants with the inability to metabolize propane (alk$^-$) selected by a lack of growth on propane, utilized all potential intermediates except acetol. This result suggests a direct relationship between the pathways for the metabolism of these growth substrates, and implicates acetol as an important intermediate in the metabolism of propane. Such a result also suggests the presence of a common enzyme, probably an oxygenase, after growth of R.rhodochrous PNKb1 on propane and acetol. Indeed SDS-PAGE analysis of cell-free extracts after growth on the above substrates

revealed the presence of similar polypeptides of approximately 68, 64 and 48 kDa which may be components of a large propane/acetol oxygenase complex. Another explanation for the dual phenotypic deficiency would be a mutation in a regulatory gene (alkR), whose product was necessary for the induction of enzyme systems required for the metabolism of both compounds. All ten alk$^-$ mutants were propane and acetol growth deficient, however, all alk$^-$ mutants were selected by a lack of growth on propane, no independent acetol-deficient phenotype was obtained. This was because of the difficulty of obtaining growth on plates where acetol was the growth substrate. Future studies would require the isolation of acetol deficient mutants (aol$^-$) independently of a propane deficiency selection.

In the metabolism of propane by R.rhodochrous PNKb1, thus far described, a common oxygenase would be required for the metabolism of propane and acetol. This may catalyze the formation of propan-1-ol and propan-2-ol from propane and also cleave acetol in a Baeyer-Villiger reaction to form acetate and reduced C_1 moiety (see Hartmans & deBont, 1986). An enzyme may catalyze both reactions as the reduction of alkyl hydroperoxides (possibly formed by the terminal and subterminal activation of propane) and Baeyer-Villiger reactions as both are mechanistically similar (see Sykes, 1975). Growth on propan-1,2-diol, which may be metabolized via acetol (see section 1.3.3.1) induced the synthesis of a specific polypeptide of approximately 53 kDa, however, it did not synthesize the polypeptides which were specific to propane and acetol-grown cells. Alk$^-$ mutants were able to utilize propan-1,2-diol as sole carbon and energy source which implies that this compound is not an intermediate in the oxidation of propane or metabolized via acetol; or there exists another pathway for the oxidation of exogenously supplied propan-1,2-diol (see section 1.3.3.1). To try and assign a

role for this compound in the metabolism of propane or whether it is metabolized *via* acetol would require future studies involving the isolation of propan-1,2-diol (*iol*$^-$) and acetol (*aol*$^-$) growth deficient mutants.

The isolation of two mutants with the inability to metabolize acetone (*ket*$^-$), indicates that this compound is not an intermediate of the subterminal oxidation of propane. This suggests that acetone is metabolized via a different pathway(s) from propane, for example carboxylation to produce acetoacetate (see section 1.3.3.2). SDS-PAGE has demonstrated that acetone-grown cells synthesized specific polypeptides of approximately 92 and 84 kDa and these may be components of an acetone oxidizing enzyme system. *ket*$^-$ mutants still possess the ability to utilize propan-2-ol as a growth substrate, thus this compound is not metabolized *via* acetone in *R.rhodochrous* PNKb1. This raises the question as to the metabolism of propan-2-ol formed at the initial step in propane oxidation, its metabolism to acetol would require a further hydroxylation at the terminal carbon to produce propan-1,2-diol and then dehydrogenation of the subterminal hydroxyl group to form acetol. This would be the required metabolic sequence if the formation of acetone is not involved. An alternative to this sequence is the initial production of propan-1,2-diol from propane by a novel propane dioxygenase, then dehydrogenation at the subterminal hydroxyl group by a secondary alcohol dehydrogenase would produce acetol (see Fig. 4.1).

Thus it may be possible that the production of the *alcB* gene is involved in the metabolism of propan-2-ol and propan-1,2-diol (see later). This could be confirmed by testing *alcB*$^-$ mutants for growth on propan-1,2-diol.

Mutant analysis has also demonstrated that terminal and subterminal oxidation pathways are distinct. The isolation of oate$^-$ and ace$^-$ phenotypes shows that utilization of terminal and subterminal intermediates respectively is blocked; but a block in the metabolism of all terminal intermediates does not preclude growth on subterminal intermediates or vice versa. It would be interesting to test whether a oate$^-$ phenotype precluded growth on primary alcohols, and whether a ace$^-$ phenotype precludes growth on secondary alcohols and even chain length fatty acids.

The involvement of propanal as an intermediate in the oxidation of propane is now confirmed by the isolation of the ald$^-$ phenotype, which also precludes growth on propan-1-ol.

If the oxidation of propane does involve the formation of a mixture of propan-1-ol and propan-2-ol, why doesn't an alcA$^-$ or alcB$^-$ mutant still grow more slowly on propane using the subterminal or terminal pathway respectively? This may be due to the in vivo build up of relatively high concentrations of propan-1-ol, propanal or propan-2-ol which would be toxic to the cell. Indeed 0.1% (v/v) of propanal or propan-2-ol supplied in the growth medium is toxic to R.rhodochrous PNKb1.

The isolation of alcA$^-$ and alcB$^-$ mutants demonstrates R.rhodochrous PNKb1 has separate alcohol dehydrogenases responsible for the metabolism of propan-1-ol and propan-2-ol. If a single enzyme was responsible for the metabolism of these alcohols then only alcAB$^-$ mutants would have been isolated. It was necessary to purify the alcohol dehydrogenase activity from propane-grown cells, because preliminary biochemical analysis of alc$^-$ mutants still demonstrated significant NAD^+-dependent propan-1-ol and propan-2-ol dehydrogenase activity. Initial experiments

showed that alcohol dehydrogenase activity was NAD^+-dependent and located in the soluble cell-free extract, no particulate $NAD(P)^+$-dependent or independent; or soluble NAD^+-independent activity was observed.

An NAD^+-dependent alcohol dehydrogenase was purified from propane-grown cells using a simple and efficient two step purification. IEF demonstrated that the purified enzyme was a single molecular species and *in situ* activity stains using non-denaturing PAGE showed that this enzyme was responsible for both propan-1-ol and propan-2-ol dehydrogenase activities. The enzyme was classed as a NAD^+-dependent secondary alcohol dehydrogenase because of its consistently higher specific activities with secondary alcohols. Its distinguishing characteristics compared to the alcohol dehydrogenases reported by Hou *et al*. (1983b) and Coleman & Perry (1985) are a lower molecular weight, dimeric subunit composition and comparatively higher k_m values.

The latter was found to be two orders of magnitude higher for propan-1-ol and propan-2-ol, 12 and 18 mM respectively. Woods (1988) raised doubts as to the physiological significance of the NAD^+-dependent alcohol dehydrogenase activity in propane-metabolism based on the relatively high k_m values measured. However, a more rigorous search of the literature showed that such values are not without precedent (see Ashraf & Murrell, 1990).

The presence of other non-specific NAD^+-dependent alcohol dehydrogenase activities has been demonstrated by a combination of enzyme assays and Western-blot analysis. For example, citrate-grown cells had relatively high propan-1-ol and propan-2-ol dehydrogenase activities, but this activity was not the NAD^+-dependent secondary alcohol dehydrogenase as

this enzyme was not synthesized. This enzyme was synthesized after growth on propane and subterminal intermediates (propan-2-ol, acetol and acetate) but not terminal intermediates (propan-1-ol, propanal and propanoate). Interestingly, the enzyme was also synthesized after growth on propan-1,2-diol in relatively high amounts, similar to those obtained for propane and acetol-grown cells. However, the enzyme was not synthesized after growth on acetone.

Biochemical analysis of *alc*$^-$ involved assaying for NAD^+-dependent alcohol dehydrogenase activity and Western-blot analysis to determine whether the secondary alcohol dehydrogenase was synthesized or had altered electrophoretic mobility. The latter suggests the synthesis of a defective enzyme as a result of mutation induced by NTG. These experiments were also undertaken to confirm whether or not the *alc*B gene product was the NAD^+-dependent secondary alcohol dehydrogenase.

However, preliminary experiments were undertaken to find conditions which supported growth of *alc*$^-$ mutants (to obtain biomass for enzymes and Western-blot analysis) but still allowed induction of the propane oxidizing system. The system was induced in the presence of propane with low concentrations of growth supporting substrates (0.035% (w/v) citrate, pyruvate and succinate), as determined by the organisms ability to form 1,2-epoxypropane from propene and the distribution of propane-specific polypeptides. Succinate was chosen as the growth supporting substrate, as in combination with 50% (v/v) propane gave an epoxidating rate, relative amounts of propane specific polypeptides and alcohol dehyrogenase activities comparable to *R.rhodochrous* PNKb1 growing on propane alone. However, low amounts of the secondary alcohol dehydrogenase were also synthesized, but even so non-specific alcohol dehydrogenase was obtained after growth on pyruvate and citrate. This background activity complicated analysis of *alc*$^-$ mutants by enzyme assay

represent a form of regulatory mutant, alkR e.g. alcA 9 and alcB 8. This means that these multiple effects are caused because a mutation has occurred at a common regulatory site (alkR) or multiple mutations have arisen due to the mode of action of NTG. The latter would be true if the propane oxidation genes are linked (see Guerola et al., 1971).

Studies with propane, propan-1,2-diol and acetol-grown cells showed that they were adapted to oxidize propane, however, only propane and acetol-grown cells had the ability to epoxidate propene. This may suggest a relationship between the metabolism of the above compounds by R.rhodochrous (see Fig. 4.1). However, another possibility is that there are two different oxygenase activities, a common propane/acetol oxygenase which can carry out epoxidation reactions and an oxygenase induced during growth on propan-1,2-diol which cannot perform these transformations. The oxidation of propane by propan-1,2-diol grown cells may be due to the fortuitous action of a non-specific oxygenase. A common feature between acetol and propan-1,2-diol is the presence of terminal and subterminal oxygen atoms on a C3 molecule and this structure may present the correct spatial arrangement for the induction of propane oxygenase(s) activity in R.rhodochrous PNKb1; indeed van der Linden & Huybregtse (1967) demonstrated that the oxidation of n-alkanes by P.aeruginosa was induced by diterminal alcohols C_5-C_8. This spatial arrangement hypothesis could easily be tested by growing R.rhodochrous PNKb1 on a range of diols and ketols, and then testing for the adaptation of propane oxidation and 1,2-epoxypropane formation from propene. SDS-PAGE would determine whether similar propane/acetol-specific polypeptides were synthesized after growth on these compounds. It would also be interesting to test if the alk$^-$ mutants are able to metabolize higher ketols in a homologous series or whether the deficiency is limited to acetol.

NADH- and NADPH-dependent acetol oxygenase activities were present in cell-free extracts obtained from propane-grown cells of R.rhodochrous PNKb1. However, only NADPH-dependent activity was detected in cell-free extracts from propan-1,2-diol and acetol-grown cells. The specific activities for the NADPH-dependent oxygenase were approximately the same for propane, propan-1,2-diol and acetol-grown cells, which suggests the presence of the same enzyme. Propane-grown cells also possessed an additional NADH-dependent oxygenase activity of approximately the same specific activity. It is tempting to speculate that the activity is derived from the NADH-dependent propane epoxidating enzyme. If this were the case it would explain the phenotype of alk⁻ mutants, which are propane and acetol metabolism deficient. However, the presence of the NADPH-dependent activity would seem to discount this. The low specific activities of acetol oxygenase obtained for propan-1,2-diol and acetol-grown cells with NADH as a cofactor may be due to fortuitous oxidation by NADH-requiring enzymes; or the NADPH-dependent enzymes ability to utilize NADH as cofactor poorly. Until further biochemical studies are carried out on these activities, e.g. purification, k_m values, inhibitor profiles, etc, it is difficult to assess their relative roles.

R.rhodochrous PNKb1 was also tested for its ability to metabolize C_2-C_8 primary and secondary alcohols, all were utilized as growth substrates. This demonstrates that its inability to utilize n-alkanes other than propane is due to limited substrate specificity of the propane oxygenase (see Woods, 1988) and not the inability to metabolize any resultant primary or secondary alcohol produced by an oxygenase. Western-blot analysis showed that the secondary alcohol dehydrogenase was synthesized after growth on propan-2-ol, pentan-2-ol and to a lesser extent on hexan-2-ol, heptan-2-ol and octan-2-ol. However, the enzyme was not

synthesized after growth on butan-2-ol and the reasons for this are not clear. The enzyme was not synthesized after growth on primary alcohols, except ethanol, and again this demonstrates the existence of separate alcohol dehydrogenase(s) for the metabolism of primary alcohols. It is probable that R.rhodochrous PNKb1 synthesizes other alcohol dehydrogenase(s) which are necessary for the metabolism of higher secondary alcohols. The latter possibility may be tested by using alcB$^-$ mutants in alcohol growth tests. It is possible that they would fail to utilize pentan-2-ol, but would utilize the other secondary alcohols.

SDS-PAGE analysis of other propane-utilizers showed a high degree of homology between members of the Rhodococcus-Nocardia complex in the synthesis of proteins after growth on propane, these included proteins which may be components of a common propane oxygenase. It would be interesting to test if the same distribution of polypeptides were obtained after growth on acetol. Only two isolates, P.butanovora and a Corynebacterium sp. showed major differences. Western-blot analysis showed the synthesis of a conserved protein of approximately 40 kDa, presumably a NAD^+-dependent alcohol dehydrogenase. Such homologies may indicate a common pathway for the oxidation of propane by members of the Rhodococcus-Nocardia complex.

Several methods were attempted to achieve genetic transformation of R.rhodochrous PNKb1 using protoplasting techniques, conjugation, transduction and electroporation. However, these methods were not successful. It is likely that these methods require acute optimization to enable the transfer of DNA into this organism. There have been recent reports on the genetic manipulation of Rhodococcus sp. by Singer & Finnerty (1988) and Hill et al. (1989), which demonstrated protoplast transformation of a Rhodococcus sp. and expression of Rhodococcus sp.

DNA in E.coli respectively. The former was only achieved by the construction of shuttle vectors with a Rhodococcus origin of replication obtained from endogenous cryptic plasmids. One of the main problems with attempting to transform R.rhodochrous PNKb1 was a lack of such plasmids. Therefore, the failure to transform may be due to either the lack of stability of foreign DNA in this organism or the transformation procedure per se.

Another problem with the potential transformation of this organism is the resistance of the cell wall to digestion by lysozyme, which reduced the efficiency of protoplast formation compared to S.coelicolor and Rhodococcus H-13A, and also the subsequent regeneration of these protoplasts. The relatively new method of transformation using electroporation would seem to be the key to this problem, especially if large field strengths could be applied.

Rhodococcus H-13A would seem the ideal choice to study the molecular biology of propane metabolism as a genetic transformation system is available (see Singer & Finnerty, 1988). However, this organism failed to utilize propane and propan-2-ol as sole carbon and energy sources, but propan-1-ol was utilized as a growth substrate.

Future studies could transform this organism with genomic DNA from R.rhodochrous PNKb1 to isolate the gene(s) responsible for propan-2-ol metabolism (alcB). The selection for such transformants would be growth of Rhodococcus H-13A on propan-2-ol. Another possibility for studying the molecular biology of propane metabolism would be to utilize Corynebacterium sp. 69 by constructing shuttle vectors using its endogenous cryptic plasmids (see Fig. 3.12) as described by Singer &

Finnerty (1988). The genetic transformation of Corynebacterium sp. is well established (Martin et al., 1987; Wolf et al., 1989).

4.2 Future Studies

The results from this study provides the basis for future studies on propane metabolism. For example, alk$^-$ mutants grown under growth-supporting, propane-inducing conditions could be used to test for functional propane oxidizing and propene epoxidating activities. Sub-classes of these mutants may also be identified using in vitro complementation studies by combining cell-free extracts, in different permutations, to assess whether propene epoxidation is regained. Studies by Macham & Heydeman (1974) used a similar technique to determine the number of components involved in the heptane mono-oxygenase from P.aeruginosa. SDS-PAGE would also reveal which specific propane polypeptides had altered electrophoretic mobility or were absent. Alk$^-$ revertents could also be tested for wider n-alkane substrate utilizing capacity and cell-free extracts of any such revertents analyzed by SDS-PAGE. The ability of any alk$^-$ revertants to utilize acetol should also be tested. This would strengthen the evidence that the metabolism of this compound requires a common enzyme system for the oxidation of both acetol and propane.

The isolation of aol$^-$ and iol$^-$ mutants is also required to test whether independently isolated acetol and propan-1,2-diol deficient mutants respectively are also alk$^-$. If so this would strongly indicate that these compounds were intermediates in the oxidation of propane by R.rhodochrous PNKb1.

The characteristics, for example inhibition and stability profiles, presence of P-450, of the NADH-dependent propane oxygenase and the NAD(P)H-dependent acetol oxygenase(s) activities require investigation to determine if the activities are as a result of one or more enzymes.

The phenotype of the alk$^-$ mutants would suggest that one enzyme is responsible for both activities. A more detailed study into the metabolism of acetol and propan-1,2-diol by cell-free extracts from propane and intermediate-grown cells would provide useful information as to their role in propane metabolism. This could include purifying the acetol monooxygenase activity from propane-grown cells.

The isolation of alcA$^-$ and alcB$^-$ mutants and Western-blot analysis has demonstrated the existence of two alcohol dehydrogenases required for the metabolism of propan-1-ol and propan-2-ol respectively. A NAD^+-dependent secondary alcohol dehydrogenase (alcB) has been purified and characterized (Ashraf & Murrell, 1990). An attempt could also be made to purify the $NAD(P)^+$-dependent/independent alcohol dehydrogenase from propane-grown R.rhodochrous PNKb1. This would aid in the biochemical analysis of alcA$^-$ mutants.

It may be possible to produce relatively high concentrations of propan-1-ol and propan-2-ol from propane using alcA$^-$ and alcB$^-$ mutants respectively, as the above are not metabolized by the respective mutants. This would provide direct evidence, in the absence of a method for purifying the propane oxygenase, that both alcohols are produced from propane, thus indicating a role for terminal and subterminal oxidation of propane by R.rhodochrous PNKb1.

The technique of electroporation perhaps provides the best hope for the eventual goal of genetic transformation of R.rhodochrous PNKb1. A wide range of conditions must be attempted using varying field strengths, electroporation buffers, and a wide range of Gram-positive plasmids. Success will enable the application of the sophisticated and precise

techniques of molecular biology to be applied to the bacterial propane metabolism.

Evidence from this work suggests that a common enzyme system is responsible for the metabolism of propane and acetol, e.g. a propane/acetol monooxygenase. Propane may be metabolized via the reduction of an activated species (possibly a hydroperoxide) and acetol via a biological Baeyer-Villiger reaction. However, the mechanisms for these reactions are different (see Sykes, 1975).

Finally, the use of NMR may provide an alternative route to the elucidation of the pathways of propane metabolism, the relative proportions of products formed *in vivo* can be determined, for example propan-1-ol and propan-2-ol (see Sanders, 1987; Narbad *et al*., 1989; Platen & Schink, 1989a).

4.3 Conclusions

The results presented in this thesis represent a significant contribution to the understanding of propane metabolism by R.rhodochrous PNKb1. The following conclusions can be made:

(1) Propane metabolism proceeds via the oxidation of terminal and subterminal carbon atoms.

(2) Mutant and SDS-PAGE analysis suggests there may be a common propane/acetol oxygenase system.

(3) The isolation of alcA$^-$ and alcB$^-$ mutants and Western-blot analysis demonstrates the presence of at least two alcohol dehydrogenases required for the metabolism of propan-1-ol and propan-2-ol.

(4) The pathway for the oxidation of acetone is not involved in the metabolism of propane or propan-2-ol.

(5) Western-blot analysis shows that NAD^+-dependent secondary alcohol dehydrogenase is synthesized after growth on propane and subterminal intermediates, propan-1,2-diol but not acetone.

(6) Biochemical analysis of alc$^-$ mutants demonstrated lower NAD^+-dependent alcohol dehydrogenase activities, but the results were complicated by the presence of non-specific dehydrogenase activities. AlcB$^-$ mutants synthesized NAD^+-dependent secondary alcohol dehydrogenase which had altered electrophoretic mobility under native conditions. This demonstrates that the alcB phenotype is due to the presence of the wild-type form of this enzyme.

(7) SDS-PAGE suggests that there is a high degree of homology between potential propane specific polypeptides in members of the Rhodococcus-Nocardia complex. Western-blot analysis also demonstrates the presence of a conserved polypeptide of approximately 40 kD after growth of these organisms on propane.

Such properties may indicate that the pathway(s) for the metabolism is similar in these bacteria.

(8) A proposed propane oxidation pathway for R.rhodochrous PNKb1 is shown in Fig. 4.1. The initial oxygenase system is indiscriminate in its insertion of oxygen into the propane molecule, leading to the formation and metabolism of terminal and subterminal oxidation intermediates. There is the possibility that the first step enzyme is a novel dioxygenase, the product formed would be propan-1,2-diol which may subsequently be metabolized to acetol (?). The further metabolism of acetol may involve a Baeyer-Villiger type reaction, the C_1 moiety may be metabolized via the reduced C_1 pool (?).

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