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THE METABOLISM OF CYCLOALKANES BY DIFFERENT SPECIES OF XANTHOBACTER

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by

Elizabeth Jean Warburton B.Sc.

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DECLARATION

This work has not been accepted in substance for any other degree and is not concurrently being submitted in candidature for any other degree.

This is to certify that the work herein was carried out by the candidate herself. Due acknowledgement has been made of all assistance received.

Signed E.J. Warburton
(Candidate)

Signed .....
(Director of Studies)

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Abstract

The metabolism of cycloalkanes by different species of Xanthobacter

E. Jean Warburton, 1989

Five members of the genus Xanthobacter were investigated with regard to their ability to metabolise cycloalkanes. Two of these microorganisms, Xanthobacter sp. and Xanthobacter autotrophicus 853 were capable of growth on the recalcitrant hydrocarbon cyclohexane. Xanthobacter autotrophicus 431 was capable of growth on cyclohexanol. Whole cell studies and enzymatic studies with cell-free extracts indicated the route of degradation of cyclohexane and cyclohexanol by X. autotrophicus 853 and X. autotrophicus 431 respectively to proceed via cyclohexane ⟶ cyclohexanol ⟶ cyclohexanone ⟶ 1-oxa-2-oxo-cycloheptane ⟶⟶ adipic acid.

Xanthobacter sp. was investigated further to gain more knowledge of the enzymes involved in cyclohexane metabolism. Cyclohexane hydroxylase was found to possess a pH optimum of 6.8 and a broad substrate specificity including alkyl substituted cycloalkanes, aromatic hydrocarbons, bicyclic terpenes and heterocyclic rings although no activity was demonstrated towards n-alkanes. The enzyme was shown to be multicomponent and cytochrome P-450 containing. Cytochrome P-450 was found to be induced concomitantly with cyclohexane hydroxylase when Xanthobacter sp. was grown on cyclohexane. Cytochrome P-450 was stabilised by the addition of glycerol (10% v/v) and was found to have a molecular weight of 58,000. Attempts to purify cyclohexane hydroxylase were unsuccessful although partial purification of cytochrome P-450 has been obtained.

Cyclohexanol dehydrogenase from Xanthobacter sp. was purified 30.4 fold and found to have a pH optimum of 10.3. Cyclohexanone was identified as the reaction product. The enzyme possessed a broad specificity for secondary alcohols, a molecular weight of 44,000 and a requirement for NAD^+ and to a lesser extent $NADP^+$. The apparent Km for NAD^+ was determined as 14.3μM.

Whole cell and enzymatic studies with Xanthobacter sp. indicated that methylcyclohexane is metabolised by the same route as cyclohexane, by hydroxylation on the ring and not by attack of the methyl substituent.

PUBLICATIONS

A.M. MAGOR, J. WARBURTON, M.K. TROWER AND M. GRIFFIN.
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A.M. MAGOR, E.J. WARBURTON, M.K. TROWER AND M. GRIFFIN.
XIV International Congress of Microbiology (1986) p.250. Microbial metabolism of cyclohexane.

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Abbreviations

BSA	-	bovine serum albumin
CFE	-	cell free extract
CoA	-	coenyzme A
DCPIP	-	dichlorophenolindophenol
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediaminetetraacetic acid
FAD	-	flavin adenine dinucleotide
FMN	-	flavin mononucleotide
glc	-	gas liquid chromatography
hplc	-	high performance liquid chromatography
Mr	-	molecular weight
MSM	-	mineral salts medium
NA	-	nutrient agar
NAD	-	nicotinamide adenine dinucleotide
NADH	-	nicotinamide adenine dinucleotide reduced
NADP	-	nicotinamide adenine dinucleotide phosphate
NADPH	-	nicotinamide adenine dinucleotide phosphate reduced
NB	-	nutrient broth
NBT	-	nitroblue tetrazolium
PAGE	-	polyacrylamide gel electrophoresis
PMS	-	phenazine methosulphate
psi	-	pounds per square inch
rpm	-	revolutions per minute
SDS	-	sodium dodecyl sulphate
TCA	-	trichloroacetic acid
TEMED	-	N,N,N',N'-tetramethylethylenediamine
tlc	-	thin layer chromatography
Tris	-	2-amino-2-hydroxymethylpropane-1, 3-diol
ve	-	elution volume
vi	-	total inclusion volume
vo	-	void volume

1. MAIN INTRODUCTION

1.1 Cycloalkanes

1.1.1 The presence of cycloalkanes and related compounds in nature

Alicyclic hydrocarbons and related compounds are present in the biosphere, both individually and as naturally occurring compounds (fig 1.1). These hydrocarbons are synthesized by animals, plants and microorganisms and can be found as components of insect secretions, plant oils, secondary metabolites of plants and lipids of microorganisms. Examples of naturally occurring alicyclic hydrocarbons include the plant lipids such as w-cyclopentyl fatty acids produced by the Flacourtiaceae (Spencer and Mangold, 1974) and the w-cyclohexyl fatty acids synthesized by the thermophilic organism Bacillus acidocaldarius (de Rosa et al., 1972). Also included in microbially produced cycloalkanes are the aflotoxins B_1 and B_2 from Aspergillus flavus (Sargeant et al., 1961) which were associated with the turkey X disease of the 1960's (Hartley et al., 1963) and gibberellic acid produced by Gibberella fujikoroi which is responsible for a disease in rice, common in Japan. Known plant sources of cycloalkanes include mono- and sesqui-terpenoids such as limonene, the principal terpenoid constituent of the oils of lemon, orange, caraway and dill, α-thujane which is found in the (-) form in eucalyptus oil and in the (+) form in origanum and certain turpentine oils. Others include α-pinene which is one of the most widely distributed monoterpenoids and commercially the most important, found in the essential oils of most conifers, and which is the chief constituent of turpentine oil, camphene which was first isolated from the essential oil of the Siberian fir and β-selinene which occurs in the fruit of the celery plant. Animal sources of alicyclic hydrocarbons include 2-methylcyclopentanone produced by the anal gland of dolichoderine ants (Wheeler et al., 1975), vitamin D_3 (5,6-cis-cholecalciferol) present in fish liver oils, the primary male and female sex hormones testosterone and oestrogen and also cholesterol, the principal sterol of mammalian tissues.

In addition to these sources of cycloalkanes a large proportion

Fig. 1.1 Examples of alicyclic hydrocarbons and related compounds present in the environment.

KEY : (a) Naturally occuring alicyclic compounds
(b) Crude oil components
(c) Pesticides.

FIG 1.1a

FIG 1.1b

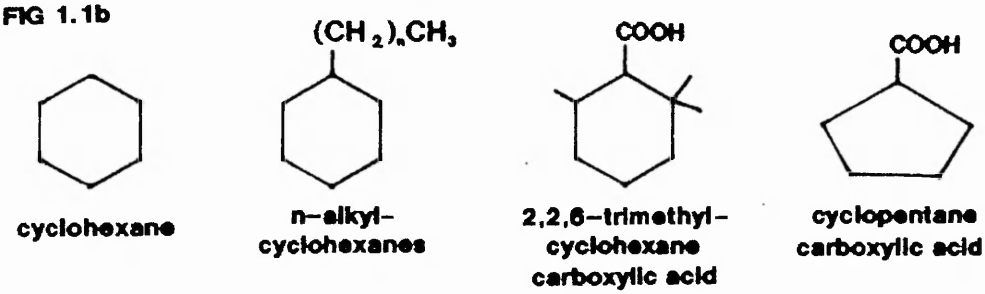
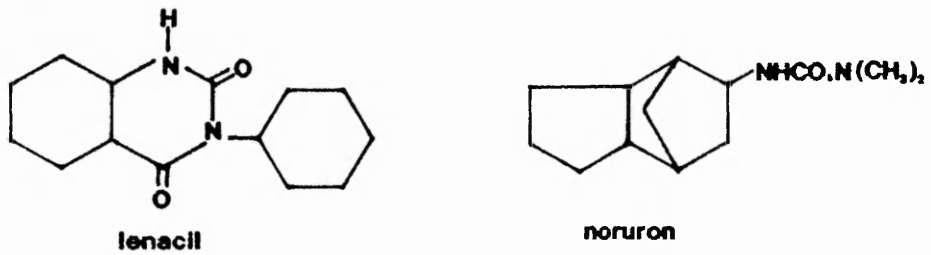


FIG 1.1c



of alicyclic hydrocarbons have been released into the biosphere due to the increased demand for fuel which has resulted in the growth of crude petroleum extraction, and also the increase in world food demand that has brought about the widespread use of fertilisers and pesticides, such as lenacil and noruron, to obtain greater food crop yields. Table 1.1 shows some of the major constituents of the gasoline fraction (b.p. 36-117°C) in selected petroleums. The increased exploitation of fossil fuels, particularly crude oil and tar which contain a significant proportion of alicyclic hydrocarbons (year book of industrial statistics, 1976) has increased the potential for environmental pollution and this has been illustrated by the Torrey Canyon and Amoco Cadiz tanker disasters and the Ixtoc Oil rig blow-out in the Gulf of Mexico. Consequently an accumulation of alicyclic hydrocarbons in particular locations such as ocean water and the air-sea interface has occurred (Ledet and Laseter, 1974; Brown and Huffman, 1976) indicating the recalcitrant nature of these compounds and their resistance to microbial attack. However, there is little evidence that cycloalkanes have accumulated to any significant extent in the microbe-rich biosphere suggesting that a definite metabolic capacity to degrade them must exist in this environment.

1.1.2 Growth of microorganisms with alicyclic hydrocarbons

1.1.2.1 Negative reports of cycloalkane degradation by microorganisms

The scarcity of reports of microorganisms capable of growth on cycloalkanes would appear to be due not to a lack of interest in this area but because of the recalcitrant nature of cycloalkanes to microbial degradation. Many workers have been unsuccessful in attempts to isolate pure cultures of micro-organisms which are capable of growth with alicyclic hydrocarbons as the sole carbon source.

Table 1.1 Some of the major hydrocarbons of the gaseous fraction (b.p. 36-117°C) in selected petroleums.

Hydrocarbon	Volume (%) Conroe, Texas	Colinga, California	Jennings, Louisiana
Alkane			
n-Pentane	0.33	0.44	1.12
n-Hexane	6.44	7.75	9.15
n-Heptane	6.90	5.94	8.42
2-Methylpentane	2.89	2.56	3.47
2,3-Dimethylhexane	0.22	1.30	2.39
Cycloalkane			
Cyclopentane	0.96	1.76	0.67
Methylcyclopentane	6.51	10.29	5.01
Cyclohexane	10.40	7.63	7.13
trans-1,2-Dimethyl cyclopentane	1.59	7.05	1.02
Methylcyclohexane	22.00	14.55	18.07
Ethylcyclopentane	2.03	4.38	2.34
Trimethylcyclopentane	3.64	8.12	4.18
Aromatic			
Benzene	3.27	2.22	3.61
Toluene	16.19	7.94	12.02

Data from Perry (1984)

Komagata et al. (1964) screened 489 yeasts and succeeded in obtaining 56 which were capable of growth on hydrocarbons, but none were capable of utilizing cycloalkanes. Pelz and Rehm (1971), using 250 soil samples and stock hydrocarbon-utilizing cultures failed to isolate any decalin utilizing organisms. Later, Pelz and Rehm (1972) reported the isolation of 6 alkane assimilating fungi from 120 soil samples and found that all 6 grew on long chain alkanes but none utilized cycloalkanes. Beam and Perry (1973, 1974a) and de Klerk and van der Linden (1974) failed to isolate organisms capable of growth on cyclohexane, nor could they demonstrate growth on cycloalkanes by 100 hydrocarbon-utilizing organisms that were screened. Tokuyama and Kaneda (1973) reported that although open-chain alkanes are susceptible to microbial action cycloalkanes are resistant to such attack. Consequently studies of the metabolism of cycloalkanes have proved difficult. Davis (1967) suggested that the resistance of cycloalkanes to microbial attack might be due to steric hindrance with regard to enzyme activity, toxicity of substrates or their insolubility in aqueous systems. However, McAuliffe (1966) showed that n-alkanes, which are readily utilized by microorganisms, are less soluble than cycloalkanes (Table 1.2).

Jones and Eddington (1968), on conducting a survey of hydrocarbon-utilizing organisms from coalfields and oil-bearing shale, reported a stimulation of oxygen consumption with the addition of cyclohexane to soil samples. Similarly, Haider et al., (1974) found that the addition of [^{14}C]-cyclohexane to soil containing mixed microbial populations resulted in the release of [^{14}C]-CO_2. These results indicated the utilization of cyclohexane in microbe containing soil samples but highlighted the failure to isolate pure strains of microorgansims that could utilize cycloalkanes as the sole source of carbon and energy. This made some workers conclude that cycloalkanes might be degraded in nature by a consortium of microorganisms involving co-oxidation.

Table 1.2 Solubility of some hydrocarbons in water at room temperature

Hydrocarbon	Grams per 10^6 grams of distilled water
Methane	24.4 ± 1.0
Propane	62.4 ± 2.1
n-Octane	0.7 ± 0.06
n-Pentane	38.5 ± 2.0
n-Hexane	9.5 ± 1.3
1-Octene	2.7 ± 0.2
Cyclopentane	156.0 ± 9.0
Cyclohexane	55.0 ± 2.3
Cycloheptane	30.0 ± 1.0
Cyclooctane	7.9 ± 1.8
Methylcyclopentane	42.0 ± 1.6
Methylcyclohexane	14.0 ± 1.2
Cyclopentene	535.0 ± 12.0
Cyclohexene	213.0 ± 10.0
1-Methylcyclohexene	52.0 ± 2.0
Benzene	1780.0 ± 45.0

Data from McAuliffe (1966)

1.1.2.2 Microbial degradation of cycloalkanes by co-oxidative systems

Mycobacterium vaccae (JOB5) was isolated by Ooyama and Foster (1965) by enrichment with 2-methylbutane. The organism was able to utilise a wide range of non-cyclic alkanes and non-proliferating cells were capable of oxidizing a number of alicyclic hydrocarbons to produce the homologous ketone. Van Ravenswaay et al., (1971) isolated a strain of Pseudomonas aeruginosa grown on n-heptane and demonstrated that cell-free extracts from this organism were capable of hydroxylating cycloalkanes to the corresponding cycloalkanols. Beam and Perry (1974a) reported the oxidation of cyclohexane by mixed microbial populations present in marine mud. These workers together with de Klerk and van der Linden (1974) also showed that if microorganisms capable of utilizing n-alkanes were provided with a cycloalkane in the presence of a cycloalkanol-utilizing organism then complete degradation of the cycloalkane could be demonstrated.

1.1.2.3 Positive reports of cycloalkane degradation by pure cultures of microorganisms

The first record of a cycloalkane growing microorganism was that of Tausz and Peter (1919) who reported the isolation of a strain of Bacterium aliphaticum liquifaciens which was able to grow with cyclohexane, methylcyclohexane, 1,3-dimethylcylohexane and 1,3,4-trimethyl cyclohexane. However, Johnson et al., (1942) re-isolated the organsims used by Tausz and Peter and found that it was not capable of growth on alicyclic hydrocarbons. It was thought that the false positive results obtained by Tausz and Peter may have been due to impurities in the substrates. Skarzynski and Cjekalowski (1946) reported a strain of Achromobacter which was capable of growth with cyclohexane. However, the substrate was prepared by the hydrogenation of benzene and was of unknown purity. Similarly Fredricks (1966) used relatively large amounts (2% (v/v)) of alicyclic hydrocarbons to obtain growth of a species of Corynebacterium on cyclohexane and methylcyclohexane. Both of these

reports are open to criticism concerning the levels of impurities in the growth substrates.

Imelik (1948) reported that a strain of Pseudomonas aeruginosa was capable of growth on cyclohexane as the sole carbon and energy source. This was the first report that took these findings further and showed that cyclohexanol, adipic acid and valeric acid were produced during the metabolism of cyclohexane. Colla and Treccani (1960) also reported a strain of Flavobacterium, which, after growth on decalin, produced pimelic and adipic acids. More recently Nocardia petroleophila was reported by Tonge and Higgins (1974) to be capable of growth on methylcyclohexane as the sole carbon source while ethylcyclohexane-utilizing bacteria have been isolated from soil (Arai and Yamada, 1969).

Three more recent reports have also occurred concerning microorganisms capable of growth on cyclohexane. Firstly, Stirling et al., (1977) isolated a Nocardia species from an estuarine mud flat, then Anderson et al., (1980) reported the isolation of a Pseudomonas species from ash-wood soil and similarly Trower et al., (1985) isolated a Xanthobacter sp. from the soil of Nottinghamshire forests.

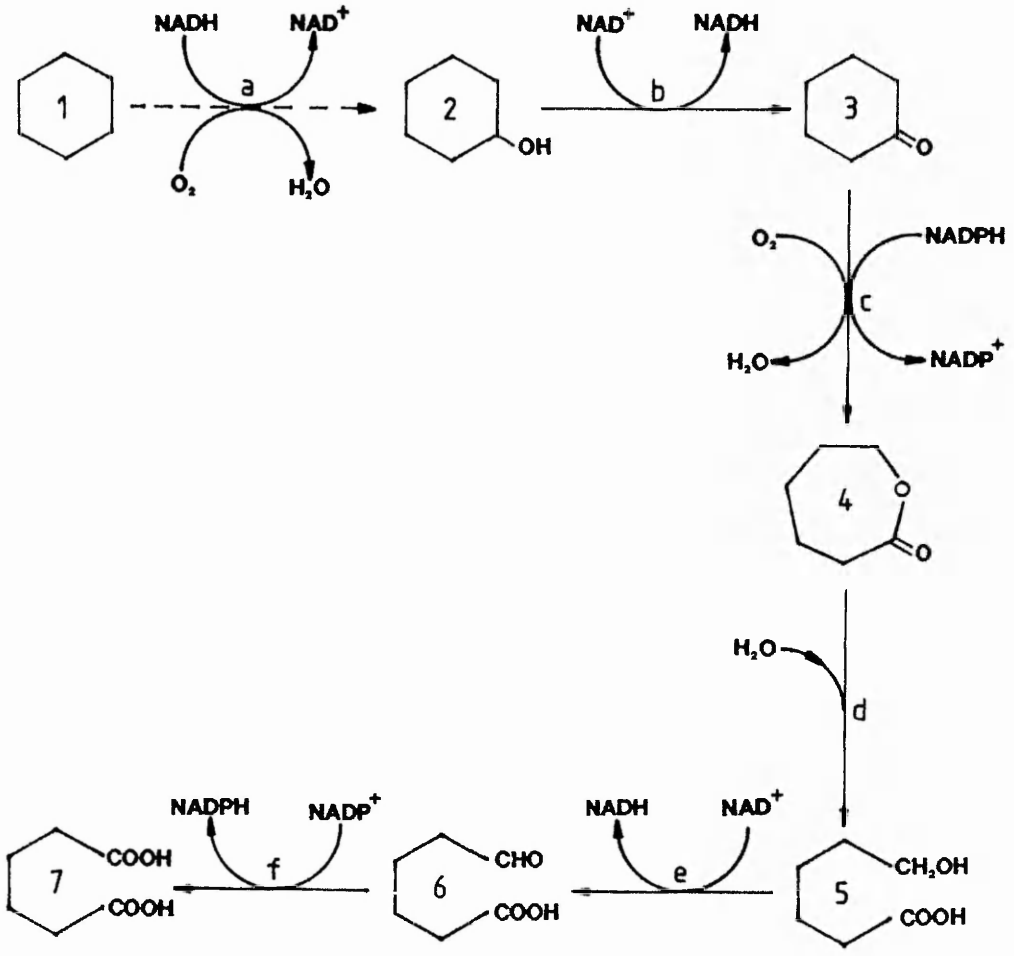
1.1.3 Cycloalkane degradation

When Stirling et al., (1977) and Anderson et al., (1980) investigated the degradation of cyclohexane by a Nocardia sp. and a Pseudomonas sp. respectively it was expected that the initial step in the pathway would involve hydroxylation of the ring to the corresponding cycloalkanol. This initial hydroxylation would be analogous to the pathways already elucidated for n-alkane oxidation (McKenna and Coon, 1970; Jurtshuk and Cardini, 1971), methane metabolism (Tonge et al., 1977; Stirling and Dalton, 1979) and the catabolism of camphor (Katagiri et al., 1968). Both Stirling et al., (1977) and Anderson et al., (1980) indicated this to be the case. This initial oxidation is an important step in cycloalkane degradation since it prepares the ring for subsequent cleavage. It was thought that the initial hydroxylation was brought about by a

mixed-function oxidase. However, Stirling et al., (1977) were unable to demonstrate cyclohexane hydroxylase activity in cell-free extracts of the Nocardia sp. whilst Anderson et al., (1980) were only able to detect very low amounts of cyclohexane hydroxylase activity in cell-free extracts of the Pseudomonas sp. Both groups of workers reported that after the initial hydroxylation of cyclohexane, the cyclohexanol so formed was degraded by the route elucidated by Donoghue et al., (1976) (Fig. 1.2) for the metabolism of cycloalkanols. More detailed investigations into the metabolism of cyclohexane by a novel Xanthobacter sp. (Trower et al., 1985) have also demonstrated that the initial step in cyclohexane degradation involves its hydroxylation to cyclohexanol. These authors were also able to detect cyclohexane hydroxylase activity in cell-free extracts thus enabling further investigation of this interesting enzyme. While investigating the metabolic fate of cyclohexane Trower et al., (1985) also looked at the possibility of a pathway involving a double hydroxylation of cyclohexane to the cyclohexandiol. This pathway had not been investigated by Stirling et al., (1977) or Anderson et al., (1980). Trower et al., (1985) indicated that the major route of metabolism of cyclohexane by Xanthobacter sp. was via hydroxylation to cyclohexanol and further oxidation of cyclohexanol to adipic acid by the pathway described by Donoghue et al., (1976). Results obtained from studies with both whole cells and cell-free extracts indicated that any alternative routes of cyclohexane dissimilation such as double hydroxylation of the ring might occur fortuitously but did not represent the major pathway of cyclohexane metabolism.

This investigation by Trower et al., (1985) into a possible double hydroxylation of the cyclohexane ring was justified as there is documented evidence that dihydroxylation of alicyclic rings or a further hydroxylation of an alicyclic alcohol or ketone can occur. Bhattacharyya and Ganapathy (1965) reported the oxidation of cyclohexene by a strain of Aspergillus niger to cyclohexanol, cyclohexanone and 3-cyclohexene-1,2-diol. Murray et al., (1974) described a strain of Nocardia which during the degradation of cyclohexanone produced 2-hydroxycyclohexanone and Higgins et al.,

Fig. 1.2 Pathway of cyclohexanol degradation by Acinetobacter NCIB9871 (adapted from the data of Donoghue et al., 1976).



Key : (----) as proposed by Anderson et al. (1980). Compounds ; (1) cyclohexane, (2) cyclohexanol, (3) cyclohexanone, (4) 1-oxa-2-oxocycloheptane, (5) 6-hydroxyhexanoate, (6) 6-oxohexanoate and (7) adipic acid.

Enzymes ; (a) cyclohexane hydroxylase, (b) cyclohexanol dehydrogenase, (c) cyclohexanone monooxygenase, (d) 1-oxa-2-oxocycloheptane hydrolase, (e) 6-hydroxyhexanoate dehydrogenase and (f) 6-oxohexanoate dehydrogenase.

(1979) showed that whole cell suspensions of the methane- grown Methylosinus trichosporium OB3b converted cyclohexane to cyclohexanol and 3-hydroxycyclohexanone.

1.1.4 Metabolism of cycloalkanols and cycloalkanones

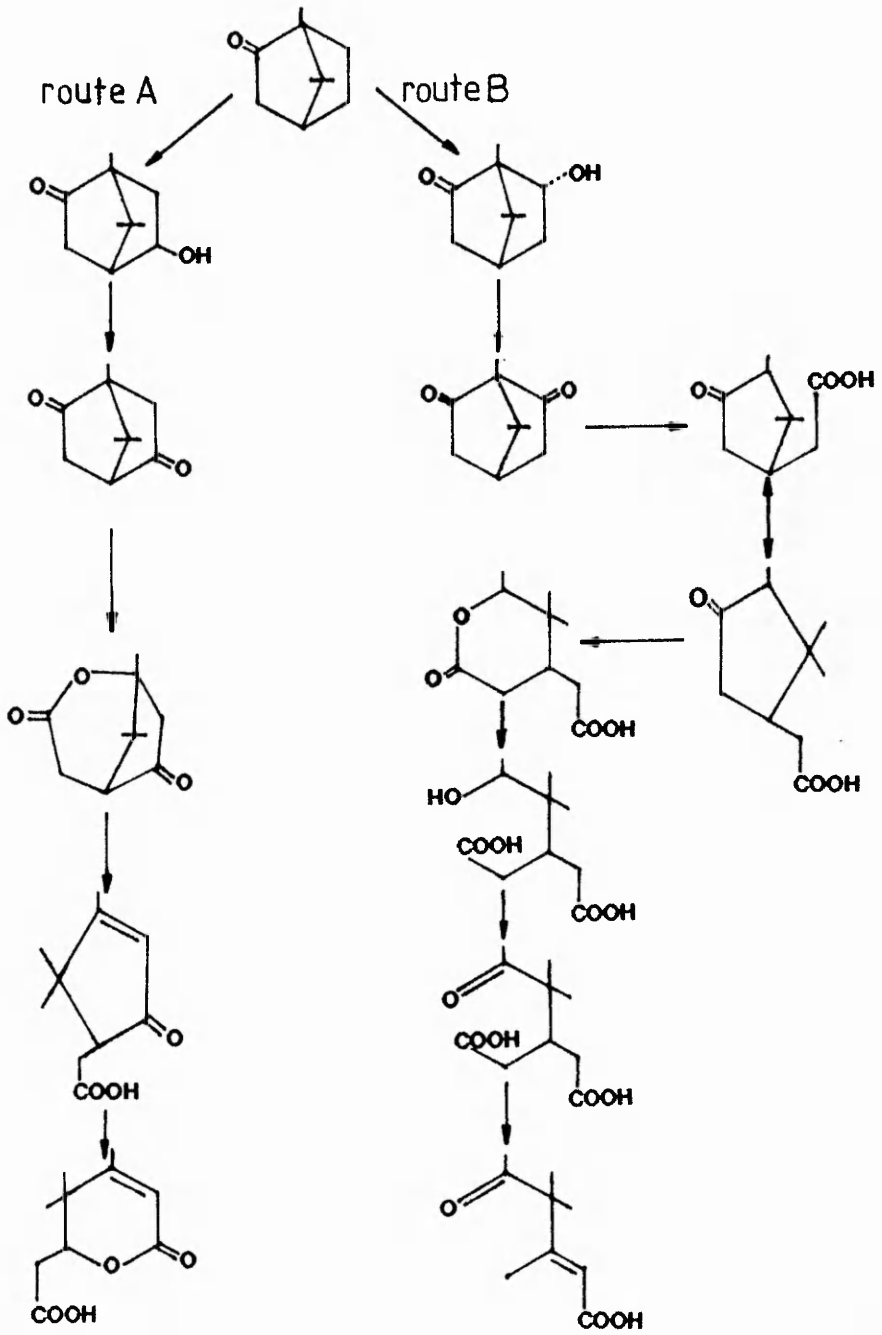
To understand the metabolism of cyclic alcohols and ketones it is necessary to know the way in which the ring is cleaved. There are two routes by which ring cleavage may be brought about. The first involves the production of a lactone from the cyclic ketone. Due to the instability of the lactone the ring is cleaved either spontaneously or by a specific hydrolase enzyme. The second method of ring cleavage is by direct hydrolytic cleavage across the carbon-carbon bond of the two substituents of a di-substituted cycloalkanone or hydroxycycloalkanone.

Reports supporting the theory of ring cleavage by formation of an unstable lactone were first published nearly three decades ago by workers investigating the microbial degradation of (+)-camphor. Bradshaw et al., (1959) elucidated a pathway for (+)-camphor degradation using Pseudomonas C1 whilst Chapman et al., (1966) using Mycobacterium rhodochrous also postulated a route for the metabolism of the same bicyclic terpene (Fig 1.3). In both pathways the presence of monooxygenase enzymes which perform a Baeyer-Villiger type reaction are essential in converting stable cycloketones to unstable lactones. These lactones are then hydrolysed enzymatically or spontaneously to form substituted carboxylic acids.

Since these first reports of (+)-camphor degradation were published the metabolic pathways of various cycloalkanols have been reported. The metabolism of cyclohexanol was reported (Norris and Trudgill, 1971) by Nocardia globerula CL1 and (Donoghue and Trudgill, 1975) by Acinetobacter NC1B 9871, also the metabolism of cyclopentanol (Griffin and Trudgill, 1972) by Pseudomonas NC1B 9872. Each of these pathways are reliant upon the conversion of the cyclic ketone to the unstable lactone by a monooxygenase to facilitate ring cleavage.

To prove the formation of these lactones, the ketone

Fig 1.3 Two pathways proposed for the microbial degradation of (+)-camphor.



Route A : Sequence of intermediates in the most direct route of (+)-camphor degradation by Pseudomonas sp. C1 (Conrad et al., 1961 ; Bradshaw et al., 1959).

Route B : Sequence of intermediates identified in the metabolism of (+)-camphor by Mycobacterium rhodochrous (Chapman et al., 1966).

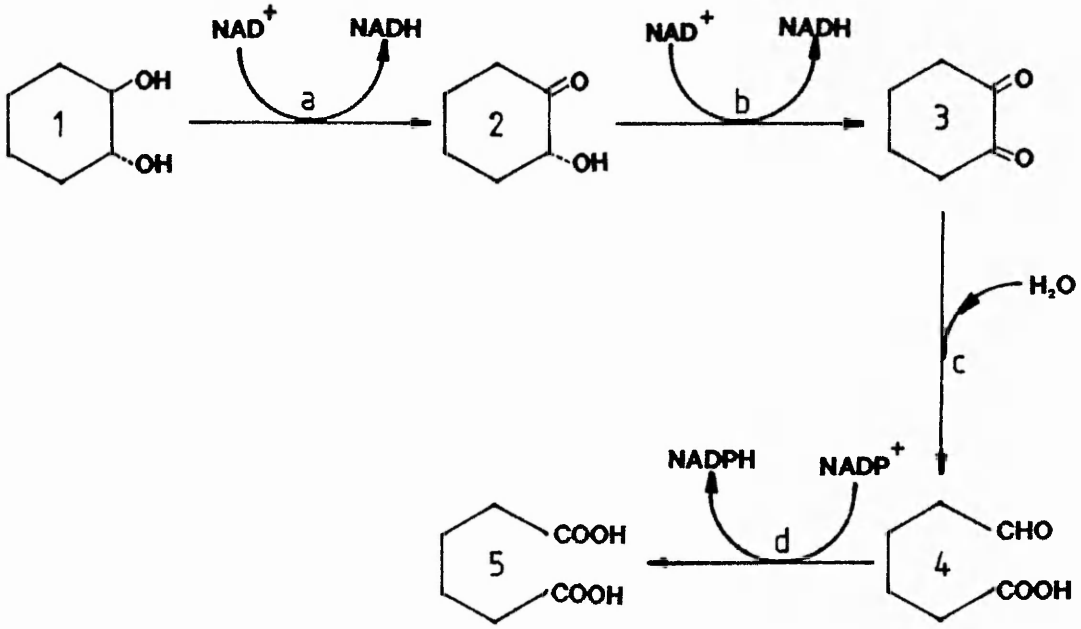
monooxygenases from the various organisms have been purified and the stoichiometric production of lactones from the appropriate ketone has been demonstrated (Norris and Trudgill, 1972; Donoghue et al., 1976; Griffin and Trudgill, 1976).

1.1.5 Metabolism of cycloalkanediols

Early studies of cycloalkanol degradation by Posternak and Reymond (1955) and Posternak et al., (1955) showed that Acetobacter suboxydans was capable of converting the isomers of cyclopentane-1,2-diol, cyclohexane-1,2-diol, cycloheptane-1,2-diol and other alicyclic diols to their corresponding monoketones. However, investigations into the further metabolism of these ketones did not occur. In 1961 Yugari isolated a strain of Pseudomonas that could utilise cyclohexane-1,2-diol. Previously, Yugari had investigated the metabolism of catechol, the aromatic counterpart of cyclohexane-1,2-diol, and found that it was cleaved by a dioxygenase enzyme. However, upon investigating the metabolism of trans-cyclohexane-1,2-diol, Yugari found that the ring was cleaved by hydrolytic fission across the carbon-carbon bond (Fig 1.4). The biochemistry of this reaction is unusual since when cyclohexane-1,2-dione is in aqueous solution its instability results in isomerisation to the monoenol form which is in equilibrium with its monohydrate (Bakule and Long, 1963). Murray et al., (1974) also proposed that the 2-hydroxycyclohexanone produced by a Nocardia species following growth on cyclohexanone was cleaved in the manner described by Yugari (1961), either directly or after dehydrogenation to cyclohexane-1,2-dione.

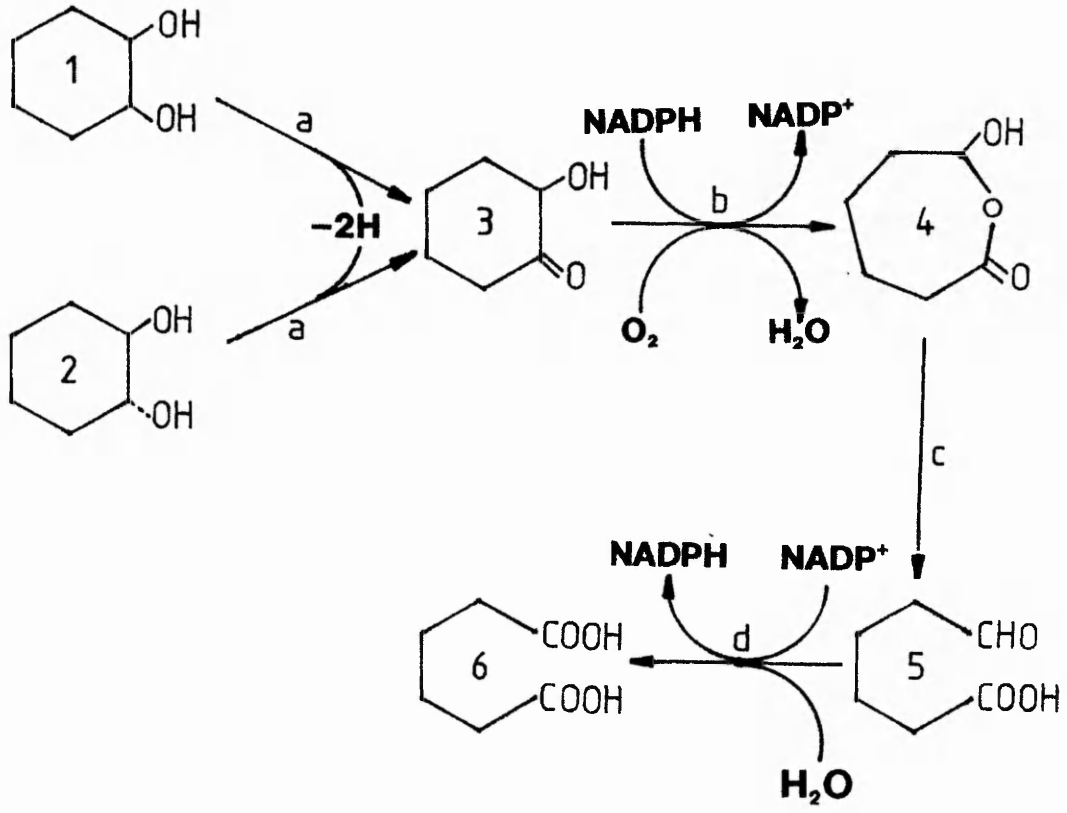
The route of cycloalkane degradation described by Yugari (1961) is dependent upon the di-hydroxylation of the alicyclic hydrocarbon whereas the route of degradation involving the formation of an unstable lactone can occur with both mono-hydroxylated cycloalkanes (Donoghue et al., 1976) and with di-hydroxylated ring compounds as shown by Donoghue and Trudgill (1973) using Nocardia globerula CL1 (Fig. 1.5). These workers reported that both cis and trans cyclohexane-1,2-diol could undergo dehydrogenation to 2-

Fig. 1.4 Proposed pathway of trans-cyclohexan-1,2 - diol degradation by a strain of Pseudomonas (adapted from the data of Yugari, (1961).



Key : Compounds; (1) trans-cyclohexan-1,2-diol, (2) 2-hydroxy-cyclohexanone, (3) cyclohexan-1,2-dione, (4) 6-oxohexanoate and (5) adipic acid. Enzymes ; (a) trans-cyclohexan-1,2-diol dehydrogenase, (b) 2-hydroxycyclohexanone dehydrogenase, (c) cyclohexan-1,2-dione hydratase and (d) 6-oxohexanoate dehydrogenase.

Fig 1.5. Proposed pathway for the oxidation of cyclohexane-1, 2-diols by Nocardia globerula CL 1 (Donoghue and Trudgill, 1973).



Key : Compounds; (1) cis-cyclohexane-1,2-diol, (2) trans-cyclohexane-1,2-diol, (3) 2-hydroxycyclohexanone, (4) 7-hydroxy-1-oxa-2-oxocycloheptane, (5) 6-oxohexanoate and (6) adipic acid. Enzymes ; (a) cyclohexane-1,2-diol dehydrogenase, (b) 2- hydroxy-cyclohexanone oxygenase, (c) spontaneous and (d) 6-oxohexanoate dehydrogenase.

hydroxycyclohexanone. This di-substituted ketone was then converted by a monooxygenase to 7-hydroxy-1-oxa-2-oxocycloheptane, an unstable lactone, which spontaneously underwent conversion to 6-oxohexanoate.

The reports indicate that considerable work has been put into investigating the method by which the cycloalkane ring is cleaved. In contrast the initial hydroxylation of the ring which is necessary to prepare it for cleavage, has not been investigated in such detail.

1.1.6 Metabolism of n-alkyl substituted cycloalkanes

Alkyl substituted cycloalkanes such as methyl-cyclopentane and methylcyclohexane make up a significant proportion of the gasoline fraction of petroleum (Table 1.1). In 1974 Tonge and Higgins reported the isolation of Nocardia petroleophila which was capable of growth on methylcyclohexane as the sole carbon source. It was found that 3-methylcyclohexanol and 3-methylcyclohexanone were excreted into the medium during growth and that the organism could utilise both of these compounds. It was therefore suggested that 3-methylcyclohexanol and 3-methylcyclohexanone were intermediates of methylcyclohexane metabolism. An ethylcyclohexane-utilizing organism, Alcaligenes faecalis strain S6B1 was isolated from soil by Arai and Yamada (1969) and it was found that the organism excreted 4-ethylcyclohexanol into the medium during growth. These findings are of interest since the initial oxidation products identified by Tonge and Higgins (1974) and Arai and Yamada (1961) which are involved in the degradation of methylcyclohexane and ethylcyclohexane respectively are analogous to those found in cyclohexane degradation (Stirling et al., 1977; Anderson et al., 1980; Trower et al., 1985). These findings therefore suggest that the metabolism of methylcyclohexane and ethylcyclohexane may be similar to that of cyclohexane. This is reinforced by the results of Stirling et al., (1977) who initially isolated a cyclohexane-utilizing Nocardia species by enrichment on methylcyclohexane. This organism was also capable of growth on ethyl- and isopropylcyclohexane. Furthermore the cyclohexane degrading

Xanthobacter species isolated by Trower et al., (1985) was capable of growth on methyl- and ethylcyclohexane.

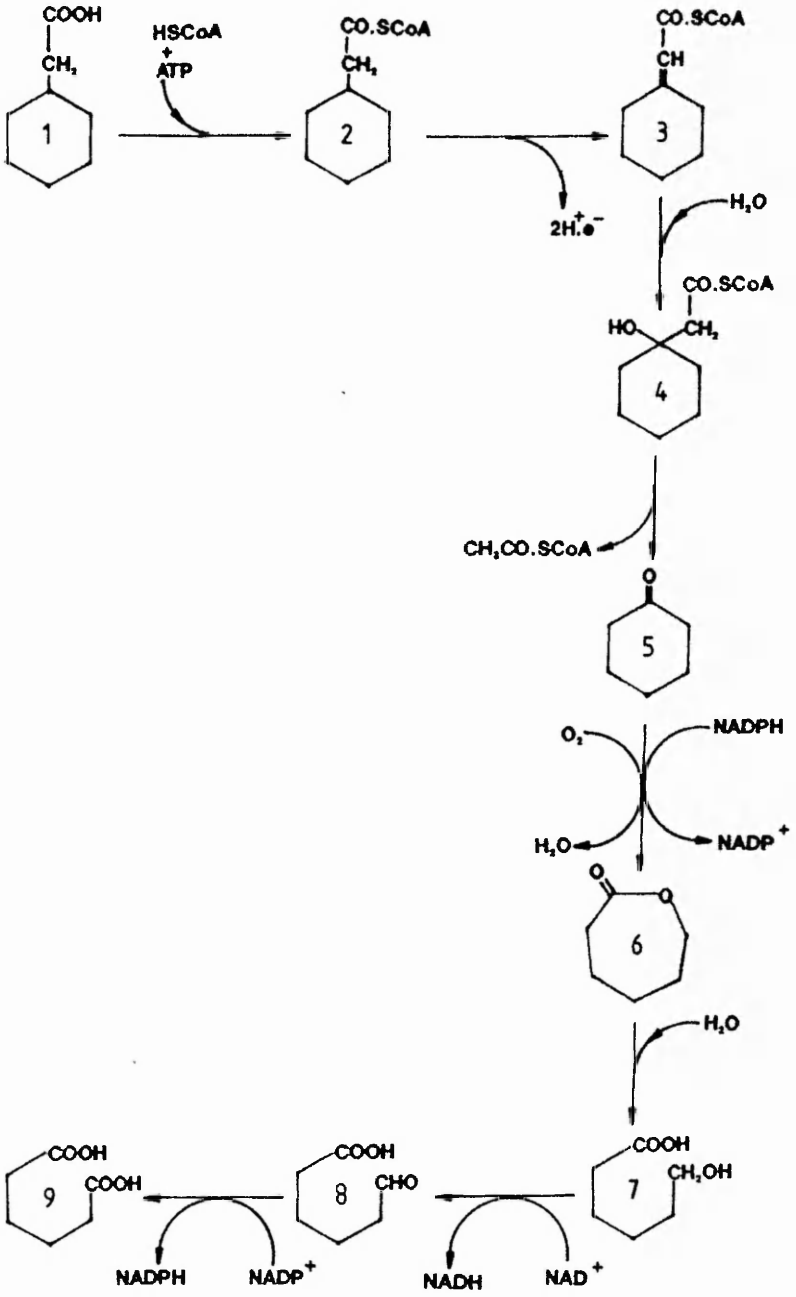
The microbial degradation of long chain substituted cycloalkanes occurs via a different route than that for short chain substituted cycloalkanes. A number of workers have reported the microbial degradation of n-alkanes (Ratledge, 1978; 1984; Atlas, 1981). In most cases n-alkane metabolism occurs via an oxidation at a methyl terminus forming an alcohol which is oxidised to the appropriate aldehyde and then to a carboxylic acid. The carboxylic acid is further metabolised by β-oxidation.

Davis and Raymond (1961) showed that the microbial degradation of n-butylcyclohexane occurred by a co-oxidation process involving two strains of Nocardia. When grown on n-octadecane these organisms co-oxidised n-butylcyclohexane to cyclohexaneacetic acid. These findings implied the activation of the n-alkyl side chain by β-oxidation with the removal of one acetyl CoA residue. Beam and Perry (1974b) using hydrocarbon-utilizing microorganisms showed that β-oxidation was involved in the degradation of the n-alkyl side chains of heptadecylcyclohexane and dodecylcyclohexane. Growth on heptadecylcyclohexane, which has an odd number of carbon atoms in the n-alkyl side chain, resulted in the formation of cyclohexane carboxyl CoA which could be further metabolised (see section 1.1.7). However, the degradation of dodecylcyclohexane, with an even number of carbon atoms in the n-alkyl side chain, resulted in cyclohexane acetic acid accumulating in the growth medium.

1.1.7 Microbial metabolism of naphthenic acids

Attempts to isolate microorganisms capable of growth on cyclohexane acetic acid and cyclohexane butyric acid have generally been unsuccessful, although Ougham and Trudgill (1978) have isolated a species of Arthrobacter which is capable of growth on cyclohexane acetic acid (fig 1.6). The cyclohexane acetic acid is converted via cyclohexane acetyl CoA to cyclohexanone and acetyl CoA. The cyclohexanone is then metabolised through the lactonisation pathway (fig 1.2).

Fig 1.6 Biodegradative pathway of cyclohexane acetic acid by Arthrobacter CA 1 (Ougham + Trudgill, 1978, 1982)



Key : Compounds; (1) cyclohexane acetic acid, (2) cyclohexane acetyl-CoA, (3) cyclohexylidene acetyl-CoA, (4) (1-hydroxycyclohexan-1-yl) acetyl-CoA, (5) cyclohexanone, (6) 1-oxa-2-oxocycloheptane, (7) 6-hydroxyhexanoate, (8) 6-oxohexanoate and (9) adipic acid.

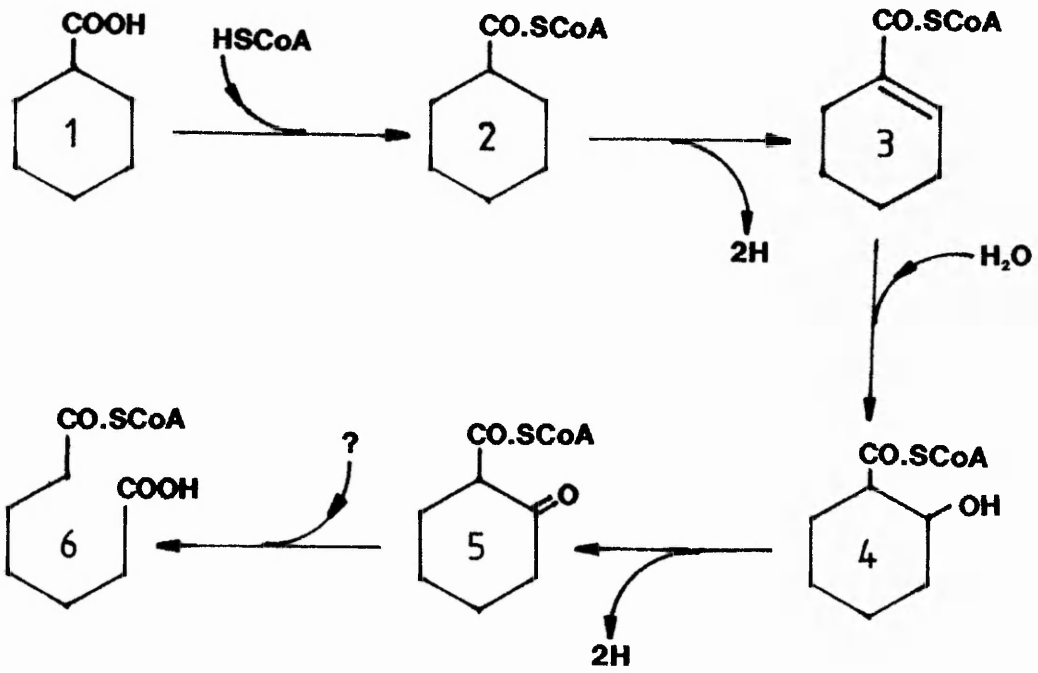
There are a number of reports of microorganisms capable of utilizing cyclohexane carboxylic acid as a sole source of carbon and energy (Beam and Perry, 1974b). Rho and Evans (1975) investigated the aerobic utilizatin of cyclohexane carboxylic acid by Acinetobacter anitratum. Using [^{14}C]-cyclohexane carboxylic acid they showed the production of radiolabelled pimelic acid and smaller amounts of 2-hydroxycyclohexanone which led them to suggest that cyclohexane carboxylic acid was metabolised by a β-oxidation pathway involving a thiolytic cleavage of 2-oxocyclohexane carboxylic acid. Blakley (1978) using an organism designated strain PRLW19 also investigated the metabolism of cyclohexane carboxylic acid and confirmed that a β-oxidation pathway analogous to that of the oxidation of fatty acids occurred (Fig 1.7).

Another route of cyclohexane carboxylic acid metabolism in aerobic systems occurs by an aromatisation pathway. This was first proposed by Blakley (1974), using a species of Arthrobacter (PRL W15), as shown in fig (1.8). The 4-hydroxycyclohexanone carboxylic acid undergoes aromatisation to form p-hydroxybenzoate. Ring cleavage then occurs in the ortho position. Kaneda (1974) using Corynebacterium cyclohexicum proposed a pathway similar to that of Blakley for the degradation of cyclohexane carboxylic acid by aromatisation. Taylor and Trudgill (1978) isolated an Alcaligenes strain W1 which metabolised cyclohexane carboxylic acid by an aromatisation pathway, however the further metabolism of p-hydroxybenzoate occurred through a meta cleavage to yield 2-hydroxy-4-carboxymuconic semialdehyde (fig 1.9). Smith and Callely (1975) employed a species of Arthrobacter which metabolised cyclohexane carboxylic acid and also showed that p-hydroxybenzoate was cleaved in the meta position.

1.1.8 Microbial metabolism of terpene hydrocarbons

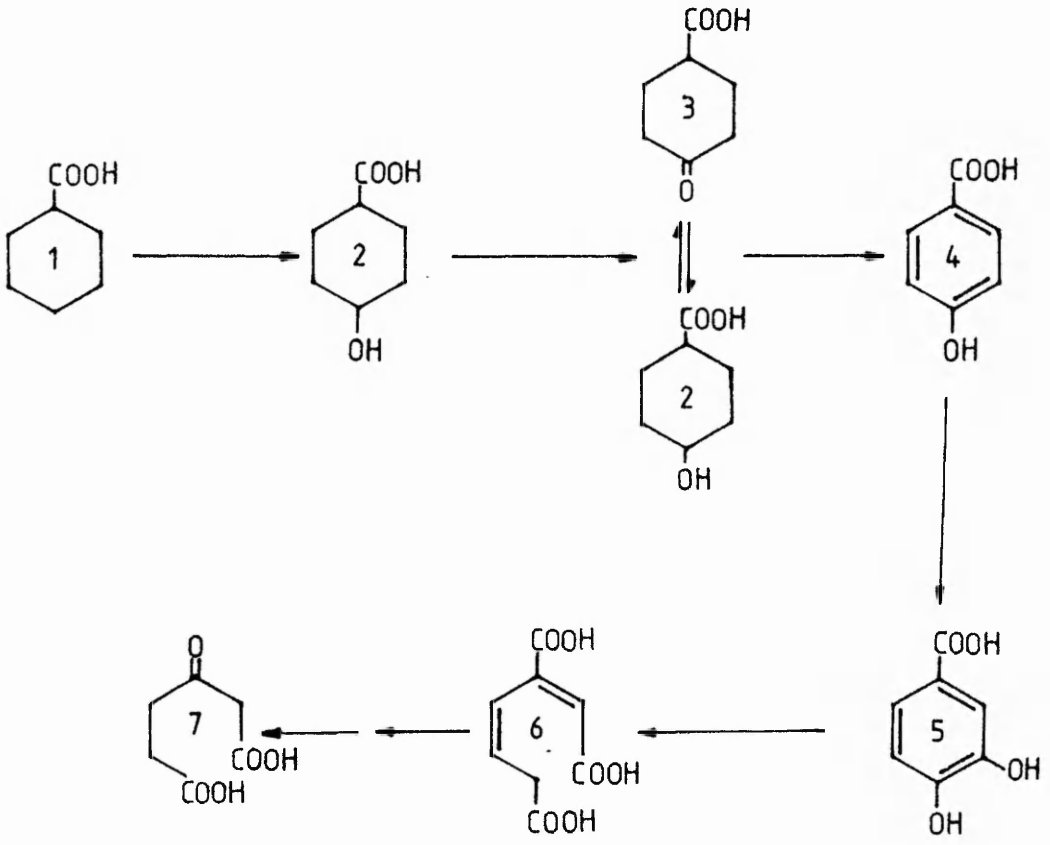
The monoterpene p-menthane can be synthesized by the hydrogenation of α-pinene or limonene. Unfortunately, little work has been performed with regard to the metabolism of this compound and those reports available do not give a suitable explanation of

Fig 1.7. Proposed biodegradative pathway of cyclohexane carboxylic acid by the bacterium PRL W19 (Blakley, 1978)



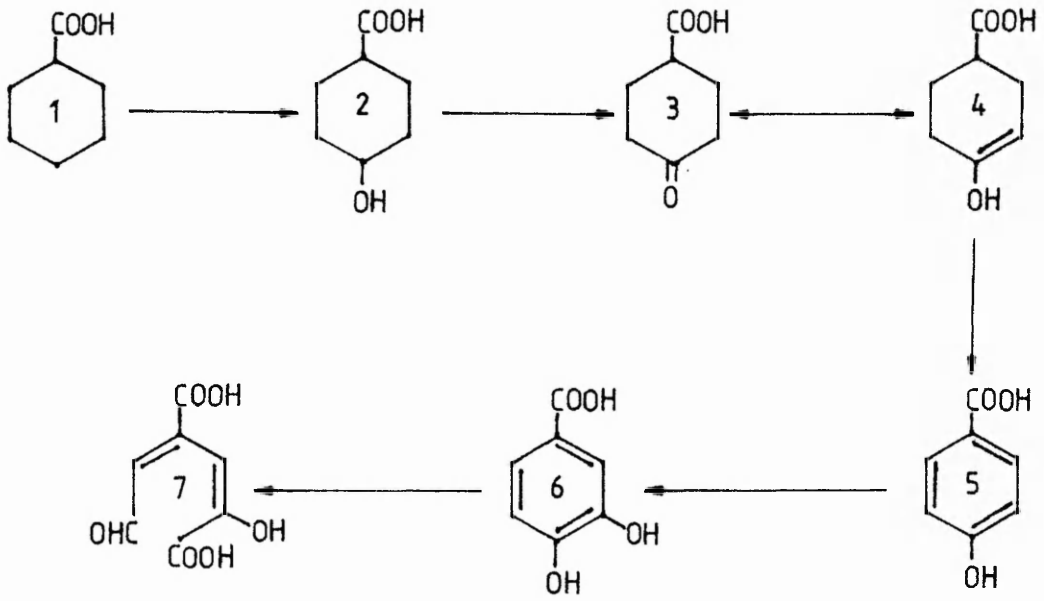
Key : Compounds ; (1) cyclohexane carboxylic acid, (2) cyclohexane carboxyl CoA, (3) Δ^{1}-cyclohexene carboxyl CoA, (4) 2-hydroxy-cyclohexane carboxyl CoA, (5) 2-oxocyclohexane carboxyl CoA and (6) pimelyl CoA.

Fig 1.8 Degradation of cyclohexane carboxylic acid via an aromatization pathway by the bacterium PRL W15 (Blakley, 1974).



Key : Compounds; (1) cyclohexane carboxylic acid, (2) 4-hydroxy-cyclohexane carboxylic acid, (3) 4-oxocyclohexane carboxylic acid, (4) p-hydroxybenzoate, (5) 3,4-dihydroxybenzoate (protocatechuate), (6) 3-carboxyhepta-2,4-dien-1,7-dioic acid and (7) 3-oxoadipic acid.

Fig 1.9 Proposed pathway for cyclohexane carboxylic acid degradation by Alcaligenes W1 (Taylor and Trudgill, 1978).



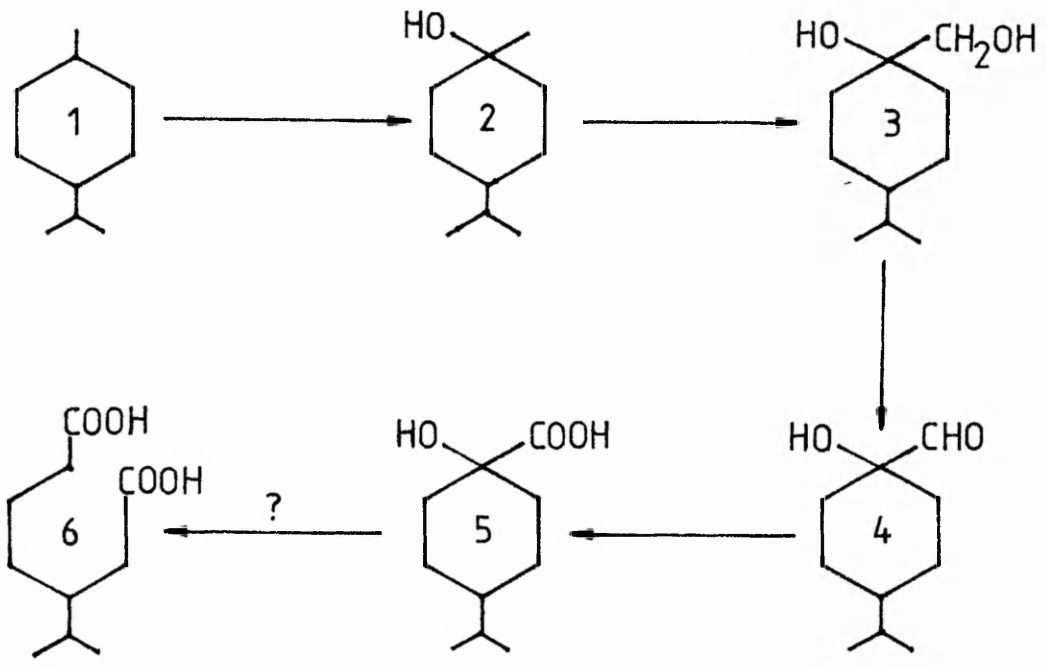
Key : Compounds; (1) cyclohexane carboxylic acid, (2) 4-hydroxy-cyclohexane carboxylic acid, (3) 4-oxocyclohexane carboxylic acid, (4) Δ'-4-hydroxycyclohexene carboxylic acid, (5) p-hydroxybenzoate, (6) 3,4-dihydroxybenzoate and (7) 2-hydroxy-4-carboxymuconic semialdehyde.

the steps involved in the cleavage of the cycloalkane ring. Tsukamoto et al., (1977) reported the isolation of a Pseudomonas mendocina which was capable of utilizing p-menthane as a sole carbon source. The organism hydroxylated the substrate, using a monooxygenase, to form cis-p-menthan-1-ol. Studies showed that cis-p-menthan-1-ol accumulated in the culture medium although levels of this compound were much lower than expected; the intermediates 1-hydroxymethyl-4-isopropylcyclohexanol and 1-carboxy-4-isopropylcyclohexanol were also found in the growth medium. These results led to the proposed sequence for p-menthane metabolism as shown in fig 1.10. One major criticism of the pathway hinges on the ring cleavage step, as a suitable reaction which can cleave 1-carboxy-4-isopropyl cyclohexanol to form 3-isopropylpimelic acid cannot be envisaged. Since the metabolite accumulation studies are as yet unsupported by subcellular enzymology the exact pathway for p-menthane metabolism remains unknown.

Various species of Pseudomonas capable of growth on limonene, 1-p-menthene and other bicyclic monoterpenes were isolated by Dhavalikar and Bhattacharyya (1966). Using one of these organisms, Pseudomonas L, they studied limonene oxidation by accumulating and identifying metabolites. This proved somewhat bewildering as it appeared that attack at carbon atoms 1, 2, 4, 6, 7 and 9 all occurred. However, only one ring cleavage metabolite, namely 3-isopropenyl pimelic acid, was detected and this led to the proposed pathway for limonene oxidation shown in fig 1.11.

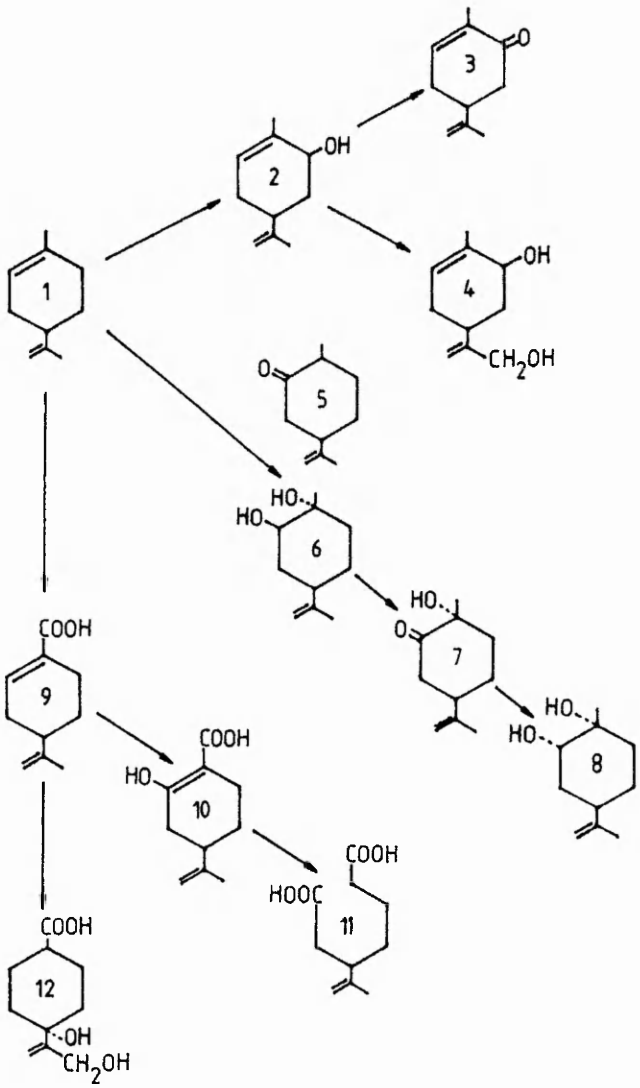
Many workers have investigated the metabolism of α- and β-pinene by microorganisms. Shukla and Bhattacharyya (1968) and Shukla et al., (1968) using an organism designated Pseudomonas strain PL proposed pathways for the metabolism and cleavage of α- and β-pinene (fig 1.12) based on the identification of metabolites accumulated in the growth medium.

Fig 1.10 Proposed degradative pathway of p-menthane by Pseudomonas mendocina - SF (Tsukamoto et al.,1977).



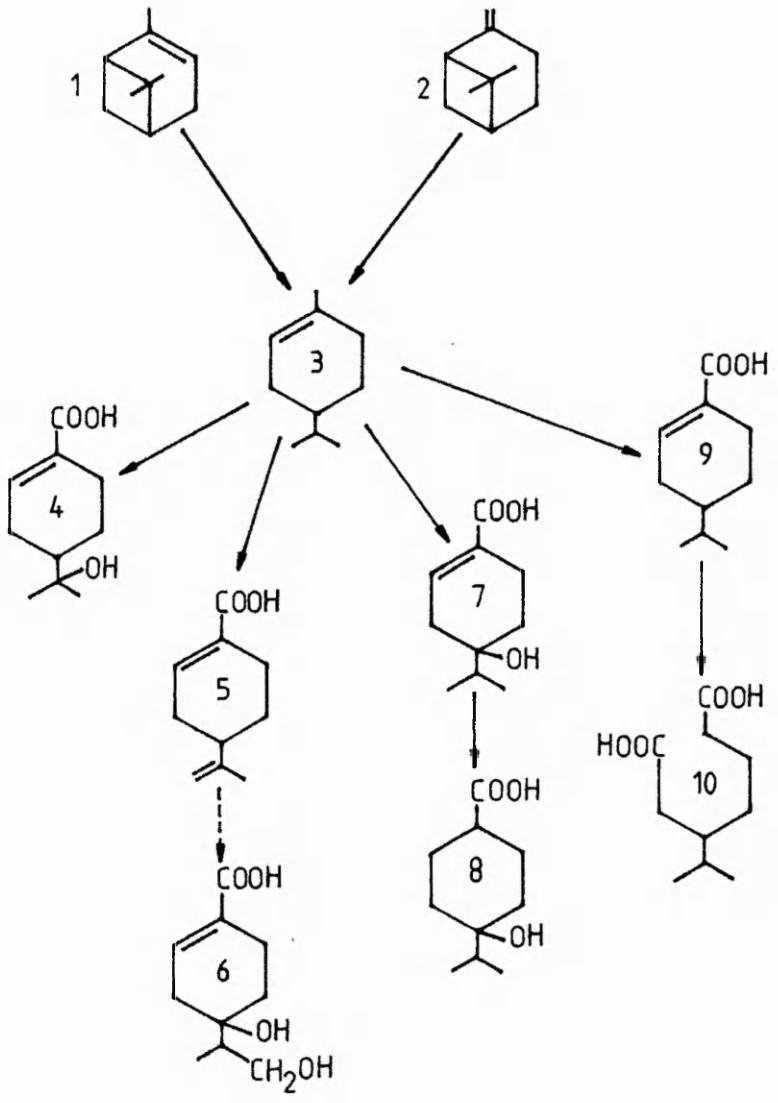
Key : Compounds; (1) p-menthane, (2) cis-p-menthan-1-ol, (3) 1-hydroxymethyl-4-isopropyl cyclohexanol, (4) 1-hydroxy-4-isopropyl cyclohexan-1-aldehyde, (5) 1-carboxy-4-isopropyl cyclohexanol and (6) 3-isopropylpimelic acid.

Fig 1.11 Proposed pathway for the oxidation of limonene by a strain of Pseudomonas (Dhavalikar and Bhattacharrya, 1966; Dhavalikar et al., 1966)



Key : Compounds; (1) limonene, (2) (+) cis-carveol, (3) (+)-carvone, (4) 1-p-menthene-6,9-diol, (5) (+)-dihydrocarvone, (6) 8-p-menthene-1,2-trans-diol, (7) 8-p-menthene-1-ol-2-one, (8) 8-p-menthene-1,2-cis-diol, (9) perillic acid, (10) 2-hydroxy-8-p-menthene-7-oic acid, (11) β-isopropenylpimelic acid, (12) 4,9-dihydroxyl-1-p-menthene-7-oic acid.

Fig 1.12 Proposed pathway for the metabolism of α- and β- pinene by Pseudomonas PL adapted from the data of Shukla and Bhattacharyya (1968) and Shukla et al. (1968).



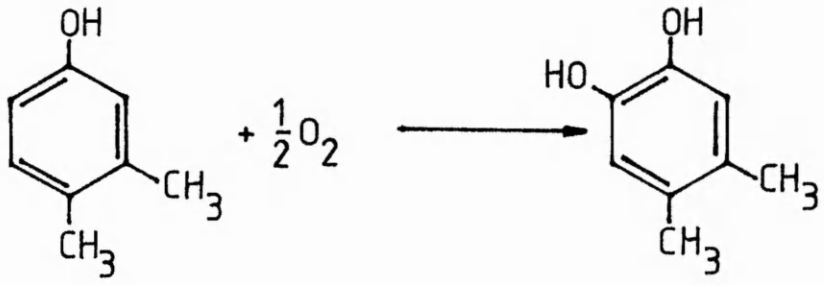
Key : Compounds; (1) α-pinene, (2) β-pinene, (3) 1-p-menthene, (4) oleuropeic acid, (5) perillic acid, (6)dihydroxyperillic acid, (7) 4-hydroxyphelandric acid, (8) 4-hydroxydihydrophellandric acid, (9) phellandric acid and (10) β-isopropylpimelic acid.

1.2 Oxygenases

The importance of oxygen in life is illustrated by the role it plays in metabolism and the generation of energy. A group of enzymes involved in catalysing reactions containing molecular oxygen are known as oxygenases. These enzymes are important in biosynthesis, transformation and degradation of essential metabolites including amino acids, sugars, lipids, porphyrins, vitamins and hormones. Oxygenases are also important in the metabolic disposal of drugs, insecticides, carcinogens, hydrocarbons and terpenes (Hayaishi, 1974). A number of pathways showing the involvement of oxygenases in the microbial degradation of hydrocarbons have already been illustrated in earlier sections of this introduction. Oxygenases are ubiquitous in nature and can be isolated from plants, animals and microorganisms, although strictly anaerobic microorganisms lack these enzymes.

The role of oxygen in enzymatic reactions was first demonstrated in 1955 by workers using radiolabelled ^{18}O. Mason et al., (1955) demonstrated the incorporation of one oxygen atom into 3,4-dimethyl phenol to form dimethyl catechol, this oxidation being brought about by phenolase (equation 1).

Equation 1



Independently Hayaishi et al., (1955) found that two atoms of oxygen were incorporated into catechol to form cis, cis muconic acid by the action of pyrocatechase (equation 2).

Equation 2

In both equations the atoms of oxygen were derived from molecular oxygen.

Oxygenases are classified into two major groups namely monooxygenases and dioxygenases of which examples are illustrated in equations 1 and 2 respectively. This division of oxygenases is dependent upon the number of oxygen atoms inserted into the substrate. The reaction catalysed by a monooxygenase involves the incorporation of one atom of molecular oxygen (dioxygen) into the substrate while the other atom of oxygen is reduced to water. In a dioxygenase reaction both atoms of molecular oxygen are incorporated into the substrate.

1.2.1 Monooxygenases

Monooxygenases may be subdivided into 'external' and 'internal' monooxygenases depending on the source of the reducing agent essential for the reaction. With external monooxygenases the reducing agent is usually a reduced pyridine nucleotide such that:

$$S + O_2 + H_2X \rightarrow SO + H_2O + X$$

(H_2X = reducing agent and S=substrate). However with an internal monooxygenase the substrate acts as the electron donor as well as the electron acceptor so that:

$$SH_2 + O_2 \longrightarrow SO + H_2O$$

(SH_2 = Substrate)

The internal monooxygenases which have been characterised so far contain flavin as the prosthetic group. Of the external monooxygenases characterised where an added reducing agent is required, the cofactors involved in these enzymes may be haem, non-haem iron, flavin, copper or pteridine.

Different types of monooxygenases are known to catalyse particular reactions, for example the lactone and ester forming enzymes are external monooxygenases containing flavin as the prosthetic group and the enzymes are usually comprised of a single protein component. However the alkyl hydroxylase systems which are also external monooxygenases are multicomponent enzymes containing a terminal oxidase protein which employs a haem or non-haem iron coenzyme as the prosthetic group.

1.2.1.1 Flavoprotein Monooxygenases

The flavoprotein monooxygenases are a group of flavoenzymes which bring about the cleavage of molecular oxygen so that one atom is incorporated into the substrate and the other converted to water. These enzymes contain either FAD or FMN as the prosthetic group. There are two main types of flavoprotein monooxygenases referred to as internal and external flavoprotein monooxygenases.

With internal flavoprotein monooxygenases the substrate provides the reducing agent. This group of enzymes perform oxidative decarboxylation examples of which include the lactate oxidative decarboxylase from Mycobacterium phlei (Takemori et al., 1974) and lysine oxidative decarboxylase from Pseudomonas fluorescens (Flashner and Massey, 1974).

The external flavoprotein monooxygenases require an external source of reducing equivalents which is generally either NADH or NADPH. The majority of external flavoprotein monooxygenases characterised have been derived from microorganisms and do not contain any other detectable organic coenzymes or transition metal ions (Nozaki and Hayaishi, 1984). These external flavoprotein monooxygenases possess the reductase and oxygenase functions necessary for the reaction on a single protein and are therefore regarded as single component systems. There are however two exceptions namely the ketone monooxygenases, 2,5-diketocamphane monooxygenase (Taylor and Trudgill, 1984) and the bacterial luciferases (Massey and Ghisla, 1983) in which the reductase and oxygenase components are separate.

The external flavoprotein monooxygenases can be grouped according to the transformation they perform. The enzymes that make up the largest percentage of this group are the aromatic or phenolic hydroxylases. In this group of reactions the substrate is an unsubstituted or substituted phenolic compound which becomes hydroxylated. Examples of such enzymes include the p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens (Howell et al., 1972), 3-hydroxybenzoate-4-hydroxylase from Pseudomonas testosteri (Michalover and Ribbons, 1973), melitotate 3-hydroxylase from Pseudomonas sp. (Strickland and Massey, 1973), 3-hydroxybenzoate 6-hydroxylase from Pseudomonas aeruginosa (Groseclose and Ribbons, 1973) and salicylate hydroxylase from Pseudomonas cepacia (Wang et al., 1984).

The other types of flavoprotein monooxygenases include a group which bring about the insertion of an atom of oxygen into a ring system to form a lactone or ester. This may be regarded as a Baeyer-Villiger type reaction where the oxygen atom is introduced into a carbon-carbon bond adjacent to a carbonyl group. Enzymes that catalyse such reactions include the cyclohexanone monooxygenase from Acinetobacter NCIB 9871 (Donoghue et al., 1976), cyclopentanone monooxygenases from Pseudomonas NCIB 9872 (Griffin and Trudgill, 1976), 2-tridecanone monooxygenases from Pseudomonas cepacia (Britton and Markovetz, 1977) and 2,5-diketocamphane monooxygenase

from Pseudomonas putida ATCC 1743 (Taylor and Trudgill, 1984).

The oxidation of long chain aldehydes to the corresponding carboxylic acids is brought about by a group of flavoprotein monooxygenases called bacterial luciferases. These enzymes have been found in Photobacterium fischeri (Gunsalus-Miguel et al., 1972), Beneckea harveyi (Hastings and Balny, 1975) and Photobacterium phosphoreum (Watanabe and Nakamura, 1976).

Another group of flavoprotein monooxygenases are responsible for the conversion of nitrogen and sulphur containing substrates to the corresponding oxides. This is found to occur in liver microsomes being catalysed by microsomal liver FAD containing monooxygenases (Poulsen and Zeigler, 1979).

A number of reviews concerning the nomenclature and characterisation of flavoprotein oxygenases have been published (Flashner and Massey, 1974; Massey and Hemmerich, 1975; Massey and Ghisla, 1983). Table 1.3 shows examples of characterised flavoprotein monooxygenases.

The mechanism of action of the external flavoprotein monooxygenases appear to comprise of two reactions namely the reductive half reaction and the oxidative half reaction. These two reactions were examined in detail by Beaty and Ballou (1981a, 1981b) using the microsomal FAD-containing monooxygenase from pig liver. The initial reductive half reaction causes the reduction of the flavin component of the enzyme by a reduced pyridine nucleotide as shown below

$$\text{E-Flavin} + \text{NAD(P)H} \longrightarrow \text{E-Flavin.}\ \text{NAD(P)H} \longrightarrow \underset{\text{NAD(P)}^+}{\text{E.Flavin}H_2}$$

During the oxidative half reaction the reduced flavin prosthetic group is re-oxidised by the molecular oxygen with the simultaneous oxygenation of the substrate.

$$\underset{\text{NADP}^+}{\text{E-Flavin}H_2} + O_2 + S \longrightarrow \text{E-Flavin} + S\text{-}O + H_2O + \text{NAD(P)}^+$$

Whilst the reductive reaction is relatively straight forward the oxidative reaction is somewhat more complex as transient flavin-oxygen intermediates are produced during the catalytic cycle. The

Table 1.3 Examples of characterised flavoprotein monooxygenases

Type	Enzyme	Source	Flavin prosthetic group	Electron donor	Reference
Oxygenative decarboxylation	Arginine oxidative decarboxylase	*Streptomyces griseus*	FAD	Internal	Olomucki *et al.* (1968)
	Lactate oxidative decarboxylase	*Mycobacterium phlei*	FMN	Internal	Takemori *et al.* (1974)
	Lysine oxidative decarboxylase	*Pseudomonas fluorescens*	FAD	Internal	Flashner + Massey (1974)
Hydroxylation of aryl groups	Salicylate hydroxylase	*Pseudomonas putida*	FAD	NADH	Yamamoto *et al.* (1965)
	Melilotate 3-hydroxylase	*Artgribacter* sp.	FAD	NADH	Levy + Frost (1966)
	Nicotine hydroxylase	*Arthrobacter oxydans*	FMN?	artificial	Hochstein + Dalton (1967)
	Imadazoleacetate monooxygenase	*Pseudomonas* sp.	FAD	NADH	Maki *et al.* (1969)
	p-Hydroxybenzoate hydroxylase	*Pseudomonas putida*	FAD	NADPH	Hesp + Calvin (1969)
	p-Hydroxygenzoate hydroxylase	*Pseudomonas putida*	FAD	NADPH	Nakamura *et al.* (1971)

Table 1.3 Cont.

Type	Enzyme	Source	Flavin prosthetic group	Electron donor	Reference
Hydroxylation of aryl groups	p-Hydroxybenzoate hydroxylase	*Pseudomonas fluorescens*	FAD	NADPH	Howell *et al.* (1972)
	3-Hydroxybenzoate 6-hydroxylase	*Pseudomonas aeruginosa*	FAD	NADH	Groseclose + Ribbons (1973)
	3-Hydroxybenzoate 4-hydroxylase	*Pseudomonas testosteri*	FAD	NADPH	Michalover + Ribbons (1973)
	Phenol 2-hydroxylase	*Trichosporon cutaneum*	FAD	NADPH	Neujhar + Gaal (1973)
	Melilotate hydroxylase	*Pseudomonas* sp.	FAD	NADH	Strickland + Massey (1973)
	4-Hydroxyphenyl-acetate 3-hydroxylase	*Pseudomonas acidovorans*	FAD	NADH NADPH	Hareland *et al* (1975)
	Orcinol hydroxylase	*Pseudomonas putida*	FAD	NADH	Ohta *et al.* (1975)
	Salicylate hydroxylase	*Pseudomonas cepacia*	FAD	NADH	Wang *et al.* (1984)

Table 1.3 Cont.

Type	Enzyme	Source	Flavin prosthetic group	Electron donor	Reference
Lactone and ester forming oxygenation	Cyclohexanone monooxygenase	Nocardia globerula CL1	FAD	NADPH	Norris + Trudgill 1972
	Cyclohexanone monooxygenase	Acinetobacter NCIB 9871	FAD	NADPH	Donoghue et al. (1976)
	Cyclopentanone monooxygenase	Pseudomonas NCIB 9872	FAD	NADPH	Griffin + Trudgill 1976
	2-Tridecanone monooxygenase	Pseudomonas cepacia	FAD	NADPH	Britton + Markovetz 1977
	2-Oxo-Δ^3-4,5-trimethylcyclopentylacetate monooxygenase	Pseudomonas putida ATCC 1743	FAD	NADPH	Ougham et al. (1983)
	2,5-diketocamphane monooxygenase	Pseudomonas putida ATCC 1743	FMN	NADH	Taylor + Trudgill 1984
Oxidation of aldehydes	Bacterial luciferase	Photobacterium fischeri	FMN	NADH	Gunsalus-Miguel et al. (1972)
	Bacterial luciferase	Beneckea harveyi	FMN	NADH	Hastings + Balny (1975)
	Bacterial luciferase	Photobacterium phosphoreum	FMN	NADH	Watanabe + Nakamura 1976
Oxidation of N- and S-containing substrates	Microsomal liver FAD containing monooxygenase	Liver microsomes	FAD	NADPH	Poulsen + Zeigler (1979)

reactive oxygen transfer species, 4α-peroxyflavin has been shown to be the initial flavin-oxygen adduct in all external flavoprotein monooxygenases investigated (Spector and Massey, 1972; Presswood and Kamin, 1976; Hussain et al., 1979; Beaty and Ballou, 1981b; Ryerson et al., 1982).

Walsh et al., (1983) studying the mechanism of action of cyclohexanone monooxygenase were able to propose a model for the catalytic cycle for the flavin prosthetic group in external flavoprotein monooxygenase reactions (fig 1.13). The 4 - α peroxyflavin (intermediate I) transfers one atom of oxygen to the substrate and itself forms 4α-hydroxyflavin (intermediate III) which in turn eliminates H_2O to form the oxidised flavin ready for the next reaction sequence.

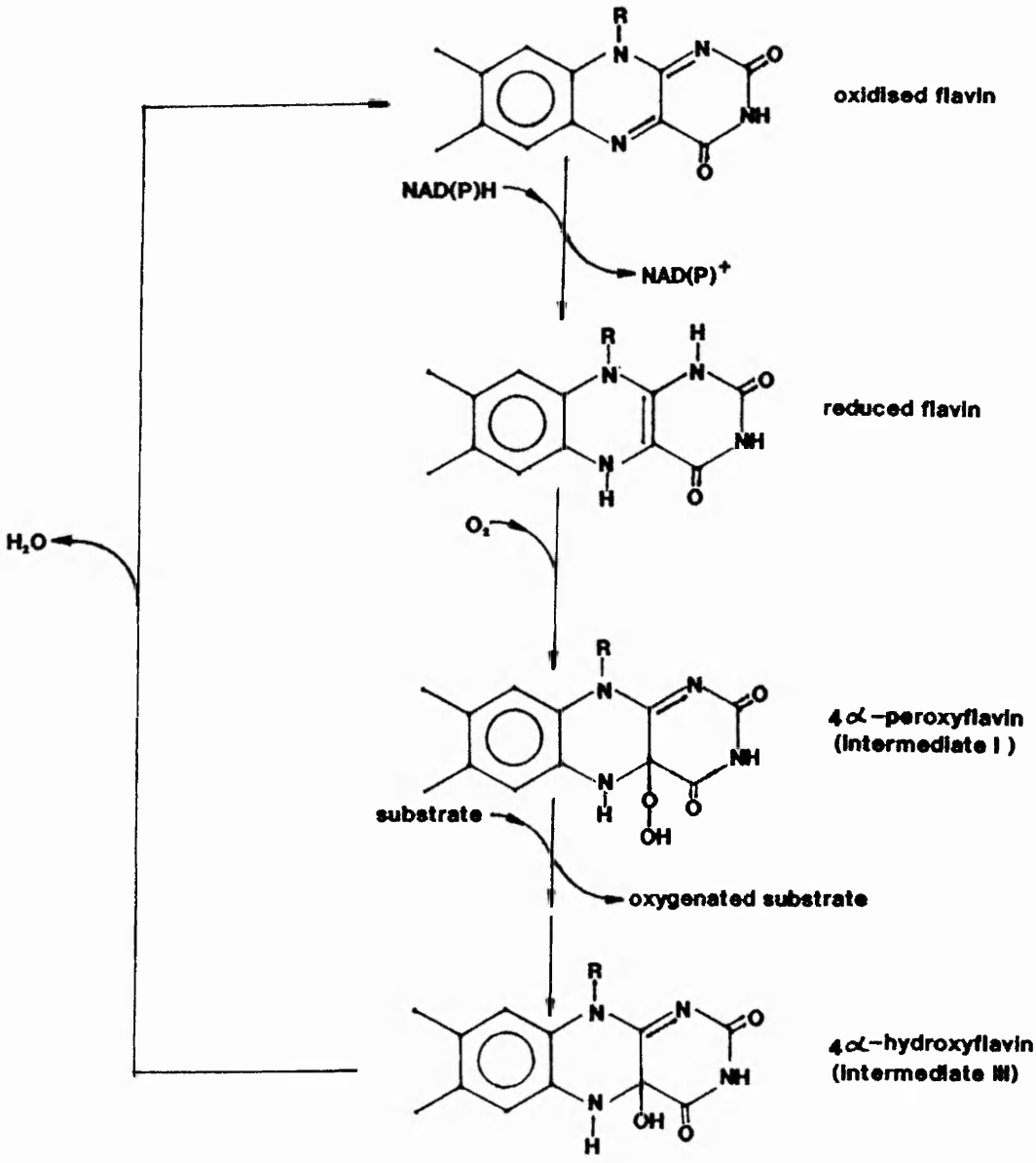
However, this model describes only the minimum catalytic cycle of the flavin prosthetic group. Studies with P-hydroxybenzoate hydroxylase (Entsch et al., 1974, 1976), phenol hydroxylase (Massey and Ghisla, 1983) and melilotate hydroxylase (Schopfer and Massey 1980) have indicated the presence of another flavin-oxygen species namely intermediate II. However, the nature of this intermediate II is not clearly understood.

1.2.1.2 Iron-containing monooxygenases

1.2.1.2.1 Haem iron-containing monooxygenases

The haem iron-containing monooxygenases, commonly referred to as cytochrome P-450-containing monooxygenases, are characterised by the fact that when reduced and complexed with carbon monoxide they display an absorbance peak at 450nm (Smith and Davis, 1980). Many cytochrome P-450 monooxygenase studies have been carried out in mammalian systems, mainly liver, but in addition to hepatic tissue, studies have been conducted with the endoplasmic reticulum of cells from the gastrointestinal tract, lung and kidneys (Smith and Davis, 1980). The cytochrome P-450 monooxygenases in these cells are responsible for the biogenesis of essential metabolites such as lipophilic and regulatory steroid hormones and in the oxygenation

Fig 1.13 Minimum catalytic cycle for the flavin prosthetic group involved in external flavoprotein monooxygenase catalysed reactions (Walsh et al., 1983)



and excretion of foreign and synthetic molecules (Gunsalus et al., 1975).

Cytochrome P-450 activity in microorganisms was demonstrated in cell extracts from Saccharomyces cerevisiae by Lindenmayer and Smith (1964). In the ensuing years two further species of cytochrome P-450 were detected, firstly by Appleby (1967) in the N_2-fixing Rhizobium japonicum and secondly by Katagiri et al., (1968) in the camphor-utilizing soil bacterium Pseudomonas putida. The discovery of the soluble cytochrome P-450 in the camphor-utilising P.putida has had a significant effect on research into this haemprotein and has contributed much of the knowledge available today regarding the molecular properties and reaction mechanism of cytochrome P-450 enzyme systems. Reports regarding the induction of cytochrome P-450 in alkane-assimilating cells of a Corynebacterium (Cardini and Jurtshuk, 1968) and a Candida tropicalis strain (Lebeault et al., 1971) have also led to investigations which suggest that n-alkanes when used as growth substrates are inducers of cytochrome P-450.

Cytochrome P-450 is the terminal component of multienzyme systems which consist of electron transport systems which channel electrons from NAD(P)H to the terminal monooxygenases. This channelling of electrons is necessary as the cytochrome P-450 is unable to accept more than one electron at a time and so therefore cannot interact directly with the reduced pyridine nucleotide which donates two electrons. The transport of electrons from the pyridine nucleotide to the haemprotein is mediated by two different mechanisms. The first mechanism of electron transport involves a single reductase protein which is associated with two flavin prosthetic groups (FAD and FMN). This flavoprotein is directly responsible for the sequential transfer of electrons from the pyridine nucleotide to the cytochrome P-450. This type of electron transport mechanism is known as a two component system and is common to the microsomal monooxygenases. The second electron transport system consists of a flavoprotein (FAD or FMN) containing reductase which couples the two electron oxidation of the reduced pyridine nucleotide to one electron reductions of an iron-sulphur protein which is the next component of the system. This iron-sulphur

redoxin catalyses the two sequential one electron transfers to the cytochrome P-450. This mechanism of electron transport is known as a three component system and is associated with mitochondrial and some bacterial monooxygenases. A comparison of the two and three component haem iron- containing monooxygenase systems is shown in fig. 1.14.

Some of the microbial two component "microsomal type" cytochrome P-450 monooxygenase systems have been characterised and examples include the n-octane w-hydroxylase from a Corynebacterium sp. (Cardini and Jurtshuk, 1970), the n-alkane w-hydroxylase from Candida tropicalis (Duppel et al., 1973) and the n-alkane w-hydroxylase from Candida guilliermondii (Schunck et al., 1978). A number of the three component cytochrome P-450 dependent monooxygenase systems have also been characterised, including the methylene hydroxylases, 25-hydroxy-cholecaliferal 1α-hydroxylase from kidney mitochondia (Kulkoski and Ghazarian , 1979) and the steroid 11β-hydroxylase from adrenal cortex mitochondria (Mitani, 1979). Table 1.4 shows examples of characterised microbial two component cytochrome P-450 monooxygenase systems. Examples of bacterial three component systems include the steroid 15 β-hydroxylase from Bacillus megaterium (Berg et al., 1976), the camphor 5-hydroxylase from Pseudomonas putida (Gunsalus and Wagner, 1978) and the methyl hydroxylase, linalool 8-hydroxylase from a Pseudomonas sp. (Wagner, 1983). Examples of three component cytochrome P-450 dependent monooxygenases are shown in Table 1.5.

Many workers have investigated the mechanism of cytochrome P-450 catalysed reactions (White and Coon, 1980). These reactions involve the transfer of two electrons from a reduced pyridine nucleotide which in turn bring about the reduction of one atom of dioxygen to water with the other oxygen atom being inserted into the substrate. The catalytic reaction sequence of cytochrome P-450 is shown in fig. 1.15 (Gunsalus et al., 1975; White and Coon, 1980; Smith and Davis, 1980). The initial step in the reaction sequence involves the hydrophobic binding of the substrate to cytochrome P-450 in the resting ferric state. This is followed by the uptake of a single electron from an associated enzyme (either flavoprotein or iron-

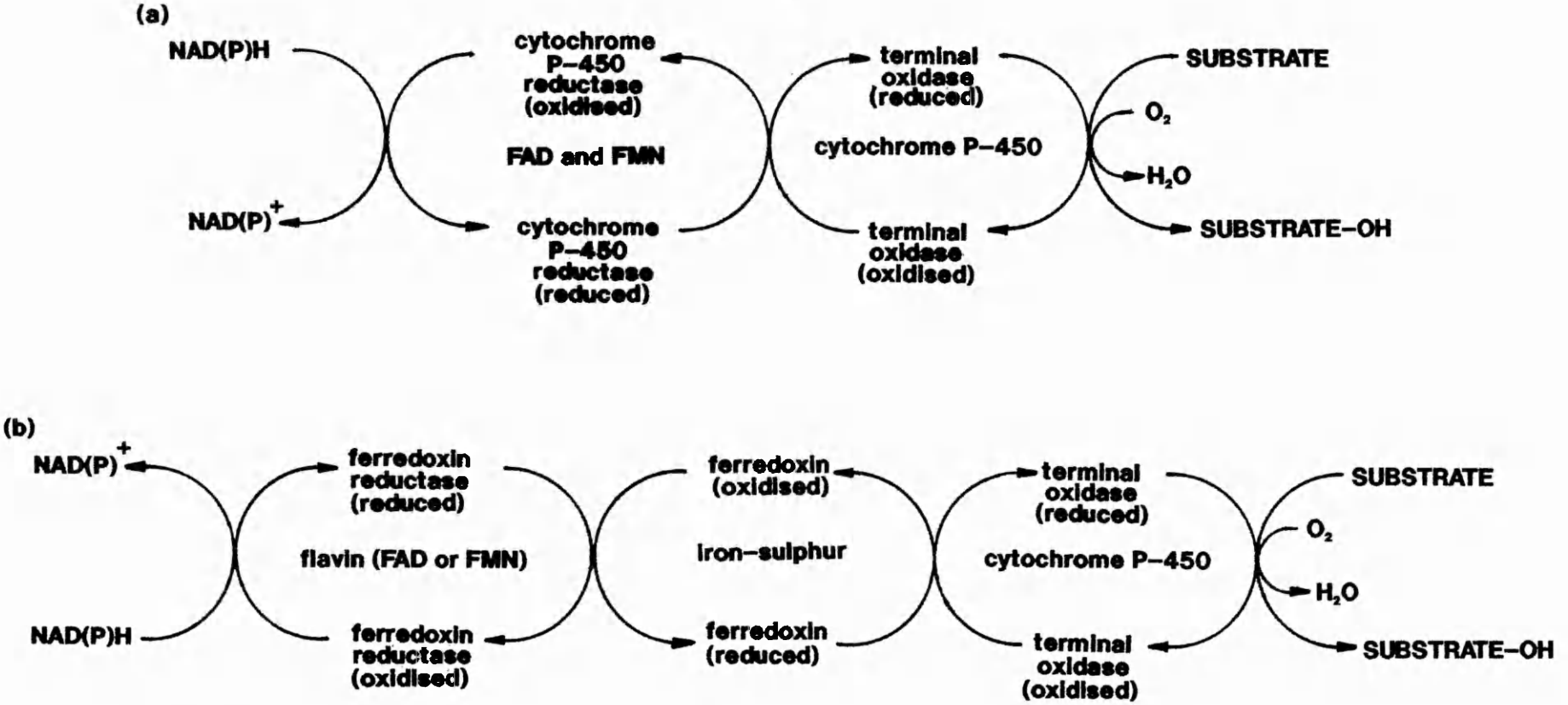


Fig. 1.14 Conceptional organisation of the mechanism of electron transfer in (a) two component and (b) three component cytochrome P-450 dependent monooxygenases.

Table 1.4 Examples of characterised microbial "microsomal type" two component cytochrome P-450 monooxygenase systems.

Enzyme	Source	Components	Electron Donor	Reference
n-octane w-hydroxylase	*Corynebacterium* sp. 7EIC	Cytochrome P-450 reductase (soluble) Cytochrome P-450	NADH	Cardini + Jurtshuk (1970)
n-alkane w-hydroxylase	*Candida tropicalis* LM7	Cytochrome P-450 reductase Cytochrome P-450 heat - stable phospholipid	NADPH	Duppel *et al.* (1973)
n-alkane w-hydroxylase	*Candida guilliermondii*	Cytochrome P-450 reductase Cytochrome P-450	NADPH	Schunck *et al.* (1978)
Progesterone 11β-hydroxylase	*Rhizopus nigricans*	Cytochrome P-450 reductase Cytochrome P-450	NADPH	Breskvar + Hudnik-Plevnik (1981)
n-alkane w-hydroxylase	*Loddermyces elongisporous*	Cytochrome P-450 reductase Cytochrome P-450	NADPH	Muller *et al.* (1982) Honeck *et al.* (1982)

Table 1.5 Examples of characterised three component cytochrome P-450 dependent monooxygenases

Enzyme	Source	Location	Components	Electron donor	Reference
Steroid 15β hydroxylase	*Bacillus megaterium* ATCC 13368	Soluble	megaredoxin reductase megaredoxin cytochrome P-450	NADPH	Berg *et al.* (1976)
Camphor 5-hydroxylase	*Pseudomonas purida* ATCC 29607	Soluble	putidaredoxin reductase putidaredoxin cytochrome P-450	NADH	Gunsalus + Wagner (1978)
25-hydroxycholecalciferol 1α-hydroxylase	Kidney mitochondria	Microsomal	ferredoxin reductase ferredoxin cytochrome P-450	NADH	Kulkoski + Ghazarian (1979)
Steroid 11β hydroxylase	Adrenal cortex mitochondria	Microsomal	adrenodoxin reductase adrenodoxin cytochrome P-450	NADPH	Mitani (1979)
Linalool 8-hydroxylase	*Pseudomonas* sp.	Soluble	ferredoxin reductase ferredoxin cytochrome P-450	NADH	Wagner (1983)

sulphur protein) producing the reduced ferrous state. The reduced haemprotein is then able to bind an oxygen molecule forming an oxyferro complex. The uptake of a second electron forms a ferric iron-coordinated peroxide complex. The splitting of the oxygen-oxygen bond results in the elimination of water, leaving a single oxygen atom bound to the iron as an oxylide radical which causes the insertion of the oxygen atom into the substrate. The product then dissociates from the active site of the enzyme resulting in the reformation of the resting ferric cytochrome ready for the next catalytic cycle.

1.2.1.2.2 Non-haem iron-containing monooxygenases

These enzymes employ iron-sulphur proteins as the terminal oxidase and those characterised are soluble, multicomponent systems of bacterial origin. However, Strijewski (1981) reported that this group of enzymes are not characterised in such detail as the cytochrome P-450 sytems. This does not necessarily mean that these non-haem iron-containing monooxygenases occur less in nature, but is probably an indication of the instability of these enzymes and reflects the difficulties encountered in purification and characterisation. However, despite these drawbacks there are some well characterised non-haem iron monooxygenase systems including the n-alkane w-hydroxylases from Pseudomonas oleovorans (Griffith et al., 1978) and Pseudomonas aeruginosa (Matsuyama et al., 1981). Also the steroid 9α-hydroxylase from a Nocardia sp. (Strijewski, 1982), the 4-methylbenzoate O-demethylase from Pseudomonas putida (Bernhart and Kuthan, 1983) and the methane monooxygenase from Methylococcus capsulatus (Bath) (Woodland and Dalton, 1984). Examples of characterised non-haem iron based monooxygenase systems are shown in Table 1.6.

The catalytic mechanism of these non-haem iron monooxygenase systems appears to be similar to that of both the two and three component cytochrome P-450 monooxygenases. As with cytochrome P-450 the non-haem iron (terminal oxidase) is unable to accept the two electrons necessary for the reaction simultaneously and so other electron transfer proteins are necessary to assist this operation.

Fig 1.15 The catalytic cycle of a cytochrome P-450-linked monooxygenase from the data of Sligar et al. (1984).

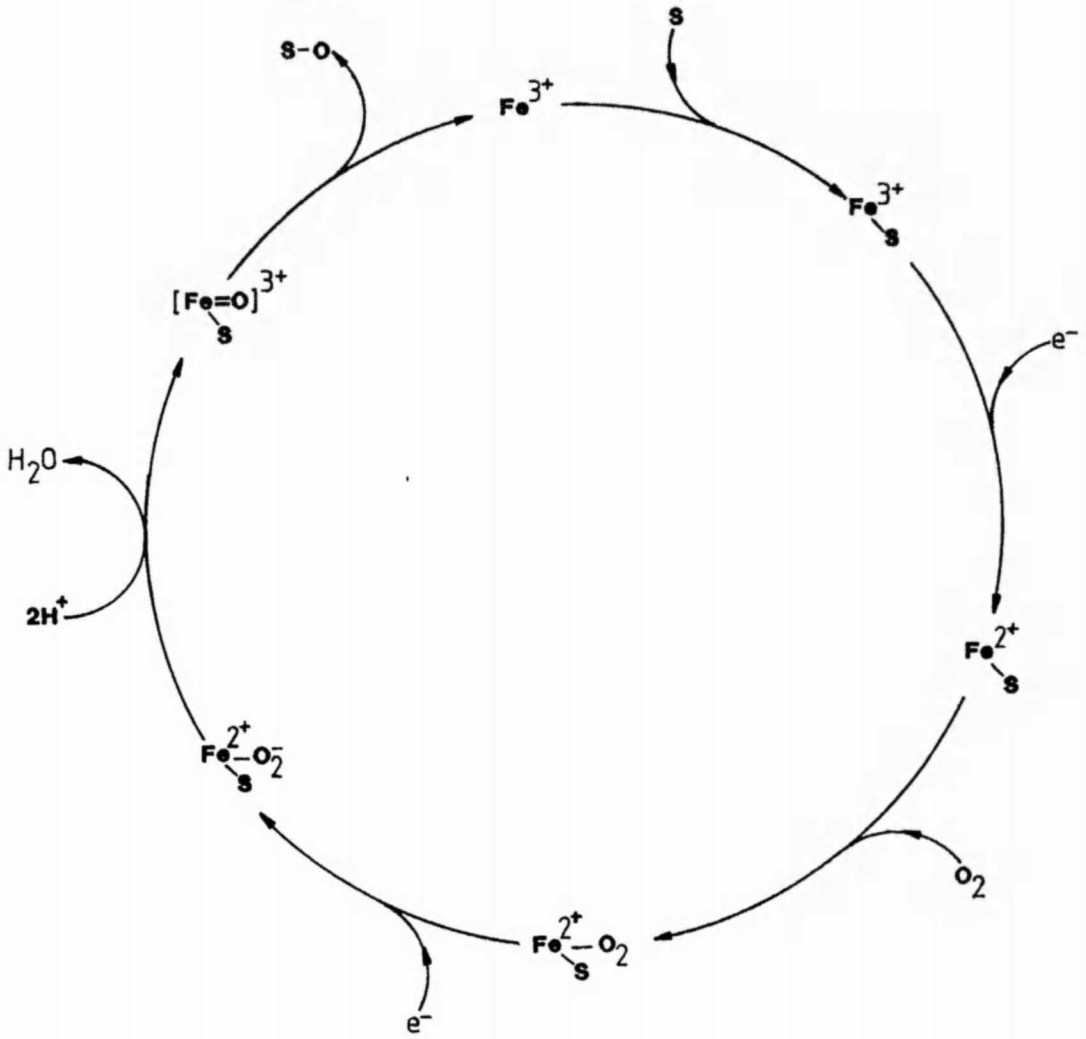


Table 1.6 Examples of characterised non-haem iron based monooxygenase systems.

Enzyme	Source	Components	Electron donor	Reference
n-alkane w-hydroxylase	*Pseudomonas oleovorans*	rubredoxin reductase rubredoxin w-hydroxylase heat-stable phospholipid	NADH	Griffith *et al.* (1978)
n-alkane w-hydroxylase	*Pseudomonas aeruginosa*	Protein III flaboprotein Protein II cytochrome C Protein I iron protein	NADPH	Matsuyama *et al.* (1981)
Steroid 9α-hydroxylase	*Nocardia* sp. M117	Flavoprotein reductase Protein II (iron-sulphur protein) Protein III (iron-sulphur protein)	NADH	Strijewski (1982)
4-methoxybenzoate O-demethylase	*Pseudomonas putida*	Putidamonooxin reductase (FMN containing iron-sulphur protein) putidamonooxin (iron/iron-sulphur protein)	NADH	Bernhart + Kuthan (1983)
Methane monooxygenase	*Methylococcus capsulatus* (Bath)	Protein C (FAD containing iron-sulphur protein) Protein B Protein A (iron-sulphur protein)	NADH	Woodland and Dalton (1984)

The reaction occurs due to the initial interaction of the reduced pyridine nucleotide with a flavoprotein/iron-sulphur component. This component mediates the two sequential one electron transfers, necessary for the reaction, through an iron-sulphur/cytochrome C protein (if present) to the iron/iron-sulphur terminal oxidase which brings about the oxygenation of the substrate.

1.3 The Genus Xanthobacter

As reported earlier in this introduction there are a number of microorganisms which are capable of degrading n-alkanes or cycloalkanes. Of particular interest in this study are microorganisms capable of growth on cycloalkanes. In this respect the genus Xanthobacter contains bacterial species which are capable of degrading both non-substituted and substituted cycloalkanes.

The genus Xanthobacter has been described in Bergey's Manual of Systematic Bacteriology, Volume 1 (Wiegel and Schlegel, 1984). Section 4 of the manual contains the Gram-negative aerobic rods and cocci and includes such families as Pseudomonadaceae, Rhizobiaceae, Methylococcaceae and five others. Also classified in this section are a number of genera including Xanthobacter, that have not been assigned to any family.

Although members of the genus Xanthobacter are designated Gram-negative, the Gram reaction is positive or variable, but the ultrastructure of the cell wall appears to be of the Gram-negative type. Other important characteristics of Xanthobacter are that they are nitrogen-fixing, hydrogen-oxidising, non-motile bacteria. They have an optimum growth temperature of 25°-30°C, are obligately aerobic, contain polyphosphate and poly-β-hydroxybutyrate (PHB) as reserve materials and produce yellow colonies due to a water insoluble carotenoid pigment, zeaxanthin dirhamnoside. The organisms occur free-living in wet soil containing decaying organic material, and also in water (Wiegel and Schlegel, 1984).

The genus Xanthobacter is newly formed and at present only two species have been described in Bergey's Manual of Systematic Bacteriology, namely Xanthobacter autotrophicus and Xanthobacter

flavus. Both of these organisms have been re-classified having previously belonged to other genera. Xanthobacter autotrophicus was first identifed by Baumgarten et al., (1974) as Corynebacterium autotrophicum but was re-classified by Wiegel et al., (1978). The natural habitat of X.autotrophicus is soil, mud and water and the organism is widely distributed in nature. There are a number of different strains of X.autotrophicus which have been identified. Xanthobacter flavus was so named by Malik and Claus (1979) having previously been misclassified by Federov and Klaininskaya (1961) as Mycobacterium flavum. The organism was isolated from turf podzol soil in the USSR and until recently no other strains had been identified. However, numerical classification, DNA base composition and DNA-DNA hydridization studies by Jenni et al., (1987) led to a number of biotin-requiring strains of Xanthobacter to be grouped with X. flavus. Although Wiegel and Schlegel (1984) described the genus Xanthobacter as containing non-motile microorgansisms, Jenni et al., (1987) reported a cluster of motile strains which probably represent another species of the genus Xanthobacter.

2. METHODS

2.1 Microorganisms

Five microorganisms were investigated in this study, namely:-

1) A Xanthobacter species isolated by Dr. M.K.Trower from soil of Nottinghamshire forests by classical enrichment techniques.

2) Xanthobacter autotrophicus (strain 853) which is a mutant of the Xanthobacter species isolated by Dr. M.K. Trower. This organism was isolated in laboratories at Trent Polytechnic, Nottingham and identified at the National Collection of Industrial and Marine Bacteria (NCIMB), Torrey Research Station, Aberdeen, Scotland.

3) Xanthobacter autotrophicus (strain 431) obtained from the Deutsche Sammlung von Mikroorganism (DSM), Gottingen, Federal Republic of Germany.

4) Xanthobacter (strain H4-14) donated to this project by Dr. J.C. Murrell, Department of Biological Sciences, University of Warwick, England.

5) Xanthobacter flavus obtained from the National Collection of Industrial and Marine Bacteria, Torrey Research Station, Aberdeen, Scotland.

2.2 Maintenance and Culture of Microorganisms

2.2.1 Maintenance of Microorganisms

All organisms were maintained on nutrient agar slopes or solid mineral salts medium (MSM) slopes containing a sole carbon source. Different carbon sources were used for the maintenance of the organisms on MSM. For Xanthobacter sp. and Xanthobacter 853, cyclohexane was provided as the carbon source, which was supplied as a vapour. For maintenance of Xanthobacter 431 on MSM, cyclohexanol

0.1% (v/v) was incorporated into the media and for Xanthobacter H4-14 and X.flavus succinate 0.2% (w/v) was provided as the carbon source. The microorganisms were subcultured at monthly intervals. Following subculture of the organsisms, the slopes were incubated at 30°C for 24-48h and after growth were stored at 4°C. The purity of the organisms was checked by plating out on nutrient agar (NA). A stock of each Xanthobacter strain was also maintained by storage in glycerol. After growth in liquid media the media was added to sterile glycerol, in the ratio 85:15 respectively and stored at -20°C.

A freeze dried stock of each Xanthobacter strain was also produced by resuspending a loopful of the desired organism in 5ml skimmed milk 20% (w/v). 0.2ml of this solution was placed in a sterile glass freeze drying ampule, covered with a cloth cap and placed on the freeze drier (Edwards High Vacuum Ltd, Crawley, Sussex). The ampules were centrifuged under vacuum for 10 min and left under vacuum for 2h at 10^{-1} torr. The ampules were constricted, placed on a secondary manifold and in turn placed on the freeze drier where they were left overnight under vacuum at 10^{-1} torr. The ampules were then heat sealed and stored in the dark at 4°C.

2.2.2 Growth of microorganisms

For growth of Xanthobacter on a sole carbon source in liquid medium, 100ml MSM in a 250ml conical flask was inoculated from a stock slope. For growth of Xanthobacter sp. and Xanthobacter 853, cyclohexane (0.1 ml) was added to the medium and the flask stoppered with a silicon bung. For growth of Xanthobacter 431, cyclohexanol (0.1ml) was added to the medium and the flask stoppered with a cotton wool bung. For growth of Xanthobacter H4-14 and X. flavus on liquid MSM the medium contained succinate 0.2% (w/v) and the flask was stoppered with a cotton wool bung. All flasks were incubated at 30°C on a gyratory shaker (200 rpm).

When growth of Xanthobacter H4-14 on solid or liquid MSM was required it was necessary to supplement the media with yeast extract

0.1% (w/v). For growth of X. flavus on solid or liquid MSM the following vitamins were added to the media :- thiamine, riboflavin, nicotinic acid, pyridoxin-HCl and calcium pantothenate each at a concentration of 1µg ml^{-1} and vitamin B_{12}, biotin and folic acid each at 0.1µg ml^{-1}.

When larger quantities of Xanthobacter were required, cells were grown in batch culture in 2, 5 or 20l impeller agitated fermenters (L.H. Engineering, Stoke Poges, Buckinghamshire) at 30°C. Volatile carbon sources such as cyclohexane were provided as a vapour through the main air supply at 0.5, 1.0 or 2.0 l min^{-1} respectively. For growth on cyclohexanol and other substituted cycloalkanes the carbon source 0.1% (v/v) was added directly to the medium. When growth on sugars and organic acids was required, the liquid MSM contained 0.2% (w/v) or (v/v) of the desired carbon source.

For growth studies involving all five species of Xanthobacter, all non-volatile growth substrates with the exception of sugars were added directly to solid and liquid MSM at a concentration of 1.0 g. l^{-1} prior to autoclaving. Sugars were autoclaved in the solid form and aseptically added to sterile MSM to give a final concentration of 2.0 g l^{-1}. Volatile carbon sources were filter sterilised through a 0.2µm polycarbonate bacterial filter (Nucleopore Inc., California, U.S.A.), and were added directly to liquid MSM at a concentration of 0.1% (w/v). Flask cultures were incubated at 30°C on a gyratory shaker (200 rpm). For solid MSM, volatile carbon sources (0.02ml) were absorbed onto sterile filter papers and placed inside the lids of the petri dishes which wre then sealed with parafilm and placed in air tight containers. The petri dishes were then incubated at 30°C.

2.3 Sterilisation

All growth media of volumes up to 2l and solid sugar growth substrates were sterilised by autoclaving at 15 lb. in^{-2} for 20 min. When sterilisation of growth media of volumes greater than 2l was required the time of autoclaving was extended to 1h at the same

pressure. Heat labile and volatile compounds were filter sterilised through a polycarbonate 0.2μm bacterial filter (Nucleopore Inc., California, U.S.A.).

2.4 Preparation of Microorganisms

2.4.1 Harvesting of microorganisms

Growth of cells was monitored by measuring the absorbance at 550nm on a Pye Unicam SP6-400 spectrophotometer (Pye Unicam, Cambridge). Cultures were generally harvested when E_{550nm} reached 1.6.

Bacteria grown in shake flasks were harvested by centrifugation at 10,000 g (10 min at 4°C). Harvesting of fermenter grown cells was by continuous centrifugation (Sharples continuous centrifuge, Pennwalt, Surrey at 14,300 g with a flow rate of 0.3ml $min.^{-1}$.

The cell pellets were washed by resuspension in 20mM phosphate buffer (pH 7.0) and then centrifuged at 10,000 g (10 min at 4°C). This process was repeated once more. Cells that were to be used directly were resuspended in 1.0-1.5 times their own wet weight of 20mM phosphate buffer (pH 7.0), and kept on ice. Cells that were not required for use immediately were stored as a pellet at -20°C.

2.4.2 Preparation of crude cell extracts

Bacterial cell suspensions were disrupted by either Ultrasonication (Soniprep 150, MSE Scientific Instruments, Sussex) for a total of 2min (6 x 20 sec bursts, allowing cooling in between, 6μm, peak to peak at 0-5°C) or by repeated passage (x2) through a French pressure cell (American Instruments Co., Maryland, U.S.A.).

The resulting cell debris was removed by centrifugation at 10,000 g (10 min at 4°C). Soluble (100,000 g supernatant) and particulate membrane fractions were prepared by ultracentrifugation of the 10,000 g supernatant at 100,000 g (60 min at 4°C).

2.5 Dry Weight Determinations

The determination of the dry weight of cell suspensions was necessary for the quantitating of reuslts from whole cell oxidation studies. A measured volume of resuspended whole cells was pipetted into a pre-weighed glass crucible and dried to constant weight at 110°C. Dry weight determinations were performed in triplicate and were corrected for the weight of buffer residue.

2.6 Enzyme Assays

All enzyme assays were performed at 30°C unless stated otherwise.

2.6.1 Cyclohexane hydroxylase

Cyclohexane hydroxylase activity was determined polarographically in the oxygen monitor by measuring the NADPH-dependent oxidation of cyclohexane. Reaction mixtures contained, in a volume of 3ml; 240 µmol phosphate buffer (pH 6.8, unless stated otherwise), 5mg protein (100,000 g supernatant), 1.0 µmol NADPH and 2µl cyclohexane.

2.6.2 Cyclohexane dehydrogenase

Cyclohexane dehydrogenase activity was measured spectrophotometrically by following the substrate stimulated reduction of NAD^+ at 340nm. Reaction mixtures contained in a volume of 1ml; 40 µmol glycine/NaOH buffer (pH 10.3, unless stated otherwise), either 0.2-0.5mg protein (100,000 g supernatant) or purified enzyme (25 µg protein), 0.5 µmol NAD^+ and 0.5 µmol cyclohexanol.

2.6.3 Cyclohexanone monooxygenase

Cyclohexanone monooxygenase activity was measured either polarographically in the oxygen monitor by following the NADPH-dependent oxidation of cyclohexanone or spectrophotometrically by following the substrate stimulated oxidation of NADPH at 340nm.

For the polarographic measurement of cylohexanone monooxygenase, reaction mixtures contained in a volume of 3ml; 560µmol glycine/NaOH buffer (pH 8.8, unless stated otherwise), 0.5-1.0 mg protein (100,000 g supernatant), 1.0 µmol NADPH and 2.0 µmol cyclohexanone.

For spectrophotometric measurement of cyclohexanone monooxygenase, reaction mixtures contained, in a volume of 1ml; 160 µmol glycine/NaOH buffer (pH 8.8, unless stated otherwise), 0.2-0.5 mg protein (100,000 g supernatant), 0.5 µmol NADPH and 0.5 µmol cyclohexanone.

2.6.4 1-Oxa-2-oxocycloheptane hydrolase

1-Oxa-2-oxocycloheptane hydrolase activity was measured by the method of Norris and Trudgill (1971) which involved monitoring the formation of the acid reaction product. 1-Oxa-2-oxocyloheptane (50 µmol) in distilled water was adjusted to pH 8.0 and then made up to a total volume of 10ml. The reaction was started by the addition of 0.1-0.5 mg protein and the reaction mixture was maintained at pH 8.0 by constant titration of 10mM NaOH against the acidic reaction product. This enzyme assay was performed at room temperature.

2.6.5 Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase activity was measured spectrophotometrically by following the substrate-stimulated reaction of $NADP^+$ at 340nm. Reaction mixtures contained, in a volume of 1ml; 80 µmol phosphate buffer (pH 7.0, unless stated otherwise), 0.2-0.5mg protein (100,000 g supernatant), 0.5 µmol NAD^+ and 0.5 µmol glucose-6- phosphate.

2.7 Determination of Cytochrome P-450 Content

Cytochrome P-450 content was measured spectrophotometrically (Beckman DU7) according to the method described by Omura and Sato (1964).

Initially a background scan from 600-350nm of 1ml *Xanthobacter* sp. cell extract (100,000 g supernatant) containing 3ul octanol was performed. To the cell extract was added a few grains of sodium dithionite and the resulting dithionite reduced cell extract then scanned once again from 600-350nm. The initial background spectrum was subtracted from this spectrum to produce the dithionite difference spectrum. The dithionite reduced cell extract was then purged with carbon monoxide (approximately 120 bubbles. min^{-1}) for 30-60 sec and then scanned once again from 600-350nm. Subtracting the dithionite difference spectrum from the dithionite carbon monoxide spectrum then produced the dithionite reduced carbon monoxide difference spectrum.

Cytochrome P-450 content was determined from this difference spectrum using a molar extinction coefficient of 91.mM^{-1}. cm^{-1} to the absorbance difference between 450 and 490 nm.

2.8 Estimation of Protein Content in Solutions

All reagents for protein determinations are listed in section 9.5.

Three different methods were employed to determine the concentration of protein in solutions. All protein determinations were carried out in duplicate.

2.8.1 Biuret assay

The Biuret assay was developed by Gornall *et al*. (1949) and was used to determine protein concentration in crude cell extracts. This assay is suitable for solutions containing 2-12mg protein. ml^{-1}.

To 1ml of an appropriately diluted protein solution was added 4ml Biuret reagent, the solution was mixed gently and allowed to

stand at room temperature for 30 min. At the same time 1ml of the appropriate buffer was treated identically and used as a blank. After standing, the absorbance of each sample was measured spectrophotometrically at 550nm against the buffer blank. To determine the protein concentration of the unknown samples a calibration graph was constructed using Bovine Serum albumin (BSA) standards ranging from 2-12mg protein. ml^{-1} which had been treated in the same manner as the unknown samples.

2.8.2 Folin-Ciocalteau assay

The Folin-Ciocalteau assay for protein determination was developed by Lowry et al. (1951). This assay is suitable for solutions containing 25-500µg protein. ml^{-1}, and was routinely used to determine the concentration of dilute protein solutions of purified enzyme.

Solutions A and B were mixed in a ratio 12.25:0.25 respectively to produce solution C. To 100µl of a suitably diluted protein solution 1ml solution C was added. The mixture was vortexed and left to stand for 20 minutes. After this, 100µl Folin-Ciocalteau Phenol reagent (diluted 1:1 with distilled water immediately prior to use) was added and the solution left for a further 20 mins. Simultaneously, 100µl of the appropriate buffer was treated identically and used as a blank. After standing, the absorbance of each sample was measured spectrophotometrically at 750nm against the buffer blank. A calibration graph was constructed using Bovine serum albumin (BSA) standards over a range 25-500µg protein. ml^{-1}. The protein concentrations of the unknown samples were determined from this calibration graph.

2.8.3 Spectrophotometric determinations

This method of estimating protein concentration was developed by Warburg and Christian (1949) and is suitable for dilute solutions of protein in the range 0.05-2.0 mg. ml^{-1}.

A suitably diluted protein sample was measured

spectrophotometrically in a 1cm light path cuvette at 260nm and 280nm against a buffer blank. The $^{E280}nm/_{E260}nm$ ratio of each sample was calculated and its protein concentration estimated using the data of Warburg and Christian.

Since this method is somewhat inaccurate it could only be used to provide a crude estimation of protein concentration and was only used to estimate the protein concentration of fractions obtained from column chromatography during enzyme purification procedures.

2.9 Product Formation Studies

2.9.1 Whole cell incubations

1g harvested, washed cells were resuspended in 10ml 100mM phosphate buffer pH 7.0 in a 50ml conical flask. The appropriate substrate (100µl) was added to the flask which was then stoppered with a silicone bung and incubated on a gyratory shaker (200 r.p.m.) for 15h, after which time the cells were pelleted by centrifugation (10,000 g 10min) and the resulting supernatant acidified to pH 2.0 with 2M HCl. Any precipitated protein was removed by centrifugation (10,000 g, 5 mins). The reaction mixture was then extracted by vortexing for 3 mins with 3 x its volume of 'Aristar' ethyl acetate, dried over anhydrous Na_2SO_4 and concentrated under a stream of N_2 gas.

2.9.2. Cell extract incubations

Incubation mixtures contained, in a volume of 3ml; 200 µ mol phosphate buffer (pH 7.0, unless stated otherwise), 5-10mg protein, any necessary cofactors and the appropriate substrate. Reaction mixtures were incubated at 30°C on a gyratory shaker (200 r.p.m.) for 1h, after which time they were acidified to pH 2.0 with 2M HCl, frozen and thawed and then the precipitated protein removed by centrifugation (10,000 g, 5 mins). The reaction mixture was extracted by vortexing for 3 mins with 3 x its volume of 'Aristar'

ethyl acetate, dried over anhydrous Na_2SO_4 and concentrated under a stream of N_2 gas.

2.9.3 Methyl ester derivatisation of acidic products

Samples to be derivatised were evaporated to dryness under nitrogen. 1ml boron trifluoride- methanol reagent was added to the residue which was then sealed under nitrogen and placed in a boiling water bath for 45 min. The sample was then cooled and extracted by vortexing for 3 min following the addition of 2ml ethyl acetate and 1ml distilled water. The sample was centrifuged (1,100 g, 10 min) to separate the solvent layers and the ethyl acetate layer removed and concentrated under a stream of nitrogen gas.

2.10 Analysis of Compounds by Chromatography

2.10.1 Gas liquid chromatography

Reaction products obtained from substrate incubations undertaken with non-proliferating cells or cell extracts from cyclohexane or methyl cyclohexane grown Xanthobacter sp. were analysed by gas liquid chromatography (glc). Extracts were analysed using a Perkin-Elmer F33 gas chromatograph (Perkin-Elmer, Connecticut, U.S.A.). The resulting peaks were integrated using a Spectra-Physics SP 4100 computing integrator (Spectra-Physics Inc., California, U.S.A.). Retention times were recorded and compared to authentic standards.

Substituted cycloalkanes were detected using the following:-

2m x 2mm glass columns packed with the following stationary phases:

A) 5% (w/w) DEGS/PS on Chromosorb W
   Injector temperature = 200°C, Oven temperature = 90°C.

B) 15% (w/w) Carbowax 20M on Chromosorb W.
   Injector temperature = 175°C, Oven temperature = 70°C with a rise of 5°C. min^{-1} to 130°C.

Methyl ester derivatised carboxylic acids were detected using the following :

2m x 2mm glass columns packed with the following stationary phases:

C) 8% (w/w) FFAPB on acid washed Chromosorb W.
Injector temperature = 250°C, Oven temperature = 100°C with a rise of 5°C. min^{-1} to 200°C.

D) 10% (w/w) Silar 5CP on Chromosorb W.
Injector temperature = 250°C, Oven temperature = 100°C with a rise of 5°C. min^{-1} to 200°C.

2.10.2 Gas chromatography/Mass spectroscopy

Reaction products from substrate incubations with non-proliferating cells and cell extracts of cyclohexane or methylcyclohexane grown Xanthobacter sp. were also analysed by gas chromatography/mass spectroscopy (GC/MS). Extracts were analysed using a Vacuum Generator 7070H gas chromotograph/mass spectrometer (performed by Trevor Blease, ICI Petrochemicals and Plastics Division).

Substituted cycloalkanes and methyl ester derivatised carboxylic acids were detected using the following column:

E) 50m x 0.5um vitreous (fused) silica FFAP capillary column.
Oven temperature initially = 100°C with a rise of 5°C.min^{-1} to a final temperature of 200°C.

2.10.3 Thin layer chromatography

The carboxylic acid reaction products from substrate incubations with non-proliferating cells and cell extracts of cyclohexane and methyl cyclohexane grown Xanthobacter sp. cells were also analysed by thin layer chromatrography. In all analyses R_F values of unknown compounds were compared to authentic standards.

For the detection of the carboxylic acids, 6-hydroxhexanoate, succinic acid, glutaric acid, adipic acid and methyl adipic acid, plates (20 x 20cm) coated with Kieselgel 60 (0.2mm thickness) (Merck, Darmstadt, W. Germany) were spotted and developed with the following solvents:

1) Benzene : Methanol : Glacial acetic acid (45:10:4 by volume) (Petrovity and Pastuska, 1962).

2) Benzene : Ethyl acetate : Formate (25:25:2 by volume) (Griffin and Trudgill, 1972).

After drying the plates were sprayed with a chromogenic reagent (section 9.5.3), the carboxylic acids present appeared yellow against a green background.

2.11 Column Chromatography

Column chromatography was performed as part of an enzyme purification process and also to determine the molecular weights of enzymes involved in cycloalkane degradation. Systems were run at 4°C, with the exception of those involving high performance liquid chromatography (HPLC) which were run at room temperature.

2.11.1 High performance liquid chromatography

2.11.1.1 Ion exchange high performance liquid chromatography

Protein purifications were assisted by the use of a Pharmacia Mono Q pre-packed MR5/5 column (5mm x 50mm). Injection was via a Rheodyne 7125 sample injector. The solvent delivery system consisted of a Waters Associates 6000A pump and a Waters Millipore M-45 pump controlled by a Waters Associates Model 660 solvent programmer. Protein samples (up to 30mg.ml^{-1}) were loaded (up to 2ml) onto the column and eluted with a linear salt gradient in the desired buffer. All solvents were pre-filtered before use (0.45 µm cellulose acetate filter, Whatman, Maidstone, Kent). The flow rate

was maintained at 0.5ml. min^{-1} and the back pressure was not allowed to exceed 500 psi. Eluted proteins were detected at 280nm using a Waters Associates Model 440 UV detector coupled to a LKB-Produktor Model 2210 2 Channel recorder. Eluant was collected on ice in suitable aliquots with an LKB-Produktor Redirac fraction collector. After use the column was purged and stored in 30% (v/v) methanol.

2.11.1.2 Size exclusion high performance liquid chromatography

Purified proteins were analysed using an LKB Ultropac TSK-GSWP (7.5 x 75mm) Precolumn connected to an LKB Ultropac TSK 3000 SW (7.5 x 600mm) size exclusion column (Mr range 1,000 - 300,000). Injection was via a Rheodyne 7125 sample injector. Solvent delivery was via a Waters Millipore M-45 pump. Protein samples and standards (0.05 - 2mg protein) were loaded (50-200µl) onto the column and eluted with HEPES buffer (section 10) pre-filtered before use (0.45 µm cellulose acetate filter, Whatman, Maidstone, Kent). The flow rate was maintained between 0.1-1.0ml. min^{-1} and the back pressure was not allowed to exceed 1000 psi. Eluted proteins were detected at 280nm using a Waters Associates Model 440 UV detector coupled to an LKB-Produktor Model 2210 2 channel recorder. The eluant was collected in suitable aliquots with an LKB-Produktor Redirac fraction collector. A solution of 0.1% (v/v) blue dextran 2,000 and 0.1% (w/v) potassium chromate in the elution buffer (10µl injected) was used to determine the column constants, void volume (Vo) and approximate inclusion volume (Vi).

After use the column was purged and stored in 0.05% (w/v) sodium azide solution.

2.11.2 Conventional size exclusion chromatography

Size exclusion gel filtration chromatography was performed using Biogel 0.5A (Biorad Laboratories, California, USA) and AcA 44 Ultrogel (LKB, Bromma, Sweden). Biogel 0.5A and AcA 44 Ultrogel were provided pre-swollen as a thick slurry and were diluted with 20mM phosphate buffer pH 7.0 before use. Both gel suspensions were

degassed on ice and packed under constant pressure. Biogel 0.5A and AcA 44 Ultrogel were both prepared for operation under ascending flow. When packed, two or three column volumes of 20mM phosphate buffer pH 7.0 were passed through the columns to stabilise the column packing and to equilibrate them with the eluting buffer.

A solution of 0.1% (v/v) blue dextran 2,000 and 0.1% (w/v) potassium chromate in 20mM phosphate buffer pH 7.0 (1ml loaded) was used to determine the column constants, void volume (Vo) and approximate inclusion volume (Vi). Protein samples applied to the columns were eluted under identical conditions.

2.11.3 Affinity chromatography

Affinity chromatography was performed using Trisacryl Blue M (LKB) which was supplied in the pre-swollen form. The Trisacryl Blue M was equilibrated with 20mM phosphate buffer pH 7.0. The slurry was degassed on ice and packed under constant pressure. When packed the column was equilibrated and stabilised by passage through the column of ten column volumes of 20mM phosphate buffer pH 7.0.

2.12 Electrophoresis

All stock solutions used in electrophoretic techniques are listed in section 9.3.

2.12.1 Polyacrylamide gel electrophoresis to determine enzyme purity.

As a routine check on enzyme purity polyacrylamide gel electrophoresis (PAGE) of protein fractions was performed on 7.5% (w/v) polyacrylamide gels with an average pore radius of 2.1nm. The method used was a modification of that originally described by Ornstein and Davies (1964).

Gel polymerisation was obtained by mixing 6ml gel buffer, 4.5ml acrylamide solution, 1ml N,N^1-methylene bis acrylamide solution and 6.5ml distilled water in a sealed Buchner flask which was evacuated

for 30 sec, followed by the addition of 2ml ammonium persulphate solution. This solution was then pipetted into six glass tubes (0.5 x 12 cm) which had been temporarily sealed at their bases. Each tube was filled to within 1cm of the rim and then overlayed with water saturated 2-butanol to exclude oxygen which prohibits polymerisation. The gels were left to stand for 1 hour after which the overlaying butanol was removed and the gels rinsed with distilled water. To the protein solution to be analysed (10-200µg) sucrose was added to form a 10% (w/v) solution which was then layered onto the gel surface. This was in turn over layered with 5µl bromophenol blue tracking dye and 5% (w/v) sucrose in tank buffer to the rim of the tube.

The tubes were placed in the electrophoresis apparatus (Shandon Southern Products Ltd., Runcorn, Cheshire) with tank buffer in the upper and lower compartments. Electrophoresis was performed at 0.5mA per tube until the sample had entered the gel (10-15 mins) and then the current was increased to 3mA per tube until the bromophenol blue tracking dye had moved to within 0.5cm of the bottom of the gel (usually about 60-90 mins).

Having removed the gels from the glass tubes they were stained in Coomassie blue staining solution for at least 2 hours followed by a number of changes of destain to remove any residual stain.

2.12.2 Polyacrylamide gel electrophoresis for in situ staining of cyclohexanol dehydrogenase activity

Polyacrylamide gel electrophoresis of cell extracts of Xanthobacter sp. and Xanthobacter 431 was performed to determine the isoenzyme pattern of cylcohexanol dehydrogenase in these organisms by in situ staining of this enzyme. The method used was similar to that of Grell et al. (1965) but with a modified staining solution. 8.5% (w/v) polyacrylamide gels were polymerised by mixing 6ml gel buffer, 6ml acrylamide solution and 6.75ml distilled water in a sealed Buchner flask, evacuating for 30 sec and then adding 3ml ammonium persulphate solution. The solution was pipetted into six glass tubes (0.5 x 12cm), which had been temporarily sealed at their

bases. Each tube was filled to within 1cm of the rim and then overlayed with water saturated 2-butanol to allow polymerisation to occur. The gels were left to stand for 1h and then the butanol was removed and the tops of the gels rinsed with distilled water. Sucrose was added to the protein sample (50-200µg) to be analysed to form a 10% (w/v) solution which was layered on top of the gels. This was sequentially overlayed by 5µl bromophenol blue tracking dye and 5% (w/v) sucrose in tank buffer to the rim of the tube.

Electrophoresis was carried out at 0.5mA per tube until the protein had entered the gel (10-15 mins) and then the current was increased to 3mA per tube until the bromophenol blue tracking dye had moved to within 0.5cm from the bottom of the gel (usually about 60-90 mins).

Having removed the gels from the glass tubes they were placed in cyclohexanol dehydrogenase staining solution for 3-4h and kept in the dark during this time period since the staining solution is light sensitive. After the bands had developed the gels were rinsed and kept in distilled water.

2.12.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis with discontinuous buffers.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with discontinuous buffers was used to determine the molecular weight of cyclohexanol dehydrogenase. The procedure utilised is a modification of that described by Laemmli (1979).

A 10% (w/v) polyacrylamide resolving gel was polymerised by mixing 19.8ml acrylamide solution, 30ml Tris-SDS buffer pH 8.8 and 10.2ml distilled water in a sealed Buchner flask. The solution was degassed under vacuum and the 15ul TEMED and 1.5ml ammonium persulphate solution then added. The gel solution was pipetted into a vertical slab electrophoresis mould (LKB) and overlayed with water saturated 2-butanol. The gel was left to polymerise for 1h, after which the overlaying solution was removed and any excess liquid absorbed with a piece of filter paper. A 3% (w/v) polyacrylamide stacking gel was prepared by mixing 2ml acrylamide solution, 10ml

Tris-SDS buffer (pH 6.8) and 8ml distilled water in a sealed Buchner flask, degassing under vacuum and then adding 5µl TEMED and 0.5ml ammonium persulphate solution. A comb was inserted into the electrophoresis mould, the stacking gel solution added and left for 1h to polyermise. The comb was then removed and the wells rinsed with tank buffer.

Protein samples (10-150 µg) for analysis were contained in 80mM Tris -HCl buffer (pH 6.8), 2% (w/v) sodium dodecyl sulphate, 100mM dithiothreitol, 10% (v/v) glycerol and 0.2% (w/v) bromophenol blue. Prior to their application, samples were boiled for three min. Protein samples were applied to the wells of the gel and overlayed with 5% (w/v) sucrose in tank buffer. Electrophoresis was performed at a constant current of 30mA, until the bromophenol blue was within 0.5cm of the gel bottom.

Following electrophoresis gels were submerged in fixative solution for 1h and then in staining solution for a minimum of 2h. The gels were then destained by frequent changes of destain solution until the residual stain had been removed.

Protein molecular weight standards (Sigma Chemical Co. Ltd., Poole, Dorset) were run simultaneously with protein samples. A calibration graph of relative mobility against $\log_{10}$ molecular weight was plotted and the molecular weights of protein samples were determined.

2.13 Fractionation and Concentration of Protein Solutions

For concentration and protein purification of cell extracts (100,000 g supernatant) from cyclohexane-grown Xanthobacter sp. it was necessary to precipitate proteins by the addition of either saturated ammonium sulphate solution (section 9.5.4) or solid ammonium sulphate.

2.13.1 Saturated ammonium sulphate solution

To determine the volume of saturated ammonium sulphate solution to be added to the protein solution to give a particular salt concentration the following equation was employed

$$V = \frac{100\ (S_2 - S_1)}{1 - S_2}$$

V = volume (ml) of saturated ammonium sulphate solution to be added.

S_1 = initial salt concentration of protein sulution.

S_2 = final salt concentration of protein solution.

S_1 and S_2 are expressed as fractions of the salt solution (eg. S_1 = 0.3 [30% saturation] and S_2 = 0.5 [50% saturation]).

Protein precipitation was performed at 4°C by the dropwise addition of saturated ammonium sulphate solution to a slowly stirring protein solution. After the appropriate volume of saturated ammonium sulphate solution had been added, the protein solution was left to stir for a further 20-30 min, after which it was centrifuged at 20,000 g (20 min, 4°). The supernatant was decanted for further salt precipitation as necessary, whilst the resulting pellet was resuspended in the desired buffer and dialysed against the same buffer to remove the residual ammonium sulphate.

2.13.2 Solid ammonium sulphate

Semi-purified and purified proteins in solution were concentrated by the addition of solid ammonium sulphate. Precipitation was performed at 4 °C.

Solid ammonium sulphate was added gradually to the protein solution, which was stirrred continuously, to give a 70% saturated solution. The protein solution was then left to stir for a further 20-30 mins, after which the solution was centrifuged at 20,000 g (20 mins, 4°C). The protein pellet was resuspended in a suitable buffer and then dialysed against the same buffer to remove the residual ammonium sulphate.

2.13.3 Ultrafiltration

Ultrafiltration was employed to concentrate dilute protein solutions. The protein solution to be concentrated was added to a 50ml ultrafiltration cell chamber (Model 52, Amicon Ltd, Woking, Surrey) containing the appropriate membrane (Diaflo Ultrafilters, Amicon Ltd) with the desired molecular weight cut-off. Pressures of between 20–40 psi N_2 were applied to the ultrafiltration cell until a suitable reduction in volume had occurred.

After use, ultrafiltration membranes were stored in 10% (v/v) aqueous ethanol at 4°C.

Concentration of dilute protein solutions of small volumes was achieved using Centrisart 1 tubes (Sartorius) containing a membrane with a molecular weight cut off of 20,000. Samples, up to 2.5ml, were placed in the tubes which were centrifuged at 1,380 g until a desired volume reduction was achieved.

2.14 Dialysis

Protein solutions were dialysed to remove residual ammonium sulphate after fractionation or concentration procedures.

Dialysis visking tubing (Scientific Instrument Centre, London) was prepared for use by boiling in an aqueous solution of 0.1M EDTA for 10–15 min. The tubing was rinsed with distilled water. Dialysis tubing that was not required for use immediately was stored in distilled water at 4°C.

3. CYCLOALKANE METABOLISM BY FIVE SPECIES OF XANTHOBACTER

3.1 INTRODUCTION

As described in section 1.3 only two species of Xanthobacter have been characterised in Bergey's Manual of Systematic Bacteriology (Wiegel and Schlegel, 1984) namely X. autotrophicus and X. flavus. However several other strains of Xanthobacter with properties different to those described for the two known species of this genus have been isolated and are under investigation. Recently a third species of Xanthobacter has been described by Jenni and Aragno (1987) which has been named X. agilis. Furthermore DNA-DNA hybridization studies performed by Jenni et al. (1981) have indicated that there may be several different species clustered within this genus.

Another species of Xanthobacter has recently been isolated from soil and characterised by Trower et al. (1985). This new species of Xanthobacter is capable of growth on cyclohexane as the sole source of carbon and energy. The isolation of such an organism is both novel and unusual as there have been few reports of organisms capable of growth on this recalcitrant hydrocarbon (Stirling et al. 1977; Anderson et al. 1980). Enzymatic studies with cell extracts of this Xanthobacter sp. showed that the major route of metabolism for cyclohexane involves the initial hydroxylation of this compound to cyclohexanol. Cyclohexanol is further oxidised via cyclohexanone to 1-oxa-2-oxocycloheptane (ε-caprolactone) which is hydrolysed to 6-hydroxyhexanoate and finally converted to adipic acid. This route of cyclohexane degradation is analogous to that described for the two other cyclohexane-growing organisms, namely a Nocardia species (Stirling et al. 1977) and a pseudomonad (Anderson et al. 1980).

Over the years the use of growth substrate specificity in newly isolated organisms has been a useful diagnostic test for speciation and identification. In the following study a number of different Xanthobacter have been compared with regard to their ability to metabolise cyclohexane and related hydrocarbons, since such an unusual genetic trait may be a feature common to this newly characterised genus. In addition, any differences seen in the ability of these organisms to metabolise cycloalkanes may be a

useful feature in distinguishing the different subgroups that exist within this genus.

The Xanthobacter chosen for comparison in this study included two strains of X. autotrophicus namely Xanthobacter 431 and Xanthobacter 853, X. flavus, the new species of Xanthobacter isolated by Trower et al. (1985), which will be referred to as Xanthobacter sp. and the Xanthobacter described by Weaver and Lidstrom (1985) which will be referred to as Xanthobacter H4-14.

3.2 RESULTS

3.2.1 Distinguishing microbiological characteristics of the different species of Xanthobacter.

A comparison of a number of distinguishing microbiological characteristics of the different species of Xanthobacter is listed in Table 3.1. The characteristics of the cyclohexane-grown Xanthobacter sp. compared with those of X. autotrophicus and X. flavus indicated this new species of Xanthobacter to be more closely related to X. autotrophicus than to X. flavus. However, as a strain of X. autotrophicus this new species of Xanthobacter appears to be relatively atypical as it possesses the ability to hydrolyse gelatin and casein, it possesses a lecithinase and a urease but lacks phoshatase activity, and is also able to utilise malonate and maltose as carbon sources.

3.2.2 Growth of Xanthobacter on cycloalkanes and other hydrocarbons

A comparison of the five species of Xanthobacter was undertaken to determine their ability to utilise cycloalkanes and their derivatives, aromatic hydrocarbons and straight chain alkanes as sole sources of carbon and energy (Table 3.2). The results showed that Xanthobacter sp., Xanthobacter 853 and Xanthobacter 431 were all capable of growth on a number of substituted cycloalkanes but only Xanthobacter sp. and Xanthobacter 853 were able to utilise the

Table 3.1 Differential characteristics of the species of the genus *Xanthobacter*

Characteristic	[a] *X. autotrophicus*	[b] *X flavus*	[c] *Xanthobacter* sp.
Growth factor requirement	-	+	-
Utilisation of			
Fructose	+	(+)	+
Propionate	+	-	+
Malonate	-	+	+
Maltose	-	+	+
Ribose	-	+	-
Phenylalanine	-	+	-
Histidine	-	+	-
Acetate	+	(+)	+
Glucose	-	+	-
Hydrolytic activity on gelatine and casein	-	-	+
Lecithinase activity	-	-	+
Urease activity	+	-	+
Phosphatase activity	+	+	-
Autotrophic growth	+	-	+

[a] Data from Bergey's Manual of Systematic Bacteriology.
[b] Data from Malik and Claus (+) = Poor growth
[c] Data from Trower (1985).

recalcitrant hydrocarbons cyclohexane and methylcylcohexane. X. flavus and Xanthobacter H4-14 were unable to grow on any of the cycloalkane derivatives provided as growth compounds. None of the Xanthobacter were able to utilise 2-hydroxycyclohexanone, the aromatic hydrocarbons benzene and toluene, or any of the straight chain alkanes (C_8-C_{16}).

The growth studies indicated that Xanthobacter sp., Xanthobacter 853 and Xanthobacter 431 showed similarities in their ability to utilise cycloalkanes and their derivatives. It was therefore decided that these three organisms should be further investigated with regard to their metabolism of cycloalkanes.

3.2.3 Whole cell oxidation of cycloalkanes and related compounds

Metabolite oxidation studies were performed using washed whole cell suspensions of cyclohexane-grown Xanthobacter sp. and X.autotrophicus 853 and cyclohexanol-grown X.autotrophicus 431 (Table 3.3).

The results showed that cyclohexane-grown Xanthobacter sp and X. autrophicus 853 were both capable of oxidising cyclohexane. The cyclohexanol-grown X.autotrophicus 431 gave a much lower rate of oxidation with cyclohexane, some 16 and 25 fold less than the rate achieved for X. autotrophicus 853 and Xanthobacter sp. respectively. All three organisms were capable of oxidising cyclohexanol, cyclohexanone, 1-oxa-2-oxocycloheptane, the methylcyclohexanols and the cyclohexandiols but all gave very low oxidation rates with the aromatic hydrocarbon toluene and no activity was detected with any of the straight chain alkanes tested.

3.2.4 The metabolism of cyclohexane by Xanthobacter sp. and Xanthobacter 853 and the metabolism of cyclohexanol by Xanthobacter 431

The next step in this investigation was to determine whether the major routes of metabolism of cyclohexane by Xanthobacter sp. and Xanthobacter 853 and of cyclohexanol by Xanthobacter 431 were

Table 3.2 Comparison of the growth of different species of *Xanthobacter* on cycloalkanes and other hydrocarbons

Growth substrate	*Xanthobacter* sp.	*Xanthobacter* 853	*Xanthobacter* 431	*Xanthobacter* H4-14	*X.flavus*
Cyclohexane	+	+	-	-	-
Cyclohexanol	(+)	+	+	-	-
Cyclohexanone	+	+	+	-	-
Methylcyclohexane	+	+	-	-	-
Cyclohexan-1,2-diol	+	(+)	+	-	-
Cyclohexan-1,3-diol	(+)	(+)	-	-	-
Cyclohexan-1,4-diol	(+)	(+)	-	-	-
Cyclohexan-1,2-dione	(+)	-	-	-	-
2-hydroxycyclohexanone	-	-	-	-	-
Benzene	-	-	-	-	-
Toluene	-	-	-	-	-
Octane	-	-	-	-	-
Decane	-	-	-	-	-
Undecane	-	-	-	-	-
Hexadecane	-	-	-	-	-

Key: + Growth
- No growth
(+) Poor growth

The ability of the different *Xanthobacter* to utilise the carbon sources provided was tested using Liquid MSM.

Table 3.3 Oxidation of Cycloalkanes and related compounds by washed cell suspensions of Xanthobacter sp., Xanthobacter 853 and Xanthobacter 431

	*Rate of oxygen uptake (μmol. $O_2 h^{-1}$ (mg dry wt organisms)$^{-1}$)		
	Xanthobacter sp. (cyclohexane-grown)	Xanthobacter 853 (cyclohexane-grown)	Xanthobacter 431 (cyclohexanol-grown)
Cyclohexane	7.5	4.7	0.3
Cyclohexanol	5.4	3.3	4.6
Cyclohexanone	5.0	8.4	3.4
1-oxa-2-oxocycloheptane	3.5	7.2	2.0
1-Methylcyclohexanol	3.5	4.2	3.6
2-Methylcyclohexanol	3.3	4.8	1.1
3-Methylcyclohexanol	5.3	7.1	2.6
4-Methylcyclohexanol	6.1	8.6	1.7
Cyclohexan-1,2-diol	8.6	9.6	2.7
Cyclohexan-1,3-diol	5.0	7.1	1.3
Cyclohexan-1,4-diol	6.0	11.5	1.1
Toluene	0.7	0.8	0.4
Heptane	ND	ND	ND
Decane	ND	ND	ND
Dodecane	ND	ND	ND
Succinate	ND	ND	ND
Adipate	ND	ND	ND
Endogenous	0.3	0.4	0.2

*Values were calculated by subtracting the endogenous rate from the rate recorded with substrate present.

ND = < 0.05 μmol O_2 h^{-1}. (mg dry wt organisms)$^{-1}$

Respiration rates were followed polarographically in the oxygen monitor at 30C. Incubation mixtures contained in a volume of 3ml; 60 μmol phosphate buffer (pH7.0), 100 μl cell suspension (approx. 2.5 mg. dry weight) and 20 μmol substrate. Water-immiscible substrates (2μl) were added directly to the incubation chamber.

comparable. The route of cyclohexane metabolism by Xanthobacter sp. has already been elucidated by Trower et al. (1985) and the enzymes involved in this pathway are summarised in Table 3.4. It was therefore necessary to undertake cell-free extract studies to characterise the enzymes employed by Xanthobacter 853 and Xanthobacter 431 in the metabolism of cyclohexane and cyclohexanol respectively.

3.2.5 Enzyme activities in cell-free extracts of cyclohexane -grown Xanthobacter 853.

3.2.5.1 Cyclohexane hydroxylase

Two different breakage methods were used to attempt to detect cyclohexane hydroxylase activity in cell-free extracts from cyclohexane-grown Xanthobacter 853. The cells were broken by either ultra-sonication or by use of the French pressure cell as described in section 2.4.2. However, incubation of cell-free extracts (10,000 g and 100,000 g supernatant) with either NADH or NADPH in the presence of cyclohexane failed to cause any cyclohexane stimulated O_2 consumption when measured in the oxygen monitor. The addition of NADH or NADPH (0.1 mM final concentration) to the cell breakage medium in order to stabilise the enzyme again failed to result in any cyclohexane hydroxylase activity being detected. This result does not correlate with those from the whole cell studies which indicated cyclohexane hydroxylase activity was present in these cells.

3.2.5.2 Cyclohexanol dehydrogenase

To determine the presence of cyclohexanol dehydrogenase activity, cyclohexane-grown Xanthobacter 853 cell free-extracts (100,000 g supernatant) were incubated in 50mM phosphate buffer, pH 7.0 with NAD^+ in the presence of cyclohexanol. The substrate stimulated reduction of NAD^+ which resulted was taken as an

indication of enzyme activity. The specific activity of cyclohexanol dehydrogenase at pH 7.0 was calculated as 0.07 μmol NAD^+ reduced. min^{-1}. $(mg\ protein)^{-1}$.

3.2.5.2.1 pH optimum of cyclohexanol dehydrogenase from Xanthobacter 853

The pH optimum of cyclohexanol dehydrogenase was estimated as 10.3 by measurement of enzyme activity over the pH range 8.5-11.5 (Fig 3.1). The specific activity of cyclohexanol dehydrogenase at pH 10.3 was calculated as 0.81 μmol NAD^+ reduced. min^{-1}. $(mg\ protein)^{-1}$.

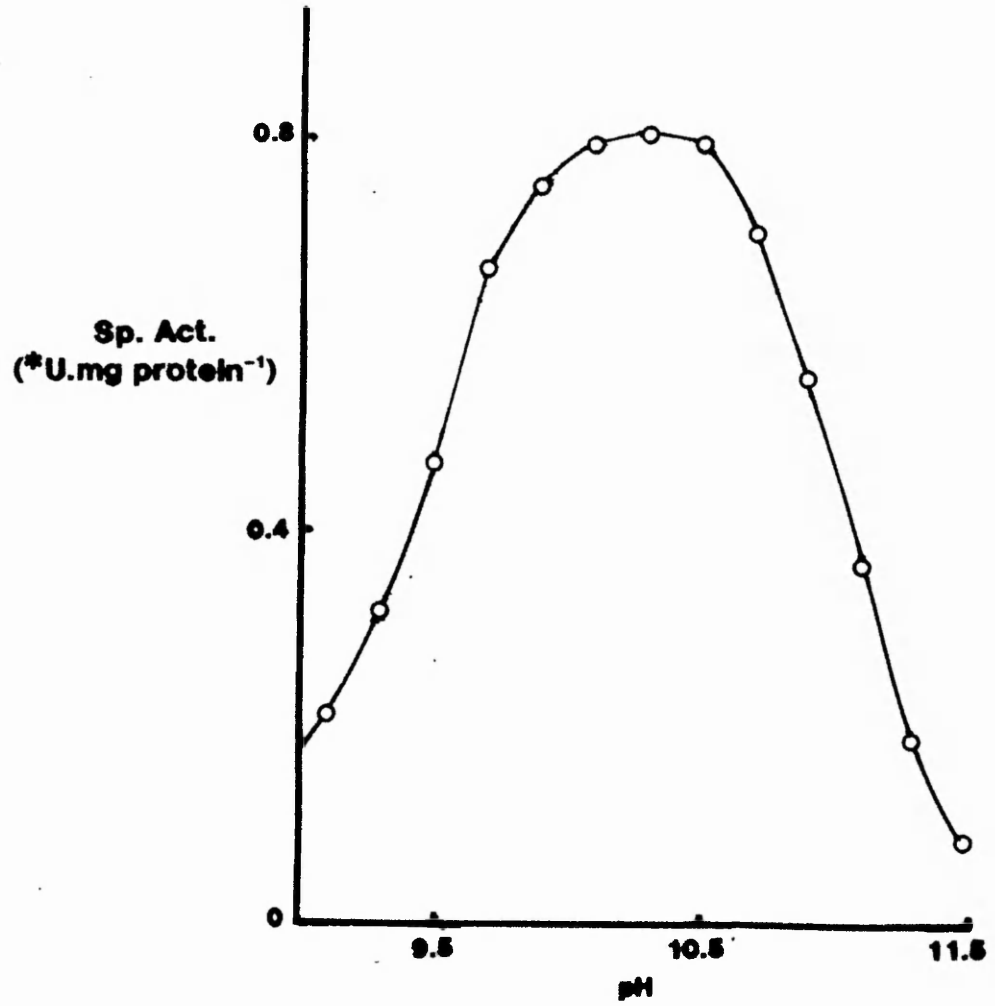
3.2.5.2.2 Cofactor specificity of cyclohexanol dehydrogenase from Xanthobacter 853

No cyclohexanol dehydrogenase activity was detected when $NADP^+$ was used as the cofactor in place of NAD^+.

3.2.5.3 Cyclohexanone monooxygenase

Cell-free extracts (100,000 g supernatant) of Xanthobacter 853 were incubated in 50mM phosphate buffer, pH 7.0 with NADPH in the presence of cyclohexanone to determine the presence of cyclohexanone monooxygenase activity. The substrate-stimulated consumption of O_2 measured in the oxygen monitor and a cyclohexanone-stimulated oxidation of NADPH monitored spectrophotometrically were taken as indications of cyclohexanone monooxygenase activity. The specific activity of the enzyme at pH 7.0 was calculated to be 0.01 μmol NADPH oxidised. min^{-1}. $(mg\ protein)^{-1}$.

Fig 3.1 pH Optimum of cyclohexanol dehydrogenase from cyclohexane-grown Xanthobacter 853.



Cyclohexanol dehydrogenase activity was measured spectrophotometrically by following the substrate-stimulated reduction of NAD^+ at 340nm. Reaction mixtures contained in a volume of 1ml; Universal buffer of the required pH (9.0-11.5), 100,000g supernatant (0.2-0.5 mg protein), 0.5 µmol NAD^+ and 0.5µmol cyclohexanol. Reactions (at 30°C) were initiated by the addition of cyclohexanol.

*One unit of cyclohexanol dehydrogenase activity is equivalent to 1 µmol NAD^+ reduced. min^{-1} at 30°C.

3.2.5.3.1 pH optimum of cyclohexanone monoxygenase from Xanthobacter 853

The pH optimum of cyclohexanone monoxygenase was found to be 8.8 by measurement of enzyme activity in the oxygen monitor over the pH range 7.5 - 10.0 (Fig. 3.2). The specific activity of the enzyme at pH 8.8 was calculated to be 0.11 µmol NADPH oxidised. $min.^{-1}$ $(mg\ protein)^{-1}$.

3.2.5.3.2. Cofactor specificity of cyclohexanone monooxygenase Xanthobacter 853

The replacement of NADPH with NADH failed to produce a cyclohexanone-stimulated consumption of O_2 when measured in the oxygen monitor indicating that cyclohexanone monooxygenase was unable to utilise NADH as a cofactor.

3.2.5.4 1-Oxa-2-oxocycloheptane hydrolase

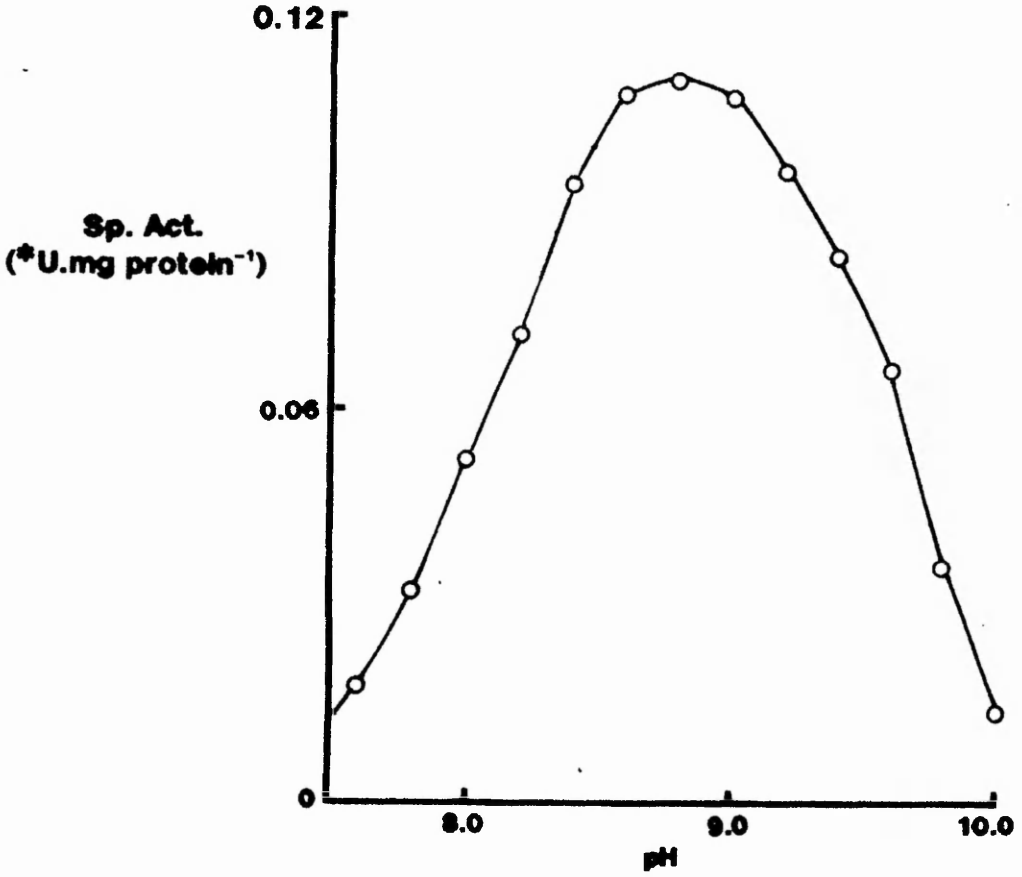
The presence of an active 1-oxa-2-oxocycloheptane hydrolase in cell extracts (100,000 g supernatant) of Xanthobacter 853 was determined by titration of 10mM NaOH against the acidic product of the reaction as described in section 2.6.4. Enzyme activity was observed at pH 8.0 and the specific activity of the cell-free extract under these conditions was found to be 23.8 µmol 1-oxa-2-oxocycloheptane hydrolysed. min^{-1}. (mg protein).$^{-1}$

3.2.6 Enzyme activities in cell-free extracts of cyclohexanol - grown Xanthobacter 431

3.2.6.1 Cyclohexane hydroxylase

To determine the presence of a cyclohexane hydroxylase, cyclohexanol-grown Xanthobacter 431 cell-free extracts (10,000 g and 100,000 g supernatants) were incubated with NADH or NADPH in the presence of cyclohexane. However, no cyclohexane-dependent

Fig 3.2 pH Optimum of Cyclohexanone monooxygenase from cyclohexane-grown Xanthobacter 853



Cyclohexanone monooxygenase was measured polarographically in the oxygen monitor. Reaction mixtures contained in a volume of 3ml; Universal buffer of the required pH (7.5 - 10.0), 100,000 g supernatant (0.5 - 1.0mg protein), 1.0 µmol NADPH and 2.0µl cyclohexanone. Reactions (at 30°C) were initiated by the addition of cyclohexanone.

*One unit of cyclohexanone monooxygenase activity is equivalent to 1 µmol NADPH oxidised. min^{-1} at 30°C.

stimulated O_2 consumption was detected with either cofactor. The inclusion of NADH or NADPH (0.1mM final concentration) into the cell breakage medium as a potential stabilising agent still failed to result in any enzyme activity being detected regardless of whether the cells were broken by ultra-sonication or by the French pressure cell. This result correlates with the whole cell studies in that cyclohexane hydroxylase is unlikely to be present in Xanthobacter 431.

3.2.6.2 Cyclohexanol dehydrogenase

Incubation of Xanthobacter 431 cell-free extract (100,000 g supernatant) with NAD^+ and cyclohexanol in 50mM phosphate buffer pH 7.0 resulted in a substrate-stimulated reduction of the NAD^+. The specific activity of the enzyme under these conditions was calculated as 0.08 μmol NAD^+ reduced. min^{-1}. (mg protein).$^{-1}$.

3.2.6.2.1 pH optimum of cyclohexanol dehydrogenase from Xanthobacter 431

The pH optimum of cyclohexanol dehydrogenase was estimated as 9.2 by measurement of enzyme activity over the pH range 8.0–10.6 (Fig 3.3). The specific activity of the enzyme at pH 9.2 was calculated to be 0.73 μmol NAD^+ reduced. min^{-1}. $(mg\ protein)^{-1}$.

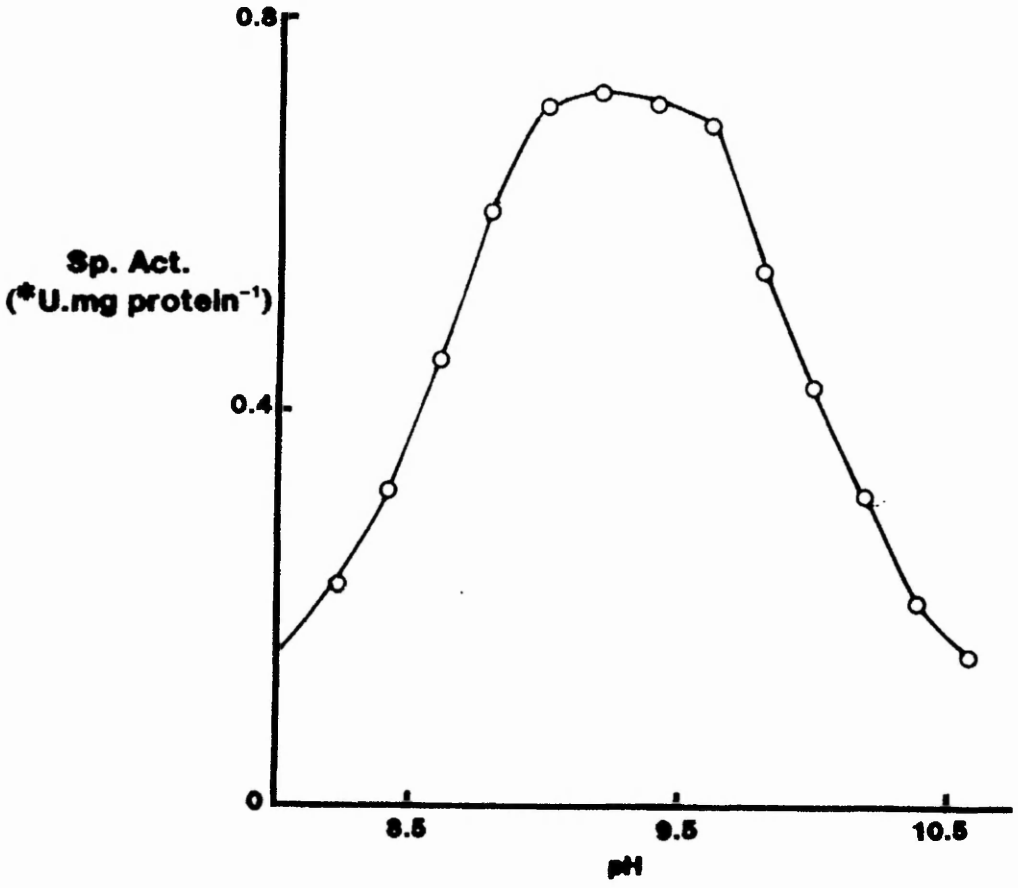
3.2.6.2.2 Cofactor specificity of cyclohexanol dehydrogenase from Xanthobacter 431

No cyclohexanol dehydrogenase activity was detected when NAD^+ was replaced by $NADP^+$ as the cofactor.

3.2.6.3 Cyclohexanone monooxygenase

Attempts to detect an active cyclohexanone monooxygenase in cell-free extracts (10,000 g and 100,000 g supernatants) of Xanthobacter 431 using both NADH and NADPH have proved unsuccessful.

Fig 3.3 pH optimum of cyclohexanol dehydrogenase from cyclohexanol-grown Xanthobacter 431.



Cyclohexanol dehydrogenase activity was measured spectrophotometrically by following the substrate-stimulated reduction of NAD^+ at 340nm. Reaction mixtures contained in a volume of 1ml; Universal buffer of the required pH (8.0-10.6), 100,000 g supernatant (0.2-0.5mg protein), 0.5 µmol NAD^+ and 0.5 µmol cyclohexanol. Reactions (at 30°C) were initiated by the addition of cyclohexanol.

* One unit of cyclohexanol dehydrogenase activity is equivalent to 1 µmol NAD^+ reduced. min^{-1} at 30°C.

When cells were disrupted by either ultra-sonication or by passage through a French pressure cell, cell-free extracts failed to produce a cyclohexanone stimulated consumption of O_2 when measured in the oxygen monitor. This situation remained unaltered when NADH or NADPH were added to the cell breakage medium as potential stabilising agents.

3.2.6.4 1-Oxa-2-oxocycloheptane hydrolase

1-Oxa-2-oxocycloheptane hydrolase activity in cell-free extracts (100,000 g supernatant) of Xanthobacter 431 was measured as described in section 2.6.4. by maintaining a buffered incubation system at a constant pH by titration with 10mM NaOH against the acidic reaction product. The enzyme was found to be active at pH 8.0 and the specific activity of the cell-free extract under these conditions was determined as 21.6 μmol 1-oxa-2-oxocycloheptane hydrolysed. min^{-1}. $(mg\ protein)^{-1}$.

A summary of the different enzyme activities measured in cyclohexane-grown Xanthobacter 853 and cyclohexanol-grown Xanthobacter 431 is shown in Table 3.4.

3.2.7 Inducibility of enzymes from cyclohexane - grown Xanthobacter 853 and cyclohexanol - grown Xanthobacter 431

Tables 3.5 and 3.6 show that following growth of Xanthobacter 853 and Xanthobacter 431 respectively on succinate, the enzyme activities obtained were at least 20 fold lower than when the organisms were grown on their respective hydrocarbons. These results indicate the inducible nature of these enzymes.

3.2.8 Characterisation of cyclohexanol dehydrogenase from Xanthobacter sp. and Xanthobacter 431

The results from the previous section indicated the cyclohexanol dehydrogenase enzymes from Xanthobacter sp. and Xanthobacter 431 to be dissimilar with regard to their pH optima (Table 3.4). It was

Table 3.4 Comparison of the enzymes involved in cyclohexane degradation by *Xanthobacter* sp. and *Xanthobacter* 853 and cyclohexanol degradation by *Xanthobacter* 431.

ENZYME	XANTHOBACTER sp.[a] (cyclohexane-grown)			XANTHOBACTER 853 (cyclohexane-grown)			XANTHOBACTER 431 (cyclohexanol-grown)		
	pH Optimum	Cofactor Specificity	Activity (U/mg protein)[b]	pH Optimum	Cofactor Specificity	Activity (U/mg protein)[b]	pH Optimum	Cofactor Specificity	Activity (U/mg protein)
CYCLOHEXANE HYDROXYLASE	6.5-7.0	NADPH NADH	7.6×10^{-3}	-	-	-	-	-	-
CYCLOHEXANOL DEHYDROGENASE	10.1-10.5	NAD^+ $NADP^+$	0.87 0.05	10.1-10.5	NAD^+ $NADP^+$	0.81 $<0.1 \times 10^{-3}$	9.0-9.4	NAD^+ $NADP^+$	0.73 $<0.1 \times 10^{-3}$
CYCLOHEXANONE MONOOXYGENASE	8.5-9.0	NADPH	0.15	8.5-9.0	NADPH	0.11	-	-	-
1-OXA-2-OXO-CYCLOHEPTANE HYDROLASE	Active at pH8.0		21.3	Active at pH8.0		23.8	Active at pH8.0		21.6

a. Data from Trower et. al. (1985)

b. One unit of enzyme activity is equivalent to 1 μmol substrate utilised. min^{-1}.

Table 3.5 Activity of enzymes involved in cyclohexane degradation after growth of Xanthobacter 853 on both cyclohexane and succinate

Enzyme	Specific activity [*U. (mg protein)$^{-1}$] in CFE from cells grown on :-	
	Cyclohexane	Succinate
Cyclohexane hydroxylase	$<0.9\times10^{-3}$	$<0.9\times10^{-3}$
Cyclohexanol dehydrogenase	0.81	$<0.1\times10^{-3}$
Cyclohexanone monooxygenase	0.11	0.005
1-oxa-2-oxocycloheptane hydrolase	23.8	0.09

Enzymes were assayed according to the procedures described in section 2.6.
*One unit of enzyme activity is equivalent to 1μmol substrate utilised. min^{-1}.

Table 3.6 Activity of enzymes involved in cyclohexane degradation after growth of Xanthobacter 431 on both cyclohexanol and succinate

Enzyme	Specific activity [*U. (mg protein)$^{-1}$] in CFE from cells grown on :-	
	Cyclohexanol	Succinate
Cyclohexane hydroxylase	$<0.9\times10^{-3}$	$<0.9\times10^{-3}$
Cyclohexanol dehydrogenase	0.73	$<0.1\times10^{-3}$
Cyclohexanone monooxygenase	$<0.9\times10^{-3}$	$<0.9\times10^{-3}$
1-oxa-2-oxocycloheptane hydrolase	21.6	0.07

Enzymes were assayed according to the procedures described in section 2.6.
*One unit of enzyme activity is equivalent to 1μmol substrate utilised. min^{-1}.

therefore decided to partially characterise these two enzymes to determine whether any further dissimilarities existed between them.

3.2.8.1 Substrate specificity of cyclohexanol dehydrogenase from Xanthobacter sp. and Xanthobacter 431

A comparison of the substrate specificities of the cyclohexanol dehydrogenase present in cell-free extracts (100,000 g supernatant) of the cyclohexane - grown Xanthobacter sp and the cyclohexanol - grown Xanthobacter 431 was undertaken (Table 3.7). Substrates tested included alicyclic and straight-chain alcohols and substituted cycloalkanols. In both cases cyclopentanol gave rise to the greatest enzyme activity. The cyclohexanol dehydrogenase from the Xanthobacter sp. was found to have a broad substrate specificity for secondary alcohols. The primary alcohol butan-1-ol was also oxidised by this enzyme. However, the cyclohexanol dehydrogenase from Xanthobacter 431 was found to possess a relatively narrow substrate range and no activity was detected towards the cyclohexandiols or butan-1-ol.

3.2.8.2 Characterisation of cyclohexanol dehydrogenase following separation of cell-free extracts by PAGE

Further differences in the two cyclohexanol dehydrogenase enzymes were shown in their relative mobilities following separation by polyacrylamide gel electrophoresis. The separation of cell extracts (100,000 g supernatant) from cyclohexane-grown Xanthobacter sp. and cyclohexanol-grown Xanthobacter 431 by polyacrylamide gel electrophoresis followed by in situ staining for cyclohexanol dehyrogenase (section 2.12.2) is shown in Fig.3.4. One major staining board was found in the cell extracts obtained from each organism. The different relative mobilities of these two bands with references to the bromophenol blue markers indicated the two enzymes to be dissimilar. A second minor staining band was also found in each of the cell extracts which proved interesting as the faint band displayed by Xanthobacter sp. showed a similar relative mobility to that of the major staining band found in Xanthobacter 431.

Table 3.7 Relative activity of cyclohexanol dehydrogenase from cyclohexane-grown Xanthobacter sp. and cyclohexanol-grown Xanthobacter 431 with different substrates

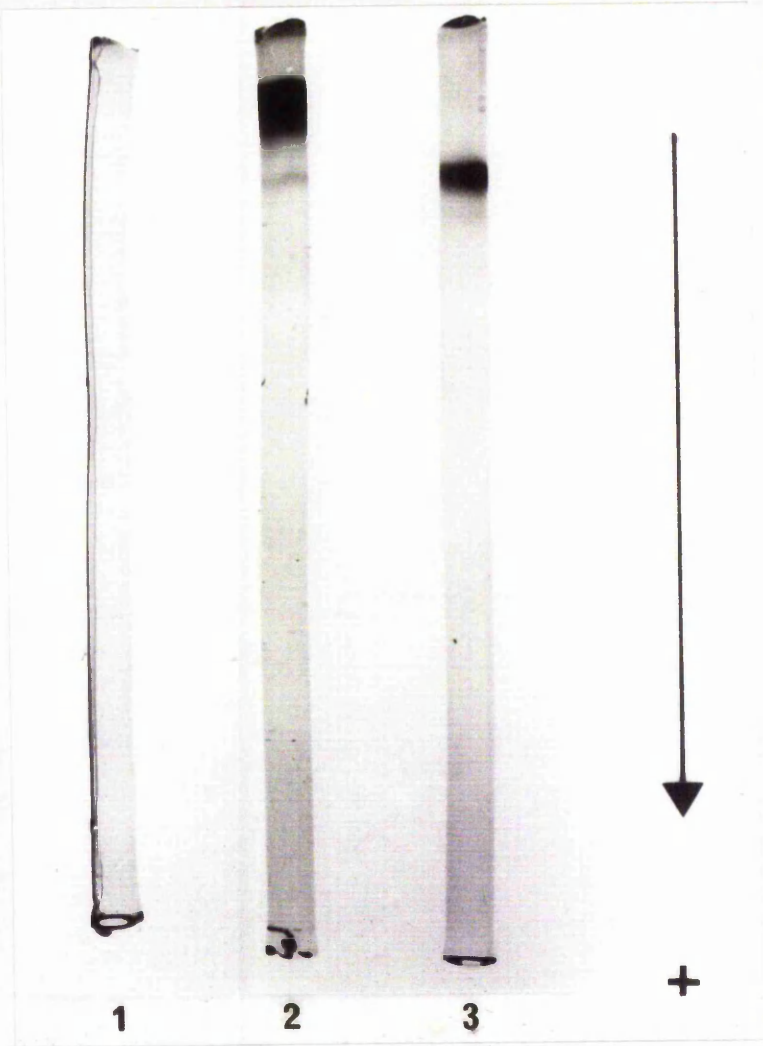
Substrate	Xanthobacter sp. (cyclohexane-grown)	Xanthobacter 431 (cyclohexanol-grown)
Cyclohexanol	[a]100	[b]100
Cyclopentanol	174	168
Butan-1-ol	22	0
Butan-2-ol	52	130
1-Methylcyclohexanol	0	0
2-Methylcyclohexanol	65	8
3-Methylcyclohexanol	99	41
4-Methylcyclohexanol	91	10
Cyclohexan-1,2-diol	24	0
Cyclohexan-1,3-diol	27	0
Cyclohexan-1,4-diol	30	0
2-Hydroxycyclohexanone	0	0

Cyclohexanol dehydrogenase activity was measured as described in section 2.6.2. Reactions were performed at the pH optimum for each enzyme. Values represent a percentage of the rate measured for each enzyme with cyclohexanol as the substrate.

[a]Xanthobacter sp. 100% activity with cyclohexanol was equivalent to 0.87 μmol. NAD reduced. min^{-1}. $(mg\ protein)^{-1}$.

[b]Xanthobacter 431 100% activity with cyclohexanol was equivalent to 0.73 μmol. NAD reduced. min^{-1}. $(mg\ protein)^{-1}$.

Fig 3.4 In situ staining of cyclohexanol dehydrogenase following separation of cell extracts of Xanthobacter sp. and Xanthobacter 431 by polyacrylamide gel electrophoresis as described in section 2.12.2



1. Control gel - stained for cyclohexanol dehydrogenase activity but without the addition of cyclohexanol.

2. 100,000 g supernatant (100µg protein) cyclohexane-grown Xanthobacter sp. stained for cyclohexanol dehydrogenase activity.

3. 100,000 g supernatant (100µg protein) cyclohexanol-grown Xanthobacter 431 stained for cyclohexanol dehydrogenase activity.

3.2.8.3 Estimation of molecular weight of cyclohexanol dehydrogenase from Xanthobacter sp. and Xanthobacter 431

To determine the approximate molecular weight of cyclohexanol dehydrogenase from cyclohexane-grown Xanthobacter sp. and cyclohexanol-grown Xanthobacter 431 gel filtration chromatography was employed. This necessitated the passage of cell-free extract (100,000 g supernatant) obtained from each organism through a previously calibrated Ultrogel AcA 44 chromatography column. The molecular weights of the proteins used to calibrate the column with their respective elution volumes are listed in table 3.8.

3.3 DISCUSSION

A variety of microbiological characteristics of the different species of Xanthobacter (Table 3.1) indicated the cyclohexane-grown Xanthobacter sp. to be more closely related to X. autotrophicus than X. flavus. This similarity between Xanthobacter sp. and X. autotrophicus is also indicated by the ability of these two species to utilise substituted cycloalkanes as growth substrates. Xanthobacter H4-14 appears to be similar to X. flavus as neither of these organisms were able to utilise any of the cycloalkanes tested.

None of the species of Xanthobacter possessed the ability to utilise the straight chain alkanes (C_8-C_{16}) as growth substrates. Growth of Xanthobacter-like bacteria with short chain alkanes (Lidstrom-O'Conner et al., 1983) and also with short chain alkenes (Ginkel et al., 1984) has been reported and this may be a further separate trait to distinguish other species of this genus which are, as yet, uncharacterised.

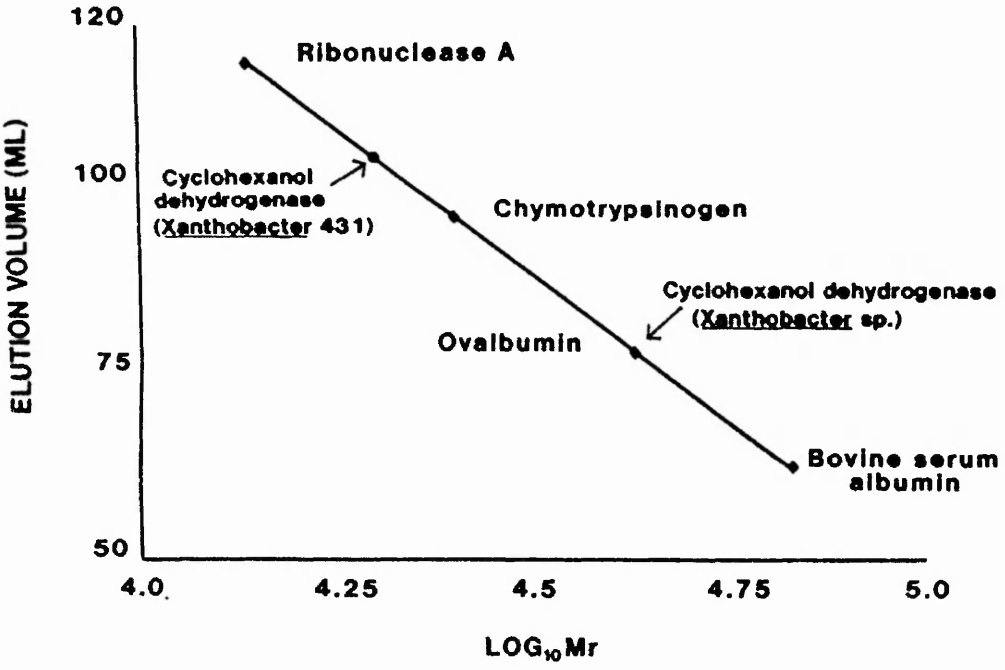
The inability of cycloalkane utilising organisms to metabolise straight chain alkanes was also noted by Stirling et al., (1977)

Table 3.8 Calibration of Ultrogel AcA 44 gel filtration column for molecular weight analysis of cyclohexanol dehydrogenase from Xanthobacter sp. and Xanthobacter 431.

Protein	Molecular weight	Elution volume (ml)
Bovine Serum Albumin	67,000	62.5
Ovalbumin	43,000	77
Chymotrypsinogen	25,000	95
Ribonuclease A	13,700	115

Calibration proteins ($5mg.ml^{-1}$) (Sigma Chemical Co. Ltd) were loaded (1ml) onto the Ultrogel AcA44 gel filtration column and eluted as described in section 2.11.2. Fractions of 3ml were collected, assayed for protein (section 2.8.3) and their respective elution volumes measured. The column constants, void volume (Vo) and approximate total inclusion volume (Vi) were determined as 44.00 and 126 ml respectively

Fig 3.5 Calibration plot for the determination of the molecular weight of cyclohexanol dehydrogenase from Xanthobacter sp. and Xanthobacter 431.



	Ve (ml)	Mr
Xanthobacter sp		
Cyclohexanol dehydrogenase	77	43,000
Xanthobacter 431		
Cyclohexanol dehydrogenase	103	20,000

100,000 g supernatant (10-12mg protein.ml^{-1}) prepared from cyclohexane-grown Xanthobacter sp. and cyclohexanol-grown Xanthobacter 431 (section 2.4.2) was loaded (2ml) onto the Ultrogel AcA 44 gel filtration column (Vo=44.0) and eluted as described in section 2.11.2. Fractions collected on ice in volumes of 3ml were assayed for protein (section 2.8.3) and cylohexanol dehydrogenase activity (section 2.6.2.). The molecular weight of each cyclohexanol dehydrogenase was determined by its elution volume.

working with a cyclohexane-degrading Nocardia sp. Conversely, there are reports of organisms capable of growth on n-alkanes which are unable to degrade cycloalkanes (Komagata et al., 1964; Pelz and Rehm, 1971; Beam and Perry, 1973). Trudgill (1978) proposed that growth on cyclic compounds may preclude growth on linear substrates and vice versa. The results obtained from the cycloalkane-degrading Xanthobacter would appear to support this proposal. None of the Xanthobacter investigated were able to utilise the aromatic compounds benzene and toluene. There is, however, a report (Anderson et al., 1980) of a cycloalkane-degrading Pseudomonas sp. which possessed the ability to utilise both short chain alkanes and aromatic compounds.

Trower et al., (1985) showed that Xanthobacter sp. degraded cyclohexane via a pathway which involved two monooxygenase reactions, the first resulted in the formation of cyclohexanol and the second catalysed the formation of 1-oxa-2-oxocycloheptane via a Baeyer-Villiger type reaction. It was therefore necessary to determine the route by which Xanthobacter 853 and Xanthobacter 431 degrade cyclohexane and cyclohexanol respectively.

Washed whole cells of Xanthobacter 853 oxidised cyclohexane, cyclohexanol, cyclohexanone and 1-oxa-2-oxocyloheptane. These same compounds were also oxidised by whole cells of Xanthobacter 431, with the exception of cyclohexane. The oxidation of 1-oxa-2-oxocycloheptane by both organisms suggests that ring cleavage is brought about by the formation of a lactone. These results suggest that the route of metabolism of cylohexane and cyclohexanol proceeds via the oxidation of cyclohexane ——➤ cyclohexanol ——➤ cyclohexanone ——➤ 1-oxa-2-oxocycloheptane ——➤ ——➤ adipic acid. This is known to be the route of oxidation of cyclohexane by Xanthobacter sp. (Trower et al., 1985) and for the majority of cyclohexanol degrading microorganisms studied by Donoghue et al., (1976). Both organisms were able to oxidise the cyclohexanediols suggesting in the case of Xanthobacter 853 that further hydroxylation of the ring may occur, although further hydroxylation of the ring is unlikely to occur in the cyclohexanol degrading Xanthobacter 431.

Unlike the cyclohexane-grown Xanthobacter sp., cyclohexane

hydroxylase activity could not be detected in cell-free extracts from the cyclohexane-grown Xanthobacter 853. However, the lack of, or low amounts of, enzyme activity with methylene hydroxylases is not an unusual occurrence as shown by Berg et al., (1976) and Anderson et al., (1980) and similarly with methyl hydroxylases (Cardini and Jurtshuk, 1968; Lebeault et al., 1971; Miura and Fulco, 1974; Marchal et al., 1982; Patel et al., 1983). Likewise cyclohexane hydroxlase activity was not detected in cell-free extracts of Xanthobacter 431. However, this was not unexpected as the organism does not grow on cyclohexane, nor would washed whole cells of this organism oxidise cyclohexane.

An inducible cyclohexanol dehydrogenase is present in cell-free extracts of all three strains of Xanthobacter. Trower et al., (1985) found that cyclohexanol dehydrogenase was located in the soluble fraction of cell-free extracts of Xanthobacter sp. Similarly, cyclohexanol dehydrogenase from Xanthobacter 853 and Xanthobacter 431 was found to be soluble and pyridine nucleotide dependent as previously reported in other cycloalkane-(Anderson et al., 1980; Stirling and Perry, 1980) and cycloalkanol-grown microorganisms (Griffin and Trudgill, 1972; Donoghue and Trudgill, 1975). The cyclohexanol dehydrogenase from Xanthobacter 853 appeared to be similar to that from Xanthobacter sp. with regard to its pH optimum (Table 3.4). However, the cyclohexanol dehydrogenase isolated from Xanthobacter 431 appeared to differ from the same enzyme in the other two Xanthobacter since it displayed a pH optimum 9.0-9.4. Further characterisation of the cyclohexanol dehydrogenase from Xanthobacter sp. and Xanthobacter 431 indicated both enzymes to be NAD^+ dependent although a small amount of activity was detectable with $NADP^+$ in the former. Cyclohexanol dehydrogenase from the cyclohexane-grown Xanthobacter sp. displayed a broad substrate range which included the methylcyclohexanols, the cyclohexandiols and also a limited amount of activity towards the primary alcohol butan-1-ol. As mentioned earlier this ability to utilise the cyclohexandiols probably indicates that other minor pathways for cyclohexane metabolism may be occurring where a double hydroxylation of the ring is brought about. This substrate specificity range is comparable to

the cyclohexanol dehydrogenase of the cyclohexanol-grown Nocardia sp. (Stirling and Perry, 1980). In contrast the cyclohexanol dehydrogenase from the cyclohexanol-grown Xanthobacter 431 showed a much more limited substrate range being unable to oxidise the cyclohexandiols and no activity was detected with the primary alochol butan-1-ol.

In situ staining for cyclohexanol dehydrogenase activity after polyacrylamide gel electrophoresis of cell-free extracts of the two organisms showed one major cyclohexanol dehydrogenase to be present in each organism. However a comparison of the relative mobilities of the two stained bands indicated the two enzymes to be dissimilar. Similarly, gel filtration chromatography indicated only one major peak of cyclohexanol dehydrogenase activity to be present in cell-free extracts from each organism. Using this technique the molecular weights from Xanthobacter sp. and Xanthobacter 431 were calculated as 43,000 and 20,000 respectively. These values can be compared to Mr 54,000 for cyclopentanol dehydrogenase isolated from a cyclopentanol-grown pseudomonad (Griffin, 1974) and Mr 34,000 for cyclohexanol dehydrogenase from the cylohexane-grown Pseudomonas sp. (Anderson et al., 1980). However, these values are quite different to that of Mr 145,000 reported for cyclohexanol dehydrogenase from the cyclohexane-grown Nocardia sp. (Stirling and Perry, 1980).

Further characterisation of the cyclohexanol dehydrogenase isolated from Xanthobacter sp. is described in section 5.

An inducible cyclohexanone monooxygenase was detected in cell-free extracts of Xanthobacter 853 and appeared similar to the same enzyme found by Trower et al., (1985) in Xanthobacter sp. Both enzymes had a pH optimum of 8.5-9.0 and a specific requirement for NADPH. The presence of a cyclohexanone monooxygenase was detected in cell-free extracts of the cyclohexane-grown Nocardia sp. (Stirling et al., 1977) and Pseudomonas sp. (Anderson et al., 1980). However, no cyclohexanone monooxygenase activity could be detected in cell-free extracts of the cyclohexanol-grown Xanthobacter 431 using either NADH or NADPH as the cofactor, although Norris and Trudgill (1972) reported cyclohexanone monooxygenase activity in cell-free extracts of cyclohexanol-grown Nocardia globerula CL1.

The finding that Xanthobacter 431 will grow on cyclohexanone and washed whole cells will oxidise this compound suggests that a cyclohexanone monooxygenase is present, but does not remain active after the cells have been disrupted. The cyclohexanone monoxygenase in Xanthobacter 431 may be a multicomponent enzyme system as is the case with the lactone-forming 2,5-diketocamphane monooxygenase isolated from Pseudomonas putida (Taylor and Trudgill, 1984). Such a multicomponnent system in Xanthobacter 431 may be inactivated during cell breakage.

All three strains of Xanthobacter possessed a soluble 1-oxa-2-oxoycloheptane hydrolase. Trower et al., (1985) demonstrated that the specific activity of 1-oxa-2-oxocycloheptane hydrolase from Xanthobacter sp. was very high compared with the activities of the other enzymes in the pathway and this was also true of the 1-oxa-2-oxo-ocycloheptane hydrolases isolated from Xanthobacter 853 and Xanthobacter 431. However, this is not an unusual feature since activities of a similar nature have been reported for 1-oxa-2-oxocycloheptane hydrolases from the cyclohexane-grown Nocardia sp. (Stirling et al., 1977) and Pseudomonas sp. (Anderson et al., 1980) as well as the cyclohexanol-grown Nocardia globerula CL1 (Norris and Trudgill, 1971). The demonstration of a 1-oxa-2-oxocycloheptane hydrolase indicates that the mechanism of ring cleavage in Xanthobacter 853 and Xanthobacter 431 is likely to be via oxidative lactone formation.

The studies show that of the different Xanthobacter species tested only Xanthobacter sp., Xanthobacter 853 and Xanthobacter 431 were able to utilise cycloalkanes. Studies with both whole cells and cell extracts indicated that the route of cyclohexane degradation by Xanthobacter 853 and cyclohexanol degradation by Xanthobacter 431 is likely to be via cyclohexane ⟶ cyclohexanol ⟶ cyclohexanone ⟶ 1-oxa-2-oxocycloheptane ⟶ adipic acid, which is the same pathway shown by Trower et al., (1985) for the degradation of cyclohexane by Xanthobacter sp. These results are consistent with those reported by Donoghue et al., (1976), Stirling et al., (1977) and Anderson et al., (1980) for the microbial degradation of cyclohexane. However, although the route of cyclohexane degradation

in Xanthobacter sp. and Xanthobacter 853 and that of cyclohexanol by Xanthobacter 431 is by the same pathway, the results indicate that certain enzymes employed by Xanthobacter 431 differ from those found in the Xanthobacter sp. and Xanthobacter 853.

4. CYCLOHEXANE HYDROXYLASE

4.1 INTRODUCTION

Trower et al., (1985) demonstrated the presence of an inducible cyclohexane hydroxylase in cell-free extracts of cyclohexane-grown Xanthobacter sp. Studies with both whole cells and cell-free extracts indicated that this enzyme catalysed the initial hydroxylation of cylohexane to cyclohexanol. However, the further characterisation of this enzyme proved difficult due to its unstable nature. Other cyclohexane hydroxylases have also been difficult to isolate. For example work with the cyclohexane-grown Nocardia sp. indicated that no cyclohexane hydroxylase was detectable in cell-free extracts (Stirling et al., 1977) while the cyclohexane hydroxylase activity demonstrated in cell extracts of a cyclohexane-grown Pseudomonas sp. lost all activity within 24 h of isolation (Anderson et al., 1980). This inherant instability of these enzymes may be explained by their complexity of structure. The alkyl hydroxylases which have been characterised to date are multicomponent systems with a terminal oxidase protein containing a haem type cytochrome P-450 or a non-haem iron component as the prosthetic group at the active centre.

The aim of these studies was to investigate further the cyclohexane hydroxylase enzyme from Xanthobacter sp. and to attempt to determine the nature of the terminal oxidase protein.

4.2 RESULTS

4.2.1 Choice of disintegration method and extraction medium used to isolate cyclohexane hydroxylase

Trower (1985) showed that cyclohexane hydroxylase activity could be detected in cell -free extracts of cyclohexane-grown Xanthobacter sp. after disruption of the cells by ultra-sonication. However, the enzyme activity obtained was low and the addition of various potential stabilisers to the breakage medium in an attempt to increase cyclohexane hydroxylase activity met with no success.

The aim of these studies was therefore to optimise the

conditions of cell disruption used to isolate cyclohexane hydroxylase and to test the use of potential stabilisers which could be added to the extraction medium to assist in obtaining greater cyclohexane hydroxylase activity.

Cells were first disrupted by sonication in 20mM phosphate buffer (pH 7.0) containing one of the following potential stabilisers, 0.1mM NADPH, 1mM NADPH or cyclohexane ($10\mu l.ml^{-1}$ cells) (Table 4.1). These different substrates of the enzyme had previously been shown by Trower (1985) to prolong the activity of the enzyme on storage at 4°C. It was found that the addition of NADPH at a final concentration of 0.1mM to the breakage medium resulted in an increased recovery of cyclohexane hydroxylase activity. The addition of NADPH at a higher concentration or cyclohexane did not bring about any further increase in cyclohexane hydroxylase activity. It was found that a further increase in cyclohexane hydroxylase activity could be obtained by partial freezing of the cells prior to and during sonication. Using this breakage procedure (addition of NADPH (0.1mM) and freezing) cyclohexane hydroxylase activity of 22.9 nmoles.min^{-1}. (mg protein $^{-1}$) was obtained compared with 7.6 nmoles. min^{-1}. (mg protein $^{-1}$) obtained by Trower (1985).

The incorporation of NADPH into the breakage medium and/or partial freezing of the cells prior to disruption did not however result in any cyclohexane hydroxylase activity being obtained in cell extracts when the cells were disrupted by the French pressure cell.

4.2.2 Determination of pH optimum of cyclohexane hydroxylase activity

An investigation into the relationship between pH and cyclohexane hydroxylase activity was undertaken using cell extracts (100,000 g supernatant) from Xanthobacter sp. (Fig. 4.1).

Table 4.1 Effect of potential stabilisers on cyclohexane hydroxylase activity when added to the breakage medium of cyclohexane-grown Xanthobacter sp.

Test	Concentration	Recovered cyclohexane hydroxylase activity following breakage (nmolO_2 consumed. min^{-1}. $protein^{-1}$)
Compound	-	7.4
NADPH	0.1mM	22.9
NADPH	1.0mM	19.8
Cyclohexane	10µl.ml^{-1} cells	10.2

Cyclohexane hydroxylase activity was measured polarographically in the oxygen monitor. Reaction mixtures contained, in a volume of 3ml; 240 µmol phosphate buffer, pH 6.8, 100,000 g supernatant (5mg protein) and 1.0 µmol NADPH. Reactions were performed at 30°C and were initiated by the addition of cyclohexane (2 µl).

Cyclohexane hydroxylase activity was measured over the pH range 6.0-7.5 and the pH optimum of the enzyme was shown to be between 6.7-6.9. The enzyme activity decreased by over 40% when the pH was adjusted 0.5 pH units either side of the pH optimum.

4.2.3 Substrate specificity of cyclohexane hydroxylase

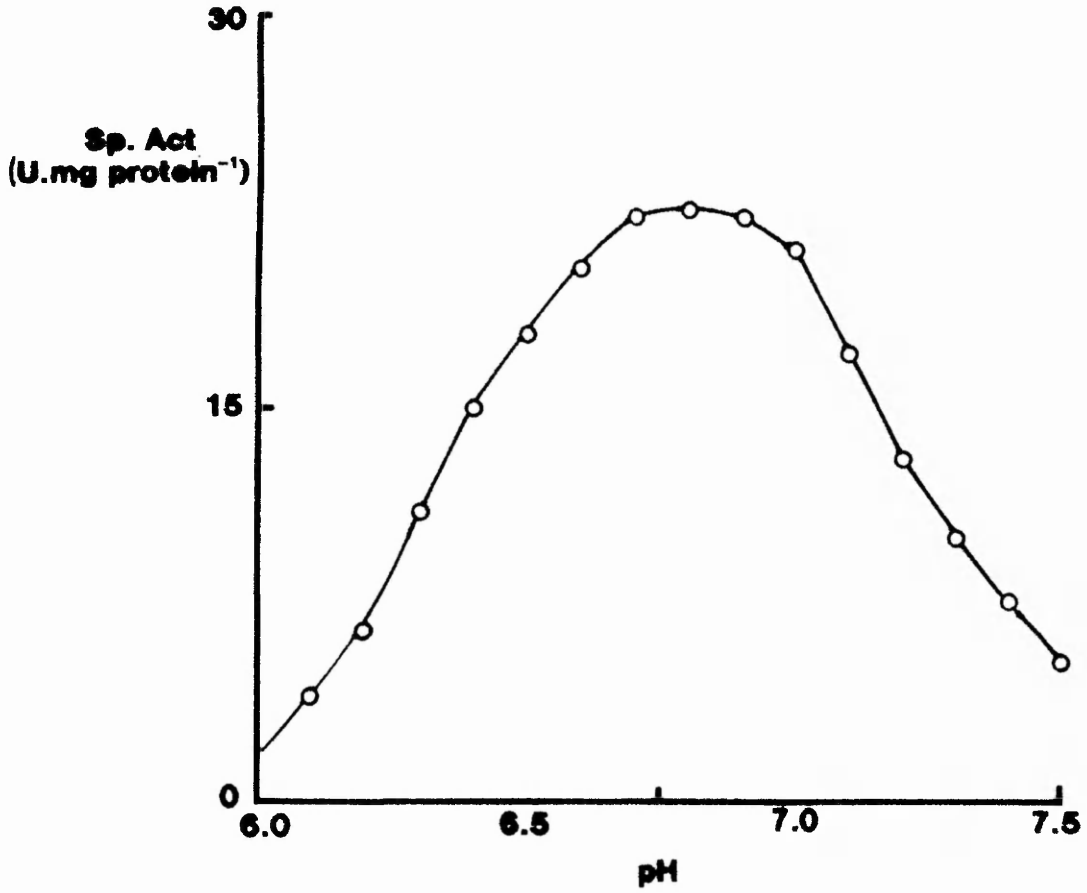
Having determined the pH optimum of cylohexane hydroxylase the enzyme was further investigated with regard to its substrate specificity. The results of these studies are shown in Table 4.2. A number of different potential substrates were tested including alkyl substituted cycloalkanes, bicyclic rings including terpenes, aromatic rings, heterocylics and straight chain alkanes. The results indicated that the enzyme has a broad specificity for ring structures with enzyme activity being especially apparent with cycloalkanes, cycloalkenes and short chain n-alkyl cycloalkanes. Substrate stimulated oxygen consumption was also noted with the aromatic hydrocarbons benzene and toluene and also with some other cyclic compounds including the heterocycle pyrrolidine and the bicyclic terpene pinane. No enzyme activity was detected with n-alkanes (C_8 - C_{16}).

4.2.4 Effect of inhibitors on endogenous NADPH oxidase and cyclohexane hydroxylase activities

A number of potential inhibitors were tested to determine their effect on the activity of NADPH oxidase and cyclohexane hydroxylase (Table 4.3).

The results indicated that cyclohexane hydroxylase showed no sensitivity to electron transport chain inhibitors, differential sensitivity to metal chelators and a high degree of sensitivity to sulphydryl group inhibitors. NADPH oxidase however showed little sensitivity towards any of the inhibitors tested. It was therefore difficult to assess from these studies whether the NADPH oxidase

Fig. 4.1 pH Optimum of cyclohexane hydroxylase from Xanthobacter sp.



Cyclohexane hydroxylase activity was measured polarographically in the oxygen monitor. Reaction mixtures contained, in a volume of 3ml; 240 µmol phosphate buffer at the required pH (6.0-7.5), 100,000 g supernatant (5mg protein) and 1.0µl mol NADPH. Reactions were performed at 30°C and were initiated by the addition of cyclohexane (2µl).

Table 4.2 Substrate specificity of cyclohexane hydroxylase in cell extracts of Xanthobacter sp.

Substrate	*Specific activity ($nmol O_2$ consumed. min^{-1}. mg $protein^{-1}$)
Cyclohexane	22.9
Cyclopentane	16.5
Cyclooctane	6.7
Cyclodecane	3.8
Methylcyclohexane	14.4
Ethylcyclohexane	3.4
Methylcyclopentane	11.9
Ethylcylopentane	9.4
Benzene	5.1
Toluene	3.9
Octane	ND
Decane	ND
Undecane	ND
Hexadecane	ND
Methylene cyclohexane	6.2
1-Methyl-1,4-Cyclohexadiene	15.7
Cyclohexene	46.5
Cyclohexene oxide	25.1
p-Menthane	ND
Pinane	10.9
Pyrrolidine	12.5
Dicyclohexyl	ND
Quadricyclane	7.8
NADPH Oxidase	2.5

Cyclohexane hydroxylase activity was assayed polarographically in the oxygen monitor at 30°C. Reaction mixtures contained, in a volume of 3ml; 240 µmol phosphate buffer (pH 6.8), 100,000 g supernatant (5mg protein) and 1.0 µmol NADPH. Reactions were initiated by the addition of the appropriate substrate (2µl).

* Calculated by subtracting the NADPH oxidase endogenous rate from that obtained in the presence of the test substrate. ND = values less than 0.9 $nmol.min^{-1}$. (mg $protein^{-1}$).

activity was coupled to the substrate stimulated cyclohexane hydroxylase activity.

4.2.5 Semi-purification of cyclohexane hydroxylase

Trower (1985) attempted semi-purification of cyclohexane hydroxylase by ammonium sulphate precipitation, ion-exchange chromatography and gel filtration chromatography but all of these procedures resulted in total loss of enzyme activity.

Since NADPH was found to stabilise cyclohexane hydroxylase activity it was decided to try and semi-purify the enzyme with the inclusion of NADPH (0.1mM) in the elution buffer (20mM phosphate buffer, pH 7.0) of a gel filtration column (Biogel 0.5-A; 51 x 1.6cm). Cell-free extract (100,000 g supernatant) (7.0ml) containing 84 mg protein and 1596 units of cyclohexane hydroxylase was loaded onto the Biogel column, eluted at a flow rate of 6ml per h and fractions of 3ml collected. All procedures were performed at 4°C. Cyclohexane hydroxylase activity was not detected in any of the resulting fractions. The eluant from the column was then pooled into three different fractions and each concentrated using an ultra filtration cell chamber (Amicon, Woking, Surrey) containing a membrane (PM10) with a molecular weight cut-off of 10,000. It was found that cyclohexane hydroxylase activity could be detected when aliquots of all three fractions were added to the reaction vessel. The fact that all three fractions were necessary to detect cyclohexane hydroxylase activity indicated that the enzyme is a multi-component system. The resulting cyclohexane hydroxylase activity was however much less (20%) than that of the original cell-free extract. The reproducibility of this experiment was very poor.

4.2.6 Demonstration of Cytochrome P-450 in cell-free extracts of cyclohexane grown Xanthobacter sp.

Since previous experiments have suggested a multicomponent nature for cyclohexane hydroxylase it was necessary to investigate

Table 4.3 The effect of various potential inhibitors on the activity of NADPH oxidase and cyclohexane hydroxylase in soluble extracts.

Inhibitor	Inhibitor concentration (mM)	*% inhibition of NADPH oxidase activity	*% inhibition of cyclohexane hydroxylase activity
Control	-	0	0
1,10-Phenanthroline	0.1	0	82
Bathophenanthroline	0.5	0	40
Bathocuproin	0.5	0	35
EDTA	1.0	0	52
α,α'-Dipyridyl	1.0	0	0
Sodium azide	1.0	0	0
Neocuproine	0.5	0	0
Potassium cyanide	1.0	0	0
Rotenone	1.0	0	6
Amytol	1.0	5	12
Sodium arsenate	1.0	12	0
p-Hydroxy-mercuribenzoate	0.1	17	98
5,5'-Dithiobis (2-nitrobenzoate)	0.1	0	99
Iodoacetamide	1.0	0	96

Reactions were measured polarographically in the oxygen monitor at 30°C. Reaction mixtures contained, in a volume of 3ml; 240 µmol phosphate buffer, pH 6.8, 100,000 g supernatant (5mg protein), 1.0 µmol NADPH and inhibitor at the required concentration. Buffer, with or without test compound, and protein were pre-incubated for 10min at 30°C, NADPH was added 1 min before the start of the reaction. Initial endogenous NADPH rates were recorded before cyclohexane addition (2µl).

*Percentage inhibition of each enzyme compared to control incubations to which no inhibitor was added.

Specific activity of NADPH oxidase control was calculated as 2.4 $nmol.min^{-1}$. $mg\ protein^{-1}$.
Specific activity of cyclohexane hydroxylase control was calculated as 22.3 $nmol.min^{-1}$. $mg\ protein^{-1}$.

this enzyme further to establish whether it was either a non-haem iron-containing monooxygenase or a cytochrome P-450 haem-containing system. The presence of cytochrome P-450 is detectable by an absorbance peak at 450nm when the enzyme is reduced and complexed with carbon monoxide (Omura and Sato, 1964). When the cell extract (100,000 g supernatant) was reduced with sodium dithionite the dithionite difference spectrum showed an absorbance peak at 420nm (Fig. 4.2a). When the reduced cell extract was complexed with carbon monoxide the dithionite reduced carbon monoxide difference spectrum displayed an absorbance peak at 450nm indicating the presence of cytochrome P-450 (Fig. 4.2b).

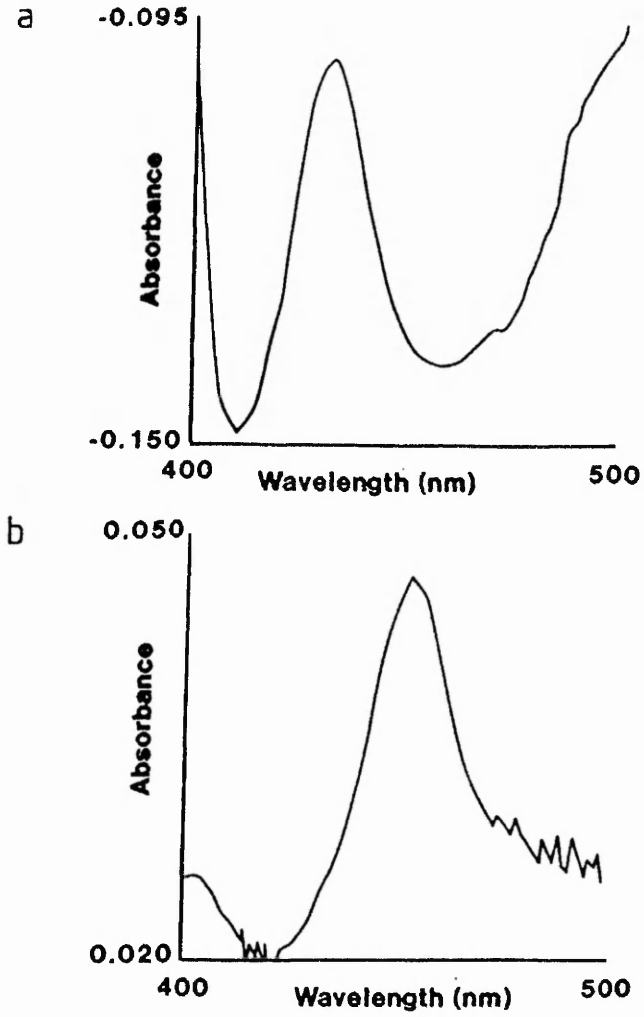
4.2.7 Correlation between the growth of Xanthobacter sp. on cyclohexane, cyclohexane hydroxylase activity and detection of cytochrome P-450.

Studies were performed to determine whether there was any correlation between the growth state of Xanthobacter sp. as determined by cell density (measured by A 550nm) following growth on cyclohexane, the activity of cyclohexane hydroxylase and presence of cytochrome P-450. The Xanthobacter sp. cells were initially grown on succinate and then transferred to cyclohexane to monitor the induction of any cyclohexane hydroxylase and cytochrome P-450. The results are shown in Fig.4.3. Below a cell density corresponding to an A 550nm of 0.5 no cyclohexane hydroxylase activity or cytochrome P-450 could be detected in cell extracts (100,000 g supernatant). However, as the A 550nm increased so to did the presence of cyclohexane hydroxylase and cytochrome P-450. At A 550nm of 1.6 maximum amounts of cyclohexane hydroxylase activity and cytochrome P-450 were obtained, after which the values of both were found to tail off slightly.

4.2.8 Inducibility of Cytochrome P-450

Since cyclohexane hydroxylase has previously been shown to be an inducible enzyme system (Trower et al., 1985) it was important to

Fig. 4.2 Dithionite difference spectrum and dithionite/ carbon monoxide difference spectrum of cell-free extracts from cyclohexane-grown Xanthobacter sp.

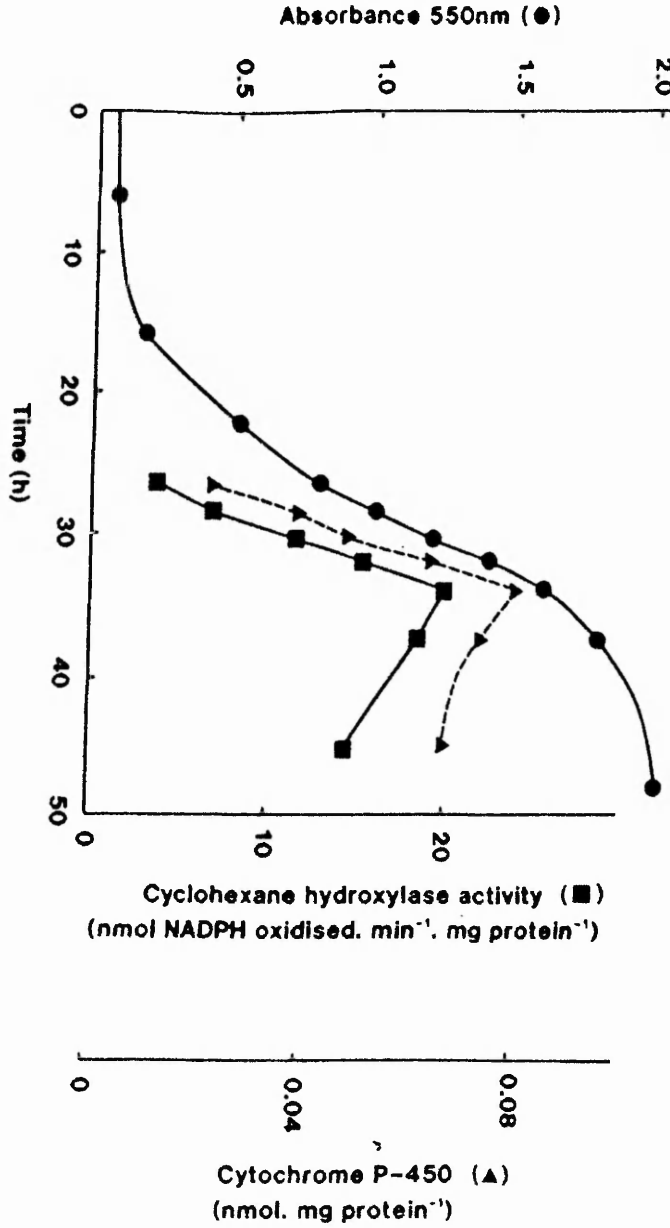


a) dithionite difference spectrum of cell-free extracts from cyclohexane-grown Xanthobacter sp.

b) dithionite/carbon monoxide difference spectrum of cell-free extracts from cyclohexane-grown Xanthobacter sp.

Spectra were obtained using a 1cm light path cuvette containing 5mg protein (100,000 g supernatant) and following the procedures described in section 2.7.

Fig 4.3 Correlation between cell density of Xanthobacter sp., activity of cyclohexane hydroxylase and presence of cytochrome P-450 following growth on cyclohexane.



Cyclohexane hydroxylase activity was monitored polarographically in the oxygen monitor following the procedure in section 2.6.1.

Cytochrome P-450 was assayed spectrophotometrically according to the procedures described in section 2.7. Protein concentrations of solutions were determined by the method described in section 2.8.2.

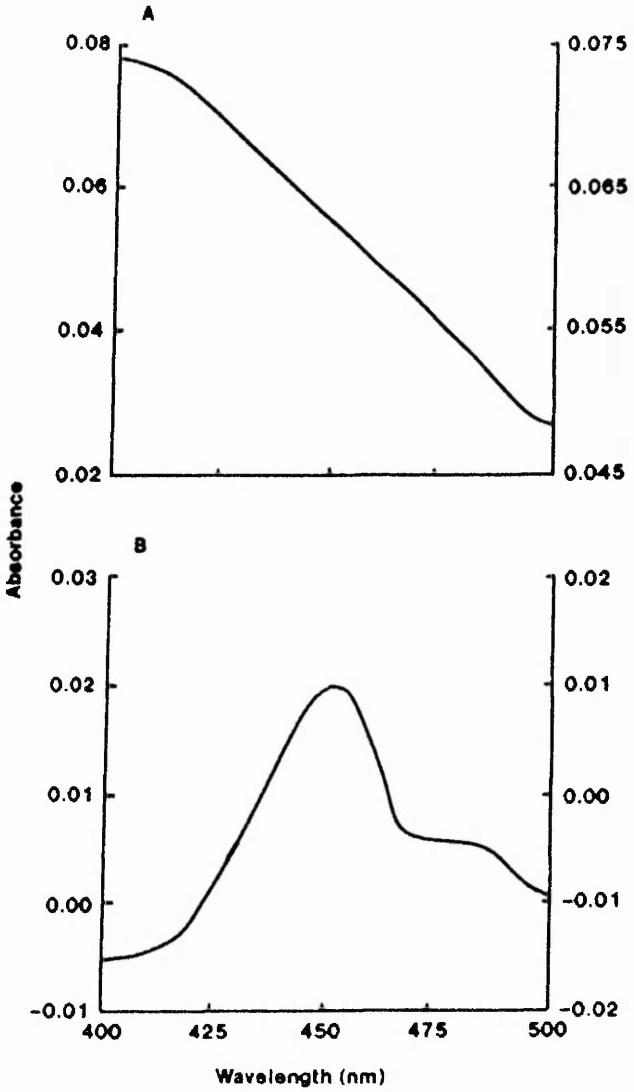
determine whether the cytochrome P-450 was also inducible. Xanthobacter sp. was therefore grown on minimal salts media containing succinate as a sole carbon source. The dithionite/carbon monoxide difference spectra of cell extracts (100,000 g supernatant) obtained from succinate grown Xanthobacter sp. is shown in Fig. 4.4a. Unlike the absorbance spectrum shown in Fig. 4.2b obtained from cells grown on cyclohexane no clear absorbance peak at 450nm was detectable. A small amount of the succinate grown cells were however retained and used as an inoculum for further growth on minimal salts media containing cyclohexane as a sole carbon source. Cell extracts (100,000 g supernatant) obtained from these cells following their growth on cyclohexane did however show a dithionite/carbon monoxide difference spectrum where a clear absorbance peak at 450nm was detectable (Fig. 4.4b). This further indicates the inducibility of cytochrome P-450 in cyclohexane grown Xanthobacter sp.

4.2.9 Stability of cytochrome P-450

When cell extracts (100,000 g supernatant) were stored at 4°C for 72h cytochrome P-450 could not be detected. Following these storage conditions the dithionite/carbon monoxide difference spectra of cell extracts did however indicate an absorbance peak to be present at 420nm which is indicative of the inactive form of cytochrome P-450.

When stored at -20°C for 20 days over 80% of cytochrome P-450 activity was observed. It was therefore necessary to try and stabilise cytochrome P-450 when stored at 4°C for further purification and characterisation purposes. A number of potential stabilisers were therefore added to freshly prepared samples of cell extract (100,000 g supernatant). These soluble extracts were then assayed for activity after 0, 24 and 72h incubation at 4°C (Table 4.4). After 72h only extracts containing glycerol 10% (v/v) or 20% (v/v) retained over 90% of the original cytochrome P-450.

Fig. 4.4 Dithionite/carbon monoxide difference spectra of cell-free extracts from succinate-grown and cyclohexane-grown Xanthobacter sp.



a) dithionite/carbon monoxide difference spectrum of cell-free extracts from succinate-grown Xanthobacter sp.

b) dithionite/carbon monoxide difference spectrum of cell-free extracts from cyclohexane-grown Xanthobacter sp.

Spectra were obtained using a 1cm light path cuvette containing 5mg protein (100,000 g supernatant) and following the procedures described in section 2.7.

Table 4.4 Effect of potential stabilizers on the presence of cytochrome P-450 in cell-free extracts of Xanthobacter sp. when stored at 4°C.

Test Compound	Concentration test compound in cell free extract	Time after initial measurement (h) 0	24	72
Control	-	100	57	0
EDTA	0.1mM	97	72	0
EDTA	1.0mM	102	81	0
DTT	0.1mM	99	59	0
DTT	1.0mM	100	54	0
NADPH	0.1mM	100	82	63
NADPH	1.0mM	98	88	57
Glycerol	10% (v/v)	103	97	95
Glycerol	20% (v/v)	100	93	91

Cell-free extracts (100,000 xg supernatant) containing the test stabilisers were assayed for cytochrome P-450 by the procedure described in section 2.7 at 0, 24 and 72h.

At time zero, cytochrome P-450 present in control sample was determined as 0.11 nmol. mg protein^{-1}.

4.2.10 Reactivation of inactive cytochrome P-420 to active cytochrome P-450

When cytochrome P-450 loses activity it is converted to cytochrome P-420 (the inactive form of cytochrome P-450). With the cytochrome P-450 obtained from Xanobacter sp. this occurs over a 72h period at 4°C. However, cytochrome P-420 can be reactivated to its P-450 form by the addition of glycerol (10% (v/v)) to the cell-free extract.

4.2.11 Estimation of the molecular weight of cytochrome P-450

The approximate molecular weight of cytochrome P-450 was determined by passage of cell-free extract (100,000 g supernatant) of cyclohexane-grown Xanthobacter sp. through a previously calibrated LKB Ultrogel AcA 44 gel filtration column. The molecular weights of the proteins used for calibration with their respective elution volumes are listed in Table 3.8. A graph (Fig. 4.5) of elution volume (Ve) against $\log_{10}$ molecular weight was plotted using the data from this table. The molecular weight of cytochrome P-450 was then determined from this plot by measurement of its elution volume.

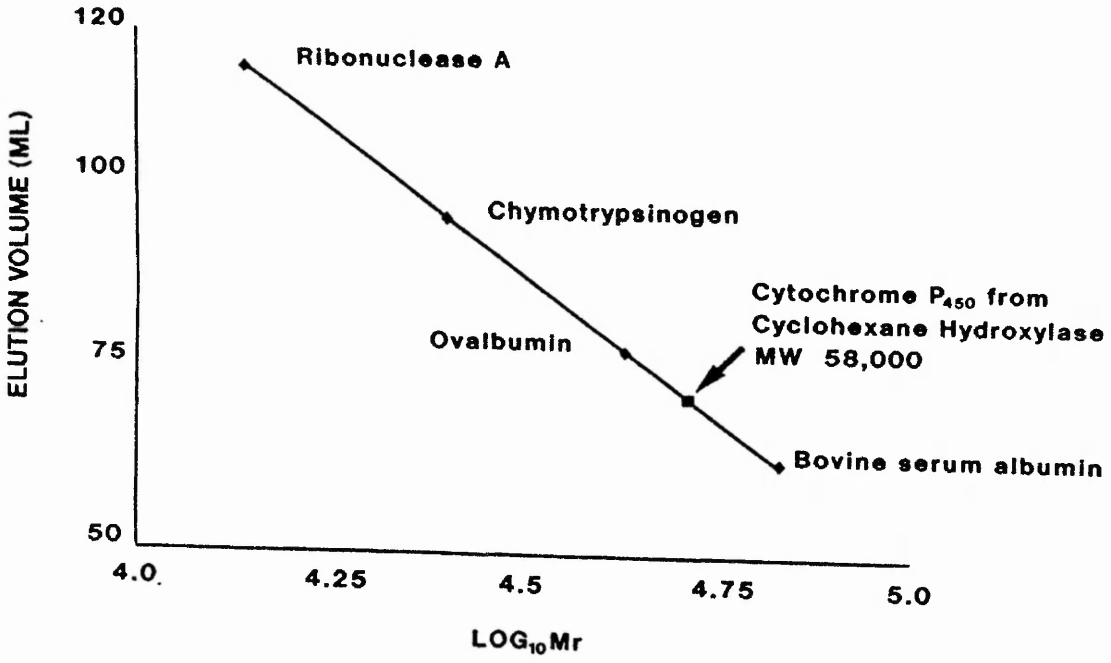
4.2.12 Semi-purification of Cytochrome P-450

All steps involved in the purification of cytochrome P-450 were performed at 4°C, unless stated otherwise.

Cytochrome P-450, present in cell extracts (100,000 g supernatant) of cyclohexane grown Xanthobacter sp., was semi-purified by a combination of ammonium sulphate precipitation, gel filtration chromatography and HPLC by anion exchange as described below.

Cell free extracts (100,000 g supernatant) from cyclohexane-

Fig. 4.5 Calibration plot for the determination of the molecular weight of cytochrome P-450 from Xanthobacter sp.



100,000 g supernatant (10 mg protein.ml^{-1}) prepared from cyclohexane-grown Xanthobacter sp. was loaded (2ml) onto the Ultrogel AcA 44 gel filtration column (Vo = 44.0) and eluted with 20mM phosphate buffer pH 7.0 containing glycerol 10% (v/v) as described in section 2.11.2. Fractions collected on ice in volumes of 3ml were assayed for protein (section 2.8.3) and the presence of cytochrome P-450. (Section 2.7) The molecular weight of cytochrome P-450 was determined by its elution volume.

grown Xanthobacter sp. cells were first precipitated by the addition of different concentrations of ammonium sulphate. The majority (76%) of cytochrome P-450 was found to be present in the precipitate corresponding to an ammonium sulphate concentration of 25-40% saturation. The protein fraction from the 25-40% ammonium sulphate saturation range was therefore pelleted by centrifugation (20,000 g, 20 min) and resuspended in 20mM phosphate buffer pH 7.0 containing 10% (w/v) glycerol. The resuspended fraction was loaded onto on LKB ultrogel AcA 44 gel filtration column and eluted with 20mM phosphate buffer pH 7.0 containing 10% (v/v) glycerol. Fractions with cytochrome P-450 concentration (nmol. mg protein) greater than 1.0 were pooled and the protein was concentrated using Centrisart 1 tubes (Sartorius) containing an ultrafiltration membrane with a molecular weight cut off of 20,000 and centrifuged at 1,380 g for 20min. The concentrated sample was dialysed against 20mM phosphate buffer pH 7.0 and loaded onto a Pharmacia Mono Q pre-packed HR 5/5 column at room temperature and eluted with 20mM phosphate buffer pH 7.0 with an increasing sodium chloride gradient over a 30 min period. The eluant was collected at 4°C. No cytochrome P-450 was recovered in the fractions eluted from the anion exchange step. The results from a semi-purification run are given in Table 4.5.

4.3 DISCUSSION

The activity of cyclohexane hydroxylase obtained, although low, was however sufficient to allow various studies involving its characterisation to be performed. These low amounts of activity are not unusual with methylene and methyl hydroxylases, as previously mentioned in section 3.3 and often little or no detectable activity is found for these type of enzymes in cell-free extracts. Similarly the instability of cyclohexane hydroxylase activity in cell-free extacts during storage is not atypical. Colby et al., (1975) demonstrated that the methane monooxygenase from Methylomonas methanica lost considerable activity after storage for 24h at 2°C, while Stirling and Dalton (1979) showed that a methane monooxygenase from Methylosinus trichosporium OB3b lost all activity when stored

Table 4.5 Semi-purification of Cytochrome P-450 from Xanthobacter sp.

	Total Volume (ml)	Total Protein (ml)	Total Cytochrome P-450 (nmoles)	Specific Cytochrome P-450 (nmoles.mg protein^{-1})	Recovery (percent)	Purification (fold)
100,000 g supernatant from disrupted cells	150	1245	138	0.11	100	1.0
$(NH_4)_2$ SO_4 fractionation 25-40% saturation	5	184	105	0.57	76	5.2
Eluate from AcA44 gel filtration	15	22	46.5	2.11	33.7	19.2

Cytochrome P-450 was measured spectrophotometrically following the procedure in section 2.7.
Protein concentration of solutions were determined by the procedures described in section 2.8.2.

for 24h at 0°C or 4°C. The addition of NADPH to the cell breakage medium and either NADPH or cyclohexane to the cell-free extract appeared to stabilise cyclohexane hydroxylase activity. When Stirling et al., (1977) and Anderson et al., (1980) tried to isolate cyclohexane hydroxylase activity from a Nocardia sp. and a Pseudomonas sp. respectively both were unsuccessful. However Anderson et al., (1980) did manage to show cyclohexane hydroxylase activity in cell-free extracts of the cyclohexane-grown Pseudomonas sp. following addition of glycerol (10% (v/v)) to the breakage medium, but the activity obtained was lost when stored at 4°C overnight. The addition of the enzyme cofactor or substrate to cell breakage medium or cell-free extract has been previously used to stabilise the activity of other comparable enzymes. For example Colby and Dalton (1978) stabilised component C of the methane monooxygenase system from M. capsulatus (Bath) by the addition of NADH (5mM), whilst Bernhardt and Meisch (1980) showed that the presence of NADH stabilised the putidamonoxin reductase component of 4-methoxybenzoate O-demethylase for several weeks under anaerobic conditions at -20°C. Some workers have reported that the presence of ferrous or ferric ions can stimulate the enzyme activity of some non-haem iron based monooxygenases. Matsuyama et al., (1981) demonstrated that the presence of ferric ions stabilised the n-alkane w-hydroxylase from Pseudomonas aeruginosa, whilst Ruettinger et al., (1974) showed that the addition of ferrous ions stimulated the activity of n-alkane w-hydroxylase from Pseudomonas oleovorans. Other workers have shown that reducing agents help to stabilise iron sulphur proteins which can be susceptible to oxidation. Gunsalus and Wagner (1978) showed that the inclusion of 10mM β-mercaptoethanol to the purification buffer of camphor-5-hydroxylase helped to stabilise the putidaredoxin component while Colby and Dalton (1978) demonstrated that the presence of sodium thioglycollate (1-10mM) or dithiothreitol (5-10mM) prevented the loss of the NADH-reductase (component C) of the methane monooxygenase from Methylococcus capsulatus (Bath).

The pH optimum of cyclohexane hydroxylase was found to be in the range 6.7 - 6.9. A number of alkyl hydroxylases have been reported

with a pH optimum in the range 6.4-7.4, including non-haem iron hydroxylases (Ruetkinger et al., 1974; Colby et al., 1975) and cytochrome P-450 containing hydroxylases (Katagiri et al., 1968; Heinz et al., 1970; Duppel et al., 1973; Miura and Fulco, 1974; Berg et al., 1976; Marchal et al., 1982).

Studies on the substrate specificity of cyclohexane hydroxylase indicated the enzyme to be mainly restricted to saturated and some unsaturated ring structures. The enzyme was active with cycloalkanes and short chain n-alkyl substituted cycloalkanes and also, but to a much lesser extent, with the aromatic hydrocarbons benzene and toluene. The enzyme was also active towards the bicyclic terpene pinane but not the di-substituted monocyclic terpene p-menthane the latter having both methyl and isopropyl substitution on the cyclohexane ring. Activity was also detected with the heterocyclic ring of pyrrolidine and also quadricyclane however no activity could be demonstrated with dicylohexyl. The inability to detect cyclohexane hydroxylase activity with any one of the straight chain n-alkanes indicates that this methylene hydroxylase is highly specific for cyclic compounds. Comparison of the substrate specificity of cyclohexane hydroxylase to the well documented methane monooxygenases (Colby et al., 1977; Stirling et al., 1979; Dalton 1981; Patel et al., 1982; Best and Higgins, 1983) would also suggest that this methylene hydroxylase is far more specific in its substrate range.

The inhibition studies showed that cyclohexane hydroxylase was particularly sensitive to sulphydryl group inhibitors. This is in keeping with the n-alkane cytochrome P-450 dependent alkyl hydroxylases from a Corynebacterium sp. (Cardini and Jurtshuk, 1970), and Bacillus megaterium (Hare and Fulco, 1975), also the soluble methane monooxygenase from Methylobacterium CRL-26 (Patel et al., 1982) and the cytochrome P-450 component of the camphor 5-hydroxylase system from Pseudomonas putida (Yu and Gunsalus, 1974), all of which were sensitive to sulphydryl group inhibitors.

Respiratory electron transport inhibitors had little effect on cyclohexane hydroxylase activity. Similarly cyanide had no inhibitory effect upon enzyme activity. This finding is in keeping

with that demonstrated for other cytochrome P-450 containing enzyme systems as reported by Cardini and Jurtshuk (1970) Heinz et al., (1970), Yu and Gunsalus (1974), Duppel et al., (1975), Hare and Fulco (1975) and Schunk et al., (1978), which were either insensitive to cyanide or inhibited only at high concentrations of the compound. However, non-haem iron monooxygenase systems show considerable variation in their sensitivity towards cyanide. This is illustrated by the soluble methane monooxygenase from Methylococcus capuslatus (Bath) which is insensitive to cyanide (Stirling and Dalton, 1979) whilst inhibition by cyanide is shown by the alkane w-hydroxylase from Pseudomonas oleovorans (Boyer et al., 1971) and the steroid 9-hydroxylase from a Nocardia sp.

The effect of metal chelating agents on cyclohexane hydroxylase activity was also found to be variable. The enzyme was greatly inhibited by the copper chelator 1,10 phenathroline and to a lesser extent by bathophenanthroline and bathocuproin. EDTA also caused inhibition while α,α'-dipyridyl and neocuproine showed no inihibitory effect upon the enzyme. This is not atypical as Dalton and Colby (1982) showed that the methane monooxygenase from Methylococcus capsulatus (Bath) was inhibited by 8-hydroxyquinoline but not by neocuproine and α,α'-dipyridyl. Stirling and Dalton (1979), Dalton (1981) and Patel et al., (1982) have shown that many soluble methane monooxygenases also display this variable pattern of inhibition by metal chelating agents.

The fractionation experiments indicated cyclohexane hydroxylase to be a multicomponent system. Studies on the kinetics of cyclohexane hydroyxlase induction during cell growth on cyclohexane demonstrated that it was paralled by the induction of a soluble cytochrome P-450. Neither cyclohexane hydroxylase activity nor the presence of cytochrome P-450 could be detected following growth on succinate. These data therefore indicate that cyclohexane hydroxylase is a multicomponent enzyme system possessing cytochrome P-450 as the terminal oxidase component. A number of these type of enzymes have already been characterised of which the methylene hydroxylases include the 5-exo-camphor hydroxylase from Pseudomonas putida (Gunsalus and Wagner, 1978), the steroid 15 β-hydroxylase

from Bacillus megaterium Berg et al., 1976), the steroid 11 β-hydroxylase from adrenal cortex mitochondria (Mitani, 1979), the 25-hydroxy-cholecalciferol from kidney mitochondria (Kulkoski and Ghazarian, 1979) and also the methyl hydroxylase, linalool 8-hydroxylase from Pseudomonas sp. (Wagner, 1983).

The inducible nature of cytochrome P-450 was also demonstrated by transferring Xanthobacter sp. from a growth medium containing succinate to that containing cyclohexane. This would appear to be typical as there are a number of reports demonstrating the inducibility of cytochrome P-450. Marchal et al., (1982) reported that the growth of Saccharomycopsis lipolytica on n-alkanes induced a hydroxylation system which contained cytochrome P-450. Duppel et al., (1973) found that the growth of Candida tropicalis on tetradecane caused the induction of a cytochrome P-450 containing enzyme system which catalysed the hydroxylation of fatty acids. Schunck et al., (1978) reported that after growth of Candida guilliermondii on n-alkanes as the sole carbon source an alkane hydroxylase and cytochrome P-450 had been induced. Breskvar and Hudnik-Plevnik (1981) demonstrated that cytochrome P-450 and NADPH cytochrome C reductase were both induced in Rhizopus nigricans and Rhizopus arrhizus when these organisms were grown in the presence of progesterone.

Investigations into the stability of cytochrome P-450 at 4°C in soluble extracts from the cyclohexane-grown Xanthobacter sp. showed that all activity was lost over a 72 h period. Various potential stabilisers were tested to determine whether they would have any effect on cytochrome P-450. The studies showed that the addition of glycerol 10% (v/v) or 20% (v/v) resulted in over 90% cytochrome P-450 activity being retained over a 72h period. Other workers have also shown it necessary to add stabilisers to reaction and purification buffers in order to retain cytochrome P-450 activity. Berg et al., (1979) added dithiothreitol (10mM) to all buffers when purifying cytochrome P-450 from Bacillus megaterium. The addition of EDTA (0.1mM) to buffers was reported by Koop et al., (1982) for the purification of isoenzymes of cytochrome P-450 from liver microsomes of ethanol treated rabbits, Kimura et al., (1978) found

it necessary to add EDTA (1mM - 5mM) to buffers for the purification of adrenodoxin reductase, adrenodoxin and cytochrome P-450 from bovine adrenal cortex. The addition of d-camphor (1mM) (the growth substrate of the organism) to extraction and purification buffers for the purification of cytochrome P-450 from Pseudomonas putida was reported by O'Keeffe et al., (1978).

The molecular weight of cytochrome P-450 from cell-free extracts of cyclohexane-grown Xanthobacter sp. as determined by gel filtration was estimated to be 58,000. This value is in keeping with others obtained for cytochrome P-450. Kappeli (1986) reported a molecular weight of 53,000 for cytochrome P-450 obtained from Lodderomyces elongisporus and 55,000 and 58,000 respectively for two forms of cytochrome P-450 from Saccharomyces cerevisiae. A molecular weight of 52,000 for cytochrome P-450 from Bacillus megaterium was recorded by Berg et al., (1979) while Appleby (1978) showed that all cytochrome P-450 subspecies from Rhizobium have molecular weights of approximately 45,000. Yu and Gunsalus (1974) and O'Keeffe et al., (1978) both investigating cytochrome P-450 of 5-exo camphor hydroxylase from different strains of Pseudomonas putida obtained molecular weights of 44,000-46,000 and 43,900-45,000 respectively.

Attempts to purify the cytochrome P-450 showed that it could be purified 19.2 fold by ammonium sulphate precipitation and AcA 44 gel filtration. However, the procedure must be investigated further to determine why all cytochrome P-450 activity was lost at the HPLC anion exchange stage of the purification. It is important to purify cytochrome P-450 completely to enable full characterisation of this component of cyclohexane hydroxylase to be carried out.

5. CYCLOHEXANOL DEHYDROGENASE

5.1 INTRODUCTION

Preliminary investigations undertaken by Trower et al., (1985) demonstrated the cyclohexanol dehydrogenase isolated from a cyclohexane-grown Xanthobacter sp. to be a soluble, inducible enzyme. This enzyme is responsible for catalysing the conversion of cyclohexanol to cyclohexanone which is then converted by a lactone forming monooxygenase to E-caprolactone thus facilitating cleavage of the alicyclic ring (Donoghue and Trudgill, 1975). The only report of a secondary alcohol dehydrogenase isolated from a non-substituted cycloalkane-growing microorganism is presently limited to the characterisation of cyclohexanol dehydrogenase isolated from the cyclohexane-growing Nocardia (Stirling and Perry, 1980). There are however a number of reports demonstrating the presence of such an enzyme in cycloalkanol-growing microorganisms including the cylopentanol-growing Pseudomonas sp (Griffin and Trudgill, 1972) and the cyclohexanol-growing Acinetobacter (Donoghue and Trudgill, 1975). However it should also be noted that Norris and Trudgill (1971) were unable to demonstrate the presence of cyclohexanol dehydrogenase activity in cell extracts of the cyclohexanol-growing Nocardia globerula CL1.

The aim of this study was to purify and characterise the cyclohexanol dehydrogenase from the cyclohexane-growing Xanthobacter sp. Comparison of its biophysical properties could then be made to the enzyme isolated from the cyclohexane-growing Nocardia sp. and to the cycloalkanol dehydrogenases isolated from cycloalkanol-growing microorganisms. Thus further information could be gained concerning the enzymes involved in the oxidation of non-substituted cycloalkanes.

5.2 RESULTS

5.2.1 Purification of cyclohexanol dehydrogenase

All cyclohexanol dehydrogenase purification steps were performed at 4°C unless stated otherwise.

Cyclohexanol dehydrogenase present in cell-free extracts of cyclohexane-grown Xanthobacter sp. was purified by a combination of ammonium sulphate precipitation, affinity chromatography and anion exchange (HPLC) as described below.

Cell-free extracts (100,000 g supernatant) from cyclohexane-grown Xanthobacter sp. cells were first precipitated by the addition of different concentrations of ammonium sulphate. The majority of cyclohexanol dehydrogenase was found to be present in the precipitate corresponding to an ammonium sulphate concentration of 50-70% saturation. The precipitated protein fraction from the 50-70% ammonium sulphate saturation range was therefore pelleted by centrifugation (20,000 g, 20min), resuspended in 20mM phosphate buffer, pH 7.0 and then dialysed against the same buffer to remove the residual ammonium sulphate. The dialysed fraction was loaded onto a Trisacryl Blue M affinity chromatography column (LKB Produktor-AB, Bromma, Sweden) and the protein eluted with a linear NAD^+ gradient in 20mM phosphate buffer, pH 7.0 (Fig 5.1). Fractions with a specific activity greater than 8 units. $(mg\ protein)^{-1}$ were pooled and the protein precipitated by solid ammonium sulphate addition (70% saturation). The precipitated protein was pelleted by centrifugation (20,000 g, 20min), resuspended in 20mM ethanolamine-HCl buffer, pH9.5, and dialysed against the same buffer to remove the residual ammonium sulphate. The dialysed sample was loaded onto a Pharmacia Mono Q pre-packed R5/5 column and the protein eluted with a linear 0.5M sodium acetate gradient in 20mM ethanolamine-HCl buffer, pH 9.5 (Fig 5.2). Fractions with a specific activity greater than 20.0 units. $(mg\ protein)^{-1}$ were pooled. If necessary the homogeneous enzyme was concentrated by solid ammonium sulphate precipitation (70% saturation). After centrifugation (20,000 g, 20min) the resulting pellet was resuspended in a small volume of chosen buffer and dialysed against the same buffer to remove the residual ammonium sulphate salt.

The results from a typical purification of cyclohexanol dehydrogenase are shown in Table 5.1.

Fig 5.1 Purification of Cyclohexanol dehydrogenase by Trisacryl Blue M affinity chromatography.

The Trisacryl Blue M affinity chromatography column (1.6 x 28cm) was loaded with 630 mg protein containing 1521 units cyclohexanol dehydrogenase. The protein was eluted at a flow rate of $10ml.h^{-1}$ by a linear NAD^+ gradient (0-7.5mM) constructed by mixing 150ml 20mM phosphate buffer, pH7.0 with 150ml 7.5mM NAD^+ in the same buffer. Eluant was collected in fractions of 5ml and the absorbance of each fraction measured at 280nm. Fractions were assayed for cyclohexanol dehydrogenase activity spectrophotometrically by following the cyclohexanol dependent reduction of NAD^+ at 340nm (Section 2.6.2).

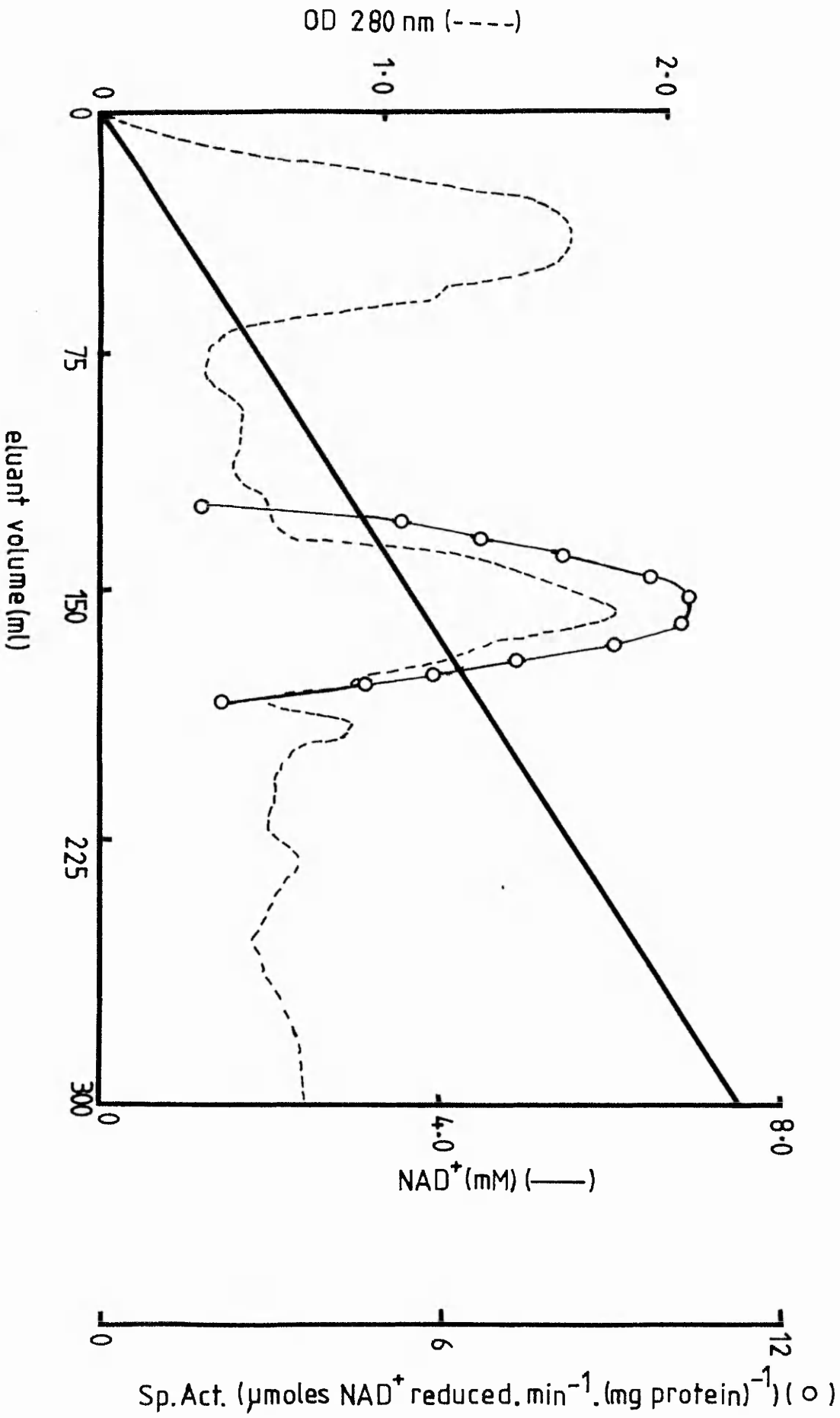

OD 280 nm (----)
0
1·0
2·0
0
75
150
225
300
eluant volume (ml)
0
4·0
8·0
NAD+ (mM) (——)
0
6
12
Sp. Act. (μmoles NAD+ reduced. min−1. (mg protein)−1) (o)

Fig 5.2 Purification of Cyclohexanol dehydrogenase by anion exchange hplc

The Pharmacia Mono Q pre-packed MR5/5 column (5 x 50mm) was loaded with 30mg protein containing 317 units cyclohexanol dehydrogenase. The protein was eluted by a linear sodium acetate gradient controlled by a Waters Associates Model 660 solvent programmer. The gradient was constructed by mixing 20mM ethanolamine-HCl buffer, pH 9.5 with 0.5M sodium acetate in the same buffer. The protein was eluted at a flow rate of $0.5ml.min^{-1}$ over a 30 min period and fractions of 0.5ml were collected. The eluant was monitored at 280nm and the fractions assayed for cyclohexanol dehydrogenase spectrophotemtrically by monitoring the cyclohexanol dependent reduction of NAD^+ at 340nm (Section 2.6.2).

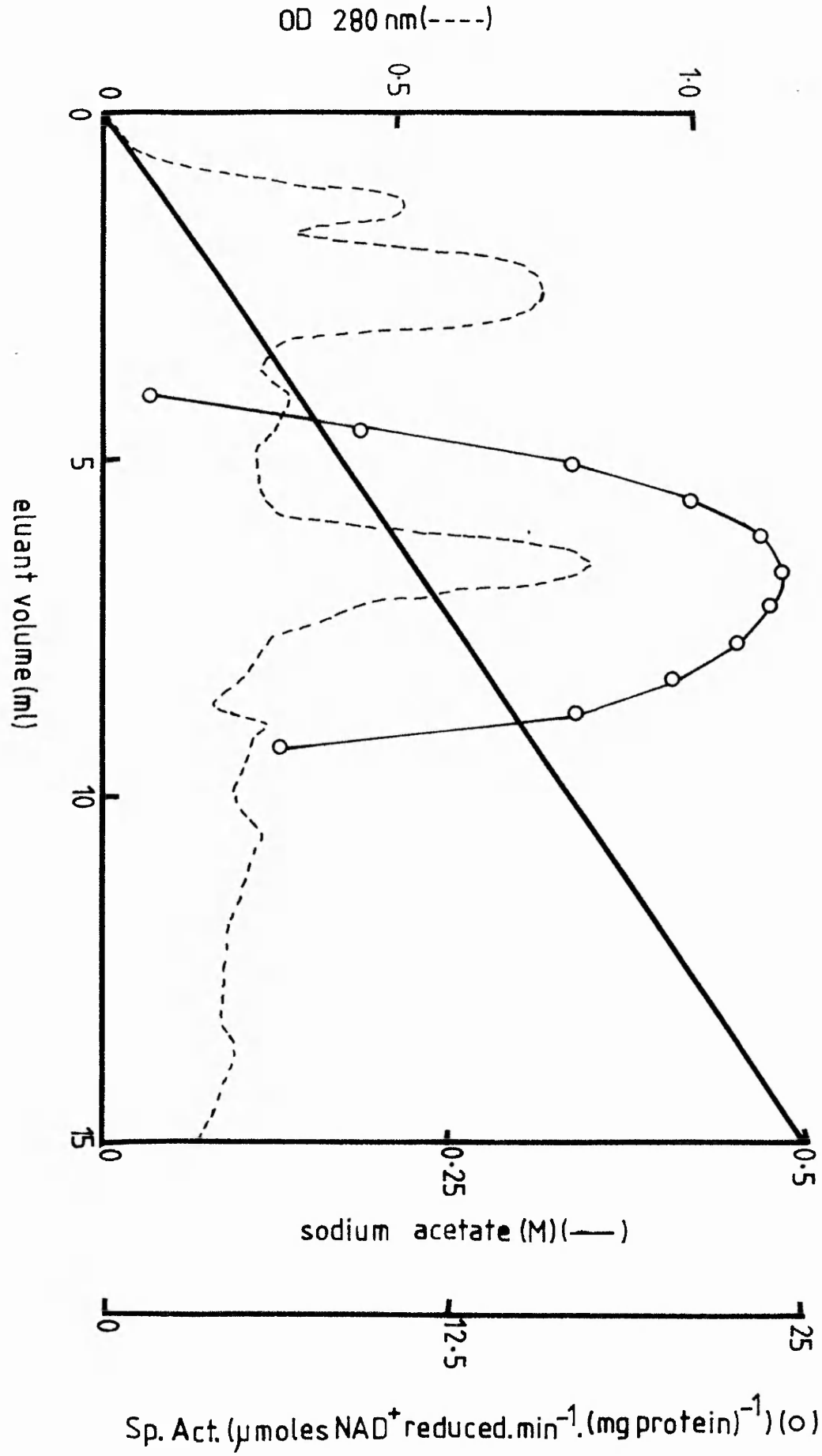

OD 280 nm(----)
0
0·5
1·0
eluant volume (ml)
0
5
10
15
sodium acetate (M)(——)
0
0·25
0·5
Sp. Act. (μmoles NAD+ reduced. min−1. (mg protein)−1) (o)
0
12·5
25

Table 5.1 Purification of cyclohexanol dehydrogenase from Xanthobacter sp.

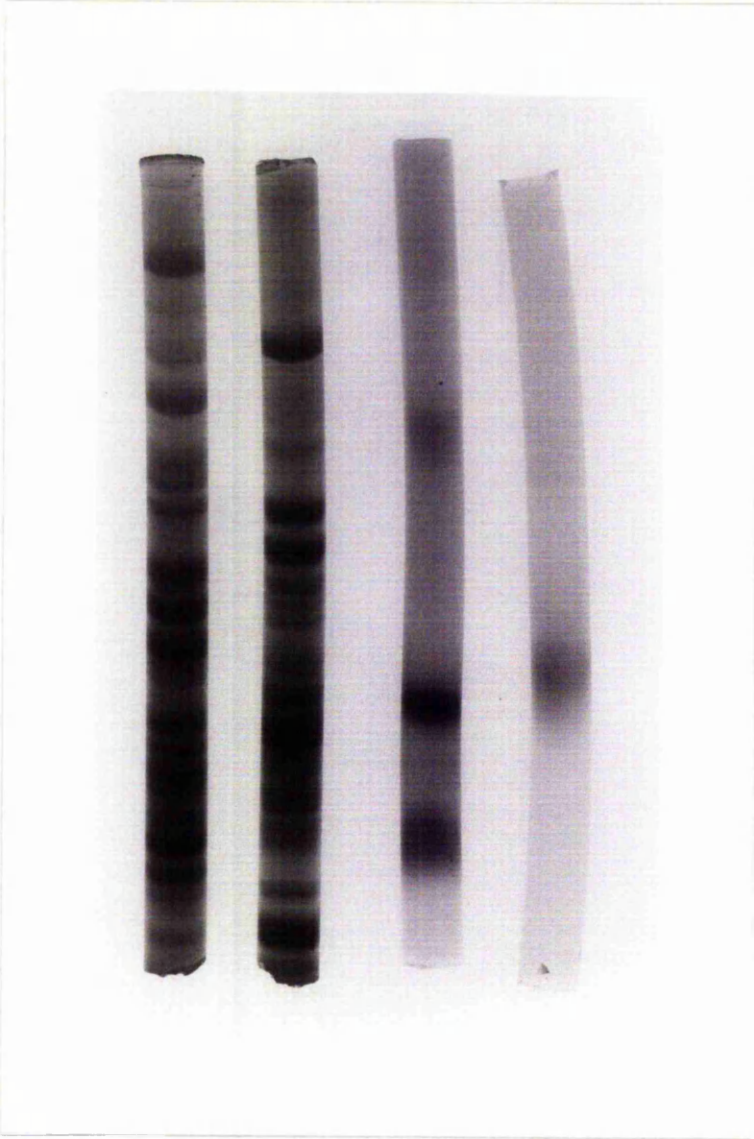
	Total Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (*U.mg protein^{-1})	Recovery (percent)	Purification (fold)
100,000 g supernatant from disrupted cells.	210	2980	2324	0.78	100	1.0
$(NH_4)_2SO_4$ fractionation 50-70% saturation	10	646	1560	2.42	67	3.1
Eluate from Trisacryl Blue M. 0-7.5 mM NAD^+	18	62	654	10.6	28	13.5
Eluate from Mono Q 0-0.5M sodium acetate	7	15.6	369	23.7	10.6	30.4

Cyclohexanol dehydrogenase activity was measured spectrophotometrically by following the cyclohexanol dependent reduction of NAD^+ (section 2.6.2).

Protein concentrations of solutions were determined as described in section 2.8.1.

Key: * - A unit of cyclohexanol dehydrogenase activity is equal to 1 μmol NAD^+ reduced. min^{-1} at 30°C.

Fig 5.3 PAGE analysis of pooled fractions from each step in the cyclohexanol dehydrogenase purification sequence.



PAGE (7.5% (w/v) polyacrylamide gels) of protein solutions from the following purification steps (1) 100,000 g supernatant from disrupted cells (200µg protein) (2) $(NH_4)_2SO_4$ fractionation (150µg protein) (3) Trisacryl Blue M (50 µg protein) and (4) Mono Q (5 µg protein) were performed according to the procedures described in section 2.12.1.

5.2.2 pH Optimum of purified cyclohexanol dehydrogenase

Studies concerning the relationship between pH and cyclohexanol dehydrogenase activity showed that the enzyme had a pH optimum of 10.3 (Fig 5.4). The enzyme activity decreased less than 10% when the pH was adjusted 0.5 pH units either side of the pH optimum.

5.2.3 Stability of cyclohexanol dehydrogenase

The purified cyclohexanol dehydrogenase was found to be stable with no detectable loss of activity after storage at 4°C for 72h. The stability of cyclohexanol dehydrogenase after incubation at elevated temperatures for different time periods is illustrated in Table 5.2. The enzyme was sensitive to heat treatment; after 1 min over 40% of enzymatic activity was lost at 55°C, 70% at 65°C and no activity was detectable after 1 min at 80°C.

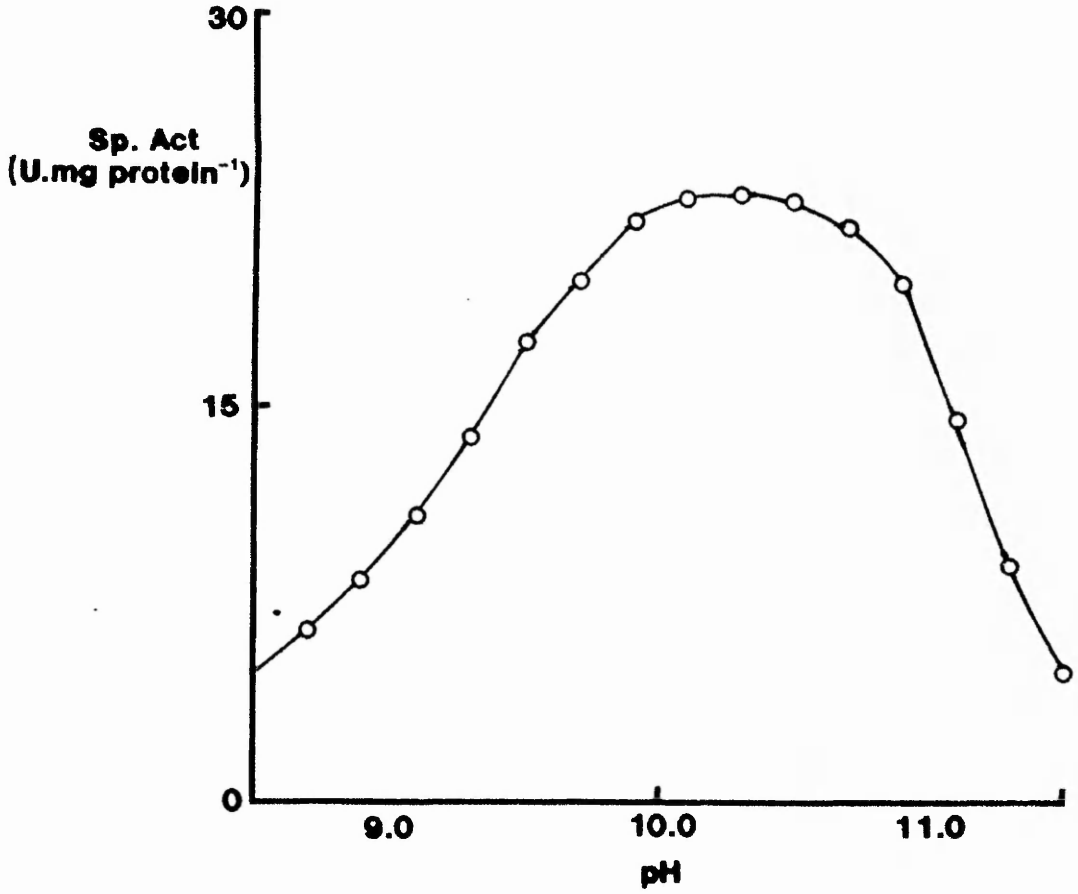
5.2.4 Cofactor specificity of cyclohexanol dehydrogenase

Various electron acceptors were tested with the purified cyclohexanol dehydrogenase. Cyclohexanol dehydrogenase activity was only detected when either NAD^+ or $NADP^+$ (0.5mM) were employed as the electron donor, with the latter giving 15% of the enzyme activity of that shown with NAD^+. Other electron acceptors tested including PMS, DCPIP, DCPIP + PMS, FMN, Cytochrome C and FAD (each at 0.5mM) all failed to bring about cyclohexanol dehydrogenase activity.

5.2.5 Identification of the cyclohexanol dehydrogenase reaction product

To determine the reaction product of cyclohexanol dehydrogenase, purified enzyme was incubated at pH 10.3 with cyclohexanol in the presence of NAD^+, and the reaction product analysed by glc. Two different glc systems were used to analyse the extracted product which was found to co-chromatograph with authentic cyclohexanone (Table 5.3).

Fig 5.4 pH Optimum of purified cyclohexanol dehydrogenase



Cyclohexanol dehydrogenase activity was measured spectrophotometrically by following the substrate stimulated reduction of NAD^+ at 340nm. Reaction mixtures contained in a volume of 1ml; Universal buffer of the required pH (8.5-11.5), 50µg enzyme, 0.5µmol NAD^+ and 1.0 µmol cyclohexanol. Reactions (at 30°C) were initiated by the addition of cyclohexanol. One unit of cyclohexanol dehydrogenase activity is equivalent to 1µmol NAD^+ reduced $.min^{-1}$ at 30°C.

Table 5.2 Heat stability of cyclohexanol dehydrogenase

Temperature (°C)	Incubation time (min)	Cyclohexanol dehydrogenase activity expressed as a percentage of the control
Control	Not incubated	*100
30	1	101.1
	5	98.3
	10	97.9
45	1	69.2
	5	56.5
	10	51.7
55	1	58.5
	5	31.7
	10	23.6
65	1	29.3
	5	15.1
	10	0
70	1	17.2
	5	0
	10	0
80	1	0
	5	0
	10	0

Reactions were measured spectrophotometrically at 30°C in a 1cm light path cuvette which contained in a volume of 1ml; 40µmol glycine/NaOH buffer (pH 10.3), 50µg of purified protein, 0.5µmol NAD^+ and 0.5µmol cyclohexanol. The enzyme was pre-incubated in the buffer at the appropriate temperature for the appropriate length of time. NAD^+ was added at the end of the incubation period and the reaction initiated by the addition of cyclohexanol. The reaction was monitored by following the reduction of NAD^+ at 340nm.
*Calculated specific activity of cyclohexanol dehydrogenase for the control was 23.0 µmoles NAD^+ reduced.min^{-1}. (mg protein)$^{-1}$.

Table 5.3 Identification of the cyclohexanol dehydrogenase reaction product

	Glc system	
	A	B
Compound	Retention time	(min)
Cyclohexanol	3.51	4.0
Cyclohexanone	2.95	2.71
Cyclohexan-1,2-dione	15.82	16.43
2-Hydroxycyclohexanone	14.68	15.95
Reaction product	2.95	2.71

A 25ml conical flask containing in a total volume of 5ml; 1mg purified cyclohexanol dehydrogenase, 200 umol glycine/NaOH buffer pH 10.3, 60μmol NAD^+ and 60μmol cyclohexanol, was incubated for 30min at 30°C. Extracts were prepared as described in section 2.9.2 and analysed by glc according to methods A and B described in section 2.10.1.

5.2.6 Molecular weight determination of cyclohexanol dehydrogenase

The molecular weight of cyclohexanol dehydrogenase was determined by size exclusion hplc and SDS-PAGE.

5.2.6.1 Size exclusion high performance liquid chromatography

To determine the approximate molecular weight of cyclohexanol dehydrogenase purified enzyme was passed through a previously calibrated TSK 3000 SW size exclusion column. Table 5.3 lists the molecular weights of the proteins used for calibration with their respective elution volumes. A graph (Fig. 5.5) of elution volume (V_e) against $\log_{10}$ molecular weight was plotted using the data from this table. From the measured elution volume of cyclohexanol dehydrogenase the molecular weight of the enzyme was determined to be 44,500.

5.2.6.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of cyclohexanol dehydrogenase

Purified cyclohexanol dehydrogenase and protein standards (Sigma) of known molecular weights were simultaneously separated by SDS-PAGE as described in section 2.12.3. A graph of relative mobility against $\log_{10}$ molecular weight (Fig. 5.6) was constructed using the resulting data from the protein standards. From this plot the molecular weight of the single protein band formed from the cyclohexanol dehydrogenase preparation was determined to be 43,000. This result was similar to that obtained by size exclusion hplc and indicates the monomeric nature of the enzyme.

5.2.7 Inhibition studies on cyclohexanol dehydrogenase

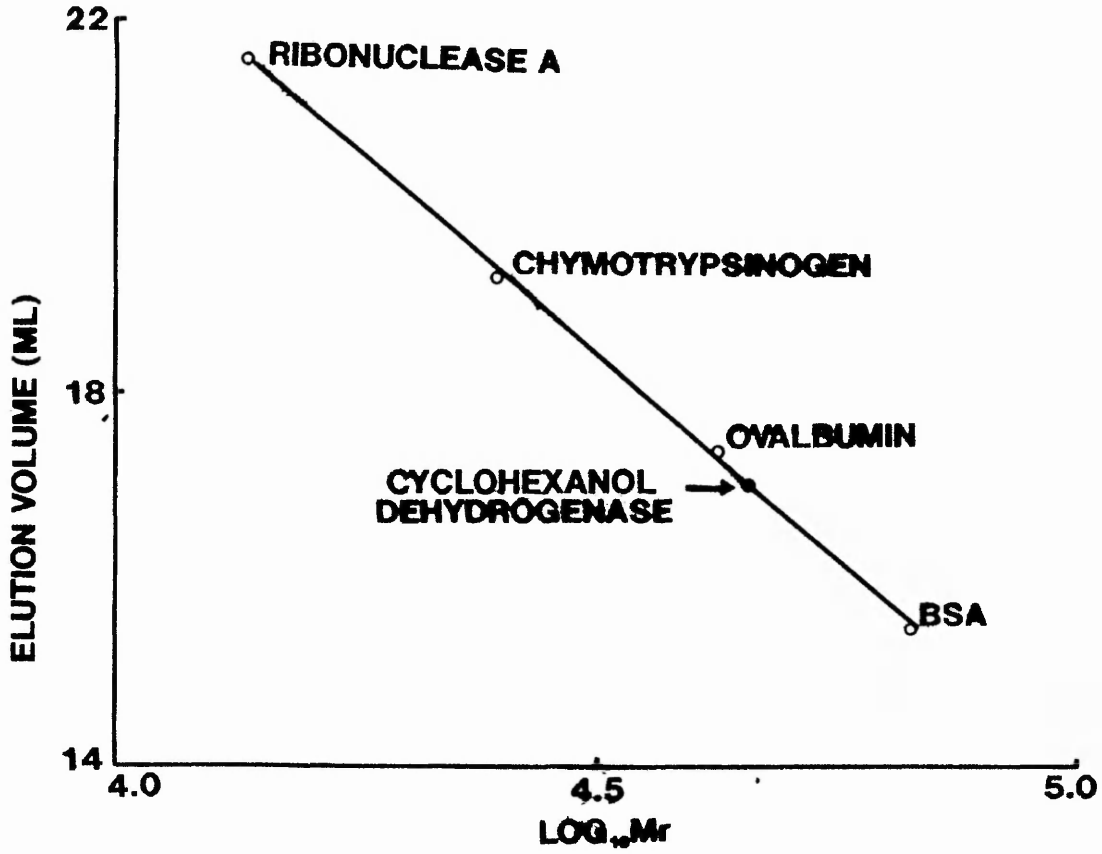
A number of potential inhibitors were tested to determine their effect on cyclohexanol dehydrogenase activity. The results of these

Table 5.4 Calibration of the TSK 3000 SW size exclusion column for molecular weight analysis of cyclohexanol dehydrogenase

Protein	Molecular weight	Elution volume (ml)
Bovine serum albumin	67,000	15.5
Ovalbumin	43,000	17.4
Chymotrypsinogen A	25,000	19.2
Ribonuclease	13,700	21.6

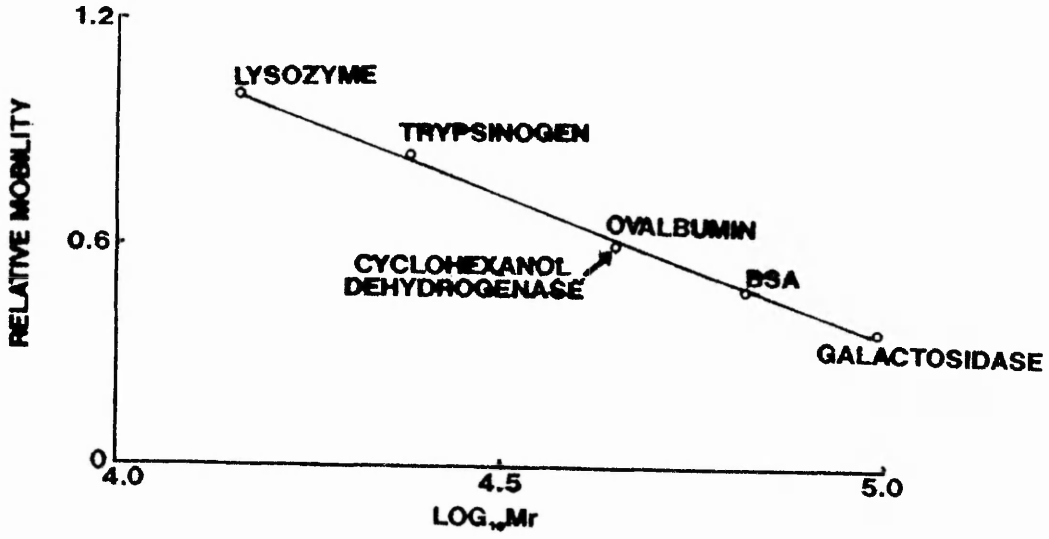
Calibration proteins ($5mg.ml^{-1}$) (Pharmacia) were loaded (200µl) onto the TSK 3000 SW size exclusion column and eluted and detected as described in section 2.11.1.2. The elution volume of each protein was recorded.

Fig 5.5 Calibration graph for the determination of the molecular weight of cyclohexanol dehydrogenase by size exclusion hplc.



Purified cyclohexanol dehydrogenase (3mg.ml^{-1}) was loaded (200µl) onto the TSK 3000 SW size exclusion column and eluted and detected as described in section 2.11.1.2. The molecular weight of cyclohexanol dehydrogenase was determined by its elution volume.

Fig 5.6 Calibration graph for the determination of the molecular weight of cyclohexanol dehydrogenase by SDS-PAGE.



SDS-PAGE of purified cyclohexanol dehydrogenase (10 µg protein per gel track) and protein calibration standards (5-50µg protein per gel track) was performed according to the procedures described in section 2.12.3.

The following equation was used to calculate relative mobilities of individual proteins.

$$R_m = \frac{\text{distance migrated by protein band}}{\text{distance migrated by bromophenol blue band}}$$

investigations are shown in Table 5.5 The results indicated that the enzyme was susceptible to metal chelating agents and somewhat differentially susceptible to sulphydryl reactive agents while electron transport inhibitors had little effect on cyclohexanol dehydrogenase activity.

5.2.8 Substrate specificity of cyclohexanol dehydrogenase

The substrate specificity of cyclohexanol dehydrogenase was investigated for a number of alcohols (Table 5.6). The enzyme showed activity with cycloalkanols and straight chain secondary alcohols but no activity was shown towards straight chain primary alcohols.

5.2.9. Steady-state kinetic investigations

5.2.9.1 Determination of the kinetic constants apparent K_m and V_{max} for cyclohexanol dehydrogenase

5.2.9.1.1 Cyclohexanol. Kinetic studies with purified cyclohexanol dehydrogenase indicated that the apparent Km for cyclohexanol was below the sensitivity measurable with the spectrophotometer employed. This indicates that the apparent Km for cyclohexanol is below 0.5μM.

5.2.9.1.2 NAD^+. Investigations undertaken on cyclohexanol dehydrogenase to calculate its apparent Km for the electron acceptor NAD^+ were performed by determining the initial reaction rates at various concentrations of NAD^+ in the presence of excess cyclohexanol. The data obtained was used to construct a Lineweaver-Burk double reciprocal plot of 1/V against 1/[S] (Fig 5.7). The resulting straight line relationship revealed an apparent Km for NAD^+ of 14.3μM and a V_{max} of 25 μmol. min^{-1}. (mg protein)$^{-1}$.

Table 5.5 Effect of potential inhibitors on cyclohexanol dehydrogenase activity

Inhibitor	Concentration (mM)	Percentage inhibition
Control	-	*0
Sodium arsenite	1.0	0
Sodium azide	1.0	0
α,α'- dipyridyl	5.0	20
	1.0	10
EDTA	1.0	20
8-hydroxyquinoline	5.0	94
	1.0	67
Bathocuproin	1.0	28
Bathophenanthroline	1.0	34
Iodoacetamide	5.0	12
p-hydroxymercuri-benzoate	1.0	100
	0.5	94
	0.1	89
5,5'-dithiobis (2-nitrobenzoate)	0.5	100
	0.1	75

Reactions were measured spectrophotometrically at 30°C in a 1cm light path cuvette which contained in a volume of 1ml; 40µmol glycine/NaOH buffer (pH 10.3), 50ug protein, 0.5µmol NAD^+ and 0.5µmol cyclohexanol. The enzyme was preincubated in the buffer with the potential inhibitor for 10min at 30°C. NAD^+ was added one minute prior to the end of the preincubation period. Reactions, initiated by the addition of cyclohexanol, were monitored by following the reduction of NAD^+ at 340nm

*Calculated specific activity of cyclohexanol dehydrogenase for the control was 22.8 µmoles NAD^+ reduced.min^{-1}.(mg $protein^{-1}$).

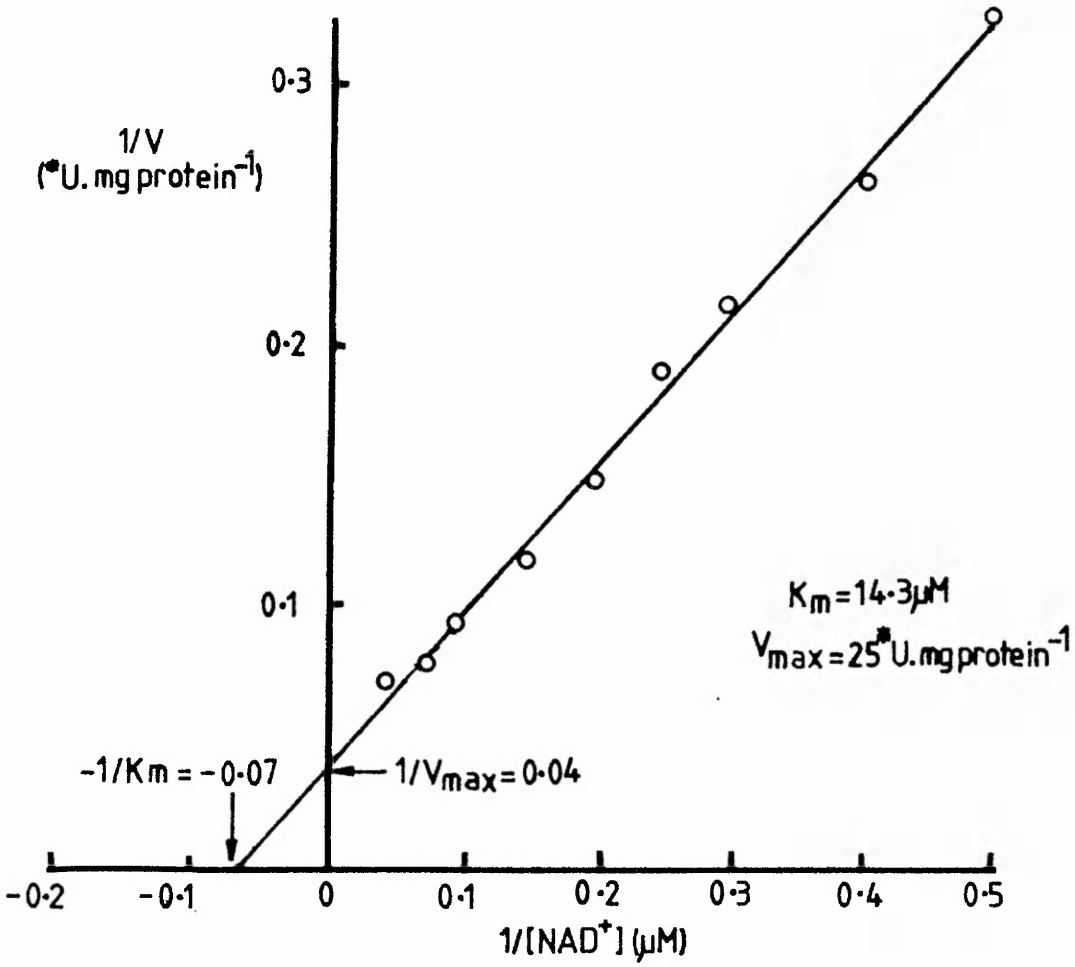
Table 5.6 Substrate specificity of cyclohexanol dehydrogenase

Substrate	Concentration (mM)	Percentage activity compared to cyclohexanol
Cyclohexanol	2.0	*100
Cyclopentanol	2.0	182
1-Methyclohexanol	2.0	0
2-Methyclohexanol	2.0	77
3-Methyclohexanol	2.0	92
4-Methyclohexanol	2.0	86
Cyclohexanol-1,2-diol	2.0	25
Cyclohexanol-1,3-diol	2.0	38
Cyclohexanol-1,4-diol	2.0	36
Menthol	2.0	0
Propan-1-ol	2.0	0
Butan-1-ol	2.0	0
Pentan-1-ol	2.0	0
Hexan-1-ol	2.0	0
Heptan-1-ol	2.0	0
Butan-2-ol	2.0	57
Heptan-3-ol	2.0	68

Reactions (at 30°C) were performed in a 1cm light path semi-micro cuvette that contained in a volume of 1ml; 25µg protein, 40 µmol glycine/NaOH buffer (pH 10.3) and 0.5 µmol NAD^+. Reactions were initiated by the addition of the desired substrate and the reduction of NAD^+ monitored spectrophotometrically at 340nm. The enzyme activity for each substrate tested is recorded as a percentage using that for cyclohexanol as a 100% reference value.

*Calculated specific activity of cyclohexanol dehydrogenase with cyclohexanol as substrate was 23.2 µmoles NAD^+ reduced. min^{-1}. (mg $protein^{-1}$).

Fig 5.7 Lineweaver - Burk plot of 1/v against 1/[S] for the reduction of NAD^+ by cyclohexanol dehydrogenase



Reactions (at 30°C) were performed in a 1cm light path semi-micro cuvette that contained in a volume of 1ml; 40μmol glycine/NaOH buffer, pH 10.3, 25μg cyclohexanol dehydrogenase and 0.5μmol cyclohexanol. Reactions were initiated by the addition of NAD^+ (2.0-20mmol) and were monitored spectrophotometrically at 340nm by following the substrate stimulated reduction of NAD^+.

*-a unit of cyclohexanol dehydrogenase activity is equal to 1μmol NAD^+ reduced.min^{-1} at 30°C.

5.3 DISCUSSION

Cyclohexanol dehydrogenase which was purified from soluble extracts of the cyclohexane-grown Xanthobacter sp. was judged to be electrophoretically pure by the formation of a single protein band on 2.25nm pore radius polyacrylamide gels. A purification factor of 30.4 was estimated after a typical purification sequence of the enzyme, which would indicate that cyclohexanol dehydrogenase constitutes 3-4% of the soluble cell protein. Such a purification value, although low, is not atypical of inducible enzymes for example the 16.1 fold purification obtained for cyclohexanone monooxygenase isolated from Xanthobacter sp. by Trower (1985), the same enzyme isolated from Nocardia globerula CL1 and purified 12.1 fold (Donoghue and Trudgill, 1976) and the 9.1 fold purification obtained from the salicylate hydroxylase isolated from Pseudomonas putida (Yamamoto et al., 1985). However, the purification value is somewhat low when compared with a purification of 126 fold obtained by Stirling and Perry (1980) for the cylohexanol dehydrogenase isolated from the cyclohexane-grown Nocardia sp.

The pH optimum of cyclohexanol dehydrogenase was determined as 10.3. This result is in keeping with other cycloalkanol dehydrogenases whose pH optima lie between 10.0-10.5 (Griffin and Trudgill, 1972; Donoghue and Trudgill, 1975; Stirling and Perry, 1980).

The electron acceptor for cyclohexanol dehydrogenase was found to be NAD^+ and to a lesser extent $NADP^+$. Various artificial and physiological electron acceptors failed to bring about cyclohexanol dehydrogenase activity. This was also true of the NAD^+ -linked cyclohexanol dehydrogenase from a cyclohexane-grown Nocardia sp. (Stirling and Perry, 1980) although for this enzyme, activity could not be demonstrated with $NADP^+$. Similarly the cyclopentanol dehydrogenase from Pseudomonas NCIB 9872 (Griffin and Trudgill, 1972) and the cyclohexanol dehydrogenase from Acinetobacter NCIB 9871 (Donoghue and Trudgill, 1975) both required NAD^+ as the electron acceptor. These latter workers also demonstrated that these cycloalkanol dehydrogenases converted the cycloalkanol to the

corresponding cycloalkanone. This was also shown for the purified cyclohexanol dehydrogenase from cyclohexane - grown Xanthobacter sp. which formed cyclohexanone as the reaction product after incubation with cyclohexanol. Similarly, Ohta et al., (1984) investigating the microbial oxidation of cyclic alcohols reported that they were converted to the corresponding ketones by Corynebacterium equi.

Size exclusion hplc and reducing SDS-polyacrylamide gel electrophoresis gave molecular weight values for cyclohexanol dehydrogenase of 44,500 and 43,000 respectively, thus indicating the monomeric nature of the enzyme. This molecular weight is comparable to that of the cyclopentanol dehydrogenase from a cyclopentanol-grown pseudomonad (Mr 54,000) characterised by Griffin (1974) and the cyclohexanol dehydrogenase from the cyclohexane-grown Pseudomonas sp. (Mr 34,000) reported by Hall (1981). However, the values for these cycloalkanol dehydrogenases are very much lower than that of Mr 145,000 obtained for the purified cyclohexanol dehydrogenase from the cyclohexane-grown Nocardia sp. (Stirling and Perry, 1980).

Enzyme inhibition studies indicated that cyclohexanol dehydrogenase was not affected by the presence of electron transport inhibitors. This is also the case with the purified cyclohexanol dehydrogenase from the cyclohexane-grown Nocardia sp. (Stirling and Perry, 1980). The enzyme displayed differential inhibition with metal chelating inhibitors, showing little inhibition with α,α'-dipyridyl and EDTA, slightly greater inhibition with the copper chelators bathocuproin and bathophenanthroline and much more inhibition with 8-hydroxyquinoline. This differential inhibition by metal chelators was also observed by Hou et al., (1980) investigating a secondary alcohol-specific dehydrogenase whilst Stirling and Perry (1980) reported that metal chelating agents had no effect on the purified secondary alcohol dehydrogenase from the cyclohexane-grown Nocardia sp. The thiol agents p-hydroxymecuri-benzoate and 5,5'-dithiobis dramatically inhibited the purified cyclohexanol dehydrogenase from Xanthobacter sp., however a greater concentration of the less potent inhibitor iodoacetamide was required to bring about any inhibition of the enzyme. This pattern

of inhibition by thiol agents has been demonstrated for other secondary alcohol dehydrogenases (Hou et al., 1980; Stirling and Perry, 1980).

Cyclohexanol dehydrogenase displayed a very broad and unique substrate specificity for secondary alcohols. The enzyme showed activity towards cycloalkanols, substituted cycloalkanols, cycloalkanediols and straight chain secondary alcohols. However, none of the primary alcohols tested could be utilised. This substrate specificity pattern is very similar to that shown by the cyclohexanol dehydrogenase from the cyclohexane-grown Nocardia sp. (Stirling and Perry 1980). These latter workers also reported that cyclohexanol dehydrogenase from Nocardia sp. had an apparent Km for NAD^+ of 2.4×10^{-5}M compared with 1.43×10^{-5}M for NAD^+ obtained with purified cyclohexanol dehydrogenase from Xanthobacter sp. Stirling and Perry were also unable to determine a K_m value for cyclohexanol using purified cyclohexanol dehydrogenase thus indicating the high affinity of these enzymes for their cycloalkanol substrates.

6. DEGRADATION OF METHYLCYCLOHEXANE BY XANTHOBACTER SP.

6.1 INTRODUCTION

The route by which Xanthobacter sp. degrades cyclohexane has been elucidated by Trower et al. (1985) and was shown to occur by the same pathway as reported for a Nocardia species (Stirling et al. 1977) and a Pseudomonas species (Anderson et al., 1980). However it is not known whether the route of metabolism for methylcyclohexane degradation by Xanthobacter sp. is the same as for cyclohexane ie. hydroxylation of the alicyclic ring or by attack of the methyl substituent.

Preliminary reports of the metabolism of short chain n-alkyl substituted cycloalkanes indicate that the initial oxidation is on the alicyclic ring (Arai and Yamada, 1969; Tonge and Higgins, 1974). Further metabolism of the methylcycloalkanol is then thought to resemble that for other cycloalkanols (Donaghue et al. 1976). However studies of the microbial degradation of long chain n-alkyl substituted cycloalkanes have indicated that attack of the n-alkyl chain occurs in preference to the alicyclic ring (Davis and Raymond, 1962; Beam and Perry, 1974b). See section 1.1.6.

In the following study two main approaches, involving studies with whole cells and cell extracts, were undertaken in order to determine the route of oxidation and metabolism of methylcyclohexane by Xanthobacter sp. Firstly cells grown on cyclohexane were investigated for their mode of oxidation of the methyl substituted cycloalkane ring and secondly, cells grown on methylcyclohexane were investigated for their route of metabolism of this substituted cycloalkane.

6.2 RESULTS

The majority of the studies in this chapter were performed with cyclohexane-grown Xanthobacter sp. and not methylcyclohexane-grown cells. The major reason for this protocol was that growth of Xanthobacter sp. on methylcyclohexane in large quantities ie/in the 5 or 20l fermenter proved to be difficult. The lower solubility and volatility of methylcyclohexane compared with cyclohexane are likely

to be the main contributors to this difficulty. Consequently, for the purpose of this chapter methylcyclohexane-grown Xanthobacter sp. was obtained by growth in shake flask cultures.

6.2.1 Reaction stoichiometry studies using cell-free extracts of cyclohexane-grown Xanthobacter sp. for methylcyclohexane oxidation

Studies using cell-free extracts from cyclohexane-grown Xanthobacter sp. were performed to investigate the reaction stoichiometry for the oxidation of possible intermediates involved in methylcyclohexane degradation. Table 6.1 indicates that the reaction stoichiometry for the oxidation of methylcyclohexane is the same as that for cyclohexane with 2 mol of oxygen being consumed for every mol of substrate added. Tables 6.2 and 6.3 indicate the reaction stoichiometry for the oxidation of the different methylcycloalkanols and methylcycloalkanones. The results demonstrate that 1 mol of oxygen was consumed for every mol of methylcycloalkanol or methylcycloalkanone added to the reaction mixture.

6.2.2 Reaction kinetics of cyclohexanol dehydrogenase and cyclohexanone monooxygenase

Investigations were carried out to determine the relative affinities of cyclohexanol dehydrogenase and cyclohexanone monooxygenase for potential intermediates resulting from methylcyclohexane oxidation. The apparent Km values were obtained by determining the initial reaction rates at various concentrations of the appropriate methylcycloalkanol or methylcycloalkanone in the presence of excess NAD^+ or NADPH respectively. The data were then used to plot Lineweaver - Burk double reciprocal graphs of 1/v against 1/[s]. The resulting straight line relationship of each graph enabled the apparent Km value to be calculated for each substrate (Fig 6.1 and 6.2).

Table 6.1 Reaction stoichiometry for the oxidation of cyclohexane and methylcyclohexane by cell-free extracts of the cyclohexane-grown Xanthobacter sp.

A. Cyclohexane added (µmol)	B. Oxygen consumed (µmol)	Ratio B/A
0.025	0.051	2.02
0.050	0.101	2.02
0.075	0.153	2.04
0.150	0.303	2.02

A. Methylcyclohexane added (µmol)	B. Oxygen consumed (µmol)	Ratio B/A
0.025	0.051	2.03
0.050	0.103	2.06
0.100	0.204	2.04
0.150	0.301	2.00

Reaction mixtures were monitored polarographically in the oxygen monitor at 30°C. Reaction mixtures contained in a volume of 3ml; 240 µmol phosphate buffer, pH 7.0, 5mg protein (100,000g supernatant), 1.0 µmol NADPH and substrate (0.025-0.150 µmol). Reactions were initiated by the addition of measured amounts of the appropriate substrate and monitored until the substrate was completely oxidised. Both substrates were dissolved in dimethylformamide to a final concentration of 0.025M so that accurate additions could be made. Dimethylformamide was found to have no effect on cyclohexane hydroxylase activity when compared to control incubations.

Table 6.2 Reaction stoichiometry for the oxidation of 2-methylcyclohexanol, 3-methylcyclohexanol and 4-methylcyclohexanol by cell-free extracts of cyclohexane-grown Xanthobacter sp.

A. 2-Methylcyclohexanol added (µmol)	B. Oxygen consumed (µmol)	Ratio B/A
0.05	0.049	0.98
0.10	0.098	0.98
0.15	0.153	1.02
0.20	0.200	1.00

A. 3-Methylcyclohexanol added (µmol)	B. Oxygen consumed (µmol)	Ratio B/A
0.05	0.052	1.04
0.10	0.106	1.06
0.15	0.208	1.04
0.30	0.298	0.99

A. 4-Methylcyclohexanol added (µmol)	B. Oxygen consumed (µmol)	Ratio B/A
0.05	0.49	0.98
0.10	0.98	0.98
0.20	0.203	1.01
0.30	0.311	1.04

Reaction mixtures were monitored polarographically in the oxygen monitor at 30°C. Reaction mixtures contained in a volume of 3ml; 240µmol phosphate buffer, pH7.0, 5mg protein (100,000g supernatant), 1.0 µmol NADPH and substrate (0.05-0.30 µmol). Reactions were initiated by the addition of measured amounts of the appropriate substrate and monitored until the substrate was completely oxidised.

Table 6.3 Reaction stoichiometry for the oxidation of 2-methylcyclohexanone, 3-methylcyclohexanone and 4-methylcyclohexanone by cell-free extracts of cyclohexane-grown Xanthobacter sp.

A. 2-Methylcyclohexanone added (µmol)	B. Oxygen consumed (µmol)	Ratio B/A
0.05	0.049	0.98
0.10	0.098	0.98
0.20	0.203	1.02
0.30	0.308	1.03

A. 3-Methylcyclohexanone added (µmol)	B. Oxygen consumed (µmol)	Ratio B/A
0.05	0.049	0.98
0.10	0.096	0.96
0.20	0.210	1.05
0.30	0.312	1.04

A. 4-Methylcyclohexanone added (µmol)	B. Oxygen consumed (µmol)	Ratio B/A
0.05	0.052	1.04
0.10	0.104	1.04
0.20	0.208	1.04
0.30	0.301	1.00

Reaction mixtures were monitored polarographically in the oxygen monitor at 30°C. Reaction mixtures contained in a volume of 3ml; 240 µmol phosphate buffer, pH7.0, 5mg protein (100,000g supernatant), 1.0 µmol NADPH and substrate (0.05-0.30 µmol). Reactions were initiated by the addition of measured amounts of the appropriate substrate and monitored until the substrate was completely oxidised.

Table 6.4 shows the apparent Km values calculated for cyclohexanol and the different methylcycloalkanols. The apparent Km for cyclohexanol was below the sensitivity measurable with the instrument. No activity was detected when 1-methylcyclohexanol was employed as the substrate. The apparent Km values for the methylcyclohexanols indicate that cyclohexanol dehydrogenase has a greater affinity for 3-methylcyclohexanol (apparent Km = 43μM) than for the other methylcycloalkanols, although there is little difference between the values for 2- and 3- methylcyclohexanol (apparent Km 72.5 μM and 43.5μM respectively).

Studies with cyclohexanone monooxygenase (Table 6.5) indicated that the apparent Km for cyclohexanone was not measurable due to the lack of sensitivity of the instrument being used (Beckman DU7). The apparent Km values calculated for the methylcyclohexanones indicated that the apparent Km for 3-methylcyclohexanone was approximately 1/3 that calculated for 4-methylcyclohexanone and approximately 2/3 that calculated for 2-methylcyclohexanone.

6.2.3 Investigation of the reaction products resulting from methylcyclohexane metabolism using cyclohexane-grown Xanthobacter species

To determine the route by which Xanthobacter sp. oxidises methylcyclohexane various studies were undertaken whereby methylcyclohexane and potential oxidation products were incubated with either washed whole cells or cell-free extracts (100,000 g supernatant). The resulting reaction products from these incubations were then analysed by either Glc, GC/MS or Tlc.

6.2.3.1 Whole cell incubations of methylcyclohexane with cyclohexane-grown Xanthobacter sp.

6.2.3.1.1 Identification of potential intermediates

Glc analysis of extracts obtained from the incubation of washed whole cells (cyclohexane-grown Xanthobacter sp.) with

Fig 6.1 Lineweaver - Burk plot of 1/v against 1/[s] for the oxidation of methylcyclohexanols by cyclohexanol dehydrogenase

Reactions (at 30°C) were performed in a 1cm light path cuvette that contained in a volume of 1ml; 40 µmol glycine/NaOH buffer, pH 10.3, 100,000 g supernatant (0.05-1.0 mg protein), 0.5 µmol NAD^+ and the required amount of cycloalkanol. Reactions were initiated by addition of the cycloalkanol and monitored spectrophotometrically by following the reduction of NAD^+ at 340nm.

Fig 6.2 Lineweaver - Burk plot of 1/v against 1/[s] for the oxidation of methylcyclohexanones by cyclohexanone monooxygenase

Reactions (at 30°C) were monitored polarographically in the oxygen monitor. Reaction mixtures contained in a volume of 3ml; 480 µmol glycine/NaOH buffer, pH8.8, 100,000 g supernatant (1.0-2.5mg protein) and 1.0 µmol NADPH. Reactions were initiated by the addition of the appropriate amount of cycloalkanone.

Fig 6.1a

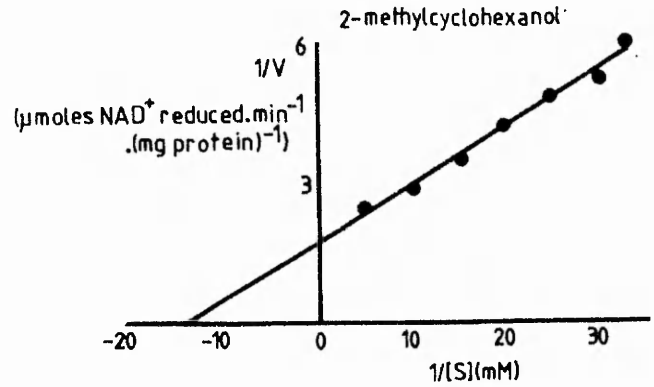


Fig 6.1b

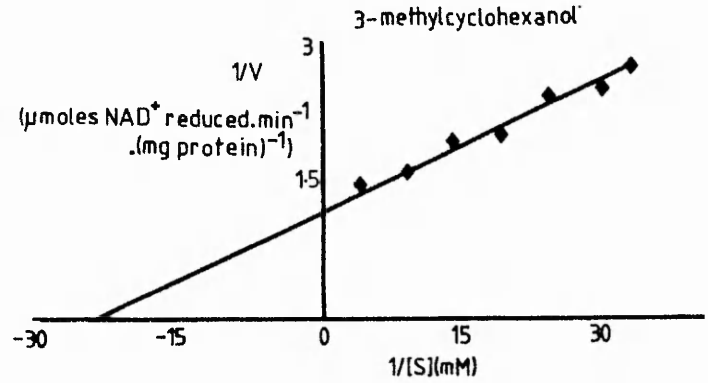


Fig 6.1c

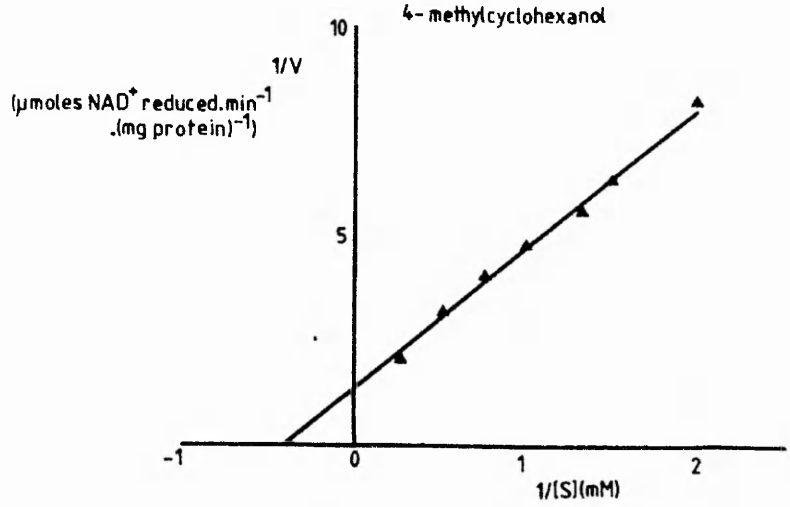


Fig 6.2

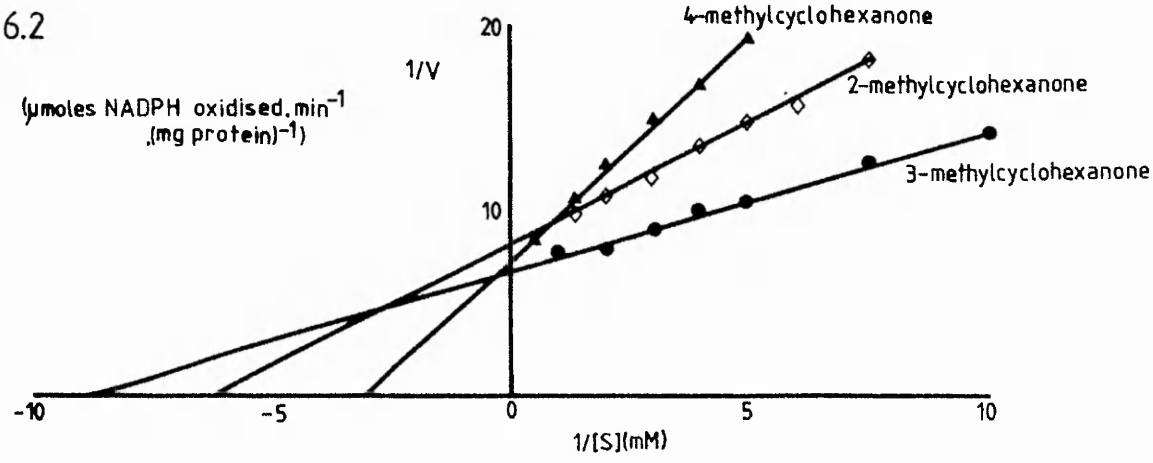


Table 6.4 Kinetic constants for cyclohexanol dehydrogenase from cyclohexane - grown Xanthobacter sp.

Substrate	Apparent Km (μM)
1-Methylcyclohexanol	ND
2-Methylcyclohexanol	72.5
3-Methylcyclohexanol	43
4-Methylcyclohexanol	2500
Cyclohexanol	ND

Reactions (at 30°C) were performed in a 1cm light path cuvette that contained in a volume of 1ml; 40μmol glycine/NaOH buffer, pH 10.3, 100,000g supernatant (0.05-1.0 mg protein), 0.4 μmol NAD^+ and the required amount of cycloalkanol. Reactions were initiated by addition of the cycloalkanol and monitored spectrophotometrically by following the reduction of NAD^+ at 340nm.

ND- representative of values below 0.1×10^{-3} μmol substrate utilised. min^{-1}. $(mg\ protein)^{-1}$

Table 6.5 Kinetic constants for cyclohexanone monooxygenase from cyclohexane-grown Xanthobacter sp.

Substrate	Apparent Km (μM)
2-Methylcyclohexanone	160
3-Methylcyclohexanone	110
4-Methylcyclohexanone	330
Cyclohexanol	ND

Reactions (at 30°C) were monitored polarographically in the oxygen monitor. Reaction mixtures contained in a volume of 3ml; 480 μmol/ glycine/NaOH buffer, pH8.8, 100,000 g supernatant (1.0-2.5 mg protein) and 1.0 μmol NADPH. Reactions were initiated by the addition of the appropriate amount of cycloalkanone.

ND - representative of values below 0.9 nmol O_2 consumed .min^{-1}. $(mg\ protein)^{-1}$

methylcyclohexane indicated the initial oxidation products to be 1-methylcyclohexanol, 3-methylcyclohexanol and 3-methylcyclohexanone (Fig 6.3 and Table 6.6). No intermediates could be detected in control incubations from which washed whole cells were omitted.

GC/MS analysis of the same sample further confirmed the presence of 3-methylcyclohexanol.

6.2.3.1.2 Identification of the dicarboxylic acid products resulting from methylcyclohexane oxidation

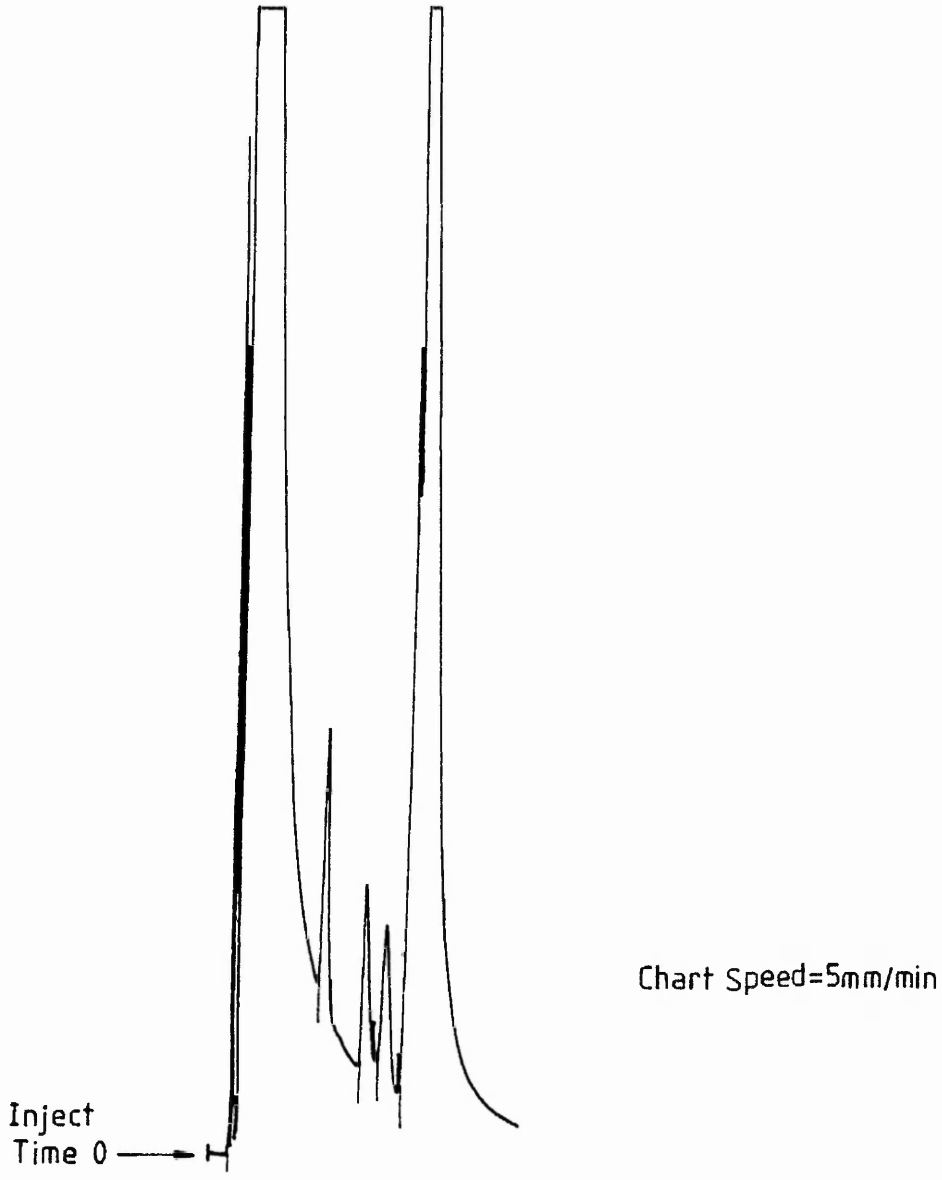
The dicarboxylic acids resulting from whole cell incubations with methylcyclohexane were extracted with ethyl acetate and then converted to their methyl esters before analysis by GC/MS (System E). Results of this analysis indicated the presence of glutaric acid, adipic acid and methyl adipic acid in the relative ratios of 6:4:3 respectively. No products could be detected in control incubations from which washed whole cells were omitted.

6.2.3.2 Cell-free extract studies with methylcyclohexane using cyclohexane-grown Xanthobacter sp.

6.2.3.2.1 Identification of potential intermediates

Glc analysis of extracts obtained from the incubation of 100,000 g supernatant with NADPH and methylcylohexane indicated the presence of 1-methylcyclohexanol. In order to increase the accumulation of any initial pathway intermediates resulting from the hydroxylase reaction an NADPH regeneration system was introduced into the incubation system. By keeping the $NADP^+$ in its reduced form it was hoped that the flux of any intermediates through the pathway by the cyclohexanol dehydrogenase would be either blocked or restricted. However, the presence of the NADPH regeneration system did not result in the accumulation of any further intermediates which could be identified in extracts from these incubations.

Fig 6.3 Identification (by glc) of reaction products resulting from the incubation of washed whole cells of cyclohexane-grown Xanthobacter sp. with methylcyclohexane



Incubation mixtures contained, in a volume of 10ml; 1.0 mmol phosphate buffer, pH 7.0, 1g (wet weight) washed cells and 100 µl methylcyclohexane. Control incubations were assayed identically with the exception that the washed cells were omitted from the incubation mixture. Reaction mixtures were incubated for 15h at 30°C, extracted as described in section 2.9.1 and analysed by glc (system A) according to the procedure described in section 2.10.1.

Table 6.6 Identification (by glc) of reaction products resulting from the incubation of washed whole cells of cyclohexane-grown Xanthobacter sp. with methylcyclohexane.

Compound	A	B
1- Methylcyclohexanol	3.6	4.3
2- Methylcyclohexanol	4.5, 4.9	5.3, 5.8
3- Methylcyclohexanol	5.5, 6.2	6.8, 7.7
4- Methylcyclohexanol	6.0, 6.7	7.0, 7.9
2- Methylcyclohexanone	4.0	4.9
3- Methylcyclohexanone	4.8	5.7
4 -Methylcyclohexanone	5.2	6.0
Reaction products from incubation of whole cells with Methylcyclohexane	3.6 5.5 6.2 4.8	4.3 6.8 7.7 5.7

Incubation mixtures contained, in a volume of 10ml; 1.0 mmol phosphate buffer, pH 7.0, 1g washed cells and 100µl methylcyclohexane. Control incubations were assayed identically with the exception that the whole cells were omitted from the incubation mixture. Reactions mixtures were incubated for 15h at 30°C, extracted as described in section 2.9.1 and analysed by glc (systems A and B) according to the procedure described in section 2.1.0.1.

Bracketed figures represent cis and trans isomers.

6.2.3.3 Incubation of cell-free extracts from cyclohexane-grown Xanthobacter sp. with methylcyclohexanols and methylcyclohexanones

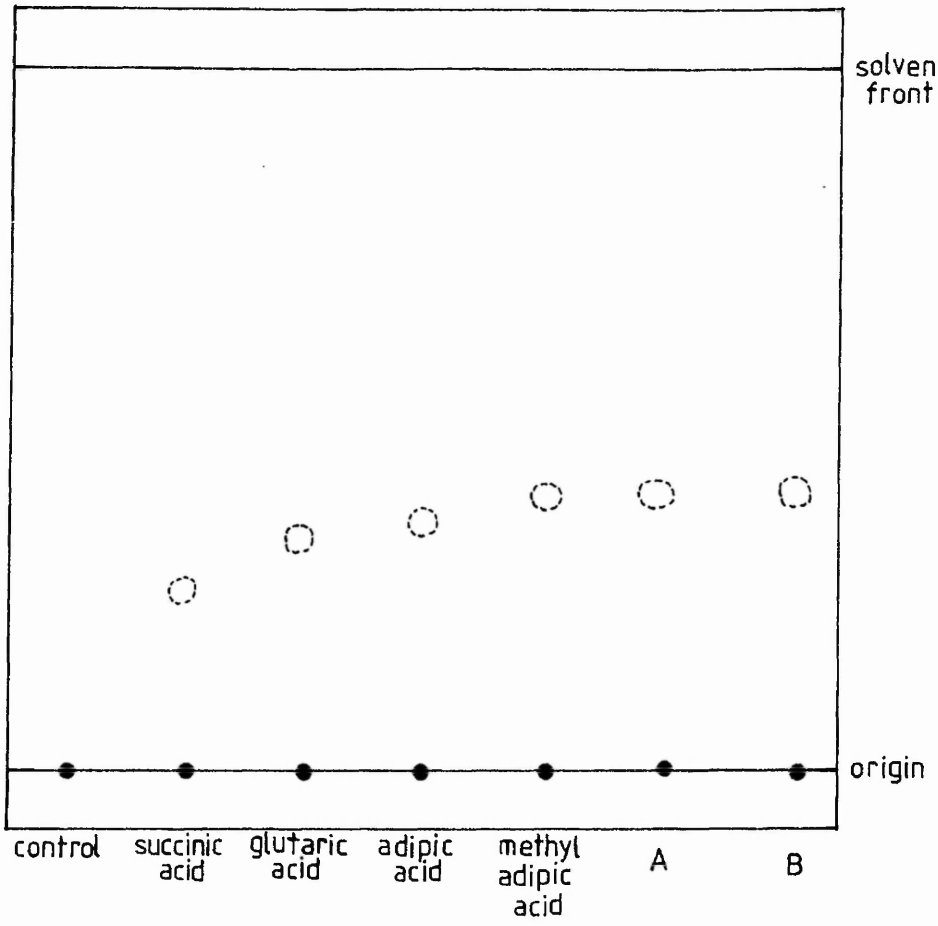
Cell-free extracts of cyclohexane-grown Xanthobacter sp. were incubated with a number of possible initial oxidation products of methylcyclohexane degradation. It was anticipated that it would be possible to compare the end products of these reactions with the end products resulting from whole cell incubations with methylcyclohexane. Cell extracts (100,000 g supernatant) were therefore incubated with, 1-, 2-, 3- and 4-methylcyclohexanol and also 2-, 3- and 4-methylcyclohexanone as substrates.

6.2.3.3.1 Identification of dicarboxylic acid end products

Ethyl acetate extracts resulting from the incubation of 100,000 g supernatant with methylcyclohexanols and methylcyclohexanones were initially analysed by tlc to determine the presence or absence of dicarboxylic acid end products from these reactions. Using as a preliminary screening technique only incubations containing 3-methylcyclohexanol and 3-methylcyclohexanone gave positive results with respect to the identification of methyl adipic acid (Fig 6.4 and Table 6.7). No products could be detected in extracts from control incubations.

Further analysis of these samples was undertaken by glc. Samples were first derivatised in order to convert any dicarboxylic acids present to their methyl esters and then analysed as previously described by glc. Extracts from 1- and 2- methylcyclohexanol incubations were found to contain both succinic acid and methyl adipic acid. Incubations containing 3-methylcyclohexanol produced succinic, adipic and methyl adipic acid while adipic acid and methyladipic acid were identified in extracts from the incubation with 4-methylcyclohexanol (Table 6.8). Glc analysis of the incubation containing 2-methylcyclohexanone indicated the presence of succinic acid and glutaric acid whilst adipic acid and methyl adipic acid were identified in extracts from incubations containing

Fig 6.4. Identification (by tlc) of reaction products resulting from the incubation of cell-free extracts with 3-methylcyclohexanol and 3-methylcyclohexanone.



A = 3-methylcyclohexanol incubation

B = 3-methylcyclohexanone incubation

Incubation mixtures contained in a volume of 3ml; 200 µmol phosphate buffer, pH 7.0, 100,000 g supernatant (5mg protein), 10.0 µmol NAD^+ and NADPH, and 10.0 µmol substrate. Control incubations were assayed identically with the exception that 100,000 g supernatant was replaced with boiled 100,000 g supernatant. Reaction mixtures were incubated for 1h at 30°C, extracted as described in section 2.9.2 and analysed by tlc (system 2) according to the procedure described in section 2.10.3.

Table 6.7 Identification (by tlc) of reaction products resulting from the incubation of cell-free extracts with 3-methylcyclohexanol and 3-methylcyclohexanone.

Compound	R_F Value	
	1	2
Standards		
Succinic acid	0.26	0.22
Glutaric acid	0.33	0.27
Adipic acid	0.35	0.29
Methyl adipic acid	0.39	0.34
Reaction product		
From 3-methylcyclohexanol	0.39	0.34
From 3-methylcyclohexanone	0.39	0.34

Incubation mixtures contained, in a volume of 3ml; 200 µmol phosphate buffer, pH 7.0, 100,000 g supernatant (5mg protein), 10.0 µmol NAD+ and NADPH, and 10.0 µmol substrate. Control incubations were assayed identically with the exception that 100,000 g supernatant was replaced with boiled 100,000 g supernatant. Reaction mixtures were incubated for 1h at 30°C, extracted as described in section 2.9.2 and analysed by tlc, (systems 1 and 2) according to the procedure described in section 2.10.3.

3- and 4- methylcyclohexanone (Table 6.9). No products could be detected in extracts from control incubations.

6.2.4. Studies with methylcyclohexanane-grown Xanthobacter sp.

A number of studies were performed to determine whether the whole cell oxidation patterns and the enzymes present in cell-free extracts from methylcyclohexane-grown Xanthobacter sp. were comparable to those found in cyclohexane-grown Xanthobacter sp.

6.2.4.1 Whole cell oxidation studies with cyclohexane-grown and methylcyclohexane-grown Xanthobacter sp.

Whole cell oxidation studies were undertaken using methylcyclohexane-grown and cyclohexane-grown Xanthobacter sp. (Table 6.10). The results showed that both sets of cells were capable of oxidising all the compounds provided as substrates which included cycloalkanes, substituted cycloalkanes, methylcyclohexanols and cyclohexandiols.

6.2.4.2 Studies with cell-free extracts from methylcyclohexane-grown Xanthobacter sp.

6.2.4.2.1 Detection of cyclohexanol dehydrogenase activity

Incubation of cell-free extracts (100,000 g supernatant) with glycine/NaOH buffer pH 10.3, NAD^+ and cyclohexanol gave rise to a substrate stimulated reduction of NAD^+ indicating the presence of cyclohexanol dehydrogenase activity.

6.2.4.2.2 pH Optimum of cyclohexanol dehydrogenase

The pH optimum of cyclohexanol dehydrogenase from methylcyclohexane-grown Xanthobacter sp. was estimated at 10.2 by

Table 6.8 Identification (by glc) of reaction products resulting from the incubation of cell-free extracts with methylcycloalkanols.

Compound	Retention time (min)	
	C	D
Standards		
Succinic acid methyl ester	7.1	9.4
Glutaric acid methyl ester	9.2	11.6
Adipic acid methyl ester	11.7	13.7
Methyl adipic acid methyl ester	12.0	14.5
Reaction products	7.1	9.4
From 1-methylcyclohexanol	12.0	14.5
	7.1	9.4
From 2-methylcyclohexanol	12.0	14.5
	7.1	9.4
From 3-methylcyclohexanol	11.7	13.7
	12.0	14.5
	11.7	13.7
From 4-methylcyclohexanol	12.0	14.5

Incubation mixtures contained, in a volume of 3ml; 200 µmol phosphate buffer, pH 7.0, 100,000 g supernatant (5mg protein), 10.0 µmol NAD^+, 10.0 µmol NADPH and 10.0 µmol substrate. Control incubations were assayed identically with the exception that 100,000 g supernatant was replaced with boiled 100,000 g supernatant. Reaction mixtures were incubated for 1h at 30°C, extracted as described in section 2.9.2, derivatised according to the procedure in section 2.9.3 and analysed by glc (systems C and D) as described in section 2.10.1.

Table 6.9 Identification (by glc) of reaction products resulting from the incubation of cell-free extracts with methylcycloalkanones.

Compound	Retention time (min)	
	C	D
Standards		
Succinic acid methyl ester	7.1	9.4
Glutaric acid methyl ester	9.2	11.6
Adipic acid methyl ester	11.7	13.7
Methyl adipic acid methyl ester	12.0	14.5
Reaction products	7.1	9.4
From 2-methylcyclohexanone	-	11.6
	11.7	13.7
From 3-methylcyclohexanone	12.0	14.5
	11.7	13.7
From 4-methylcyclohexanone	-	14.5

Incubation mixtures contained, in a volume of 3ml; 200 µmol phosphate buffer, pH 7.0, 100,000g supernatant (5mg protein), 10.0 µmol NADPH and 10.0 µmol substrate. Control incubations were assayed identically with the exception that 100,000 g supernatant was replaced with boiled 100,000 g supernatant. Reaction mixtures were incubated for 1h at 30°C, extracted as described in section 2.9.2, derivatised according to the procedure in section 2.9.3. and analysed by glc (systems C and D) as described in section 2.10.1.

Table 6.10 Oxidation of cycloalkanes and related compounds by washed cell suspensions of cyclohexane-grown and methylcyclohexane-grown Xanthobacter sp.

Substrate	% Oxygen uptake Growth substrate	
	Cyclohexane	Methylcyclohexane
Cyclohexane	[a]100	[b]100
Cyclohexanol	72	55
Cyclohexanone	67	90
1-oxa-2-oxocycloheptane	47	81
Methylcyclohexane	55	53
Ethycycyclohexane	18	12
1-methylcyclohexanol	47	57
2-methylcyclohexanol	44	66
3-methylcyclohexanol	71	44
4-methylcyclohexanol	81	56
Cyclohexan-1,2-diol	115	83
Cyclohexan-1,3-diol	67	51
Cyclohexan-1,4-diol	80	43

Respiration rates were followed polarographically in the oxygen monitor at 30°C. Incubation mixtures contained in a volume of 3ml; 60 µmol phosphate buffer (pH 7.0), 100µl cell suspension (approx 2.5 mg dry weight) and 20 µmol substrate. Water immiscible substrates (2ul) were added directly to the incubation chamber.

[a] Cyclohexane-grown Xanthobacter sp. 100% activity with cyclohexane was equivalent to 7.5 µmol O_2 consumed. h^{-1} (mg dry $weight^{-1}$).

[b] Methylcyclohexane-grown Xanthobacter sp. 100% activity with cyclohexane was equivalen to 8.3 µmol O_2 consumed. h^{-1}. (mg dry $weight^{-1}$).

measurement of enzyme activity over the pH range 8.0-11.6. The specific activity of the enzyme measured at pH 10.2 was 0.72 μmol NAD^+ reduced. min^{-1}. $(mg\ protein)^{-1}$.

6.2.4.2.3 Cofactor specificity of cyclohexanol dehydrogenase

Cyclohexanol dehydrogenase activity was detected when NAD^+ was supplied as the cofactor. A much reduced level (24%) of cyclohexanol dehydrogenase activity was detected when NAD^+ was replaced with $NADP^+$ as the cofactor.

6.2.4.2.4 Substrate specificity of cyclohexanol dehydrogenase

The substrate specificity of cyclohexanol dehydrogenase in cell-free extracts of methylcyclohexane-grown Xanthobacter sp. was investigated and compared to that from cyclohexane-grown Xanthobacter sp. (Table 6.11). The substrate specificity of methylclohexane-grown Xanthobacter sp. was similar to that of cyclohexane-grown Xanthobacter sp., but with a number of substrates, in particular cyclopentanol and the cyclohexandiols the relative dehydrogenase activity was greater with methycyclohexane-grown cells compared to cyclohexane-grown cells.

6.2.4.2.5 Detection of cyclohexanone monooxygenase activity

Incubation of cell-free extract (100,000 g supernatant) with glycine/NaOH buffer pH8.8, NADPH and cyclohexanone gave rise to the substrate stimulated consumption of O_2 when measured in the oxygen monitor indicating the presence of cyclohexane monooxygenase activity.

Table 6.11 Relative activity of cyclohexanol dehydrogenase from cyclohexane-grown and methylcyclohexane-grown Xanthobacter sp. towards different substrates.

Substrate	Relative cyclohexanol dehydrogenase activity	
	Growth substrate	
	Cyclohexane	Methylcyclohexane
Cyclohexanol	[a]100	[b]100
Cyclopentanol	174	266
1-Methylcyclohexanol	0	0
2-Methylcyclohexanol	65	86
3-Methylcyclohexanol	99	130
4-Methylcyclohexanol	91	134
Cyclohexan-1,2-diol	24	65
Cyclohexan-1,3-diol	27	109
Cyclohexan-1,4-diol	30	115
2-Hydroxycyclohexanone	0	0

Reaction mixtures contained in a volume of 1ml; glycine/NaOH buffer pH 10.2, 100,000g supernatant (0.1g protein), 0.5 µmol NAD+ and 0.5 µmol appropriate substrate. Reactions (at 30°C) were initiated by the addition of substrate and the subsequent reduction of NAD^+ monitored spectrophotometrically at 340nm.

[a] Calculated specific activity of cyclohexanol dehydrogenase with cyclohexanol as substrate was 0.79 µmol. min^{-1}. $(mg\ protein)^{-1}$.

[b] Calculated specific activity of cyclohexanol dehydrogenase with cyclohexanol as substrate was 0.72 µmol. min^{-1}. $(mg\ protein)^{-1}$.

6.2.4.2.6 pH optimum of cyclohexanone monooxygenase

The pH optimum of cyclohexanone monooxygenase from methylcyclohexane-grown Xanthobacter sp. was estimated at pH 8.8 by measurement of enzyme activity over the pH range 7.5-10.5. The specific activity of the enzyme measured at pH 8.8 was 0.11 μmol NADPH oxidised.min^{-1}. (mg protein)$^{-1}$.

6.2.4.2.7 Cofactor specificity of cyclohexanone monooxygenase

The replacement of NADPH with NADH failed to produce a cyclohexanone stimulated consumption of O_2 when measured in the oxygen monitor indicating that cyclohexanone monooxygenase was unable to utilise NADH as a cofactor.

6.2.4.2.8 Substrate specificity of cyclohexanone monooxygenase

The substrate specificity of cyclohexanone monooxygenase in cell-free extracts of methylcyclohexane-grown Xanthobacter sp. was investigated and compared to that from cyclohexane-grown Xanthobacter sp. (Table 6.12). The results indicated similar patterns of substrate specificity for both types of cells with neither being able to oxidise cyclohexan-1, 3-dione.

A comparison of some of the properties of the cyclohexanol dehydrogenase and cyclohexanone monooxygenase present in cyclohexane-grown and methylcyclohexane-grown Xanthobacter sp. is shown in Table 6.13.

6.2.4.3 Identification of reaction products resulting from the incubation of washed whole cells of methylcyclohexane-grown Xanthobacter sp. with methylcyclohexane

Glc analysis of ethyl acetate extracts obtained from the incubation of whole cells (1g wet weight methylcyclohexane-grown Xanthobacter sp.) with methylcyclohexane (100 μl) indicated 1-

Table 6.12 Relative activity of cyclohexanone monooxygenase from cyclohexane-grown and methylcyclohexane-grown Xanthobacter sp. towards different substrates.

Substrate	Relative cyclohexanone monooxygenase activity	
	Growth substrate	
	Cyclohexane	Methylcyclohexane
Cyclohexanone	[a]100	[b]100
Cyclopentanone	75	95
2-Methylcyclohexanone	91	57
3-Methylcyclohexanone	118	80
4-Methylcyclohexanone	110	82
Cyclohexan-1,2-dione	58	73
Cyclohexan-1,3-dione	0	0
Cyclohexan-1,4-dione	89	92
2-Hydroxycyclohexanone	68	57

Reactions were monitored polarographically in the oxygen monitor at 30°C. Reaction mixtures contained, in a volume of 3ml; glycine/NaOH buffer pH 8.8, 100,000 g supernatant (0.5 mg protein), 1.0 µmol NADPH and 2.0 µmol appropriate substrate. Reactions were initiated by the addition of substrate.

[a] Calculated specific activity of cyclohexane monooxygenase with cyclohexanone as substrate was 0.13 $\mu mol.min^{-1}$. $(mg\ protein)^{-1}$.

[b] Calculated specific activity of cyclohexanone monoonxygenase with cyclohexanone as substrate was 0.11 $\mu mol.min^{-1}$. $(mg\ protein)^{-1}$.

Table 6.13 Comparison of cyclohexanol dehydrogenase and cyclohexanone monooxygenase from cyclohexane-grown and methylcyclohexane-grown Xanthobacter sp.

	Cyclohexane-grown Xanthobacter sp.	Methylcyclohexane-grown Xanthobacter sp.
Cyclohexanol dehydrogenase		
pH optimum	10.3	10.2
Cofactor specificity	NAD^+ $NADP^+$	NAD^+ $NADP^+$
Cyclohexanone monooxygenase		
pH optimum	[a]8.8	8.8
Cofactor specificity	[a]NADPH	NADPH

[a] Data from Trower et al (1985)

Enzymes were assayed according to the procedures described in section 2.6.

methylcyclohexanol to be the only detectable intermediate of the reaction. No intermediates could be detected in control incubations from which the washed cells had been omitted.

No dicarboxylic acid products following derivatisation to their methyl esters could be detected in extracts from the incubation. Similarly no dicarboxylic acid products could be detected in control incubations from which the whole cells had been omitted.

6.2.4.4 Identification of reaction products resulting from the incubation of cell-free extracts from methylcyclohexane-grown Xanthobacter sp. with methylclohexane

Glc analysis of extracts obtained from the incubation of 100,000 g supernatant (5mg protein) with NADPH (10 μmol) and methylcyclohexane (10 μmol) indicated the presence of 1-methylcyclohexanol. No dicarboxylic acid products following derivatisation to their methyl esters could be detected in extracts from the incubation.

No intermediates or dicarboxylic acid products could be detected in control incubations in which the 100,000 g supernatant was replaced with boiled cell-free extract.

6.3 DISCUSSION

The objective of the work described in this chapter was to investigate the metabolism of methylcyclohexane by Xanthobacter sp. Difficulties were however encountered in obtaining large quantities of methylcyclohexane-grown cells that would allow substantive studies using cell-free extracts. To overcome this problem a number of investigations were undertaken using both whole cells and cell-free extracts from cyclohexane-grown cells. The rationale behind this approach was that if the metabolism of methylcyclohexane by methylcyclohexane-grown Xanthobacter sp. was via the same route as that operating for the metabolism of cyclohexane ie/cyclohexane ⟶ cyclohexanol ⟶ cyclohexanone ⟶ 1-oxa-2-oxocyloheptane ⟶ ⟶

adipic acid; then cells grown on cyclohexane should have the capacity to oxidise methylcyclohexane using the enzymes induced for cyclohexane metabolism and vice versa. This hypothesis assumes that cyclohexane-grown cells are as permeable to methylcyclohexane as they are to cyclohexane and that methylcyclohexane-grown cells are permeable to cyclohexane.

Reaction stoichiometry studies using cell-free extracts of cyclohexane-grown Xanthobacter sp. indicated the oxidation of methylcyclohexane to be similar, stoichiometrically, to that found for cyclohexane. In both cases 2 mol of oxygen were consumed for every mol of substrate added. This suggests that one mol of oxygen is required for the initial hydroxylation reaction and the second atom of oxygen, like that for cyclohexane oxidation, is inserted into the ring to form a lactone. Confirmation of this comes from the reaction stoichiometry found for the oxidation of methylcycloalkanols and methylcycloalkanones where 1 mol of oxygen was consumed for every mol of substrate added. This indicates that in all these cases one atom of oxygen is inserted into the ring to form a lactone. The evidence is therefore consistent with the theory that methylcyclohexane may be degraded by hydroxylation on the alicyclic ring and not by attack on the methyl substituent.

Further studies were undertaken to try and determine the actual route of methylcyclohexane degradation. Investigations were carried out to determine the regiospecificity of cyclohexane hydroxylase ie/in which position on the ring does the enzyme hydroxylate in relation to the methyl substituent. The relative affinities of cyclohexanol dehydrogenase and cyclohexanone monooxygenase towards potential intermediates of methylcyclohexane degradation were determined by calculation of the apparent Km values for these different substrates. Examination of the methylcyclohexanols and methylcyclohexanones indicated that 3-methylcyclohexanol was the preferred substrate of cyclohexanol dehydrogenase while cyclohexanone monooxygenase had greatest affinity for 3-methyl-cyclohexanone. These results would therefore suggest that Xanthobacter sp. hydroxylates methylcyclohexane in the meta position.

The detection of 1-methylcyclohexanol in extracts from whole cell and cell-free extract incubations of methylcyclohexane with cyclohexane-grown Xanthobacter sp. is somewhat puzzling as it would appear to be a "dead-end" metabolite possibly resulting as a consequence of the tertiary carbon being the most susceptible to oxidation. 1-Methylcyclohexanol is not a substrate for cyclohexanol dehydrogenase and concentrations of 0.06mM or above are necessary to stimulate further hydroxylation by cyclohexane hydroxylase activity when using cell-free extracts of cyclohexane-grown Xanthobacter sp. The accumulation of 3-methylcyclohexanol and 3-methylcyclohexanone during whole cell incubations with methylcyclohexane further indicates that cyclohexane hydroxylase hydroxylates methylcyclohexane in the meta position. These results are similar to those of Tonge and Higgins (1974) who reported the presence of 3-methylcyclohexanol and 3-methylcyclohexanone in the growth media of Nocardia petroleophila following growth on methylcyclohexane.

The whole cell incubation studies which yielded glutaric, adipic and methyl adipic acid as end products may indicate that several pathways are operating during methylcyclohexane metabolism. The identification of methyl adipic acid would indicate that methylcyclohexane is metabolised in the same manner as cyclohexane and that the methyl substituent has no direct involvement in the initial route of degradation of this compound.

The identification of glutaric and adipic acid from methylcyclohexane is not easily explained. The production of glutaric acid from methylcyclohexane indicates that two carbons have been removed from the compound possibly as acetyl CoA. To produce adipic acid from from methylcyclohexane involves the removal of one carbon atom. This could possibly be achieved by α-oxidation followed by w-oxidation as indicated in Fig. 6.5.

The identification of methyl adipic acid by tlc analysis of incubations of cell-free extracts with 3-methylcyclohexanol and 3-methylcyclohexanone correlates with the GC/MS analysis of whole cell incubations with methylcyclohexane which indicated methyl adipic acid to be one of the end products.

The glc analysis of extracts from cell-free extract

incubations with methylcycloalkanols and methylcycloalkanones indicates a variety of products to be present. 1-,2- and 3-methylcyclohexanol and 2-methylcyclohexanone incubations all indicated succinic acid to be present. The presence of this compound suggests that three carbon atoms have been removed from methyladipic acid. This may have been brought about by β-oxidation causing the elimination of propionyl CoA and giving rise to succinic acid. Fig 6.5 indicates that succinic acid may be formed from 2- and 3-methylcyclohexanol and 2-methylcyclohexanone.

1- and 2-methylcyclohexanol also produced methyl adipic acid which was shown to be present in extracts from the methylcyclohexane whole cell incubations. The route by which 1-methylcyclohexanol is oxidised to succinic acid and methyl adipic acid is unknown and is cause for further investigation as 1-methylcyclohexanol is not utilised by cyclohexanol dehydrogenase and only by cyclohexane hydroxylase when present at a concentration of 0.06mM or above. However, these studies using both whole cells and cell-free extracts indicate it to be a potential intermediate of methylcyclohexane oxidation.

The 2-methylcyclohexanone incubation produced glutaric acid as a reaction end product. This also correlates with the GC/MS finding from the whole cell incubations with methylcyclohexane. It is possible that glutaric acid is formed only when hydroxylation of methylcyclohexane occurs at the ortho position. Further investigations are required to confirm this.

The 3- and 4-methylcyclohexanol and 3- and 4- methyl-cyclohexanone incubations all gave rise to the same end products namely adipic acid and methyladipic acid, the presence of which correlate with the methylcyclohexane whole cell incubations.

Not many experiments were performed with methylcyclohexane-grown Xanthobacter sp. mainly due to the difficulty in growing large quantities of the microorganism with this carbon source. Initial studies with methylcyclohexane-grown Xanthobacter sp. involved a comparison of whole cell oxidation patterns and the enzymes present in cell-free extracts with those of cyclohexane-grown Xanthobacter sp. The whole cell oxidation studies indicated that methyl-

cyclohexane-grown Xanthobacter sp. oxidised cycloalkanes and related compounds in a similar fashion to cyclohexane-grown Xanthobacter sp. These initial results therefore suggested that a major route for methylcyclohexane degradation by this organism is likely to be by a route analogous to that described by Trower et al. (1985) for cyclohexane-grown Xanthobacter sp. ie/cyclohexane → cyclohexanol → cyclohexanone → 1-oxa-2-oxocycloheptane → → adipic acid. This is the same pathway described by Stirling et al. (1977) for the degradation of cyclohexane by a Nocardia sp. and by Anderson et al. (1980) for the degradation of the same alicyclic hydrocarbon by a Pseudomonas sp. Confirmation of such a route came from further studies using cell-free extracts of methylcyclohexane-grown Xanthobacter sp. which were found to contain comparable amounts of cyclohexanol dehydrogenase to that found in the cyclohexane-grown organism. The enzyme was found to be similar with regard to pH optimum and cofactor specificity and furthermore the substrate specificities of cyclohexanol dehydrogenase from methylcyclohexane-grown and cyclohexane-grown Xanthobacter sp. were found to be comparable.

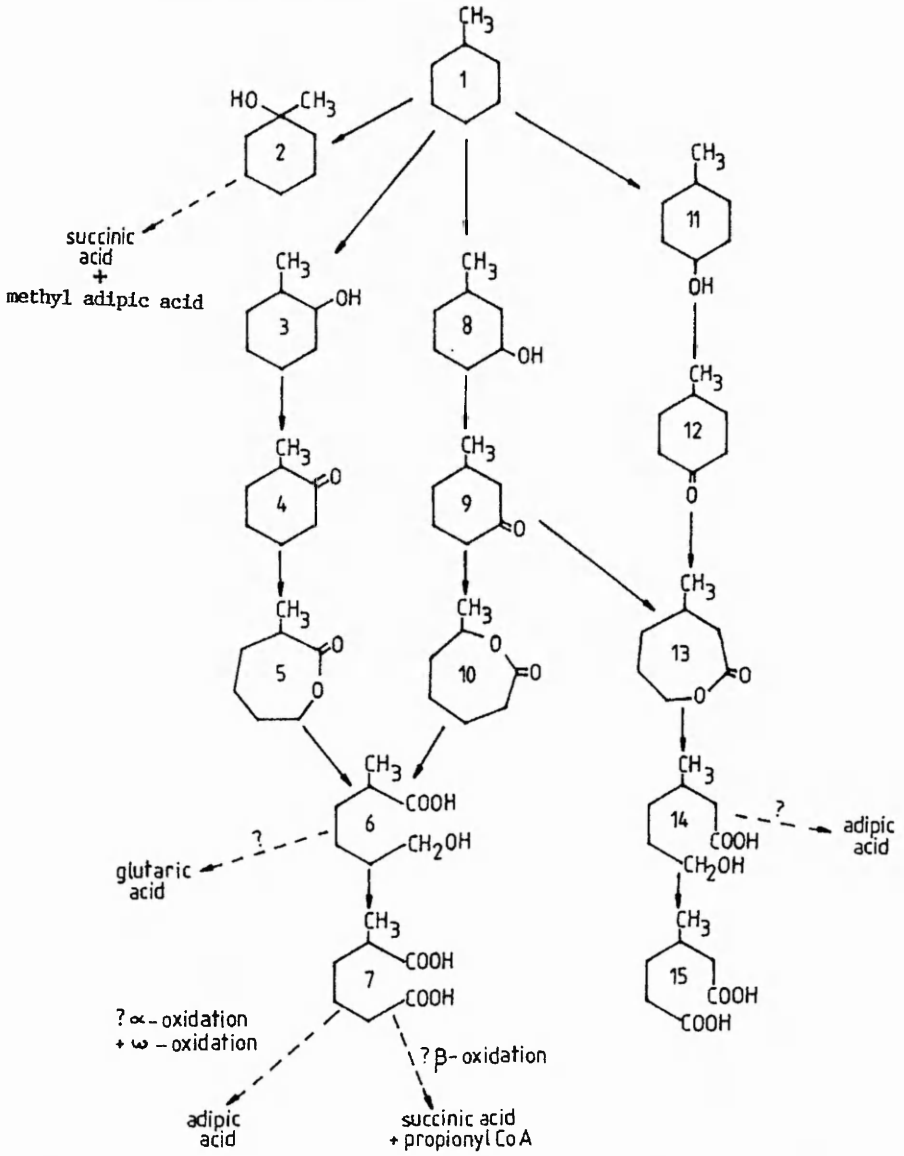
Similarly the cyclohexanone monooxygenase found in cell-free extracts from methylcyclohexane-grown Xanthobacter sp. was found to be comparable to the same enzyme found in cyclohexane-grown Xanthobacter sp. (Trower et al., 1985) with regard to pH optimum and cofactor specificity. The substrate specificities of the two oxgenase enzymes isolated from methylcyclohexane-grown and cyclohexane-grown Xanthobacter sp. were also found to be comparable.

The whole cell and cell-free extract incubations of methylcyclohexane-grown Xanthobacter sp. with methylcyclohexane indicated 1-methylcyclohexanol to be the only detectable intermediate and in both cases no acidic end products could be detected. These results compare with those gained from incubations using cyclohexane-grown Xanthobacter sp. The difficulties in explaining the presence of this metabolite have been eluded to earlier in this discussion.

In conclusion it would appear that a number of pathways may be operating in the degradation of methylcyclohexane by Xanthobacter

sp. (Fig. 6.5). However, the results do indicate that one major route of metabolism of methylcyclohexane by Xanthobacter sp. occurs via the same pathway described by Trower et al. (1985) for the degradation of cyclohexane. Furthermore studies regarding the regiospecificity of cyclohexane hydroxylase in methylcyclohexane metabolism indicate that the enzyme brings about hydroxylation at the tertiary carbon and meta position of the alicyclic ring.

Fig 6.5 Proposed routes for the oxidation of methylcyclohexane by Xanthobacter sp.



Key: Compounds; (1) methylcyclohexane, (2) 1-methylcyclohexanol, (3) 2-methylcyclohexanol, (4) 2-methylcyclohexanone, (5) 1-oxa-2-oxo-3-methylcycloheptane, (6) 2-methyl-6-hydroxyhexanoate, (7)2-methyladipic acid, (8) 3-methylcyclohexanol, (9) 3-methylcylcohexanone, (10) 1-oxa-2-oxo-7-methylcycloheptane, (11) 4-methylcyclohexanol, (12) 4-methyclylohexanone, (13) 1-oxa-2-oxo-4-methylcycloheptane, (14) 3-methyl-6-hydroxyhexanoate and (15) 3-methyladipic acid.

7. GENERAL DISCUSSION

The initial aim of this project was to investigate the metabolism of cycloalkanes by different species of Xanthobacter. A more detailed study was also undertaken on the Xanthobacter sp. isolated by Trower et al. (1985) with regard to its ability to metabolise cyclohexane and methylcyclohexane.

Wiegel and Schlegel (1984) who described the new genus of Xanthobacter in Bergey's Manual of Systematic Bacteriology referred to two species of this genus, X.autotrophicus and X.flavus, both of which had previously belonged to different genera. Section 3 of this thesis investigated the ability of different Xanthobacter to degrade cycloalkanes. The use of substrate utilisation to determine speciation within genera is a useful technique widely employed in bacterial classification. The ability of Xanthobacter to degrade cycloalkanes was therefore employed as a potential method to determine the different subgroups that exist within this genus. The ability to degrade cycloalkanes and related compounds appeared to be restricted to X.autotrophicus and related strains including Xanthobacter sp. These studies may be of interest to other workers who are presently investigating the speciation of the genus Xanthobacter (Jenni et al., 1987; Jenni and Aragno, 1987). These workers using DNA-DNA hybridisation and numerical analysis have recently identified a further species of Xanthobacter named X.agilis.

Given the scarcity of reports of cycloalkane - degrading micro-organisms it is necessary to consider the factors that enabled Trower et al. (1985) to isolate a species of Xanthobacter capable of growth on cyclohexane. As mentioned in section 1.1.2.3. There are only a few reports of microorganisms with the capacity to degrade cycloalkanes although the reasons for this phenomenom are not clear. However, these alicyclic hydrocarbons do not accumulate in the terrestrial environment, implying that a mechanism for their degradation does exist. Suggestions have been put forward to explain the lack of successful attempts to isolate cycloalkane-degrading microorganisms. Trudgill (1978) suggested that it may be necessary

to implement specialised enrichment techniques in order to isolate microorganisms capable of utilising cycloalkanes. However the Xanthobacter sp. reported by Trower et al. (1985) was isolated by classical enrichment techniques and no specialised enrichment procedures were necessary.

Some workers (Beam and Perry, 1974[a]; de Klerk and van der Linden, 1974) suggested that cycloalkane degradation may be brought about by commensalism and co-oxidation. This is understandable considering the 'artificial' conditions which are set up in the laboratory upon isolation of an organism, whereas in nature these microorganisms exist as mixed populations. However, the fact that Trower et al. (1985) were able to isolate a bacterium capable of autonomous growth on cyclohexane indicates that microbial metabolism of cycloalkanes is not always the result of commensalism or co-oxidation.

It has also been suggested (Trudgill, 1978) that organisms capable of degrading cycloalkanes are only present in areas which contain sufficient quantities of these carbon sources. Trower et al. (1985) isolated the Xanthobacter sp. from the soil of a north Nottinghamshire forest. In this area there are two potential sources of alicyclic hydrocarbons. The first comes form plant metabolites which may have accumulated during the life of the forest and the second from the large quantities of fossil fuels present in this coal mining area. The presence of alicyclic plant metabolites may have helped in facilitating the cycloalkane degrading ability of microorganisms. It may be argued however that this is unlikely owing to the complex structure of many plant metabolites. However, section 4.2.3 does indicate the cyclohexane hydroxylase from Xanthobacter sp. to have a broad substrate specificity, a point in favour of plant metabolites serving as effectors of cycloalkane degradation. The presence of sub-terrestrial fossil fuels may have caused seepage of cycloalkanes into the terrestrial environment exerting a positve pressure on microorganisms present to degrade these compounds. The Pseudomonas sp. reported by Anderson et al. (1980) was isolated from mixed cultures obtained from the same area as the Xanthobacter sp.

This may indicate that microorganisms with this unusual capacity to degrade cycloalkanes are limited in their distribution and that this area of north Nottinghamshire does possess positive effectors for alicyclic hydrocarbon degradation. It is interesting that when Trower et al. (1985) isolated the Xanthobacter sp. they did not also detect the Pseudomonas sp. isolated by Anderson et al. (1980) and vice versa.

The important cyclohexane hydroxylase responsible for the initial attack on cyclohexane was shown to be a soluble cytochrome P-450 containing enzyme system. Further studies demonstrated it to be a 3-component (Type II cytochrome P-450) monooxygenase. The enzyme system is thought to contain a ferredoxin and a ferredoxin reductase - like component which have recently been identified by assaying with cytochrome C (T. Worrall - personal communication). Current work involves purifying the cytochrome P-450 with the aim of reconstituting cyclohexane hydroxylase activity using ferredoxin and ferredoxin reductase obtained commercially. This would allow further examination of the substrate specificity and inhibition patterns of the enzyme and also allow identification of its catalytic products. Future work will involve the purification and characterisation of the ferredoxin and ferredoxin reductase components of the enzyme system.

Studies on the degradation of methylcyclohexane by Xanthobacter sp. indicated that this short chain alkyl substituted cycloalkane is degraded by hydroxylation of the alicyclic ring and not by attack of the methyl substituent. This route of oxidation is supported by the knowledge that Xanthobacter sp. is unable to grow on either the long chain n-alkyl cycloalkanes or their oxidation products,namely cyclohexane acetic acid and cyclohexane carboxylic acid. These investigations have indicated that the major route of methyl-cyclohexane degradation is by the same catabolic route as cyclohexane with ring cleavage being brought about via oxidative lactone formation. However, it would appear that other pathways involving the intermediate 1-methylcyclohexanol may also be in

operation during methylcyclohexane degradation and further investigations are necessary in order to clarify this point.

The unusual metabolic capacity of Xanthobacter sp. to degrade cycloalkanes might render it suitable for industrial exploitation. The organism might be employed industrially for the manufacture of high value compounds or compounds which are dangerous or difficult to produce by conventional chemical techniques. The possibility of using Xanthobacter sp. for the production of dicarboxylic acids and substituted dicarboxylic acids in particular adipic acid, a nylon precursor, has already been investigated by Trower (1985).

The fact that cyclohexane hydroxylase possesses a fairly broad substrate specificity (section 4.2.3) suggests that the potential for biotransformations with Xanthobacter sp. is high. One great advantage of using enzymes as oppose to conventional chemical techniques is the higher degree of stereo - and regioselectivity available. Whilst cyclohexane hydroxylase does possess a broad substrate specificity it is unlikely that this enzyme could be employed for the biotrans formation of such complex substituted cycloalkanes as steroids. However the methylene hydroxylation of less complex alicyclic compounds, especially those which result in the production of valuable cyclic monoterpene alcohols, may be of considerable interest to the pharmaceutical or flavour and fragrance industry.

8. REFERENCES

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9. APPENDIX

9.1 SUPPLIERS

9.1.1 Chemicals

Chemicals used were of the purest grades and obtained from the following sources:

Air Products Ltd., Crewe, Staffordshire. Nitrogen.

Aldrich Chemical Co., Gillingham, Dorset. Cyclodecane, cyclohexan - 1,3-diol, cyclohexan-1,2-dione, cyclohexan-1,3-dione, cyclohexan-1, 4-dione, cyclohexanol, cyclohexene oxide, cyclooctane, cyclopentane, cyclopentanol, decane, dicyclohexyl, dodecane, ethylcyclohexane, ethylcyclopentane, 1-methyl-1, 4-cyclohexadiene, methylcyclohexane, 1-methylcyclohexanol, 2-methylcyclohexanol, 3-methylcyclohexanol, 4-methylcyclohexanol, 2-methylcyclohexanone, 3-methylcyclohexanone, 4-methylcyclohexanone, methylcyclopentane, methylcyclopentanol, methylene cyclohexane, octane, pyrrolidine and quadricyclane.

Alltech U.K. Ltd., Carnforth, Lancs. Carbowax 20M, Chromosorb W, DEGS/PS, FFAP and Silar 5CP.

British Drug Houses (BDH) Chemicals Ltd., Poole, Dorset. 'Aristar' : cyclohexane, cyclohexene and ethyl acetate. 'Analar' : adipic acid, ammonium persulphate, ammonium sulphate, amytal, benzene, bromocresol green, butan-1-ol, butan-2-ol, $CaCl_2.2H_2O$, $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, dimethylformamide, EDTA, ethanol, $FeSO_4.7H_2O$, Folin-Ciocalteau phenol reagent, glucose-6-phosphate, glutaric acid, glycerol, glycine, H_3BO_3, HCl, hexadecane, 8-hydroxyquinoline, KH_2PO_4, K_2HPO_4, methanol, methyladipic acid, $MgSO_4.7H_2O$, $MnSO_4$ $4H_2O$, NaH_2PO_4, Na_2HPO_4, $Na_2HPO_4.12H_2O$, $Na_2MoO_4.2H_2O$, N,N'-methylene bisacrylamide, phenolindo-2,6-dichlorophenol, potassium chloride, potassium chromate, potassium cyanide, propan-1-ol, sodium acetate,

sodium arsenate, sodium chloride, sodium hydroxide, sodium potassium tartrate, sodium tartrate, succinic acid, sucrose, sulphosalicyclic acid, N,N,N^1,N^1-tetramethylethylene diamine (TEMED), toluene, trichloroacetic acid (TCA) and $ZnSO_4.7H_2O$. 'Biochemical' : acrylamide and sodium dodecyl sulphate.

Bio-Rad Laboratories, California, U.S.A. Biogel 0.5-A.

Difco Laboratories, Mitchigan, U.S.A. Bacto-agar, nutrient agar (NA), nutrient broth (NB), skimmed milk and yeast extract.

Foster and Co., Nottingham. Carbon monoxide.

Koch-Light, Massachusetts, U.S.A. Cyclohexan-1,2-diol, cyclohexan-1,4-diol, cyclohexanone, 1-oxa-2-oxocycloheptane and undecane.

LKB Produktor - AB, Bromma, Sweden. Trisacryl Blue M and AcA 44 Ultrogel.

Merck, Darmstadt, West Germany. Kieselgel 60 (0.2mm thickness) and cellulose (0.1mm thickness) tlc plates (20 x 20 cm).

Pfaltz and Bauer Inc., Connecticut, U.S.A. 2-Hydroxycyclohexanone.

Pharmacia, Uppsala, Sweden. Blue dextran 2000 and protein molecular weight standards.

Sigma Chemical Co Ltd., Poole, Dorset. Antiform A emulsion, bathocuproine, bathophenanthroline, biotin, calcium pantothenate, coomassie brilliant blue G-250, cytochrome C, α,α'-dipyridyl, 5,5'-dithiobis (2-nitrobenzoate), dithiothreitol, ethanolamine, fatty acid free bovine serum albumin (BSA), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), folic acid, glucose-6-phosphate

dehydrogenase, N-2-hydroxyethylpiperazine-N^1-2-ethane sulphonic acid (HEPES), p-hydroxymercuribenzoate, iodoacetamide, (-) menthol, NAD^+, NADH, $NADP^+$, NADPH, neocuproine, nicotinic acid, nitroblue tetrazolium (NBT), PAGE blue 83, 1,10-phenanthroline, phenazine methosulphate (PMS), protein molecular weight standards, pyridoxin-HCl, riboflavin, rotenone, thiamine, Tris and vitamin B_{12}.

9.1.2 Hardware

Autoclave : Model 225E. Laboratory Thermal Equipment, Oldham, Lancs.

Centrifuges:

		Rotor	Tube vol. (ml)	Max. force (xg)
(i)	MSE Centuar 1. MSE Scientific Instruments, Crawley, Sussex	-	15	1,100
(ii)	MSE Mistral 3000	-	2.5	1,380
(iii)	Sharples continuous centrifuge Pennwalt Ltd., Surrey.	-	-	14,300
(iv)	MSE Europa 24M.	43115-113	6x500	18,000
(v)	MSE 21 high speed centrifuge	43114-143 43115-112	8x50 6x300	49,640 31,770
(vi)	Beckman L870 ultracentrifuge Beckman Instruments, California, U.S.A.	Ti70	8x38.5	504,000

Computing integrater. Model SP 4100. Spectra - Physics Inc., California, U.S.A.

Dialysis visking tubing. Scientific Instruments Centre, London.

Disposable Petri dishes. Sterilin, Teddington.

Electrophoresis apparatus

(i) Disc electrophoresis apparatus. Shandon Southern Products Ltd., Runcorn, Cheshire.

(ii) Vertical electrophoresis apparatus. Model 2001. LKB Produktor - AB, Bromma, Sweden.

Fermentation equipment. 502D and 1000 series fermenters, 2, 5 and 201 fermentation vessels. L.H. Engineering Co. Ltd., Stoke Poges, Bucks.

Filters

(i) Whatman No.1 cellulose and 0.45 μm cellulose-nitrate. Whatman, Maidstone, Kent.

(ii) 0.2μm Polycarbonate bacterial filter. Nucleopore Inc., California, U.S.A.

Freeze drier. Edwards EF03. Edwards High Vacuum, Crawley Sussex.

French Pressure cell. American Instrument Co., Maryland, U.S.A.

Gas liquid chromatograph. Model F33, Perkin-Elmer, Conneticut, U.S.A.

Gyratory shaker Gallenkamp orbital incubator, Galllenkamp, London.

HPLC Equipment

(i) Columns. Guard column. Ultropac TSK-GSWP (7.5 x 75mm) precolumn. Size exclusion column. Ultropac TSK 3000 SW (7.5x600mm). LKB Produktor-AB, Bromma, Sweden.

Anion exchange column. Mono Q pre-packed HR 5/5 (5 x 50 mm). Pharmacia, Uppsala, Sweden.

(ii) Sample injector. Model 7125. Rheodyne, Cocati, California, U.S.A.

(iii) Pump-Model M-45. UV detector-Model 440. Waters Associates Inc., Massachusetts, U.S.A. Pump-Model 6000A. Solvent programmer-Model 660. Waters Millipore, Massachusetts, U.S.A.

Magnetic stirrer Model PC-353, Corning Ltd., Essex.

Oxygen monitor. Yellow Springs Instrument Co., Ohio, U.S.A.

pH Meter. Model PW 9409. Phillips Pye Unicam, Cambridge.

Protein purification equipment

(i) Micropump. Gilson minpuls. 2., Anachem, Luton.

(ii) Fraction collector - Redirac 2112. Column equipment. LKB Produktor - AB, Bromma, Sweden.

Sonicator. Soniprep 150. MSE Scientific Instruments, Crawley, Sussex.

Spectrophotometers

(i) Pye Unicam SP6-400. Pye Unicam, Cambridge.

(ii) Beckman DU-7. Beckman, California, U.S.A.

Ultrafiltration equipment

(i) Centrisart ultrafiltration unit SM 132 49E (Mr cut-off 20,000). Sartorius Instruments Ltd., Belmont, Surrey.

(ii) Ultrafiltration cell chamber-Model 52.
Ultrafilters - diaflo XM100 A (Mr cut-off 100,000) and PM10 (Mr cut-off 10,000). Amicon Ltd., Woking, Surrey.

Vacuum pump. Edwards High Vacuum, Crawley, Sussex.

X-press Biox-AB, Stockholm, Sweden.

9.2 MICROBIAL GROWTH MEDIA

9.2.1 Mineral Salts media

Liquid mineral salts media

	$g.l^{-1}$
$Na_2HPO_4.12H_2O$	4.5
KH_2PO_4 (anhydrous)	1.0
$(NH_4)_2SO_4$	1.8
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.04

Trace element solution 1.0 $ml.l^{-1}$

Solid mineral salts media

	$g.l^{-1}$
Na_2HPO_4	1.56
K_2HPO_4	1.90
$(NH_4)_2SO_4$	1.80
$MgSO_4.7H_2O$	0.20
$CaCl_2.2H_2O$	0.01
Bacto-agar	12.50

Trace element solution 1.0 $ml.l^{-1}$.

The trace element solution was prepared as follows:-

Solution A :- 10g $FeSO_4.7H_2O$ + 9ml concentrated H_2SO_4 made up to 150 ml with distilled water.

Solution B

	$g.l^{-1}$
$CuSO_4.5H_2O$	0.40
H_3BO_3	0.28
$MnSO_4.4H_2O$	2.00
$ZnSO_4.7H_2O$	2.00
$CoCl_2.6H_2O$	0.40
$Na_2MoO_4.2H_2O$	0.40

The final trace element solution was prepared by mixing 15ml solution A with 50ml solution B and then made up to 1l with distilled water. This solution was kept at 4°C.

9.2.2 Nutrient media

Nutrient agar (NA) and Nutrient broth (NB) were prepared as instructed by the manufacturers (Difco Laboratories, Mitchigan,

U.S.A.). Once prepared, the media was incubated at 30°C for 48 hours to check for contamination before storing at 4°C.

9.3 Electrophoresis Stock Solutions

9.3.1 Polyacrylamide gel electrophoresis (PAGE) for protein analysis

Gel buffer

A solution of 4.56g Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) and 0.17ml TEMED (N,N,N',N'-tetra-methyl-1,2-diaminoethane) was titrated to pH 8.9 with 0.2M HCl and made up to 100ml with distilled water.

Acrylamide solution

33% (w/v) acrylamide in distilled water.

N,N^1-methylene bisacrylamide solution

2.25% (w/v) N,N^1-methylene bisacrylamide solution in distilled water.

Ammonium persulphate solution

0.75% (w/v) ammonium persulphate solution in distilled water prepared immediately prior to use.

Tank buffer

A solution of 2.85g glycine dissolved in 800ml distilled water was titrated to pH 8.2 with 0.2M Tris (2-amino-2-hydroxymethylpropane-1,3-diol) and made up to 1l with distilled water giving a 38mM glycine solution.

Tracking dye

A solution of 0.05% (w/v) bromophenol blue and 10% (w/v) sucrose in tank buffer.

Gel stain for protein

0.25% (w/v) Coomassie brilliant blue G-250 in a mixture of methanol : distilled water : glacial acetic acid (5:5:1 by volume).

Gel destain

Mixture of methanol : glacial acetic acid : distilled water (3:1:7 by volume).

9.3.2 Polyacrylamide gel electrophoresis (PAGE) for in situ staining of cyclohexanol dehydrogenase activity

Gel buffer

A solution of 6.06g Tris (2-amino-2-hydroxymethyl propane-1,3-diol) and 0.23ml TEMED (N,N,N^1,N^1-tetramethyl-1,2-diaminoethane) was titrated to pH 7.5 with acetic acid and made up to 100ml with distilled water.

Acrylamide solution

30.0g acrylamide and 0.8g N,N^1-methylene bisacrylamide dissolved in distilled water and made up to 100ml with distilled water.

Ammonium persulphate solution

0.56% (w/v) ammonium persulphate in distilled water prepared freshly immediately prior to use.

Tank buffer

A solution of 6.06g Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) was titrated to pH 7.5 with acetic acid and made up to 1l with distilled water.

Tracking dye

A solution of 0.05% (w/v) bromophenol blue and 10% (w/v) sucrose in tank buffer.

Gel stain for cyclohexanol dehydrogenase activity

A solution of 17mM glycine NaOH buffer (pH 10.3) for Xanthobacter sp. cell extracts or 17mM glycine/NaOH buffer (pH 9.2) for Xanthobacter autotrophicus 431 cell extracts, 0.1M cyclohexanol, 2mM NAD^+, 0.4mM Nitro blue tetrazolium and 0.1mM phenazine methosulphate.

9.3.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis with discontinuous buffers

Acrylamide solution

30% (w/v) acrylamide and 0.8% (w/v) N,N^1-methylene bisacrylamide made up in distilled water.

Resolving gel buffer

A solution of 90.83g Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) and 2g Sodium dodecyl sulphate titrated to pH 8.8 with HCl and made up to 1l with distilled water.

Stacking gel buffer

A solution of 30.28g Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) and 2g sodium dodecyl sulphate titrated to pH 6.8 with HCl and made up to 1l with distilled water.

Ammonium persulphate solution

1% (w/v) ammonium persulphate solution prepared freshly immediately prior to use.

Tank buffer

A solution of 15.15g Tris (2-amino-2-hydroxymethyl-propane-1,3-diol), 72g glycine and 5g SDS was titrated to pH 8.3 with HCl and made up to 5l with distilled water.

Fixative

37g Sulphosalicyclic acid, 50g TCA and 2% (v/v) acetic acid in 500ml of 1:1 methanol : water mixture.

Gel stain for protein

1.25g PAGE Blue 83 was dissolved in a small volume of methanol and filtered under vacuum. The filtrate was made up to 180ml with methanol, to which 50ml glacial acetic acid and 670 ml distilled water were added.

Gel destain

Mixture of methanol : glacial acetic acid : distilled water (3:1:7 by volume).

9.4 Preparation of Compounds

9.4.1 Preparation of 6-hydroxyhexanoate

Preparation of 6-hydroxyhexanoate was by alkaline hydrolysis of 1-oxa-2-oxocycloheptane as described by Norris and Trudgill (1971).

A solution of 1mmol 1-oxa-1-oxocyloheptane in 16ml 1.2M NaOH was incubated in a boiling water bath for 20 mins, and after cooling on ice 20ml of 20mM phosphate buffer (pH 7.0) was added. The pH of the solution was adjusted to pH 7.0 by the addition of 2.0M HCl and the volume made up to 50ml with 20mM phosphate buffer (pH 7.0). The 6-hydroxyhexanoate solution (20 $\mu mol.ml^{-1}$) did not contain any detectable residual lactone.

9.5 Specialised Reagents and Solutions

9.5.1 Biuret reagent for protein assay

A solution of 1.5g Copper sulphate pentahydrate and 6.0g Sodium potassium tartrate was made in 500ml distilled water. To this solution 300ml of 10% (w/v) sodium hydroxide was added and the resulting solution made up to 1l with distilled water.

9.5.2 Folin-Ciocalteau reagents for protein assay

Solution A

0.4% (w/v) sodium hydroxide, 2.0% (w/v) sodium carbonate and 0.02% (w/v) sodium tartrate in distilled water.

Solution B

0.5% (w/v) copper sulphate pentahydrate in distilled water.

9.5.3 Chromagenic reagent for the identification of carboxylic acids on thin layer chromatography plates

Bromocresol green 0.7% (w/v) in 9.5% (v/v) aqueous ethanol adjusted to pH 6.0 with 0.1M Sodium hydroxide.

10.5.4 Saturated ammonium sulphate solution for protein precipitation

400g Ammonium sulphate was dissolved in 500ml 20mM phosphate buffer (pH 7.0). The solution was filtered through a Whatman No.1. disc. (Whatman, Maidstone, Kent) to remove any insoluble material and adjusted to pH 7.0 with 0.5M sodium hydroxide. The resulting solution was stored at 4°C.

9.6 Preparation of Buffer Solutions

9.6.1 Phosphate buffers

200mM Phosphate buffer pH 7.0

A stock buffer solution (200mM) was prepared by dissolving 28.4g Na_2HPO_4 in distilled water and titrating to pH 7.0 with 200mM $NaH_2PO_4.2H_2O$ (31.2g l^{-1}) and making up to 1l with distilled water. This stock buffer solution was diluted with distilled water when other buffer concentrations were required.

100mM phosphate buffer pH 6.0-7.5

Stock solutions

A) 200mM Na_2HPO_4 (28.39 $g.l^{-1}$)

B) 200mM KH_2PO_4 (27.22$g.l^{-1}$)

Required pH	ml A	ml B
6.0	5.75	44.25
6.1	7.00	43.00
6.2	8.25	41.75
6.3	10.00	40.00
6.4	12.50	37.50
6.5	15.50	34.50
6.6	18.75	31.25
6.7	22.00	28.00
6.8	25.00	25.00
6.9	30.00	20.00
7.0	31.50	18.50
7.1	35.00	15.00
7.2	36.50	13.50
7.3	40.00	10.00
7.4	42.25	7.75
7.5	42.75	7.25

9.6.2 20mM Ethanolamine - HCl buffer pH 9.5

1.222g ethanolamine in distilled water was titrated to pH 9.5 with HCl and made up to 1l with distilled water.

9.6.3 HEPES buffer pH 7.4

8.057g Sodium sulphate decahydrate, 2.979g N-2-Hydroxyethyethyl piperazine-N^1-2-ethane sulphonic acid, 93mg EDTA and 12.5ml glycerol in 150ml distilled water was adjusted to pH 7.4 with NaOH and made up to 250ml with distilled water.

9.6.4 0.25M Sodium acetate buffer pH 5.0

20.51g Sodium acetate was dissolved in distilled water and the pH adjusted to 5.0 with acetic acid. The solution was then made up to 1l with distilled water.

9.6.5 Glycine/NaOH buffers

50mM Glycine/NaOH buffer pH 9.2 and 10.3

0.375 glycine was dissolved in 25ml distiled water and titrated to pH 9.2 or 10.3 with 20mM NaOH as required and made up to 100ml with distilled water.

200mM Glycine/NaOH buffer pH 8.8

1.5g glycine was dissolved in 25ml distilled water. The pH of the solution was adjusted to 8.8 with 0.2M NaOH and made up to 100ml with distilled water.

9.6.6 Universal buffers pH 6.0 - 11.6

A stock solution of 6.008g Citric acid, 3.893g anhydrous potassium dihydrogen orthophosphate, 1.769g basic acid and 5.266g diethylbarbituric acid were contained in 1l of distilled water. An aliquot of this stock solution was titrated to the required pH with 0.2M NaOH.

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Characterisation of cyclohexane hydroxylase; involvement of a cytochrome P-450 system from a cyclohexane grown *Xanthobacter sp.*

(Ring methylene hydroxylase; Type II Cytochrome P-450 system)

E J Warburton[1], A M Magor[1], M K Trower[2] and *M Griffin[1]

[1]Department of Life Sciences, Trent Polytechnic,

Clifton Lane, Nottingham, NG11 8NS, UK.

(Tel: 0602-418418)

[2]MRC Molecular Genetics Unit,

Hills Road, Cambridge, CB2 2QH, UK.

* Corresponding Author.

1.

ABSTRACT

Growth of a Xanthobacter sp. on cyclohexane as the sole source of carbon and energy leads to the induction of a soluble, NADPH-dependent cyclohexane hydroxylase. Studies with this enzyme have indicated it to possess cytochrome P-450 as the terminal oxidase. Cytochrome P-450 was shown to be induced concomitantly with cyclohexane hydroxylase activity following growth of Xanthobacter sp. on cyclohexane. The enzyme has a broad substrate specificity which includes alkyl substituted cycloalkanes, aromatic hydrocarbons, a bicyclic terpene and heterocyclic rings. Cyclohexane hydroxylase was shown to be sensitive to sulphydryl group inhibitors and to show differential sensitivity to metal chelators.

2.

INTRODUCTION

An important step in the microbial metabolism of cyclohexane is its initial hydroxylation to cyclohexanol [1]. Further metabolism of cyclohexanol then proceeds via a pathway whereby the ring is opened by the formation of an unstable lactone [2]. A number of micro-organisms are capable of growth on cyclohexanol and other cycloalkanols [3,4,5]. In contrast a scarcity of reports exist for the autonomous growth of micro-organisms on the more recalcitrant cyclohexane. With the exception of a cyclohexane growing Xanthobacter sp. little success has been achieved in characterising the initial key enzyme-cyclohexane hydroxylase [6,7].

Characterisation of bacterial ring methylene hydroxylases has so far been limited to those enzymes involved in the hydroxylation of bicyclic and multi ring structures [8] of which the 5-exo camphor hydroxylase isolated from a Pseudomonas putida ATCC 29607 has served as one of the best characterised examples [9].

We previously indicated that the cyclohexane hydroxylase isolated from a cyclohexane growing Xanthobacter sp. was a soluble NADPH-requiring mono-oxygenase enzyme [1]. In the present report we have further

characterised this enzyme and present evidence to indicate that this inducible enzyme contains a cytochrome P-450 component as its terminal oxidase.

3. MATERIALS AND METHODS

3.1 Organism and growth - The bacterium used in this study was previously identified as a Xanthobacter sp. [1]. Growth of this Xanthobacter on cyclohexane was according to the procedures described by Trower et al. [1].

3.2 Preparation of cell free extracts - Cell extracts were prepared by a modification of the method described by Trower et al. [1]. Protein concentration was estimated by the biuret method of Gornall et al. [10]

3.3 Cyclohexane hydroxylase activity - Cyclohexane hydroxylase was assayed using the procedure described by Trower et al. [1] except reactions were performed at pH 6.8.

3.4 Cytochrome P-450 assay - Cytochrome (Cyt) P-450 was assayed by reduced CO-difference spectrophotometry as described by Omura and Sato [11] using a Beckman DU7 spectrophotometer.

3.5 Induction of cytochrome P-450 by cyclohexane - Xanthobacter sp. was grown on 400 ml minimal salts medium containing succinate as the sole carbon source. These cells were then transferred to a 5 l fermenter (L.H. Engineering Co. Ltd., Stoke Poges, Bucks.) containing minimal medium to which cyclohexane was supplied as a vapour through the main air supply which was set at 1.0 $l.min^{-1}$. Samples (200 ml) were removed periodically for measurement of their cell density (A550nm), cyclohexane hydroxylase activity and cytochrome P-450 content.

3.6 Chemicals - NADPH was purchased from Boehringer Mannheim, Lewes, East Sussex, U.K. All other chemicals were purchased from BDH Ltd., Poole, Dorset, U.K. and were of the highest commercial grade available.

4. RESULTS AND DISCUSSION

Various extraction methods (X-press, French pressure cell and ultrasonication) were investigated to achieve optimum yields of cyclohexane hydroxylase activity. Conditions finally chosen involved the addition of NADPH (0.1 mM) to the unbroken cell suspension, partial freezing of the cell suspension and ultrasonication using the conditions described by Trower et al. [1]. Subsequent measurement of cyclohexane hydroxylase in cell extracts (100,000 g supernatant) demonstrated a cyclohexane dependent stimulation in O_2 consumption (specific activity 22.9 nmol NADPH oxidised min^{-1} $(mg\ protein)^{-1}$), at least twice that previously reported [1].

To determine the inducibility of cytochrome P-450, cell extracts (100,000 g supernatant) from log phase cultures (A 550 nm = 1.6) grown on sodium succinate, which lacked detectable cyclohexane hydroxylase activity, produced a dithionite/CO-difference spectrum without an absorbance peak at 450 nm (Fig 1a). In contrast, cell extracts from cyclohexane-grown cultures produced a reduced CO-difference spectrum with an absorbance peat at 450 nm, indicative of the induction of cytochrome P-450 by growth on cyclohexane (Fig 1b)

Experiments were performed to determine whether there was any correlation between the induction of cytochrome P-450 and cyclohexane hydroxylase activity following growth of Xanthobacter sp. on cyclohexane. Cytochrome P-450 and cyclohexane hydroxylase activity were detected during the early exponential phase of growth on cyclohexane. Both continued to increase rapidly and concomitantly during exponential growth (Fig 2). The two components reached maximum levels at A 550 nm (80 pmol.mg $protein^{-1}$ for cytochrome P-450; 19.9 nmol NADPH oxidised min^{-1} $(mg\ protein)^{-1}$ for cyclohexane hydroxylase activity). After this both components began to decline slowly but again in parallel.

Considering the well established multicomponent nature of other methylene hydroxylases [8, 9, 12, 13] our data are therefore consistent with the idea that the terminal oxidase component of cyclohexane hydroxylase is a cytochrome P-450. The soluble nature of the cytochrome P-450 suggests that cyclohexane hydroxylase

may have a number of properties in common with two other bacterial ring methylene hydroxylases, the camphor 5-exohydroxylase from Pseudomonas putida ATCC 29607 [9] and the steroid 15 β-hydroxylase from Bacillus megaterium ATCC 13368 [8], each of which are three component systems with a soluble cytochrome P-450 as their terminal oxidase component.

A number of inhibitors were tested to determine their effect on the endogenous NADPH oxidase and cyclohexane hydroxylase activity present in cell extracts (100,000 g supernatant) from cyclohexane-grown Xanthobacter sp.

Cyclohexane hydroxylase activity showed little sensitivity to electron transport inhibitors KCN, rotenone, amytal and sodium arsenate (all at 1 mM). Cyclohexane hydroxylase activity showed a high degree of sensitivity to sulphydryl reagents 0.1 mM p-hydroxymercuribenzoate, 0.1 mM 5,5'-dithiobis-2-nitrobenzoate and 1.0 mM iodoacetamide giving 98, 99 and 96% inhibition respectively, a property shared by the cytochrome P-450 component of the camphor 5-exohydroxylase and by other multimeric oxygenases which contain an iron-sulphur protein in their electron transport chain [14, 15]. Differential sensitivity was shown to metal chelators with 0.1 mM phenanthroline, 0.5 mM bathophenanthroline and 1.0 mM EDTA leading to 82%, 40% and 52% inhibition respectively, but 1.0 mM α,α'-dipyridyl and neocuproine had no effect on enzyme activity. This inhibition pattern is common amongst haem and non-haem containing multicomponent monooxygenases, indeed the spectrum of activity shown by cyclohexane hydroxylase towards these inhibitors closely resembles that shown by many soluble methane monooxygenases [16, 17, 18]. In contrast NADPH oxidase showed little sensitivity towards any of the above inhibitors tested. It was therefore difficult to assess whether NADPH oxidase was coupled to the substrate stimulated cyclohexane hydroxylase activity.

Substrate specificity studies on cyclohexane hydroxylase were undertaken on cell extracts (100,000 g supernatant) since preliminary studies to purify this enzyme have met with loss of activity. The initial hydroxylation of the

cycloalkane ring has previously been shown to be the rate limiting step of any further reactions which may be taking place and is representative of cyclohexane hydroxylase activity [1].

A wide range of ring containing compounds were utilised (Table 1), however no activity was shown towards dicyclohexyl, p-menthane or any of the straight chain alkanes (C_8-C_{16}). No O_2 was released from any of the incubation mixtures when catalase was added indicating the absence of H_2O_2, hence all active substrates were likely to be acting as true substrates of the enzyme.

This substrate range is much narrower than the microsomal cytochrome P-450 isolated from liver [19] and the soluble methane monooxygenases isolated from methylotrophic bacteria [17, 18, 20, 21]. It is presently difficult to compare this substrate specificity to that of similar ring methylene hydroxylases since little information is available concerning their substrate specificity patterns. This is in contrast to the considerable amount of information present on the individual redox components which make up the 5-exo camphor hydroxylase enzyme [9]. It will therefore be of considerable interest to compare these components to those present in the cyclohexane hydroxylase enzyme in future studies.

ACKNOWLEDGEMENTS

We wish to thank the SERC Biotechnology Directorate, Bush Boake Allen Limited and ICI Petrochemicals and Plastics Division for financial support towards this work.

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Table 1 Substrate specificity of cyclohexane hydroxylase in cell extracts of *Xanthobacter* sp.

Substrate	*Specific activity (nmol O_2 consumed.min^{-1}.mg $protein^{-1}$)
Cyclohexane	22.9
Cyclopentane	16.5
Cyclooctane	6.7
Cyclodecane	3.8
Methylcyclohexane	14.4
Ethylcyclohexane	3.4
Methylcyclopentane	11.9
Ethylcyclopentane	9.4
Benzene	5.1
Toluene	3.9
Octane	<0.9
Decane	<0.9
Undecane	<0.9
Dodecane	<0.9
Hexadecane	<0.9
Methylene cyclohexane	6.2
1-Methyl-1,4-cyclohexadiene	15.7
Cyclohexene	46.5
Cyclohexene oxide	25.1
p-Menthane	<0.9
Pinane	10.9
Pyrrolidine	12.5
Dicyclohexyl	<0.9
Quadricyclane	7.8
NADPH Oxidase	2.5

Legend to Table 1 Cyclohexane hydroxylase activity was assayed according to the procedure described in the Methods section using 100,000 g supernatant as the enzyme source. Reactions were initiated by the addition of the appropriate substrate (2µl).

*Calculated by subtracting the NADPH oxidase endogenous rate from that obtained in the presence of the test substrate.

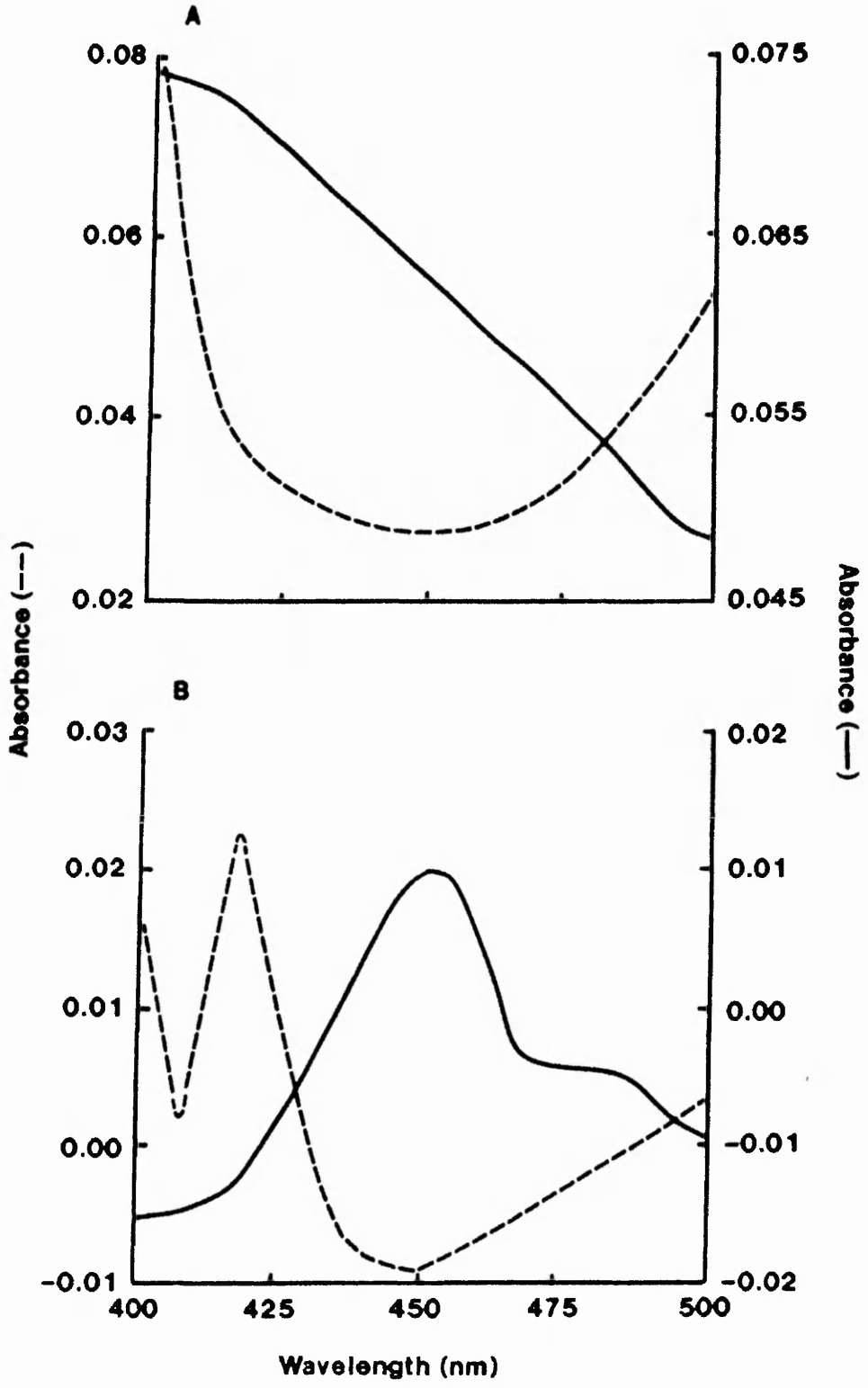

Fig.1. CO-difference spectra of 100,000 g supernatant (0.5-1.0 mg protein) from Xanthobacter sp. grown on a) sodium succinate and b) cyclohexane. The sample cuvette was reduced with solid sodium dithionite and the spectrum recorded (...). CO was then bubbled through the cuvette and the resulting spectra recorded following subtraction of the reduced spectrum. (___)

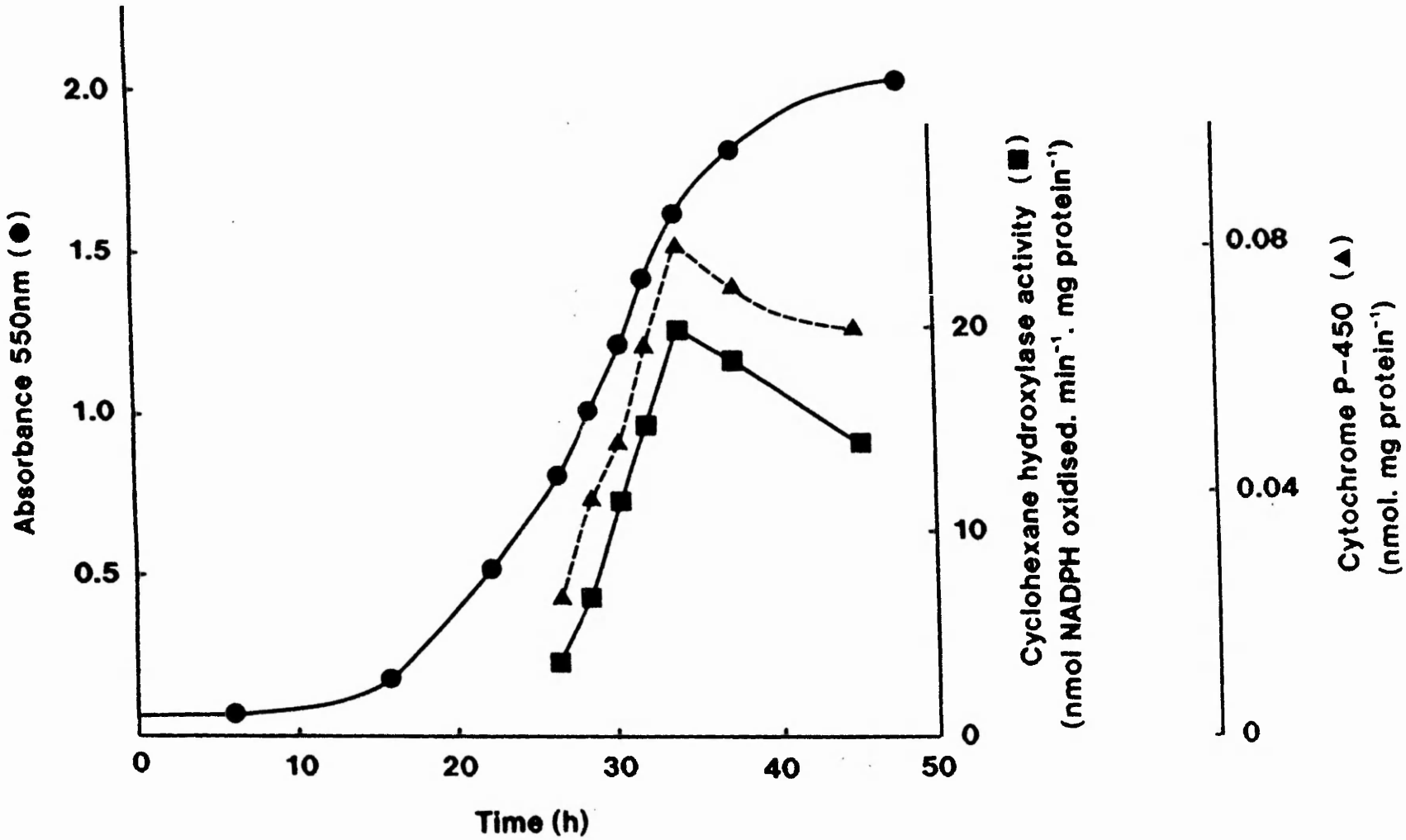

Fig.2. Correlation between the induction of cytochrome P-450 and cyclohexane hydroxylase activity following growth of Xanthobacter sp. on cyclohexane.

GENERAL POSTERS
P.G4: MICROBIAL PHYSIOLOGY AND METABOLISM
Thursday 11th September - Medical School, Second Floor

P.G4-31 3,4-Hydroxyphenilacetic dioxygenase in the aromatic compound degradation pathway from Escherichia coli C.

J.Martín-Checa, R.Balaña-Fouce, M.L.Martín and A.Garrido-Pertierra.
Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense, Madrid 28040. SPAIN.

The ability of Escherichia coli to grow in a wide range of carbon and nitrogen sources, has been amply demonstrated. The microbial degradation of aromatic compounds is very important because plants synthesize a large number of aromatic substances and many man-made chemicals used as detergents and pesticides. Both 3 & 4-Hydroxyphenylacetate are oxidized to 3,4-diHydroxyphenylacetate which in turn reoxidized cleaving the aromatic ring, thus forming 5-carboxymethyl-2-hydroxymuconate by a presumable rate-limiting enzyme: 3,4Hydroxyphenylacetic dioxigenase. 3,4-Hydroxyphenylacetate dioxigenase from Escherichia coli C extracts, grown in 4-Hydroxyphenylacetate as sole carbon source, has been purified and characterized by conventional methods of chromatography. The enzyme shows a relatively high affinity for its substrate (0.15 mM) and is strongly inhibited by dopamine (0.005 mM) in optima activity conditions. Maximum activity was found in Tris-HCl buffer 0.1 M, pH 8.0 at 30°C. Molecular weight of the native enzyme calculated by gel filtration chromatography has been estimated at about 230,000 ± 10,000 and the Stokes radium at 51.0 Å.

P.G4-32 Microbial Metabolism of Cyclohexane

A.M.MAGOR, E.J.WARBURTON, M.K.TROWER and M.GRIFFIN
Department of Life Sciences, Trent Polytechnic, Nottingham, UK.

The metabolism of cyclohexane by a cyclohexane-growing Xanthobacter sp. involves the initial hydroxylation of cyclohexane to cyclohexanol by a cyclohexane hydroxylase. Analysis of this enzyme indicates it to be soluble, inducible, NADPH dependent and has a stoichiometric requirement for O_2 consistent with cyclohexanol formation. The pH optimum for the enzyme is 6.5 and its activity may be stabilised at 4°C by the presence of NADPH or cyclohexane. Cyclohexane hydroxylase has a very narrow substrate specificity restricted to closely related cycloalkanes with no activity shown towards n-alkanes. Initial characterisation of the genes responsible for cyclohexane degradation indicate that they are likely to be chromosomally encoded since no plasmid DNA has been isolated from Xanthobacter sp. using a variety of techniques. Conjugation of the Xanthobacter sp. with E.coli K12 strains carrying RP4 and its derivative RP301 have resulted in the successful transfer of these plasmids into the recipient Xanthobacter sp. The potential for using RP301 as a broad host range vector for cloning the genes responsible for cyclohexane degradation is presently being explored. Attempts have been made to isolate pathway mutants using MNNG, EMS and transposon mutagenesis.

P.G4-33 Metebolism of Alkyl-substituted Aromatics by Rhodococcus rhodochrous: A Modified β-ketoadipate Pathway.

N. C. BRUCE and R. B. CAIN: Department of Agricultural Biology, University of Newcastle, Newcastle upon Tyne, U.K.

Alkyl-substituted aromatics are commonly found in petroleum and coal tar, and in Gram-negative bacteria these are usually catabolised via the "meta"-cleavage pathway. "Ortho"-fission of methyl-substituted phenols and benzoates in eubacteria is of rare occurrence, because in such cases degradation is not completed. The reaction sequence is blocked at one of the later reactions, resulting in the production of the "dead-end" metabolite γ-methymuconolactone. Thus, the catechol 1,2-dioxygenase and the muconate cycluisomerase can be shown to accept substrates with a methyl substituent, but muconolactone isomerase can not if that substituent is in the γ-position.

p. Toluate induced cells of Rh. rhodochrous, however, use a modified "ortho"-fission pathway for degrading 4-methylcatechol to completion. The γ-methylmuconolactone intermediate is produced, as seen in Gram-negative bacteria but Rh. rhodochrous synthesises a new "isomerase" which interconverts γ-methylmuconolactone to (-)-β-methylmuconolactone; this enzyme is present at only low activities in benzoate-grown cells and virtually absent when the cells are grown with succinate or glucose. The shift of the methyl group to the β-position now permits isomerisation of the substrate to the enol-lactone and the catabolic pathway is completed by hydrolysis of this enol-lactone to 4-methyl-3-oxoadipate.

The lactone "isomerase" has been purified to homogeneity and its properties examined.

P.G4-34 Production of Bacilysin by *Bacillus subtilis* strain AI4 and Adaptation of the Producer Strain and Other Bacilli to Growth in Its Presence.

A.V.H.FITZGERALD and A.W.BUNCH: Biological Laboratory, University of Kent, Canterbury, UK.

Microorganisms often produce secondary metabolites with antimicrobial activity and may be sensitive to them at particular stages of growth in batch culture. Bacilysin is an example of a naturally occurring dipeptide antibiotic which exploits transport systems present in target organisms to reach the site of action. It is produced continuously during growth in batch culture by *Bacillus subtilis* strain AI4 when succinate replaces glucose as the carbon source. Agar diffusion bioassay has shown that cells grown on glucose are resistant to bacilysin though under other conditions they appear to be sensitive. Other *B. subtilis* strains develop resistance more rapidly than the *Staphylococcus aureus* strain used routinely to assay activity. Peptidase enzymes do not appear to play an important role and there are indications that modification of peptide transport systems may be involved.

P.G4-35 Ethylene Formation in Batch and Continuous Cultures of *Escherichia coli* strain B SPA0.

N.F. SHIPSTON AND A.W. BUNCH: Biological Laboratory, University of Kent at Canterbury, Canterbury, Kent.

Ethylene is produced as a secondary metabolite by *Escherichia coli* strain B SPA0 where its formation is associated with catabolism of the primary metabolic precursor methionine. The ethylogenic pathway offers a model system in which to study microbial secondary metabolism. Ethylene is a simple, gaseous compound and freely diffuses across the cell boundary. Its release is therefore not subject to transport regulation. Earlier studies have suggested that ethylogenesis could play an important role in nitrogen metabolism in *E. coli* under conditions of nitrogen limitation. The activity and regulation of the pathway in relation to nitrogen assimilatory processes is examined in batch and continuous culture under conditions of carbon and nitrogen limitation.

P.G4-36 Influence of growth rate on phage propagation in chemostat cultures of *Streptococcus cremoris*

A. STERKENBURG and J.T.M. WOUTERS: Laboratorium voor Microbiologie, Universiteit van Amsterdam, Amsterdam, The Netherlands.

Lactic streptococci used as starter bacteria for cheese-making are susceptible to attack by lytic bacteriophages. To understand the development of phages in this milk fermentation process, pure cultures of *Streptococcus cremoris* SK112 were infected with phage 11G and the phage propagation was studied. In milk the efficiency of plaquing of this phage was 3-4 times higher than that found in M17 broth. This was found with stationary phase cells, irrespective of the conditions under which these had been cultured, and was due apparently to inherent physico-chemical properties of milk. The parameters of phage propagation varied considerably with growth rate of the bacteria. When lactose-limited chemostat cultures with a high (0.7 h^{-1}) or average (0.4 h^{-1}) growth rate were infected with phage, up to 4 successive bursts of phages were observed with latent periods and burst sizes of 30 min and 78 plaque-forming units (PFU) per infected cell, respectively. At lower growth rate (0.1 h^{-1}) the latent period increased to 1 h, whereas the burst size became 8 PFU per infected cell. The latter values of these parameters were also found in slow-growing amino acid-limited cultures. Comparable kinetics of phage propagation were observed in mixed populations of the sensitive strain and a phage-resistant derivative.

P.G4-43 Morphological and biochemical changes in thermosensitive (ts) cell division mutants of Streptococcus faecium.

M.M. Lleò and P. Canepari. Istituto di Microbiologia dell' Università di Genova. Genova, Italy.

In order to study the regulatory mechanisms of bacterial morphology we have isolated 115 ts mutants of S. faecium unable to divide at 42°C. Of these mutants, 25% do not show morphological alterations while 75% show different morphological changes at the non permissive temperature: rods and short filaments (26%), lemon-like forms (28%) and partially elongated elements (21%).
When the maximum extent of elongation has been reached (aprox. after 90 min. of incubation at 42°C), apparently normal septa are formed and short chains of spherical cells appear. These cells can only partially separate, but further divisions do not occur.
It has been observed that the longer is the incubation of mutants at the non permissive temperature the longer is the time necessary for division resumption at the permissive temperature.
These data together with others concerning macromolecular synthesis and cell wall turnover has led to the conclusion that the wall produced at 42°C must be remodeled at 30°C.

P.G4-44 Purification of a compound that stimulates cell division in Streptococcus faecium.

M.M. Lleò and P. Canepari. Istituto di Microbiologia dell' Università di Genova. Genova, Italy.

We have previously identified a compound present in the culture supernatant of S. faecium parental strain that restore division of thermosensitive cell division mutants at the non permissive temperature (42°C). This compound can be precipitated and concentrated from the parental broth culture either with acetone or with TCA and renatured with 6M guanidine-HCl.
Concentrated material was applied to a column of Sephadex G-100 and cell division stimulatory activity was recovered in the first of the three peaks that could be eluted. Further purification was performed with ionic exchange chromatography to obtain a pure compound as revealed by SDS polyacrylamide gel electrophoresis. Molecular weight was calculated in about 70,000 daltons. This compound was thermolabile and trypsin sensitive. Stimulatory activity on cell division was maintained for 3 days at 4°C in the presence of BSA.
In an attempt to determine the activity of this compound we have also studied the lytic activity of the parental supernatants on whole cells and purified walls of S. faecium and Micrococcus luteus.

P.G4-45 The cyanide oxygenase Pseudomonas fluoroscens (biotype II) N.C.I.M.B. 11764

RICHARD L. JONES & C.J. KNOWLES, University of Kent at Canterbury, Canterbury, Kent, CT2 7NJ.

We have shown that the Pseudomonas fluorescens is able to convert cyanide to carbon dioxide and ammonia via an NAD(P)H+H$^+$ dependent oxygenase. The enzyme system responsible is inducible by free cyanide or metal-bound cyanide ($NiCN_4$), and enables the organism to utilise cyanide as a nitrogen source for growth.

The oxygenase is significantly different from other C_1/C_2 type oxygenases reported e.g. methane mono-oxygenase, in having a very restricted substrate range. None of the following were oxygenated by the cyanide oxygenase; gaseous hydrocarbons (saturated and unsaturated); amines; amides; alcohols; aromatics and nitriles.

Initial studies investigated the induction of the enzyme system by adding varying concentrations of potassium cyanide to batch grown cultures at different points in the growth curve. This showed two peaks of induction.

To obtain higher enzyme yields as a prerequisite to purification, cultures were grown using an exponential feeding gradient of potassium cyanide as the nitrogen source. The storage of the cyanide oxygenase activity was also investigated extensively, using several temperatures and a variety of chemical additions, to obtain sufficient enzyme for purification.

The data indicates at least two subunits in the system i.e. dependence of specific activity on protein concentration and the requirement of two specific ammonium sulphate cuts for activity. The cyanide oxygenase is not constitutive under any of the growth conditions tried.

There is also evidence that induction of this novel enzyme is repressed by ammonia.

P.G4-46 Control of Nitrogenase and Respiratory Activities by NH_4-Ions and Sucrose in Azotobacter vinelandii.

U.MONTER, R.SANN and J.OELZE: Institut Biol. II (Mikrobiologie), Univ. Freiburg, Freiburg, FRG

Previous investigations demonstrated control of nitrogenase (N_2ase) solely through the supply with NH_4^+-ions of chemostat cultures of different diazotrophs. This communication shows that the specific N_2ase activity of A. vinelandii depends not only on the NH_4^+-supply but also on the supply with the energy and carbon source sucrose. Likewise, the specific respiratory activity depends on the supply with NH_4^+-ions and sucrose. However, the effects of sucrose on N_2ase and of NH_4^+-ions on respiration are more general through their influence on steady state protein levels in chemostat cultures. In order to eliminate this variable control of N_2ase and respiration is presented best by the molar ratio of sucrose per NH_4^+ (C/N). N_2ase activity becomes detectable at a C/N of about 3.5 and it increases as C/N is increased. Respiration increases with low C/N, but it becomes already constant as N_2ase appears. ELISA revealed that N_2ase activity and N_2ase compound proteins appear concomitantly.

P.G4-47 Comparative Study of the Metabolism of Cycloalkanes by Three Species of Xanthobacter

E.J.WARBURTON, A.M.MAGUR and M.GRIFFIN: Department of Life Sciences, Trent Polytechnic, Nottingham, UK.

The ability of three species of Xanthobacter to metabolise cyclohexane and its derivatives has been compared. Xanthobacter flavus was unable to utilize any of the cycloalkanes under investigation. Xanthobacter autotrophicus was unable to utilize cyclohexane but was able to grow with a limited range of substituted cycloalkanes including cyclohexanol and cyclohexanone. Comparison of previously isolated cyclohexane growing Xanthobacter sp. with X.flavus and X.autotrophicus indicated it to be closely related to X.autotrophicus. Studies with cell free extracts have indicated that the route of metabolism for cyclohexanol by X.autotrophicus is the same as that shown for the cyclohexane growing Xanthobacter sp., proceeding via cyclohexanol → cyclohexanone → ε-caprolactone → adipic acid. A comparison of the cyclohexanol dehydrogenase found in the X.autotrophicus with that found in the cyclohexane growing Xanthobacter sp. indicated these enzymes to be distinctly different from one another on the basis of substrate specificity, molecular weight and pH optima. The cyclohexanone monooxygenase enzymes found in the two bacteria were also found to be different when the pH optima and cofactor specificity of the two enzymes were compared.

P.G4-48 Blocking Bdellocyst Outer Wall Formation Does Not Block Further Cyst Development. JOHN J. TUDOR: St. Joseph's University, Philadelphia, PA U.S.A.

Bdellovibrio sp. strain W is unique among the bdellovibrios in that, in addition to vegetative growth, it is capable of producing a resting stage, termed a bdellocyst. During development, a thick layer of peptidoglycan is deposited around the cyst. This layer of peptidoglycan constitutes the outer cyst wall. It has been shown that sporulation in Bacillus is blocked by antibiotics which inhibit peptidoglycan synthesis. It was therefore of interest to determine the effect of various inhibitors of peptidoglycan synthesis on bdellovibrio encystment. Addition of penicillin to encysting cultures led to production of cysts that were spherical rather than the normal kidney-shape. These spherical cysts were produced upon addition of penicillin up to 90 minutes following infection of the prey cells. Addition of penicillin after that time resulted in normal-shaped cysts. Also, pretreatment of bdellovibrios with penicillin for more than 2 hours resulted in development of spherical cysts after penicillin was withdrawn. Ultrastructure studies of these spherical cysts reveal the absence of the outer cyst wall, whereas inclusion material which is characteristic of cysts had accumulated. When penicillin was added 90 min post-infection, spherical cysts developed, but they were surrounded by a very thin cyst wall. These findings indicate that the bdellovibrios can continue to encyst even in the absence of peptidoglycan synthesis and produce a wall-less cyst.

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Comparative Study of the Ability of Three *Xanthobacter* Species To Metabolize Cycloalkanes

ANN M. MAGOR, JEAN WARBURTON, MICHAEL K. TROWER, AND MARTIN GRIFFIN*

Department of Life Sciences, Trent Polytechnic, Clifton, Nottingham NG11 8NS, England

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The ability of three species of *Xanthobacter* to metabolize cyclohexane and its derivatives has been compared. *Xanthobacter flavus* was unable to utilize any of the cycloalkanes under investigation. *X. autotrophicus* was unable to utilize cyclohexane but was able to grow with a limited range of substituted cycloalkanes, including cyclohexanol and cyclohexanone. Comparison of a previously isolated cyclohexane growing *Xanthobacter* sp. with *X. flavus* and *X. autotrophicus* indicated it to be closely related to *X. autotrophicus*. Studies with cell-free extracts have indicated that the route of metabolism for cyclohexanol by *X. autotrophicus* is the same as that shown for the cyclohexane growing *Xanthobacter* sp., proceeding via cyclohexanol→cyclohexanone→ ε-caprolactone→ → adipic acid. A comparison of the cyclohexanol dehydrogenase found in *X. autotrophicus* with that found in the cyclohexane-growing *Xanthobacter* sp. indicated these enzymes to be distinctly different from one another on the basis of substrate specificity, molecular weight, and pH optima. The cyclohexanone monooxygenase enzymes found in the two bacteria were also found to be different when the pH optima and cofactor specificity of the two enzymes were compared. Preliminary genetic studies on the cyclohexane-growing *Xanthobacter* sp. have indicated that there are no plasmids present in this bacterium. The presence of RP4 in the *Xanthobacter* sp. can be detected following its conjugation with an RP4-carrying *Escherichia coli* strain.

Members of the genus *Xanthobacter* are gram-negative, nitrogen-fixing, hydrogen-oxidizing, nonmotile bacteria. To date, only two species have been described in *Bergey's Manual of Systematic Bacteriology* (22); these are *Xanthobacter autotrophicus*, previously known as *Corynebacterium autotrophicum* (23), and *Xanthobacter flavus*, known as *Mycobacterium flavum* prior to reclassification by Malik and Claus (15). However, DNA-DNA hybridization studies of the genus *Xanthobacter* performed by Jenni et al. (12) have indicated that there may be several different species clustered within this genus.

A *Xanthobacter* species capable of growth on cyclohexane as the sole carbon and energy source was isolated and characterized in this laboratory by Trower et al. (20). The isolation of an organism with an efficient metabolic pathway for the degradation of cyclohexane is both novel and unusual since there have been few reports of microorganisms capable of growth upon this hydrocarbon (1, 18). Enzymatic studies with cell extracts of this *Xanthobacter* sp. showed that cyclohexane is degraded by a route that involves the initial hydroxylation of cyclohexane to cyclohexanol. Further oxidation of cyclohexanol occurs via cyclohexanone to 1-oxa-2-oxocycloheptane (ε-caprolactone) to 6-hydroxyhexanoate (6-hydroxycaproate) and finally to adipic acid (20). This pathway is analogous to that described for the two other cyclohexane-growing organisms, a *Nocardia* species (18) and a pseudomonad (1).

In the following study we compared the ability of three different species of *Xanthobacter* to metabolize cyclohexane and its related compounds since such an unusual genetic trait may be a feature common to this newly characterized genus. Furthermore, any differences seen in the ability of these bacteria to metabolize cycloalkanes may be a useful feature in distinguishing the different subgroups which may exist in this genus. In addition, we have carried out preliminary investigations into the genetic organization of cyclohexane degradation to establish whether the genes responsible for this catabolic pathway are carried on a plasmid or are chromosomally encoded.

MATERIALS AND METHODS

Isolation, maintenance, and culture of microorganisms. A species of *Xanthobacter* capable of growth on cyclohexane was isolated from the soil of local Nottinghamshire forests by classical enrichment techniques, using cyclohexane as a sole carbon source (20). *X. autotrophicus* (strain DSM 431) was obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Gottingen, Federal Republic of Germany. *X. flavus* was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland. The bacteria were grown on either liquid or solid mineral salts medium (MSM) containing the required carbon source as described by Trower et al. (20). For maintenance of bacteria on solid MSM *Xanthobacter* sp. was grown on cyclohexane, *X. autotrophicus* was grown on cyclohexanol, and *X. flavus* was grown on succinate. For growth of *X. flavus* the MSM also contained the following vitamins: thiamine, riboflavin, nicotinic acid, pyridoxin-HCl, and calcium pantothenate, each at a concentration of 1 μg/ml; and vitamin B_{12}, biotin, and folic acid, each at 0.1 μg/ml.

The requirement of complex growth factors by the cyclohexane-growing *Xanthobacter* sp. was tested by the method of Holding and Collee (10). Determination of the possession of lecithinase, urease, and phosphatase activity by the cyclohexane-growing *Xanthobacter* sp. and of the ability of this bacterium to hydrolyze geletin and casein was carried out by the NCIMB.

Preparation of washed cell suspensions. The cell cultures were harvested in the late log phase of growth by centrifugation (10,000 × *g* for 10 min at 4°C), washed twice in 20 mM KH_2PO_4–Na_2HPO_4 buffer (pH 7.0), and then suspended in the same buffer (1.5 times the cell volume). Suspended cells were either used directly or stored at −20°C until required.

* Corresponding author.

Preparation of cell extracts. Resuspended cell suspensions were disrupted by ultrasonication (MSE Soniprep 150) for a total of 2 min (20-s bursts; amplitude, 6 μm at 4°C). Cell debris was removed by centrifugation at 10,000 × *g* for 10 min at 4°C (10,000 × *g* supernatant). Soluble (10,000 × *g* supernatant) and membrane fractions were prepared by centrifugation of the 10,000 × *g* supernatant at 100,000 × *g* for 60 min at 4°C. Protein concentrations were determined by the biuret assay of Gornall et al. (5), with bovine serum albumin as a standard.

Measurement of oxygen uptake. Oxygen consumption by whole cells was measured polarographically, using a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) at 30°C. Incubation mixtures contained (in 3 ml) 60 μmol of phosphate buffer (pH 7.0), 20 μmol of substrate, and 50 μl of cell suspension. Cyclohexane and other water-immiscible substrates (2 μl) were added directly to the incubation mixtures.

Enzyme assays. Cyclohexane hydroxylase activity was assayed polarographically by measuring the NADPH-dependent oxidation of cyclohexane in the oxygen electrode. Reaction mixtures contained (in 3 ml) 240 μmol of phosphate buffer (pH 6.5 or 7.5), 0.3 μmol of NADPH, 1.0 to 3.0 mg of protein, and 2 μl of cyclohexane.

Cyclohexanol dehydrogenase activity was measured spectrophotometrically (Beckman Du-7) as previously described (2). The reaction mixture contained (in 1 ml) 40 μmol of glycine-NaOH buffer (pH 10.2) for the *Xanthobacter* sp. or 40 μmol of glycine-NaOH buffer (pH 9.2) for *X. autotrophicus*, 0.3 μmol of NAD^+, 0.1 to 0.5 mg of protein, and 1 μmol of cyclohexanol.

Cyclohexanone monooxygenase activitiy was assayed polarographically in the oxygen electrode by measuring the cyclohexanone-dependent oxidation of NADH or NADPH. Reaction mixtures contained (in 3 ml) 480 μmol of glycine-NaOH buffer (pH 8.8) and 0.3 μmol of NADPH for the *Xanthobacter* sp. or 150 μmol of phosphate buffer (pH 7.5) and 0.3 μmol of NADH for *X. autotrophicus*, 0.5 mg of protein, and 2 μmol of cyclohexanone.

1-Oxa-2-oxocycloheptane hydrolase activity was measured by the method of Norris and Trudgill (16). 1-Oxa-2-oxocycloheptane (50 μmol) in distilled water was adjusted to pH 8.0 and then made up to a total volume of 10 ml. The reaction was started by the addition of 0.1 to 0.5 mg of protein, and the reaction mixture was maintained at pH 8.0 by constant titration of 10 mM NaOH against the acidic reaction product.

Estimation of enzyme pH optima. Enzyme pH optima were determined as described previously (1).

Polyacrylamide gel electrophoresis. Cell extracts (100,000 × *g* supernatant) were fractionated by polyacrylamide rod gel electrophoresis (8.5% [wt/vol] polyacrylamide) (8). For measurement of in situ cyclohexanol dehydrogenase activity a technique similar to that of Grell et al. (6) was used but with a modified staining solution. The staining solution (in 22 ml) contained 17 mM glycine-NaOH buffer (pH 10.2) for *Xanthobacter* sp. cell extracts or 17 mM glycine-NaOH buffer (pH 9.2) for *X. autotrophicus* cell extracts, 0.1 M cyclohexanol, 2 mM NAD^+, 0.4 mM Nitro Blue Tetrazolium, and 0.1 mM phenazine methosulfate. The gels were stained for 3 to 4 h. For detection of protein gels were stained for 1 h in 0.2% (wt/vol) Coomassie brilliant blue G in aqueous 7.5% (vol/vol) glacial acetic acid–18% (vol/vol) methanol.

Molecular weight determination. Molecular weight determinations were performed by gel filtration, using Ultrogel AcA 44 (LKB Instruments Ltd., Selsdon, Croydon, Surrey, England). Cell extracts (100,000 × *g* supernatant) were eluted from the column (65 by 1.6 cm) with 20 mM phosphate buffer (pH 7.0). The column was calibrated with a Pharmacia gel filtration molecular weight calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden) containing RNase A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), and bovine serum albumin (67,000).

Isolation of plasmid DNA. DNA was isolated from cyclohexane-grown *Xanthobacter* sp. by the method of Wheatcroft and Williams (21). Plasmid DNA preparations were purified by using freeze-thaw-generated sucrose gradients as described by Wheatcroft and Williams (21). Other large-scale plasmid isolations used included the methods of Hansen and Olsen (9) and Guerry et al. (7).

Small-scale plasmid DNA isolations were performed on single colonies of *Xanthobacter* sp. from MSM plates with cyclohexane as the growth substrate or 1-ml MSM cultures grown with cyclohexane, using the methods of Kado and Liu (13) and an adaption of the Wheatcroft and Williams method (21).

Agarose gel electrophoresis. DNA fragments were separated by agarose gel electrophoresis, using Tris-borate buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0), on a horizontal apparatus at 75 V for 3 h or 20 V overnight, using 0.7% (wt/vol) agarose (agarose "10"; BDH Chemicals Ltd.) and 0.5 μg of ethidium bromide per ml. The loading buffer (added at 0.1 volume of sample) consisted of 30% (wt/vol) Ficoll, 0.2 M EDTA, and 50 μg of bromophenol blue per ml. Gels were visualized on a transilluminator (Ultraviolet Products Ltd.). Photographs were taken with type 667 Polaroid film.

Conjugation. Matings were performed with overnight nutrient broth donor cultures and late-log-phase recipient cultures of *Xanthobacter* sp. in cyclohexane MSM. Filter matings were carried out by mixing 25 μl of both donor and recipient cells on a filter (0.45-μm pore size) and placing the filter on nutrient agar. The procedure was the same for donor and recipient controls. Plates were incubated overnight at 25°C, after which cells were washed from the filter with saline and dilutions were plated to selective media. Viable counts were performed on donor cultures.

Materials. Aristar and Analar cyclohexane were obtained from BDH (British Drug House), Poole, Dorset, England. Cyclohexanol, cyclohexanone, *cis,trans*-cyclohexan-1,2-diol, *cis,trans*-cyclohexan-1,3-diol, and *cis,trans*-1,4-diol were purchased from Aldrich Chemical Co., Gillingham, Dorset, England. 2-Hydroxycyclohexanone was purchased from Pfaltz and Bauer, Inc., Stamford, Conn. 1-Oxa-2-oxycycloheptane was obtained from Koch-Light, Coinbrook, England. NADPH, NADH, $NADP^+$, and NAD^+ were supplied by Boehringer Mannheim, Lewes, East Sussex, England. Nitro Blue Tetrazolium and phenazine methosulfate were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, England.

RESULTS

Distinguishing microbiological characteristics of the different species of *Xanthobacter*. Comparison of the microbiological characteristics of the cyclohexane-growing *Xanthobacter* sp. with those previously obtained for *X. flavus* and *X. autotrophicus* indicated this new species to be more closely related to *X. autotrophicus* than to *X. flavus* (Table 1). As a strain of *X. autotrophicus* it is, however, relatively atypical since it demonstrates the ability to hydro-

lyze gelatin and casein, possesses a lecithinase and a urease, lacks phosphatase activity, and has the ability to use malonate and maltose as carbon sources.

Growth of the different *Xanthobacter* species on cyclohexane and related compounds. A comparison of the ability of the three different species of *Xanthobacter* to grow on cyclohexane and related compounds indicated that only the *Xanthobacter* sp. was capable of growth with the recalcitrant hydrocarbon cyclohexane. *X. autotrophicus*, when tested for growth on a range of substituted cycloalkanes, was able to utilize cyclohexanol, cyclohexanone, and cyclohexan-1,2-diol as the sole carbon source. Unlike the cyclohexane-growing *Xanthobacter* sp. (20), *X. autotrophicus* was unable to utilize methylcyclohexane, cyclohexan-1,3-diol, cyclohexan-1,4-diol, or cyclohexan-1,2-dione as a carbon source. *X. flavus*, unlike *X. autotrophicus*, was unable to utilize any of the substituted cycloalkanes tested.

The ability of the three species of *Xanthobacter* to grow on the aromatic hydrocarbons benzene and toluene and the straight-chain alkanes (C_6 and C_{16}) was also tested, but none of the *Xanthobacter* species were able to utilize these compounds as growth substrates.

Comparison of the route of metabolism for cyclohexanol by *X. autotrophicus* and the cyclohexane-growing *Xanthobacter* sp. Since both the cyclohexane-growing *Xanthobacter* sp. and *X. autotrophicus* appeared to be closely related with respect to not only their ability to metabolize specific organic acids and sugars but also their ability to metabolize substituted cycloalkanes, comparative studies on these two bacteria were further extended.

(i) Metabolite oxidation. The ability of washed cells of the cyclohexanol-grown *X. autotrophicus* to oxidize potential cyclohexanol metabolites and other substituted cycloalkanes is shown in Table 2. Comparison of these results with those obtained from the cyclohexane-grown *Xanthobacter* sp. indicated considerable similarity in their ability to oxidize cyclohexanol, cyclohexanone, 1-oxa-2-oxocycloheptane (ε-caprolactone), and cyclohexanol-1,2-diol. Neither *X. autotrophicus* nor the cyclohexane-grown *Xanthobacter* sp. was found to be capable of oxidizing adipic acid.

(ii) Enzyme activities in cell extracts of cyclohexanol-grown *X. autotrophicus*. To confirm that the major routes of metabolism of cyclohexanol by *X. autotrophicus* and the cyclohexane-grown *Xanthobacter* sp. were comparable and to assess any similarities in the key enzymes involved in this pathway, studies were undertaken with *X. autotrophicus*, using crude cell extracts.

Incubation of cell extracts (10,000 × *g* and 100,000 × *g* supernatants) from the cyclohexanol-grown *X. autotrophicus* with either NADH or NADPH in the presence of cyclohexane failed to cause any cyclohexane stimulation in O_2 consumption when measured in the oxygen monitor. This confirmed the studies with whole cells in that cyclohexane hydroxylase is unlikely to be present in *X. autotrophicus*.

Enzyme assays carried out to determine the presence of a cyclohexanol dehydrogenase, a cyclohexanone oxygenase, and a ε-caprolactone hydrolase indicated each of these enzymes to be present in the cell extracts (10,000 × *g* supernatant) of the cyclohexanol-grown *X. autotrophicus* (Table 3). The finding that much lower levels (20- to 100-fold) of enzyme activity were detected in cells grown with succinate as the carbon source (data not shown) indicated the inducible nature of these enzymes.

Preliminary studies with the cyclohexanol dehydrogenase and the cyclohexanone oxygenase concerning their cofactor specificity and pH optimum indicated that these enzymes were unlikely to be closely related to the same enzymes found in the cyclohexane-grown *Xanthobacter* sp. (Table 3). Comparison of these enzymes from the two different *Xanthobacter* spp. indicated that cyclohexanol dehydrogenase demonstrated greater specificity for NAD^+ than $NADP^+$ in each case. However, the cyclohexanol dehydrogenase from the cyclohexane-grown *Xanthobacter* sp. displays a pH optimum of 10.1 to 10.5, whereas the optimum pH found for the *X. autotrophicus* enzyme was 9.0 to 9.4. Comparison of the cyclohexanone oxygenase isolated from the two species of *Xanthobacter* also indicated that this enzyme was different in the two microorganisms. The enzyme isolated from the cyclohexanol-grown *X. autotrophicus* is specific for NADH and has a pH optimum of 7.2 to 7.7. In contrast, the enzyme isolated from the cyclohexane-grown *Xanthobacter* sp. is specific for NADPH and displays a pH optimum of 8.5 to 9.0.

Characterization of cyclohexanol dehydrogenase from *X. autotrophicus* and the cyclohexane-grown *Xanthobacter* sp. A comparison of the substrate specificities of the cyclohexanol dehydrogenase present in cell extracts (100,000 × *g* supernatant) of the cyclohexanol-grown *X. autotrophicus* and the cyclohexane-grown *Xanthobacter* sp. is shown in Table 4. The substrate which gave rise to the greatest activity for both enzymes was cyclopentanol. The cyclohexanol dehydrogenase from the *Xanthobacter* sp. was found to have a broad specificity for secondary alcohols, and the primary alcohol butan-1-ol was also oxidized by this enzyme. In contrast, the cyclohexanol dehydrogenase from *X. autotrophicus* was found to have a relatively narrow substrate range and demonstrated no activity towards the cyclohexandiols or butan-1-ol.

Further differences in the two cyclohexanol dehydrogenase enzymes was shown in their respective molecular weights and in their relative mobilities following separation by polyacrylamide gel electrophoresis.

The separation of cell extracts (100,000 × *g* supernatant)

TABLE 1. Differential characteristics of the species of the genus *Xanthobacter*

Characteristic	*Xanthobacter* sp.[a]	*X. autotrophicus*[b]	*X. flavus*[c]
Growth factor requirement	−	−	+
Utilization of			
Fructose	+	+	(+)
Propionate	+	+	−
Malonate	+	−	+
Maltose	+	−	+
Ribose	−	−	+
Phenylalanine	−	−	+
Histidine	−	−	+
Acetate	+	+	(+)
Glucose	−	−	+
Hydrolytic activity on gelatin and casein	+	−	−
Lecithinase activity	+	−	−
Urease activity	+	+[d]	−
Phosphatase activity	−	+	+
Autotrophic growth	+	+	−[e]

[a] Tests on *Xanthobacter* sp. were carried out according to the procedures described in Materials and Methods.
[b] Data from *Bergey's Manual of Systematic Bacteriology* (22).
[c] Data from Malik and Claus (25). (+) Poor growth.
[d] 71 to 89% of strains are positive.
[e] Autotrophic growth occurs only below 5% O_2 atmosphere.

TABLE 2. Comparitive oxidation of potential cyclohexanol metabolites and other hydrocarbons by washed cell suspensions of *Xanthobacter* sp. and *X. autotrophicus*[a]

Substrate	Rate of oxygen uptake (μmol of O_2 h^{-1} mg [dry wt] of organisms^{-1})	
	Xanthobacter sp. (cyclohexane grown)	*X. autotrophicus* (cyclohexanol grown)
Cyclohexane	7.5	0.3
Cyclohexanol	5.4	4.6
Cyclohexanone	5.0	3.4
1-Oxa-2-oxocycloheptane	3.5	2.0
1-Methylcyclohexanol	3.5	3.6
2-Methylcyclohexanol	3.3	1.1
3-Methylcyclohexanol	5.3	2.6
4-Methylcyclohexanol	6.1	1.7
Cyclohexan-1,2-diol	8.6	2.7
Cyclohexan-1,3-diol	5.0	1.3
Cyclohexan-1,4-diol	6.0	1.1
Toluene	0.7	0.4
Heptane	<0.05	<0.05
Decane	<0.05	<0.05
Dodecane	<0.05	<0.05
Succinate	<0.05	<0.05
Adipate	<0.05	<0.05

[a] Values shown were calculated by subtracting the endogenous rate from the rate recorded with the substrate present. Incubation mixtures contained (in 3 ml) 60 μ mol of phosphate buffer (pH 7.0), 100 μl of cell suspension (2.5 mg, dry weight), and 20 μ mol of substrate. Water-immiscible substrates (2 μl) were added directly to the incubation chamber.

from the cyclohexanol-grown *X. autotrophicus* and the cyclohexane-grown *Xanthobacter* sp. by polyacrylamide gel electrophoresis followed by in situ staining for cyclohexanol dehydrogenase is shown in Fig. 1. Only one major staining band was found in the cell extracts obtained from either organism, but the different relative mobilities of these two bands with reference to the bromophenol blue markers indicated the two enzymes to be dissimilar. A further very faint staining band was also found in each of the cell extracts; interestingly, the faint band displayed by the *Xanthobacter* sp. proved to have a relative mobility similar to that of the major staining band found in *X. autotrophicus*.

Estimation of the molecular weights of the two cyclohexanol dehydrogenase enzymes was obtained by comparing the elution volumes of the different enzymes with those obtained for protein standards of known molecular weight following separation of cell extracts (100,000 × *g* supernatant) by Ultrogel AcA44 chromatography. By using this technique the molecular weight of the cyclohexanol dehydrogenase from the cyclohexanol-grown *X. autotrophicus* was found to be 20,000 ± 3,000 (n = 3), while that from the *Xanthobacter* sp. was shown to be 43,000 ± 3,000 (n = 3). Only one band of cyclohexanol dehydrogenase activity could be detected in the column eluents obtained from cell extracts of either *Xanthobacter* species.

Preliminary genetic studies with the cyclohexane-grown *Xanthobacter* sp. The scarcity of reports concerning the ability of microorganisms to grow on cyclohexane compared with those found to grow on cyclohexanols suggests that this unusual metabolic ability may be related to the possession of a degradative plasmid by these microorganisms. Such a finding is not without precedent since plasmids of this kind have been shown to be responsible for a number of degradative pathways (4). In this report, two closely related species of *Xanthobacter* have been demonstrated: one grows on cyclohexanol; the other has the capacity to grow on cyclohexane. It is therefore possible that either the gene(s) responsible for the cyclohexane hydroxylase or other genes that may be required for cyclohexane degradation are present on a degradative plasmid in the cyclohexane-growing *Xanthobacter* sp.

To test this hypothesis a variety of techniques suitable for the isolation of large degradative plasmids, including the Hansen and Olsen procedure (9), the cleared lysate procedure (7), and the procedure of Wheatcroft and Williams (21), were used to detect the presence of plasmid DNA in extracts of the cyclohexane-grown *Xanthobacter* sp. By these techniques no plasmid DNA was detected in this organism. Figure 2 shows agarose gel electrophoresis of DNA extracted from *Xanthobacter* sp. by the Wheatcroft and Williams method (21). The results obtained from this technique clearly show the presence of only chromosomal DNA following separation of the extracted DNA by sucrose gradient centrifugation. Furthermore, no plasmids were detected in *Xanthobacter* sp. by using the rapid plasmid screening procedures of Kado and Liu (13), Eckhardt (3), and Holmes and Quigley (11).

To confirm the methodology used for plasmid isolation and, perhaps more importantly, its application to the *Xanthobacter* species of interest, the broad-host-range plasmid RP4 was conjugated into the cyclohexane-growing *Xanthobacter* sp. from an *Escherichia coli* strain, J53. Transconjugants were selected by growth on cyclohexanone in the presence of kanamycin (25 μg/ml) and tetracycline (10 μg/ml). The presence of RP4 in the *Xanthobacter* recipients

TABLE 3. Comparison of enzymes involved in cyclohexane/cyclohexanol degradation of *Xanthobacter* sp. and *X. autotrophicus* after growth on cyclohexane and cyclohexanol, respectively[a]

Enzyme	*Xanthobacter* sp.[b]			*X. autotrophicus*		
	pH optimum	Cofactor specificity	Activity (U/mg of protein)[c]	pH optimum	Cofactor specificity	Activity (U/mg of protein)[c]
Cyclohexane hydroxylase	6.2–6.7	NADPH	7.5×10^{-3}			
Cyclohexanol dehydrogenase	10.1–10.5	NAD^+	0.87	9.0–9.4	NAD^+	0.73
		$NADP^+$	0.05		$NADP^+$	$<0.1 \times 10^{-3}$
Cyclohexanone monooxygenase	8.5–9.0	NADPH	0.15	7.2–7.7	NADH	0.42
					NADPH	$<0.9 \times 10^{-3}$
1-Oxa-2-oxo cycloheptane hydrolase	7.8–8.1		21.3	8.0		21.6

[a] Enzyme activity and pH optima were measured according to the procedures described in Materials and Methods.
[b] Data from Trower et al. (20).
[c] One unit of enzyme activity equals 1 μmol of substrate utilized per min.

could be clearly detected (data not shown) by the rapid screening procedure of Wheatcroft and Williams (21).

DISCUSSION

Comparison of a variety of microbiological characteristics obtained for the cyclohexane-growing *Xanthobacter* sp. with those reported for *X. autotrophicus* (23) and *X. flavus* (15) indicate this new *Xanthobacter* sp. to be closely related to *X. autotrophicus*.

This relationship to *X. autotrophicus* is further reflected in the ability of these two species of *Xanthobacter* to use substituted cycloalkanes as growth substrates, a property which is not shown by *X. flavus*. The metabolism of cycloalkanes or their substituted analogs may therefore be a characteristic trait of differing strains of *X. autotrophicus*. Indeed *X. autotrophicus* NCIMB 10811 is also capable of growth on cyclohexanol (NCIMB, personal communication).

None of the species of *Xanthobacter* were able to grow with either the straight-chain alkanes (C_6 and C_{16}) or the aromatic hydrocarbons toluene and benzene as growth substrates. Growth with short-chain alkanes (14) and, more recently, short-chain alkenes (C. G. van Ginkel, K. A. Dekker, and J. A. M. de Bont, Proc. 100th Ordinary Meet. Soc. Gen. Microbiol., 1984) has, however, been reported for *Xanthobacter*-like bacteria and may be a separate characteristic representative of other as yet uncharacterised species of this genus.

Similarities between *X. autotrophicus* and the cyclohexane-grown *Xanthobacter* sp. could also be demonstrated with respect to their mode of metabolism of cyclohexanol. Like the cyclohexane-grown *Xanthobacter* sp., the cyclohexanol-grown *X. autotrophicus* possesses an inducible cyclohexanol dehydrogenase, a cyclohexanone oxygenase, and a ε-caprolactone hydrolase. The presence of these enzymes indicate that the route of metabolism for cyclohexanol is likely to proceed via oxidation of cyclohexanol ⟶ cyclohexanone → ε-caprolactone ⟶ adipic acid. This route of metabolism is the major pathway used by the *Xanthobacter* sp. during growth on cyclohexane (20) and has been demonstrated for other cycloalkanol-utilizing microorganisms (16).

TABLE 4. Relative activity of the cyclohexanol dehydrogenase from *Xanthobacter* sp. and *X. autotrophicus* with different substrates[a]

Substrate	% Activity	
	Xanthobacter sp.	*X. autotrophicus*
Cyclohexanol	100	100
Cyclopentanol	174	168
Butan-1-ol	22	0
Butan-2-ol	52	130
1-Methylcyclohexanol	52	0
2-Methylcyclohexanol	65	8
3-Methylcyclohexanol	99	41
4-Methylcyclohexanol	91	10
Cyclohexan-1,2-diol	24	0
Cyclohexan-1,3-diol	27	0
Cyclohexan-1,4-diol	30	0
2-Hydroxycyclohexanone	0	0

[a] Cyclohexanol dehydrogenase activity was measured according to the procedures described in Materials and Methods. Reactions were performed at the pH optimum for each enzyme. Substrates were added at a concentration of 1 mM. Values represent a percentage of the rate measured for each enzyme with cyclohexanol as the substrate.

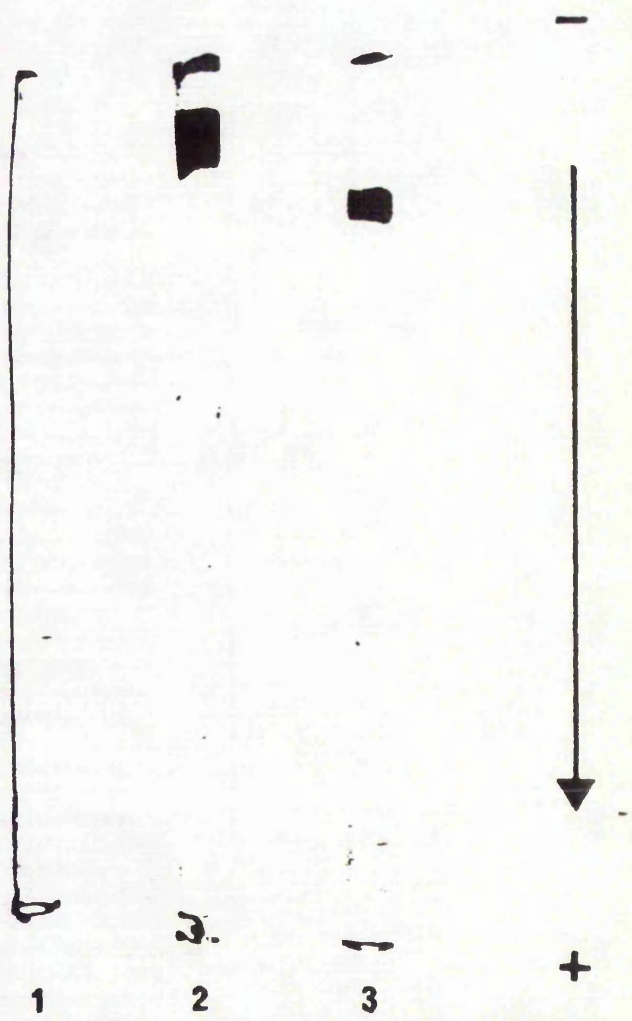

FIG. 1. In situ activity staining for cyclohexanol dehydrogenase following separation of crude cell extracts by polyacrylamide electrophoresis. Cell extracts (100,000 × *g* supernatant containing 50 to 100 μg of protein) were fractionated by polyacrylamide gel electrophoresis by the procedure described in Materials and Methods, using a current of 3 mA per gel tube. The photograph shows gels stained for cyclohexanol dehydrogenase activity according to the procedures described in the text. Gel 1, Control gel of fractionated extracts from the cyclohexanol-grown *Xanthobacter* sp. stained for cyclohexanol dehydrogenase activity but without addition of cyclohexanol; gel 2, cell extracts from the cyclohexane-grown *Xanthobacter* sp.; gel 3, cell extracts from the cyclohexanol-grown *X. autotrophicus*.

Characterization of the cyclohexanol dehydrogenase from each of the species of *Xanthobacter* with respect to pH optima and molecular weight indicated the two enzymes to be distinctly different from one another. A similar feature shared by the two enzymes was their preferred requirement of NAD^+ for enzyme activity, a property displayed by other secondary alcohol dehydrogenases which have so far been characterized (16, 17). Analysis of cell extracts from the cyclohexane-grown *Xanthobacter* sp. and the cyclohexanol-grown *X. autotrophicus* by polyacrylamide gel electrophoresis and gel filtration chromatography indicated only one major cyclohexanol dehydrogenase enzyme to be present. Characterization of the enzyme from the cyclohexane-grown

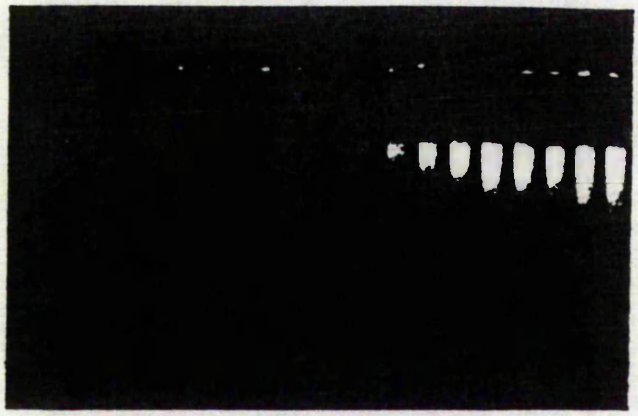

FIG. 2. Agarose gel electrophoresis of DNA from *Xanthobacter* sp. following its fractionation on sucrose density gradients. DNA extracts from the cyclohexane-grown *Xanthobacter* sp. were fractionated by centrifugation on sucrose density gradients according to the procedure of Wheatcroft and Williams (21). The collected fractions were then analyzed (20 μl) by agarose gel electrophoresis for 15 h, using the procedures described in Materials and Methods. The photograph shows an ethidium bromide-stained gel of sucrose fractions. The lanes, left to right, indicate fractions of decreasing sucrose density.

Xanthobacter sp. with respect to substrate specificity indicated this enzyme to have a broad substrate range for secondary alcohols and, in addition, a small amount of activity towards the primary alcohol butan-1-ol. This type of substrate range is comparable to the cyclohexanol dehydrogenase isolated from a cyclohexane-growing *Nocardia* sp. (18), the only other secondary alcohol dehydrogenase characterized from a cyclohexane-growing microorganism. In contrast, the cyclohexanol dehydrogenase from the cyclohexanol-grown *X. autotrophicus* displayed a more limited substrate range for secondary alcohols and demonstrated no activity towards butan-1-ol.

The other key enzyme involved in cyclohexanol metabolism, the lactone-forming cyclohexanone oxygenase, was also found to be different in the two *Xanthobacter* species. The most striking difference was the finding that the *X. autotrophicus* enzyme was specific for NADH. This is a particularly interesting feature since of the lactone- and ester-forming oxygenases so far characterized only one other oxygenase, the 2,5-diketocamphane monooxygenase from *Pseudomonas putida*, is specific for NADH. The latter enzyme is unusual with respect to other lactone-forming monooxygenases in a number of features since it carrys flavin mononucleotide rather than flavin adenine dinucleotide as the prosthetic group and is a multicomponent enzyme possessing separable reductase and oxygenase components (19). Interestingly, the cyclohexanone monooxygenase isolated from the cyclohexane-growing *Xanthobacter* sp. also possesses flavin mononucleotide as its prosthetic group (20) but, unlike the 2,5-diketocamphane monooxygenase, is specific for NADPH and not NADH and is not a multicomponent enzyme. Hence the cyclohexanone oxygenase enzymes isolated from the two *Xanthobacter* species, although different from one another, each appear to show unusual characteristics for this group of enzymes and merit further investigations with regard to their properties.

Our inability to detect the presence of plasmid DNA in the cyclohexane-growing *Xanthobacter* sp. would indicate that the genetic information responsible for the added capacity of this particular *Xanthobacter* sp. to grow on cyclohexane is not carried on a degradative plasmid. However, the presence of a large undetected plasmid cannot be excluded. It is also possible that such a plasmid may be destroyed during the physical procedures used to isolate it; however, our ability to detect the presence of plasmid RP4 in the *Xanthobacter* sp. would seem to dispel this idea.

The finding that shuttle vectors, such as RP4, may be transferred into this *Xanthobacter* sp. by conjugation with RP4-carrying *E. coli* J53 is, however, an important discovery in itself, since this vector or its derivatives have the potential to be used as broad-host-range cloning vehicles in the further characterization of the genes responsible for cyclohexane degradation.

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