THE UTILIZATION OF C_{14}-C_{16} N-ALKANES BY MICROORGANISMS

by

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Summary

Microorganisms capable of growth using n-alkanes as sole carbon and energy source were isolated from the environment. Subsequently selected organisms from those isolated were subjected to morphological and biochemical surveys that involved electron microscopy and assays of enzymes concerned with n-alkane degradation and assimilation. The alcohol and aldehyde dehydrogenases associated with the growth of a pseudomonad on n-alkanes were partially purified and shown to be NAD(P) independent.

Genetic manipulations were attempted to produce mutants of a coryneform bacterium, by chemical and U.V. mutagenesis, that were blocked at specific points in the n-alkane degradation pathway which were shown to be chromosomally borne. Appropriate selection procedures were devised and mutations identified by product accumulation by whole cells. Evidence is also presented for the presence of a large plasmid that may carry genes for n-alkane assimilation in a pseudomonad under investigation.

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Acknowledgements

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Declaration

I hereby declare that this thesis was composed by myself and has not been accepted in any previous application for a degree. The work represented was all done by myself. All sources of information have been acknowledged by reference.

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CHAPTER ONE

INTRODUCTION

I.1 Organisms capable of growth using n-alkanes

I.1.1 Sources

The occurrence of microorganisms capable of growth using n-alkanes as sole carbon and energy source is widespread. In a survey of microorganisms from soil and underlying shale, n-alkane degrading organisms were found at every level (Jones and Edington, 1968). Perry and Scheld (1968) also found a wide variety of n-alkane utilizing organisms when screening microorganisms isolated from soil using non-hydrocarbon substrates.

Marine and freshwater environments have proved to be rich sources of n-alkane utilizing microorganisms, particularly when polluted with petroleum products. Austin et al. (1977), from surveys of n-alkane degrading microorganisms in aquatic environments concluded that a wide variety of taxa are responsible for the degradation of petroleum. However, there appeared to be a predominance of Pseudomonas, Mycobacterium or Nocardia species, depending upon the aquatic niche investigated. Ecological studies were confused further by the observation of Calomiris et al. (1976) that enrichment procedures significantly influence the range of organisms isolated. Recent interest in degradation of petroleum in aquatic environments centres upon the possibility of using microorganisms to disperse and degrade oil spills; the

subject has been reviewed recently by Atlas (1977).

Thermophilic microorganisms capable of growth at the expense of n-alkanes have been isolated by Merkel et al. (1978 Some of the organisms isolated had an optimum growth temperature of 60°C and were unable to utilize substrates other than n-alkanes and 1-alkenes (Merkel, Underwood and Perry, 1978 The capacity to grow only at the expense of a limited range of saturated or unsaturated hydrocarbons is rare except in the case of methylotrophs (Anthony, 1975), although some isolated cases have been reported (e.g. Bertrand et al., 1976).

Because of the wide range of bacteria that have been shown to utilize n-alkanes, the characteristic is of only limited taxanomic value. This was illustrated by Grange (1974) in a study of 50 strains of Mycobacterium. He concluded that there was considerable variation within species as well as between species when the range of shorter chain n-alkanes that could be utilized was tested. Teh and Lee (1973) had shown similar variations in four isolates of Cladosporium resinae. In yeasts, however, n-alkane degradation was found to be a sufficiently distinct property to aid in the identification of species (Bos and De Bruyn, 1973).

From such work it is evident that the ability to degrade n-alkanes is not confined to any particular group of micro-

organism nor to any specific environment. This observation reflects the considerable amounts of n-paraffinic hydrocarbon that are produced and consumed in the biosphere. n-alkanes are known to occur in the tissues of plants and animals and in the cellular lipids of microorganisms, it is only under exceptional circumstances that oil and coal deposits build up having escaped complete microbial decomposition.

Davis (1967) in an extensive review of selected aspects of petroleum microbiology pointed out that in most soils the high molecular weight n-alkane content is relatively stable; usually in the range of 0.5 - 5 μg/g of soil. From a 50 kg sample of New Mexico soil 78 mg of heavy n-alkane were recovered. Under laboratory conditions a sample of the same soil was enriched with some of the extracted hydrocarbon, increasing the amount of n-alkane present from approximately 1.5 μg/g to about 30 μg/g of soil. When incubated at 15% humidity for two months no change in the hydrocarbon content was observed. However, when nutrient mineral salts were added a decrease in the soil paraffins occurred. It was concluded that the microbial degradation of n-alkanes in the soil was limited by deficiencies of nutrient minerals, particularly nitrogen and phosphorus.

Since Fuhs (1961) reviewed the organisms known to that date to utilize n-alkanes, the frequency of reports of n-alkane degrading bacteria, fungi and yeasts increased rapidly

as interest in the subject widened. A similar list would now be of such proportions as to be impractical, nonetheless, reviews by Klug and Markovetz (1971) and Shennan and Levi (1974) present sufficient examples to illustrate the range of organisms involved. The present discussion however will be limited to those organisms that predominate in recent and current work on n-alkane utilization.

I.1.2 Bacteria

It was observed by Klug and Markovetz that the number of bacteria known to utilize n-alkanes far exceeded the number of yeasts and filamentous fungi. They suggested that this merely reflected the bias of workers towards bacteria; more recent work indicates that this was the case.

Of the enormous number of bacteria isolated that are capable of utilizing n-alkanes relatively few genera have been used for detailed study of growth at the expense of these substrates.

Pseudomonas strains have been used for a wide range of investigations into hydrocarbon utilization. Their common occurrence and their ability to grow to high cell densities on a variety of substrates make Pseudomonas species convenient organisms for such studies. Also of interest is the existence of plasmid-borne catabolic pathways in many Pseudomonas strains. With respect to n-alkane metabolism

Table I

Bacteria that utilize n-alkanes and investigations of n-alkane degradation.

Investigation	Genus
Enzymic attack of n-alkane substrates	Pseudomonas
	Corynebacterium
	Acinetobacter
	Nocardia
Metabolic pathways and regulation	Arthrobacter (Corynebacterium)
	Pseudomonas
	Acinetobacter
Morphology	Flavobacterium
	Brevibacterium
	Acinetobacter
Cellular composition	Acinetobacter
	Mycobacterium
	Brevibacterium
	Micrococcus
	Corynebacterium
Surfactants	Pseudomonas
	Arthrobacter
	Corynebacterium
	Brevibacterium
	Nocardia
	Mycobacterium

the OCT plasmid of P. putida (Chakrabarty et al., 1973) which carries the genes for an inducible n-alkane hydroxylase and primary alcohol dehydrogenase (Grund et al., 1975) is of particular interest.

Strains of Acinetobacter have been extensively studied in relation to changes in structure and lipid composition involved in adaptation to n-alkane metabolism.

Strains of Nocardia, Mycobacterium and Corynebacterium have also been used to investigate various aspects of n-alkane degradation. The predominance of these genera above others is probably due to their common occurrence amongst n-alkane-degrading isolates. Table I gives an indication of the range of bacteria that have been used and the nature of the different work done.

I.1.3 Yeasts

The amount of work done on n-alkane utilizing yeasts has increased rapidly over the last fifteen years. Much of the increased interest shown can be attributed to industrially sponsored research on organisms that have some commercial potential.

As with other n-alkane degrading microorganisms, yeasts that utilize paraffinic hydrocarbons can be readily isolated from garden or agricultural soils, as well as from sources where oil spillage has occurred. An insight into the range

of yeasts that utilize n-alkane substrates was obtained by screening cultures from the Central bureau voor Schimmel-cultures using n-decane and n-hexadecane as growth substrates (Scheda and Bos, 1966). It was found that the majority of strains of Pichia, Debaromyces and Torulopsis as well as the better documented strains of Candida were positive when tested.

Shennan and Levi (1974) compiled an extensive list of organisms when reviewing the growth of yeasts on hydrocarbons. Strains from the genus Candida however have recently received most attention. This may hardly be surprising when 63 of the 155 species cited by Shennan and Levi were from this genus.

Recently much work has centred upon biotechnological aspects of growth on hydrocarbons and has been concerned with such problems as oxygen transfer, substrate interaction and transfer and the behaviour of cells under various cultural conditions. Other considerations not directly related to these problems have received relatively little attention. Important exceptions to this general view are the isolation and reconstitution in vitro of the hydroxylation systems for n-alkanes from Candida guillermondii and Candida tropicalis which will be discussed later.

Table II outlines genera of yeasts cited by Shennan and Levi as containing n-alkane utilizing species.

Table II

Genera of Yeasts cited by Shennan and Levi (1974) as containing n-alkane-utilizing species.

Ascomycetous Yeasts	Fungi Imperfecti
Debaromyces	Brettanomyces
Endomycopsis	Candida
Hansenula*	Rhodotorula
Kluyveromyces*	Selenotila
Lodderomyces	Sporidiobolus
Metschinkowia	Sporobolomyces
Pichia	Torulopsis
Saccharomyces*	Trichosporon
Schizosaccharomyces	
Schwanniomyces	
Wingea	

*Bos and de Bruyn (1973) concluded that these genera were devoid of n-alkane utilizing species.

Basidiomycetous Yeasts
Leucosporidium
Rhodosporidium

I.1.4 Filamentous fungi

The observation by Miyoshi (1895) that Botrytis cinerea would attack paraffin is one of the earliest examples of n-alkane utilization in any microorganism. However, many more species of filamentous fungi are now known to grow at the expense of n-alkanes. Klug and Markovetz (1971) reviewed the genera of moulds that grow on n-alkanes and alk-1-enes; those mentioned and the substrates tested are shown in Table III. In a more recent study by Bemmann and Troger (1975) nearly 300 strains of mould were tested for their ability to grow at the expense of a mixture of n-alkanes (C_{12} - C_{21}).

Sources of n-alkane degrading moulds are similar to bacteria and yeasts with isolates from soil being easily obtainable. Rynearson and Peterson (1965) isolated 20 cultures that grew with paraffin as sole carbon source by a direct soil-baiting method, the strains represented species of Aspergillus, Chaetomium, Penicillium, Syncephalastrum and Cunninghamella.

Jet fuel (Prince, 1961; Edmonds and Cooney, 1967) and diesel fuel (Flippin et al., 1964; Koval et al., 1966) have also been reported to support growth of filamentous fungi. Contamination of fuel lines with fungus has caused blockages and given rise to the need for fuel filters in some engines.

Apart from the mode of growth of filamentous fungi other

Table III

Genera of Filamentous Fungi cited by Klug and Markovetz (1971) as being able to utilize n-alkanes.

Genus	Chain lengths of n-alkane substrates
Absidia	Not given
Acremonium	C_{13}
Aspergillus	C_1-C_{18}
Botrytis	C_9-C_{10}
Cephalosporium	C_1-C_{18}
Chaetomium	Paraffin
Chloridium	Not given
Cladosporium	C_{11}-C_{14}
Colletotrichum	C_{13}
Cunninghamella	C_{10}-C_{18}
Dematium	C_1-C_{14}, C_{16}
Epicoccum	C_1-C_{14}, C_{16}
Fusarium	C_1-C_{18}
Gliodadium	C_1-C_{14}, C_{16}
Graphium	C_1-C_{14}, C_{16}
Helicostylum	C_{12}-C_{18}
Helminthosporum	C_{10}-C_{18}
Monilia	C_{13}
Mucor	C_1-C_{14}, C_{16}
Oidoidendron	Not given
Paecilomyces	C_{11}-C_{14}, C_{16}
Penicillium	C_1-C_{14}, C_{16}
Rhizopus	C_{12}-C_{14}
Scolecobasidium	Not given
Spicania	C_{10}-C_{18}
Syncophalastrum	Paraffin
Trichoderma	C_1-C_{14}, C_{16}

important differences between other n-alkane-degrading microorganisms have been noted. In particular, Walker and Cooney (1973a) found that the n-alkane-oxidizing apparatus in fungi is constitutive rather than inducible.

As with yeasts however studies have mostly been confined to specific areas such as identification of primary oxidation products. Biochemical knowledge of n-alkane metabolism in general is lacking.

I.2 Toxicity of lower n-alkanes

The number of microorganisms capable of growth on n-alkanes shorter than nonane (C_9H_{20}) is much smaller than that of the microorganisms that can utilize n-alkanes of greater chain length. Lower alkanes (i.e. shorter than C_9) are relatively soluble but tend to have toxic effects, higher alkanes exhibit lower toxicity but are extremely insoluble in water (Table IV). It generally appears that increasing solubility of n-alkanes leads to increasing toxicity and this factor is harder to overcome than transfer problems with highly insoluble substrates.

Opinions vary as to what causes lower n-alkanes to be toxic. It is known that alkanes are able to adsorb to membranes and bind to hydrophobic membrane proteins. The total effect is likely to be a combination of membrane disruption and protein inactivation.

Table IV

Solubility of n-alkanes in water at 25°C.

n-alkane	Chain length	Molar concentration of saturated solution
Hexane	6	1.1×10^{-4}
Octane	8	5.8×10^{-6}
Decane	10	3.1×10^{-7}
Dodecane	12	1.7×10^{-8}
Tetradecane	14	9.8×10^{-10}

From Einsele and Fiechter (1971).

When studying the toxicity of lower alkanes in yeasts, Gill and Ratledge (1972) found that the toxic effect could be reduced by addition of longer chain n-alkanes. The explanation of this phenomenon was given as the partition of the short chain alkanes into the more insoluble, immiscible higher alkane phase, effectively reducing their concentration in the medium. It was found that under these conditions n-alkanes that were normally toxic could be oxidized by organisms that utilized long chain molecules. Such results have been used to explain the ability of some microorganisms to grow at the expense of motor oils.

Observations by Finnerty et al. (1962) suggest that the physical character of n-alkane substrates as well as the metabolic capability of the microorganisms may affect growth. Using strains of Micrococcus it was shown that the range of shorter chain substrates that could be used could be expanded if the growth temperature was lowered. One strain able to use n-undecane as a minimum length growth substrate at 25°C could also utilize n-decane at 20°C. Such decreases in temperature affect the solubility and vapour pressure of alkane substrates. It can also be argued however that such decreases in temperature may have a more profound effect on the biochemical activity of the organisms and upon the fluidity of the cell membrane.

Further evidence for the solubility of lower n-alkanes

being responsible for toxicity was obtained by Johnson (1964). It was shown that the number of organisms capable of growth on n-hexane increased if the hydrocarbon concentration was kept below saturation.

I.3 Substrate interaction

I.3.1 Initial interaction between cells and n-alkane substrates

Before any substrate can be degraded it must be made available to the microorganism concerned. However, for an organism to grow on long chain n-alkanes which are only poorly soluble it is necessary for cellular contact with the substrate to be maximized. Three mechanisms for uptake of sparingly soluble n-alkanes by microorganisms have been proposed: (i) by direct contact of cells with large oil drops, (ii) by direct contact of cells with submicron drops of oil in the 'accommodated' form, and (iii) by direct uptake of hydrocarbon dissolved in the aqueous phase (Nakahara et al., 1977).

Although some workers have reported that uptake of dissolved hydrocarbon was significant when n-undecane and n-dodecane were used as substrates (Erdtsieck and Rietema, 1969) Aiba et al. (1969) and Aiba and Huang (1970) considered it to be negligible when using n-hexadecane. They observed

that the ratio of n-alkane in solution to the amount consumed during growth was approximately 10^{-6}.

The large amount of work done on yeasts by several workers left opinion divided as to which of the remaining two alternatives was dominant. Velankar et al. (1974, 1975) suggested that submicron droplets were essential for growth on hydrocarbons and proposed the mechanism outlined in figures 1 and 2. High rates of growth on n-alkanes were explained by large drops of oil acting as reservoirs for the diffusion of substrates into micelles. When the micelles were immediately adjacent to a cell rapid transfer of hydrocarbon could take place. Transport of hydrocarbons was dependent upon the amounts within the micelle and the number of micelles present. Support for this model comes from the work of numerous researchers. Yoshida and Yamane (1974) found that when growing yeasts on a colloidal emulsion of n-paraffins the growth rate was directly proportional to the concentration of submicron droplets. When submicron droplets and an oil phase were present the growth rate was still solely dependent upon the submicron droplet concentration.

Other workers have observed that in many fermentations the bulk of cells appear to be associated with large drops of oil. Nakahara et al. (1977) noted that Candida lipolytica when grown on n-hexadecane had 70% of the cells associated

Figure 1

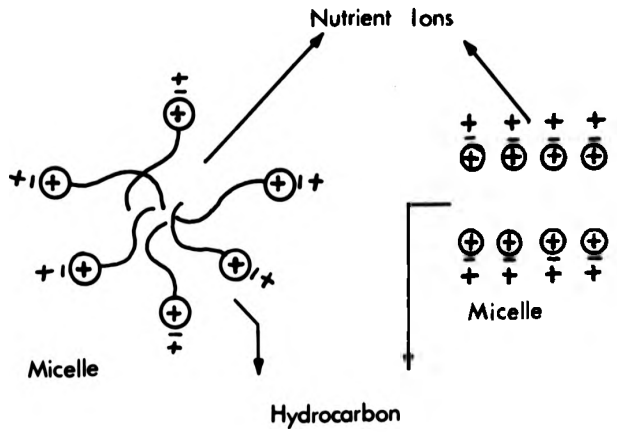
Possible structure of micelles.

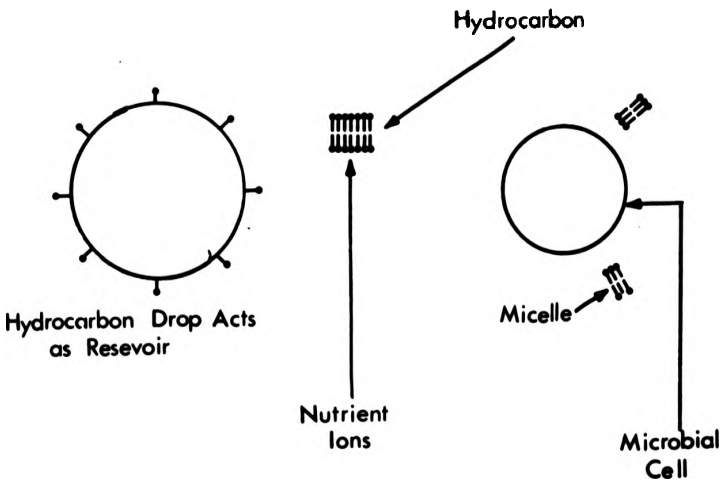
Velankar *et al*. (1974).

Figure 2

Model for uptake of n-alkanes by microbial cells.

Velankar *et al*. (1974).


Nutrient Ions
Micelle
Micelle
Hydrocarbon


Hydrocarbon
Hydrocarbon Drop Acts as Resevoir
Micelle
Nutrient Ions
Microbial Cell

with oil. Einsele et al. (1975) showed that microemulsions did build up around cells growing on n-alkane and pointed out that these microemulsions were formed at the water - oil interface of an emulsified oil fraction. One interpretation of these results is that the association of cells with large oil drops may be due to the cells' requirement for the formation of microemulsions.

The role of large oil drops and submicron droplets may change as a fermentation proceeds. The observation by Katinger (1973) that the interfacial area per unit of the oil fraction increased as cells grew may be accounted for by an increase in the number of submicron droplets which have a larger surface area relative to volume. Goma et al. (1973) noted that drop size decreased during the growth of Candida lipolytica and that 'pseudosolubility' increased. They also observed that the phenomenon was caused by cells and appeared to exhibit a specificity for utilized substrates.

Cellular affinity for hydrocarbons was investigated by Miura et al. (1977). Several species of hydrocarbon utilizing yeasts were compared with each other and with other non-hydrocarbon utilizing species. Adsorption was only observed in those organisms that could degrade hydrocarbons. The degree of adsorption varied between species and it was suggested that the ability to grow at the expense of large droplets directly was only present in some yeasts. This

work confirmed the finding of Einsele et al. (1975) who observed the adherance of submicron drops to alkane-utilizing yeasts but not on yeasts grown on glucose.

The predominance of research into yeast is due to their potential as a source of single cell protein and the possibility of producing useful transformation products from n-alkanes on an industrial scale (Ratledge, 1970). The situation is complicated considerably when other micro-organisms are considered since their behaviour often differs markedly from that of yeasts. Rosenburg et al. (1980) have recently done a simple survey of adherance of bacteria to hydrocarbons. Widely varying organisms were used and it was shown that the majority of types that did not grow on hydrocarbons showed little affinity for the non-aqueous layer. There were however some exceptions; Staphylococcus aureus and early stationary phase Serratia marcescens adhered strongly to a variety of hydrocarbons. It was also noted that an Acinetobacter sp. tested adhered not only to substrates but also to a number of compounds it was incapable of metabolizing. These results were attributed to the hydrophobic nature of the cells. Such observations agree with the work of Kennedy et al. (1975) who investigated the fine structure of an Acinetobacter species growing on n-alkanes using electron microscopy. They also noted adhesion of hydrocarbon to cells and demonstrated the

accumulation of hydrocarbon inclusions within the cytoplasm.

Rosenburg *et al.* (1980) also noted the low affinity of *Pseudomonas aeruginosa* for hydrocarbons despite its ability to use them. This had previously been noted by Hisatsuka *et al.* (1975) and emphasizes the danger of generalizations concerning hydrocarbon uptake in such a wide variety of organisms.

I.3.2 Emulsification of n-alkane substrates

The dispersion of oil in the aqueous phase and the increase in interfacial area between oil drops and water occurs in fermentations involving both yeasts and bacteria when grown on hydrocarbons. In all cases investigated such emulsification was greater than could be explained by simple agitation and was thought to be induced by the presence of cells. Many examples of cultures producing emulsifying agents or surfactants affecting the dispersion of oil substrates in the aqueous phase have been reported. Suzuki *et al.* (1969) observed the formation of trehalose lipid by strains of *Arthrobacter paraffinicus* when grown on n-alkanes. It was postulated that such glycolipids may play a role in hydrocarbon utilization. Strains of several other species able to grow on n-alkanes were also screened and many were found to produce lipids containing trehalose in significant quantities (see Table V). Although it was mentioned that trehalose lipid had been reported prevously in organisms

Table V

Surfactants associated with microorganisms grown on n-alkanes (Cooper and Zajic, 1980)

Surfactants	Genus
Trehalose lipids	Arthrobacter
	Mycobacterium
	Brevibacterium
	Corynebacterium
	Nocardia
Phamnolipids	Pseudomonas
	Torulopsis
Sophorose	Torulopsis
Fatty acids and Neutral lipids	Corynebacterium
	Pseudomonas
	Mycococcus
	Acinetobacter
	Candida
	Aspergillus
	Penicillium
Polysaccharide lipid complex	Candida
	Arthrobacter
Lipopeptides	Candida
	Corynebacterium

growing on non-hydrocarbon substrates, the comparison between the tested strains growing on alternative substrates was not done. However, the trehalose lipid when isolated from Arthrobacter paraffinicus did have significant surfactant activity when added to a mixture of oil and an aqueous solution.

Further evidence for the role of trehalose-containing glycolipid in n-alkane utilization was obtained by treating cultures of the organism growing on hydrocarbons with penicillin. Free trehalose and α-branched β-hydroxy fatty acids were produced. It was suggested that these compounds were the precursors of the active glycolipid and that penicillin inhibited the formation of the final product. The accumulation of these precursors was accompanied by the suppression of both growth and n-alkane consumption.

Hisatsuka et al. (1971) isolated from Pseudomonas aeruginosa a growth stimulant which acted specifically on cells growing on n-alkanes. When added to cells growing on glucose no effects were observed whereas when added to cells growing on n-alkanes the lag phase was decreased and the growth rate increased. The compound was identified as a rhamnolipid and shown to be identical in structure to a glycolipid previously isolated from a similar organism grown on glycerol-bacteropeptone medium. It was shown to possess strong surface activity and emulsifying power but did not appear to affect the growth rate of several other organisms

able to grow on n-alkanes.

Further work by Hisatsuka, Nakahara and Yamada (1972) demonstrated the stimulating effect of a 'protein-like' activator molecule produced when Pseudomonas aeruginosa was grown on n-alkanes. It was also shown that this activator interacted with the rhamnolipid previously isolated. The phenomenon was observed even when sufficient amounts of rhamnolipid were present and stimulation of growth occurred only with n-alkane substrates. Work by the same group (1975) suggested the existence of a complex relationship between the rhamnolipid, the protein-like activator and bivalent cations. Treatment of cells of Pseudomonas aeruginosa with EDTA decreased their ability to oxidize hydrocarbon substrates, oxidation of soluble substrates however was not affected. The ability to oxidize unemulsified n-alkanes was restored by addition of the protein-like activator. It was postulated that removal of bivalent cations from the cells with EDTA caused changes in conformation of the cell wall and released the protein-like activator into the medium.

Studies by Itoh et al. (1971) and Itoh and Suzuki (1972) confirmed the findings of Hisatsuka et al. (1971) concerning the formation of rhamnolipid by n-alkane grown Pseudomonas aeruginosa but identified two different types that differed in one rhamnose residue. Working with mutants of Ps. aeruginosa deficient in the ability to utilize n-alkanes it was shown

that growth on such substrates could be restored by addition of either of the two rhamnolipids to the culture. The mutants were shown to be unable to produce rhamnolipids.

Zajic et al. (1977) investigated a biopolymer produced by Corynebacterium hydrocarboclastus when grown on n-alkanes. The polymer was also found to be present when the organism was grown on some sugars, notably fructose, glucose and mannitol. However the final concentrations obtained with these substrates were not as great as the optimum amounts produced during growth on some n-alkanes. The nature of the polymer did not vary with the growth substrate used but the surface activity and emulsifying power did. This observation in association with the unusual relationship between polymer concentration and surface tension led to the hypothesis that the crude preparation was heterogeneous and that variations in behaviour reflected variations in its relative composition.

In yeasts glycolipids associated with sophorose have been identified and interaction of cells with n-alkanes has been shown to involve a lipopolysaccharide attached to the cell surface.

More recently Kappeli and Finnerty (1979) attributed the enhanced solubility of hexadecane in the growth medium of hexadecane grown Acinetobacter sp. to the accumulation of extracellular vesicles. The particles produced were shown

to have a phospholipid-rich lipo-polysaccharide-rich composition with proteins similar to those found in the Acinetobacter outer membrane.

Many of the substances isolated from cultures of organisms grown on n-alkanes that have some effect upon the dispersal of substrates throughout the medium have also been reported to occur in cultures growing on other substrates. Whether surfactants produced by cultures grown on n-alkanes are a direct response to demands imposed by insoluble substrates in many cases has yet to be demonstrated.

I.4 Transport and uptake of n-alkanes by cells

The uptake of n-alkanes or other highly insoluble substrates by microorganisms, may be facilitated by initial substrate interactions at an extracellular level. The subsequent transport of n-alkane substrates into cells has been shown in many cases to be rapid. In yeasts the ability to transport n-alkanes into the cell is vital because of the localization of the degradative enzymes in the microsomal and mitochondrial membranes. Ultramicroscopic pores were shown by Kozlova et al. (1973) to be present in n-alkane-utilizing yeasts and Munk et al. (1969) had suggested that such pores might account for the appearance of n-alkanes within cells 60 seconds after introduction to cultures. Earlier work by Ludvik et al.

(1968) had demonstrated that such pores were absent in glucose grown cells. The correlation between submicroscopic pores and n-alkane uptake in yeasts may not provide the total mechanism involved. Davidova *et al*. (1975) showed that octadecane was able to penetrate into all membranes of *Candida utilis* within three minutes at 30^{o}C despite the inability of the organism used to utilize n-alkanes. Such observations may be accounted for by the solubility of n-alkanes in the hydrophobic regions of cellular membranes. Ratledge (1978) indicated that little evidence existed for the hypothesis that active transport of n-alkanes across membranes occurred. Hydrocarbon uptake appeared to be by diffusion since it was unaffected by inhibitors of ATP synthesis. It was postulated that diffusion was in fact the mechanism by which hydrocarbons entered cells and that interaction between the substrate and surfactants produced by the cells themselves contributed to a process of 'facilitated diffusion'.

In the case of hydrocarbon uptake it would appear that the transport of n-alkanes into cells does not pose problems of the same magnitude as those that have been shown to arise in the initiation of cell adhesion to the substrate. As has been discussed, cells have overcome the problems of initial interaction with the substrate by synthesis of surfactants and also by apparent chemotaxis (McLee and

Davies, 1972) and modifications to the chemical and structural nature of the cell surface which will be discussed later.

I.5 Initial oxidation of n-alkanes

I.5.1 Possible routes for oxidation of n-alkanes

In most biological systems that are able to degrade n-alkanes or similar saturated carbon residues, the substrate is initially converted to a primary alcohol. This is achieved by direct hydroxylation of a terminal methyl group. The reaction is catalyzed by complex hydroxylases which, in those cases investigated, consist of at least three different components.

Although less common, reports of subterminal attack of n-alkanes to yield secondary alcohols and the detection of products derived from subterminal hydroxylation are numerous. Lukins and Foster (1963) showed that *Mycobacterium smegmatis* produced methyl ketones from corresponding short-chain n-alkanes (C_2-C_6) and Fredericks (1967) identified several ketones and corresponding secondary alcohols when *Pseudomonas aeruginosa* oxidized n-decane. Klein *et al.* (1968) and Klein and Hemming (1969) demonstrated the ability of an *Arthrobacter* species to transform n-decane, n-dodecane,

n-tetradecane and n-hexadecane to n-alkan-2-, -3-, and -4- ols and the corresponding ketones. In these experiments however n-alkane substrates were co-oxidized and were unable to serve alone as sole carbon or energy source.

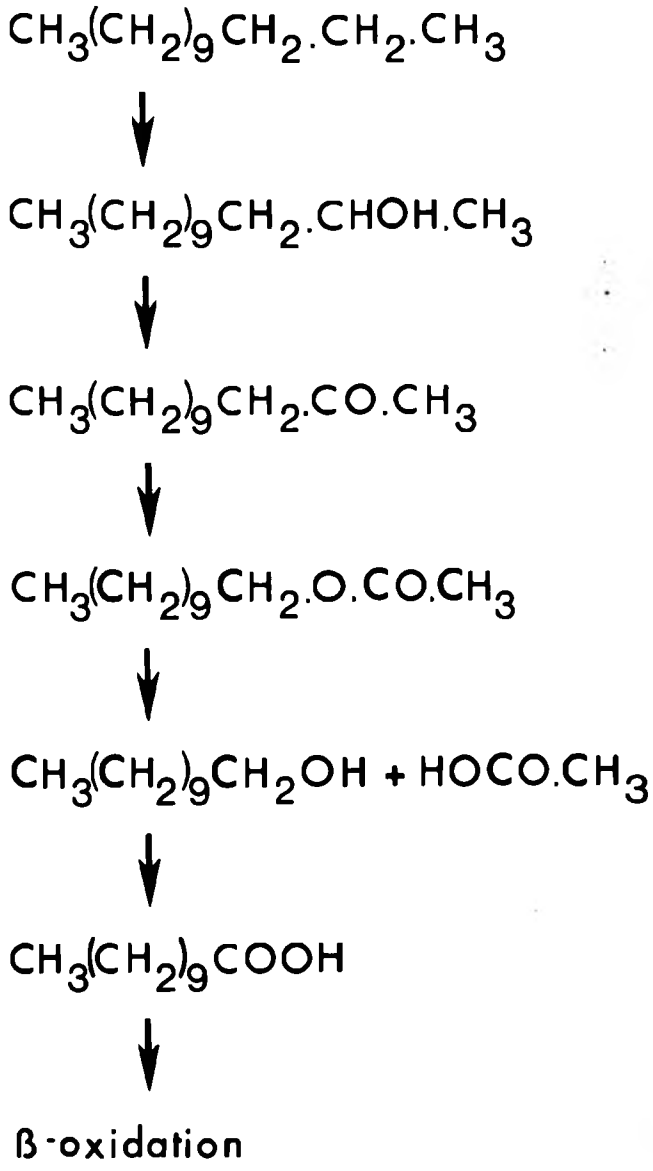
Forney and Markovetz (1970) demonstrated subterminal oxidation by Pseudomonas aeruginosa of tridecane to tridecan-2-ol and undecan-1-ol. They suggested a possible degradative pathway shown in figure 3. Shum and Markovetz (1974a, 1974b) confirmed undecyl acetate as an intermediate in this pathway by a detailed study of undecyl acetate esterase in Pseudomonas cepacia. They found that the enzyme was induced by growth on undecyl acetate, tridecan-2-one and tridecan-2-ol but not on undecan-1-ol.

In filamentous fungi terminal hydroxylation is predominant in n-alkane oxidations. Allen and Markovetz (1970) in a study of species of Cunninghamella and Penicillium demonstrated that the Penicillium species attacked n-alkane substrates subterminally and proposed a pathway similar to that in figure 3 for n-alkane degradation. Allen et al. (1971) showed further that n-tetradecane was attacked by a Penicillium species to give a mixture of subterminally hydroxylated products. This finding was confirmed independently by Pelz and Rehm (1973) who also found products of subterminal oxidation of n-alkanes in a species of

Figure 3

Pathway of n-alkane degradation after subterminal attack.

Forney and Markovetz (1970).


CH3(CH2)9CH2.CH2.CH3
CH3(CH2)9CH2.CHOH.CH3
CH3(CH2)9CH2.CO.CH3
CH3(CH2)9CH2.O.CO.CH3
CH3(CH2)9CH2OH + HOCO.CH3
CH3(CH2)9COOH
ß-oxidation

Verticillium. Ratledge (1978) in a summary of subterminal oxidations of n-alkanes suggested that the phenomenon may be more widespread than previously thought.

Alk-1-enes have been reported by several groups as intermediates in the oxidation of n-alkanes. McKenna and Kallio (1965) prompted by the inconclusive work of Senez and Azoulay (1961) and Chouteau et al. (1962) pointed out that such a system would be thermodynamically unfavourable. An NAD^+-linked dehydrogenase would have to work under anaerobic conditions against an unfavourable equilibrium constant. Despite these reservations Parekh et al. (1977) purified an NAD^+-dependent n-alkane dehydrogenase together with a reduced NADP-dependent n-alkane hydroxylase from a Pseudomonas species grown anaerobically on n-alkanes. The mechanism shown in figure 4(a) was proposed for the anaerobic conversion of n-alkane to fatty acid. Ratledge (1978) indicated that anaerobic growth on n-alkanes is rare and that the organism used by Parekh et al. (1977) was a special case since it grew very poorly aerobically. An alternative to this view was that the alk-1-ene was perhaps produced from a hypothetical intermediate between n-alkane and alcohol if the oxidation of the intermediate became rate limiting. The proposed scheme would then become that in figure 4 (b).

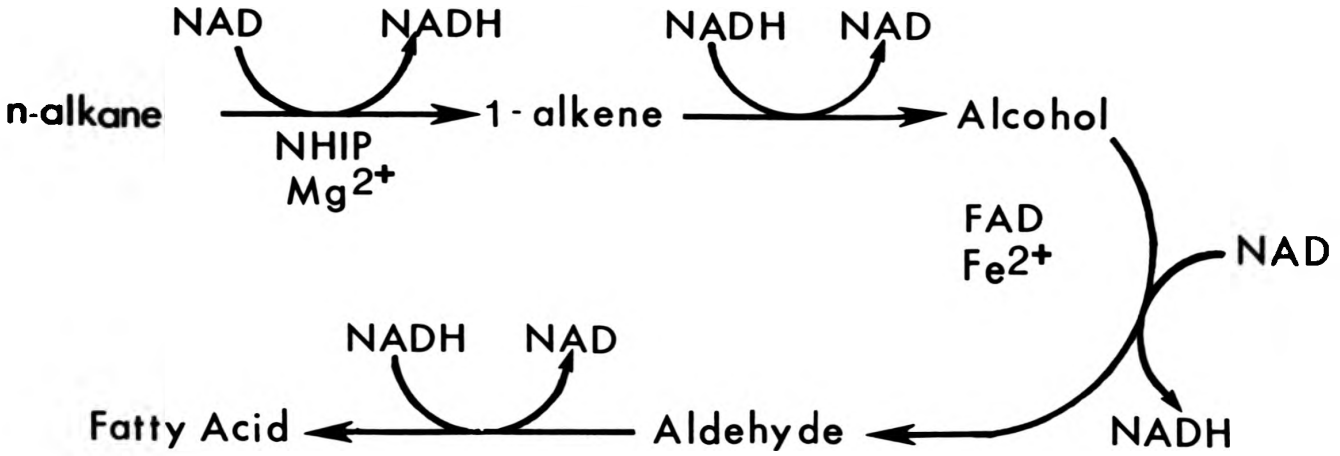
Abbott and Casida (1968) also noted the production of

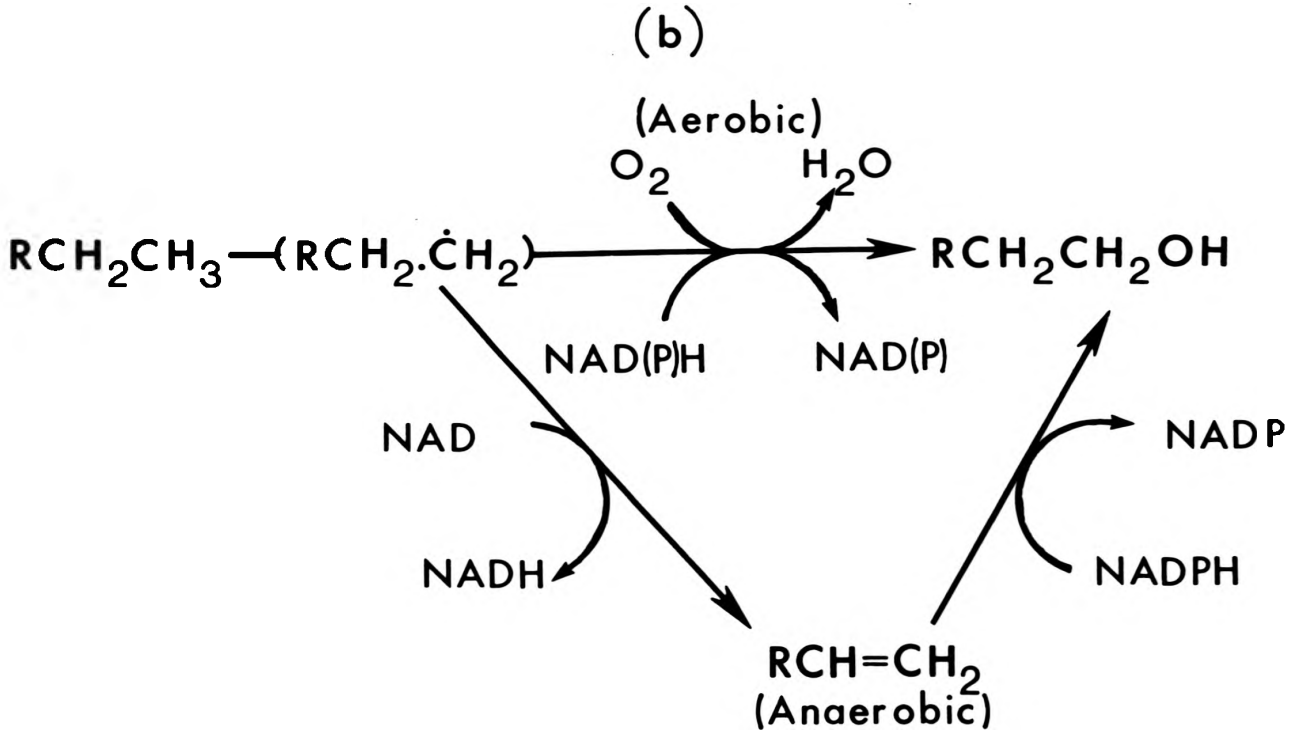
Figure 4

(a) Scheme for anaerobic conversion of n-alkanes to fatty acids (NHIP = non-haem iron protein).
Parekh *et al*. (1977).

(b) Modified scheme in which 1-alkenes are produced when oxidation of an intermediate becomes rate limiting.
Ratledge (1978).

(a)


NAD
NADH
NADH
NAD
n-alkane
1-alkene
Alcohol
NHIP
Mg^{2+}
FAD
Fe^{2+}
NAD
NADH
NAD
Fatty Acid
Aldehyde
NADH


(b)
(Aerobic)
O_2
H_2O
RCH_2CH_3
$RCH_2.\dot{C}H_2$
RCH_2CH_2OH
NAD(P)H
NAD(P)
NAD
NADH
NADP
NADPH
$RCH=CH_2$
(Anaerobic)

internal monoalkenes by Nocardia salmonicolor. Glucose-grown suspensions of resting cells oxidized n-hexadecane to a mixture of internal cis-hexadecenes. The major product when using n-hexadecane as substrate was 7-hexadecene. When using n-octadecane, 9-octadecene was the major product. Such unsaturated products accumulated only in trace amounts when cells were grown on n-alkanes and it was postulated that the insertion of the double bond represented an early step in a novel pathway of aliphatic hydrocarbon degradation. This dehydrogenation did not appear to be associated with the synthesis of unsaturated fatty acids. Further intermediates in the proposed degradative pathway were not identified.

In many cases work with whole cells tended to suggest that 1-alkenes were not intermediates in the oxidation of n-alkanes although the double bond was shown to be attacked when 1-alkenes were introduced into n-alkane degrading systems. Markovetz et al. (1967) obtained 13-tetradeceneoic acid when growing Pseudomonas aeruginosa on 1-tetradecene. This indicated that the double bond was not attacked by the organism and was unlikely to be an intermediate in n-alkane oxidation. However, small amounts of tetradecan-2-ol were also produced.

Klug and Markovetz (1968) found evidence for three

mechanisms for oxidation of 1-alkenes when investigating the degradation of hydrocarbons by Candida lipolytica: (i) Methyl group oxidation to produce ω-unsaturated primary alcohols, (ii) subterminal oxidation to give ω-unsaturated secondary alcohols and (iii) double-bond oxidations to produce 1-2-epoxides and 1,2-diols. The latter products were not detected when cells were grown on n-alkanes.

Allen and Markovetz (1970) noted that when strains of Cunninghamella and Penicillium were grown on 1-tetradecene 13-tetradecenoic acid and unsaturated derivatives of subterminal oxidation were produced by the two organisms respectively. These results show that the unsaturated carbons were not attacked in these strains of fungi.

Overall there is little substantiated evidence for the involvement of a 1-alkene intermediate in the oxidation of n-alkanes.

Of the microbial systems for the degradation of n-alkanes investigated the majority appear to involve terminal hydroxylation of a methyl group as an initial oxidation of the substrate. Two systems which are relatively well understood are the cytochrome P-450 system from Candida tropicalis and the rubredoxin system of Pseudomonas oleovorans. both of which will be discussed more fully.

I.5.2 The cytochrome P-450 hydroxylating system

The term cytochrome P-450 refers to a group of haem proteins that have several characteristics in common. An important diagnostic feature is the formation of carbon monoxide complexes that have a major absorption band at about 450 nm. Such proteins occur widely in nature and have been identified in most animal tissues and in plants. Their chief function is thought to be the hydroxylation of lipophilic substrates.

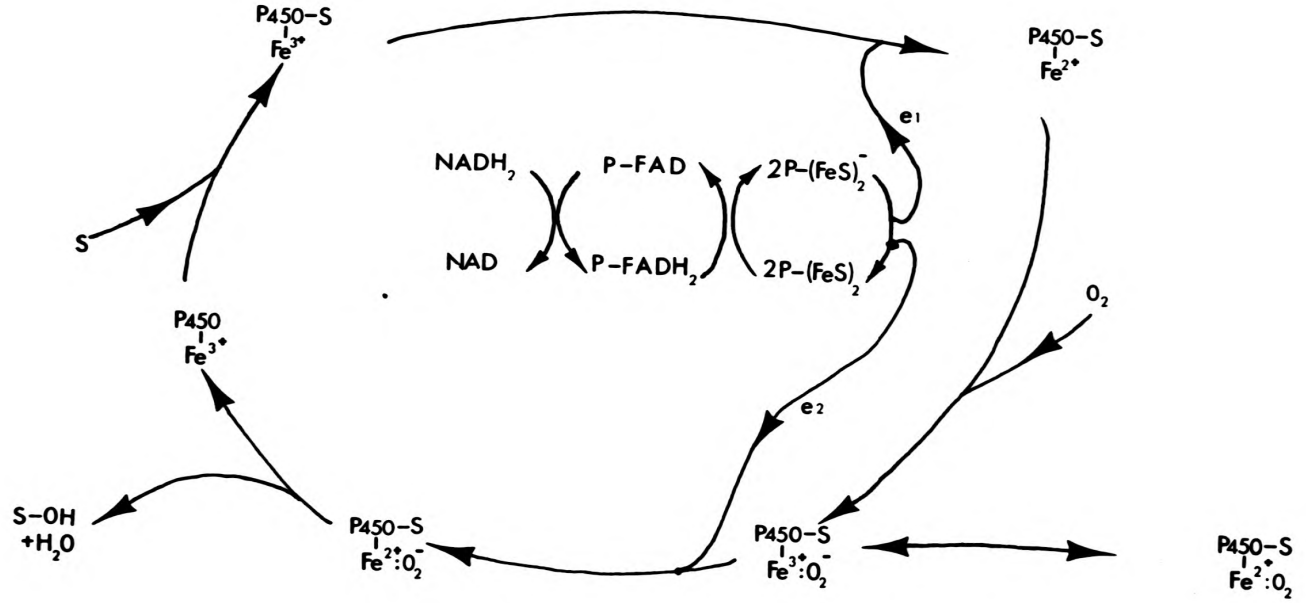
Of several bacterial cytochrome P-450 systems to have been reported, the most highly purified and characterized is the camphor hydroxylation system of Pseudomonas putida (see figure 5). This model system, together with rat-liver microsomal cytochrome P-450, have proved to be of great value in elucidating many aspects of substrate binding and in particular oxygen activation, in reactions involving relatively inert hydrophobic substrates. The subject has been extensively reviewed by Gunsales et al. (1974) and White and Coon (1980).

The first bacterial cytochrome P-450 system associated with the hydroxylation of n-alkanes was reported by Cardini and Jurtshuk (1968, 1970) in a Corynebacterium species strain 7E1C grown at the expense of n-octane. Unlike the liver microsomal system which had already been described, the

Figure 5

Cytochrome P-450 oxygenation-reduction cycle. A postulated native system. (S = substrate molecule.)

Gunsales *et al*. (1974).


P450–S
Fe3+
P450–S
Fe2+
e1
NADH2
P-FAD
2P-(FeS)2-
NAD
P-FADH2
2P-(FeS)2
S
O2
P450
Fe3+
e2
S–OH
+H2O
P450–S
Fe2+:O2-
P450–S
Fe3+:O2-
P450–S
Fe2+:O2

electron donor was NADH rather than NADPH. Electron transfer to the cytochrome was shown to involve a flavoprotein dehydrogenase. The Corynebacterium also differed from the liver microsomal and Pseudomonas putida systems in that no iron-sulphur protein was identified and reductive coupling to the cytochrome was unclear. Atmospheric oxygen was shown to be incorporated into the primary alcohol products using $^{18}O_2$ and the equivalence of NADH to oxygen and products conformed to the stoichiometry of a monooxygenase.

More recently the occurrence of a cytochrome P-450 in several strains of Acinetobacter after growth on n-alkanes has been demonstrated (Asperger et al., 1981). The presence of the cytochrome P-450 was shown to be dependent upon the presence of n-hexadecane in the medium. Also of interest was the observation that only seven of the fifteen strains investigated showed the diagnostic absorbance peak at 450 nm when reduced and in the presence of carbon monoxide.

The n-alkane hydroxylases of Candida guillermondii (Schunk et al., 1978; Müller et al., 1979) and Candida tropicalis (Lebeault et al., 1971) have both been shown to involve a cytochrome P-450 moiety. In Candida tropicalis Duppel et al. (1973) determined that the entire hydroxylation system was NADPH dependent and consisted of a cytochrome

P-450, an NADPH-cytochrome P-450 reductase and a heat stable lipid fraction. The yeast reductase and lipid fractions could be replaced by corresponding fractions obtained from rat liver microsomes. As far as the lipid was concerned, optimal activity was obtained by addition of a yeast lysophosphatidylethanolamine. Under the conditions of preparation the fractions appeared to be soluble. Lebeault et al. (1970) had been misled into suggesting the presence of an NAD^+ dependent n-alkane dehydrogenase by the observation of anaerobic NAD^+ reduction in the presence of n-decane. Re-examination by Gallo et al. (1973) concluded that the observed NAD^+ reduction could be attributed to impurities contaminating the n-decane used in assays. The hydroxylation system that was obtained agreed with the observations of Duppel et al. (1973). The enzyme was NADPH dependent and contained cytochrome P-450. The hydroxylation function however was localized on microsomal membranes in close association with alcohol and aldehyde dehydrogenases. Gallo et al. (1976) in a comparison of methods of preparation of microsomal fractions observed the cytochromes P-450 and b_5 were specifically induced by growth on n-alkanes. It was also noted that glucose grown cells contained low levels of NADPH cytochrome c reductase.

Subsequent work by Bertrand et al. (1979a) investigated

the cytochrome P-450 n-alkane hydroxylase of Candida tropicalis in terms of its individual components. An NADPH cytochrome c reductase was detected and shown to be identical with that produced by Saccharomyces cerevisiae and glycerol grown Candida tropicalis. In n-alkane grown Candida tropicalis however the concentration of the protein was three to four times higher than in glycerol grown cells. It was concluded that such an enzyme was probably involved directly in n-alkane hydroxylation. It was impossible initially to show conclusively that the NADPH-cytochrome c reductase was capable of reducing cytochrome P-450 since the cytochrome itself had not been sufficiently purified.

Studies of the purified NADPH cytochrome c reductase showed that it was a flavoprotein with an apparent molecular weight of 67,000 daltons. It contained one mole of FMN and one mole of FAD per mole of protein. Under the appropriate conditions the enzyme would reduce beef-heart cytochrome c, dichlorophenolindolphenol and ferricyanide. NADPH was oxidized in the presence of beef heart cytochrome c and 2 methyl-1,4-naphthoquinone (menadione).

The partial purification of the Candida tropicalis cytochrome P-450 by Bertrand et al. (1979b) allowed the reconstruction of hydroxylation activity in vitro. The cytochrome P-450 was released from microsomal membranes by

gentle treatment with mild detergents. Use of more severe treatments caused significant conversion of the cytochrome P-450 to its denatured form; cytochrome P-420. Subsequent fractionation of the hydroxylase components was achieved by various chromatographic techniques including hydrophic interaction chromatography and DEAE-cellulose chromatography.

It was shown that the individual components as fractionated had very little hydroxylase activity but that a mixture of the cytochrome P-450 and the NADPH cytochrome c reductase reconstituted a hydroxylating activity with a specific activity of 1.25 units. The addition of a lipid fraction was not required. It was suggested that this lack of requirement for lipid was due to either the non-ionic detergents used in the purification stages which may have had the same effect as a lipid moiety or the presence of residual lipids in the fractionated cytochrome P-450. It is important to note that the units of activity employed for n-alkane hydroxylation effected by the cytochrome P-450 system were very small. It is doubtful whether activities expressed in nanomoles per minute per milligramme of protein may be considered to reflect those encountered in intact cells. The labile nature of the enzyme system as a whole and the insolubility of the substrates were major obstacles to the isolation and purification of preparations

with high activity.

More recently Riege *et al*. (1981) and Honeck *et al*. (1982) have demonstrated conclusively the involvement of cytochrome P-450 in n-alkane hydroxylation on *Lodderomyces elongispora* (formerly *Candida guillermondii*). Both the cytochrome P-450 and the NADPH-cytochrome P-450 reductase were purified to electrophoretic homogeneity and used to reconstitute hydroxylation activity using n-hexadecane as substrate.

The purified cytochrome P-450 had absorption maxima at 555 nm, 523 nm and 417 nm indicating the presence of a protohaeme group. The molecular weight of the enzyme was estimated to be 53,000 and contained one mole of haem per mole of protein. The oxidized form of the protein exhibited a low-spin type absorption spectrum analogous to other low-spin type cytochrome P-450 from other organisms but the absorption peak of the CO-complex was relatively low at 447 nm. The cytochrome P-450 was reduced by NADPH in the presence of the cytochrome P-450 reductase and the substrate which could be replaced by 'tween 20'.

The purified NADPH cytochrome P-450 reductase was shown to be similar to that of *Candida tropicalis* and contained one mole of both FAD and FMN per mole of protein. The molecular weight was estimated to be 79,000.

The activity of the reconstituted enzyme was 0.8

nmoles of product per nmole of cytochrome P-450 per minute, but was enhanced to 5.64 when the reaction was supplemented with a non-ionic detergent. In this respect also, this system bears significant similarities to other systems that have been studied.

Cytochrome P-450 is a widespread and important hydroxylation enzyme with a variety of roles in nature including the initial oxidation of n-alkanes. In bacterial systems that degrade n-alkanes however, cytochrome P-450 has been implicated only in the two cases previously mentioned.

I.5.3 The Rubredoxin-linked ω-hydroxylase of *Pseudomonas putida*

The only bacterial n-alkane hydroxylase that has been extensively purified and characterized is the rubredoxin-linked system isolated from *Pseudomonas putida* (previously called *P. oleovorans* (Nieder and Shapiro, 1975)) outlined in figure 6. Baptiste *et al*. (1963) reported the preparation of soluble cell-free extracts capable of oxidizing n-octane to octan-1-ol. Kusunose *et al*. (1964) demonstrated that similar extracts would also ω-oxidize a series of fatty acids. The hydroxylating activity was shown to require the presence of dioxygen, ferrous ions and NADH. It was resolved into two protein components both required for the formation of octan-1-ol or ω-hydroxy fatty acids (Gholson *et al*., 1963;

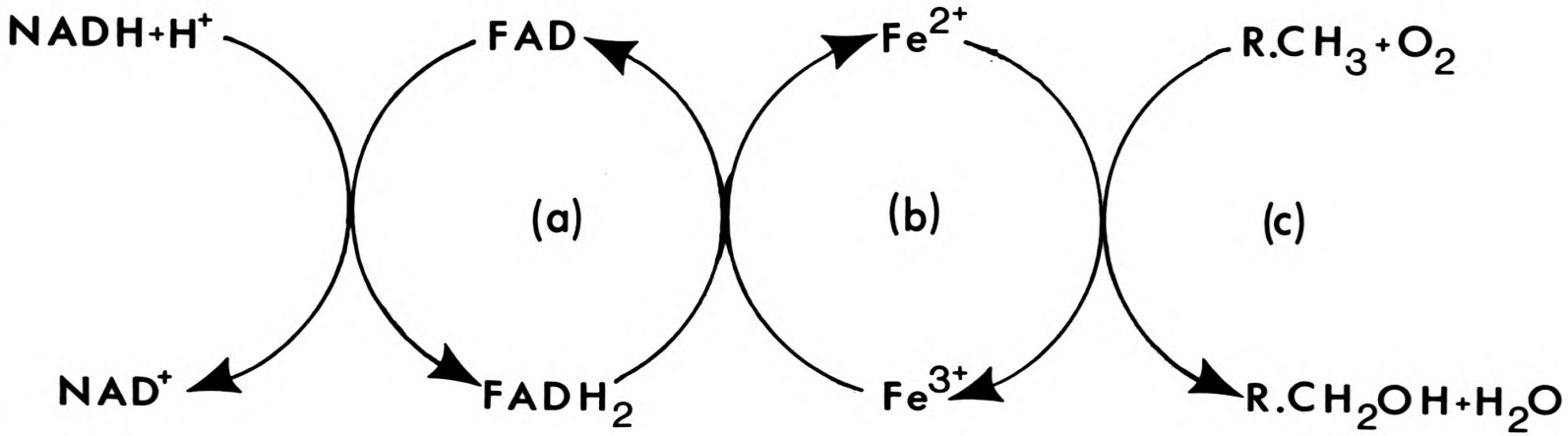
Figure 6

The rubredoxin-linked ω-hydroxylase of *Pseudomonas putida*.

(a) Cytochrome C reductase

(b) Rubredoxin

(c) Hydroxylase.


NADH+H+
FAD
Fe2+
R.CH3+O2
(a)
(b)
(c)
NAD+
FADH2
Fe3+
R.CH2OH+H2O

Coon et al., 1964). Peterson et al. (1966) further separated the ω-hydroxylation system into three essential protein components. These were identified as (i) a highly purified rubredoxin-like protein containing non-haem iron but no inorganic sulphide, (ii) an NADH-rubredoxin reductase and (iii) the ω-hydroxylase.

The ability of rubredoxin to act as an electron carrier was demonstrated by the reduction of cytochrome c by NADPH in the presence of spinach NADPH-ferredoxin reductase (Peterson et al., 1967). When the bacterial NADH-rubredoxin reductase replaced the NADPH-ferredoxin reductase, NADH rather than NADPH was required for the reaction to proceed. Neither of the reductases alone were able to reduce cytochrome c.

It was suggested that rubredoxin acted in a similar manner in the ω-hydroxylation system. Both rubredoxin and a reductase were required with the ω-hydroxylase for substrate hydroxylation and homogeneous preparations of the rubredoxin retained all the activity in the hydroxylation system attributed to partially purified preparations. In addition to this evidence it was shown that under comparable conditions the rate of electron transfer from NADH to cytochrome c was more than enough to account for the rate of electron transfer to oxygen in the presence of the ω-hydroxylase.

Further work was done on various properties of the Ps. putida rubredoxin by Peterson and Coon (1968). The

apparent molecular weight determined by gel filtration was 12,800. Two atoms of iron per molecule were shown to be present and evidence was presented that when enzymically reduced each iron atom accepted one electron. Apart from a comparatively high molecular weight, the rubredoxin from Ps. putida appeared to share many properties with rubredoxins from other sources. The rubredoxins from Clostridium pasteurianum and Peptostreptococcus elselenii, however, were poor substitutes for the Pseudomonas rubredoxin in the ω-hydroxylation system. It was assumed that there was a specificity in the transfer of electrons from the rubredoxin to the ω-hydroxylase since all the rubredoxins used were readily reduced by NADPH in the presence of spinach NADPH-ferredoxin reductase.

Whereas in Ps. putida a role for the rubredoxin had been established in a pathway involving molecular oxygen, the role of rubredoxins in the anaerobic organisms from which they had previously been isolated had not been established.

Improved methods employed by Lode and Coon (1971) showed that the rubredoxin molecule was a single polypeptide chain with a molecular weight of 19,000. It differed in its amino-acid composition from other rubredoxins which did not contain arginine and histidine. The molecule also appeared to differ from the rubredoxin previously isolated by Peterson and Coon (1968) in that it contained only one

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atom of iron. It was found however that the form which contained one iron atom;(1Fe)-rubredoxin, was easily converted to (2Fe)-rubredoxin; a form that contained two iron atoms. Whereas both iron atoms in the (2Fe) form were able to accept electrons from a donor such as dithionite, it was shown that only one of the atoms was effective in the transfer of electrons to the ω-hydroxylase.

The NADH-rubredoxin reductase associated with the ω-hydroxylation system of Pseudomonas putida was isolated and purified to homogeneity by Ueda et al. (1972). The enzyme had previously been too labile for extensive purification and was eventually stabilized by the addition of 10% glycerol to all buffers. The purified protein was shown to contain a single polypeptide chain with a molecular weight of 55,000. A prosthetic group was dissociated from a sample of the purified enzyme and shown by thin-layer chromatography to be FAD. It was estimated that the FAD was present at the level of one mole per mole of purified enzyme. Further investigation of the properties of the protein showed that no metals were detectable by atomic absorption spectrophotometry and that the amino acid composition of the protein showed a high content of hydrophobic residues. The purified enzyme was able to catalyze the rubredoxin-dependent reduction of cytochrome c by NADH.

It was noted that the isolated NADH rubredoxin reductase was similar to proteins demonstrated to be present

in other hydrocarbon degrading systems that had been studied such as the Corynebacterium cytochrome-P450 system reported by Cardini and Jurtshuk (1968, 1970). Other hydroxylation systems were also mentioned as possessing similar reductases. The previously discussed Camphor hydroxylation system from Pseudomonas putida in particular contains a putidoredoxin reductase which contains FAD, has a molecular weight of 43,500 and is composed of a single polypeptide chain. The amino acid composition of the two enzymes was also found to be similar.

The purification and properties of the ω-hydroxylase protein of Ps. putida were investigated initially by McKenna and Coon (1970). It was found that the enzyme contained no cytochrome P-450 and in this respect it was unlike enzymes of similar function in liver microsomes that had been isolated previously. Although a highly purified form of the protein was not obtained, the results suggested that the ω-hydroxylase was a non-haem iron protein; only trace amounts of flavin and haem were detected. Other major differences between the bacterial system and the hepatic cytochrome P-450 were found in the nature of chemicals that were inhibitory. Cytochrome P-450 was severely inhibited by carbon monoxide but was insensitive to cyanide concentrations up to 10 mM. The bacterial rubredoxin system however was inhibited by cyanide and was apparently

unaffected by carbon monoxide.

The substrate specificity of the partially purified ω-hydroxylase was investigated using a variety of substrates that contained methyl groups. It was shown that optimal activity was obtained with n-alkanes with chain lengths of C_8 or C_9. The optimal chain length for the ω-hydroxylation of fatty acids was found to be C_7. Cyclohexane and some of its methyl derivatives were also effective substrates whereas highly branched molecules such as 2,2,4-trimethyl pentane andpristane were attacked at barely significant rates. In contrast to the cytochrome P-450 system isolated from liver microsomes which was capable of hydroxylation of several drugs containing N-methyl and O-methyl groups as well as n-alkanes and fatty acids, the rubredoxin system was shown to be inactive towards benzphetamine and hexobarbital. In none of these experiments were the products identified. Activity was determined on the basis of NADH disappearance.

Further work by May and Abbott (1972 ; 1973) demonstrated that the same enzyme system that hydroxylated n-alkanes in Ps. putida was also capable of the conversion of terminal olifins to their corresponding 1,2-epoxides. Conditions for hydroxylation and epoxidation were similar and both reactions required the presence of the three components of ω-hydroxylation system. Both reactions were inhibited by

cyanide and exhibited a similar pH dependence. It was suggested, despite the impurity of the ω-hydroxylase preparations used, that epoxidation and hydroxylation were mechanistically similar and may involve the same species of activated oxygen.

Ruettinger et al. (1977) reported the purification of the Ps. putida ω-hydroxylase to electrophoretic homogeneity. It was confirmed that the enzyme was a non-haem iron protein which contained one iron atom and one cysteine residue per polypeptide chain of molecular weight 40,800. The enzyme was found still to be capable of both epoxidation and hydroxylation.

It was shown that the inhibition by cyanide was reversible and that enzyme activity was restored upon its removal by dialysis. The iron could be removed from the enzyme by dialysis against EDTA in the presence of a reducing agent. Enzyme activity could be restored by addition of ferrous ions to the apohydroxylase.

I.5.4 Substrate specificity in n-alkane hydroxylases

The two n-alkane hydroxylase systems that have been discussed are chemically and physically distinct from one another but have the same physiological function. Variations in substrate specificity between the two systems and between systems that are more closely related indicate that different enzymes have adapted to similar physiological

functions in different ways.

The cytochrome P-450 monooxygenase from Bacillus megaterium was shown to catalyze the hydroxylation of long chain fatty acids, amides and alcohols. It was inactive against n-alkanes and fatty acid methyl esters (Miura and Fulco, 1974; 1975). The products of the reactions catalysed by this system were all subterminally hydroxylated.

As with the ω-hydroxylase from Ps. aeruginosa the enzyme system was shown to catalyze the epoxidation of long chain unsaturated fatty acids (Ruettinger and Fulco, 1981). Subsequent work by Matson and Fulco, (1981) using hydroxy fatty acids as substrates, has given us an insight into the nature of the hydrophobic substrate binding site. The observations on substrate specificity by the cytochrome-P-450 from Bacillus megaterium contrast markedly from those isolated from other sources that act on long chain saturated substrates. The nature of the reactions catalyzed however were similar and required an activated oxygen species of similar energy. Constraints upon substrate specificity are therefore likely to be dependent upon the ability of the enzyme to bind the hydrophobic substrate .

The methane monooxygenase in Methylococcus capsulatus hydroxylated methane to methanol under physiological conditions (Colby and Dalton, 1976). The organism is an obligate methylotroph and will only grow at the expense of methane or methanol as carbon and energy source. However,

the monooxygenase has a wide substrate specificity and will hydroxylate aromatic compounds, long and medium-chain-length n-alkanes, olefins and compounds that contain O- and N-methyl groups (Colby et al., 1977). The enzyme has been partially purified and shown to be a three component system. It is however distinct from the cytochrome P-450 or rubredoxin hydroxylases (Colby and Dalton, 1978). It has been demonstrated that other methylotrophs have mono-oxygenases that are able to catalyze a similar range of reactions although there appear to be some differences in the physical and chemical properties of the enzymes.

In order to take advantage of an abundant carbon source many organisms - perhaps independently - have developed systems which may effect the initial oxidation of saturated hydrocarbons. The cytochrome P-450 and rubredoxin hydroxylases and the methane monooxygenases of the methylotrophic bacteria are three examples of the development of parallel systems for the degradation of hydrocarbons. The similarity of the reactions catalyzed and the wide range of substrates that may be transformed suggest that the generation of activated oxygen may be achieved by similar mechanisms whereas differences occur in the nature of the hydrophobic substrate binding site. It was postulated by Stirling and Dalton (1981) that the physiological demands of such hydroxylases may have resulted in an active site which was

able to bind a range of substrates giving products for which the metabolism of the cell was not equiped to cope.

I.6 Pathways of n-alkane degradation

I.6.1 Alcohol and aldehyde dehydrogenases

After the initial oxidation of n-alkanes by micro-organisms further oxidation renders primary alcohol products suitable for conversion into the carbon skeletons of cell material or for the production of energy. Several alcohol and aldehyde dehydrogenases have been associated with n-alkane degradation and the production of fatty acids from primary alcohols. It was pointed out by Ratledge (1978) however that such enzymes occur in many organisms that may not degrade n-alkanes. Tassin and Vandecasteel (1971, 1972) demonstrated the presence of an NADP-dependent alcohol dehydrogenase in Pseudomonas aeruginosa strain 196 Aa grown on glucose. Subsequent purification of the enzyme activity revealed the existence of several constitutive alcohol dehydrogenases; all dependent upon NAD^+ or $NADP^+$ and all with high affinity for long chain alcohols (Tassin; Celier and Vandecasteele, 1973). Similar enzymes had previously been reported in other strains of Pseudomonas aeruginosa by Vanderlinden and Huygbregste (1969) and by Azoulay and Heydeman (1963). When Ps. aeruginosa 196 Aa was grown on hydrocarbons (n-hexadecane), another alcohol dehydrogenase

was detected. In contrast to those characterized previously the enzyme was membrane bound and was not NAD(P)-dependent. The product of oxidation was an aldehyde corresponding to the alcohol substrate and the reaction required the artificial electron acceptor phenazine methosulphate (PMS). The high affinity for long chain alcohols and the absence of the enzyme in glucose grown cells led to the conclusion that it was involved in degradation of hydrocarbons. The requirement for an electron acceptor with a high redox potential such as PMS in preference to NAD was suggested to have a physiological value as it may facilitate the oxidation of substrates present at low concentrations by displacement of the equilibrium towards aldehyde formation (Tassin, Celier and Vandecasteele, 1973).

Studies on the aldehyde dehydrogenases of Pseudomonas aeruginosa yielded similar results (Guerillot and Vandecasteele, 1977). Two soluble constitutive enzymes were identified, one NAD-dependent and the other NADP-dependent. The first enzyme had a high affinity for middle and short chain aldehydes and the second had a high affinity for longer chain aldehydes. Growth on hydrocarbons induced the synthesis of a third aldehyde dehydrogenase which was membrane bound and NAD-dependent. The physiological function of the constitutive enzymes was unclear but it was noted that the occurrence of non-specific aldehyde dehydrogenases of ill-defined function was widespread.

In Pseudomonas putida, capable of growth on octane or nonane , due to the presence of the OCT plasmid, a specific NAD-independent alcohol dehydrogenase induced by n-alkane substrates was identified (Grund et al., 1975; Benson and Shapiro, 1976). It was also demonstrated however that at least two alcohol dehydrogenases were coded on the chromosome. Plasmid-free mutants unable to grow on octanol or nonanol were still able to grow at the expense of shorter chain alcohols. In other wild-type strains the plasmid borne alcohol dehydrogenase was shown to be redundant. No alcohol dehydrogenase-defective mutants were isolated amongst strains selected for inability to grow on n-alkanes unless they also carried a chromosomal mutation that blocked growth on octanol or nonanol. The alcohol dehydrogenases coded for by the chromosome differed from the particulate n-alkane-induced enzyme since they were NAD-dependent and soluble. The plasmid encoded alcohol dehydrogenase was suggested to act on surface or membrane bound substrate because of its particulate nature whereas the constitutive chromosomal enzymes could only act on soluble substrates within the cell. Wild-type and some octanol-negative strains without the plasmid did contain the constitutive NAD-dependent octanol dehydrogenase. The octanol mutants were therefore cryptic for octanol oxidation.

The OCT plasmid was shown to encode an n-alkane

hydroxylase and an inducible alcohol dehydrogenase that was distinct from other alcohol dehydrogenases already in the cell. Organisms capable of growth on n-alkanes however were shown to be dependent upon a chromosomal aldehyde dehydrogenase.

In yeasts, specific alcohol and aldehyde dehydrogenases that are connected directly with n-alkane utilization have been reported. In Candida tropicalis two groups of enzyme were identified one of mitochondrial origin (Lebeault et al., 1970) and one of microsomal origin (Gallo et al., 1973a; Gallo, Roche, Aubert and Azoulay, 1973b). Both were induced by growth on n-alkanes. Microsomal alcohol and aldehyde dehydrogenases were also found in Candida intermedia by Liu and Johnson (1971). In contrast to the alcohol dehydrogenase coded for by the OCT plasmid in Pseudomonas oleovorans, the substrate specificity of the enzyme induced by growth on n-alkanes differed greatly from the yeast alcohol dehydrogenase normally found and was NAD-dependent.

In filamentous fungi enzymes of n-alkane oxidation are constitutive and alcohol and aldehyde dehydrogenases are consequently not subject to induction by the growth substrate. Walker and Cooney (1973b) detected NAD(P)-linked alcohol and aldehyde dehydrogenase activities in Cladorsporium resinae. The substrate specificity was wide but only crude protein extracts were used. There may be, as has been demonstrated in

other cases, more than one alcohol or aldehyde dehydrogenase present.

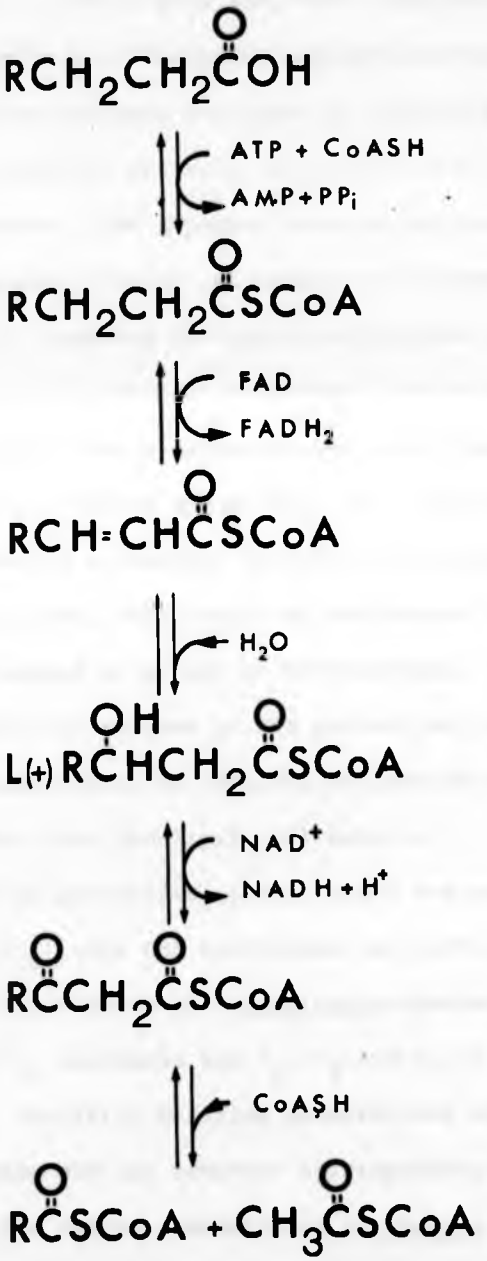
I.6.2 β-oxidation

The subsequent oxidation of fatty acids synthesized from n-alkane substrates is in many cases assumed to be via β-oxidation. There is however little evidence to substantiate this view. Finnerty and Makula (1975) in a review of microbial lipid metabolism, pointed out that on the whole mechanisms elucidated in animal tissues have been transferred to bacterial systems with relatively little experimental justification. In Escherichia coli however, β-oxidation enzymes (figure 7) were detected indepenently by Overath and Raufuss (1967) and Weeks et al. (1969). The pathway was found to be induced by growth on fatty acids although low levels of activity were also found in organisms not grown on fatty acids. Klein et al. (1971) determined that a closely linked fatty acid transport system was also induced at the same time as the β-oxidation enzymes. Both had the same substrate specificity and were only induced by fatty acids with chain lengths greater than C_8. Activation of C_2 to C_{19} mono-carboxylic acids was also reported by Trust and Millis (1971) in two strains of Pseudomonas grown on hexodecanoic acid.

Whether such an enzyme system may be induced by growth on hydrocarbons remains unclear. Duvnjak et al. (1970)

Figure 7

Reactions of β-oxidation based on Finnerty and Makula (1975).

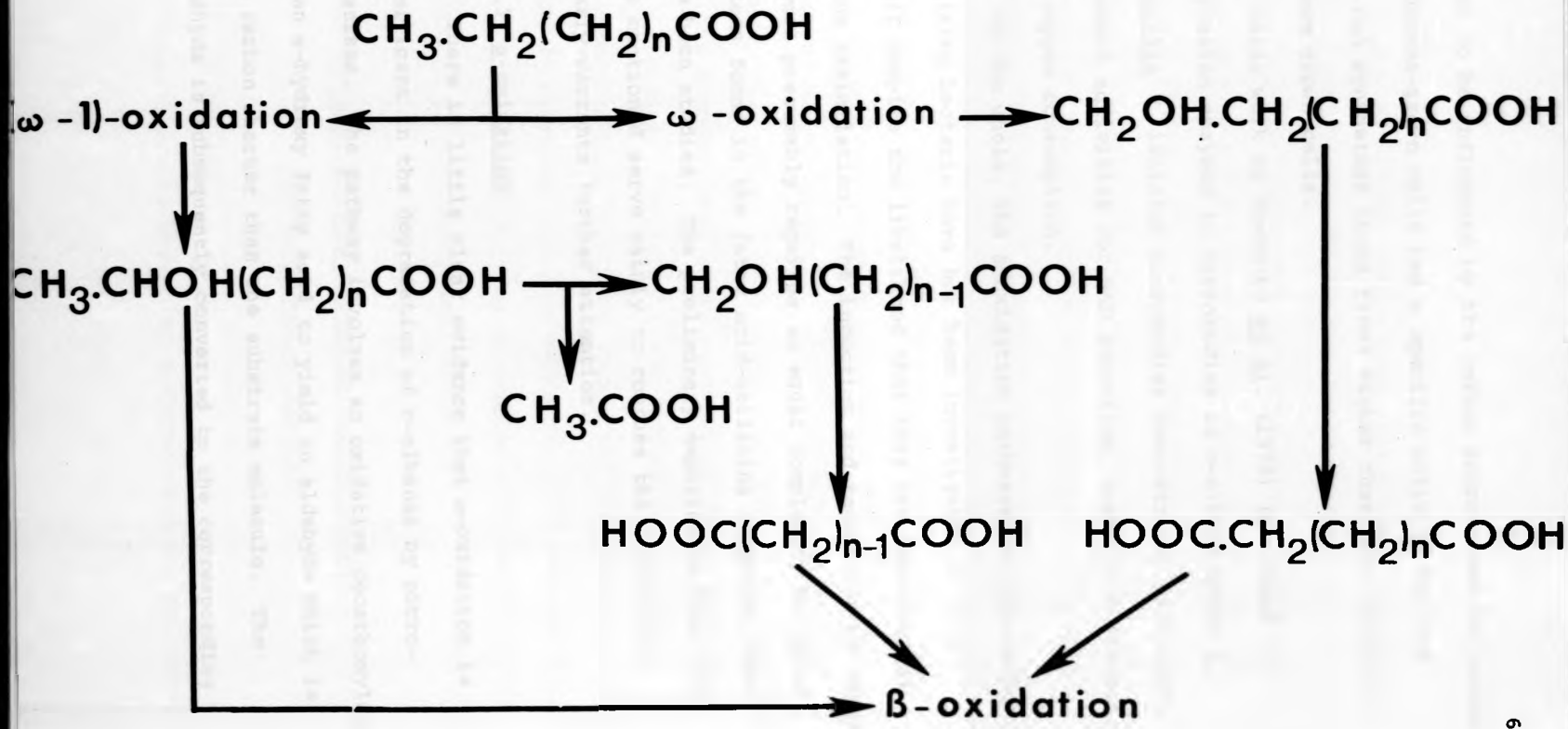

RCH2CH2COH
ATP + CoASH
AMP+PPi
RCH2CH2CSCoA
FAD
FADH2
RCH=CHCSCoA
H2O
L(+) RCHCH2CSCoA
OH
NAD+
NADH + H+
RCCH2CSCoA
CoASH
RCSCoA + CH3CSCoA

investigated the activation of fatty acids by hydrocarbon-grown Candida tropicalis. Mitochondrial particles were found to activate both long-chain saturated and unsaturated fatty acids in the presence of ATP and Coenzyme A. The ability to activate two types of substrate was attributed to the possible presence of two distinct fatty-acyl-CoA synthetases. The expected association between levels of β-oxidation activity and growth on hydrocarbons was not evident. Measured fatty-acyl-synthetase levels were not increased by growth on n-alkanes. At the same time it was pointed out that ω-oxidation may occur leading to the synthesis of dioic acids (fig. 8). Such products would be activated by a specific diacyl-CoA synthetase. If this were the case, fatty acyl-CoA synthetase levels would not be influenced by growth on hydrocarbons. Elevated levels of subsequent enzymes in the pathway may occur since these would still be required to provide the sole route of carbon into non-lipid cell material.

Trust and Millis (1970a) noted the presence of a (C_7 to C_{14}) acyl CoA synthetase, an acetyl CoA synthetase, and an acetokinase in a Torulopsis species capable of growth on C_{12}-C_{20} n-alkanes and C_2, C_3 and C_9 to C_{20} monocarboxylic acids. Partially purified preparations of the acyl-CoA synthetase did not activate corresponding dioic acids and unlike the system demonstrated in Candida tropicalis did

Figure 8

Possible routes for degradation of dioic acids produced by ω- or (ω-1)-oxidation.


CH3.CH2(CH2)nCOOH
(ω -1)-oxidation
ω -oxidation
CH2OH.CH2(CH2)nCOOH
CH3.CHOH(CH2)nCOOH
CH2OH(CH2)n-1COOH
CH3.COOH
HOOC(CH2)n-1COOH
HOOC.CH2(CH2)nCOOH
ß-oxidation

appear to be influenced by the carbon source used for growth. Hexadecane-grown cells had a specific activity for the acyl-CoA synthetase three times higher than that found in glucose grown cells.

Later work by Kawamoto *et al*. (1978) localized the β-oxidation enzymes to microbodies in n-alkane-grown *C. tropicalis* I isolated microbodies demonstrated palmitate-dependent activities for NAD reduction, acetyl-CoA formation and oxygen consumption.

On the whole, the β-oxidation pathways in hydrocarbon-utilizing bacteria have not been investigated in great detail despite the likelihood that they are important in carbon assimilation. The induction and regulation of these enzymes presumably requires an equal complexity to those systems found in the fatty acid-utilizing organisms that have been studied. The preliminary experiments that have been mentioned serve mainly to confuse the issue which clearly warrants further attention.

I.6.3 α-oxidation

There is little clear evidence that α-oxidation is significant in the degradation of n-alkanes by micro-organisms. The pathway involves an oxidative decarboxylation of an α-hydroxy fatty acid to yield an aldehyde which is one carbon shorter than the substrate molecule. The aldehyde is subsequently converted to the corresponding

fatty acid (fig. 9).

Yano *et al*. (1969) pointed out that whereas α-oxidation had been demonstrated in higher plants, mammalian tissues and yeasts, the pathway had only been detected indirectly in bacteria by the presence of α-hydroxy fatty acids in bacterial lipids. Evidence was then presented for the α-hydroxylation of palmitic acid to α-hydroxypalmitate by growing and resting cultures of *Corynebacterium simplex* grown on a rich glucose medium. It was found that small but significant amounts of radioactivity from ^{14}C-labelled substrate were associated with pentadecanoic acid. When the organisms were grown on pentadecane they readily converted α-hydroxypalmitic acid to pentadecanoic acid (Yano *et al*., 1971). It is quite clear from these experiments that the enzymes involved in α-oxidation are present in these organisms. Ratledge (1978) indicates that the occurrence of high levels of even-chain fatty-acids in the lipids of organisms grown on odd chain n-alkanes may suggest that lipids are modified by controlled α-oxidation in the absence of *de novo* fatty acid synthesis.

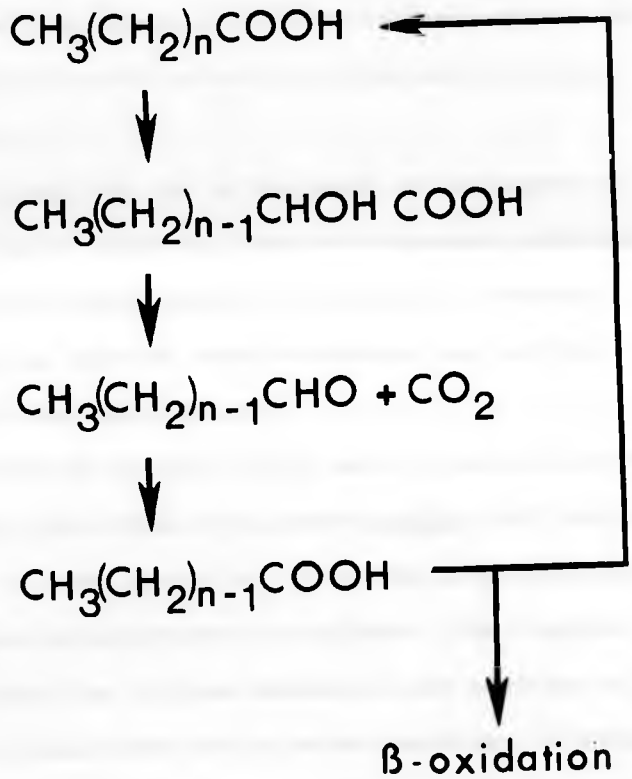
I.7 Adaptations that arise in microorganisms during growth on alkanes

I.7.1 Metabolism

Adaptations to growth on n-alkanes involve not only the

Figure 9

α-oxidation of fatty acids.


CH3(CH2)nCOOH
CH3(CH2)n-1CHOH COOH
CH3(CH2)n-1CHO + CO2
CH3(CH2)n-1COOH
ß-oxidation

induction of degradative enzymes concerned with initial oxidation of the growth substrate, but also a range of changes in the overall metabolic condition of the cell. Whitworth and Ratledge (1975) pointed out that the overall metabolic state of Candida strain 107, when grown on n-alkanes was lipolytic and gluconeogenic whereas the same organism when grown on glucose under carbon limitation was glycolytic and lipogenic.

As with other fields in the study of the growth of microorganisms on n-alkanes, the use of yeasts predominates and particularly when considering prokaryotic systems, generalizations from the results obtained may only be applied with some reservation.

Whitworth and Ratledge (1975) made an analysis of the intermediary metabolism of the yeast Candida (107) which accumulates a large proportion of its dry weight as lipid when grown on carbohydrates or n-alkanes. The organism is constitutive for n-alkane metabolism and addition of n-alkanes to glucose grown cells caused immediate inhibition of glucose transport and metabolism (Gill and Ratledge, 1973).

During growth on glucose and hydrocarbons it was shown that glucose metabolism was through the pentose phosphate pathway which would result in rapid production of NADPH. With glucose as growth substrate the NADPH could be consumed in fatty acid synthesis since there appeared to be an

uninhibited flow of carbon from glucose to acetyl CoA. Fatty acid synthesis could therefore proceed whenever there was a supply of glucose. Growth on n-alkanes, however, resulted in the inhibition of fatty acid synthesis and carbon was shown to flow from the substrate via acetyl CoA, oxaloacetate and pyruvate into pentoses and hexoses. Under these conditions carbon flow was unidirectional due to inhibition of pyruvate kinase and increased activity of phosphoenolpyruvate kinase and fructose 1,6-bisphosphatase.

The enzymes of the tricarboxylic acid cycle were found to be relatively unaffected by changes of growth substrate although citrate synthase was present at levels significantly higher than in baker's yeast particularly during growth on n-alkanes. Enzymes of the glyoxylate bypass were assumed to be present when cells were grown on n-alkanes due to the presence of isocitrate lyase although malate synthetase was not detected. This pathway would provide an additional route for acetyl CoA synthesized by the β-oxidation of fatty acids to enter into the cell metabolism and has been shown to be induced during the growth of several yeasts and bacteria on n-alkanes (Trust and Millis, 1970; Hilderbrant and Weide, 1973, 1974a, 1974b; Kleber and Aurich, 1973; Kleber, 1978).

The observation that addition of n-alkanes to a glucose grown culture of Candida 107 inhibited glucose transport,

was thought by Gill and Ratledge (1973) to be a result of feedback inhibition whereby n-alkanes or their derivatives acted on a key enzyme such as acetyl CoA carboxylase, which caused an eventual inhibition of the transport system. Teh (1975) observed similar effects in Cladosporium resinae when cultures were challenged with short chain n-alkanes that could not be used as growth substrates. It was concluded that the effect of the n-alkanes in this case was directly upon the transport system. Inhibition of glucose uptake was shown to occur before the loss of any cellular protein or potassium which also accompanies the addition of these n-alkanes to C. resinae cultures. The temperature dependence of the phenomenon together with the known ability of n-alkanes to readily bind with the hydrophobic regions of proteins suggested that contact of the n-alkanes with the cell membrane caused a perturbation of the arrangement of glucose transport proteins. The inability of longer chain n-alkanes, which were capable of supporting growth, to inhibit glucose uptake was thought to be due to their decreased solubility.

Siporin and Cooney (1976) found that n-hexadecane was used preferentially when C. resinae was provided with n-hexadecane and glucose. When hexadecane-grown cells were transferred to glucose-containing medium, glucose could be recovered quantitatively. This indicated that n-alkane

grown cells were unable to grow on glucose and it was subsequently shown that hexokinase, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase and succinate dehydrogenase activities were all absent. These enzymes were detected in glucose-grown cells but activity was rapidly lost on addition of n-hexadecane to cell-free extracts. It was evident from this that n-hexadecane can inhibit the activity of enzymes involved in glucose metabolism. By use of the growth inhibitor cycloheximide it was demonstrated that the synthesis of the enzymes was not inhibited by n-hexadecane. The depletion of intracellular n-hexadecane in n-alkane-grown cells transferred to glucose medium that contained cycloheximide allowed the detection of the four marker enzymes at the same levels as in cells transferred to glucose medium without inhibitor despite the absence of protein synthesis.

In an analysis of glucose phosphorylating enzymes in n-alkane- and glucose-grown Candida tropicalis, Hirai et al. (1977) found that hexokinases were regulated and induced by the presence of sugars. The transfer of cells from growth on glucose to growth on n-alkane resulted in a loss of hexokinase activity. There was also a glucokinase present but this enzyme was shown to be constitutive. When a comparison was made with C. lipolytica it was found that both the hexokinase and glucokinase present were constitutive.

It was therefore unclear as to what the metabolic significance of the observed changes in C. tropicalis were unless the control of glucose metabolism during growth on n-alkanes differs between the two organisms.

In other organisms growth on n-alkanes was shown not to be preferential and that metabolism of hydrocarbons could be inhibited by the presence of alternative substrates. The growth of Pseudomonas aeruginosa was investigated by Dalhoff and Rehm (1975; 1976a; 1976b) who found that acetate, succinate, fumarate, glycolate and malonate all severely inhibited the oxidation of n-tetradecane and the utilization of its primary oxidation products including tetradecanoic acid. Other effects noted were that tetradecanal inhibited the utilization of tetradecanol whereas the utilization of tetradecanal was inhibited by addition of n-tetradecane, also the oxidation of added tetradecanoic acid was found to be repressed by the presence of n-tetradecane, tetradecanol and tetradecanal. It was concluded that these observations demonstrated that the inhibitory effects were due to enzyme inhibition rather than adaptation since oxidation rates were unaffected by chloramphenicol. The co-incubation of Ps. aeruginosa with glucose or glucose-6-phosphate and n-tetradecane, inhibited alkane oxidation, protein synthesis and CO_2 production for several hours but the inhibition was removed totally by the addition of cyclic AMP. It was

found that the phosphorylated sugars were the active repressors although no real explanation of their action was given.

An obvious pathway for investigation when considering modifications of cellular metabolism in response to n-alkane utilization would be that of fatty acid biosynthesis. It would seem to be a waste of metabolic energy for fatty acids to be synthesized from the C_2 pool and formed directly from n-alkane oxidation. Such control of fatty acid biosynthesis would be dependent upon the nature of the lipid composition of the organism itself and how this is reflected by the chain length of the n-alkane substrate. For organisms that grow on short chain n-alkanes, the advantage of obtaining fatty acids by direct oxidation of growth substrate would be less because major elongation of the molecules would have to be achieved before incorporation into cellular lipids.

Gill and Ratledge (1973) pointed out that addition of fatty acids to Lactobacillus plantarum does inhibit acetyl-CoA carboxylase which is the first enzyme of fatty acid biosynthesis (Weeks and Wakil, 1970). They were also the first to show that this enzyme is almost completely repressed in Candida 107 grown on n-alkanes. Less than 2% of the activity of acetyl-CoA carboxylase found in glucose-grown cells was present in cells grown on n-alkanes. Subsequent enzymes in the pathway were not repressed to the same extent but it was nonetheless concluded that fatty acid biosynthesis

did not occur in n-alkane grown cells. These observations were supported by several workers. Sampson and Finnerty (1974) found repression of fatty acid biosynthesis in n-alkane-grown Acinetobacter H01N and Mishina et al. (1976a, 1976b) in an analysis of acetyl-CoA carboxylase from Candida lipolytica found that enzyme levels in n-alkane-grown cells were only 16%-18% of the levels found in cells grown on glucose. The same effect was found when cells were grown on oleic and linoleic acids.

Ratledge (1978) expressed surprise that the repression of fatty acid biosynthesis reported by Mishina et al. (1976b) was not greater and attributes the observed activities in the n-alkane grown cells to the presence of yeast extract in the medium that may have caused some derepression of the enzyme.

Decreases in the amount of de novo fatty acid synthesis were also indicated when Mycobacterium convolutum was grown C_{13}-C_{17} n-alkanes (Ascenzi and Vestal, 1979). Cells grown on long chain n-alkanes incorporated between 15 and 85 pmoles of acetate per µg of lipid into the fatty acids, whereas acetate- or propane-grown cells incorporated 280 and 255 pmoles of acetate per g of lipid respectively. Fatty acid synthesis was demonstrated to be inhibited by hexadecane, hexadecanoic acid and hexadecanoyl CoA at the level of Acetyl-CoA carboxylase. The most potent inhibitor was

hexadecanoyl-CoA which caused 65% inhibition at a concentration of 50 μM in extracts of acetate-grown cells, hexadecane and hexadecanoic acid inhibited up to 37% and 39% respectively under similar conditions but concentrations of 1 mM were required. Such data suggested that hexadecanoyl CoA acted as the intracellular inhibitor of acetyl-CoA carboxylase. Levels of acetyl-CoA carboxylase were also found to be twenty-five times less in cells grown on C_{14} to C_{15} n-alkanes when compared to acetate or propane-grown cells. It was concluded from these observations coupled with the high concentrations of free fatty acids in n-alkane-grown cells that de novo fatty acid synthesis was controlled by repression of acetyl-CoA carboxylase synthesis and inhibition of acetyl-CoA carboxylase activity by high levels of acyl-CoA esters derived from the fatty acid pool.

Similar repression of acetate incorporation into fatty acids was demonstrated by Hallas and Vestal (1978) in Mycobacterium convolutum grown on solid n-alkane substrates. The acetate incorporation into other forms of cellular carbon was unaffected. The observed changes in both the fatty acid profiles and acetate incorporation were found to be consistent with the results of other studies in which a number of bacteria and eukaryotic cells were grown on a variety of n-alkanes (Dunlap and Perry, 1967, 1968; Cooney and Proby, 1971; King and Perry, 1975; Makula and

Finnerty, 1968a, 1968b; Patrick and Dugan , 1974; Thorpe and Ratledge, 1972; Vestal and Perry, 1971).

There are many reports that indicate that in numerous cases the fatty acids of lipids of n-alkane-grown organisms do not correspond with the chain length of the substrate. Iida et al. (1980) observed that in Candida rugosa the major fatty acid products after growth on even chain n-alkanes were $C_{18:2}$, $C_{18:1}$, $C_{16:0}$ irrespective of the chain length of the substrate. When the cells were grown on odd-chain n-alkanes the proportion of odd-chain fatty acids was high, reaching 77%-88% in pentadecane- or heptadecane-grown cells. It was concluded that the produced fatty acids were metabolised by β-oxidation or inserted into cellular lipids directly or after elongation with C_2 units. It was not explained how the 12%-23% of fatty acids that were even-chained during growth on odd-chain substrates were obtained. Roy et al. (1978) found in Endomycopsis lipolytica that during a 12 hour incubation with n-alkanes, resting cultures rapidly consumed the substrate and formed $C_{18:2}$, $C_{18:1}$, $C_{16:1}$ and $C_{16:0}$ fatty acids. As with the work of Iida et al. (1980), the products were formed irrespective of the chain-length of the substrate. With n-tridecane and n-pentadecane the corresponding fatty acids appeared only after the C_{16} and C_{18} products had reached peak levels and started to decline. A metabolic scheme was proposed

involving initial oxidation of substrates to their corresponding fatty acids followed by elongation through a malonyl ester independent system followed by decarboxylation by α-oxidation.

The differences of processing of fatty acids between organisms was illustrated by Fukui *et al.* (1976) in a comparison of the fatty acids of *Candida tropicalis* and *Candida lipolytica* grown on a variety of individual n-alkanes. It was found that the organisms differed mainly in their responses to n-undecane and n-dodecane. Growth on n-undecane enhanced the C_{17} fatty acid content of *C. tropicalis* but increased the C_{16} content of *C. lipolytica*. Growth on n-dodecane increased the C_{12} fatty acid content of *C. lipolytica* but not of *C. tropicalis*. In general growth on C_{13}-C_{18} n-alkanes enriched the corresponding fatty acid contents of both cells. Shorter chain n-alkanes were converted to fatty acids and underwent subsequent elongation. The enrichment of even-chain fatty acids during the growth of *C. lipolytica* on n-undecane was thought to indicate *de novo* fatty acid synthesis. It was concluded that the relative activities of incorporation, elongation and *de novo* synthesis of fatty acids differed with the substrate, the organism and the stage of growth of any given culture. This view was taken further by Tanaka *et al.* (1976) in experiments on *Candida lipolytica*. The fatty acid composition during growth of

cells on n-alkaneswas investigated in the presence of a specific inhibitor of de novo fatty acid synthesis with n-alkanes up to dodecane, growth was inhibited and indicated that de novo fatty acid biosynthesis did occur. With n-alkanes above n-tetradecane little inhibition occurred and presumably elongation mechanisms which were unaffected by the inhibitor were able to produce longer chain fatty acids. With n-tridecane both de novo synthesis and chain elongation occurred.

Work concerned with respiratory activity during the growth of microorganisms on n-alkanes has been almost exclusively confined to yeasts and is reviewed in some detail by Ratledge (1978). An important observation was made that during growth on n-alkanes not all oxidation of the substrate is coupled with oxidative phosphorylation (Bell, 1972). It was proposed that this only occurred when the alkane had been oxidized to the level of acetate. It followed from this argument that degradation of fatty acids derived from n-alkanes may not occur in the mitochondria of yeasts. Gallo and Azoulay (1974) showed that re-oxidation of NADH and NADPH formed in the initial oxidation of n-alkanes occurred in microsomal preparations and was not linked to mitochondrial respiration. In this case the hydrocarbon has the same energy potential as the corresponding fatty acids. Gallo and Azoulay (1975) calculated the grams of cells formed per mole of ATP generated from the substrate (Y_{ATP}), and

showed that both palmitate and hexadecane gave the same value of approximately 4 g cells per mole of ATP. The low value of the Y_{ATP} considering the high theoretical energy potential of the substrate could be explained if as Bell (1972) suggested, the β-oxidation of palmitate was not energy producing. The biomass produced from 1 g of carbon whether in glucose or hexadecane was the same despite the considerable difference in the energy content of the two substrates. The extra energy of the hydrocarbon substrate was dissipated as heat which is a feature of growth on n-alkanes.

The initial oxidation of fatty acids was not localized in the mitochondria of yeast but was found to be associated with peroxisomes which were reported to increase significantly in number during the growth of C. tropicalis on n-alkanes (Osumi, 1974; Kapelli, et al. (1975) and were shown by Kawamoto et al. (1977, 1978a, 1978b, 1979), Ueda et al. (1982) and Yamada et al. (1980) to contain the enzymes of higher alcohol oxidation, fatty acid degradation and part of the glyoxylate cycle as well as increased amounts of catalase. The increased presence of catalase was thought to participate in the degradation of hydrogen peroxide generated in the acyl CoA oxidase reaction (Fig. 10(a)) as was postulated by Schneider et al. (1976).

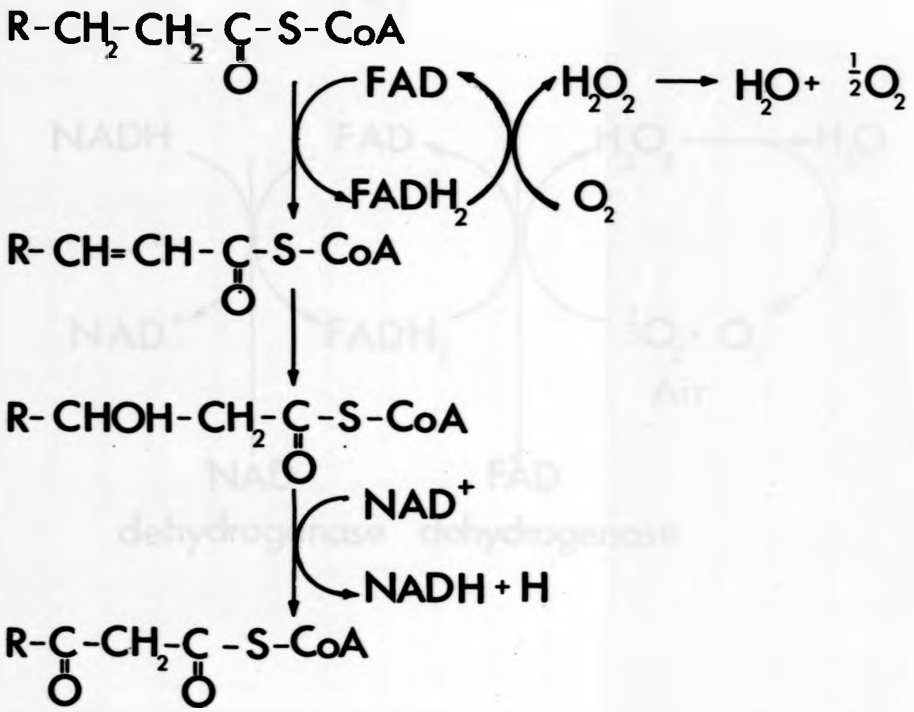
Ratledge (1978) proposed that the high catalase activity may indicate a system for the dissipation of reduced NAD^+

Figure 10

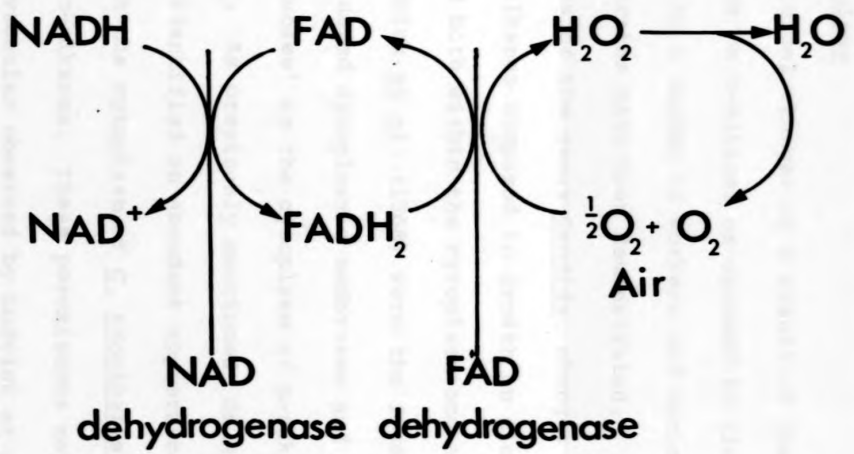
(a) Hypothetical involvement of catalase in degradation of fatty acids in Candida tropicalis during growth on n-alkanes. Schneider et al. (1976).

(b) System for dissipation of reduced NAD^+ proposed by Ratledge (1978).

(a)


R-CH2-CH2-C-S-CoA
O
FAD
FADH2
H2O2
H2O + ½O2
O2
R-CH=CH-C-S-CoA
O
R-CHOH-CH2-C-S-CoA
O
NAD+
NADH + H
R-C-CH2-C-S-CoA
O O

(b)


NADH
FAD
H_2O_2
H_2O
NAD^+
$FADH_2$
$\frac{1}{2}O_2 + O_2$
Air
NAD
dehydrogenase
FAD
dehydrogenase

and FAD (fig.10(b)). NADH and FADH reduction would not be coupled to ATP synthesis and the increased oxygen demand of n-alkane grown cells could be accounted for by the oxygen required by the flavoprotein dehydrogenase.

I.7.2 Morphology

Morphological changes as a result of the growth of microorganisms on n-alkanes as opposed to glucose have been investigated by a number of workers and various adaptations to such substrates have been demonstrated.

In yeasts of the genus Candida, changes induced by growth on n-alkanes compared to growth on glucose have been observed both within the cytoplasm and at the cell surface. Ludvick et al. (1968) were the first to note deeply invaginated cytoplasmic membranes and an increase in 'lipid vacuoles' in the cytoplasm of n-alkane-grown C. lipolytica. As previously mentioned, Osumi et al. (1974) identified an abundant appearance of peroxisomes in the cytoplasm of C. tropicalis in response to growth on n-alkanes. These peroxisomes may correspond to the lipid vacuoles observed by Ludvick et al. (1968) in C. lipolytica. The catalytic activities associated with peroxisomes indicate that they have an indispensible role in the assimilation of n-alkanes (Fukui and Tanaka 1979(a), 1979(b), 1981) and few such organelles have been observed in yeasts grown on other substrates.

Cell surface alterations in response to growth on n-alkanes were reported in C. tropicalis by Kapelli et al. (1978). Kapelli and Feichter (1977) had noted chemical alterations of the cell surface of organisms grown on n-alkanes and identified a polysaccharide-fatty acid complex which was absent in glucose grown cells. Electron microscopy of the surface-structure showed a radial arrangement of wall polymers with protruding evaginations whereas glucose-grown cells were smooth.

Differences in the ultrasctructure of n-alkane-grown bacteria have also been demonstrated. Inclusions within the cytoplasm of a Flavobacterium species (Atlas and Bartha, 1972) and a Brevibacterium species (Atlas and Heintz, 1973) were observed after growth on crude oil and were thought to contain large amounts of lipid. In an Acinetobacter species (strain H01 N, previously Micrococcus cenficans) large intracytoplasmic inclusions were identified after growth on n-alkanes (Kennedy et al., 1975). It was suggested from evidence of Gas chromatography and X-ray crystallography that these inclusions contained unchanged hydrocarbon. Further work on the same organism by Scott and Finnerty (1976), investigated the ultrastructure of hydrocarbon-grown cells and demonstrated that the inclusions previously observed were enclosed by an atypical monolayered membrane structure. Isolated inclusion particles were analysed and shown to contain both polar and neutral lipids of which

unchanged hydrocarbon formed only a minor part.

Scott et al. (1976) indicated that as well as cytoplasmic inclusions in Acinetobacter H01 N, intracytoplasmic membranes may also form in response to n-alkanes. A unique membrane fraction was isolated from hydrocarbon grown cells that was distinct from the inner and outer membrane fractions normally associated with nutrient broth-grown organisms. These findings were subsequently confirmed by Scott and Finnerty (1976) using electron microscopic evidence. The isolated intracytoplasmic membranes were found to contain more phospholipid than other membrane fractions. They did not, however, appear to differ from inner membrane fractions in any enzymic activities nor in electrophoretic banding patterns. The function of such membranes consequently remains unclear. It was noted that neither the inclusions nor the intracytoplasmic membranes were present in glucose-grown cells.

In filamentous fungi few studies of ultrastructure have been done in relation to growth on n-alkanes. Cundell et al. (1976) investigated the growth of a Penicillium species on peptone and on n-hexadecane. n-Alkane grown cultures grew as pellets around hydrocarbon droplets. Thin sections of hyphae indicated the presence of large vacuoles which were absent during growth on peptone.

Walker and Cooney (1973(a)) demonstrated that Cladosporium resinae contained unchanged hydrocarbon when

grown on C_{12}-C_{16} n-alkanes and Cooney *et al*. (1980) showed that the organism was able to accumulate hydrocarbons before oxidizing them. Walker and Cooney (1973b) had already noted that little internal structure was visible during growth of *C. resinae* on glucose whereas cells grown on n-hexadecane contained large globules when observed using phase-contrast microscopy. Further work by Smucker and Cooney (1981) examined ultrastructural changes associated with growth on hydrocarbons. Changes noted when cells were transferred to growth on n-alkanes included thinner cell walls in both hyphae and spores, the appearance of large vacuoles in cells and the synthesis of microbodies. Some vacuoles in hydrocarbon grown cells contained electron-dense inclusions which were absent in vacuoles of glucose-grown cells and which were consequently thought to be associated with some aspect of n-alkane metabolism. The observed microbodies were associated, as in *Candida tropicalis*, with an increase in catalase activity and it was suggested that despite structural differences, they might also be involved in hydrocarbon metabolism. It was pointed out by Smucker and Cooney (1981) however that microbodies did not contain enzymes for initial oxidation of n-alkanes which had been shown to be constitutive (Walker and Cooney, 1973a).

I.8 Hydrocarbons, bacteria and industry

I.8.1 General interests in microbial degradation of hydrocarbons

The considerable interest in microbial degradation of hydrocarbons stems from widely different aims, many of which have been reviewed in recent literature (e.g. Fukui and Tanaka, 1980). As has been mentioned, transformations of hydrocarbons into useful products on an industrial scale has led to extensive research in fermentation processes and enzyme pathways of n-alkane oxidation. In addition to these direct transformations of hydrocarbon substrates, other metabolites that are over-produced during growth of some organisms on n-alkanes are also of commercial interest (Ratledge, 1977). The production of single-cell protein and the degradation of oil spills by the use of bacteria are also fields of investigation and investment.

The reasons for such intense activity with organisms that in many cases are not genetically well understood and which, owing to the insoluble nature of growth substrates, cause considerable problems for large-scale fermentations, are two-fold. The products that are under investigation can be produced by existing industrial processes using chemical means, by fermentation or from vegetable or animal sources. Efficient methods of producing high-cost products from

abundant,relatively low-cost materials such as hydrocarbons, are being sought. The motivation for the development of industrial biotechnology in these cases lies in more economical production processes using cheaper starting materials.

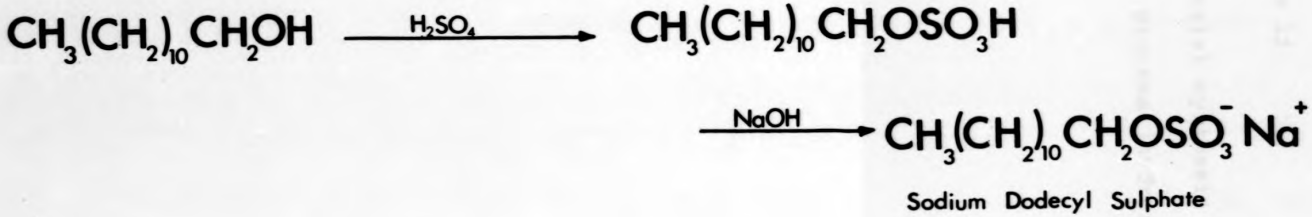
I.8.2 Long-chain primary alcohols and the present work

Long-chain primary alcohols are of economic and industrial importance as starting materials in the production of detergents (figure 11). Existing industrial processes for synthesis of primary alcohols involve the hydroformylation of 1-alkenes (figure 12). The process is only 70% efficient and also produces branched alcohols which reduce the quality of the detergent end-products.

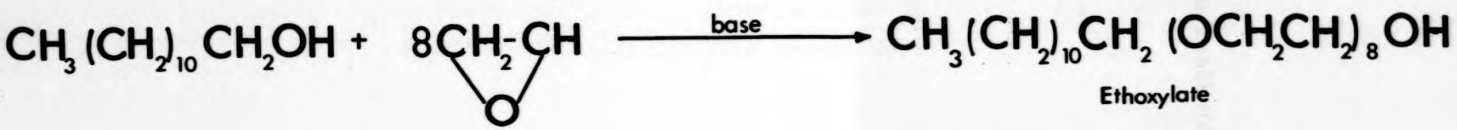
The inefficiency of the process and the high cost of the 1-alkane starting materials make it desirable to find alternative methods of synthesizing long chain primary alcohols. It is known that many microorganisms that are able to grow at the expense of n-alkanes achieve the initial oxidation of the growth substrate directly to produce primary alcohols. Studies of this reaction in carefully chosen organisms may prove useful from several points of view. Apart from the possibility of using microorganisms to effect the transformation of hydrocarbons in a fermentation process, better understanding of the proteins involved may allow the isolation of enzyme systems that can terminally

Figure 11

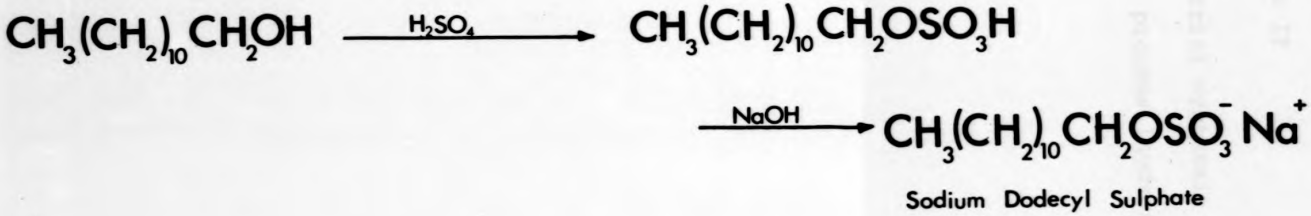
Examples of detergents synthesized from primary alcohols.

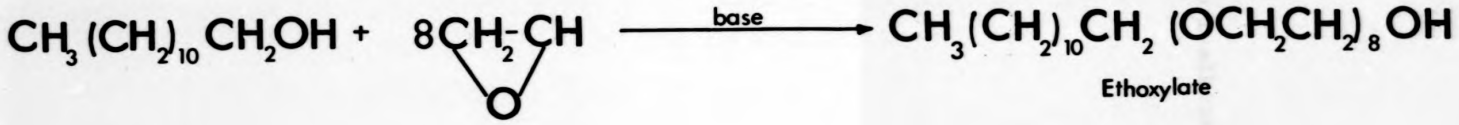


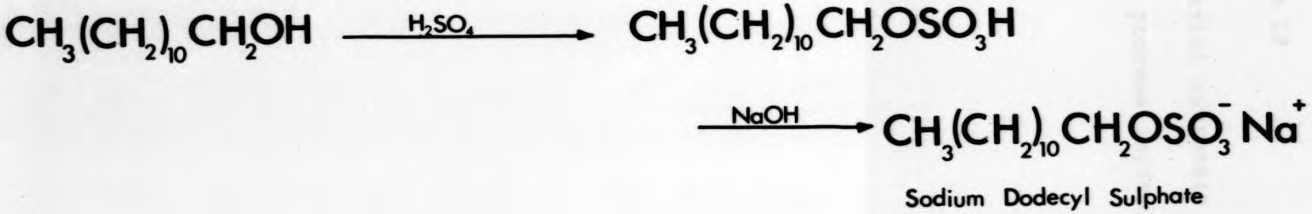
Sodium Dodecyl Sulphate



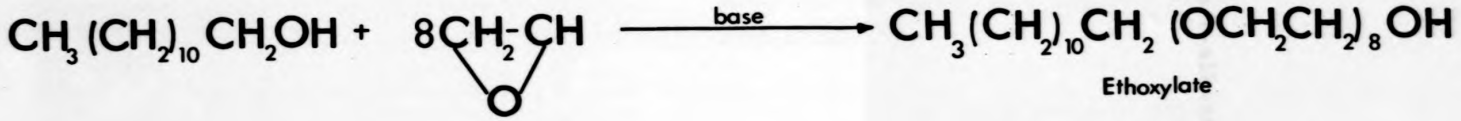
Ethoxylate


CH3(CH2)10CH2OH
H2SO4
CH3(CH2)10CH2OSO3H
NaOH
CH3(CH2)10CH2OSO3- Na+
Sodium Dodecyl Sulphate


CH3(CH2)10CH2OH + 8CH2-CH
O
base
CH3(CH2)10CH2 (OCH2CH2)8OH
Ethoxylate



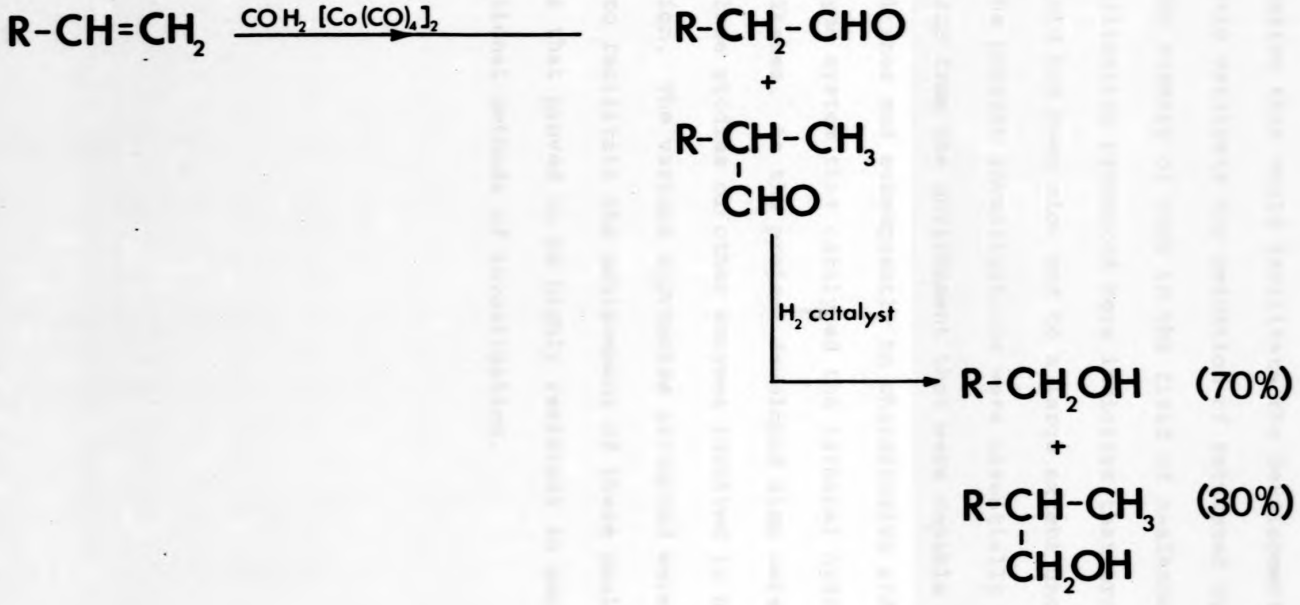
Sodium Dodecyl Sulphate



Ethoxylate

Figure 12

Industrial synthesis of alcohols from 1-alkenes by the 'Oxo' process (hydroformylation).


R-CH=CH2
CO H2 [Co(CO)4]2
R-CH2-CHO
+
R-CH-CH3
CHO
H2 catalyst
R-CH2OH (70%)
+
R-CH-CH3 (30%)
CH2OH

hydroxylate n-alkanes in an immobilized state. Investigations into the mechanistic aspects of enzyme action could provide information that would facilitate the development of synthetic catalysts for oxidation of saturated hydrocarbons.

The summary of work in the field of n-alkane degradation and utilization presented here indicates that progress in the field has been slow and to a large extent uncoordinated.

The present investigations were essentially to isolate organisms from the environment that were capable of growth on n-alkanes and subsequently to characterize and purify an enzyme system that catalyzed the terminal hydroxylation of n-alkanes. As the project developed aims were broadened to include studies of other enzymes involved in n-alkane oxidation. The various approaches attempted were used in order to facilitate the achievement of these goals in systems that proved to be highly resistant to many conventional methods of investigation.

CHAPTER TWO

MATERIALS AND METHODS

II.1 Enrichment and isolation of microorganisms growing on n-alkanes

The initial aim of these experiments was to isolate from the environment microorganisms capable of growth using C_{12}-C_{16} n-alkanes as sole energy and carbon source.

i Media and Materials.

The standard medium used for isolation of n-alkane utilizing microorganisms was one devised for the study of obligate methylotrophs by Dalton and Whittenbury (1976), and will be referred to as Ammonium-Mineral Salts medium (AMS). It was prepared as in Table VI a. The 5% stock phosphate buffer was prepared as in Table VI c. autoclaved separately and added to the medium when both had cooled to below 50°C.

The n-alkanes used as carbon sources were autoclaved at 121°C and 15 lb in^{-2} and added to liquid cultures to a final concentration of 0.5% (v/v). Dispersion of the oil throughout the aqueous phase was achieved initially by violent agitation and subsequent shaking on a rotary shaker.

Agar plates of AMS were made by adding agar to a concentration of 1.5% (w/v). The alkane substrates were provided by one of two methods. The first was to add 0.05 ml of the appropriate substrate to the surface of the agar and to spread it into a thin layer covering the

Table VI

(a) Ammonium mineral salts medium (AMS)

Chemical	Quantity
NH_4Cl	0.5 g
$MgSO_4$	1.0 g
$CaCl_2.2H_2O$	0.2 g
Fe EDTA (3.8% soln in H_2O)	0.1 ml
Trace elements soln*	1.0 ml
$NaMoO_4$ (500 mg/ml in H_2O)	0.5 ml
H_2O	1000 ml

(b) *The composition of the trace elements solution was as follows:

Chemical	Quantity
Na_2 EDTA	250 mg
$FeSO_4.7H_2O$	500 mg
$ZnSO_4.7H_2O$	400 mg
$MnCl_2.4H_2O$	20 mg
HBO_3	10 mg
$CaCl_2.6H_2O$	50 mg
$CuSO_4.5H_2O$	200 mg
$NiCl_2.6H_2O$	10 mg
H_2O	1000 ml

(c) 5% stock phosphate buffer pH 6.8

Chemical	Quantity
Na_2HPO_4	4.97 g
$KH_2PO_4.12H_2O$ or	10.74 g
KH_2PO_4	3.90 g

Dissolved in 100 ml of distilled H_2O and then made up to 300 ml. 20 ml added per litre of AMS.

cont'd..

Table VI continued

(d) L-broth

Chemical	Amount
Bacto-tryptone	10 g
Yeast extract	5 g
NaCl	10 g
$CaCl_2$	0.5 g
Distilled H_2O	1000 ml

plate. The second was to place a sterile 9.0 cm filter paper into the lid of the plate and apply 0.5 ml of the substrate to it. The first method relied on direct contact between cells and liquid substrate. the second relied on substrate in the vapour phase.

When pure cultures had been isolated it was found convenient to maintain them on L-agar plates (Table VI (d)).

All inorganic salts were obtained from British Drug Houses Ltd. and were of analytical grade. Specially pure n-undecane, n-dodecane, n-tetradecane and n-hexadecane were also obtained from British Drug Houses Ltd.. n-Tridecane and n-pentadecane were obtained from Koch-Light. Bacto - agar and nutrient agar were obtained from Difco Ltd. The filter papers used to supply substrates to plate cultures were Whatman No. 1.

ii Enrichment procedures.

Enrichment procedures were the same for all samples except for the differences required for the initial treatment of liquid and solid samples.

Solid samples were dispersed in sterile AMS and 1.0 ml of the resulting suspension was used to inoculate 50 ml of AMS in a 250 ml conical flask. A further quantity of the samples was dispersed in the substrate and 0.25 ml of this suspension was added to the 50 ml already inoculated.

This was done because some of the more oily samples were not readily dispersed in the aqueous medium.

Liquid samples were used to inoculate flasks directly. 1.0 ml of the sample was added to 50 ml of AMS in a 250 ml conical flask after which 0.25 ml of the appropriate substrate was introduced.

All inoculated flasks were placed on a rotary shaker at 30°C and incubated until the cultures were visibly turbid.

The substrates for which this method was used were n-alkanes between and including n-undecane and n-hexadecane.

From the flasks in which growth had occurred 1.0 ml aliquots were taken to inoculate further 50 ml quantities of fresh AMS supplied with the appropriate substrate. Several passages of each culture were made in this way to ensure that growth was at the expense of the supplied substrate.

Isolations were also attempted from samples by direct spreading onto AMS plates. Solid samples were dispersed in sterile AMS as previously described and serial dilutions were made of the resulting suspension down to 10^{-8} using sterile AMS. 0.1 ml of each dilution was spread onto a plate containing substrate on the surface of the agar. Liquid samples were treated similarly except that they were diluted directly. The range of substrates was the same as for liquid enrichments.

The plates were incubated at 30°C until colonies appeared. Individual colonies of distinct morphology were picked off and streaked on to fresh AMS plates supplied with the appropriate substrate. After several passages of single colonies from these plates pure cultures of each cell type were readily obtained as judged by using light microscopic examination.

The liquid enrichments were plated out and pure cultures obtained in a similar manner.

iii Choice of organisms for further study.

The choice of organisms picked from those isolated, for further study, was based upon several factors. Required characteristics considered to be of major importance were (a) the ability to grow on n-alkanes within the range C_{12}-C_{16}, (b) to give high yields of biomass when grown on n-alkanes and (c) that the ability of organisms to degrade n-alkanes should be stable.

II.2 Morphological studies of n-alkane-grown organisms

Morphological characteristics of the organisms chosen for further study were investigated to determine whether any major differences could be observed in cells grown at the expense of n-alkanes. Using electron microscopy, comparisons were made between the morphology of cells grown on n-alkanes and cells grown on alternative substrates. The alternative substrates used were L-broth and acetate in AMS.

i External structure

The structure of whole cells grown on different substrates was observed by staining with Uranyl acetate. 10 ml cultures of the organisms to be used were grown using AMS/n-tetradecane, AMS/acetate and L-broth media. Each of the cultures was stained in the same manner.

One drop of mid-exponential cultures was placed onto a copper formvar-coated grid and left for five minutes. The majority of liquid was removed and one drop of an aqueous solution of uranyl acetate (1% w/v) was placed on the grid. This was immediately removed using a piece of filter paper, and replaced by a drop of distilled water that was left for two minutes. The water drop was removed and the grid left to dry thoroughly for twenty minutes. The stained cells were then ready to be viewed in the electron microscope.

ii Internal structure

(a) Fixation.

Internal structures of cells grown on differing substrates were investigated by the preparation and viewing of ultra-thin sections. The method of preparations was a modified form of that used by Kellenburger (1958) and outlined by Glauert (1965) and Glauert and Phillips (1965). Cells were grown on an appropriate substrate in 50 ml cultures. Exponentially growing cells were centrifuged down and washed twice in distilled water. The cells were then fixed for two hours in 2% glutaraldehyde in Kellenburger buffer (KB) (Table VII, b). Samples were washed after glutaraldehyde fixation and resuspended in osmium tetroxide fixative (Table VII, c). 0.1 ml of Tryptone medium was added for each millilitre of osmium tetroxide fixative used. The samples were left overnight at room temperature before being diluted with KB (8 ml /ml fixative) and spun down. The resulting pellets were resuspended in a minimal volume of molten noble agar (1.5% w/v) which was then spread as a thin layer onto a glass microscope slide and allowed to cool. The solid agar was cut into small cubes which were washed in 0.5% uranyl acetate in KB for two hours. The agar cubes were finally washed twice in KB.

(b) Dehydration.

Samples were dehydrated by washes in progressively more

Table VII

Solutions required for fixation of samples prior to embedding for preparation of thin sections.

(a) Veronal Acetate Buffer (VB)

Chemical	Quantity
Sodium veronal	2.94 g
Sodium acetate (hydrated)	1.94 g
Sodium chloride	3.40 g
Distilled water to give 100 ml final volume	

(b) Kellenburger buffer (KB)

Chemical	Quantity
VB	5 ml (28 ml)
Distilled water	13.0 ml (72 ml)
1M Calcium chloride	0.45 ml (1.4 ml)
(adjust to pH 6.0 using concentrated hydrochloric acid)	

(c) Kellenburger Osmium tetroxide fixative

1 g osmium tetroxide in 100ml Kellenburger buffer.

(d) Tryptone medium (sterilized by filtration)

Chemical	Quantity
Bactotryptone	1.0 g
Sodium hloride	0.5 g
Distilled water	100 ml

concentrated aqueous solutions of absolute ethanol. A procedure that was satisfactory for all samples was that shown in Table VIII.

(c) Embedding.

The dehydrated samples were washed twice in propylene oxide. Samples were then put into a 50% v/v mixture of propylene oxide and Araldite resin (Table IX) without accelerator and agitated gently overnight. The propylene oxide-Araldite mixture was replaced by Araldite resin minus accelerator and the samples agitated for one day. During this period the resin was changed twice.

The agar cubes were finally placed in the bottom of an embedding capsule and covered with complete araldite resin including accelerator. The araldite-embedded samples were placed at 60°C and left for 48 h for polymerization to occur.

When the blocks had hardened sufficiently they were trimmed with razor blades and thin sections were cut using a Reichert OMU2 ultramicrotome. The thin sections were taken up onto a copper grid and viewed using a Joel JEM 100 electron microscope operating at 60 kV.

iii Analysis by light microscopy

Light microscopy was used to assess the average size of the organisms and to investigate the nature of growth

Table VIII

Procedure for dehydration of fixed and stained samples prior to embedding for electron microscopy

% (v/v) Absolute Ethanol	Period of wash (min)
5	5
10	10
30	15
50	15
70	30
95	30
100	30
100	30

Table IX

Composition of Araldite resin used for embedding dehydrated samples for preparation of thin sections.

Chemical	Quantity (g)	
Resin (C7212)	4.9	
Hardener (Dodecyl succinic anydride)	4.9	
Plasticizer (Dibutyl phthalate)	0.05-0.1	mixed before additon of accelerator
Accelerator (Benzyldimethylamine)	0.15-0.2	

on n-alkanes by the use of slide cultures. All photomicrographs were obtained from an Olympus UHT microscope fitted with an Olympus PM-6 camera unit using Kodak Panatomic X film.

Slide cultures were prepared using a version of the method of Dow and France (1980). AMS agar was autoclaved and allowed to cool to below 50°C. Phosphate buffer was added aseptically and two drops of the complete medium were placed on to a sterile microscope slide placed inside a sterile petri-dish. A sterile cover slip was applied to produce a thin film of agar. After 5-10 minutes at room temperature the cover slip was removed after excess agar had been trimmed away. The small square of agar remaining was inoculated with 10 µlof an exponentially growing culture. A fresh cover slip was placed on the agar and sealed onto the slide using paraffin wax. The slide cultures were incubated at 30°C.

No additional substrate was provided in the AMS agar used because it was considered that sufficient n-alkane was provided in the inoculum. In order that the culture should not become oxygen limited during the experiment small grooves could be cut into the agar to allow the retention of air bubbles under the cover slip.

II.3 Routine growth of cells in batch culture and preparation of cell extracts

When large amounts of an isolated strain were required the cells were grown up in LH fermenters in either 5 or 15 litre quantities. The cultures were grown from 100 ml inocula and were maintained at a constant temperature of 30°C and at constant pH by automatic addition of alkali (the pH was usually maintained between 6.6 and 6.8).

The medium was aerated vigorously using electrical air pumps, air flow was usually about 700 ml/minute for 5 litre cultures and 1500 ml/minute for 15 litre cultures. The medium used was AMS and the growth substrate (normally n-tetradecane) was provided at a concentration of 0.5% (v/v).

Growth was followed by increase in optical density of the cultures at 620 nm. Interference from the oil phase appeared to be less at this wavelength than at 540 nm. Assessment of growth using optical densities was not completely satisfactory because great care was required to ensure that cells and oil were properly dispersed. As the age of the cultures increased the increasingly dispersed oil phase contributed to a greater extent to the overall extinction. However adequate growth curves could be obtained.

Cells were harvested by centrifugation during mid- or late-exponential growth using an MSE 'High Speed 18' centrifuge

fitted with continuous flow rotor.

Pelleted cells were washed twice and resuspended in a minimal volume of 20 mM Tris-HCl buffer pH 7.4 containing 1 mM dithiothreitol and 10% v/v glycerol. All manipulations were done at 4°C unless otherwise stated.

DNAase I obtained from Sigma was added to the thick cell suspension to a final concentration of 10 µg/ml. The cells were broken by two passages through the French pressure cell at 138 MPa. Cell breakage was followed by light microscopy.

The resulting cell extract was centrifuged at 10,00 x g to remove remaining whole cells. The pellet was discarded and the supernatant assayed for the enzyme activities of interest before further fractionation.

Initial fractionation was achieved by centrifugation. Extracts were spun at 144,000 x g for 2 h . This effectively removed membranes from the soluble protein fraction.

The soluble fraction was retained and the particulate fraction was resuspended in the breakage buffer. Cell extracts and fractionated samples were stored at -70°C after freezing by dropwise addition into liquid nitrogen.

II.4 Enzyme assays

i n-Alkane hydroxylase

Several approaches were made to the detection of n-alkane hydroxylase activity. These included (a) substrate disappearance, (b) product appearance, (c) substrate stimulated consumption of NAD(P)H and (d) substrate stimulated consumption of oxygen.

(a) & (b) Substrate disappearance and product appearance.

Table X gives the variety of substrates that were used in assays based on the disappearance of substrates or the detection of products. In crude extracts it was thought that n-alkanes may have been oxidized beyond the expected alcohols and several products had to be considered.

Assay procedures were all based on a similar protocol which was varied according to the extracts and substrates used. The reaction volume was 10 ml including substrates and contained 0.1 mg to 5.0 mg of protein in 20 mM Tris-HCl buffer pH 7.4. Assays were done at 30^{o}C in 5 ml conical flasks sealed with size 37 Suba Seals (W. Freeman & Co. Ltd., Yorks). The flasks were incubated in a New Brunswick Gyrotory water bath (Model 976) shaking at 400 oscillations per minute in order to disperse insoluble substrates. Reactions were started by addition of excess donor (50 µl of 100 mM NAD(P)H).

Samples from assay mixtures were taken at five minute intervals or the complete assay mixture was extracted with

Table X

Substrates for n-alkane hydroxylases

Substrate	Expected products	Means of detection
n-Hexadecane n-Tetradecane n-Tridecane n-Dodecane n-Undecane n-Decane n-Octane n-Heptane n-Hexane n-Butane	1^{o} or 2^{o} alcohols, Aldehydes, Ketones, Fatty acids.	GLC/FID
Hexadecanoic acid Pentadecanoic acid Tetradenoic acid Tridecanoic acid Dodecanoic acid Decanoic acid Octanoic acid	ω-hydroxy-fatty acids, ω-1 hydroxy-fatty acids	GLC/FID
1-Tetradecene 1-Hexadecene	1,2 expoxides, Alken-1-ols	GLC/FID
Octadiene	1,2 epoxide 1,2-7,8 die-poxide	GLC/FID
Cyclohexane	Cyclohexanol	GLC/FID
^{14}C-decane	As for alkanes above	Scintillation counting

diethyl ether and analysed for product appearance or substrate disappearance after a set period (usually 15 or 30 minutes). In cases where large volumes of substrate were used samples for analysis were taken from the non-aqueous phase where the products were expected to reside. Small amounts of substrate (1 μmole) used when assays of substrate disappearance were attempted were added as a solution in acetone in order to increase substrate dispersion. In such experiments aqueous samples were taken and tested for the levels of added substrate at intervals over a set period.

In all experiments associated with direct assays of substrates or products, reaction mixtures were analyzed using gas-liquid chromatography (GLC) in association with flame ionization detectors (FID). A Pye-Unicam GCV chromatograph GLC/FID apparatus was used in conjunction with a Hewlett-Packard 3380A chart recorder integrator. All the columns and phases used for GLC were obtained from Phase Separations Ltd. Alcohols, aldehydes, alkanes, alkenes and epoxides could be separated using 10% OV-101 on Chromosorb W.HP 80-100 mesh in a 1 m glass column of 4 mm internal diameter. Acids could be detected directly using 5% DEGS on Gas Chrom Q 100-120 mesh in a 0.5 m column of 4 mm internal diameter. Rates of nitrogen flow and temperatures used were varied within the limits stipulated by the manufacturers according to the substrates used in assays.

An assay based upon detection of ^{14}C labelled products

was attempted using n[1-^{14}C]-decane obtained from Amersham Radiochemicals. 1 ml assays were done as described containing 10 μCi of the labelled substrate. After 30 minutes 10 μg each of unlabelled n-decane, decan-1-ol, decanal and n-decanoic acid were added and the assay mixture was extracted with 3 x 1 ml washes with ether. The ethereal solution was retained and evaporated at as low a temperature as possible to a volume of 100 μl. The remaining solution was subjected to thin layer chromatography using silica gel 60 spread as a 0.2 mm layer on 20 x 20 cm aluminium TLC plates (Merck) and the solvent system of Makula et al. (1975) which contained diethyl ether, benzene, ethanol and glacial acetic acid in the ratio 40:50:2:0.2 (v/v/v/v). The solvent was allowed to run 17 cm before the plate was removed and air dried. The separated fatty derivatives were visualized using a spray continuing 1% iodine in methanol and identified against standards run on the same plate. The different spots were scraped away from the plate and put into 5 ml of a scintillation mixture containing 5 gl^{-1} 2,5-diphenyloxazone (PPO) and 0.3 gl^{-1} p-bis 2-(5-phenyl-oxazoyl)-benzene (POPOP) dissolved in toluene. Scintillation counting for the presence of ^{14}C was done using a Packard Tri-Carb Liquid Scintillation Spectrometer set at 13% gain with the windows set at 50-0.

(c) Substrate stimulated NAD(P)H consumption.

Assays based on substrate stimulated NAD(P)H oxidation were done in 1.5 ml quartz cuvettes and were followed spectrophotometrically at a wavelength of 340 nm.

Assays were done in 1.0 ml and contained 20 µmoles Tris-HCl buffer pH 7.5, 1 µmole dithiothreitol, 0.1 to 1.0 mg of protein and 0.2 µmoles NAD(P)H. The initial rate of oxidation of the added NAD(P)H was followed by monitoring the decrease in absorbance at 340 nm using a Pye-Unicam SP-800. 5 µl of the substrate was added and the rate of NAD(P)H oxidation was followed for a further 5 minutes.

(d) Substrate stimulated oxygen consumption.

Substrate stimulated oxygen uptake was investigated using the Rank oxygen electrode system in conjunction with a Servo-Scribe chart recorder.

Whole cells and cell extract were used. Whole cells were grown up in 500 ml batch cultures and were harvested at mid-exponential phase. Cells were centrifuged down at 10,000 x g, washed twice and resuspended in 20 mM Tris-HCl buffer pH 7.0 to give an optical density at 540 nm of between 3 and 5. 0.5 ml of the whole cell suspension was placed in an oxygen electrode apparatus which contained 1.5 ml of air saturated buffer maintained at 30°C. The initial rate of oxygen consumption was noted. 5 µl of the appropriate buffer was added and the rate of oxygen uptake was noted until all the

oxygen had been exhausted.

Cell extracts were used in similar experiments. Assay volumes were 2.0 ml and contained 40 µmoles Tris-HCl buffer pH 7.5, 2 µmoles dithiothreitol and 0.5 to 1.5 mg of protein. 5 µl 100 mM NADH was added and the initial rate of oxygen consumption was noted. 5 µl of substrate was added and the rate of oxygen uptake followed until all the oxygen had been used.

Using oxygen uptake as an indicator, inhibitors known to inhibit previously isolated n-alkane hydroxylases were used, these included piperonyl butoxide, carbon monoxide and potassium cyanide. Piperonyl butoxide was added as an undiluted liquid in 1 µl quantities, carbon monoxide was added as a saturated solution of the assay buffer and potassium cyanide was added as 100 mM solution in water.

ii Alcohol and aldehyde dehydrogenases

Two assays for alcohol and aldehyde dehydrogenases were used. The first tested the presence of NAD(P)-linked enzymes and the second for phenazine methosulphate (PMS)-linked enzymes.

(a) NAD(P)-linked alcohol and aldehyde dehydrogenases.

NAD(P)-linked enzymes were assayed spectrophotometrically. The reduction of NAD(P) was followed by monitoring the change in absorbance at 340 nm in response to the addition of the appropriate substrate in the presence of protein extracts.

Assays were done at 30^{o}C in a reaction volume of 1.5 ml. The reactions were followed in 1.5 ml quartz cuvettes which contained 30 µmoles Tris-HCl buffer pH 7.5, 1.5 µmole dithiothreitol and 10 µmoles NAD^{+} or NADP. The reaction mixture was bubbled with nitrogen for 30 seconds before addition of 0.1 to 1.0 mg of protein extract. The reaction was commenced by addition of the appropriate substrate and mixed by gentle inversion of the cuvette. The change in absorbance at 340 nm was followed for 10 minutes in a Pye-Unicam SP-800 spectrophotometer.

(b) PMS-linked alcohol and aldehyde dehydrogenases.

The assay used for PMS-linked alcohol and aldehyde dehydrogenases was based upon the method of Tassin, Celier

and Vandecasteele (1973). The reaction volume was 1.5 ml. Assays were done at 30°C in 1.5 ml glass cuvettes and contained 30 µmoles Tris-HCl buffer pH 7.5, 1.5 µmoles dithiothreitol, 0.25 mg PMS, 0.0325 mg dichlorophenolindol-phenol (DCPIP), 75 µmoles NH_4Cl, 15 µmoles KCN and 5 µl substrate. Before addition of protein the reaction mixture was bubbled with nitrogen for 30 seconds. The reaction was commenced by addition of 0.1 to 1.0 mg of protein and followed by monitoring the change in absorbance at 600 nm in a Pye-Unicam SP800 spectrophotometer against a blank containing all the reagents except substrate.

In addition to the spectrophotometric assays for alcohol and aldehyde dehydrogenases it was also possible to follow reactions by the detection of accumulated products or the disappearance of alcohol or aldehyde substrates. Similar 1.5 ml assays were done as above in 5 ml conical flasks sealed with a size 37 Suba seal. The flasks were maintained at 30°C in a New Brunswick Gyrotory shaking water-bath (Model 976) and shaken at 360 oscillations per minute. Samples were periodically removed for analysis by GLC as described previously. In PMS-linked assays it was unnecessary to add DCPIP when this method was used. It was also unnecessary for any of the assays to be done anaerobically.

iii Isocitrate lyase

The isocitrate lyase assay used was that described by Dixon and Kornberg (1959). The method is dependent upon measuring formation of glyoxylate in the presence of isocitrate and protein. Glyoxylate is converted to its phenylhydrazone derivative which is detected by its absorbance at 324 nm.

Assays were done at 30°C in a volume of 1.5 ml. Reactions were followed in 1.5 ml quartz cuvettes and contained 30 μmoles Tris-HCl buffer pH 7.5, 10 μmoles phenylhydrazine hydrochloride, 0.05 μmoles cysteine hydrochloride, 5 μmoles $MgCl_2$ and 0.1 mg protein. The reaction was started by addition of 1 μmole potassium isocitrate (threo) and followed by measuring the absorbance at 324 nm in a Pye-Unicam SP-800 spectrophotometer over a period of five minutes against a reagent blank which lacked only the isocitrate substrate.

One unit of isocitrate lyase will catalyze the formation of 1 μmole of glyoxylic acid phenylhydrazone per minute. The molar extinction of glyoxylic acid phenylhydrazone at 324 nm was determined to be 1.7×10^4 (Dixon and Kornberg, 1959). One unit of enzyme in the described assay system catalyzes a A_{324} of 11.55 per minute. Therefore a A_{324} of 0.100 per minute is equivalent to 0.0087 units.

iv Acetyl CoA carboxylase

The acetyl CoA carboxylase assay used was a linked assay in which ADP produced by the reaction was used to drive production of pyruvate from phosphoenolpyruvate by pyruvate kinase. The pyruvate produced was converted to lactate by lactate dehydrogenase at the expense of NADH. The conversion of NADH to NAD^+ was followed spectrophotometrically by monitoring the change in absorbance at 340 nm (Matsuhashi, 1969).

Assays were done in a volume of 1.5 ml at 30°C. The reactions were done in 1.5 ml quartz cuvettes and contained 30 µmoles Tris-HCl buffer pH 7.5, 15 µmoles $MgCl_2$, 5 µmoles ATP, 0.3 µmoles NADH, 35 µmoles $NaHCO_3$, 1.5 µmoles phosphoenol pyruvate (trisodium salt), 1 µl lactate dehydrogenase/ pyruvate kinase mixture (Sigma). The reaction was started by addition of 0.1 µmoles of acetyl CoA and the rate of decrease of absorbance at 340 nm was followed in a Pye-Unicam SP-800 spectrophotometer for 5 minutes.

v Fatty-Acyl-CoA synthetase

The assay of fatty acyl-CoA synthetase was done by the determination of CoA sulphydryl disappearance according to the method of Grunert and Phillips (1951).

Assays were done in small test tubes in a total volume of 0.2 ml. Reaction mixtures contained 10 µmoles Tris-HCl buffer pH 7.4, 2.5 µmoles $MgCl_2$, 1 µmole KBH_4 (from freshly prepared solution), 0.4 µmoles CoA, 1 µmole ATP, 0.2 µmoles potassium octanoate pH 8.0 and 100-500 µg protein. Assays were incubated at 30°C for 15 minutes under nitrogen. A second tube was prepared containing 2 ml saturated NaCl solution, 0.4 ml NaCN-Na_2CO_3 solution (0.44 g NaCN and 21.2 g Na_2CO_3 per 100 ml of water) and 0.4 ml nitroprusside reagent (2.7 g nitroprusside per 100 ml of water). The resulting solution was poured into the assay reaction and mixed. The absorbance at 520 nm was read immediately using a Pye-Unicam SP-800 against a reagent blank in which potassium octanoate had been omitted.

A change in 0.1 µmole of SH concentration in 3 ml is equivalent to an absorbance reading of 0.200.

II.5 Polyacrylamide gel electrophoresis

Whole cell protein was subjected to polyacrylamide gel electrophoresis in order to determine whether any gross changes in protein composition could be detected as a result of growth on n-alkanes.

i. Preparation of samples

Cells were grown in 100 ml cultures at 30°C. The growth media used were AMS/glucose, AMS/acetate, AMS/n-alkane (n-tetradecane) and L-broth.

Cells were grown to late exponential phase, centrifuged down and resuspended in a minimal volume of 20 mM Tris-HCl pH 7.5. The cells were broken by a single passage through the French pressure cell at 138 MPa and remaining whole cells and cell debris were removed by centrifugation at 10,000 x g for 10 minutes.

The crude preparations were centrifuged at 40,000 x g for 2 hrs to produce a clear supernatant. The soluble fraction was decanted and retained. The particulate fractions were washed in the breaking buffer and the wash supernatants were discarded. The pellets were resuspended in a minimal volume of breaking buffer.

Protein contents were determined by the standard Bio-Rad protein assay.

Lipids were extracted from the particulate fractions by

two washes in chloroform. The chloroform was removed and a volume of the fractions containing 100 µg protein was removed and diluted at least 1 in 5 with loading (LB) buffer containing 125 mM Tris-HCl, 10 mM β-mercaptoethanol, 4% (w/v) SDS and 10% sucrose.

These samples were supplemented with a trace of bromophenol blue and boiled for two minutes before loading onto the gel.

The soluble protein fractions were ready for immediate use. Samples containing 100 µg of protein were taken and diluted in the same manner as the particulate fractions with LB. Trace amounts of bromophenol blue were added and the diluted samples were boiled for two minutes before loading onto gels.

Unused soluble and particulate proteins could be stored for short periods at -20°C.

ii. Polyacrylamide gels and electrophoresis

The protein samples were run on SDS 5-20% polyacrylamide linear gradient gels using the discontinuous buffer system of Laemmli (1970).

The gradient gels were formed with two solutions each of 27 ml . The first solution contained 5% (w/v) acrylamide and 0.133% (w/v) bisacrylamide, the second solution contained 20% (w/v) acrylamide and 0.1 % bisacrylamide. The two

solutions both contained 0.1% (w/v) SDS, 0.2% (w/v) linear polyacrylamide and 375 mM Tris-HCl pH 8.8.

10 µl TEMED and 100 µl of 10% (w/v) ammonium persulphate were added to the two solutions immediately before the gel was prepared. The gradient was then formed by pumping the 5% solution into the 20% solution at half the rate that the 20% solution was simultaneously pumped into the gel plates. Once poured the gel was overlaid with butan-2-ol and allowed to polymerize for at least 2 hrs. The gels could be left overnight at this stage if they were covered to avoid evaporation of the butan-2-ol.

The top of the gel was washed thoroughly with water until no butan-2-ol remained. A stacking gel solution was prepared which contained 4.0% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 0.1% (w/v) SDS and 15 mM Tris-HCl pH 6.8.

To the stacking gel solution was added 15 µl TEMED and 100 µl of 10% (w/v) ammonium persulphate. A small amount of the complete solution was used to wash the top of the resolving gel. The remaining solution was then poured onto the top of the resolving gel. A Teflon slot former was inserted and the stacking gel was left to polymerized for at least 1 hr. After polymerization the slot former was removed and the gel mounted in the electrophoresis tank.

Both electrode buffers contained 25 mM Tris-base, 192 mM glycine and 0.1% (w/v) SDS. The pH was normally about 8.6.

Before loading the gels underwent pre-electrophoresis at 20 milliamps for 30 minutes. The protein samples were loaded onto the gel. Gels were run at 10 milliamps for 16 h which was sufficient for the bromophenol blue to have just run off the end of the gel.

iii Staining of polyacrylamide gels

Gels were stained by immersion in a staining solution which contained 0.25% (w/v) Coomassie Blue (R250), 50% (v/v) methanol and 7% (v/v) glacial acetic acid for 2 hrs. The gels were then destained with several changes of a destaining solution containing 40% (v/v) methanol and 7% (v/v) acetic acid in distilled water.

iv Photography of gels

The gels were photographed after staining using an SMC Pentax-M camera fitted with a 50 mm FZ lens, and Kodak Panatomic 'X' film.

II.6 Preliminary genetic studies

The aims of these experiments were two-fold. Initially they were to determine whether the n-alkane degradation pathway genes were located chromosomally or on a plasmid. Secondly attempts were made to generate mutants that were defective specifically in the initial oxidation enzymes of n-alkane assimilation.

i Curing experiments with acridine orange and ethidium bromide

Both acridine orange and ethidium bromide are reagents that intercalate with DNA and can cause curing of plasmids carried by microorganisms.

The following procedures were used. 10 ml aliquots of L-broth were prepared and adjusted to pH 7.6 with 0.1 M NaOH before sterilization. A 5 mg/ml solution of acridine orange in L-broth was also prepared and sterilized by filtration. A range of concentrations of acridine orange in L-broth were made from zero to 500 µg/ml. Each concentration of acridine orange was inoculated with 0.05 ml of a culture of the organisms under investigation. The inoculated flasks were incubated at 30°C with constant shaking until the control sample (zero acridine orange) had grown to late exponential phase. The inhibition of growth increased with increasing concentration of acridine orange in the growth medium.

The concentration immediately below that which caused total inhibition of growth was used. The culture was serially diluted and spread onto L-agar plates to obtain single colonies. A sample of 500 colonies was then plated onto AMS/acetate and AMS/n-alkane agar and scored for growth on each substrate. A high percentage of non-auxotrophic colonies unable to grow at the expense of n-alkane was considered to be indicative of the pathway being plasmid borne.

Similar procedures were adopted using ethidium bromide in the place of acridine orange.

ii Mutagenesis

(a) Selective media and screening for mutants.

Before organisms were subjected to mutagenic treatments, effective means of screening mutants had to be found.

After mutagenic treatments all samples were screened in the same manner. Cultures were serially diluted and spread onto AMS/acetate agar plates to obtain single colonies. These were then replica plated onto AMS/n-alkane plates and scored for growth. Those colonies unable to grow on n-alkane were plated out and retained.

The mutants thus obtained were characterized by identification of accumulated products when n-alkanes were introduced to cultures growing on acetate or L-broth. In

some cases no products accumulated from the n-alkane because of the repression of the n-alkane degradation enzymes by the presence of the alternative growth substrate. The blocks induced in the pathway of these organisms were screened by scoring for the ability to grow at the expense of n-alkane primary oxidation products.

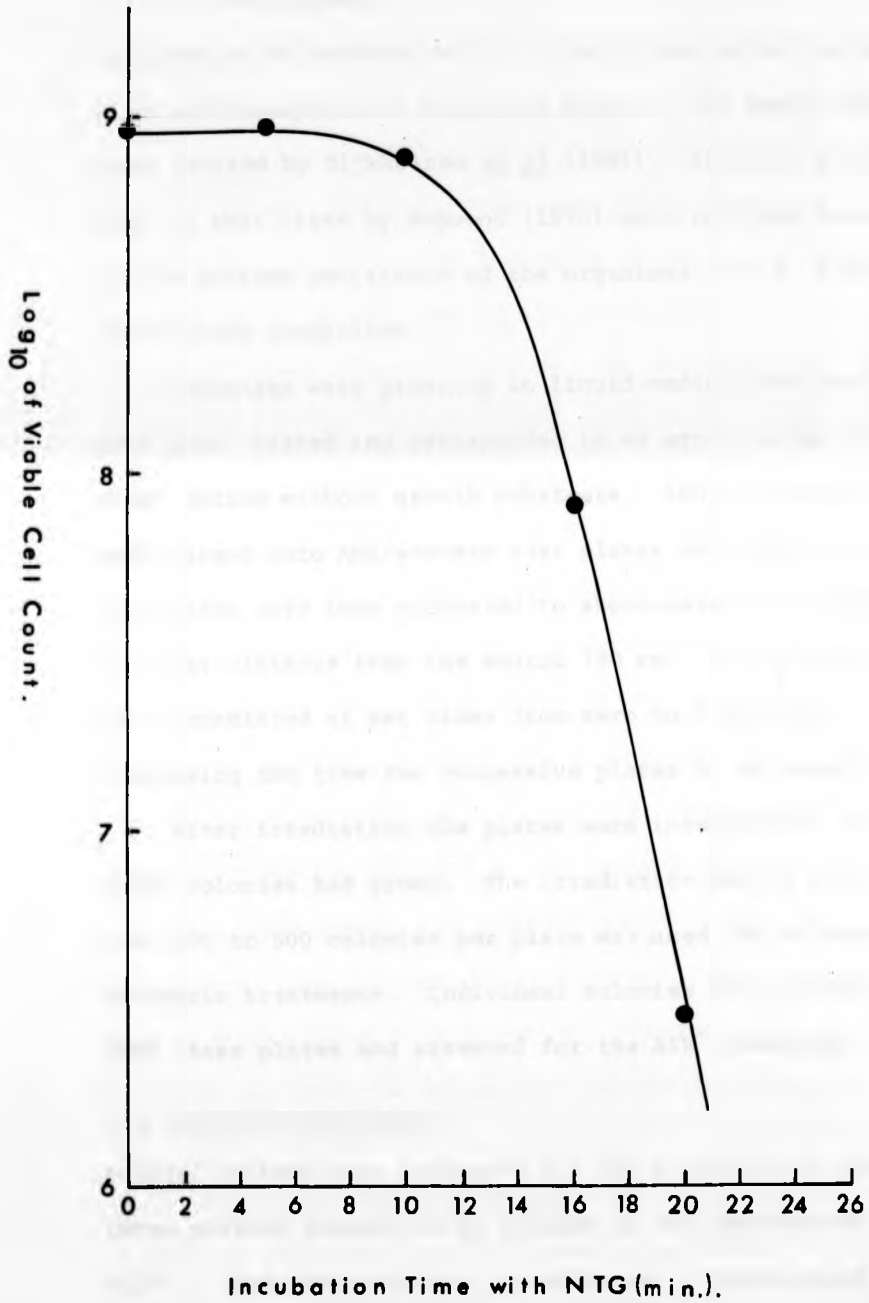
(b) NTG mutagenesis.

The conditions for mutagenesis using nitrosoguanidine (NTG) were determined empirically. 10 ml cultures were grown overnight in L-broth, the cells were spun down and resuspended in an equal volume of fresh medium made 30 μg/ml with respect to NTG. The resulting cell suspension was incubated at 30°C with shaking and aliquots were removed at 5 minute intervals for viable count analysis (fig 13). In subsequent experiments cultures were incubated for periods that resulted in 90% mortality. The cells were then spun down and washed three times in AMS medium without growth substrate before being serially diluted and plated out to single colonies on AMS/acetate agar plates. In some cases the number of auxotrophs generated was assessed by plating onto L-agar plates.

Using the colonies obtained from AMS/acetate plates colonies were picked onto AMS/acetate and AMS/n-alkane plates to screen for their inability to grow on n-alkanes (Alk^-).

Figure 13

Killing curve produced by treatment of C16/8S with NTG.


9
8
7
6
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Log10 of Viable Cell Count.
Incubation Time with NTG (min.).

(c) U.V. mutagenesis.

Mutagenesis by short-wave U.V. light proved effective only with the Gram-positive organisms tested. The method used was that devised by Dijkhuisen *et al*.(1981). Standard procedures such as that cited by Hopwood (1970) were not used because of the extreme resistance of the organisms to U.V. light under those conditions.

Organisms were grown up in liquid medium (AMS/acetate), spun down, washed and resuspended in an equal volume of fresh medium without growth substrate. 100 µl aliquots were spread onto AMS/acetate agar plates and allowed to dry. The plates were then subjected to short-wave U.V. radiation at a set distance from the source (30 cm). The plates were irradiated at set times from zero to 3 minutes increasing the time for successive plates by 30 seconds.

After irradiation the plates were incubated at 30^{o}C until colonies had grown. The irradiation period that left 200 to 500 colonies per plate was used for subsequent mutagenic treatments. Individual colonies were picked from these plates and screened for the Alk^{-} phenotype.

iii Plasmid preparation.

Several methods were attempted for the isolation of the large plasmid thought to be present in the pseudomonad TC15-1. The most reliable procedure was a combination of

the cell lysis method of Mosek (1980, personal communication) and the sucrose gradients used by Wheatcroft and Williams (1981).

50 ml cultures were grown up on AMS/n-tetradecane to late exponential phase. The cells were spun down, washed in 10 mM Tris-HCl pH 7.4 containing 1 mM EDTA (T.E.) and resuspended in 0.312 ml 50 mM Tris-HCl pH 8.0 containing 5 mM EDTA and 50 mM NaCl (TES), 25% (w/v) sucrose. To the resulting cell suspension was added 0.312 ml TES containing 2 mg/ml lysozyme and 1 mg/ml DNAase-free RNAase A (Sigma). The mixture was incubated at 37°C for 10 minutes and on ice for a further 5 minutes. The cells were lysed by addition of 0.312 ml of a 2.4% (w/v) solution of sodium laurosarcosinate in water. The lysed cells were gently drawn up and down a small pipette to partially disrupt the chromosome. When the mixture appeared homogeneous it was extracted with a 25:24:1 mixture of phenol, chloroform and isoamyl alcohol.

Remaining phenol was removed by two washes with chloroform. The volumes of the reagents used could be scaled up for larger quantities of cells. The resulting DNA was suitable for analysis by agarose gel electrophoresis.

Plasmid isolations were attempted using sucrose gradients and caesium chloride-ethidium bromide gradients. Sucrose gradients were formed by the method described by Wheatcroft and Williams (1981). 12.5 ml aliquots of 20% (w/v) solution

of sucrose in sterile distilled water were put into 17 ml nitrocellulose centrifuge tubes and slowly frozen. The gradients were formed by allowing the tubes to thaw slowly at room temperature.

1.5-2.0 ml aliquots of the crude DNA preparations were layered onto the gradients and spun at 100,000 x g for 1 hr. The gradients were fractionated after centrifugation by piercing the bottom of the tubes and collecting 0.5 ml aliquots by gravity. Fractions were screened for the presence of plasmid by agarose gel electrophoresis.

Caesium chloride gradients were made by increasing the volume of the crude DNA preparations to 15.0 ml with T.E. buffer and adding 17.0 g of solid caesium chloride. When the caesium chloride had dissolved completely 250 µl of a 5.0 mg/ml solution of ethidium bromide was added. The resulting solutions were placed into 25 ml centrifuge tubes and spun at 140,000 x g for 72 hrs. Gradients were fractionated by piercing the bottom of the tube and collecting plasmid bands visualized by ultraviolet light. Ethidium bromide was removed from fractions by washing with isopropanol saturated with a saturated solution of caesium chloride in T.E. Caesium chloride was removed by extensive dialysis against T.E. buffer.

CHAPTER THREE

RESULTS AND DISCUSSION

III.1 Isolation of Microorganisms

III.1.1 Enrichment and isolation of organisms from the environment

Soil and water samples were taken from a variety of areas in the vicinity of Leamington Spa, Warwickshire. Using the procedures already described, the organisms in Table XI were isolated.

Strains were numbered in the following manner:

Substrate used for isolation —— C16/1S —— Method of enrichment (S = direct plating, L = liquid)
Isolate number

A soil sample was also obtained by Dr. H. Dalton from an oil-field in Texas. Organisms isolated from this source were numbered in a similar manner but prefixed with the letter 'T'. The letter indicating the method of enrichment was omitted.

A variety of organisms were isolated from the different sources but many were unable to maintain substained growth on minimal medium with n-alkanes as sole carbon and energy source and were consequently lost. Those that remained all had the ability to grow on n-alkanes even after long periods of maintainance on media with non-alkane growth substrates.

It was noted that microorganisms were isolated from all

Table XI

Organisms isolated during the present work.

Strain	Source	Gram-stain	Cell type
C11/1L	(a)	+ve	Long irregular rods
C12/2S	(a)	+ve	Rods
C12/3L	(c)	+ve	Rods
C12/4S	(a)	-ve	Short rods
C12/5S	(a)	+ve	Rods
C14/1S	(a)	-	Yeast
C14/2S	(a)	+ve	Variable-rods and coccoid
C14/3S	(c)	-ve	Short rods
C14/6S	(c)	+ve	Rods
C14/1L	(a)	+ve	Long irregular rods
C14/3L	(a)	+ve	Variable-rods and coccoid
C16/3L	(a)	-	Yeast
C16/7S	(b)	-ve	Cocci
C16/8S	(c)	variable	Club-shaped rods
C16/2L	(b)	+ve	Long irregular rods
TC14-1	(d)	+ve	Long irregular rods
TC14-3	(d)	-ve	Short rods
TC14-4	(d)	+ve	Variable-long and short rods
TC14-6	(d)	+ve	Short rods
TC15-1	(d)	-ve	Motile rods
TC16-1	(d)	-	Yeast

(a) Activated sludge (Finham Sewage works)

(b) Grand Union Canal (Leamington Spa)

(c) Flavel Garage Forecourt (Leamington Spa)

(d) Texas oil-field soil sample

the samples taken but significant contamination with oil products was only present in those taken from the garage forecourt and Texan oil-field. It was also found that there was considerable duplication between the methods of enrichment and organisms from liquid enrichment were also isolated by direct plating.

III.1.2 C16/8S and TC15-1

i Growth characteristics

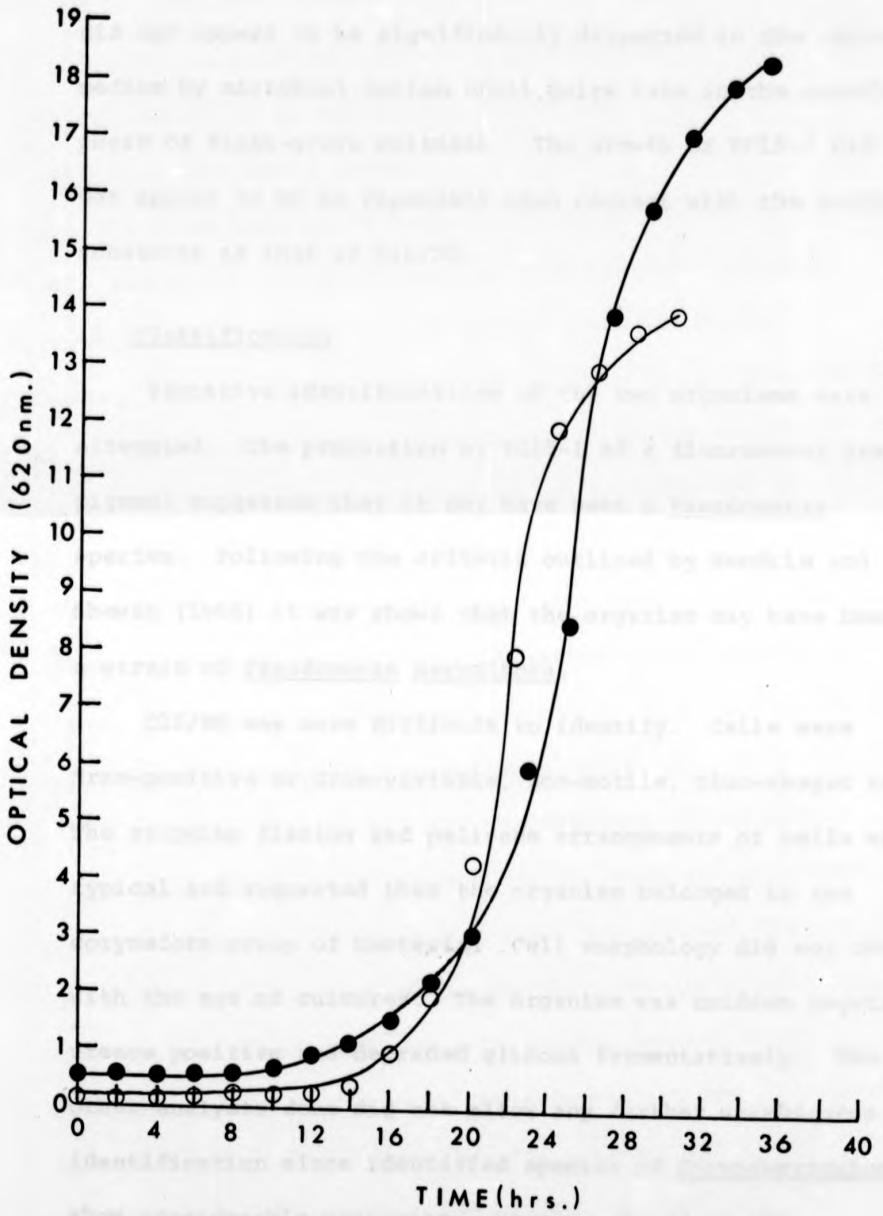
The organisms chosen for further study was C16/8S and TC15-1. Compared with the other strains isolated, growth on n-alkanes was particularly vigorous. In batch culture the growth curves in figure 14 were obtained. It is seen that the optical density obtained with cultures of C16/8S was higher than that obtained with TC15-1. This was due partly to the extremely efficient emulsification of the n-alkane substrates by C16/8S. It was found that C16/8S was able to rapidly disperse oil throughout the aqueous medium during growth. Another reason for the high optical density may have been the yellow pigmentation of the organism which could be recovered from the n-alkane phase after growth had ceased. When cellular yields were assessed on the basis of wet cell weight however, no significant differences between the two organisms were found.

TC15-1 unlike C16/8S, did not produce surfacants that

Figure 14

Growth curves obtained for batch cultures of C16/8S and TC15-1 during growth on n-alkanes.

o TC15-1 • C16/8S.


OPTICAL DENSITY (620nm.)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
0
4
8
12
16
20
24
28
32
36
40
TIME(hrs.)

were able to disperse the n-alkane substrates. The oil phase did not appear to be significantly dispersed in the aqueous medium by microbial action until quite late in the growth phase of flask-grown cultures. The growth of TC15-1 did not appear to be as dependent upon contact with the n-alkane substrate as that of C16/8S.

ii Classification

Tentative identifications of the two organisms were attempted. The production by TC15-1 of a fluorescent green pigment suggested that it may have been a Pseudomonas species. Following the criteria outlined by Hendrie and Shewan (1966) it was shown that the organism may have been a strain of Pseudomonas aeruginosa.

C16/8S was more difficult to identify. Cells were Gram-positive or Gram-variable, non-motile, club-shaped rods. The snapping fission and palisade arrangements of cells were typical and suggested that the organism belonged to the coryneform group of bacteria. Cell morphology did not change with the age of cultures. The organism was oxidase negative, urease positive and degraded glucose fermentatively. The other analyses done did not allow any further unambiguous identification since identified species of Corynebacterium show considerable variation. However, based on the morphological characteristics which were distinctive C16/8S was tentatively identified as a Corynebacterium species.

III.1.3 Growth measurements of n-alkane-grown organisms

There are difficult inherent problems associated with the estimation of growth of microorganisms in liquid medium containing two liquid phases.

Rapid estimations of culture growth are required when cells are grown for preparation of cell extracts and must parallel the increase in cell number. Normal optical density readings may be considerably distorted by dispersion of one phase in another. Also the interference caused by the oil may not remain constant due to the presence of microbially produced surfactants.

With the two organisms under investigation, the nature of the problems involved in rapid estimation of growth in liquid culture were different. This was due to the different types of interaction with the growth substrate. TC15-1 had a low affinity for the oil and the majority of the cells remained in the aqueous phase. The emulsification effects produced by the cells were also small and allowed the oil to be removed by low speed centrifugation. The growth of flask cultures could be estimated using normal optical density measurements due to the small interference by the oil phase. In fermenter-grown cultures however, violent agitation caused much greater dispersion of the oil resulting in higher backgrounds when using optical density measurements. The relatively small contribution to the dispersion of the

oil phase made by cell surfactants still allowed satisfactory optical density readings to be obtained. Great care was taken that the contribution of the oil phase to the optical density measurements was constant. 10 ml samples were removed from the culture and mixed for 30 s using a 'Whirlimix'. 100 µl of the mixed sample was rapidly transferred to 9.9 ml of sterile n-alkane free medium. The resulting mixture was agitated for 1 minute on the 'Whirlimix'. A 1 ml sample was taken and placed in a 1.5 ml glass cuvette and the optical density was read immediately at 625 nm. Using a Pye-Unicam SP800 spectrophotometer in initial experiments, readings were made at 540 nm and at 625 nm. Serial dilutions of the samples taken from the fermenter were also made down to 10^{-7} and 100 µl aliquots were plated onto AMS/n-tetradecane agar plates and onto L-agar plates. Both sets of plates were incubated at 30°C until colonies appeared.

It was found using these procedures that readings taken at 625 nm were more stable and more reproduceable than those at 540 nm. Both sets of readings appeared to parallel the increase in cell number demonstrated by the viable counts from the agar plates.

Similar experiments were done with C16/8S and similar results were obtained except for much higher background contributions from the oil phase. The readings however could not be made in the absence of the oil because it was shown

that all the cells present were residing within the non-aqueous phase. The satisfactory results obtained may be explained to some extent by the nature of the emulsion formed by the action of C16/8S on the oil. As will be discussed later, it seems likely that the high affinity for the growth substrate demonstrated by the organisms requires an emulsion in which relatively large droplets are stabilized. It is observed in cultures of C16/8S that significant formation of emulsified suspensions occurs rapidly after inoculation. Further fragmentation of the oil drops may be caused by increase in the number of cells within the oil itself but the resulting increase in optical density may be attributed to the cells.

Apart from viable counts which were used to assess the effectiveness of the other methods attempted, other procedures were tested. These are summarized in Table XA.

The methods used for rapid and routine estimations of microbial growth on n-alkanes in liquid culture are satisfactory providing considerable care was taken to take truly representative samples from the cultures. This was a problem with many of the methods used apart from the other setbacks outlined in Table XA.

TABLE XA

Estimations of growth of cultures on n-alkanes in liquid medium.

Method	Comment
Optical density measurements	Satisfactory care was taken when samples were processed. The most rapid method used.
Viable counts	Accurate and used for estimating effectiveness of other methods. Slow and unsatisfactory for assessment of the state of cultures for extracts.
Dry weight determination	Extremely unreliable with small samples especially during early stages of growth. The methods were slow and the growth substrate was difficult to remove.
Growth substrate disappearance	This was impractical owing to the large amounts of growth substrate present in order to avoid carbon limitation. Significant quantities remained when cultures had reached stationary phase. Taking representative samples and alkane extraction proved to be difficult and results were variable.
Carbon dioxide production	This method was considered. It is possible to determine the amount of CO_2 in the exhaust gas from fermenters. It was unsatisfactory for flask cultures. It was thought that this parameter would be affected by too many other criteria to be used on a routine basis.

III.1.4 Discussion

The widespread occurrence of the ability of micro-organisms to utilize n-alkanes as sole carbon and energy source was reflected in the relative ease with which a variety of isolates were obtained from different sources during the present work. It was found that organisms could be isolated from all the samples taken although only two were significantly contaminated with oil products.

With the bacterial strains isolated it was found that the ability to grow to high cell densities on n-alkanes was variable. However, most of the strains were able to grow on all the alkane substrates of interest and differences noted in the efficiency of growth in liquid were not as pronounced during growth on plates. Variation was also noted in the association of the organisms with the n-alkane substrates. Some were able to grow in the aqueous medium whereas the majority were found to be confined to the oil phase. In many cases negligible emulsification of the n-alkane substrates occurred and it seems likely that this, in conjunction with a high affinity for the oil-phase which contributed only 0.5% of the total culture volume, limited the possible growth of the organisms.

The observation that the majority of organisms isolated had a high affinity for the substrate raises the question of whether the isolation methods used were selective for

organisms that displayed this type of interaction. The variety of isolation methods attempted and the resulting duplication of isolated strains tend to suggest that highly hydrophobic n-alkane-utilizing organisms were predominant in the samples taken.

The identification of the organisms C16/8S and TC15-1 was only attempted to the level of genus since this part of the work was not considered of major importance. With TC15-1 the production of distinctive green fluorescent pigment during growth on certain media including L-broth and the Gram-negative nature of the motile cells suggested strongly that the organism was a pseudomonad. However with C16/8S which was tentatively identified as a Corynebacterium largely on the basis of Gram-stain reaction, morphology and growth characteristics there are difficulties due to the taxonomic diversity in the coryneform group of bacteria.

III.2 Mode of growth on n-alkanes

III.2.1 Growth in relation to substrate

Following the isolation of the organisms C16/8S and TC15-1 and the initial studies already described, investigations of the growth of the two organisms on n-alkane substrates were done on a comparative basis.

Microscopic studies showed that there were considerable differences between the interaction of C16/8S and TC15-1 with n-alkane substrates. C16/8S was confined to the non-aqueous phase to such an extent that when harvested by centrifugation, cells migrated upwards and collected at the interface between the oil and the aqueous medium. No cells could be spun down to form a pellet. This type of growth on n-alkanes was always observed and it appeared that growth would not occur unless intimate contact between the cells and the oil phase was established. Figures 15 and 16 show the typical appearance of cells of C16/8S during growth on n-alkanes. It is observed that no cells occurred outside the oil droplets.

TC15-1 was found to have a much lower affinity for the non-aqueous phase when grown on n-alkanes than was shown by C16/8S. When harvested by centrifugation cells would form a pellet in the normal manner and negligible amounts would migrate with the oil phase. It was observed that

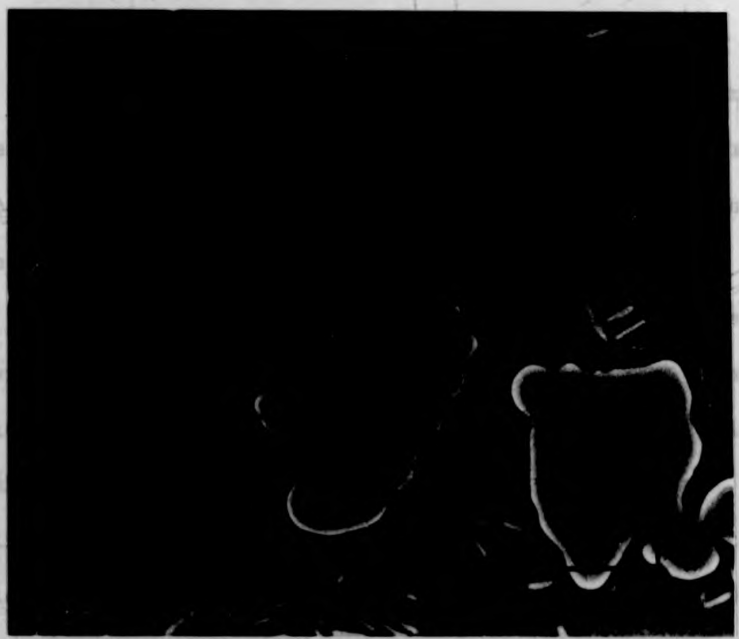
Figures 15 and 16.

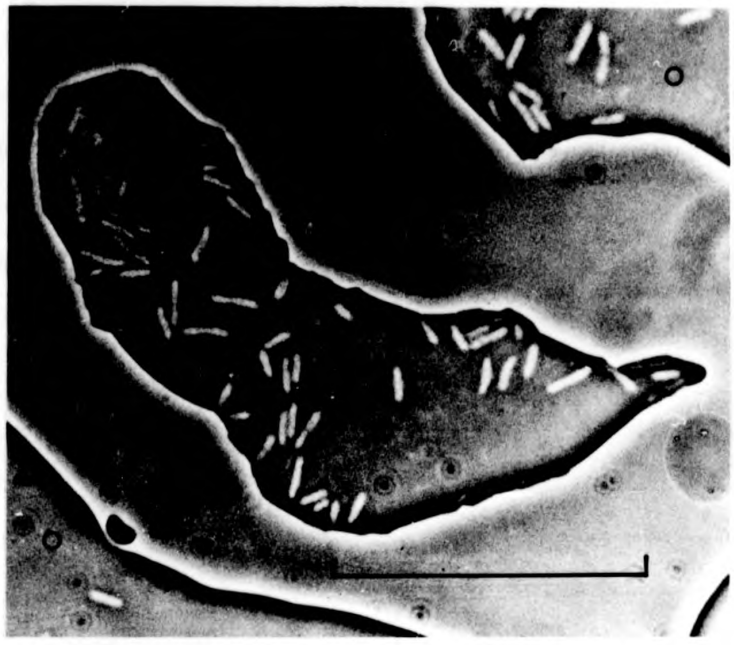
Typical appearance of C16/8S during growth on n-alkanes.

(Bar = 10 μ).

A = aqueous phase

O = oil phase





the majority of cells were found within the aqueous medium during growth on n-alkanes and did not appear to have any direct contact with the droplets of the growth substrate. There were however large accumulations of cells at the surfaces of larger oil droplets. The typical appearance of the two types of growth of TC15-1 are shown in figures 17 and 18.

Slide cultures were done to further demonstrate the different patterns of growth of C16/8S and TC15-1 on n-alkanes. Also, owing to the motility of the organism TC15-1 it was of interest to investigate whether chemotaxis towards n-alkane substrates occurred which might counteract the low affinity of the cells for hydrocarbons and account for the accumulation of cells around large oil drops.

The slide cultures of C16/8S confirmed the earlier observations that all cells were confined within oil drops. The snapping fission typical of coryneform bacteria was also observed (figure 19).

The opposite trends were observed when TC15-1 was grown in slide culture. Growth occurred both within the medium without direct contact with the substrate and also at the surface of n-alkane droplets. After 240 minutes the cells were still growing but their arrangement within the field of view changed. The dispersal of cells that was observed was due to a high proportion of the organisms becoming motile (figure 20).

Figure 17

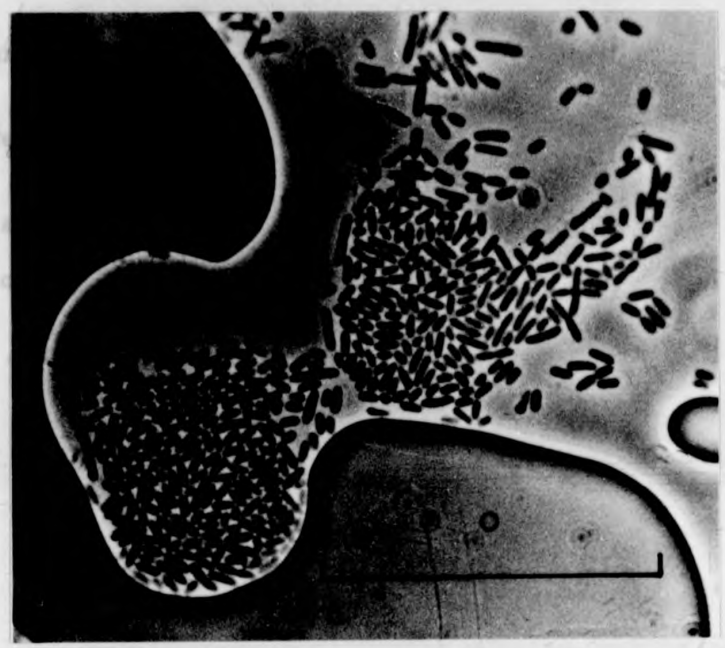
Typical growth of TC15-1 near n-alkane droplets.

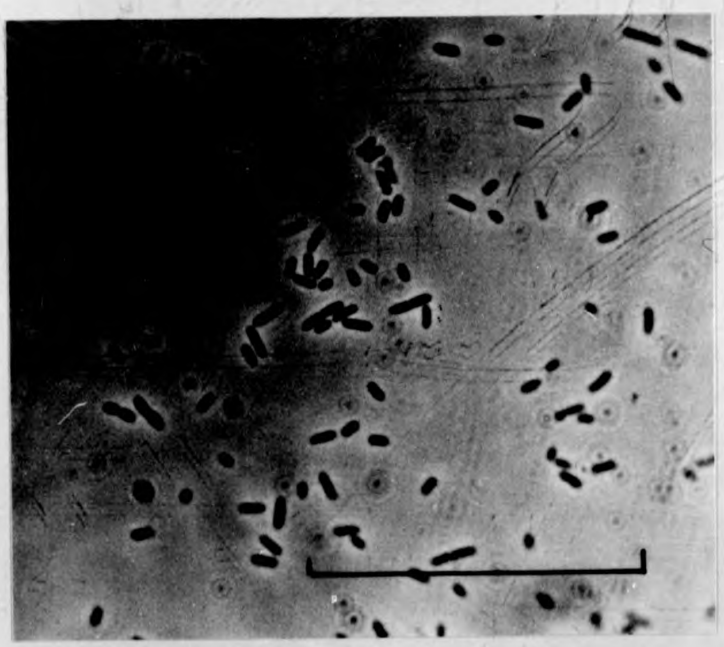
Figure 18

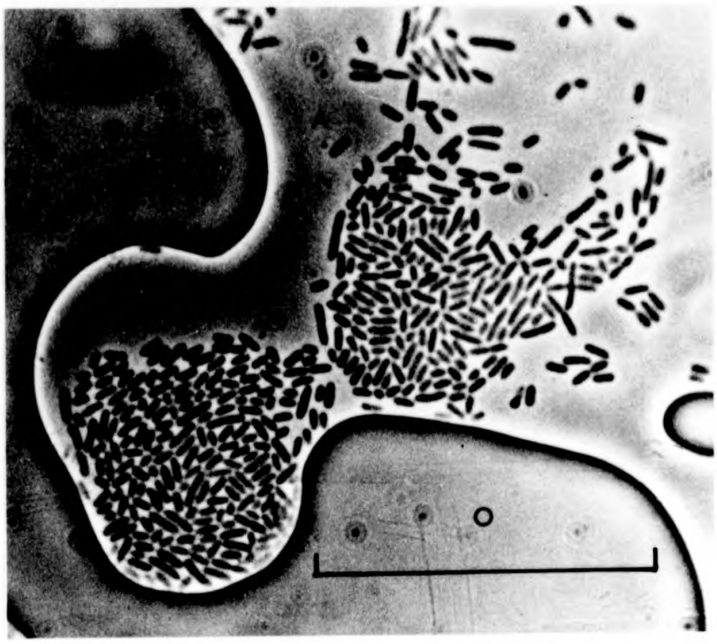
Growth of TC15-1 in the aqueous medium with n-alkane growth substrate. (Bar = 10 μ).

A = aqueous phase

O = oil phase







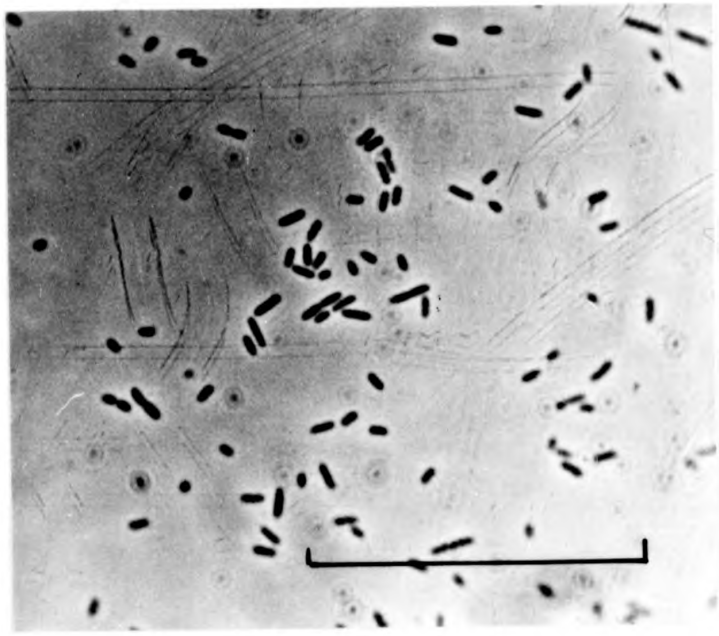
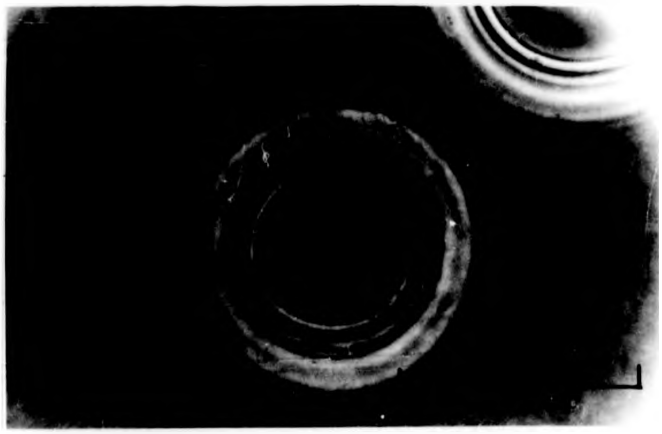


Figure 19

Slide culture of C16/8S grown on n-alkanes.

A = aqueous phase

O = oil phase



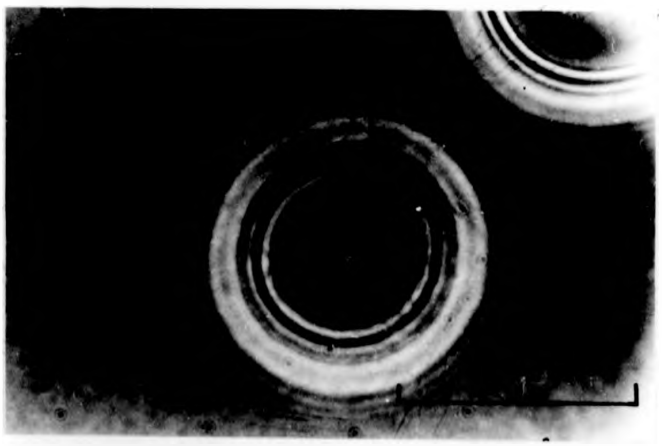
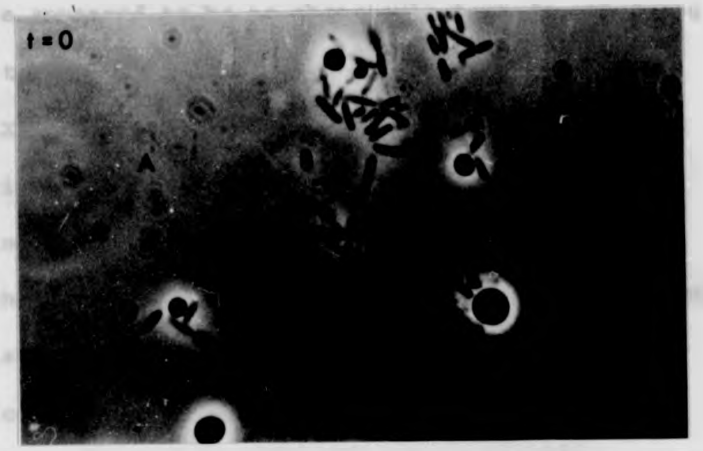


Figure 20

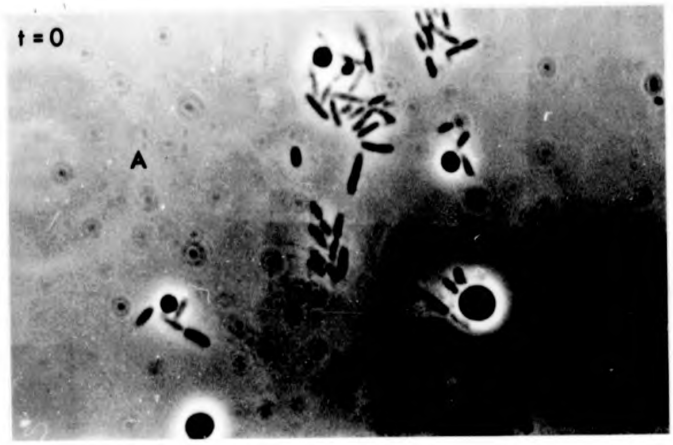
Slide culture of TC15-1 grown on n-alkanes.

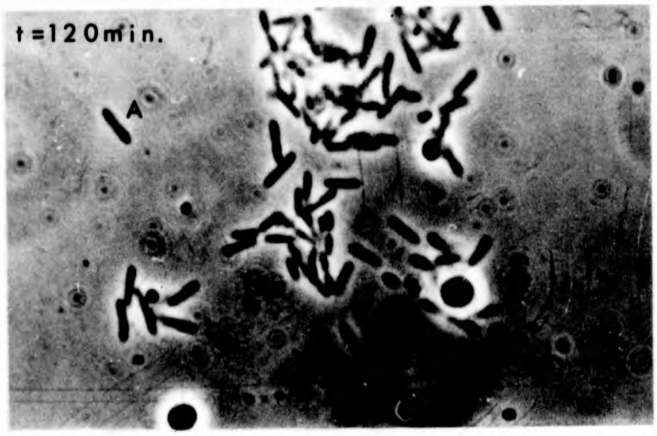
A = aqueous phase

O = oil phase


t=0


t=120min.


t=0
A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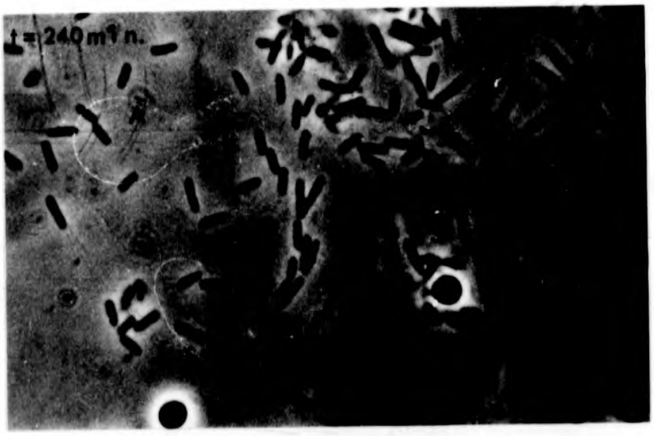
A


t=240min.

There appeared to be no chemotaxis towards oil drops demonstrated by the motile cells of TC15-1. The oil droplet 'X' in figure 20 remained undisturbed throughout the experiment which was done over a total period of 480 minutes. The accumulation of cells around other oil drops such as 'O' in figure 20 appeared to be due to growth of organisms already present at their surfaces in conjunction with the production of fewer motile progeny. It was observed in other cases that when contact was made with n-alkane droplets, cells tended to remain at the surface of the n-alkane.

The use of slide cultures also allowed the calculation of generation times for the organisms C16/8S and TC15-1 during growth on n-alkanes. Using the following equations the results in table XI were obtained.

(i) $n = 3.3 \log b/B$

(ii) $G = t/n$

n = Number of generations; b = Number of cells after time 't'; B = Number of cells at time zero; G = Generation time; t = time period (minutes).

The generation times obtained for the two organisms agreed well with the growth curves obtained previously. The growth rates of both C16/8S and TC15-1 were lower than

Generation times of n-alkane grown C16/8S and TC15-1.

(a) C16/8S

Time (min)	Number of cells	Number of generations (n)	Mean Generation time (G) (min)
0	9	0	
120	14	0.63	194
240	21	1.21	

(b) TC15-1

Time (min)	Number of cells	Number of generations (n)	Mean Generation time (G) (min)
0	51	0.97	122
120	101		

the growth rates obtained using rich media such as L-broth. However the differences in the growth rates of C16/8S and TC15-1 grown on n-alkanes reflected the growth rates of the two organisms on rich medium. It was shown that the doubling time in exponentially growing cultures of TC15-1 in L-broth was 30 minutes at 30°C. Under similar conditions the doubling time of C16/8S was significantly longer at 65 minutes.

III.2.2 Morphology

Morphological studies of the organisms C16/8S and TC15-1 were done to investigate whether any adaptations to growth on n-alkanes could be observed and whether any changes that might occur could be related to the different interactions of the two organisms with the growth substrate.

The studies of external structure by electron microscopy indicated that no gross morphological changes occurred as a result of growth on n-alkanes. Under all the growth conditions tested C16/8S appeared as club-shaped rods which varied in shape and length. TC15-1 cells were invariably short rods which were motile by means of a single polar flagellum.

Thin sections showed that there were some morphological differences in internal structure that could be attributed to growth on n-alkanes in both C16/8S and TC15-1.

Figure 21 shows the typical appearance of cells of C16/8S when grown on (a) L-broth, (b) acetate and (c) n-tetradecane. In all the cells shown the smudged effect around the surfaces of cell walls is probably due to protein material protruding from the cell surface. The thick outer cell wall typical of Gram-positive bacteria was apparent when C16/8S was grown on all the tested substrates. It was however more pronounced and electron-dense in acetate grown cells (figure 21(b)). Also common to all cells of C16/8S regardless of growth substrate, were mesosomal structures. In figure 21(b) they are clearly visible as invaginations of the cytoplasmic membrane and electron dense coiled tube-like structures within the cytoplasm. The appearance of these structures was dependent upon the plane of the sections observed. In many cases they were not visible at all as in figures 21(a) and 21(c).

In n-tetradecane-grown cells of C16/8S there were inclusions within the cytoplasm that were absent when the cells were grown on L-broth or acetate. A typical example of C16/8S grown on n-alkane is shown in figure 21(c). Close examination of a number of examples indicated that the inclusions were not membrane-bound and that their average size was variable between 5 and 30% of the total area of the cytoplasm.

Also of interest and in sharp contrast to the organism

Figure 21

Electron micrographs of C16/8S grown on different substrates.

(a) Nutrient broth grown

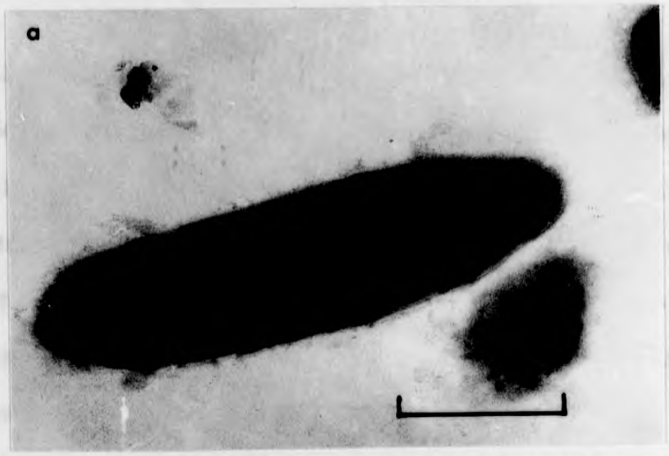
(b) Acetate grown

(c) n-alkane grown.

(Bars = 0.5 μ).

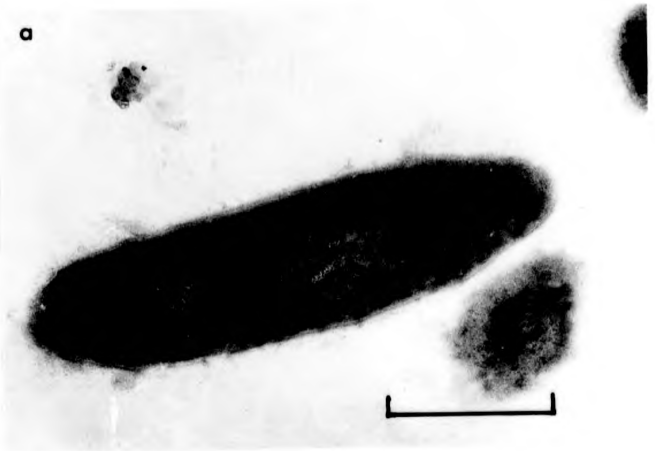
m = mesosome

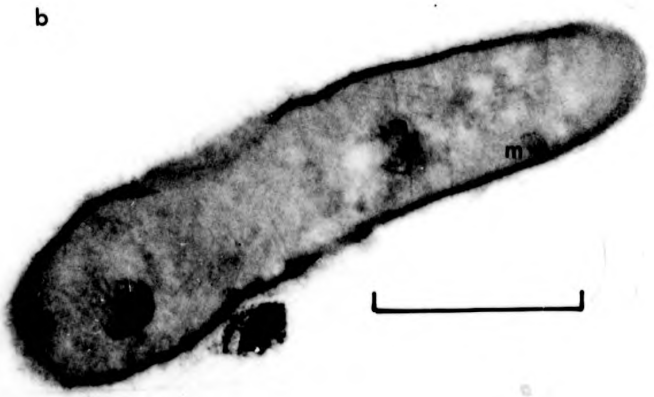
i = inclusion


a


b


c


a


b
m


c

TC15-1, was the process of cell division in C16/8S. In figures 23(a) and 23(c) it is seen that the division of TC15-1 is characterized by invagination of the cell wall. In C16/8S no invagination of the cell wall occurs and division is achieved by direct formation of a transverse septum (figure 22). This form of division is a characteristic of coryneform bacteria.

A comparison of the morphologies of TC15-1 cells grown on L-broth, acetate and n-tetradecane showed that there was little difference between the cells grown on the non-alkane substrates. In TC15-1 there was no evidence of the mesosomes observed in C16/8S.

Two major differences were observed between n-alkane grown cells and L-broth- and acetate-grown cells of TC15-1. The first was the appearance in n-alkane grown cells of small inclusions within the cytoplasm. Unlike the cytoplasmic inclusions observed in C16/8S, those in TC15-1 were always small and were present in low numbers; usually two or three per cell. Close observation of the inclusions in TC15-1 indicated that they were surrounded by electron-dense areas that appeared to have a distinct membrane-like structure. Examination of dividing cells showed that the cytoplasmic inclusions in TC15-1 were distributed between daughter cells so that each cell carried at least one inclusion when division was complete.

Figure 22

Electron micrograph of C16/8S showing typical appearance of a division septum. (Bar = 0.5 μ).

Figure 23

Electron micrographs of TC15-1 grown on different substrates.

(a) Nutrient broth growth.

(b) Acetate grown.

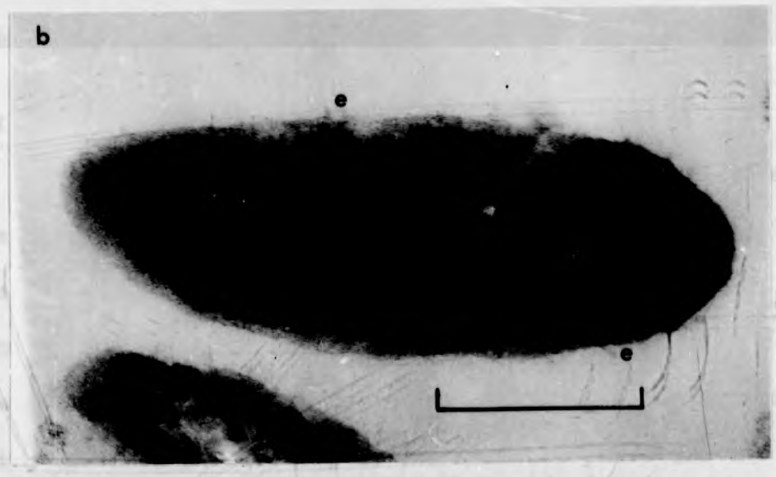
(c) n-alkane grown.

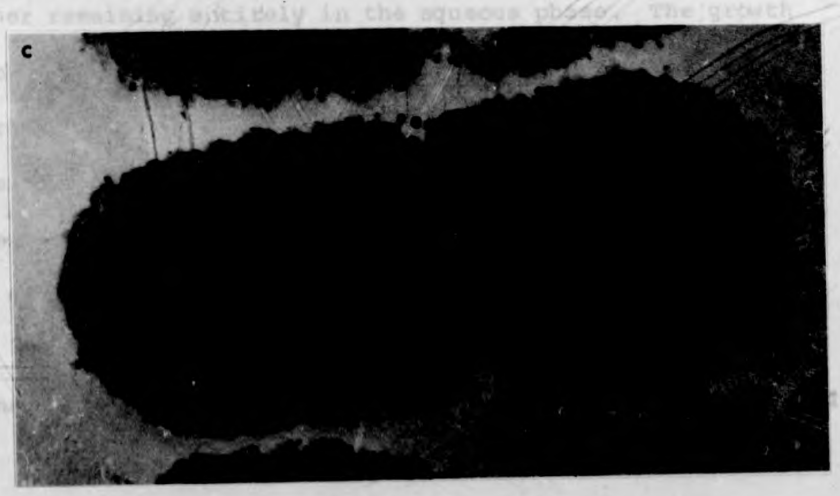
(Bars = 0.5 μ).

e = extrusion

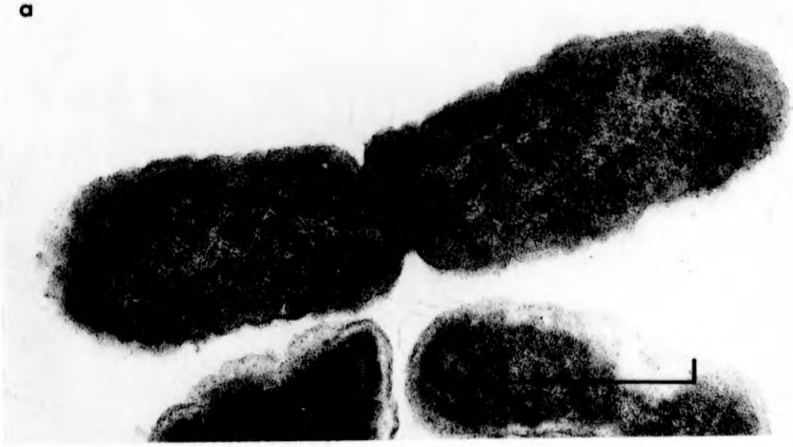
i = inclusion


a

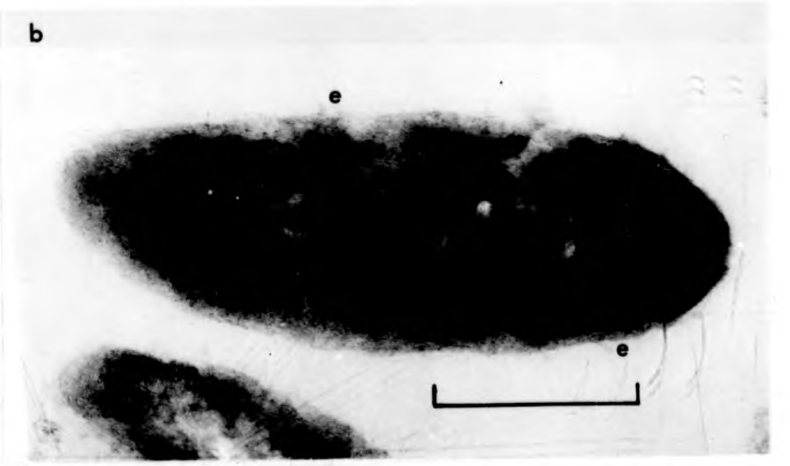

b


c

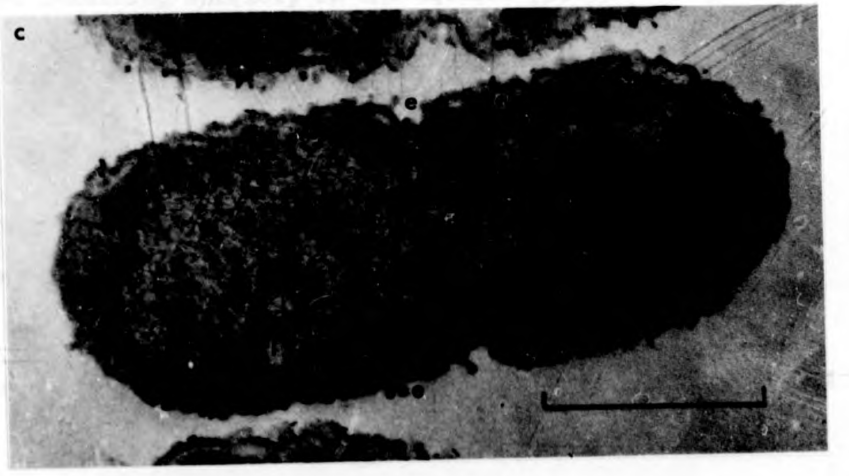
a



b



c



The second difference was the presence of extracellular vesicles attached to the outer surface of n-alkane-grown cells. Smaller extracellular particles were also observed on acetate grown cells and more rarely on L-broth grown cells, those associated with growth on n-alkanes however were more numerous and morphologically distinct. The vesicles of n-alkane grown cells appeared to be surrounded by membrane and to contain cellular material since their contents were more electron-dense than the surroundings.

III.2.3 Discussion

The observations made in the present work illustrate the ability of different organisms to interact with n-alkane substrates in different ways. The differences between the affinity of the coryneform C16/8S and the pseudomonad TC15-1 for n-alkane substrates results in one being confined exclusively to the n-alkane phase and the other remaining entirely in the aqueous phase. The growth problems of the two organisms are consequently different. Strain C16/8S which is confined to the non-aqueous phase is limited by the accessibility of water-soluble nutrients whereas strain TC15-1 which remains in the aqueous phase is effectively carbon limited.

A survey of the literature indicates that despite considerable interest in microbially produced surfactants and

lipids, relatively little attention has been focussed on how the surfactants produced by bacteria relate to the growth requirements of the organisms themselves. Admittedly, as has already been discussed, it has been shown that mutants of Pseudomonas aeruginosa that are unable to produce rhamnolipids are unable to grow at the expense of n-alkanes and that many other organisms appear to be dependent upon surface-active products that cause the dispersal of insoluble substrates. For the organisms C16/8S and TC15-1, the observation that microbially produced surfactants increase the interfacial area between the two phases has differing significance. In the pseudomonad TC15-1 the accessibility of the substrate is increased by the formation of micro-emulsions which occur in high concentrations in the vicinity of larger n-alkane droplets but are also dispersed throughout the medium. This would account for the observed accumulation of cells of TC15-1 around larger substrate droplets with which they do not appear to make contact as well as the significant number of cells that occur free in the medium. In the coryneform C16/8S the increase in interfacial area between the two phases is desirable in order to allow the contact of a maximum number of cells with an interface so that nutrients soluble in the aqueous phase may be transferred. The cells are confined to the non-aqueous layer by the extremely hydrophobic nature of the cell wall. In such a

case the formation of microemulsions is not beneficial because the cells are unable to utilize submicroscopic droplets for growth. In these two cases the action of the surfactant produced must consequently be different. Pseudomonads and yeasts produce surfactants that stabilize extremely small droplets whereas the coryneform bacteria and other bacteria that have highly hydrophobic cell walls have surfactants that allow the formation of stable relatively large droplets with large surface areas. Evidently such differences in the actions of surface active agents would require differences in their chemical nature. From this point of view it may be argued that the surfactants produced by organisms in response to growth on n-alkane substrates generally complement the requirements of the cell in the way they affect the substrate. Therefore the nature of the surfactants produced by cells growing on n-alkanes is subject to selective pressure and not purely fortuitous.

The morphological changes observed when cells of C16/8S were grown on n-alkanes indicate the accumulation of material within the cytoplasm. This result agrees with the observation made by other workers who have noted intracellular inclusions in various organisms grown on n-alkanes. The nature of the material within the inclusions in C16/8S remains unclear and warrants further investigation.

In TC15-1 the membrane-bound inclusions within the cytoplasm of n-alkane grown cells did not resemble the large accumulations of material observed in C16/8S . The structures were small in size and their distribution in cells growing on n-alkanes appeared to be controlled. These observations suggest that the appearance of such inclusions may be associated with particular functions involved in n-alkane metabolism. Isolation of these inclusions and investigations of their contents and catalytic activities could prove to be of great value.

The extracellular vesicles attached to the surface of n-alkane grown cells of TC15-1 may have some functional significance since they appeared to be morphologically distinct from those observed on cells grown on non-alkane substrates. Close examination suggested that these structures were extrusions of the outer membrane through the cell wall. A possible function for such vesicles may be to provide hydrophobic areas for binding of n-alkane submicron droplets on the surface of the apparently hydrophilic cell exterior. Such extrusions occur in large numbers on n-alkane grown cells.

III.3 Enzymes associated with growth on n-alkanes

III.3.1 n-alkane hydroxylase

During the present work it was attempted to produce cell extracts that showed accumulation of primary alcohols from n-alkanes. Using both C16/8S and TC15-1 numerous assays were done and various methods of preparation of cell extracts were used.

With C16/8S the growth of the organism within the oil phase resulted in extracts that contained large amounts of n-alkane. Assays in which alcohols were detected using GLC analysis were done using endogenous substrate due to its presence in large excess. A method was devised to remove endogenous substrate in which harvested cells were washed in n-heptane and lyophilized. The dried cells were then resuspended in the breaking buffer and extracts were made in the normal manner. Using cells prepared in this way assays using alternative substrates could be attempted although small amounts of growth substrate remained.

Assays based on oxygen electrode analysis of oxygen consumption in response to added substrates proved to be impractical both for whole cells and for cell extracts. Endogenous rates of oxygen consumption were extremely high and no enhancement of oxygen uptake was observed when n-alkanes were added. Similar results were obtained whether endogenous substrates were present or removed as described.

When cell extracts of C16/8S were used for assays in the oxygen electrode it was found that there were high levels of non-specific NADH oxidase activity. High rates of oxygen uptake were observed when NADH was added to the reaction vessel and no enhancement was noted when n-alkane substrates were introduced. It was thought that some of the endogenous oxygen consumption may have been due to the presence of n-alkane hydroxylase activity on endogenous substrate. Analysis by GLC however revealed no accumulation of oxidation products and the use of inhibitors known to affect other n-alkane hydroxylases did not reduce the rate of oxygen uptake. Because of the NADH oxidase activity observed in the oxygen electrode, substrate stimulated NADH disappearance on a spectrophotometric basis was ruled out.

Using all the substrates listed and the methods described, assays were done under varying conditions of pH and temperature. NADH and NADPH were used as electron donors and different metal ions were introduced in order to determine whether a specific metal was required for activity.

The same range of assays was done using whole cells and cell extracts of TC15-1. Despite the low affinity for n-alkane substrate demonstrated by the organism which made harvesting of cells and preparation of cell extracts much easier, similar results were obtained. In neither organism was it possible to demonstrate n-alkane hydroxylase activity.

III.3.2. Cytochrome P-450 in C16/85 and TC15-1

Investigations were done using whole-cell preparations to determine whether the n-alkane hydroxylases of C16/8S or TC15-1 were associated with a cytochrome P-450-type system.

Culture of 500 ml were grown up in flasks on AMS/acetate, AMS/n-tetradecane and L-broth. Cells were harvested by centrifugation during late exponential growth. The harvested cells were washed once and resuspended in 2 ml 20 mM Tris-HCl buffer pH 7.4. 1 ml of the resulting thick cell suspensions was put into each of two 1.5 ml glass cuvettes with 1 cm lightpaths, one to act as reference and the other as sample. Both the reference and the sample cuvettes were fully oxidized by bubbling air through the cell suspensions for 1 minute.

A difference spectrum case-line was then prepared using a Pye-unicam SP-800 spectrophotometer which scanned from 380 nm to 600 nm. Crystals of sodium dithionite were added to the sample cuvette. The sample was then scanned against the reference cuvette from 380 nm to 600 nm to give an oxidized/reduced difference spectrum. To detect the cytochrome P-450-carbon monoxide complex, gaseous carbon monoxide was bubbled through the sample cuvette. An oxidized/reduced-CO-treated difference spectrum was then obtained.

The results of these experiments showed that in IC15-1 there were no differences between the oxidized versus reduced spectrum and the oxidized versus reduced-CO-treated spectrum in the region of 450 nm. This indicated that the hydroxylation system probably did not involve a cytochrome P-450. Spectra prepared using cells grown on the alternative substrates appeared to be very similar to those obtained using n-alkane-grown cells.

Similar results were obtained with C16/8S although initial experiments with the wild-type organism were inadequate due to the intense yellow pigmentation which served to mask any changes that might have occurred at 450 nm. During the experiments on mutagenesis however, a non-pigmented mutant was obtained which made analysis easier. The results using this strain also indicated that the cytochrome P-450 system was absent in n-alkane-grown cells.

It is possible to speculate that in the pseudomonad TC15-1, the hydroxylation system may be associated with other pigments that are not detectable using whole cells such as the rubredoxin system of Ps. putida. In C16/8S which has been tentatively described as a Corynebacterium the only hydroxylation system studied in detail contained cytochrome P-450. However, as was mentioned previously, it has been shown in Acinetobacter that n-alkane grown strains of the same genus do not all contain cytochrome P-450 and it is possible that a novel hydroxylase system is involved.

III.3.3 Alcohol and aldehyde dehydrogenases

Initial experiments using the spectrophotometric assays described showed that DCPIP was reduced in the presence of PMS and crude cell extracts when primary alcohols were added to the assay mixture. The assays at this stage were not quantitative and the results for TC15-1 and C16/8S are shown in Table XII .

Further work was done exclusively on extracts of TC15-1. This was because cell extracts could be prepared without the presence of contaminating growth substrate. It appeared that the procedure devised for the removal of growth substrate from cells of C16/8S resulted in a loss of alcohol dehydrogenase activity in cell-extracts.

It was found using GLC analysis that in TC15-1 the alcohol dehydrogenase was closely associated with an aldehyde dehydrogenase and that assays yielded corresponding fatty-acids when alcohols were added to cell extracts in the presence of PMS. No aldehyde intermediates could be detected. It was also observed that aldehydes added to assays in the place of alcohols were untouched and no acids were accumulated. The activities of crude cell extracts are given in Table XIII. Activities are expressed in nanomoles of octanoate formed per minute per milligramme of protein. The extracts were found to be active with longer chain primary alcohols such as dodecan-1-ol and tetradecan-1-ol but owing to the insolubility in water of these substrates compared with

Table XII

Induction of PMS-linked alcohol dehydrogenase by growth on n-alkanes in C16/8S and TC15-1

Growth substrate	Organism	
	C16/8S	TC15-1
Alkane ($C_{14}H_{30}$)	+	+
Acetate	-	±
Glucose	ND	±
L-broth	-	-

+ = Presence of significant activity indicated by PMS reduction

± = Presence of small amounts of activity

- = No detectable activity

ND = Not determined

Table XIII

PMS-linked conversion of alcohol to acid by crude extracts of TC15-1.

Growth substrate	Fraction	Enzyme activity (nmoles product/ min/mg protein)
n-Alkane ($C_{14}H_{30}$)	Supernatant	0
	Pellet	233.7
Acetate	Supernatant	0
	Pellet	1.6
Glucose	Supernatant	0
	Pellet	5.8

the shorter chain-length octan-1-ol they were not used in routine assays. No activity was observed using secondary alcohols. The results show a significant increase in the activity detected when cells were grown on n-alkanes. It was also evident that the enzymes of interest were located in the membrane fraction.

Further purification of the alcohol dehydrogenase was attempted. It was found that 90% of the activity could be released from the membrane by sonication. 5 ml volumes of particulate fraction containing 25 to 50 mg of protein were sonicated using an MSE sonicator at maximum amplitude for 5 minutes. Samples were maintained at 4^{o}C by immersion of the container in an ice-methanol bath during sonication, which was done in 20 second bursts with intervening rest periods of 30 seconds. The resulting suspensions were spun at 144,000 x g for 3 h to remove particulate debris. The resulting red-brown protein solution was retained and assayed for alcohol dehydrogenase activity.

The fractionation procedures used were done on a preliminary basis and no large-scale purifications were achieved in the time available. The solubilized protein was subjected to DEAE cellulose chromatography. DEAE cellulose (Sigma) was prepared by washing in 1M HCl followed by neutralization with 1M NaOH and further washes with

distilled water until the pH was 7.0. The washed DEAE cellulose was resuspended in 20 mM Tris-HCl chromatography buffer pH 7.5 containing 1 mM dithiothreitol and 10% (v/v) glycerol. It was found using 30 cm columns of 2.5 cm internal diameter loaded with 500 mg of solubilized protein, that the alcohol dehydrogenase did not bind to DEAE cellulose and was recovered in the unbound eluate.

Further fractionation was done by gel filtration using 100 cm columns of 2.5 cm internal diameter with G-125 and G-200 medium Sephadex. The dry Sephadex (Sigma) was swollen for at least 48 hrs at $4^{o}C$ in the chromatography buffer described. It was found that the alcohol dehydrogenase activity was not retained by G-125 Sephadex but was fractionated to some extent using the G-200 column.

During all the purification procedures described the alcohol and aldehyde dehydrogenase activities could not be separated and fatty acids were produced from alcohols with no detectable accumulation of aldehyde intermediates. However when ammonium sulphate precipitation was used after initial DEAE chromatography it was found that alcohol dehydrogenase activity was located in the 40-60% saturation precipitate. It was also observed that no aldehyde dehydrogenase activity was present and could not be detected in any of the ammonium sulphate precipitates using both PMS- and NAD^{+}-

linked assays. The specific activity of the recovered alcohol dehydrogenase was shown to be significantly diminished.

Results of preliminary purification procedures are shown in Table XIV.

III.3.4 The induction of n-alkane degradation enzymes in TCl5-1

The previously described assays indicated that the n-alkane degradation enzymes of TCl5-1 were induced by the presence of n-alkanes. Further investigations were done to determine whether the enzymes could be switched on in the presence of alternative substrates and to find out at which levels expression was controlled.

Initially 500 ml batch cultures of TCl5-1 were grown in AMS medium using glucose, acetate and n-tetradecane as sole carbon and energy sources. Cultures were also grown on acetate and glucose in the presence of n-tetradecane at a concentration (v/v) of 0.5%. Cells were harvested during mid-exponential phase and cell extracts were prepared. Assays were done for the presence of the alcohol dehydrogenase previously shown to be induced by growth on n-alkanes. The results are shown in Table XIVA(a). It is seen that the enzyme system is not significantly induced by the presence of n-alkanes during growth on

acetate or glucose, but is induced by growth on n-alkanes alone. These results indicated that the alternative growth substrates were able to repress the expression of the enzymes of n-alkane degradation. The nature of the repression was further investigated using 3'-5'-cyclic AMP (cAMP).

Similar experiments were done using cultures of TC15-1 grown on acetate and glucose in the presence or absence of n-tetradecane. To all the cultures cAMP (Sigma) was added to a final concentration of 0.05 M. Cells were harvested during mid-exponential growth and cell extracts were prepared. The results of the assays of alcohol dehydrogenase assays done are shown in table XIVA(b). The addition of n-alkane to glucose- or acetate-grown cultures of TC15-1 clearly causes expression of the n-alkane degradation enzymes in the presence of cAMP.

The observation of the effects of cAMP on the expression of the n-alkane degradation pathway as demonstrated by the induction of specific alcohol and aldehyde dehydrogenases, indicates that the system as a whole is under catabolic repression. Low levels of cAMP in cells grown on glucose or acetate do not allow expression of n-alkane degrading enzymes even in the presence of inducing amounts of n-tetradecane. Artificially elevating the cAMP levels allowed induction to occur.

The clear evidence for catabolite repression of the

n-alkane degrading genes may have further implications. The metabolic state of a cell changes when a transition from growth on glucose to growth on n-alkanes occurs. It would be of interest to determine whether similar controls are involved in the switch from glycolytic to lipolytic metabolism. For this reason preliminary assays were attempted on TC15-1 grown on glucose, acetate and n-alkanes.

III.3.5 Isocitrate lyase and other enzyme assays

During the work with TC15-1 other enzyme activities were assayed in n-alkane grown cells and compared to glucose- and acetate-grown cultures. The assays attempted were (a) isocitrate lyase, (b) acetyl CoA carboxylase, and (c) fatty acyl CoA synthetase.

For all the assays except isocitrate lyase unsatisfactory results were obtained and activities were extremely small or undetected. The results for isocitrate lyase activity are shown in Table XV. It is seen that significant induction of isocitrate lyase activity was obtained when cells were grown on acetate and n-alkane in comparison to glucose-grown cells.

III.3.6 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used to compare protein banding patterns of soluble and particulate fractions of TC15-1 cells grown on different substrates. An example of the resulting gels is shown in figure 24.

Table XIV

Partial purification of alcohol/aldehyde dehydrogenase from n-alkane grown TC15-1

Stage of purification	% recovery of total activity	Product from Octan-1-ol	activity n-moles product/ min/mg protein
Crude extract	100	Octanoate	58.5
Particulate fraction	100	Octanoate	253.7
Sonication	90	Octanoate	261.3
DEAE cellulose	87	Octanoate	345.0
G-125	80	Octanoate	423.3
G-200	72	Octanoate	790.4
Ammonium Sulphate	ND	Octanal	150

ND = not determined

TABLE XIVA

(a) PMS-linked Alcohol dehydrogenase induction in the particulate fraction of acetate-, glucose- and n-alkane-grown cells of TC15-1.

Growth substrate	Enzyme activity (nmoles octanoate produced/min/mg protein)
Acetate	1.1
Glucose	3.2
n-Alkane	257.3
n-Alkane + Acetate	16.3
n-Alkane + Glucose	12.7

(b) The effect of cAMP on the induction of n-alkane degrading enzymes in the presence of alternative growth substrates. All cultures were grown in the presence of 0.05 M cAMP.

Growth substrate	Enzyme activity (nmoles octanoate produced/min/mg protein)
Acetate	0.8
Glucose	4.2
n-Alkane + Acetate	300.0
n-Alkane + Glucose	273.6

Table XV

Induction of Isocitrate Lyase (IL) in TC15-1 by growth on n-alkanes

Growth substrates	Fraction	Units of IL activity/ mg protein
Glucose	Supernatant	0.0013
	Pellet	0.0000
Acetate	Supernatant	0.1609
	Pellet	0.0000
n-Alkane ($C_{14}H_{30}$)	Supernatant	0.0827
	Pellet	0.0000

Figure 24

Polyacrylamide gel electrophoresis of proteins from TC15-1.

Lane a = Standards

Lane b = Particulate fraction glucose grown

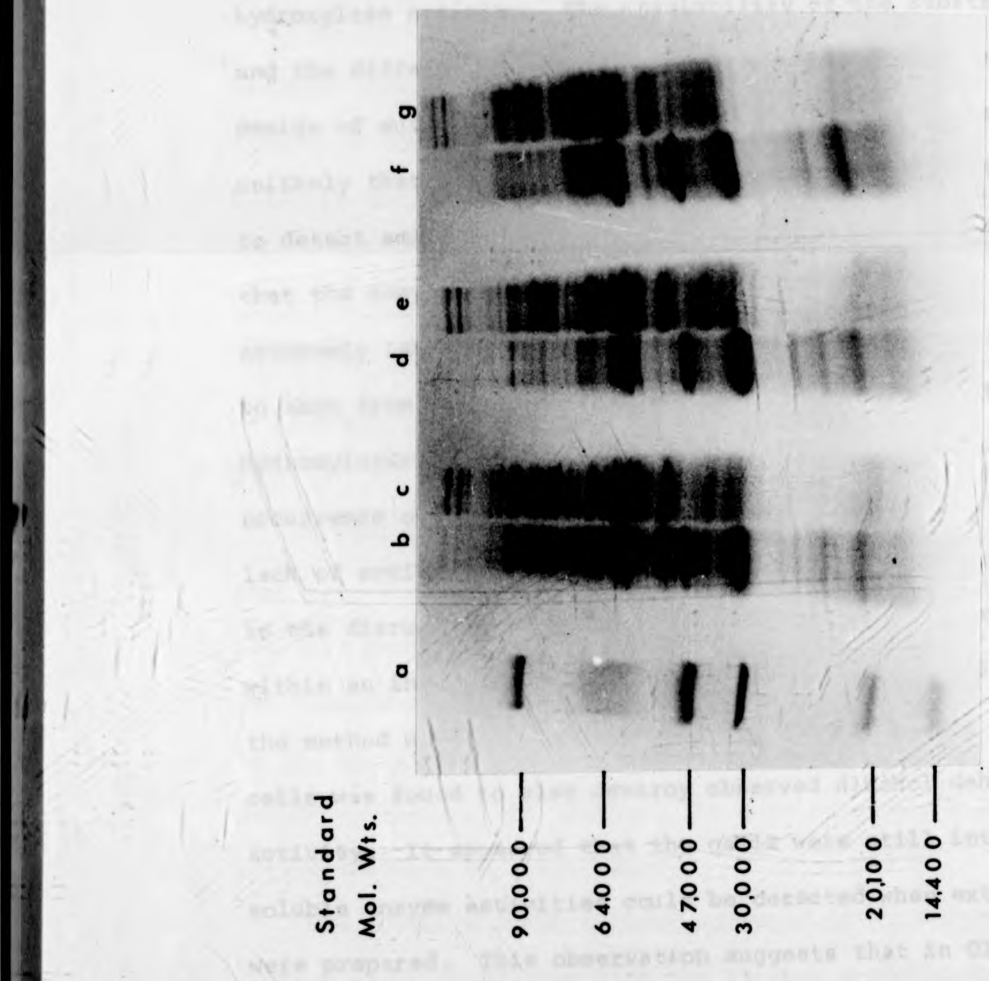
Lane c = Soluble fraction, glucose grown cells

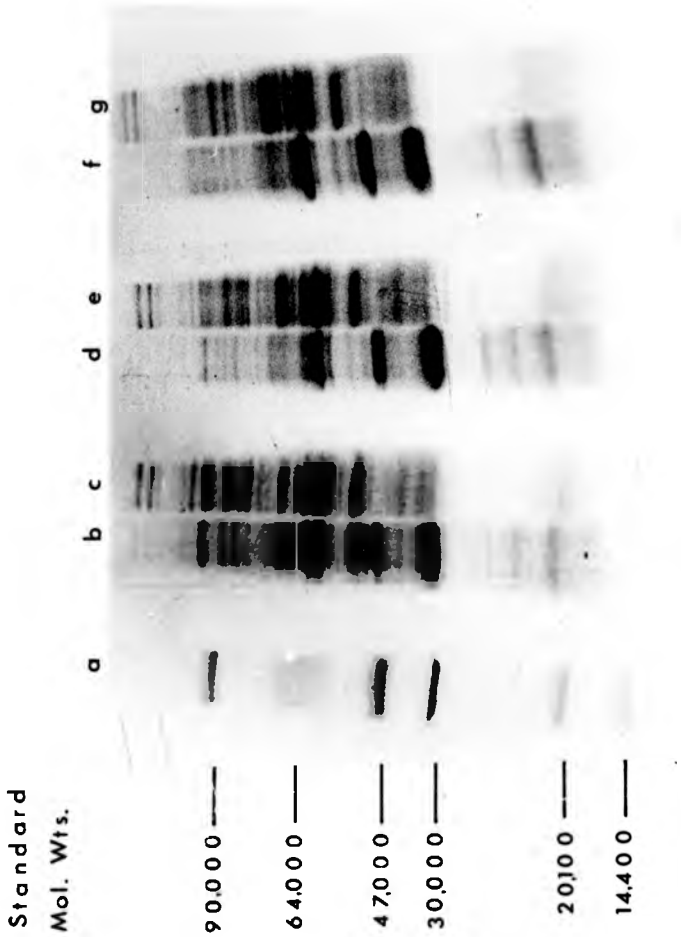
Lane d = Particulate fraction, n-alkane grown cells

Lane e = Soluble fraction n-alkane grown cells

Lane f = Particulate fraction, acetate grown cells

Lane g = Soluble fraction acetate grown cells


a
b
c
d
e
f
g
Standard
Mol. Wts.
90,000
64,000
47,000
30,000
20,100
14,400


Standard
Mol. Wts.
90,000
64,000
47,000
30,000
20,100
14,400
a
b
c
d
e
f
g

III.3.7 Discussion

The difficulties with enzyme assays encountered when cells were grown on n-alkanes were highlighted by the lack of any positive results with attempts to detect n-alkane hydroxylase activity. The insolubility of the substrate and the differences between the two organisms used made design of suitable assays much harder. However it is unlikely that all of the assays used were too insensitive to detect small amounts of activity and it must be concluded that the enzyme system in both C16/8S and TC15-1 was extremely labile and sensitive to cell disruption. It can be seen from the literature that relatively few n-alkane hydroxylases have been isolated and this suggests that the occurrence of such labile systems is not uncommon. The lack of activity when cell extracts were prepared may be due to the disruption of a structure that can only be maintained within an intact cell membrane or cell wall. In C16/8S the method used to remove endogenous substrate from harvested cells was found to also destroy observed alcohol dehydrogenase activity. It appeared that the cells were still intact and soluble enzyme activities could be detected when extracts were prepared. This observation suggests that in C16/8S the n-alkane degradation enzymes were situated either in the cell membrane or cell wall and were sensitive to the toxic effects of the n-heptane used for washing away the endogenous

substrate.

Clearly in the face of such labile enzyme systems much more work is required before they may be isolated and indirect investigations of their activities were attempted in the studies that followed.

The results of the investigations of the alcohol and aldehyde dehydrogenases involved in n-alkane degradation indicated that the initial step of assimilation is a terminal hydroxylation of the growth substrates. In both C16/8S and TC15-1 PMS-linked alcohol dehydrogenases were induced by growth on n-alkanes. Further studies on TC15-1 showed that a particulate fraction catalyzed the PMS-linked conversion of alcohols directly to the corresponding fatty acid. No similar activity was found for secondary alcohol substrates.

TC15-1 differed from other Pseudomonas aeruginosa strains that have been reported since the aldehyde dehydrogenase activity produced in response to n-alkanes appeared to be PMS-dependent. Tassin and Vandecasteele (1971) showed that in Ps. aeruginosa 196 Aa the corresponding aldehyde dehydrogenase was NAD^+-linked. In both cases the induced activities were membrane bound.

The aldehyde dehydrogenase activity in TC15-1 was found to be closely associated with the alcohol dehydrogenase. Alcohol substrates were converted rapidly to their corresponding fatty acids in the presence of PMS and no aldehyde intermediates

could be detected. Aldehydes added to assays under the same conditions were not transformed. These results suggested that either the active site of a separate aldehyde dehydrogenase enzyme was situated in a complex such that it could only receive its substrate from the alcohol dehydrogenase or that a single enzyme was responsible for both reactions. During the first stages of purification of the alcohol dehydrogenase the alcohol and aldehyde dehydrogenase activities could not be separated. However further physical purification showed that the alcohol dehydrogenase activity could be isolated but that as a consequence the aldehyde dehydrogenase activity was totally lost. The work on the purification of the alcohol dehydrogenase was in its early stages and clearly further work is necessary. It would be of interest to determine whether the alcohol dehydrogenase was responsible for both the reactions involved in the formation of fatty acids from alcohols. The present evidence is conflicting. The corresponding disappearance of substrate and appearance of product suggest that only one enzyme is involved. Physical purification techniques indicated that at least the alcohol dehydrogenase activity could be isolated. However, such an observation may be due to a change in a single enzyme as a result of the purification procedures, particularly since no aldehyde dehydrogenase activity could be retrieved. It

was hoped that purification of the enzymes in the n-alkane degradation pathway and studies of the kinetics of the reactions that were catalyzed may have allowed questions about the hydroxylase activity to be answered indirectly. The presence of alcohol dehydrogenases that exhibit high activity and have high affinity for long-chain alcohol substrates in association with the observation that alcohol intermediates in n-alkane metabolism are not observed under normal circumstances suggest that the hydroxylation of n-alkane substrates may be a rate limiting factor during growth.

The induction of isocitrate lyase activity in n-alkane grown cells was demonstrated in TC15-1. The presence of this enzyme indicated that the organisms have a functioning glyoxylate cycle during growth on n-alkanes and agrees with the observations of other groups already mentioned. The glyoxylate cycle allows n-alkane grown cells to introduce acetyl CoA into the tricarboxylic acid cycle in order to produce energy and carbon for cellular biosynthesis. It can be postulated that in n-alkane grown cells the acetyl CoA produced from β-oxidation is the only source of carbon the organisms have for synthesis of amino acids, sugars and bases. It was not investigated however, whether the acetyl CoA introduced into the glyoxylate cycle was also the only source of ATP available to the cell, as had been suggested for other organisms.

In TC15-1 the levels of isocitrate lyase in n-alkane grown cells were not as high as in acetate-grown cells in which the glyoxylate cycle is also the only route of carbon into cellular material. It may be that this was due to differences in culture conditions since the difference in activities between the cultures was not large. However, in acetate-grown cells the growth substrate is also the starting material for fatty acid biosynthesis. This is not the case in n-alkane-grown cells which are probably able to make fatty acids directly from the n-alkane growth substrate and this may be reflected in an attenuation of isocitrate lyase expression. The assays which attempted to determine whether fatty-acid synthesis is in fact repressed in n-alkane grown TC15-1 were not successful and require further work. There is much conflicting evidence concerning the presence of fatty-biosynthesis in other n-alkane-grown organisms and it would be of interest to determine whether this function is repressed in TC15-1 and if so to what extent.

Also of interest in the context of the control of cellular carbon within n-alkane-grown cells were the levels of the enzymes of β-oxidation. In TC15-1 the levels of the first enzyme of the pathway were assayed in n-alkane-, glucose- and acetate-grown cultures. It was indicated that there were elevated levels of fatty acyl CoA synthetase

in n-alkane-grown cells compared with almost negligible activity found in the other samples. This finding however in conjunction with the high activity of alcohol and aldehyde dehydrogenases induced by growth on n-alkanes and the presence of isocitrate lyase indicate strongly that the pathway of carbon into n-alkane grown cells of TC15-1 is through β-oxidation of fatty acids and the introduction of acetyl CoA into the glyoxylate cycle. As previously pointed out growth on n-alkanes requires cells to be lipolytic and gluconeogenic. Studies of the enzymes of glycolysis and carbohydrate metabolism compared with glucose grown cells would be required for a complete picture of carbon flow in n-alkane grown cells.

It is seen from the polyacrylamide gel electrophoresis analysis of TC15-1 that individual bands change as a result of growth on different substrates, but no large-scale redistribution of the proteins signified by gross changes in the banding pattern were observed. Further studies of this kind could be done using non-denaturing gels and two dimensional gel systems in which changes in the polypeptide patterns may be more evident.

III.4 Genetic analyses and manipulation

III.4.1 Curing experiments

Curing experiments to determine whether the n-alkane degradation pathway was plasmid borne were done initially using the organism C16/PS.

Cells were grown on L-broth with increasing concentrations of acridine orange at pH 7.6. It was found that the organism was very sensitive to the acridine dye and that growth was severely inhibited at a concentration of 1 µg/ml. Higher concentrations inhibited growth totally.

The organisms that grew up in the presence of acridine orange at the most severely inhibiting concentrations were serially diluted and plated onto L-agar to obtain single colonies. When 1000 colonies were picked onto AMS/agar supplied with n-alkane all the colonies tested grew. The procedure was repeated several times with similar results.

The same experiment was done using the pseudomonad TC15-1. In this case the cells were extremely resistant to both acridine orange and ethidium bromide and would grow in concentrations of both dyes that caused precipitates to form in L-broth. Clearly no meaningful results could be obtained.

Using mitomycin C according to the method of Chakrabarty *et al*. (1973) further curing experiments were attempted. 2 ml aliquots of L-broth in 10 mm test-tubes were supplemented with mitomycin C at 2 µg increments from

0-16 µg/ml. The test-tubes were inoculated with approximately 10^5 cells from a culture of TC15-1 grown in L-broth, and incubated at 30°C with shaking. When appreciable growth had occurred the cultures were serially diluted and plated out onto L-agar to obtain single colonies. These were picked onto AMS/n-tetradecane plates and AMS/glucose plates and scored for the loss of the ability to grow on n-alkanes.

TC15-1 proved to be extremely sensitive to mitomycin C and total inhibition was caused at concentrations of 6 µg/ml and above. Significant growth occurred in cultures which contained 2 µg/ml and 4 µg/ml of mitomycin C after a lag phase of 24 hrs. These cultures were treated as described and the culture which contained 6 µg/ml and displayed no perceptible growth was plated out undiluted (Table XVI).

The results of scoring for loss of n-alkane utilization are shown in Table XVI and compared to the frequency of occurrence of auxotrophic mutants.

The highest frequencies of auxotrophic mutants were obtained using mitomycin C at a concentration of 2 µg/ml. At this concentration it was also found that a high frequency of Alk^- mutants were generated. When 4 µg/ml of mitomycin C was used comparable growth occurred but the lag phase was longer than for lower concentrations. The number of auxotrophic mutants obtained was lower and no Alk^- mutants were isolated. Mitomycin C concentrations higher

Table XVI

Effects of mitomycin C on the organism TC15-1 with respect to growth on n-alkanes.

Concentration mitomycin C (μg/ml)	Viable counts (cells/ml)*	Frequency of Alk^- phenotype	Frequency of auxotrophs
0	5×10^9	$<10^{-8}$**	$<10^{-8}$**
2	1.6×10^9	1.6×10^{-3}	1.6×10^{-3}
4	2.4×10^9	$<10^{-4}$	9×10^{-4}
6	0	ND	ND

* Cells per ml after three days incubation.

** Assessed previously using replica plating.

ND Not determined.

than 4 μg/ml caused total mortality of the inoculum.

III.4.2 Mutants of C16/8S

The mutagenic treatments used on C16/8S were adjusted empirically to produce high frequencies of auxotrophic mutants with single mutations.

Because of the very high frequency of killing using the U.V. irradiation method described, the mutants produced have been demonstrated elsewhere to carry multiple mutations (Dijkhuisen et al., 1981).

The frequency of auxotrophic mutants obtained using both NTG and U.V. mutagenesis are shown in Table XVII and compared to the frequency of Alk^- mutants. It was observed that the frequency of Alk^- mutants in comparison to the frequency of auxotrophic mutants was low and also appeared to vary to a greater extent between experiments. However these results were not unexpected and several mutants were obtained that were unable to grow at the expense of n-alkanes.

The Alk^- mutants were screened for accumulation of primary oxidation products when n-alkanes were introduced to cultures grown on acetate or L-broth. 10 ml cultures were grown up overnight, spun down and resuspended in an equal volume of fresh medium. The cultures were then supplemented with 1.0 ml of n-tetradecane and incubated with vigorous shaking at 30°C. Samples were taken at

Table XVII

Mutagenesis using C16/8S.

Frequencies of auxotrophic and Alk^- mutations using NTG and U.V. mutagenesis.

Treatment	% Killing	Frequency of auxotrophic mutants	Frequency of Alk^- mutants
NTG	90	7×10^{-2}	$<3 \times 10^{-3}$
U.V.	>99.999	8.5×10^{-2}	$<2 \times 10^{-3}$

hourly intervals from the oil phase for analysis by GLC.

In all cases the rapid emulsification of the oil which was normally observed in wild-type cells occurred and the substrate was quickly dispersed throughout the medium. Samples were taken from the oil after a short period of centrifugation which served to separate the two phases.

Most of the mutants screened in this manner showed no accumulation of products. One however, which was numbered strain 3615, was able to accumulate significant quantities of tetradecan-1-ol.

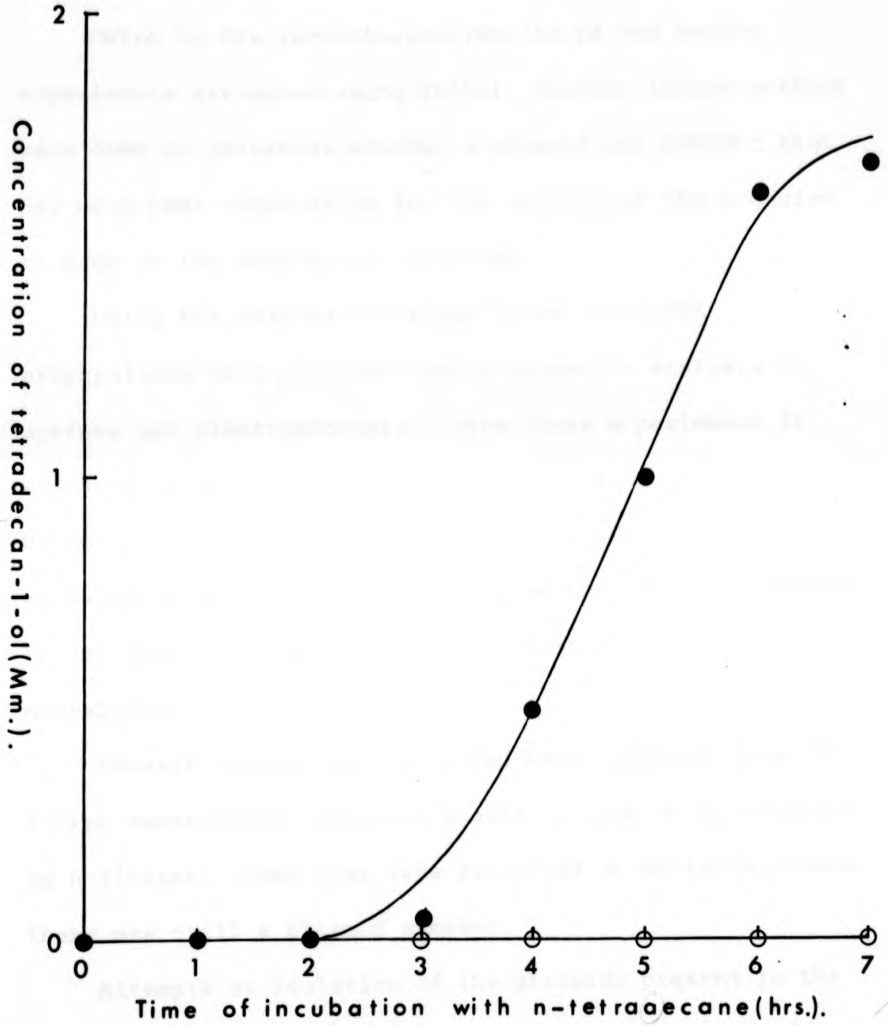
The pattern of accumulation of alcohol by C15/8S strain 3615 is shown in figure 25. The wild-type organisms treated in the same manner did not accumulate any alcohol at all.

The strains produced that were Alk^- proved to be unstable and had such high reversion rates that they could not be maintained. They would rapidly revert to the wild-type Alk^+ phenotype and cease to accumulate primary oxidation products. This was true of mutants produced by both NTG and U.V. mutagenesis. Attempts to generate mutants by methods that caused deletions in DNA proved unsuccessful because the organism was highly sensitive to the chemical agent used (Nitrous acid).

Figure 25

The accumulation of tetradecan-1-ol by cells of C16/8S strain 3615 when supplied with n-tetradecane during growth on L-broth.

● strain 3615 o wild-type.


2
1
0
Concentration of tetradecan-1-ol(Mm.).
0
1
2
3
4
5
6
7
Time of incubation with n-tetraaecane(hrs.).

III.4.3 The large plasmid of TC15-1

Owing to the inconclusive results of the curing experiments attempted using TC15-1, further investigations were done to determine whether a plasmid was present that may have been responsible for the ability of the organism to grow at the expense of n-alkanes.

Using the methods described crude total DNA preparations were prepared and subjected to analysis by agarose gel electrophoresis. From these experiments it was evident that a large plasmid was present that ran behind the chromosomal DNA (figure 26). The plasmid appeared to be very stably maintained and could be detected in cultures grown on a variety of substrates including non-alkanes.

Several strains of TC15-1 had been isolated from the curing experiments that were unable to grow at the expense of n-alkanes. When they were subjected to analysis however there was still a plasmid present.

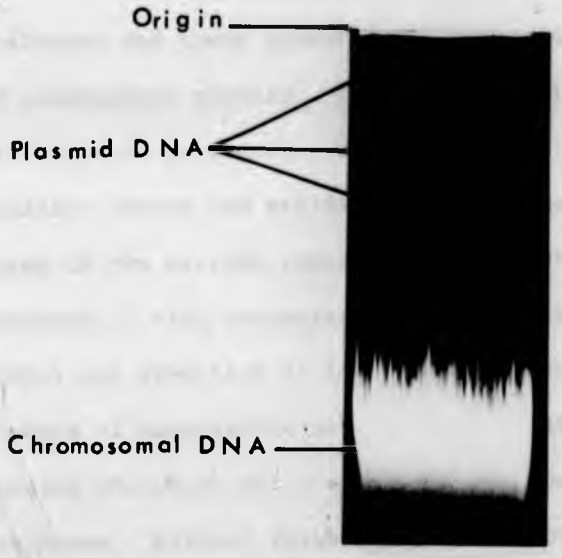
Attempts at isolation of the plasmids present in the wild-type strains and Alk^- mutants for the purposes of restriction analysis had been started. Insufficient plasmid was purified to allow these studies to be completed.

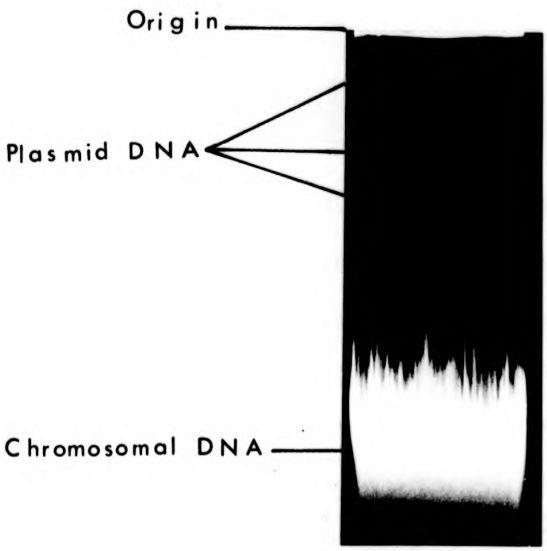
III.4.4 Discussion

The experiments in this section were done primarily to obtain mutants of the organism C16/85 which might allow

Figure 26

0.5 % agarose gel of a crude preparation of total DNA from n-alkane grown cells of TC15-1.


Origin
Plasmid DNA
Chromosomal DNA


Origin
Plasmid DNA
Chromosomal DNA

the elucidation of the pathway of n-alkane degradation and also to determine whether the genetic information for the pathway was plasmid borne in both C16/8S and TC15-1.

The curing experiments using C16/8S indicated that the catabolic pathway for n-alkane metabolism was probably chromosomally borne. Curing agents to which the organism was sensitive did not destroy the ability to grow on n-alkanes and there appeared to be no significant background of auxotrophic mutants. In TC15-1 the situation was more difficult to interpret. The standard curing agents such as acridine orange and ethidium bromide were unsatisfactory owing to the extreme resistance of the organism to their presence at high concentrations. Using mitomycin C to which TC15-1 was sensitive at low concentrations, significant numbers of auxotrophic mutants were obtained as well as strains that had lost the ability to grow at the expense of n-alkanes. Without further investigations it was impossible to tell whether the desired phenotype was due to plasmid loss. The experiments did produce a high frequency of Alk^- phenotypes at a mitomycin C concentration of 2 μg/ml and it was subsequently shown that the wild-type TC15-1 did in fact contain at least one large plasmid. In Alk^- strains generated by treatment with mitomycin C however it was shown that plasmids had not been lost. The curing experiments were therefore inconclusive since the use of

mitomycin C proved to be ineffective as a curing agent and produced high numbers of spurious mutations.

The question as to whether the plasmid which was demonstrably present in TC15-1 was associated with n-alkane degradation remains. The existence in Pseudomonas species of plasmids that carry catabolic pathways such as those coding for toluene and xylene degradation (Williams and Murray, 1974), naphthalene degradation (Dunn and Gunsalus, 1973) and n-octane degradation (Chakrabarty et al., 1973) suggest that the enzymes for higher n-alkane degradation may also be plasmid borne. It may also be pointed out however that not all strains of Pseudomonas aeruginosa that have been isolated have carried plasmids whereas they were almost invariably capable of growth on n-alkanes. This suggests a chromosomal location of the degradation pathway genes in this organism. These arguments are not mutually exclusive. Recent work by Jeenes et al. (1982(a), 1982(b)) suggest that with TOL plasmids there may be reversible exchange of genetic information between the plasmid and the host chromosome. The occurrence of strains that carry chromosomal pathways for n-alkane degradation need not imply the non-existence of plasmids that carry an identical function. Clearly the large plasmid of TC15-1 may not be involved in n-alkane degradation, but further work to investigate its function is required. The mitomycin C experiments did serve

to show that mutants of TC15-1 may be isolated that are unable to grow at the expense of n-alkanes. If such mutants could be generated by insertion of transposons, the position of the genes may be located unambiguously. It is also important to attempt mating experiments to investigate whether the plasmid is transmissible and whether the ability to grow on n-alkanes is transferred with it. In such experiments the recipient strains must be chosen carefully. It was shown that in Pseudomonas putida n-alkane degradation encoded by the OCT plasmid was dependent upon host-determined aldehyde dehydrogenase.

The mutagenesis experiments using C16/8S did produce Alk^- mutants which were capable of accumulating primary oxidation products when supplied with n-alkanes during growth on alternative substrates.

Such experiments showed that in C16/8S the pathway of n-alkane degradation involves the terminal hydroxylation of substrates. It was also evident that the enzymes of the pathway were not repressed by the presence of alternative growth substrates. The lag phase observed before alcohol accumulation commenced may have been the time taken for the pathway to become induced. The fact that any alcohol accumulated was also significant. It indicated that the organism C16/8S did not carry multiple alcohol dehydrogenases capable of further metabolizing the products of the n-alkane hydroxylase.

The reasons for the accumulation of alcohol decreasing after a period of six hours were not investigated. The n-alkane substrate was present in large quantities but it is possible that the culture, either became energy- or oxygen-limited or may have been affected by toxic effects of the accumulated product. Energy limitation seems an unlikely explanation since cultures were supplied with fresh rich medium at the start of the experiments which provided ample substrates for the generation of NADH. The possibility of oxygen limitation was greater, the experiments were done in sealed flasks in order to allow vigorous agitation. The increased oxygen demand as a result of the induction of the n-alkane hydroxylase may have resulted in a rapid depletion of the oxygen available.

Toxic effects of the alcohol products on the activity of the n-alkane hydroxylase cannot be ruled out. Under normal circumstances alcohols are rapidly metabolized and do not accumulate, high concentrations may inhibit the n-alkane hydroxylase. To test these possibilities stable mutants would have to be generated.

The instability of the Alk^- mutants isolated was a major problem. It proved impossible to maintain strains that did not revert to the Alk^+ phenotype with prohibitively high frequencies. Such problems could probably be overcome by using different methods of mutagenesis although initial

experiments with nitrous acid were unsuccessful. From an industrial point of view an organism that can be manipulated to accumulate primary alcohols without further metabolism would be of considerable interest.

CHAPTER FOUR

GENERAL DISCUSSION

IV General Discussion

The present work has covered a wide range of investigations into different aspects of n-alkane utilization by microorganisms.

It was demonstrated that n-alkane utilizing organisms are ubiquitous and may be easily isolated from the environment regardless of whether sampling sites are contaminated with petroleum products. Diverse species could be shown to grow at the expense of n-alkanes as sole carbon and energy source.

The studies of growth on n-alkanes of two different organisms highlighted the diversity of interactions with the insoluble growth substrates that were possible. It was also seen that different structural adaptations to growth on n-alkanes could occur.

The major problem that was encountered and which was not overcome despite the large part of the work devoted to it, was the demonstration and isolation of n-alkane hydroxylation enzymes from n-alkane-grown microorganisms. The variety of assays attempted under a wide range of conditions showed no accumulation of primary alcohols or other oxidation products when cell extracts were incubated with n-alkane substrates. It was also shown that no

accumulation of primary oxidation products occurred in the medium of n-alkane-grown cultures. The only indication that the organisms under investigation possessed an n-alkane degradation pathway that involved terminal hydroxylation of the growth substrate was the identification of enzymes responsible for other reactions in the pathway that were also induced by growth on n-alkanes. Mutants were also generated that could accumulate primary alcohols in the growth medium.

It may be argued that much of the lack of success in this area of the work was due to the choice of organisms used for study. The screening of a wider variety of micro-organisms from different sources using different criteria may have yielded bacterial strains that were more amenable to the investigations attempted. Under circumstances in which time was limited, lengthy screening procedures which may not have been any more fruitful were sacrificed when two potentially suitable organisms were found. The organisms chosen for further study both grew vigorously on n-alkanes and were subsequently shown to assimilate them by the pathway of interest. It is clear from the investigations done however that the n-alkane hydroxylase in both strains was highly labile in disrupted cells.

The implications of such work from an industrial point of view are significant. The findings of the present

investigations suggest that n-alkane hydroxylases are often highly labile in cell-free systems and the work of other groups indicate that they are multicomponent enzymes that require expensive electron donors. The prospect of immobilized enzyme systems that are able to terminally hydroxylate n-alkanes on an industrial scale must be bleak. The possibility of immobilized whole-cell systems may be greater, particularly with organisms that can be grown on alternative substrates and still induce the n-alkane hydroxylation enzyme when presented with n-alkanes or other inducers. Organisms such as C16/8S which normally grows preferentially on n-alkanes and which has a high affinity for the substrate due to the hydrophobic nature of the cell wall would be useful in this context. Stable mutants would have to be generated so that substrates could not be degraded beyond the required product.

In the short term it seems that the genetic manipulation of whole-cell systems may be able to provide organisms that can be used to convert alkanes to alcohols on an industrial scale. Mechanistic studies with the more long term aim of developing synthetic catalysts still continue and recent work has been done on the mechanism and stereochemistry of the ω-hydroxylase of Pseudomonas oleovorans using chiral methyl groups (Capsi et al., 1981).

The investigations of the fate of n-alkane substrates

after initial hydroxylation in the organism TC15-1 allowed a pathway of carbon into non-lipid cellular material to be postulated. However as with much of the present work avenues for further study are numerous.

The difficulties encountered in basic procedures essential for the work undertaken were mostly unforeseen. In C16/8S extremely high affinity for the substrates when grown on n-alkanes made harvesting of cells very difficult. To obtain cell extracts that were n-alkane free was almost impossible except by methods which were found to damage enzyme activity. In TC15-1 which was far more amenable from the point of view of preparation of cell-free extracts, problems were caused by the low affinity of cells for the substrate when studies of n-alkane degradation were attempted. The results obtained although disappointing from the point of view of the initial aims were satisfactory for a project that was started from basic isolation of the organisms used and attempted to investigate a system that has been consistently demonstrated to be intractable.

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