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Fungal Oxidation Of Gaseous Alkanes

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FUNGAL OXIDATION OF GASEOUS ALKANES

by

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ABSTRACT

Reports of fungi which grow at the expense of gaseous alkanes have been few and usually of an incidental nature. The frequency of this ability amongst the various fungal taxa is unknown. Little direct information is available on the biochemical mechanisms of gaseous alkane assimilation by fungi.

Eleven new cultures were isolated from sewage by natural enrichment upon natural gas as sole carbon and energy source. They all grew well upon ethane, propane, n-butane and conventional carbohydrate carbon sources under aerobic conditions but not upon methane nor upon any liquid n-alkanes. These cultures were identified as belonging to the genera Acremonium, Graphium and Phialophora. Representative cultures were described in detail.

An Acremonium isolate was selected for metabolic studies on gaseous alkane oxidation. Warburg respirometry and [1,2-^{14}C] ethane tracer experiments with resting-cell suspensions indicated that gaseous alkane growth-substrates were oxidized via a terminal attack which required molecular oxygen and involved production of primary alcohol, aldehyde and fatty acid intermediates but not olefines.

Studies on cell-free extracts of ethane-grown Acremonium cells employing [1,2-^{14}C] ethane indicated that the enzyme (system) responsible for the initial fixation reaction

with ethane was predominantly microsomal and probably contained a haemoprotein, NADPH was required for ethane fixation. Ethane probably undergoes initial hydroxylation to ethanol in a reaction catalysed by an enzyme system of the monooxygenase type.

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TABLE OF CONTENTS

LIST OF PHOTOGRAPHIC PLATES

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS (used in the text)

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CoA	Coenzyme A
EDTA	Ethylenediamine tetraacetic acid
E^{mM}_{340}	Millimolar extinction coefficient (340 nm)
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced form of NAD^+
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of $NADP^+$
P-450	Cytochrome P-450
Pi	Inorganic phosphate
R	Alkyl group
Tris	Tris (hydroxymethyl) amino methane

CHAPTER 1

INTRODUCTION

The economic importance of those compounds which make up the normal alkane or paraffin homologous series cannot be overstated in 1974. Petroleum hydrocarbon deposits have largely replaced coal as the principal energy resource and as the source of raw materials for the chemical industry in the twentieth century. The n-alkanes are without doubt the most valuable component of these deposits. Natural gas, consisting principally of gaseous n-alkanes, is the most sought-after petroleum fraction of all.

The n-alkanes consist of unbranched saturated chains of carbon atoms; all the carbon valencies are involved in bonding to either carbon or hydrogen atoms. A general empirical formula can be written for all members of the series, C_nH_{2n+2}. The n-alkanes collectively are relatively inert hydrophobic compounds. Physical properties such as water solubility, melting point and boiling point are usually closely correlated with the value of n. It can readily be seen from Table 1-1 that only the first four members of the series are gases at room temperature (25°C). Compounds C_5 to C_{17} inclusive are liquids of decreasing volatility; C_5 to

Table 1-1. The n-alkane hydrocarbons[a].

Empirical formula	Systematic name	Melting point °C	Boiling[b] point °C
CH_4	methane	-182.5	-161.5
C_2H_6	ethane	-183.3	- 88.6
C_3H_8	propane	-189.9	- 44.5
C_4H_{10}	n-butane	-138.3	- 0.5
C_5H_{12}	n-pentane	-129.7	36.0
C_6H_{14}	n-hexane	- 95.0	68.0
C_7H_{16}	n-heptane	- 91.0	98.4
C_8H_{18}	n-octane	- 56.5	125.0
C_9H_{20}	n-nonane	- 51.0	151.0
$C_{10}H_{22}$	n-decane	- 29.7	174.1
$C_{11}H_{24}$	n-undecane	- 25.0	195.0
$C_{12}H_{26}$	n-dodecane	- 9.6	216.0
$C_{13}H_{28}$	n-tridecane	- 5.5	243.0
$C_{14}H_{30}$	n-tetradecane	6.0	253.5
$C_{15}H_{32}$	n-pentadecane	10.0	270.5
$C_{16}H_{34}$	n-hexadecane	18.0	287.0
$C_{17}H_{36}$	n-heptadecane	22.0	303.0
$C_{18}H_{38}$	n-octadecane	28.0	306.0
$C_{19}H_{40}$	n-nonadecane	32.0	330.0
$C_{20}H_{42}$	n-eicosane	36.8	343.0
$C_{21}H_{44}$	n-heneicosane	40.0	363.0

Table 1-1 (cont'd.)

Empirical formula	Systematic name	Melting point °C	Boiling point[b] °C
$C_{22}H_{46}$	n-docosane	44.0	327.0
⋮			
C_{30+}			

[a]Data from Handbook of Chemistry and Physics (1964).

[b]At one atmosphere pressure.

C_{10} n-alkanes are major components of automotive gasoline; the higher boiling liquid n-alkanes are major components of kerosene and jet fuel. Compounds from C_{18} on up the series to C_{30+} are solids, the paraffin waxes.

Hydrocarbons are rarely considered in a biological context, let alone as carbon substrates for microbial growth. The hydrophobic nature of hydrocarbon compounds in general and n-alkanes in particular and the absence of reactive functional groups makes them seem most unsuitable substrates for enzymic attack. Textbooks of microbiology and biochemistry have generally given little if any attention to hydrocarbon-utilizing microbes and their metabolism. There is ample evidence however that hydrocarbons are significant components of the biological carbon cycle in nature. Weete (1972) summarized the widespread natural occurrence of paraffin waxes (C_{16}-C_{39}) in higher plants, algae, fungi, bacteria and even animals. Present day petroleum hydrocarbon deposits are believed to be of biological origin (Davis 1967). The major classes of compounds found in such deposits (aromatics, alicyclics, branched chains, straight chains, saturates and unsaturates etc.) are all subject to biodegradation, some more readily than others. The n-alkanes are in fact the most readily degraded hydrocarbons of all.

Miyoshi's report (1895) of a mould, Botrytis cinerea, growing upon paraffin wax in all probability marked the beginning of hydrocarbon microbiology. Söhngen (1906) followed soon after with a report of a bacterium, Bacillus methanica,

growing upon methane. The next half century however saw relatively little activity in this field. The literature for that period was reviewed by Zobell (1950).

The past twenty years has seen a marked increase in interest in this field. Interest has been focussed particularly upon microorganisms which can utilize n-alkanes as sole carbon and energy sources and the metabolic machinery employed to assimilate and dissimilate such unconventional substrates.

Much of this kind of research has important practical applications; the large oil companies have been a major source of funding. A very topical example is research into biodegradability of crude oil spills at sea and on land. Considerable interest has been shown in recent years in industrial fermentations using n-alkanes as raw materials for microbial synthesis of marketable metabolites. Bulk culture of microbes upon n-alkanes as a method of single-cell-protein production is being researched by several oil companies, notably the French division of British Petroleum. The abundance of certain ethane and propane-utilizing bacteria in soil has been used as an indicator in oil prospecting (Davis 1967). On the negative side certain fungi have become a problem by growing in the fuel tanks of jet aircraft on fuel contaminated with water, where they cause clogging and corrosion.

A preliminary literature search revealed that considerable effort had been expended in the area of bacterial growth

on and metabolism of liquid n-alkanes. Frequent reports had also appeared on growth of moulds and yeasts upon liquid n-alkanes but there were few metabolic studies. The report by Zajic et al. (1969) of a mould which grew upon certain n-alkane components of natural gas pinpointed an area of apparent neglect in hydrocarbon microbiology. Until then reports of fungal growth upon gaseous n-alkanes had been few, usually superficial and largely incidental. No information was available upon gaseous n-alkane metabolism in fungi.

The present study was undertaken in an attempt to fill this apparent gap in our knowledge and also as an adjunct to the work in progress in Dr. Zajic's laboratory on single-cell-protein production by bulk culture of fungal tissue on natural gas. The intent was to follow the initial pathway of carbon assimilation from gaseous n-alkane growth substrates in moulds. A principal interest was the nature of the first biological 'fixation' reaction of the hydrocarbon in the cell.

The literature survey which follows is of necessity rather broad in scope. Little specific information is available on fungal assimilation of gaseous n-alkanes. However reports on the metabolism of other micro-organisms on liquid n-alkanes are highly relevant since one might reasonably expect similar mechanisms to operate upon gaseous alkane homologues. Therefore in addition to fungal metabolism, bacterial metabolism and certain relevant work with

mammalian systems will be covered, since prokaryotes and eukaryotes have many biochemical pathways in common.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Introduction:

No attempt has been made here to review the literature on this subject in its entirety. The reader is referred to the books of Beerstecher (1954) and Davis (1967) for comprehensive accounts of petroleum microbiology. McKenna and Kallio (1965) and Van der Linden and Thijsse (1965) exhaustively reviewed the literature of microbial metabolism of the major classes of petroleum hydrocarbons. More recently the metabolism of single carbon compounds, particularly methane, was reviewed by Ribbons et al. (1970) and the C_{2+} aliphatic hydrocarbons by Klug and Markovetz (1971).

2.2. Organisms and substrate specificity:

Reports of the ability or inability of microbes to utilize various n-alkanes are legion. As pointed out by Klug and Markovetz (1971), caution should be exercized in interpreting such growth studies in terms of substrate specificity. A positive test for ability to utilize a particular alkane is usually informative; the reverse however is not always true. Usually growth studies on a range of hydrocarbons are carried

out under uniform conditions. A culture's inability to use a particular alkane may not necessarily be due to a metabolic inadequacy. With the lower members, $C_5 - C_{10}$ especially, problems of toxicity have been encountered. Such compounds are more soluble than higher homologues and higher concentrations are therefore encountered in solution. Johnson (1964) found that more organisms would grow on n-hexane if the concentration in the medium was kept below saturation. It was noted by Davis (1967) that when alkanes such as n-hexane or n-heptane are agitated in the liquid phase, growth of cultures is often retarded, possibly due to the solvent or cell permeation effect of these effective lipid solvents. When such compounds are supplied as vapours however growth is often possible.

2.2.1. Prokaryotes:

The ability of eubacteria and actinomycetes to utilize n-alkanes is widespread and not confined to a few select genera. This ability has been found in species of Pseudomonas, Mycobacterium, Corynebacterium, Brevibacterium, Desulphovibrio, Micrococcus, Arthrobacter, Achromobacter, Nocardia and many others. Virtually all members of the normal alkane series (from methane on up to the paraffin waxes) are used by one or more microbes.

With a few notable exceptions, little taxonomic significance can be seen in the apparent substrate specificity exhibited by various cultures with respect to n-alkanes.

Some species can grow on a wide range of homologues, others on only one or two. Methane utilizers can be cited as a special case however. The majority of bacteria growing on methane belong to genera of obligate methyl users, that is they will grow only upon methane or methanol. These genera, revieweđ by Ribbons et al. (1970) are Methylococcus, Methylosinus, Methylocystis, Methylobacter, Methanomonas and Methylomonas.

In a reorganization of the taxonomy of this group of bacteria, Foster and Davis (1966) assigned the prefix 'Methylo' to obligate methyl users. The genus Methylomonas includes forms previously referred to as Pseudomonas methanica. A few reports have appeared however of bacteria able to grow on methane and higher alkanes. Perry (1968) reported Brevibacterium isolates which grew on C_1 - C_8 n-alkanes and Kozlova et al. (1969) reported species of Mycobacterium, Pseudomonas, Bacillus and Actinomyces which grew upon methane or higher homologues.

Many reports have appeared of utilization of long chain liquid alkanes (C_{12} - C_{18}), less of the C_5 to C_{11} range, still fewer of the gaseous alkanes and hardly any of paraffin wax utilization (C_{19+}). To a large extent this reflects the greater effort applied in some areas. Technical problems probably dissuade many from working with gaseous or solid alkanes. However recent reports of cultures of Pseudomonas, Azotomonas and Serratia using paraffin waxes by Chakravarty et al. (1972) and Amin et al. (1973) indicate that such

microbes are probably not as uncommon as is generally supposed. It is probably true to say however that reports of the use of low molecular weight volatile liquid alkanes are more scarce because of problems of higher solubility and toxicity.

2.2.2. Eukaryotes:

The ability of genera of moulds and yeasts to utilize alkanes is as widespread as in the eubacteria and actinomycetes.

2.2.2.1. Yeasts:

Only one report has appeared of a yeast using gaseous alkanes, that of Kormendy and Wayman (1974) who isolated a strain of Candida utilis which will grow on n-butane. Reports of growth on n-alkanes below C_{10} are very rare. There is some evidence however that this might be more a deficiency in methodology than in metabolism. Yoshida et al. (1971) found no growth of C. tropicalis on C_6, C_7 or C_8 when the alkane was supplied to the medium as a liquid or as vapour sufficient to saturate the medium. However, when levels of vapours were supplied which produced sub-saturation levels of hydrocarbon, growth took place. Solvent action upon the cell membranes was proposed as an explanation. Klug and Markovetz (1971) summarized much of the previous literature on yeast growth on liquid hydrocarbons. Species of Debaryomyces, Endomyces, Hansenula, Torulopsis, Monilia,

Candida, Rhodotorula, Trichosporon, Pichia, Mycotorula, Cryptococcus and Saccharomyces were found to have this ability.

Nyns et al. (1968a) reported species of the genera Debaryomyces, Pichia, Hansenula, Rhodotorula, Torulopsis, Candida, Sporobolomyces, Brettanomyces, Saccharomyces and Schizosaccharomyces able to grow upon C_{10} - C_{16} n-alkanes. Singh et al. (1970, 1972) reported use of n-alkanes of this same fraction by Trichosporon pullulans and Endomycopsis lipolytica as did Merdinger and Merdinger (1970) for Pullularia pullulans. Kato et al. (1971) reported assimilation of C_{10} - C_{17} n-alkanes by Candida species from marine sediments at low temperature (12°C) in artificial sea water. It was shown by Hirai et al. (1972) that the choice of glucose or hydrocarbon substrate affected the dimorphism of a Candida tropicalis strain. C_{10} - C_{12} n-alkanes favoured true mycelium development, C_{13} - C_{16} favoured pseudomycelium development and glucose-grown cultures grew as true yeasts.

One of the most extensive surveys of alkane utilization in yeasts was that of Bos and de Bruyn (1973). All the type strains mentioned in 'The Yeasts' (Lodder 1970) and others too from the Delft collection were tested for ability to grow upon either n-octane, n-decane or n-hexadecane. Positive tests for use of one or more alkanes (no distinction was made between them) were found in species of the following genera: Debaryomyces, Endomyces, Lodderomyces, Melschnikowia, Pichia, Saccharomycopsis, Schwanniomyces,

Wangea (Ascomycetes); Leucosporidium, Rhodosporidium (Basidiomycetes); Sporidiobolus, Sporobolomyces, Candida, Rhodotorula, Selenotila, Torulopsis, Trichosporon (Fungi Imperfecti). The ability was most common in Candida species, followed closely by Torulopsis.

Growth of yeasts upon paraffin waxes has not often been reported; Yamada and Yogo (1970) reported a Candida tropicalis strain which would assimilate paraffin wax consisting of C_{25} - C_{37} n-alkanes (melting point 62.5°C) as well as liquid alkanes. Champagnat (1965), reporting progress on single-cell protein production from yeasts grown on gas oil, noted de-waxing of this fraction by preferential utilization of waxes.

As in the prokaryotes, little taxonomic significance can be seen in the ability of yeasts to use hydrocarbons; it is found in all the major yeast taxa. Many genera are quite heterogeneous in this respect as evidenced by the study of Bos and de Bruyn (1973). However good correlation was found in the latter work between the alkane-utilizing ability of an imperfect yeast and that of its perfect state. Among the perfect genera, Kluyveromyces, Saccharomyces and Hansenula had no alkane-utilizing species (S. elongisporus, reported by Scheda and Bos (1966) to use alkanes, has since been transferred to the genus Lodderomyces). Hence inability to use alkanes in imperfect species may indicate natural groupings. Ability or inability to use alkanes was consistent for all strains of a particular species.

2.2.2.2. Moulds:

For a long time the ability to use methane as a sole carbon and energy source was generally regarded as being restricted to a small specialized group of obligate methyl-requiring bacteria which had more in common with autotrophs than other heterotrophs. Subsequently Zavarsina (1968) reported three Cephalosporium isolates able to grow on methane; the evidence presented however was somewhat equivocal and this ability was lost after serial subculture on carbohydrate media. Adamse et al. (1972) reported the isolation of two fungi able to grow at the expense of methane or ethane. Descriptions of these fungi however were not very precise; one was described as having brown conidia the other as having green ones and resembling Penicillium janthinellum.

Reports of moulds using C_2 - C_4 n-alkanes are scarce. Dworkin and Foster (1958) and Kester (1961) reported Fusarium and Acremonium isolates growing upon propane and ethane. Davis (1967) referred to the infrequent appearance of moulds upon plates intended for isolation of ethane-oxidizing bacteria as an aid to oil prospecting; no attempt however was made to identify them. The first serious work on moulds using gaseous alkanes was that of Zajic et al. (1969) describing the isolation of a Graphium species able to grow upon ethane, propane or n-butane. Volesky and Zajic (1970, 1971) investigated bulk culture of this isolate upon gaseous alkanes as a source of single-cell protein. From the same laboratory (Davies et al. 1973, 1974) came reports of further

Graphium isolates, cultures of Phialophora and Acremonium (sensu Gams) which grew upon the same substrates. McClee et al. (1972) isolated species of Graphium, Penicillium and Allescheria which used n-butane.

As with the yeasts, and prokaryotes, most reports have been of moulds using C_{10} - C_{18} n-alkanes, again reports of growth upon C_5 - C_9 n-alkanes are scarce. The higher liquid n-alkanes are the principal constituents of jet aviation fuel and many fungi have been isolated from fuel tanks contaminated with water. The principal mould from this source is Cladosporium resinae which has been isolated many times, e.g. Nicot and Zakartchenko (1966). Parberry (1969) reported a perfect ascomycete state for this mould, Amorphotheca resinae. Species of the following genera have been reported to use the C_{10} - C_{18} petroleum fraction: Cladosporium, Hormodendron (now believed synonymous with Cladosporium, Baron 1968), Fusarium, Penicillium, Trichoderma, Aspergillus, Cephalosporium, Colletotrichum, Acremonium, Monilia, Botrytis, Helminthosporium, Dematium, Epicoccum, Gliocladium, Graphium, Paecilomyces, Spicaria, Alternaria, Verticillium, Periconia, Chloridium, Pullularia, Gliberella, Amorphotheca (perfect state of Cladosporium resinae), Cunninghamella, Mucor, Rhizopus, Zygorhynchus; Absidia, Circinella Mortierella, Helicostylum (Klug and Markovetz 1971; Zaichenko and Koval 1966; Darby et al. 1968; Pelz and Rehm 1972; Cerniglia and Perry 1973; Nyns et al. 1968b).

The ability to utilize paraffin waxes has been reported more often in the moulds than in any other microbes. Be-

sides the early report of Miyoshi (1895) for Botrytis, Tausson (1925) described an Aspergillus niger isolate able to grow upon paraffin wax and Hopkins and Chibnall (1932) found an Aspergillus versicolor strain able to grow at the expense of C_{23} - C_{34} solid n-alkanes. Rynearson and Peterson (1965) isolated species of Aspergillus, Chaetomium, Penicillium, Syncephalastrum and Cunninghamella by a direct soil-baiting technique, using buried paraffin rods. Fergus (1966) isolated the following thermophilic fungi able to grow upon paraffin wax (melting point 48°C) at 55°C: Chaetomium thermophile, Mucor pusillus, and Stilbella thermophila. Fusarium moniliforme was reported by Navikova et al. (1969) to produce extracellular enzymes active upon solid paraffin though their data are somewhat equivocal.

Once again little taxonomic significance can be discerned in the ability of filamentous fungi to utilize n-alkanes as can be seen from Table 2-1. Nyns et al. (1968b) came to the same conclusion, noting only that the ability seemed more prevalent in the Mucorales and Moniliales, particularly in the genera Aspergillus and Penicillium in the latter order.

2.3. Hydrocarbon uptake:

The usual method of uptake of carbon substrates by bacteria and fungi is from solution. Soluble substrates can be taken up directly. Insoluble ones, such as the carbohydrate cellulose, may need to be converted to a soluble form by extracellular en-

Table 2-1. Mould genera reported to grow on n-alkanes.

Class	Order	Genus
Zygomycetes		
	Mucorales	
		Absidia
		Circinella
		Cunninghamella
		Helicostylum
		Mortierella
		Mucor
		Rhizopus
		Syncephalastrum
		Zygorhynchus
Ascomycetes		
	Eurotiales	
		Amorphotheca
	Hypocreales	
		Gibberella
	Sphaeriales	
		Chaetomium
Deuteromycetes		
	Melanconiales	
		Colletotrichum
	Moniliales	
		Acremonium
		Allescheria

Table 2-1 (cont'd.)

Class	Order	Genus
		Alternaria
		Aspergillus
		Botrytis
		Cephalosporium
		Chloridium
		Cladosporium
		Dematium
		Epicoccum
		Fusarium
		Gliocladium
		Graphium
		Helminthosporium
		Monilia
		Oidiodendron
		Paecilomyces
		Penicillium
		Periconia
		Phialophora
		Pullularia
		Scolecobasidium
		Sepedonium
		Spicaria
		Stilbella
		Trichoderma

zymes before uptake can take place. In the case of n-alkane substrates, most reports indicate that the hydrocarbon molecule enters the cell unchanged and is then metabolized. Cooney and Walker (1973) showed that cells of Cladosporium resinae, growing on C_{12} - C_{16} n-alkanes, accumulated the unchanged substrate within the cell.

An alternate view is the one previously cited, that of Navikova et al. (1969), reporting breakdown of paraffin wax by extracellular enzymes of Fusarium moniliforme; however these results are open to question.

How then are n-alkane molecules taken up into a cell? To answer this question one must consider the solubility of these compounds in water. The molecular weight of an alkane has a profound effect upon its solubility. The gases, methane, ethane, propane and n-butane are the most soluble members of the series; their solubilities are close to that of oxygen, which is generally agreed to be taken up from solution. Uptake of dissolved n-alkane gases can adequately account for microbial growth. Ribbons and Michalover (1970) provided indirect evidence of uptake of dissolved methane. Oxygen uptake by a washed cell suspension of Methylococcus capsulatus was demonstrated by polarography when a solution of methane in buffer was provided.

The low molecular weight liquid n-alkanes are relatively soluble in water but solubility decreases logarithmically with increasing carbon number over the range of C_5 - C_{10} (McAuliffe 1969). Uptake of hydrocarbon from solution

is adequate to explain growth on many of these substrates, in fact examples have been cited where sub-saturation levels support growth (section 2.2) and saturated solutions are inhibitory. From C_{11} on up the series, the theoretical linear relationship between the log of solubility and carbon number does not hold in practice. Starting with n-undecane the phenomenon of 'accommodation' is encountered. This has been described by McAuliffe (1969) and Yoshida and Yamane (1971). An aqueous solution can contain more hydrocarbon than a theoretical saturated solution without formation of an emulsion. A change occurs from true solution (molecular dispersion) to 'accommodation' of sub-micron aggregates of hydrocarbon molecules at C_{11} and continues (McAuliffe 1969) at least up to C_{28}, allowing a given volume of water to carry a hydrocarbon load far in excess of what would be possible by true solution.

Yoshida et al. (1971, 1973) and Yoshida and Yamane (1971), working with Candida tropicalis growing on n-hexadecane, produced evidence that hydrocarbon is taken up by the cells in the form of these sub-micron (0.1 - 0.8 μm diameter) aggregates and that exponential growth depends upon their formation. The prevailing view in the literature however is that the higher molecular weight liquid n-alkanes are taken up by direct contact between cells and hydrocarbon in emulsions.

It was suggested by Johnson (1964) that uptake involved direct contact and that hydrocarbons became incorporated

into the phospholipid micelle of the cell membrane. Ludvik et al. (1968), from an electron microscope study, claimed that hydrocarbons penetrated the cell wall of Candida lipolytica and came to lie between it and the cell membrane, which was more convoluted and thus had a greater surface area in hydrocarbon-grown than in glucose-grown cells. The authors debated whether hydrocarbon was entering the cell unchanged by pinocytosis or was being oxidized by enzymes upon the membrane surface. Later work by the same group (Munk et al., 1969) indicated that such uptake was proportional to cell lipid and non-specific. A mixture of utilizable n-alkanes and other non-utilizable hydrocarbons entered and left the cell rapidly. Bos and de Boer (1967), from electronmicrographs, pointed out a dark outer layer to the cell wall of Candida cloacae grown on n-hexadecane, which was absent in glucose-grown cells even when shaken with hydrocarbon. The authors suggested that this was a lipophilic layer which facilitated cell contact with oil droplets.

Several workers have demonstrated the importance of the oil-water interface in facilitating oil-cell direct contact during microbial growth in emulsions. Bakhuis and Bos (1969), working with Candida lipolytica, grown on jet fuel, showed that growth rates were highest when oil droplets were smaller or larger than the yeast cells themselves. Where the droplets were approximately the same size as the cells, growth was very slow. This was explained in terms of greater oil-

cell contact with droplets appreciably larger or smaller than the cells themselves. Cells clustered on the surface of the larger oil droplets, while the smaller droplets clustered on the surface of the cells.

Erdtsieck and Reitsma (1969) also showed the importance of the oil-water interface for Candida lipolytica growing on C_{13+} n-alkanes. Coty et al. (1971) showed that a yeast, Pichia, and a bacterium, Brevibacterium, grew well in 'water-in-oil' emulsions as well as in the more usual 'oil-in-water' emulsions. McLee and Davies (1972) demonstrated that cells of a Torulopsis species clung to the surface of oil droplets in shake flasks. Growth was linear yet no growth took place in a fermenter, where, according to the authors, high shear forces knocked cells off the oil droplets and prevented growth.

Wang and Ochoa (1972) provided the most convincing evidence for the significance of the oil-water interface. Working with Candida intermedia growing on n-hexadecane in a small fermenter, they were able to accurately measure the oil-water interfacial area and vary it by changing the impellor speed and thus the droplet size. It was found that growth was exponential up to a point then changed to linear. The break point was always found to be at a particular value of interfacial area per unit volume, regardless of impellor speed or of cell or hydrocarbon concentration. Growth was limited not by absolute oil concentration but by the area of available interface.

Hisatsuka et al. (1972) demonstrated that Pseudomonas aeruginosa, growing on hydrocarbon, produced an emulsifier, probably a rhamnolipid, which increased the interfacial area. The same authors later produced evidence for a 'protein-like activator' produced by this culture, which stimulated alkane oxidation. They suggested it might be a carrier for facilitating entry of alkanes into the cell.

Yamada and Yogo (1970), working with Corynebacterium hydrocarboclastus and Candida tropicalis, and Amin et al. (1973), working with Pseudomonas, Azotomonas and Serratia species, found that submerged growth upon emulsified paraffin wax was linear not exponential. Chakravarty et al. (1972), in an extension of the latter work, constructed a mathematical model which fitted the observed kinetics of growth of a Pseudomonas sp. on pure n-eicosane. Microscopic examination revealed that cells did not adhere to particles. The authors concluded that uptake was from solution, not from direct contact. The production of a metabolite by the cells themselves was claimed to aid solubilization and uptake from solution.

It seems likely that hydrocarbons can be taken up from solution and by direct contact in different situations although little is known of the mechanisms involved in either instance. It is debatable whether there is any real difference between uptake of hydrocarbon by direct contact with oil in emulsions and uptake from accommodated molecular aggregates. If one considers the size range of 0.1 to 0.8 μm diameter proposed by Yoshida and Yamane (1971) for such

aggregates, they are quite large when compared with yeast or bacterial cells, which are both usually in the 0.5 - 10 μm size range. A spherical molecular aggregate of 0.1 μm diameter would have approximately 9.16×10^5 molecules of n-hexadecane (calculated from molecular volume and Avogadro's number) and one of 0.8 μm diameter, 4.69×10^8 molecules. Such a situation is quite distinct from that in a true solution where molecules exist singly in a state of true molecular dispersion. McAuliffe (1969) demonstrated that the wax n-eicosane exists as an accommodation in water as evidenced by the reduction in total hydrocarbon of a 'solution' achieved by passage through a 0.45 μm membrane filter. This being so, it seems possible that uptake of solid n-alkanes takes place by a form of direct contact rather than from solution as postulated by Chakravarty et al. (1972).

2.4. Biochemical pathways of n-alkane oxidation:

This section is concerned principally with the initial pathway followed by the n-alkane carbon skeleton before it enters the mainstream of conventional intermediary metabolism. Details of enzyme systems will be dealt with later.

The vast majority of n-alkanes have in common certain characteristics of chemical structure which are reflected in the often identical or similar pathways found for many individual compounds in different organisms. They consist of linear chains of methylene groups, terminated at either end by a methyl group; hence all the carbon to carbon bonds are

either methylene to methylene or methylene to methyl. The lower members of the series C_1 - C_3 are atypical and unique in structure and their pathways are best considered separately from the rest. Methane has no carbon to carbon bond and can, for convenience, be thought of as a methyl group attached to a hydrogen atom. Ethane has no methyl to methylene or methylene to methylene bonds, only methyl to methyl. Propane has methyl to methylene but no methylene to methylene bonds. None of these three alkanes has any isomers therefore the prefix 'n' is unnecessary.

For convenience the pathways of C_{5+} n-alkanes will be considered together. n-Butane will be considered with ethane and propane since all three are gases. Methane metabolism has more in common with autotrophic metabolism than it does with that of other alkanes and will be treated separately.

Since n-alkanes are amongst the most reduced of all carbon compounds, assimilation of such compounds into the oxygenated compounds which make up living cells is accomplished by oxidative pathways. Evidence presented in the literature for a particular n-alkane assimilative pathway usually falls into one of two categories:

a) Isolation of metabolic intermediates when the n-alkane substrate is supplied to either growing-cells or 'resting-cells' (cells suspended in a medium incapable of supporting growth) which have previously been grown upon that n-alkane.

b) Ability of such resting cells or in vitro systems derived from them to oxidize hypothetical intermediates without any lag period. It is usually assumed that such cells would have the necessary enzymes for the whole pathway. Growth of n-alkane-utilizing microbes is often possible on intermediates of n-alkane oxidation. However lack of growth upon a particular compound does not necessarily mean that it is not an intermediate. The situation can be complicated by such factors as toxicity at concentrations required for growth (e.g. low molecular weight aldehydes), low water solubility and problems of uptake. None of these factors are encountered when such a compound exists only as a fleeting intermediate in the cell.

Rapid oxidation of compounds which are not intermediates of n-alkanes assimilation can take place, usually resulting in accumulation of products which cannot be metabolized further. Such oxidation can often be attributed to enzymes principally active upon intermediates of the growth substrates but also able to metabolize compounds of similar structure. Foster (1962b) found that an obligate methane utilizer, Pseudomonas methanica, would oxidize ethane, propane and n-butane to accumulated products. He coined the term 'cooxidation' for such oxidation in the presence of a growth substrate. This phenomenon of 'cometabolism' has since been demonstrated in a wide variety of n-alkane utilizers. Normally the initial attack upon the 'cosubstrate' is of the same nature as that upon the n-alkane growth substrate.

Raymond et al. (1971) reviewed this subject in some detail.

Quite frequently conflicting evidence appears in the literature indicating different pathways in the same organism for an n-alkane growth substrate. Identification of the major pathway(s) of carbon assimilation is often difficult, for example, in Pseudomonas aeruginosa as will be discussed in detail later.

2.4.1. C_{5+} n-alkanes:

Of primary interest in studies of alkane metabolism is the nature of the first biochemical reaction with the hydrocarbon. Is the initial attack upon a terminal methyl carbon or upon one of the sub-terminal methylene carbons? What is the first stable product of this reaction? The various pathways proposed for initial n-alkane oxidation are outlined in Figure 2-1.

The pathway which has been most often demonstrated is that following steps 10, 4 and 5. The first stable product of n-alkane oxidation is the corresponding primary alcohol, produced by the incorporation of an atom of molecular oxygen into the substrate. This is further oxidized by appropriate dehydrogenases to the corresponding aldehyde and fatty acid in turn. The initial attack here is of course upon the terminal methyl group.

Many examples of this pathway have been found in bacteria. Buswell and Jurtshuk (1969) showed that a Corynebacterium sp. oxidized n-octane by the terminal route via

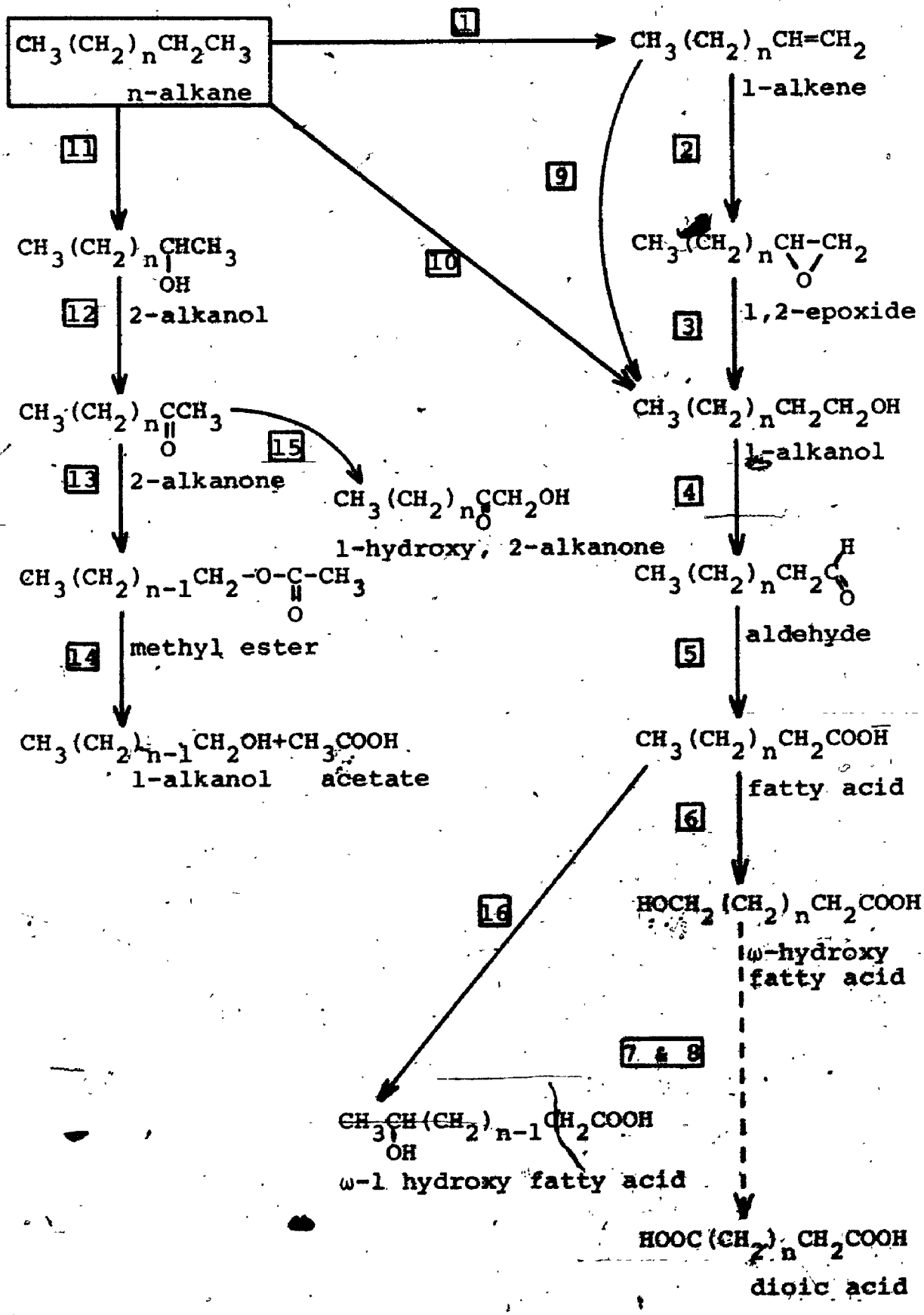


Figure 2-1. Summary of initial pathways of n-alkane oxidation.

1-octanol, octanal and octanoate as did Baptist et al. (1963) for a pseudomonad. Treccani (1962) demonstrated production of hexanoate and heptanoate from n-hexane and n-heptane respectively by Pseudomonas aeruginosa and species of Achromobacter, Nocardia, and Mycobacterium. Production of the corresponding primary alcohol and fatty acid from n-hexadecane by a thermophilic Bacillus sp. was reported by Hart et al. (1968). Suzuki and Ogawa (1972) showed the accumulation of corresponding primary alcohols in cultures of Arthrobacter paraffineus grown on n-alkanes.

In several cases it has been shown that fatty acids produced from initial alkane oxidation are further metabolized by β-oxidation to yield progressively shorter chains and acetate (acetyl CoA) units. Fredricks (1967) demonstrated that Mycobacterium rhodochrous growing upon n-decane produced 1-decanol, decanal, decanoate, octanoate, hexanoate, butyrate and acetate. Hankin and Kolatukudy (1968) showed terminal oxidation of n-nonacosane followed by β-oxidation in Micrococcus cerificans.

It is not uncommon to find that carbon skeletons produced by terminal oxidation of n-alkane substrates are incorporated directly into cell lipid unchanged. Stewart et al. (1959) and Stewart and Kallio (1959) showed that Micrococcus cerificans, when grown upon n-hexadecane produced an ester, cetyl palmitate, with C_{16} alcohol and fatty acid moieties. Incorporation of molecular $^{18}O_2$ into this ester was also reported by Stewart's group. Finnerty et al. (1962),

Stevenson et al. (1962) working with Micrococcus cerificans and Davis (1964) with Nocardia salmonicolor showed a relationship between the fatty acid and/or alcohol moieties of cell esters (including triglycerides) and the alkane substrates. A similar relationship was found by Dunlap and Perry (1967) for a Mycobacterium sp.

This same pathway has been demonstrated many times in alkane-utilizing fungi. Lirova (1968) showed that n-hexadecane was oxidized via the corresponding primary alcohol, aldehyde and fatty acid by Candida tropicalis and C. rugosa as did Singh et al. (1972) for Endomycopsis lipolytica. Allen and Markovetz (1970) showed a terminal attack upon n-tetradecane by Cunninghamella blakesleeana as did Walker and Cooney (1973) for Cladosporium resinae oxidizing n-dodecane and n-hexadecane. Pelz and Rehm (1973) found that three members of the Mucorales, Cunninghamella echinulata, Absidia glauca and a Mucor sp. all oxidized n-dodecane, n-tridecane and n-tetradecane via a terminal attack.

Quayle (1967) suggested that β-oxidation is a likely general fate of fatty acids produced by terminal alkane oxidation and that the acetate (acetyl CoA) fragments so produced are further metabolized via the glyoxylate pathway. Trust and Millis (1970) produced support for this hypothesis when they showed increased levels of isocitrate lyase, a key enzyme in this pathway, in two pseudomonads, a Mycobacterium and a Torulopsis species grown on n-alkanes.

In fatty acids with an even number of carbon atoms,

the whole chain can be metabolized via β-oxidation into two-carbon fragments. When an odd-numbered chain is encountered however a three-carbon fragment of propionate (propionyl Co A) remains after β-oxidation. The most likely fate of propionate is the methyl malonate pathway. Thijsse and Van der Linden (1963) showed the presence of an adaptive enzyme system for propionate in Pseudomonas aeruginosa; n-heptane-grown cells could metabolize propionate; n-hexane-grown cells could not. The authors suggested that n-heptane was oxidized to heptanoate which passed twice through the β-oxidative spiral to yield propionate which then passed to the methyl malonate pathway. Hexanoate was completely degraded to acetate by three cycles of β-oxidation so no pathway for a three carbon fragment was needed. Vestal and Perry (1969) however found methyl malonyl Co A mutase, a key enzyme in the methyl malonate pathway, to be constitutive in a Brevibacterium sp., whatever the growth substrate.

A variant on the terminal oxidative pathway is diterminal oxidation. After oxidation of the alkane to the corresponding fatty acid a second ω-hydroxylation of the terminal methyl group takes place, followed by further oxidation yielding a dioic acid by steps 6, 7 and 8 in Figure 2-1. Kester and Foster (1963), showed diterminal oxidation of C_{10} - C_{14} n-alkanes but not of any alkanes below C_{10} in a Corynebacterium sp. They suggested it was only a minor pathway. Diterminal oxidation of n-octane was shown in a Pseudomonas sp. by Ali Khan et al. (1963) and in Pseudomonas oleovorans

by Coon's group (Baptist et al. 1963; Gholson et al. 1963) in the latter case the same enzyme was shown to be responsible for the hydroxylation of n-octane and the ω-hydroxylation of octanoic acid.

Yeasts too show diterminal oxidation. Ogina et al. (1965) showed production of C_{11}, C_9, C_7 and C_5 dioic acids from n-undecane by a Pichia species, which indicates diterminal oxidation followed by β-oxidation. Yamada and Torigoe (1966) reported production of C_9 and C_{10} mono and dioic acids from a mixture of C_9 - C_{18} n-alkanes. Uchio and Shiio (1972) showed that Candida cloacae, growing on acetate, could cooxidize n-hexadecane to produce a dioic acid. Torulopsis magnoliae was reported by Tulloch et al. (1962) to produce ω and ω-1 hydroxy acids from C_{16} - C_{24} n-alkanes.

Initial attack can also take place at a sub-terminal methylene carbon atom rather than at the terminal methyl carbon as in step 11 (Fig. 2-1) producing a secondary alcohol. Further oxidation of the functional group yields a carbonyl group as before; this time however, a ketone results in step 12 instead of an aldehyde.

Lukins and Foster (1963) showed production of 2-pentanone and 2-hexanone from n-pentane and n-hexane respectively by Mycobacterium smegmatis and 2-undecanone from n-undecane by M. rhodochrous. This indicates initial attack at the α or penultimate carbon atom. Fredricks (1967) demonstrated initial attack at the 2, 3, 4, and 5 carbon atoms of n-decane by Pseudomonas aeruginosa to produce 2, 3, 4 and 5-

decanols and decanones; 2-decanone predominated. Markovetz' group (Forney et al. 1967; Forney and Markovetz 1968, 1969, 1970) showed, with whole cells and cell-free extracts of Pseudomonas aeruginosa, that 2-tridecanone, produced by sub-terminal attack upon n-tridecane, could be further metabolized to 1-undecanol and acetate (steps 13 and 14, Fig. 2-1). Klug and Markovetz (1967) found that besides the major mono-terminal pathway in Candida lipolytica, sub-terminal oxidation could also occur; secondary alcohols corresponding to the C_{14} - C_{18} n-alkane substrates were found. Again in a minor way, sub-terminal attack was found in Torulopsis gropengiesseri on C_{12} - C_{24} alkanes by Jones and Howe (1968). The work of Allen and Markovetz (1970) indicates that in a Penicillium sp., grown on n-tetradecane, oxidation is principally sub-terminal; 2-tetradecanol, 1-dodecanol, 2-tetradecanone and 4-tetradecanone were identified from such cultures. Pelz and Rehm (1973) found production of secondary alcohols and ketones when C_{12}, C_{13} and C_{14} n-alkanes were supplied to Aspergillus flavus, A. ochraceus, A. niger, Penicillium javanicum, a Penicillium sp., a Verticillium sp. and a non-ascosporogenous yeast.

Proposals have appeared in the literature for schemes involving initial dehydrogenation of n-alkanes to 1-alkenes from which alcohols are derived by subsequent steps (steps 1, 2, 3 and 9, Fig. 2-1). Such reports have been the subject of some controversy and therefore will be covered in section 2.5 in a discussion of the mechanism of the initial "fixation"

reaction of the alkane molecule.

2.4.2. C_2 - C_4 n-alkanes:

With ethane, no possibility other than a terminal attack exists; both carbon atoms of the molecule are terminal. Propane or n-butane however offer both terminal and sub-terminal sites of attack.

Davis et al. (1956), working with Mycobacterium paraffinicum, implicated ethylene, ethanol, acetaldehyde and acetate as possible intermediates of ethane. Leadbetter and Foster (1959, 1960) showed that Pseudomonas methanica, an obligate methane user, would cooxidize other gaseous alkanes, to produce ethanol, acetaldehyde and acetate from ethane; 1-propanol, propionaldehyde propionate and acetone from propane; and 1-butanol, butyraldehyde, butyrate and 2-butanone from n-butane. By their use of deuterated ethane (C_2D_6) they indicated that primary alcohols were not produced via hydration of a 1-alkene intermediate (see section 2.5).

It is difficult to say which is the major pathway followed by bacteria which utilize propane for growth. Lukins and Foster (1963) found evidence for both terminal and sub-terminal oxidation of propane in mycobacteria. Sub-terminal oxidation seemed to involve acetone and acetol (1-hydroxyacetone) and involved incorporation of molecular oxygen after acetol. Propylene was oxidized by propane-grown cells but would not support growth. Both acetone and 1-propanol supported growth. Klausmeier et al. (1958)

worked with a Mycobacterium sp. grown on propane which would oxidize propane, propylene, 1-propanol, 2-propanol but not propionate. The authors suggested that propylene is the first stable product which is hydrated to 1-propanol which then undergoes isomerisation to 2-propanol which is further degraded via acetone.

Pabst and Brown (1967) on the other hand, also working with a Mycobacterium sp., found that their culture would grow on propane, 2-propanol and 1-propanol but that acetone was only cooxidized. They suggested that possibly the initial attack is sub-terminal and the secondary alcohol so produced is then converted to 1-propanol by an isomerase. Perry's work with a Brevibacterium (1968) indicates sub-terminal oxidation. Cells grown on 2-propanol or acetone oxidized n-alkanes but those grown on 1-propanol or 1-butanol would not. 2-Propanol-grown cells also oxidized acetol without lag. Later work by Vestal and Perry (1969) with [2-^{14}C] propane indicated oxidation via 2-propanol, acetone and acetol to produce acetate eventually after CO_2 evolution.

2.4.3. Methane:

The literature on methane-oxidizing bacteria up to 1970 was extensively covered by Ribbons et al. (1970) in a review encompassing all heterotrophic metabolism of one-carbon substrates. The dissimilative pathway of methane oxidation was elucidated in the early work on Pseudomonas methanica and Methanomonas methanooxydans:

$$CH_4 \longrightarrow CH_3OH \longrightarrow HCHO \longrightarrow HCOOH \longrightarrow CO_2$$

The first step, most workers agree, is essentially the same as the monooxygenase systems found in many other alkane-oxidizing microbes (Leadbetter and Foster 1959; Ribbons and Michalover 1970; Higgins and Quayle 1970). Whittenbury however (1971) suggested that this scheme cannot fit the facts. Citing known data for the stoichiometry of methane oxidation and cell synthesis:

$$CH_4 + O_2 \longrightarrow \begin{cases} \text{cell constituents} \\ CO_2 \end{cases}$$

and assuming that a monooxygenase does not give rise to energy conservation, it was pointed out that the theoretical amount of [H] and thus ATP produced is insufficient for the needs of the organism when it is remembered that 2 [H] are required (as $NADH + H^+$) per mole of CH_4 oxidized to methanol by a monooxygenase (see Sect. 2.5):

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

Whittenbury (1971), citing unpublished work, suggested an alternative pathway by which methanol could be derived from methane without a monooxygenase and without reduced co-enzyme:

$$2CH_4 + O_2 \longrightarrow \underset{\text{dimethyl ether}}{CH_3OCH_3} + H_2O \qquad \text{(i)}$$

$$CH_3OCH_3 + H_2O \longrightarrow CH_3OH + HCHO + 2[H] \qquad \text{(ii)}$$

Such a scheme requires no reducing equivalents for the initial attack upon methane and would give a higher net yield of [H]. In support of this scheme he cites Leadbetter and Foster's data (1959) showing that $^{18}O_2$ incorporation into methane-grown bacterial cells was only half that expected from the results of work with ethane and propane-grown cells. They further claimed that dimethyl ether was oxidized by methane-grown cells and used as a sole carbon and energy source by many methane-utilizers and that this compound was detected in cultures grown on methane but not in those grown on methanol. They suggested that ethane-utilizers do not employ this method of attack since ethane-grown cells would not oxidize or utilize dimethyl or diethyl ether.

It is difficult to evaluate such data second hand; yet it cannot of course be ignored. Conflicting data comes from the work of Ribbons and Michalover (1970) who showed that a cell-free-system of Methylococcus capsulatus took up O_2 and oxidized NADH in the stoichiometic proportions one would expect in a monooxygenase in the presence of methane. Moreover both oxygen uptake and NADH oxidation were proportional to the methane supplied. Profound differences in the metabolism of methane and other alkanes are apparent when one examines subsequent steps in the oxidation and assimilation of

this one-carbon compound.

Ribbons et al. (1970) pointed out the controversy that exists as to the nature of the enzymes catalysing the $CH_4 \longrightarrow CO_2$ conversion. For example, for the oxidation of methanol, different workers have proposed conventional NAD^+ - dependent dehydrogenases, $NAD(P)^+$ - independent dehydrogenases (possibly with a pteridine prosthetic group) and even an H_2O_2 - dependent alcohol peroxidase.

Ribbons et al. (1970) considered an NAD^+ - dependent methanol dehydrogenase unlikely because of the redox potential (E_0 = -0.182 volts at pH 7.0) of the $CH_3OH/HCHO$ half reaction. Such enzymes active upon formaldehyde and formate however are considered quite feasible, the redox potentials of the HCHO/ ($HCOO^- + H^+$) and ($HCOO^- + H^+$) /CO_2 half reactions are -0.45 and -0.46 volts respectively and have frequently been demonstrated.

A recent paper by Patel et al. (1972) describes an enzyme in Methylococcus capsulatus and Pseudomonas M27 which will oxidize methanol and formaldehyde to formate and contains a pteridine prosthetic group.

In the metabolism of C_{2+} n-alkanes, little problem was encountered in accounting for carbon assimilation by conventional metabolic pathways. Thanks largely to the work of Quayle and co-workers (Kemp and Quayle 1967; Lawrence et al. 1970; Lawrence and Quayle 1970) the incorporation of methane into cell constituents is also understood in some detail. In species of Methylomonas, Methylobacter and Methylococcus

one-carbon units from methane are assimilated at the formaldehyde level of oxidation through the allulose pathway, as outlined in Figure 2-2 which bears a remarkable resemblance to the Bassham-Calvin cycle of CO_2 fixation in higher plant photosynthesis. The first step involves condensation of formaldehyde with a pentose phosphate (ribose 5-phosphate) to form a hexose phosphate (allulose 6-phosphate) which undergoes epimerisation to yield fructose 6-phosphate which after further phosphorylation to fructose, 1,6-diphosphate is split into two triose phosphate units which are readily shuttled into the mainstream of metabolism. The acceptor, ribose-5-phosphate, is regenerated from fructose-6-phosphate and triose phosphate by a series of transaldolase and transketolase rearrangements very similar to those involved in the phosphogluconate pathway or Bassham-Calvin cycle. A net production of one molecule of triose phosphate per three molecules of formaldehyde is achieved.

Species of Methylosinus, Methylocystis and Methanomonas methanooxydans (Lawrence and Quayle (1970) favour including the latter species in the genus Methylosinus) were found to utilize the serine pathway for the assimilation of one carbon compounds. Heptinstall and Quayle (1970) first found this pathway in Pseudomonas AM1, a methanol oxidizer. The serine pathway is outlined in Figure 2-3. Bacteria using this pathway have high levels of hydroxypyruvate reductase rather than hexose phosphate synthetase, which is found in those with the allulose pathway. It is not yet clear

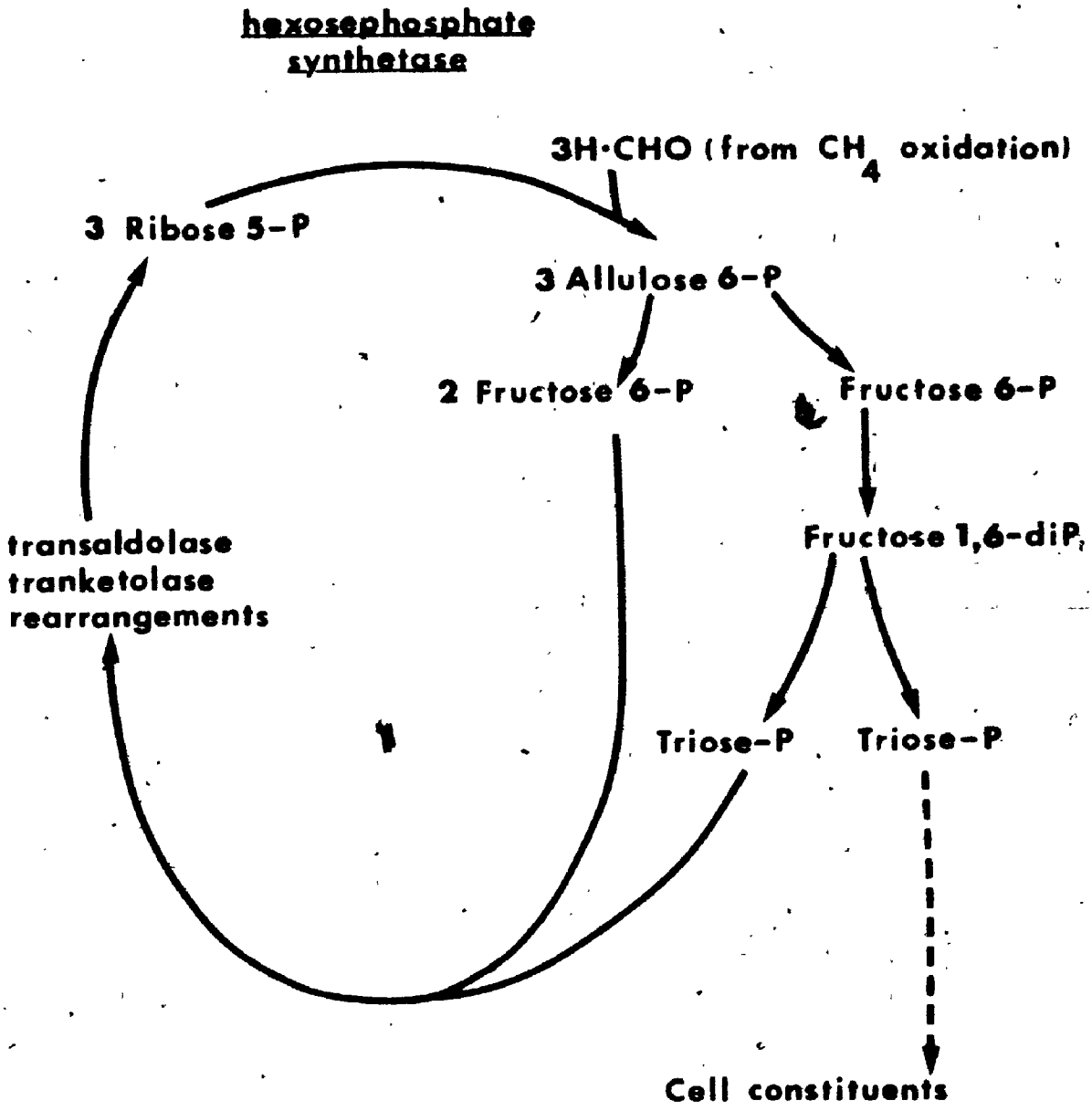
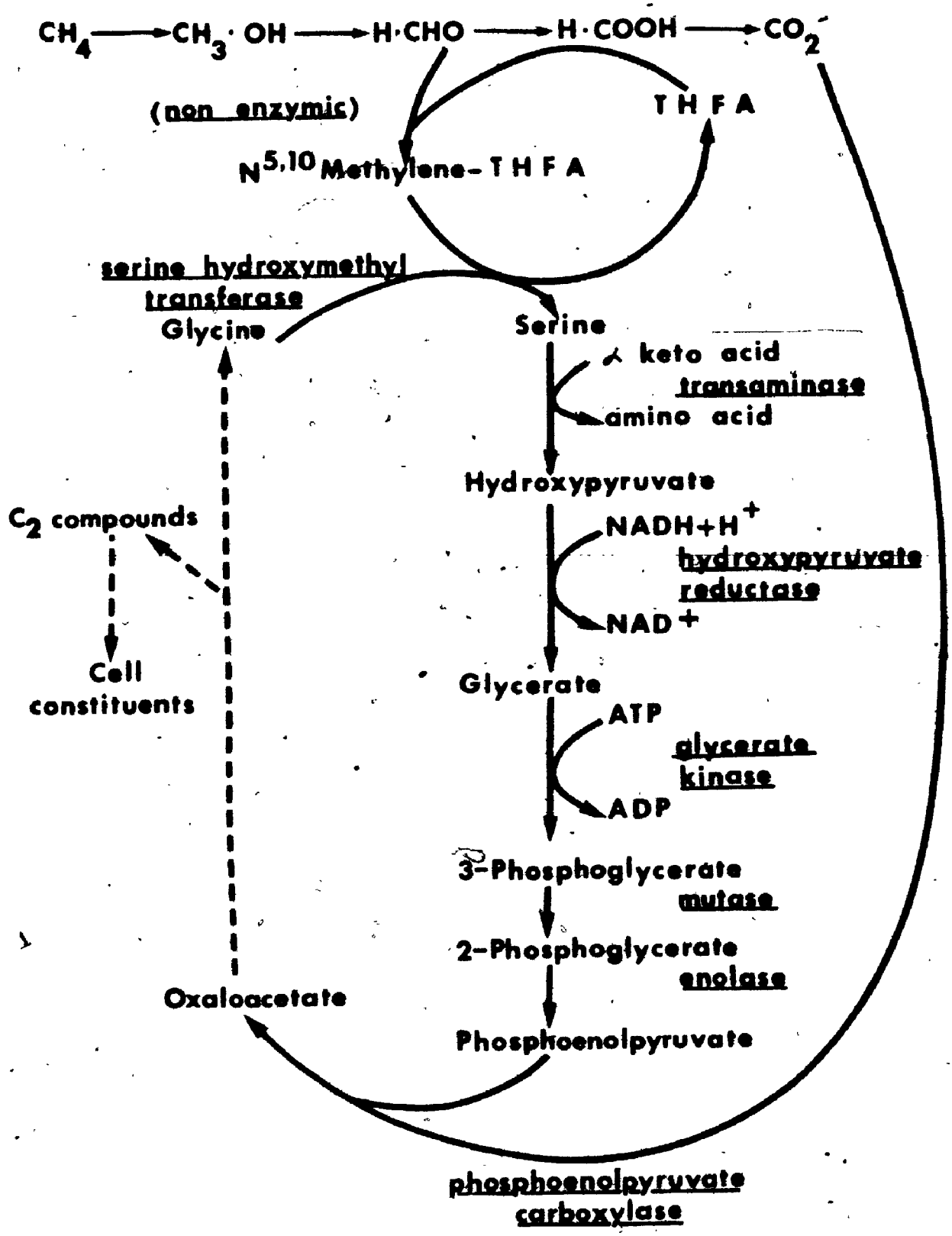


Figure 2-2. Assimilation of one-carbon units through the allulose pathway (after Ribbons et al. 1970).



THFA = tetrahydrofolic acid

Figure 2-3. Assimilation of one-carbon units through the serine pathway (after Ribbons et al. 1970).

exactly how glycine is regenerated from oxaloacetate.

Electron microscope studies of methane-oxidizing bacteria by Proctor and Norris (1969), Davies and Whittenbury (1970) and de Boer and Hazeu (1972) revealed two basic types of internal membrane structure amongst these bacteria. Species of Methylomonas, Methylococcus and Methylobacter, have type I membrane structure, that is, well organized stacks of vesicles. Species of Methylosinus and Methylocystis have type II membrane structure, that is membranes in pairs either at the periphery of the cell or running through it. Lawrence and Quayle (1970) found that bacteria with type I membrane systems use the allulose pathway and those with type II the serine pathway.

In their review Ribbons et al. (1970) raised doubts as to the validity of reports of microbes able to grow at the expense of methane or methanol facultatively. They made the point that obligate methyl users bear more resemblance to autotrophs than to other heterotrophic bacteria and possess in common with other autotrophs complex internal membrane systems and phosphatidyl choline in the cell phospholipid.

Bearing in mind that, like most autotrophs, obligate methyl users can assimilate many organic compounds (though not as sole carbon and energy sources) and that they synthesize cell constituents from either hexose-phosphates or three-carbon acids (serine), Ribbons et al. (1970) suggested that the basis of obligate methyl dependency is not a loss of enzymes of central metabolic pathways. They argue rather

that, like autotrophs, these bacteria are dependent upon a particular reaction for energy conservation, possibly formate oxidation. NADH oxidase systems (that is the whole electron transport chain from NADH oxidation with O_2 as ultimate electron acceptor) are absent or at very low levels in these bacteria hence normal levels of ATP production from oxidation of tricarboxylic acid cycle intermediates is not possible. In the absence of exogenous methane or methanol low levels of NADH oxidase must be expected to account for ATP production from endogenous reserves (poly-β-hydroxybutyrate has been found).

In the light of recent reports, it is no longer tenable that ability to use methane or methanol is restricted to obligate methyl users. Ogata et al. (1969, 1970a, 1970b) demonstrated high yields of a yeast of the genus Kloeckera grown on methanol; the same culture grows very well on carbohydrates. Similar reports have been made by Sahm and Wagner (1972) for an isolate of Candida boidinii and by Asthana et al. (1971) for Torulopsis glabrata. With this in mind one cannot lightly dismiss the earlier report of Zavarsina (1968) or the more recent one of Adamse et al. (1972) of fungi growing upon methane and other more conventional substrates.

It seems reasonable to postulate that there are two groups of organisms able to use methane and/or methanol as sole carbon and energy sources, obligate 'methyl' users, such as Methylomonas, dependent upon a particular energy

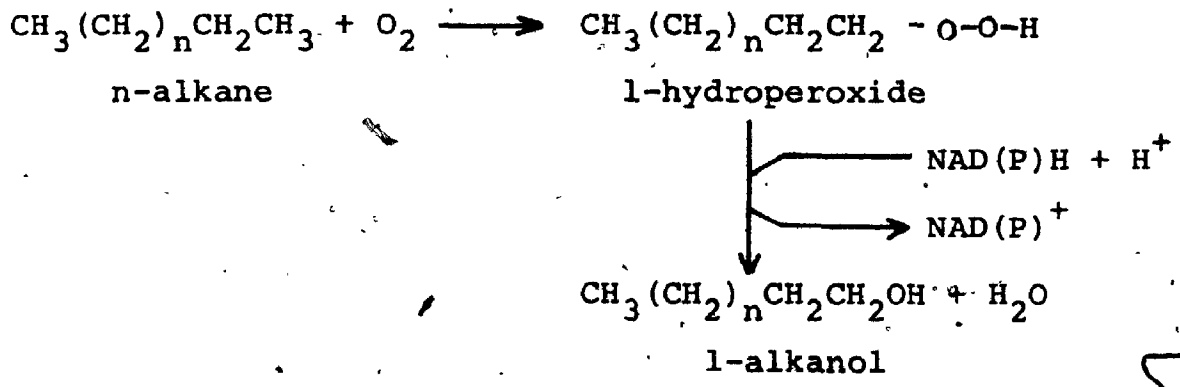
source, and facultative users, such as Kloeckera, with no such dependency. The existence of such facultative users in the fungi makes previous reports of facultative bacteria seem more reasonable.

2.5. Alkane fixation reactions:

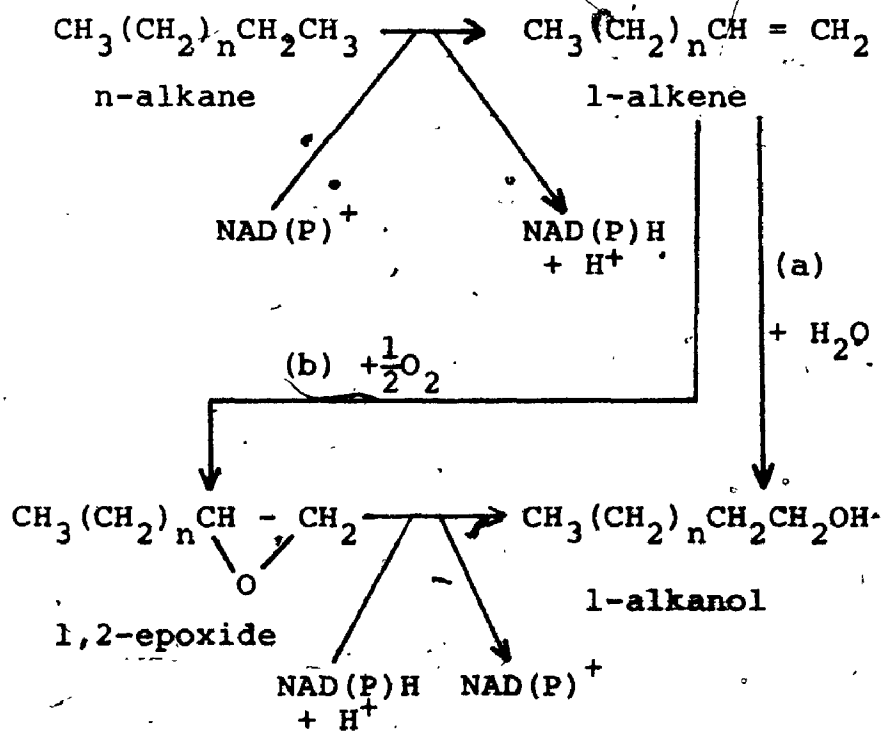
Interest in hydrocarbon metabolism has naturally centered upon the nature of the first biochemical reaction of the hydrocarbon substrate. There has been general agreement that one of the intermediates is an alcohol, primary after terminal attack or secondary after sub-terminal attack. Opinions differ however as to how such an alcohol is produced. Is it the product of the initial fixation reaction of the alkane molecule or of a subsequent reaction? Is the oxygen atom, introduced into the substrate molecule, derived from molecular oxygen, water or another source?

The principal schemes for derivation of primary alcohol intermediates from n-alkanes (omitting the dimethyl ether pathway suggested for methane) are as follows:

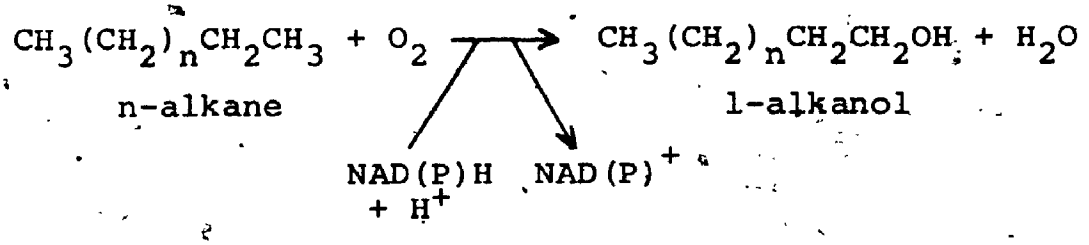
1. Hydroperoxide formation:



2. Dehydrogenation: (see also Fig. 2-1, steps 1, 2, 3 and 9)



3. Hydroxylation: (see also Fig. 2-1, step 10)



No biochemical mechanism was known for direct incorporation of molecular oxygen into a substrate before the discovery in 1955 of a new class of enzymes, the oxygenases, by Hayafshi et al. (1955) and Mason et al. (1955).

The involvement of molecular oxygen in initial hydrocarbon oxidation by bacteria was shown by the early work of Foster's group (Foster 1962a) and by Stewart et al. (1959) by means of deuterated substrates and $^{18}O_2$. Leadbetter and Foster (1960) proposed the initial formation of 1 or 2 - hydroperoxides by a free radical equilibrium between the terminal and sub-terminal carbon atoms. The 1 or 2 - hydroperoxides, once formed, would be reduced to primary and secondary alcohols respectively. McKenna and Kallio in their review (1965) cite much of the evidence of Kallio's group for hydroperoxide intermediates. Their conclusions are mainly based upon the ability of alkane-grown cells and enzyme preparations therefrom to metabolize 1-hydroperoxides. The biochemical formation of a 1-hydroperoxide from an n-alkane however has never been demonstrated.

The majority of microbes oxidizing hydrocarbons are

obligate aerobes. Reports have appeared however of anaerobic n-alkane degradation, for example, that of Rosenfeld (1947) and Davis and Yarborough (1966). Many claims have appeared of initial n-alkane oxidation steps without the participation of molecular oxygen. In such schemes the alkane undergoes dehydrogenation to the corresponding 1-alkene by an alkane dehydrogenase acting in conjunction with a pyridine nucleotide coenzyme as in scheme 2 (p. 45). From an energetic standpoint, dehydrogenation is extremely unlikely (Johnson 1964; McKenna and Kallio 1965). For example for the reaction

$$\text{n-heptane} + NAD^+ \rightleftharpoons \text{1-heptene} + NADH + H^+$$

Johnson (1964) calculated that $\Delta G'$ (pH 7.0) = +15.98 Kcal per mole and K eq. = 2×10^{-12}. It was concluded that direct n-heptane oxidation by NAD^+ was therefore impossible.

Van der Linden and Thijsse (1965) warned against uncritical acceptance of many early reports of anaerobic n-alkane oxidation. They pointed out that many such claims were based upon dye reduction in Thunberg tubes and quite probably oxygen was not completely excluded, nor were alkane substrates as free from oxygenated impurities as they might be. Azoulay et al. (1963) proposed NAD^+ - dependent dehydrogenation of n-heptane by Pseudomonas aeruginosa followed by epoxidation and subsequent reduction to a primary alcohol as in scheme 2(b) (p. 45). Wagner et al. (1967) claimed

dehydrogenation of hexadecane by a Nocardia sp. Iizuka et al. (1968, 1969) and Tida and Iizuka (1970, 1971) produced evidence to support initial dehydrogenation of n-decane by Candida rugosa and subsequent hydration to 1-decanol by a hydrase enzyme analogous to fumarase as in scheme 2(a) (p. 45).

The above reports cannot lightly be dismissed especially since in each of them the 1-alkene intermediate has been positively identified. In the case of the latter work formation of 1-decanol from 1-decene has also been shown. Evidence that the oxygen of 1-decanol comes from water was claimed from $^{18}O_2$ - mass spectometry data but detailed results were not presented. Both Van der Linden and Thijsse (1965) and Klug and Markovetz (1971) in their reviews presented evidence against 1-alkenes as intermediates in alkane oxidation. It was pointed out that 1-alkenes often produce different intermediates from their corresponding n-alkanes when metabolized. Often the saturated end of a 1-alkene is attacked preferentially. Bruyn (1954) noted that 1-alkenes, oxidized by Candida lipodytica, gave rise, not to the corresponding primary alcohol, but to a 1,2 - diol. The scheme of Azoulay and co-workers involved molecular oxygen uptake and is not in conflict with much of the earlier data using $^{18}O_2$. Azoulay's hypothesis is not supported however by data of Huybregtse and Van der Linden (1964) who found that 1,2-epoxides were produced from 1-alkenes but not from n-alkanes. May and Abbott (1972, 1973) found epoxidation of 1-alkenes by

a Pseudomonas enzyme system known to hydroxylate n-alkanes and fatty acids.

Most evidence points to scheme 3 (p. 46) as the principal mode of attack in microbial n-alkane-oxidizing-systems. The enzymes involved belong to that class known variously as monooxygenases, mixed function oxidases or hydroxylases. An atom of molecular oxygen is interposed between a carbon atom and a hydrogen producing a hydroxyl group; the remaining oxygen atom is reduced by the essential reduced pyridine nucleotide. The net result is a 1:1:1 stoichiometry of n-alkane, coenzyme and oxygen identical to that found in scheme 1 (p. 45) for the n-alkane to alcohol conversion.

Coon and co-workers (Gholson et al. 1963; Baptist et al. 1963; Peterson et al. 1966, 1967; Peterson and Coon 1968) worked intensively upon a soluble monooxygenase system, from Pseudomonas oleovorans, capable of terminal hydroxylation of n-alkanes, such as n-octane, or ω-hydroxylation of fatty acids, such as lauric acid. The system requires NADH and molecular oxygen and consists of three protein fractions: (a) a non-haem-iron-protein, rubredoxin, (b) NADH-rubredoxin reductase (believed to be a flavin enzyme) and (c) a hydroxylase. The reductase and rubredoxin were believed to be part of an electron transport chain from NADH to the hydroxylase. A similar system was found by Kusunose et al. (1967a, 1967b) in Pseudomonas denitrificans (originally referred to as Ps. desmolytica). Later work by Peterson et al. (1969) indicated that the hydroxylase moiety was in fact not soluble but

particulate. A similar three protein monooxygenase was described in Pseudomonas aeruginosa by Peterson et al. (1967) and more recently by Van Ravenswaay Claasen and Van der Linden (1971) in the same organism.

Much of the early work upon monooxygenases was upon systems bound to the microsomes of mammalian liver homogenates; these were active upon alkanes, fatty acids and certain drugs. In all mammalian systems it was found that there was a requirement for NADPH rather than NADH; such systems were very sensitive to carbon monoxide inhibition. The hydroxylase in mammalian systems was a haemoprotein, cytochrome P-450, so called because of a characteristic absorption peak at 450 nm for a carbon monoxide difference spectrum of the reduced cytochrome. The pseudomonad systems were not sensitive to carbon monoxide and did not involve cytochrome P-450.

Cardini and Jurtshuk (1970) however found that n-octane hydroxylation in a Corynebacterium species required NADH, molecular oxygen and two proteins one of which had the spectral properties of cytochrome P-450; the other was a flavoprotein. The system was also carbon monoxide sensitive. Ribbons and Michalover (1970) demonstrated a membrane bound system from Methyloccus capsulatus, which appeared to be an NADH-dependent monooxygenase, active upon methane and ethane.

In depth studies of the enzyme mechanisms of initial n-alkane oxidation are rare, so it cannot be predicted with any certainty whether cytochrome P-450 hydroxylase systems are uncommon amongst n-alkane-oxidizing bacteria or not.

There is probably a lot to be learned from an analogous system in Pseudomonas putida which oxidizes camphor. Gunsalus and co-workers have obtained highly purified preparations of a camphor monooxygenase system (Tsai et al. 1971) consisting of three proteins: (a) cytochrome P-450, (b) putidaredoxin (a non-haem-iron-protein) and (c) a flavoprotein, NADH-putidaredoxin reductase, and have characterized these components in some detail.

Early work on alkane oxidation by Candida tropicalis by Azoulay and co-workers indicated the activity of an NAD^+-dependent alkane dehydrogenase (Lebeault et al. 1969) active upon n-decane in the mitochondria. Later work by the same group (Gallo et al. 1971) demonstrated the activity of an NADPH-dependent hydroxylase system bound to the microsomes, consisting of cytochrome P-450 and NADPH-cytochrome P-450 reductase (normally in such studies NADPH-cytochrome c reductase activity upon mammalian cytochrome c is taken as evidence of the presence of NADPH-cytochrome P-450 reductase activity). Membrane-bound NAD^+-dependent alcohol and aldehyde dehydrogenases were also found in the microsomes.

Recently (Gallo et al. 1973a, 1973b), Azoulay and co-workers published a retraction of their earlier work, to the effect that there is no alkane dehydrogenase in C. tropicalis, only a microsomal cytochrome P-450 hydroxylase system. Earlier results apparently were due to oxygenated impurities in their n-decane which brought about reduction of NAD^+. A similar system from another C. tropicalis strain was inves-

tigated in some depth in Coon's laboratory (Lebeault et al. 1971; Duppel et al. 1973). This system, which hydroxylated n-hexadecane and brought about ω-hydroxylation of laurate was readily solubilized and was remarkably similar to a mammalian microsomal system also under investigation by the same group (Lu et al. 1969; Strobel et al. 1970; Strobel and Coon 1971). The system is NADPH-dependent and contains two proteins, cytochrome P-450 and NADPH- cytochrome P-450 reductase. There is also a requirement for a heat stable lipid normally found in the membrane to which the enzyme system is bound. In the case of the liver system phosphatidyl choline is the heat stable lipid; other phospholipids had varying effects. In the yeast system, lysophosphatidylethanolamine extracted from yeast cells, was most effective.

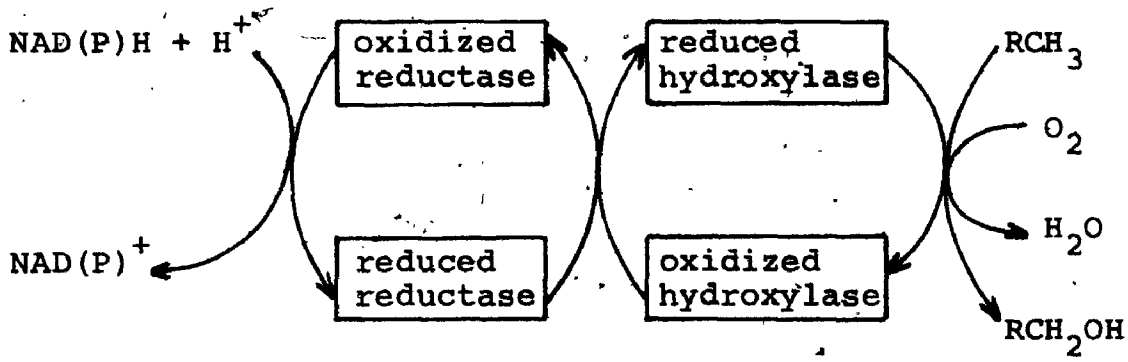
A similar hydroxylase system to that of C. tropicalis has been shown in a Torulopsis sp. by Heinz et al. (1970). Oleic acid was hydroxylated by a microsomal system requiring molecular oxygen and NADPH. It would seem from these data that fungal and mammalian hydroxylase systems contain cytochrome P-450, require NADPH rather than NADH and are microsomal.

One can however cite apparent exceptions to this generalization. Liu and Johnson (1971), working with an n-decane-hydroxylase system from a Candida intermedia homogenate, found activity in the 10,000 g sedimentable particulate fraction, not in the 105,000 g sedimentable (microsomal) fraction. Decanol and decanal dehydrogenase activity

(NAD^+-linked) were found in both particulate fractions. After determining that the mitochondria (normally sedimenting at 10,000 g) had no hydroxylase activity, the authors concluded that such activity resided in the 'cell membrane'. No attempt was made to investigate the effect of coenzymes on hydroxylase activity or the possibility of cytochrome P-450 involvement.

In a recent paper by Walker and Cooney (1973) it was suggested that either monooxygenase activity or intermediate 1-hydroperoxide formation were involved in n-hexadecane oxidation by Cladosporium resinae. The C_6, C_{12} and C_{16} alkanes and corresponding primary alcohols, aldehydes and fatty acids were oxidized by the 10,000 g supernatant of a crude homogenate. Oxygen uptake in the presence of the n-alkanes was found to be stimulated by NADH and NADPH but not NAD^+ or $NADP^+$. 1-Hexadecanol was established as an intermediate of n-hexadecane oxidation. The authors claim cytochrome P-450 is probably not involved in n-hexadecane oxidation since an inhibitor (piperonyl butoxide) specific for this cytochrome was ineffective. No mention was made for a test for carbon monoxide inhibition.

It was recognized quite early that monooxygenases active upon n-alkanes were not single enzymes but enzyme systems of two or more proteins interacting with each other and with the substrates, usually forming a short electron transport chain, often written as:



In three-protein systems a non-haem-iron-protein would be placed between the reductase and the hydroxylase. Such sequences however were very much conjectural; the actual temporal sequence of interactions was unknown.

Recent work, in particular from the laboratories of Coon and Gunsalus, has done a lot to indicate exactly how these components interact. Much of this work has been with mammalian and non-hydrocarbon cytochrome P-450 hydroxylase systems but is highly relevant to microbial alkane systems.

Gunsalus et al. (1972) have worked with an inducible system from Pseudomonas putida responsible for the following reaction:

$$\text{D-camphor} + \text{NADH} + H^+ + O_2 \longrightarrow \text{exo-5-OH-camphor} + NAD^+ + H_2O$$

The three protein components, flavoprotein reductase, putidaredoxin and cytochrome P-450 hydroxylase have all been obtained in pure form, a goal which has eluded workers with other hydroxylase systems. Working with these pure compon-

ents Gunsalus et al. have been able to trace a sequence of events for binding of cytochrome P-450 with its substrates, camphor and oxygen, principally by differences in absorption spectra of enzyme-substrate complexes.

For many years there has been speculation about the involvement of an active form of oxygen in oxygenase reactions. Such an active form has been shown to be formed by several diverse biological enzyme systems. This is the superoxide radical, an anion produced by addition of an extra electron to an oxygen molecule:

$$O_2 + e^- \longrightarrow O_2^{\cdot -}$$

Superoxide production has been demonstrated from milk-xanthine oxidase, certain flavoproteins and by non-haem-iron redox proteins similar to rubredoxin and putidaredoxin (Knowles et al. 1969; Bállou et al. 1969; Misra and Fridovich 1971). Another enzyme is known, superoxide dismutase, which acts upon superoxide in the following manner:

$$2O_2^{\cdot -} + 2H^+ \longrightarrow O_2 + H_2O_2$$

Strobel and Coon (1971) working with a solubilized rat liver microsomal hydroxylase system (active upon n-alkanes, fatty acids and certain drugs) consisting of cytochrome P-450, NADPH-cytochrome P-450 reductase and phosphatidyl choline, showed that superoxide dismutase could inhibit

hydroxylation of the drug benzphetamine. Furthermore a superoxide generating system (xanthine + xanthine oxidase) was shown to couple with cytochrome P-450 in the absence of NADPH and the reductase to hydroxylate benzphetamine; phosphatidyl choline was found to be essential for coupling. Superoxide dismutase was found to abolish coupling, adding further confirmation of the possible involvement of superoxide in hydroxylation. Caution is urged by the authors in that superoxide might not be involved directly but that a secondary product may in fact be the active species. The tentative scheme proposed by Strobel and Coon (1971) is outlined in Figure 2-4.

Oxidized cytochrome P-450 combines with the substrate (R.H), it is then reduced in the presence of phosphatidyl choline by the reductase before combining with molecular oxygen in a ternary complex. Intramolecular electron transfer then yields superoxide bound to oxidized cytochrome P-450. In the last step superoxide attacks the substrate while a second electron comes from the reductase or from a second molecule of superoxide. Steps (b), (c) and (d) can be eliminated by a supply of free O_2^- from xanthine oxidase and step (e) blocked by superoxide dismutase.

Gunsalus et al. (1972) proposed an almost identical sequence for their system except that electrons from NADH passed from the reductase to putidaredoxin before reduction of the enzyme-substrate complex. They proposed that the second electron is also donated directly by putidaredoxin.

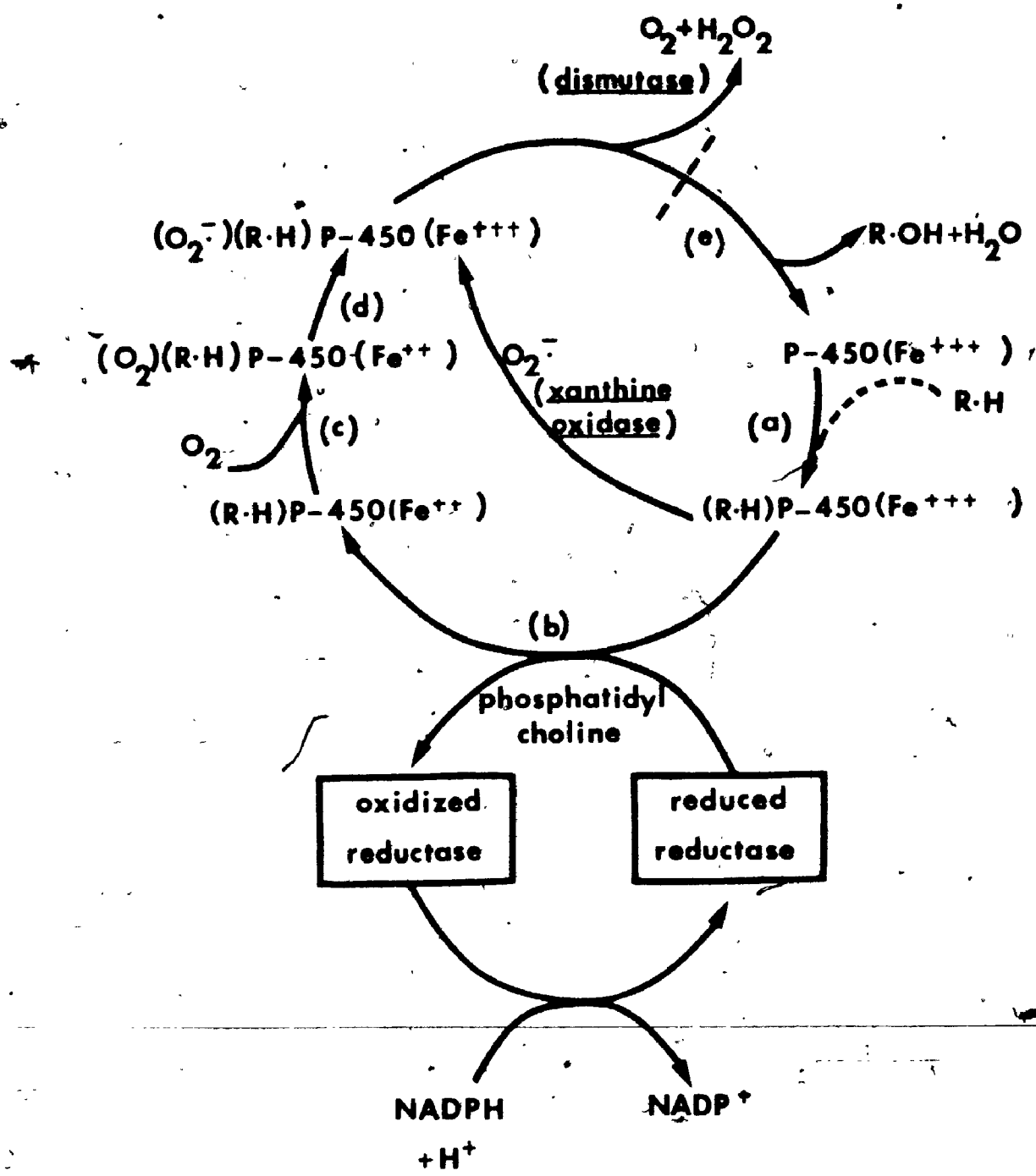


Figure 2-4. Proposed reaction mechanism for substrate hydroxylation by a mammalian liver microsomal system (after Strobel and Coon 1971).

It was reported by May and Abbot (1972, 1973) that the non-cytochrome P-450 hydroxylase system of Pseudomonas oleovorans would also catalyse 1-alkene epoxidation in the presence of NADH and O_2. Van der Linden and Thijsse (1965) discussed this phenomenon in their review, citing several instances where cells which could introduce a hydroxyl group at a particular position would also bring about epoxidation of a double bond at the same position. Functional degradation of 1-alkenes via 1,2-epoxides however was discounted. May and Abbot could suggest no functional significance for epoxidation in Ps. oleovorans.

In a later paper however May et al. (1973) found that the rubredoxin and reductase components of this system, in the presence of NADH and O_2, generated superoxide anions. Oxidation of epinephrine to adrenochrome was used as an assay. Furthermore it was found that in the complete hydroxylase system (including the hydroxylase itself), epinephrine oxidation and 1-alkene epoxidation are mutually competitive. Possible involvement of superoxide or a secondary product in epoxidation is therefore indicated.

Such data point to the possible involvement of superoxide in alkane hydroxylation by this system. However in an earlier paper Coon's group (Boyer et al. 1971) showed that rubredoxin and reductase, in the presence of NADH, reduced 1-octyl hydroperoxide to 1-octanol. The authors, noting that hydroperoxide intermediates from alkane oxidation have never been isolated, suggested that the hydroxylase catalyses

formation of bound hydroperoxide from n-octane and O_2 and that this is then stoichiometrically reduced by the NADH - reductase and rubredoxin components of the system.

$$\underset{\text{n-alkane}}{RH} + O_2 \xrightarrow{\text{hydroxylase}} \underset{\text{1-hydroperoxide}}{R\text{-}O\text{-}O\text{-}H} + NADH + H^+$$

$$\xrightarrow[\text{reductase}]{\text{rubredoxin}} \underset{\text{1-alkanol}}{ROH} + H_2O + NAD^+$$

It is difficult to evaluate these results in the light of recent evidence implicating reductase and rubredoxin in superoxide production. Quite possibly hydroperoxide reduction to 1-alkanol is of the same anomalous nature as 1,2-epoxidation of 1-alkenes. Hydroperoxide could conceivably be reduced by superoxide anions produced by the reductase-rubredoxin system while retaining the overall stoichiometry outlined above.

$$NADH + H^+ + 2O_2 \xrightarrow[\text{rubredoxin}]{\text{reductase}} NAD^+ + 2O_2^{\cdot -} + 2H^+$$

$$2O_2^{\cdot -} + 2H^+ + ROOH \longrightarrow ROH + 2O_2 + H_2O$$

This hypothesis could easily be tested by (a) looking for hydroperoxide reduction in the absence of oxygen and (b) testing for any possible effect of superoxide dismutase

upon hydroperoxide reduction in the presence of O_2.

CHAPTER 3

ISOLATION AND IDENTIFICATION OF FUNGI FROM CONTINUOUS ENRICHMENT SYSTEMS

3.1. Introduction:

The primary aim of this work was a physiological study of the assimilation of gaseous hydrocarbons when supplied as sole carbon and energy sources by fungi. Since the strain of Graphium which had been isolated by Zajic et al. (1969) by enrichment culture on natural gas grew rather slowly and produced a heterogeneous mass of mycelial pellets in submerged culture it was not ideal for the intended physiological studies. Therefore a search was made for additional isolates capable of assimilating gaseous hydrocarbons, one or more of which might prove to be a more suitable metabolic model than the original Graphium isolate. It was hoped to find a culture which would produce rapid homogeneous vegetative growth in submerged culture on natural gas.

3.2. Materials and methods:

3.2.1. Natural gas enrichment:

The method used was very similar in principle to that

used by Zajic et al. (1969) to isolate their Graphium species. Five separate continuous enrichment systems were operated simultaneously; each inoculated with different source material. Each system consisted of a closed 4-litre Erlenmeyer flask containing 1500 ml of Coty's (Coty 1967) mineral salts medium which had the following composition per litre of medium: $(NH_4)_2SO_4$-1.0g, K_2HPO_4-1.0g, $MgSO_4 \cdot 7H_2O$-0.5g, $CaCl_2$-0.1g, NaCl-0.1g, $FeSO_4 \cdot 7H_2O$-0.01g in tap water (reagent grade chemicals); the pH was adjusted with 2N HCl to the required value. A mixture of 50% natural gas and 50% air was bubbled through each system. Agitation of the medium was effected by means of magnetic stirrers which also aided in the dispersion of gas bubbles.

Natural gas for these systems was taken from the domestic line supplied by the Union Gas Company, Chatham, Ontario. A typical analysis of this gas supply (courtesy of Union Gas) revealed the following major components by volume: methane - 85.17%, ethane - 5.99%, propane - 2.54%, n-butane - 0.59%; the balance being made up of nitrogen, carbon dioxide, helium and traces of volatile liquid hydrocarbons.

Throughout the course of the enrichment procedure fresh medium was pumped in daily by means of a multi-channel peristaltic pump and the effluent was harvested continuously by positive gas pressure displacement at the same rate, thus effecting continuous dilution. Initially the retention time of each system was held at ten days and the pH of the feed medium adjusted to 6.5. Subsequently the dilution rate was

increased to give a retention time of 4.7 days and the feed medium pH was adjusted to 5.5.

Each system received an inoculum of 5% by volume raw sewage taken from one of five different points in an activated sludge sewage treatment plant. All five systems were run continuously for six weeks. In principle, the continuous dilution process should wash out all micro-organisms except those which can grow upon a medium of simple inorganic nutrients with gaseous hydrocarbons as sole carbon and energy sources. These organisms should be progressively 'enriched' in the effluent with time. Low initial pH of the feed medium and use of an ammonium sulphate nitrogen source served to keep the pH of the systems low (3.5 $\pm$ 0.3 after two weeks), thereby favouring fungi at the expense of bacteria. Present also in the effluents were secondary microorganisms which could not utilize hydrocarbons but subsisted upon metabolites of the hydrocarbon utilizers.

After two weeks, attempts were made to isolate individual fungi from the effluent of each system by the following methods. Samples of effluent were treated for 30 seconds in a Waring Blendor to break up hyphal clumps into smaller fragments. Each sample was washed three times by centrifugation from sterile Coty's medium (pH 5.5) for two minutes at 1000 g. Soluble organic nutrients and most bacteria are removed with the supernatant by this method. Dilutions of the resulting suspensions were made and 0.1 ml aliquots spread over the surface of petri plates of Coty's agar

(pH 5.5). Replicate plates of each dilution of the sample were incubated at room temperature (22-25°C) in desiccators, gassed manometrically as follows:

1. Methane - 40%, air - 60%.
2. Ethane - 40%, air - 60%.
3. Air (control).

The plates were examined regularly for growth. When significant growth of fungal colonies occurred, serial transfers were made to fresh medium until pure cultures were obtained. These were thereafter maintained on stock slants of Coty's agar incubated in an atmosphere of 40% ethane in air.

3.2.2. Pure methane enrichment:

As only ethane-utilizing but no methane-utilizing fungi were isolated from natural gas enrichments (see results section 3.3), a further attempt was made to find methane-utilizers using 30% methane in air instead of natural gas in the enrichment system. A closed system was set up using a small (15 litre) fermenter containing Coty's medium as before with continuous dilution. A mixture of 30% methane in air was bubbled through the system. A fixed volume of gas was recycled by a small diaphragm pump and replaced at regular intervals. Initially raw sewage was used as inoculum and the system was operated for six weeks. A second run employed, as inoculum, mud from a local bog where methane production by anaerobic bacteria is abundant and where the possibility of natural selection of methane-utilizers was

thus enhanced. Effluent dilutions from these systems were plated out as before and incubated in desiccators of 40% methane in air.

Except where otherwise stated gaseous alkanes used were C.P. grade from Matheson of Canada Ltd., Whitby, Ontario.

3.2.3. Description and identification:

For comparative morphological studies and identification, each isolate was inoculated onto replicate plates of standard media such as malt agar, corn meal agar and Czapek's agar. These plates were then incubated in air, in the dark at 25°C. Observations and measurements were made and photographs taken of unstained wet mounts and of preparations stained with polychrome blue, examined by bright field and phase contrast microscopy.

Each isolate for convenience was assigned a code number; isolate 3E/1 for example was isolated from enrichment system number three on a plate incubated in an ethane and air atmosphere. It can be seen from Table 3-1 that three groups can be distinguished on the basis of gross colony morphology. The Graphium isolates were indistinguishable from the Graphium isolates of Zajic et al. (1969). They were not described further since the taxonomic position of this ill-defined genus is now under review in this laboratory.

3.2.4. Growth upon gaseous alkanes:

Isolates 3E/1, 4E/3, 4E/5, 5E/3 and 5E/4 were inoculated

Table 3-1. Isolates from natural gas enrichment systems.

Code number	Gross colony morphology[a]	Identification[b]
1E/3	White, wooly	Graphium sp.
1E/4	"	"
1E/7	"	"
2E/1	Flat, yellow, powdery	Acremonium sp.
3E/1	"	"
4E/3	"	"
4E/4	"	"
4E/5	"	"
5E/2	Olive-grey, wooly	Phialophora jeanselmei
5E/3	"	"
5E/4	"	"

[a]On Coty's agar in an atmosphere of ethane and air.

[b]The question of the taxonomic position and nomenclature of these isolates is discussed in section 3.4.1.

to plates of Coty's agar, pH 5.0. Three replicate plates for each isolate were incubated in desiccators gassed with the following atmospheres for 14 days in the dark at room temperature (22-25°C):

1. Methane - 40%, air - 60%.
2. Ethane - 40%, air - 60%.
3. Propane - 40%, air - 60%.
4. n-Butane - 40%, air - 60%.
5. Air (control).

Plates were then scored for growth by mycelial proliferation relative to the air control.

3.2.5. Submerged culture:

3.2.5.1. Media:

Previous work on Graphium by Zajic et al. (1969) revealed very low growth rates and final fields for submerged culture on natural gas and Coty's medium. An attempt was made to find a more suitable medium and a formula was devized which had the following composition per litre of medium: $NaNO_3$-2.0g, K_2HPO_4-1.0g, $MgSO_4.7H_2O$-0.5g, KCl-0.1g, $CaCl_2$-0.1g, $FeSO_4.7H_2O$-0.01g, micronutrient stock solution-1ml (see below), in distilled water. Micronutrient stock solution: $Na_2B_4O_7.10H_2O$-150 mg, $(NH_4)_2\ MoO_4.4H_2O$-90 mg, $ZnSO_4.7H_2O$-1 g, $MnCl_2.4H_2O$-60 mg, $CuSO_4.5H_2O$-350 mg, acidified distilled water to 1ℓ. This medium will henceforth be referred to as N.M. (nitrate-micronutrients) medium. It was

a modification of Coty's medium (Coty 1967) and Fries' minimal medium (Cooke 1966).

3.2.5.2. Submerged culture in shake flasks on natural gas:

Five hundred-millilitre Erlenmeyer flasks each received 100 ml of liquid medium; they were then plugged with cotton plugs, covered with cheesecloth, secured with elastic bands (to protect flask necks from dust) and autoclaved for 15 minutes at 15 psi. Submerged cultures were normally initiated with loops of conidia from ethane-grown slants and thereafter inoculation was accomplished by pipetting 5 ml of a five-day-old shake flask culture. Inoculated flasks were placed in clamps in a large gas-tight 'plexi-glas' box, mounted on a gyratory shaker. A constant slow stream of 50% natural gas, 50% air was passed through this box during incubation. The shaker was operated at 200 rpm at room temperature (22-25°C).

For growth rate studies, three replicate flasks were harvested by filtration of cells onto pre-weighed 0.45 µm millipore membrane filters, which were washed with distilled water and dried overnight at 105°C before estimation of dry weights. The pH of the culture filtrate was also measured from one flask at each sampling time.

3.2.5.3. Submerged culture in a small fermenter on natural gas:

Shake flask studies were restricted to an environmental

shaker located in a laboratory supplied with high-pressure natural gas where precise temperature control was not possible. To compensate for this inadequacy, in several growth rate experiments a small scale fermenter was used in order to attain reproducible temperatures from one experiment to the next. A Chemap fermenter (Chemap A.G., 8708 Männedorf, Switzerland) was used in all these studies. The fermenter contained eight litres of medium which had been sterilized by its internal steam lines (temperature of medium raised to 121°C for 15 minutes, equivalent to 15 psi in an autoclave) and which had been cooled before inoculation.

A fermenter run was initiated by inoculation with the combined contents of four five-day-old shake flask cultures (100 ml each). The inoculum was added aseptically through a silicone rubber septum. The temperature of the medium was maintained at 25°C. A mixture of 1.0 l min^{-1} natural gas and 1.5 l min^{-1} air (measured by rotameters) was delivered to the internal sparger, after sterilization by passage through a sterile fibre-glass-wool filter. Adequate mixing of the medium and dispersion of gas bubbles was ensured by impeller blades rotating at 800 rpm. A serious problem in many runs was foaming caused by release of surfactant material from cells and by the vigorous agitation and sparging. When this happened, growth was prematurely terminated because cells were removed from the medium into the foam. This problem was overcome by the addition of a silicone-based antifoam, 'Hodag F-28', to the medium. Samples of

100 ml were removed aseptically at various time intervals by positive gas pressure and dry weight and pH were determined as before.

3.2.5.4. Attempt at submerged culture on liquid n-alkanes:

The ability of a representative isolate, 3E/1 to grow at the expense of higher molecular weight members of the n-alkane homologous series was tested. The following hydrocarbons were tested: n-pentane, n-hexane, n-heptane, n-octane, n-nonane, n-decane, n-undecane, n-dodecane, n-tridecane, n-tetradecane, n-pentadecane and n-hexadecane. One millilitre of one of the previously mentioned filter-sterilized liquid hydrocarbons was added aseptically to each plugged 500 ml - Erlenmeyer flask containing 100 ml of N.M. medium (pH 5.0). Three replicates were prepared in each case. Control flasks contained no hydrocarbon. Each flask was inoculated with 5 ml of a five-day-old shake flask culture grown on natural gas. The hydrocarbon flasks and controls were shaken at room temperature (22-25°C) in air. After seven days flasks were harvested and dry weights of mycelia taken as previously described. This experiment was carried out in two parts due to space limitations on the shaker; alkanes C_5 to C_{10} inclusive were tested first, then alkanes C_{11} to C_{16}. A hydrocarbon - free control was included in each run since different inocula were used.

3.3. Results:

3.3.1. Natural gas enrichment:

Microscopic examinations of effluents initially revealed mixed populations of fungi, bacteria and protozoa in all systems. As time progressed and the effluent pH dropped and stabilized at about 3.5, an increasing dominance of fungi was observed in these populations. Eleven fungal cultures able to grow at the expense of pure ethane as a sole carbon and energy source were isolated from these systems. No significant growth was ever observed on plates incubated in methane and air mixtures.

3.3.2. Methane enrichment:

No significant growth was observed in the methane enrichment system with either source of inoculum, nor was any found on the plates incubated in methane and air mixtures.

3.3.3. Isolates:

Of the three groups of isolates listed in Table 3-1, the flat yellow powdery and the olive-grey wooly cultures will be described in detail since they differed from previously described strains which grow on n-alkanes.

3.3.3.1. Yellow cultures:

The five yellow cultures are very similar morphologically. Two representative cultures are described in detail.

Colony after 14 days on 2% malt agar, 22 mm diameter, flat, lacking aerial vegetative mycelium, dull, yellow-brown above and upon reverse side; surface powdery with slight sheen, covered by abundant conidiophores. Vegetative hyphae septate, branched, 1.6 - 4.7 µm wide, average 2.8 µm, showing frequent anastomoses, hyphal walls usually hyaline; older hyphal segments contain vacuoles, lipid globules and an abundance of dense yellow pigmented material; pigment diffusing into agar giving colony yellow-brown colour. Sporulation abundant over entire colony surface, especially dense in centre. Conidiophores (Pl. 3-1 C, D) erect and solitary; either single sporogenous cells or branched, septate, 15.4 - 121.5 µm high, average 43.8 µm; branching usually in whorls, often irregular, one to four whorls per conidiophore usually one or two, no main axis; usually a single cell from parent hypha to first whorl of branches, 9.7 - 44.5 µm long, swelling distally at whorl up to 5.7 µm wide, average 3.7 µm, narrow basal 'waist' from 1.5 µm, average 2.2 µm. Conidiogenous cells phialides, solitary or terminal branches of conidiophores at any whorl, one to five per whorl, usually two or three, elongate, tapering distally, 11.3 - 38.1 µm long, average 21.2 µm, average 2.7 µm, at widest point 1.6 µm at tip, sometimes ending in small indistinct collarette (Pl. 3-2 A). Conidia unicellular, ellipsoidal, slightly apiculate, hyaline to pale yellow, 3.2 - 10.9 x 2.6 - 4.5 µm, average 6.9 x 3.3 µm (Pl. 3-1 B), produced in

Colony after 14 days on 2% malt agar, 22 mm diameter, flat, lacking aerial vegetative mycelium, dull, yellow-brown above and upon reverse side; surface powdery with slight sheen, covered by abundant conidiophores. Vegetative hyphae septate, branched, 1.6 - 4.7 µm wide, average 2.8 µm, showing frequent anastomoses, hyphal walls usually hyaline; older hyphal segments contain vacuoles, lipid globules and an abundance of dense yellow pigmented material; pigment diffusing into agar giving colony yellow-brown colour. Sporulation abundant over entire colony surface, especially dense in centre. Conidiophores (Pl. 3-1 C, D) erect and solitary; either single sporogenous cells or branched, septate, 15.4 - 121.5 µm high, average 43.8 µm; branching usually in whorls, often irregular, one to four whorls per conidiophore usually one or two, no main axis; usually a single cell from parent hypha to first whorl of branches, 9.7 - 44.5 µm long, swelling distally at whorl up to 5.7 µm wide, average 3.7 µm, narrow basal 'waist' from 1.5 µm, average 2.2 µm. Conidiogenous cells phialides, solitary or terminal branches of conidiophores at any whorl, one to five per whorl, usually two or three, elongate, tapering distally, 11.3 - 38.1 µm long, average 21.2 µm, average 2.7 µm, at widest point 1.6 µm at tip, sometimes ending in small indistinct collarette (Pl. 3-2 A). Conidia unicellular, ellipsoidal, slightly apiculate, hyaline to pale yellow, 3.2 - 10.9 x 2.6 - 4.5 µm, average 6.9 x 3.3 µm (Pl. 3-1 B), produced in

PLATE 3-1

A. 4E/3 conidiophores bearing spore balls on Coty's agar in ethane/air atmosphere, x 450. B. 4E/3 conidia from malt agar, stained with polychrome blue, x 2800. C. Typical branched conidiophore of 4E/3 bearing phialides with developing conidia on Coty's agar in ethane/air atmosphere, phase contrast, x 1600. D. 4E/3 conidiophore of greater complexity than C on malt agar, three degrees of branching, stained with polychrome blue, x 1500.


A
B
C
D

2 4

OF/DE

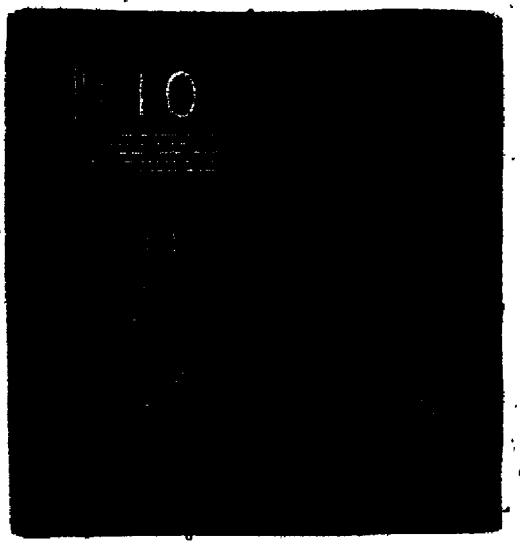


PLATE 3-2

A. Phialides of 4E/3 showing indistinct collarettes (c) on Coty's agar in ethane/air atmosphere, phase contrast, x 3000. B. Solitary unbranched phialide of 4E/3 on corn meal agar, phase contrast, x 1700. C. Conidia of 3E/1 from malt agar, stained with polychrome blue, x 2800. D. 3E/1 conidiophores arising from aerial hyphal ropes on malt agar, stained with polychrome blue, x 300.


A
B
C
D

basipetal succession from phialide tip, accumulating as gloeoid spore balls, up to 23.2 µm, diameter, average 16.8 µm, at phialide tips (Pl. 3-1 A), coalescing in older parts of colony.

Morphology upon Coty's agar in ethane and air atmosphere essentially the same as that upon malt agar, growth less dense. Upon Czapek's agar, growth denser, colony colourless at fourteen days, later becoming lemon yellow to olive, conidiophores abundant but of lesser complexity than upon malt, either single phialides or once branched conidiophores. Colonies upon corn meal agar colourless, sporulation sparse, branched conidiophores absent, solitary phialides only (Pl. 3-2 B).

Cultures 2E/1 and 4E/4 are indistinguishable in morphology from 4E/3; they do, however, show minor differences in pigmentation on certain media.

4E/5

Culture 4E/5 is similar to 4E/3 in gross colony morphology but displays greater complexity of branching in the conidiophores. Conidia are somewhat narrower and slightly reniform.

3E/1

Colony after 14 days on 2% malt agar similar to that of 4E/3, 22 mm diameter, flat, slightly more aerial mycelium than 4E/3, deep golden yellow above, dull brown on reverse side; surface cottony, powdery with slight sheen, covered by abundant conidiophores often arising from ropes of twisted

aerial hyphae (Pl. 3-2 D). Vegetative hyphae similar to 4E/3, but slightly narrower, 1.1 - 3.9 µm wide, average 2.3 µm. Sporulation abundant over entire colony surface, denser in centre. Conidiophores similar to 4E/3, solitary phialides or a whorled branching pattern, 25.0 - 129.6 µm high, average 62.3 µm; 'foot cell' 8.9 - 20.2 µm long, swelling at whorl, up to 5.7 µm wide, one to five phialides per whorl, usually two or three. Phialides elongate, tapering distally, 11.3 - 56.7 µm, average 28.4 µm, averaging 2.2 µm at widest point 1.6 µm at tip, sometimes ending in an indistinct collarette. Conidia unicellular, ellipsoidal, slightly apiculate, hyaline to pale yellow, smaller than 4E/3, 3.2 - 7.3 x 1.9 - 3.6 µm, average 4.8 x 2.5 µm (Pl. 3-2 C), again produced in basipetal succession accumulating in gloeoid spore balls at phialide tips, up to 28.4 µm diameter, average 19.5 µm.

As with 4E/3, growth on Coty's agar in an ethane and air mixture very similar to that upon malt but a little less dense. Upon Czapek's agar growth more dense; lemon yellow to khaki, pigmentation developed earlier than in 4E/3; sporulation abundant; aerial hyphal ropes bearing conidiophores more prominent and conidiophores less complex than those upon malt agar. Wider, flat, colourless, spreading colony produced upon corn meal agar; sporulation sparse; conidiophores of lesser complexity than upon malt or Czapek's agar but not solitary phialides, usually once branched, two or three phialides per whorl; aerial hyphal ropes absent.

3.3.3.2. Olive-grey cultures:

Cultures 5E/2, 5E/3, and 5E/4, all belong to the same species; the first two are morphologically indistinguishable, the third differs slightly in gross colony morphology.

5E/3

Colony after 14 days on 2% malt agar, 25 mm diameter, dense, compact, woolly, olive-grey above, olive-black on reverse side. Vegetative hyphae branched, septate, 1.1 - 3.1 µm wide, average 2.2 µm; cell wall pigmentation hyaline to light green in young and aerial hyphae, becoming darker green to olive-black in older and submerged hyphae. Sporulation abundant over entire colony surface. Conidia unicellular, ellipsoidal, slightly apiculate, 4.1 - 8.3 x 2.0 - 3.2 µm, average 5.1 x 2.3 µm, hyaline to light green (Pl. 3-3 B), produced in basipetal succession from a small peg (about 1.3 x 0.8 µm, not exceeding 2.5 µm long, too small to measure accurately) at the conidiogenous locus of the conidiogenous cell. Conidiogenous cells either intercalary, indistinguishable from vegetative cells, 8.1 - 30.0 x 1.6 - 2.8 µm, average 18.1 x 2.0 µm (Pl. 3-3 C) with sporogenous pegs usually near septum or lateral constituting a lateral branch or terminating a lateral branch, more or less elongate, expanding distally, clavate to globose, 4.1 - 17.8 µm long x 1.6 - 3.7 µm at widest point, 1.1 - 1.6 µm at base, average 9 x 2.8 x 1.4 µm; conidiogenous pegs usually slightly displaced at the apex (Pl. 3-3 D); rarely two conidiogenous pegs on one conidiogenous cell; occasionally lateral conidio-

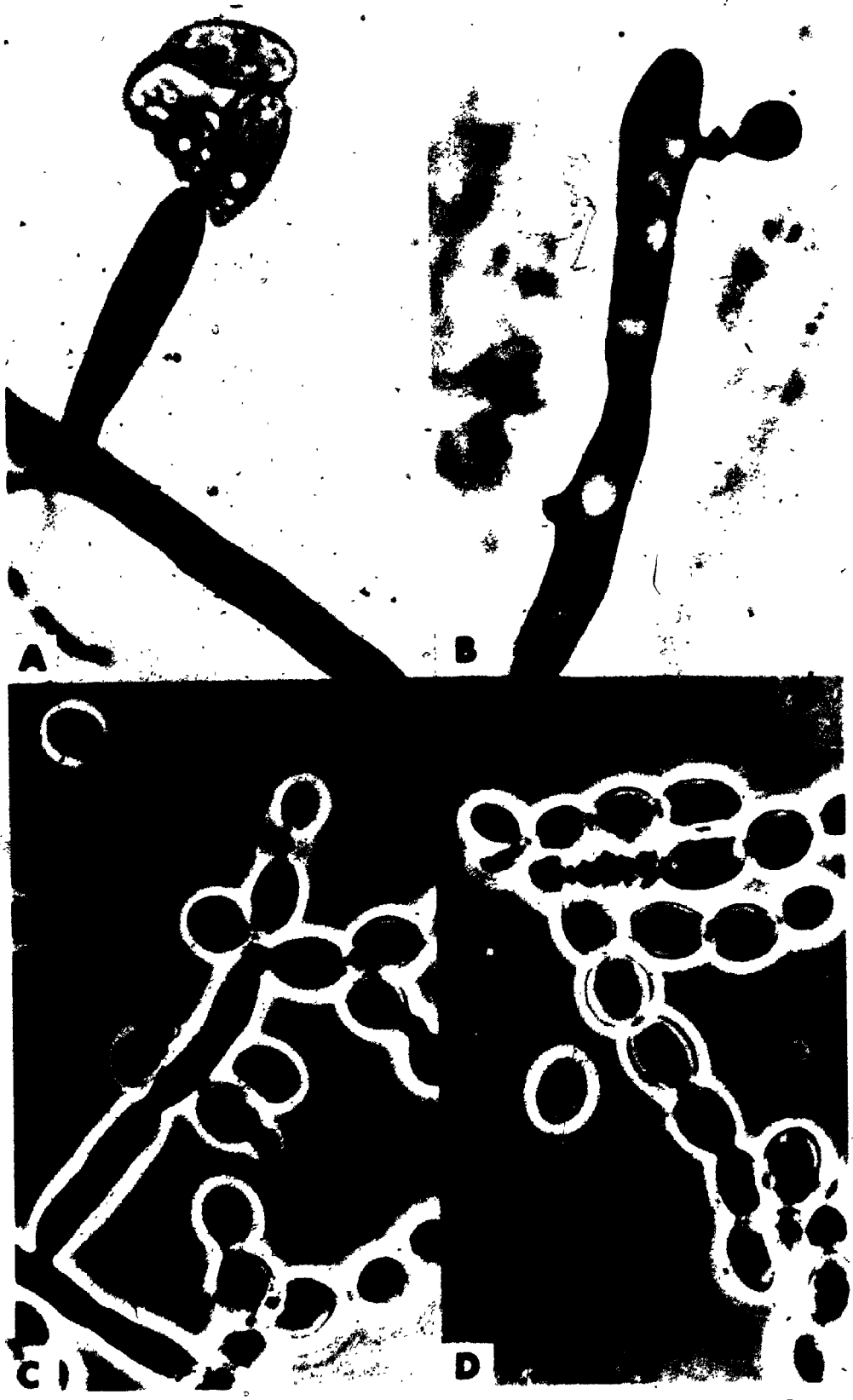
PLATE 3-3

A. Clusters of 5E/3 conidia at sporogenous loci on cultures grown on Coty's agar in ethane/air atmosphere, x 450. B. 5E/3 conidia from malt agar, stained with polychrome blue, x 2800. C. Slide culture of 5E/3 on malt agar. Intercalary sporogenous cell showing 'peg', stained with polychrome blue, x 2500. D. Lateral sporogenous cell of 5E/3 showing 'pegs' on malt agar, phase contrast, x 2800.


C
D

PLATE 3-4

A. Slide culture of 5E/3 on malt agar. Lateral sporogenous cell showing cluster of conidia, stained with polychrome blue, x 2800. B. 5E/3 sporogenous cell with collarette on Coty's agar in ethane/air atmosphere, phase contrast, x 3100. C. Yeast-like growth of 5E/3 by budding, phase contrast, x 1500. D. Later stage of yeast-like growth of 5E/3 showing chains of budding cells, phase contrast, x 1500.


A
B
C
D

genous cell proliferating through conidiogenous locus producing a secondary conidiogenous cell; indistinct collarette sometimes observed (Pl. 3-4 B). Conidia accumulating at conidiogenous locus embedded in slime to form a gloeoid spore ball (Pl. 3-3 A, 3-4 A); adjacent spore balls often coalescing to form large slimy spore masses.

Colony appearance on Czapek's agar and on Coty's agar in ethane and air mixtures similar to that on malt agar. On corn meal agar, colony thinner with paucity of aerial hyphae. Sporulation more sparse than on malt agar. Occasional irregularities of morphology observed upon malt agar more frequent upon Coty's agar in ethane and air atmospheres, especially in older parts of colony; up to four or five conidiogenous points clustered together on one cell; proliferation of conidiogenous cells more frequent. In submerged cultures in shake flasks of Coty's medium in 50% natural gas, 50% air atmosphere culture yeastlike, olive-black, growth by budding (Pl. 3-4 C, D).

3.3.4. Growth upon gaseous alkanes:

A comparison of growth of representative isolates upon the gaseous alkanes is summarized in Table 3-2. Ethane, propane and n-butane support vigorous growth of all the cultures tested in that order of preference. No significant growth could be detected upon methane however.

Table 3-2. Comparison of the ability of isolates to grow upon gaseous alkanes.

	Relative proliferation of mycelium on plates of Coty's agar in atmospheres of 40% alkane in air[a]				
	Acremonium 3E/1	Acremonium 4E/3	Acremonium 4E/5	Phialophora jeanselmei 5E/3	Phialophora jeanselmei 5E/4
Methane	0	0	0	0	0
Ethane	+++++	+++++	+++++	+++++	+++++
Propane	++++	++++	++++	++++	++++
n-Butane	+++	+++	+++	+++	+++

[a]Scored on a scale of 0 to +++++, 0 corresponding to growth on the control, in atmospheres of air only, +++++ to that on ethane.

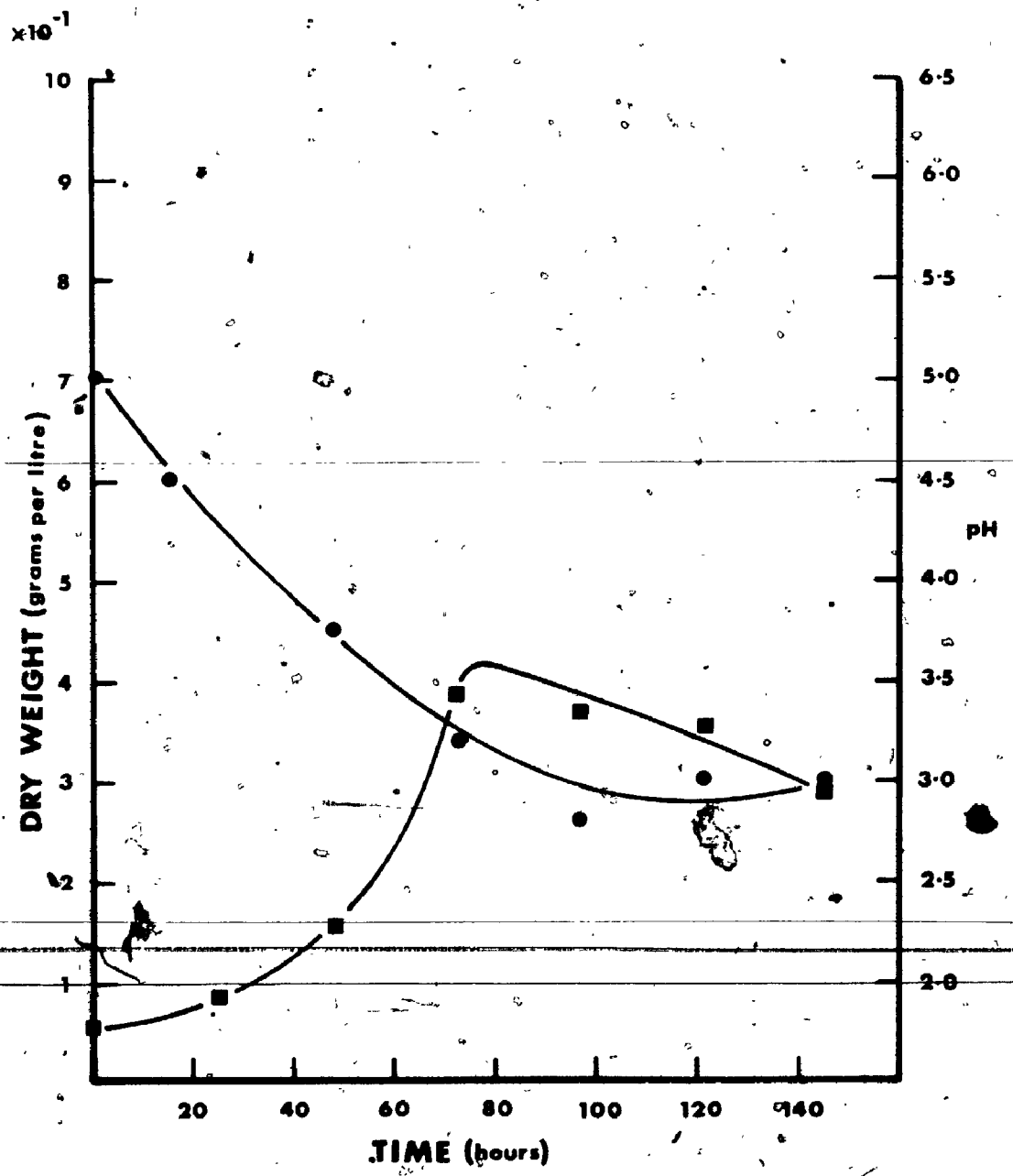
3.3.5. Submerged growth upon natural gas:

Isolates 5E/3 and 5E/4 produced a vigorous homogeneous yeast-like growth in submerged culture on Coty's or N.M. medium. However due to the possibility of a health hazard (see section 3.4.1.) from this species no further work was carried out with it.

Isolate 4E/3 when grown on Coty's medium (pH 5.5) grew very slowly (Fig. 3-1) indeed and produced a final yield of mycelial tissue of less than 400 milligrams per litre after 75 hours. Growth is accompanied by a steep drop in the pH of the medium (probably associated with utilization of the $(NH_4)_2SO_4$ nitrogen source), followed by a final rise in pH, accompanying the final decrease in dry weight; this latter rise might indicate autolysis of cells and release of basic nitrogenous compounds (Cochrane 1958). It was likely that the low pH (down to 2.8) produced in Coty's medium inhibited growth once the tolerance limit was reached. For this reason growth of 4E/3 was compared in N.M. medium and in Coty's medium in shake flasks. Table 3-3 shows dry weight in the two media after seven day's growth; N.M. medium gave three times the yield of Coty's.

Figure 3-2 shows growth of the same culture in the fermenter in N.M. medium (pH 5.5) with an inoculum grown on Coty's medium. Growth is much more rapid and attains a much higher yield than was the case with Coty's medium (note dry weight scale difference between Fig. 3-1 and 3-2). After an initial lag period a linear vegetative growth phase could be

Figure 3-1. Submerged growth of Acremonium 4E/3 on natural gas in a fermenter, 25°C, Coty's medium. -■- dry weight of cells; -●- pH of culture filtrate.


x10⁻¹
10
9
8
7
6
5
4
3
2
1
DRY WEIGHT (grams per litre)
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
2·5
2·0
pH
20
40
60
80
100
120
140
TIME (hours)

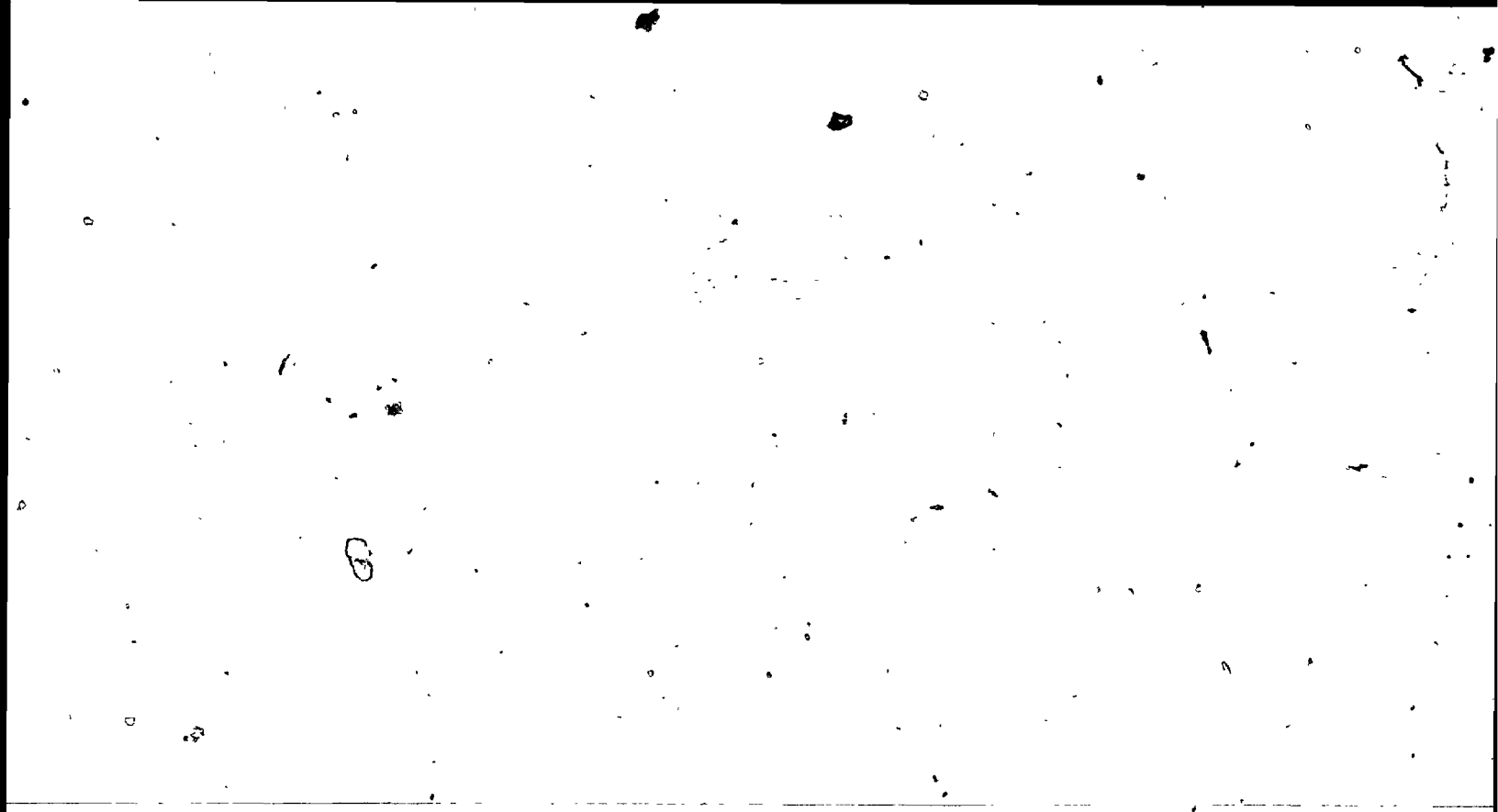


Figure 3-2. Submerged growth of Acremonium 4E/3 on natural gas in a fermenter, 25°C, N.M. medium. -■- dry weight of cells; -●- pH of culture filtrate.

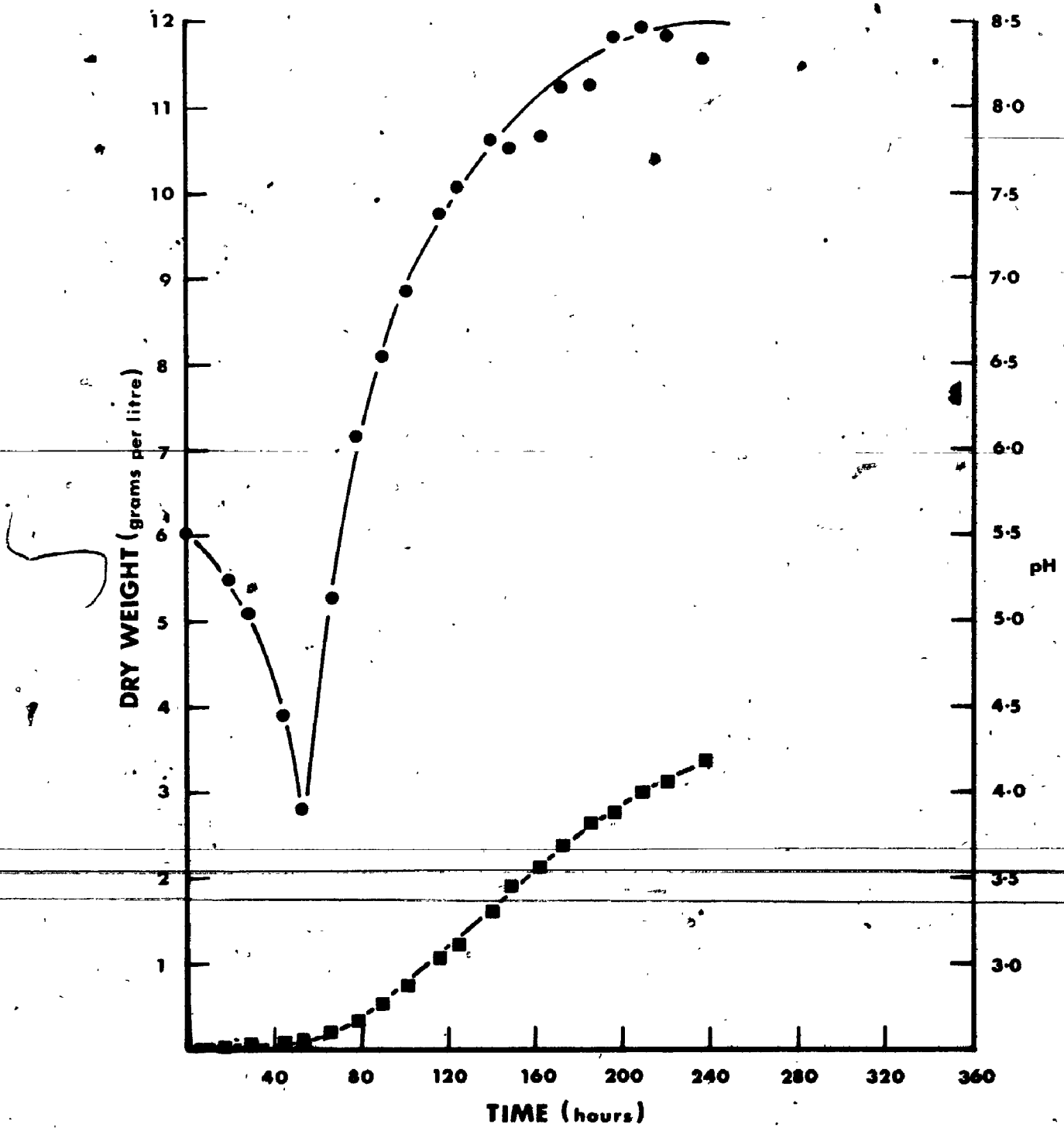

DRY WEIGHT (grams per litre)
pH
TIME (hours)
12
11
10
9
8
7
6
5
4
3
2
1
8·5
8·0
7·5
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
40
80
120
160
200
240
280
320
360

Table 3-3. Comparison of growth of Acremonium 4E/3 on two media in an atmosphere of 50% natural gas in air.

Mean dry weight of mycelium (mg) per 100 ml medium[a] and Standard Deviation after seven days growth in shake flasks

Coty's medium	N.M. medium
49.3 (± 1.3)	142.3 (± 15.5)

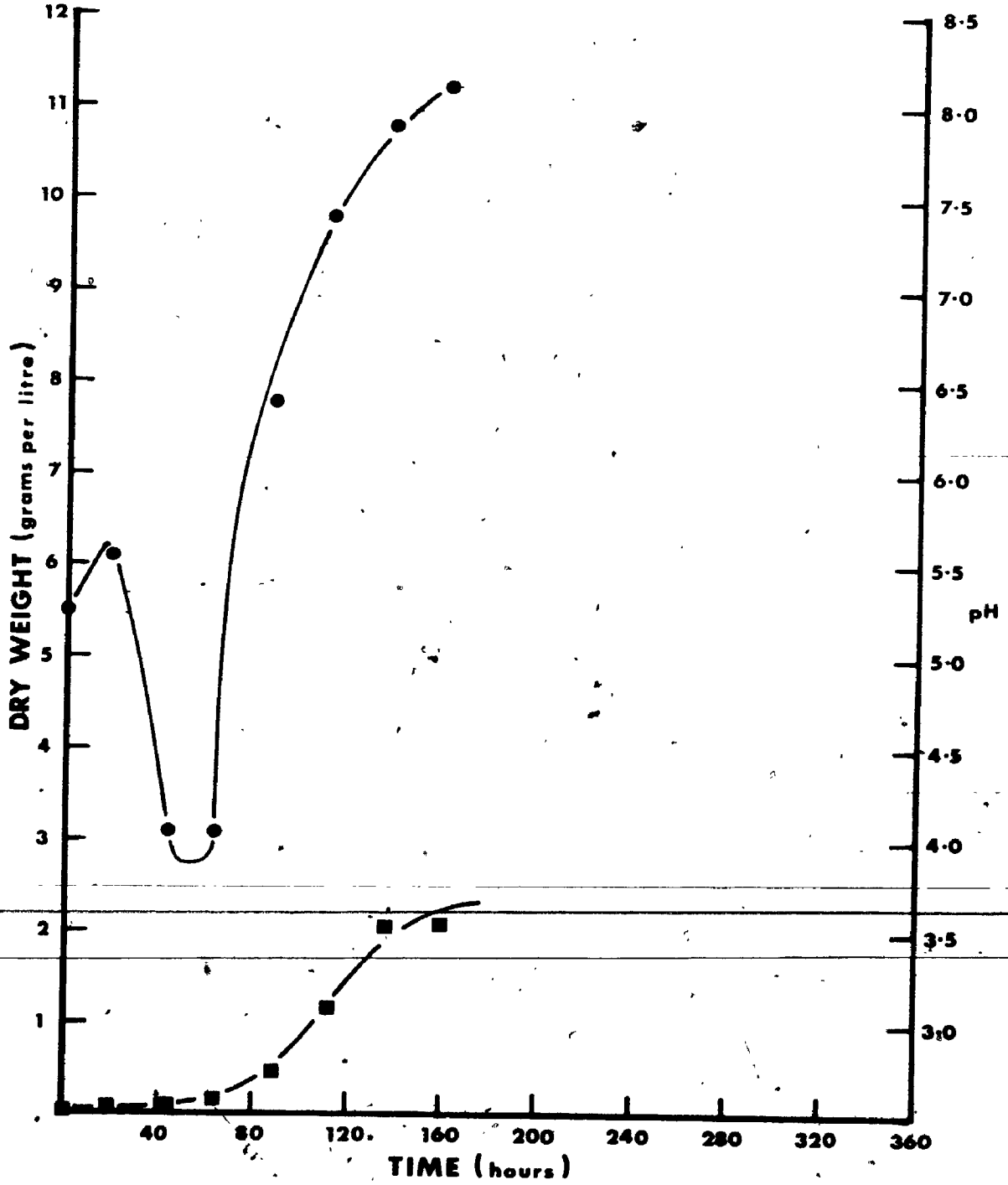
[a]Three replicate flasks.

recognized with a growth rate of 24 mg l^{-1} hr^{-1} compared to an estimated 13 mg l^{-1} hr^{-1} for Coty's medium. Towards the end of the run, the growth rate was beginning to decrease but a total yield almost ten times that on Coty's medium had been achieved. The pH of the medium showed an initial drop, probably due to the release of acid associated with uptake of residual $(NH_4)_2SO_4$ from the inoculum; the pH then climbed rapidly to a maximum of 8.4 probably due to uptake of NO_3^- ions and release of free Na^+ ions into the medium (Cochrane 1958). It has been well documented that many fungi when supplied with both NH_4^+ and NO_3^- will use the NH_4^+ preferentially first (Cochrane 1958).

Microscopic examination of samples from this fermenter run revealed that the biomass consisted almost entirely of conidia. Vegetative hyphae were almost completely absent. Soon after inoculation conidiophores of varying complexity, much like those seen on solid media, were produced and henceforth produced conidia continually; these were never observed to germinate during a run. With this culture the same type of growth was also observed in shake flasks. The morphologically identical isolates, 2E/1 and 4E/4 and the similar isolate 4E/5 followed the same pattern of growth.

Isolate 3E/1 produced sparse growth on Coty's medium. Figure 3-3 shows growth in a fermenter on N.M. medium (pH 5.5) inoculated with a culture grown on Coty's medium. The pattern of dry weight increase with time and pH change is similar to that of 4E/3. Growth was again linear in the

Figure 3-3. Submerged growth of Acremonium 3E/1 on natural gas in a fermenter, 25°C, N.M.,medium. -■- dry weight of cells; -●- pH of culture filtrate.


DRY WEIGHT (grams per litre)
12
11
10
9
8
7
6
5
4
3
2
1
8·5
8·0
7·5
7·0
6·5
6·0
5·5
pH
5·0
4·5
4·0
3·5
3·0
40
80
120
160
200
240
280
320
360
TIME (hours)

active vegetative phase (100 - 120 hours), and was more rapid (40 mg l^{-1} hr^{-1}) but was terminated prematurely by severe foaming in the fermenter; cells were lifted out of the medium by the foam and onto the baffles and walls of the fermenter.

Figure 3-4 illustrates a second run with 3E/1. N.M. medium was employed again (pH 5.0); the inoculum this time was grown on N.M. medium and was slightly basic thus raising the initial pH of the medium. The medium also received an initial 2 ml of antifoam and further aseptic addition of 2 ml at 90 hours and 6 ml at 110 hours. In this run the initial lag phase was shortened. The linear growth phase was extended considerably and the growth rate increased (67 mg l^{-1} hr^{-1}); foaming was entirely suppressed. A final yield of 9.7 grams per litre was achieved after 320 hours. The pH of the medium rose rapidly, reaching a maximum of 8.7 at one stage. A microscopic examination revealed a homogeneous suspension of vegetative mycelium with hardly any conidia and no evidence of pellet formation.

The growth pattern of 3E/1 in shake flasks was very similar to that in the fermenter as can be seen in Figure 3-5 (N.M. medium, pH 5.0, N.M.-grown inoculum) the growth rate however is somewhat lower (35 mg l^{-1} hr^{-1}) in the linear phase. Again a microscopic examination revealed a homogeneous suspension of mycelium. Where S.D. bars (Standard Deviations) are omitted in this figure they were covered by the symbols for the points. The yield in shake flasks indicate that the high yield in Figure 3-4 was not due to assimilation of the antifoam.

The Graphium isolates, including the original one

Figure 3-4. Submerged growth of Acremonium 3E/1 on natural gas in a fermenter, 25°C, N.M. medium. -■- dry weight of cells; -●- pH of culture filtrate.

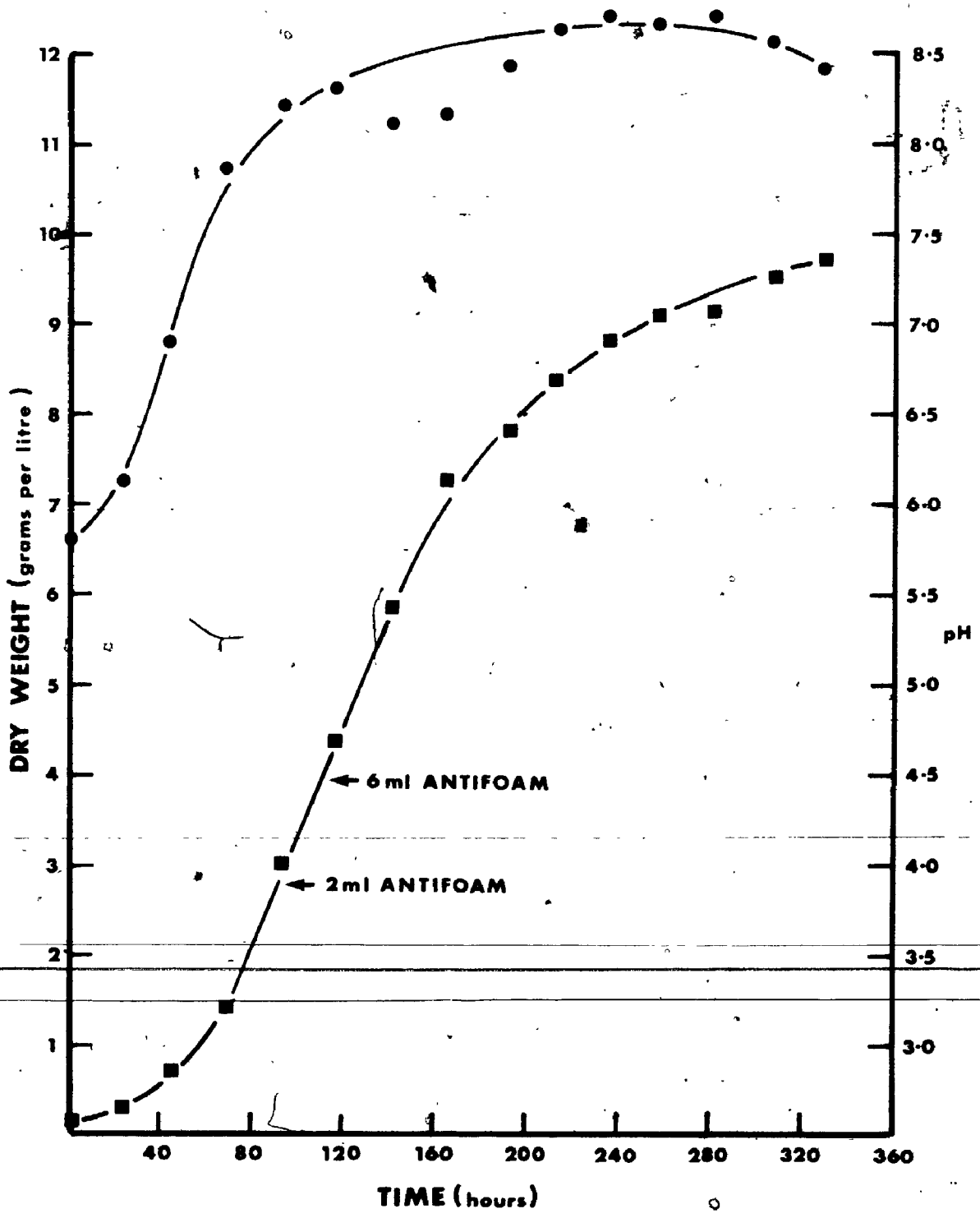
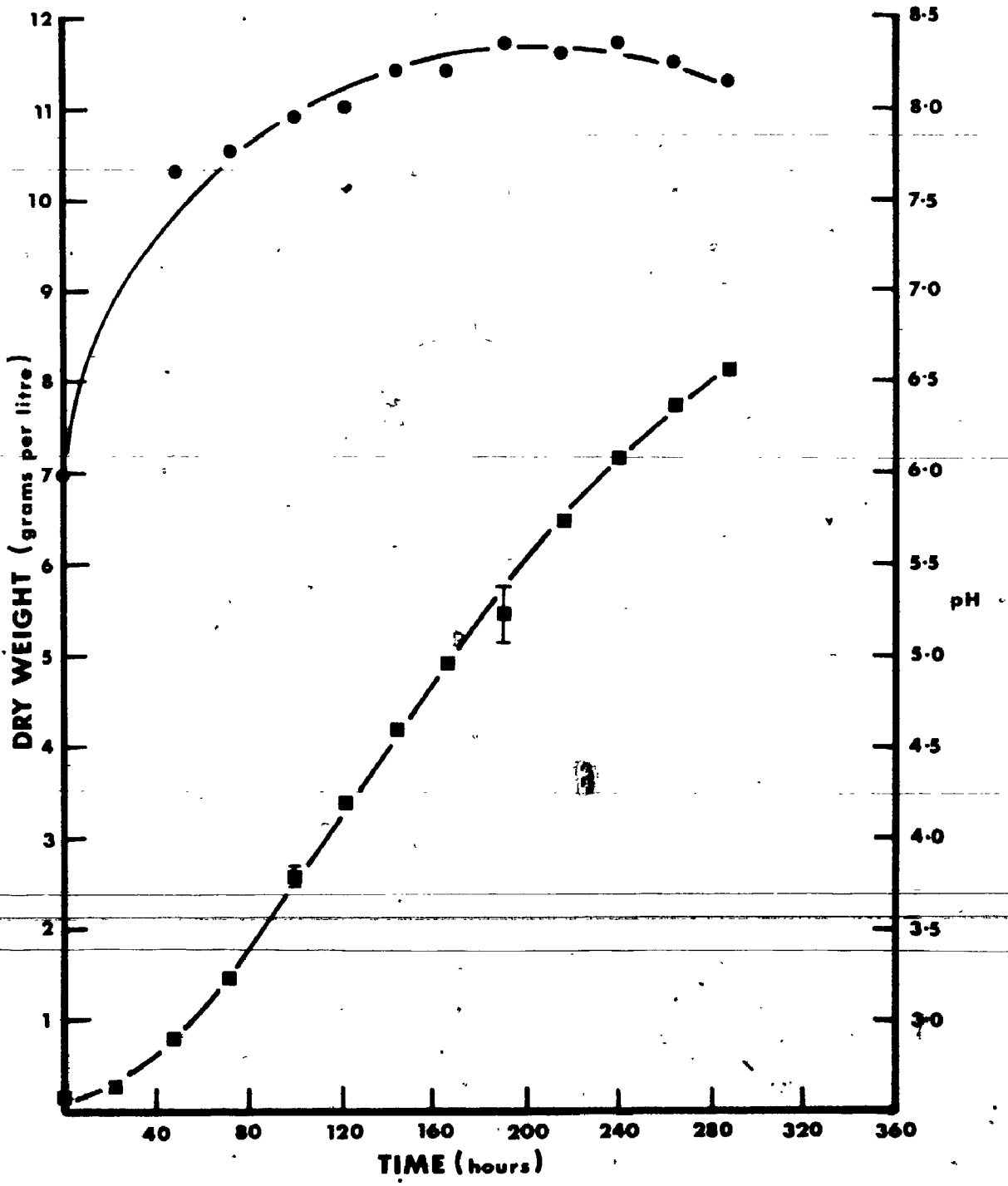

DRY WEIGHT (grams per litre)
12
11
10
9
8
7
6
5
4
3
2
1
8·5
8·0
7·5
7·0
6·5
6·0
5·5
pH
5·0
4·5
4·0
3·5
3·0
6 ml ANTIFOAM
2 ml ANTIFOAM
40
80
120
160
200
240
280
320
360
TIME (hours)

Figure 3-5. Submerged growth of Acremonium 3E/1 on natural gas in shake flasks, room temperature, N.M. medium.
-■- dry weight of cells (mean of three replicates, Standard Deviation given when not covered by point);
-●- pH of culture filtrate.


DRY WEIGHT (grams per litre)
pH
TIME (hours)
12
11
10
9
8
7
6
5
4
3
2
1
8·5
8·0
7·5
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
40
80
120
160
200
240
280
320
360

isolated by Zajic et al. (1969), were tested for their ability to grow in N.M. media. Growth was almost non existent; large hyphal clumps formed in shake flasks, while in the fermenter no growth at all took place, the inoculum merely depositing itself on the baffles above the medium. Curiously all the Graphium isolates grew very well on N.M. media solidified with 2% agar as did all the other cultures.

3.3.6. Growth of isolate 3E/1 on liquid n-alkanes:

It can readily be seen from Table 3-4 that no growth was observed upon any of the liquid normal alkanes. The lower molecular weight members might possibly have served as growth substrates at lower concentrations. n-Pentane particularly is very volatile and probably would soon evaporate from the culture flasks. Another problem with these volatile liquids is that they are excellent lipid solvents and might partially extract lipid from the inoculum.

3.4. Discussion:

3.4.1. Taxonomic position of isolates:

Assignment of the yellow isolates to a particular form genus presents some difficulty. They do however fit into section IV of Hughes' (1953) classification of the Hyphomycetes or into the Phialosporae of Barron (1968), their sporogenous cells being clearly phialides sensu Hughes. Other characteristics, namely unicellular, hyaline to lightly

Table 3-4. Growth of Acremonium 3E/1 on liquid n-alkanes.

Mean dry weight of mycelium[a] (mg) per 100 ml medium and Standard Deviation after seven days in shake flasks			
Experiment I		Experiment II	
Control	6.0 (± 0)	Control	10.0 (± 0)
n-Pentane	6.7 (± 0.6)	n-Undecane	10.7 (± 0.6)
n-Hexane	5.0 (± 0)	n-Dodecane	10.3 (± 1.2)
n-Heptane	5.0 (± 0)	n-Tridecane	10.7 (± 0.6)
n-Octane	4.3 (± 0.6)	n-Tetradecane	11.0 (± 1.0)
n-Nonane	4.7 (± 0.6)	n-Pentadecane	12.0 (± 1.7)
n-Decane	6.7 (± 1.2)	n-Hexadecane	11.7 (± 1.2)

[a]Three replicate flasks.

pigmented conidia sliming down to form gloeoid spore balls and elongated tapering phialides would seem to restrict the choice to Cephalosporium, Gliocladium or Verticillium.

Allocation of a culture to one or other of these genera depends primarily upon the branching pattern of the conidiophores. These cultures do not display patterns typical of any one of these genera; branching is not typically absent, penicillate or verticillate. Dr. Aubé (1971) an authority on the genus Verticillium, examined these isolates and ruled out this genus as a possibility.

A marked resemblance was noted between these isolates and imperfect states of the pyrenomycete genus Nectria described in a paper by Booth (1959). The author noted that this genus has conidial states assigned to many form genera including Cephalosporium, Fusarium, Gliocladium and Verticillium. Particular stress was laid upon the close interrelationship between such form-genera and the lack of any clear line of demarcation between them. It must be emphasized that form genera are taxa of convenience and do not necessarily imply close natural relationships between included species in the same way that perfect genera do. The converse also applies; Booth (1959) in reference to the imperfect states of Nectria says, "The use of conidial names should not be allowed to obscure the close relationship that exists between these species, nor the fact that no real fundamental distinction exists."

Assignment of these isolates to a particular form genus

(no perfect state having been found) is therefore open to some debate. Dr. Booth (1971) after examining these isolates confirmed that they are indeed imperfect states of the 'Episphaeria' group of Nectria species.

In a recent monograph on 'Cephalosporium - like' fungi, Gams (1971) includes species formerly assigned to Cephalosporium in a much enlarged and revised version of the old genus Acremonium. This now includes a wide variety of forms in three sections Simplex, Gliomastix and Nectrioidea. The latter group includes the 'Cephalosporium - like' conidial states of Nectria. All five yellow isolates clearly belong in this latter section, their conidiophores fitting the 'basitonous' branching pattern referred to by Gams. They do not however agree closely enough with any of his descriptions for positive identification to a particular species. It is preferred therefore to refer to these isolates as Acremonium 3E/1, 4E/3 etc. at this time.

Cultures 5E/2, 5E/3 and 5E/4 closely resemble Cooke's description of Phialophora jeanselmei (Langeron) Emmons in his papers (Cooke 1962a, 1962b) on the taxonomy of the so-called 'black yeasts'. Conidia produced on pegs on intercalary conidiogenous cells correspond to Cooke's 'radulaspores'; the pegs, on which they are borne, correspond to his 'spicules', found mainly in younger colonies. The more usual lateral conidiogenous cells correspond to cells which Cooke interprets as phialides, noting the occurrence of a tubular tip, which, with age, become weakly funnel-

shaped with the development of an indistinct collar (collarette). The ability of this species to produce a yeast-like stage in submerged culture was noted as was its widespread occurrence in sewage, soil, wood pulp and clinical cases of mycetoma in man. Cooke considered the following species synonyms of P. jeanselmei (Langeron) Emmons:

Torula jeanselmei Langeron

Torula bergeri Langeron

Margarinomyces heteromorphum (Nannf.) Mangenot.

Sporotrichum gougerotii sensu Borelli

Wang (1966) in a study of the morphological characteristics of this species, while agreeing that it was synonymous with Margarinomyces heteromorphum (Nannf.) Mangenot, produced evidence that conidia of numerous isolates of the species isolated from wood pulp, sewage and clinical mycetoma were not phialospores but annellospores. She described annellated growing tips of conidiogenous cells and also basal frills on conidia, typical of annellospores. Wang (1966) removed the species from the genus Phialophora since it did not have collarettes and suggested it might belong in section III of Hughes' classification of the Hyphomycetes (Hughes 1953) consisting of species with annellophores. She was however undecided as to its true generic position but considered it advisable to use the original name Torula jeanselmei Langeron for the meantime, while at the same time admitting that it is not a true Torula since the chains of phragmosphores found in T.

herbarum, the type species, were absent.

Dr. Wang (1971) examined cultures of isolates 5E/3 and 5E/4 and was of the opinion that they were Torula jeanselmei Langeron despite the apparent absence of elongated tips on the conidiogenous cells. Studies herein however did not produce any evidence of annellations, conidial frills or elongated conidiogenous tips although Wang (1966) did report that they were often indiscernible by light microscopy.

Schol-Schwartz (1968) included P. jeanselmei as one of 28 synonyms for Rhinocladiella mansonii (Castell.) Schol-Schwartz which include those mentioned by Cooke. She regarded R. mansonii as an extremely polymorphic species which could exhibit as many as four different patterns of conidiation including phialospore and annellospore production. A perfect state was found in only one of 60 isolates examined, Dictyotrichiella mansonii Schol-Schwartz.

The true taxonomic position of isolates 5E/2, 5E/3 and 5E/4 remains in doubt. While the isolates described herein are quite variable the kind of polymorphism documented by Schol-Schwartz was not found. Lateral conidiogenous cells would seem quite clearly to be phialides in the sense of Hughes (1953) and Barron (1968) producing a basipetal series of rapidly maturing conidia from the apex while remaining the same length, since no evidence for the existence of annellophores has been found. This being so, these isolates seem best assigned to Phialophora under the broader definition of the genus including forms without pronounced

collarettes, formerly called Margarinomyces. The name Phialophora jeanselmei (Langeron) Emmons is therefore preferred at this time. A culture of isolate 5E/3 has been deposited with the American Type Culture Collection under the name Phialophora jeanselmei, accession number ATCC26272.

3.4.2. Selection of a suitable culture for physiological experiments:

It was hoped to investigate the metabolism of gaseous alkanes by working both with natural gas-grown whole cells and enzyme preparations prepared from them. Since several cultures were available with which to work a decision had to be made as to which was most suitable for this work. Ideally one would like to be able to produce homogeneous suspensions of metabolically active cells in as short a time as possible by a method which would lend itself to the establishment of a simple regular routine.

The Phialophora jeanselmei isolates were ruled out. Although there was no positive evidence of pathogenicity in these particular isolates there was certainly an element of risk involved not only to the researcher but to co-workers in the same laboratory.

The Graphium isolates did not lend themselves to routine shake flask culture since very low yields of heterogeneous mycelial pellets were produced on Coty's or N.M. medium.

Acremonium 2E/1, 4E/3, 4E/4 and 4E/5 produced good

yields of homogeneous tissue but this consisted almost entirely of spores which one would not expect to be metabolically active.

Acremonium 3E/1 met all the above mentioned requirements. It grew very rapidly in shake flask culture, producing a homogeneous suspension of mycelium with little sporulation at room temperature. This isolate was therefore used in all subsequent experiments.

CHAPTER 4

MANOMETRIC STUDIES ON NATURAL GAS GROWN-RESTING CELLS OF ACREMONIUM 3E/1

4.1. Introduction:

Much information about the metabolic pathways of particular growth substrates can be obtained by the "resting cell technique" (see section 2.4). Such substrates and their metabolic intermediates should be metabolized without lag since the necessary enzymes are already present (assuming no problems such as those discussed in section 2.4).

Warburg respirometry is particularly useful in the study of uptake of gaseous substrates such as gaseous alkanes by resting cells. Oxidation of postulated intermediates can be followed by measuring oxygen uptake by the cells when such compounds are supplied.

4.2. Materials and methods:

4.2.1. Resting cells:

Cells for the following experiments were cultured as previously described (3.2.5.2) in shake flasks of N.M. medium (pH 5.0) at room temperature (25 ± 2°C) on natural

gas as the sole source of carbon and energy. Cells were routinely harvested five days after inoculation. A preliminary growth curve study (Fig. 3-5) indicated that cells at this time were in the middle of the active linear growth phase. In this way it was hoped to standardize the physiological age of cell populations as much as possible from one experiment to the next.

Variations in growth conditions did occur which were impossible to control. For example, besides minor variations in room temperature and inoculum, the natural gas supply itself was subject to variation in composition. Analyses supplied by the Union Gas Company indicated that the major growth substrate ethane could vary from 4.27% to 5.99% by volume of the total natural gas supply. Levels of sulphur compounds present in the gas stream (such as H_2S and various mercaptans) are also subject to fluctuation and might conceivably reach inhibitory concentrations.

Cells were harvested by centrifugation in a refrigerated centrifuge equipped with a swinging bucket rotor at 1000 g for five minutes. A "resting cell" suspension in sterile, ice-cold, 0.02 M potassium phosphate buffer (pH 7.0) was prepared by centrifugation and resuspension four times in this same buffer to remove residual growth medium. While the suspension was stored on ice, three 5 ml samples were filtered on to tared filter papers, dried at 105°C and weighed to determine the cell concentration. The suspension was then diluted with buffer to the required concentration

(one milligram dry weight per millitre unless otherwise specified).

4.2.2. Warburg manometry:

Gas uptake measurements were made with a Gilson circular Warburg apparatus (Gilson Medical Electronics Co., Middleton, Wisc.) using the methods of Umbreit, Burris and Stauffer (1964). Respirometer systems (flask plus manometer) were calibrated to the 250 mm mark on the manometers to allow measurement of as much gas uptake as possible.

Since in many experiments uptake of two gases was measured simultaneously some discussion of principles is useful. Uptake of a gas or gases into the fluid phase (cell suspension) of a closed respirometer system is measured by the pressure decrease, indicated by a fall in the level of manometer fluid (in millimetres) when the volume of the system is kept constant. This value is converted to microlitres of gas taken up (reduced to N.T.P.) by multiplication by a flask constant specific for each individual system. The value of this constant depends upon the gas and fluid volumes of the system, the water bath temperature, the specific gravity of the manometer fluid (Brodie's fluid) and the solubility of the gas in question (usually oxygen) at the temperature employed. Microlitres of gas at N.T.P. can of course be readily converted to micromoles of gas by dividing by 22.4 (one micromole of a gas at N.T.P. occupies 22.4 microlitres).

Where alkane and air atmospheres were employed in Warburg systems, oxygen and gaseous alkane were assumed to be taken up simultaneously. In such situations the exact uptake of each gas in microlitres at N.T.P. could not be calculated with the same precision that could be relied upon with oxygen uptake alone, since it was not known in what proportions the two gases were taken up. The error is introduced by the difference in solubility of the two gases. Considering α (the Bunsen absorption coefficient, ml gas per ml fluid at one atmosphere) for the gaseous alkanes at 30°C, the values for methane, ethane, propane and n-butane are respectively 0.0289, 0.0362, 0.0288 and 0.0233 (International Critical Tables 1928). These values are very close to that of oxygen at the same temperature (α = 0.0261).

Taking an example, the flask constant for oxygen uptake in a given system, k_{O_2} at 30°C, was calculated as 1.390; for uptake of ethane (greatest difference in solubility), $k_{C_2H_6}$ = 1.394. In practice an average constant k_{av} was used, the mean of k_{O_2} and $k_{C_2H_6}$, in the example k_{av} = 1.392. For a hypothetical case where a 150 mm drop in manometer fluid is measured and 50 mm is due to ethane uptake and 100 mm to oxygen uptake:

$$50 \times 1.394 = 69.7 \text{ µl ethane}$$

$$100 \times 1.390 = \underline{139.0} \text{ µl oxygen}$$

$$\underline{208.7} \text{ µl total}$$

Estimation using k_{av} = 150 x 1.392 = 208.8 µl total

Error = 208.8 - 208.7 = 0.1 µl

The error introduced is only an increase of 0.047% over the true value and can be ignored for present purposes.

For experiments involving gaseous alkane and air atmospheres, prior to gassing, individual flasks received 3 ml of resting cell suspension (1.0 mg dry weight per ml), and 0.2 ml of 20% (wt/vol) KOH in the centre well to absorb CO_2 (plus a small piece of fluted filter paper to aid absorbtion). Atmospheres of 10% alkane in air were prepared in advance manometrically and dispensed to the Warburg systems by a combination of manometry and water displacement, after the method of Umbreit, Burris and Stauffer (1964). After ten minutes of temperature equilibriation in the water bath, measurement of gas uptake commenced after release of positive pressure (from gas expansion due to warming) via the stopcocks.

The procedure followed for measuring oxygen uptake in the presence of suspected intermediates differed in the following respects. The atmosphere in the flasks was air. Four micromoles of substrate was added to the sidearm dissolved in 0.2 ml of buffer. The main compartment of each flask received 1.8 ml of cell suspension containing 2 mg cells. After temperature equilibriation with open stopcocks for ten minutes the experiment was initiated by closing the stopcocks and tipping the contents of the sidearm into the main compartment.

Three replicate systems were run for each treatment in an experiment. In all cases endogenous oxygen uptake was measured simultaneously in an air atmosphere without added substrate. In graphical presentations of such data endogenous values were subtracted from all treatments to show substrate-dependent gas uptake.

All chemicals used in these experiments were reagent grade. Water was redistilled in an all glass apparatus. Gaseous hydrocarbons, unless otherwise specified were Matheson C.P. grade. Nitrogen and oxygen, where used, were of the highest purity available (food grade and medical grade respectively) from Canadian Liquid Air Ltd., London, Ontario.

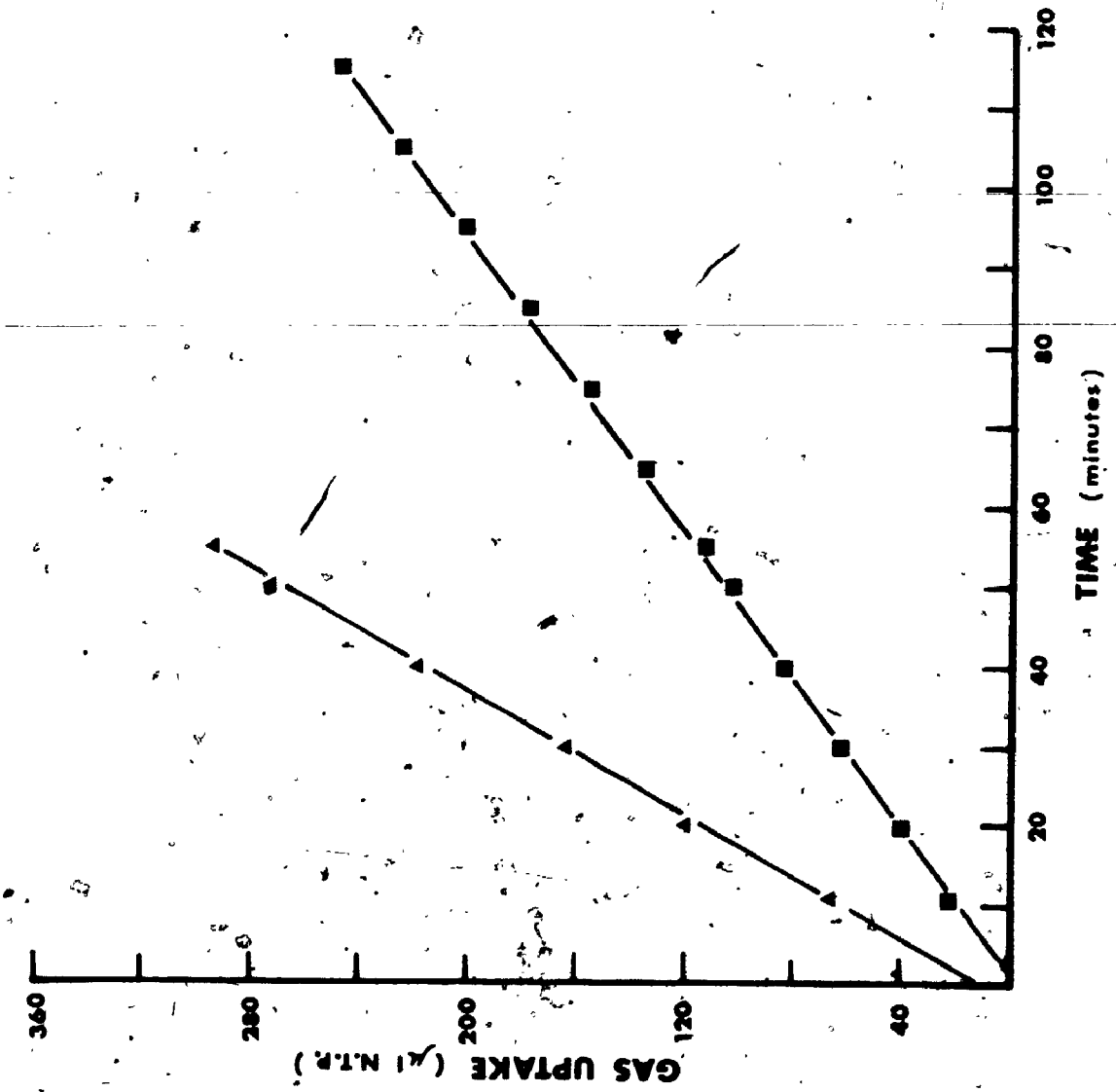
4.3. Results and discussion:

In the accompanying figures, each point of a graph in any given experiment is the mean of three replicates from which the mean endogenous control has been subtracted.

4.3.1. Uptake of gaseous alkanes:

Preliminary manometric experiments were run to determine suitable experimental conditions. Figure 4-1 demonstrates gas uptake of 1.0 mg ml^{-1} and 2.0 mg ml^{-1} cell concentrations at 25°C in pH 7.0 phosphate buffer (0.02 M) in an atmosphere of 10% ethane in air. Uptake was linear with time at both concentrations. Substrate-dependent rates of gas uptake expressed per milligram of cells did not differ appreciably for the two cell concentrations;

Figure 4-1. Effect of cell concentration upon gas uptake in an atmosphere of 10% ethane, 90% air by resting cells of Acremonium 3E/1 at 25°C. -▲- 2 mg ml^{-1} resting cells; -■- 1 mg ml^{-1} resting cells; endogenous oxygen uptake subtracted (2 mg ml^{-1} - 32 µl hr^{-1}; 1 mg ml^{-1} - 14 µl hr^{-1}).


GAS UPTAKE (μl N.T.P.)
360
280
200
120
40
TIME (minutes)
20
40
60
80
100
120

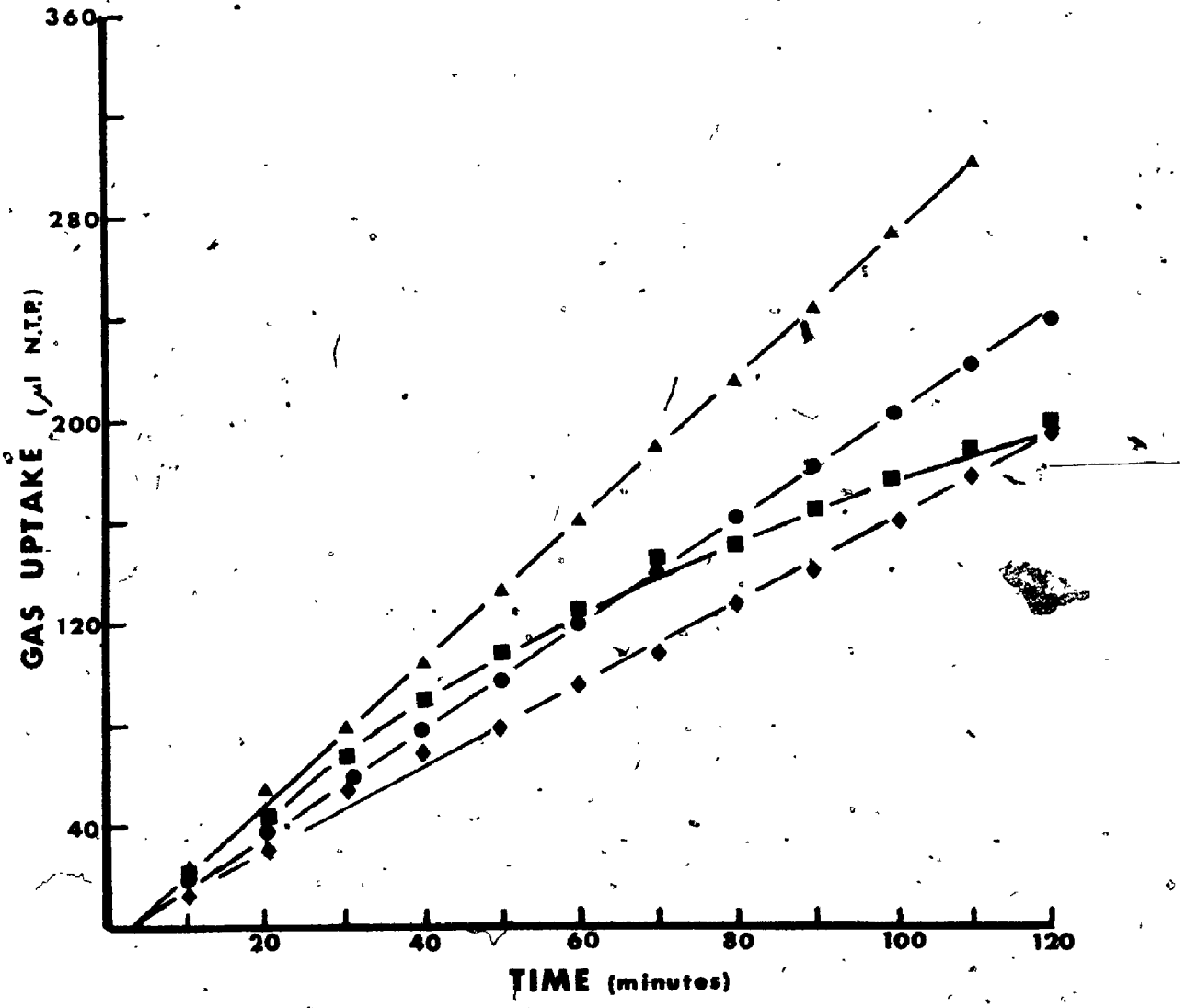
rates of 55.2 and 52.5 µl mg^{-1} hr^{-1} respectively for 2.0 and 1.0 mg ml^{-1} cell concentrations were found. Uptake is apparently proportional to cell concentration over this range. Endogenous rates were less than 10% of the substrate-dependent rates; 5.4 and 4.6 µl mg^{-1} hr^{-1} respectively were recorded for 2.0 and 1.0 mg ml^{-1} cell concentrations. A concentration of 1 mg ml^{-1} was adopted for all future experiments.

Figure 4-2 shows the effect of temperature upon gas uptake in an atmosphere of 10% ethane in air at pH 7.0. Linear gas uptake was observed at 20, 25 and 30°C, with rates of 32.0, 41.6 and 56.0 µl mg^{-1} hr^{-1} respectively. At 35°C a progressive decrease in the rate of gas uptake with time was noted. Such a temperature is apparently above the optimum; the declining curve indicates heat denaturation of an enzyme or enzymes at this temperature. The apparent optimum temperature, 30°C, was employed in all future experiments.

It can be seen from Figure 4-3 that variations in pH over the range 6.0 to 7.5 (in 0.02 M potassium phosphate buffer) had little effect upon the rate of gas uptake in ethane and air atmospheres. Uptake was slightly higher at pH 6.0, but for the sake of consistency with previous data, it was decided to continue using buffer of pH 7.0 in subsequent experiments.

The ability of resting cells to take up different gaseous alkanes was compared in two different experiments.

Figure 4-2. Effect of temperature upon gas uptake by resting cells of Acremonium 3E/1 in an atmosphere of 10% ethane, 90% air. -■- 35°C; -▲- 30°C, -●- 25°C; -◆- 20°C; each point the mean of three replicates; endogenous oxygen uptake subtracted (20 & 25°C - 14 µl hr^{-1}; 30°C - 18 µl hr^{-1}; 35°C - 20 µl hr^{-1}).


360
280
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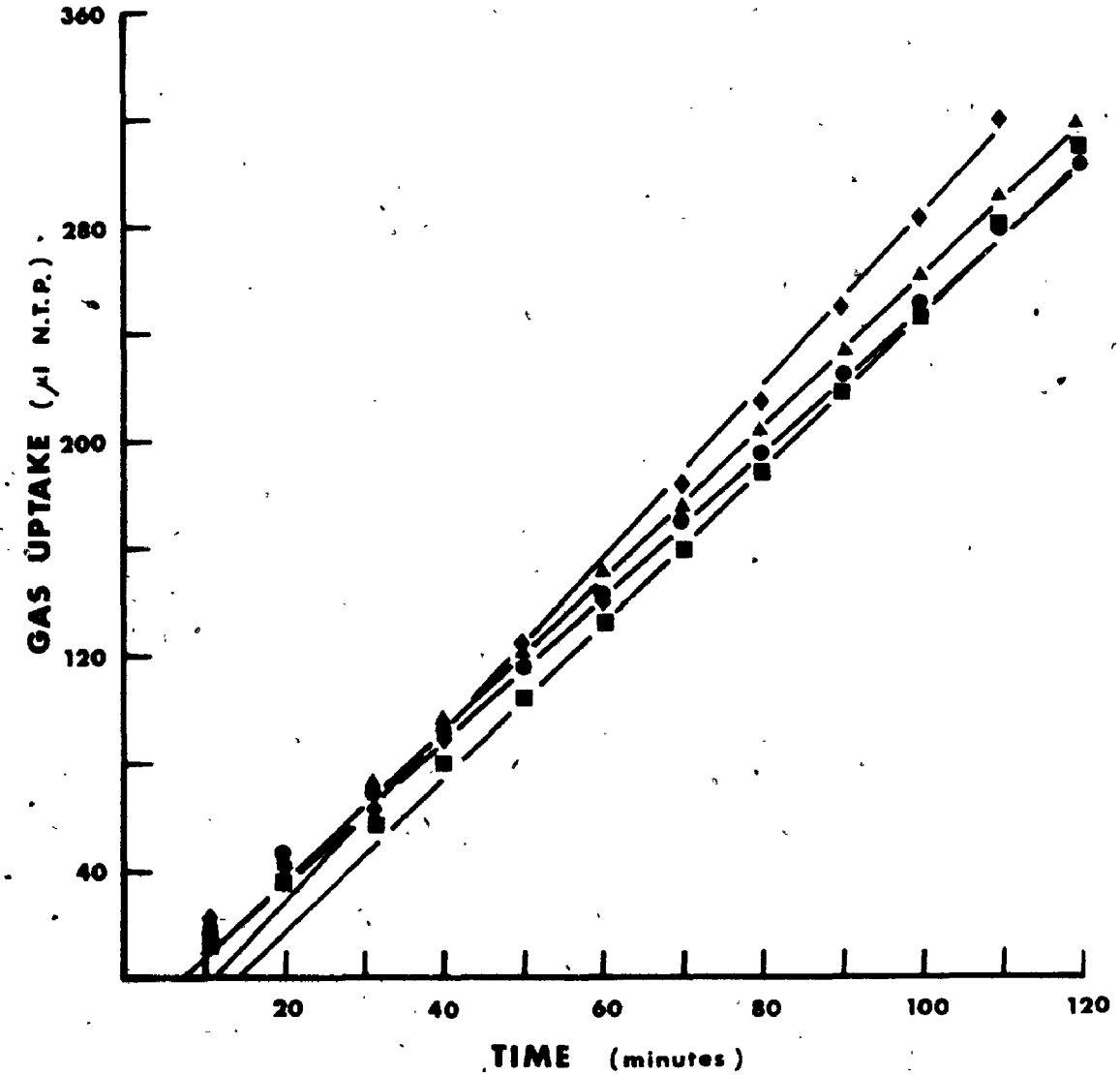
120
40
GAS UPTAKE (µl N.T.P.)
20
40
60
80
100
120
TIME (minutes)

Figure 4-3. Effect of pH upon gas uptake by resting cells of Acremonium 3E/1 in an atmosphere of 10% ethane, 90° air at 30°C. -◆- pH 6.0; -●- pH 6.5; -▲- pH 7.0; -■- pH 7.5; each point the mean of three replicates; endogenous oxygen uptake subtracted (pH 6.0, 6.5, 7.0 & 7.5 - 12 µl hr^{-1}).


360
280
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120
40
GAS UPTAKE (µl N.T.P.)
20
40
60
80
100
120
TIME (minutes)

Data from a representative experiment are presented in Figure 4-4.

Rapid linear gas uptake took place in atmospheres containing 10% ethane, propane or n-butane, as might be expected, since all three support growth. Gas uptake in an atmosphere of the non-growth substrate, methane, was only slightly higher than endogenous. Rates of gas uptake in atmospheres containing propane, n-butane and methane were respectively 86.6%, 67.6% and 6.7% of that recorded in ethane and air atmospheres (means of two experiments).

No gas uptake was recorded when methane, ethane, propane or n-butane were supplied in the absence of oxygen (10% alkane, 90% nitrogen). To further demonstrate an oxygen requirement for alkane uptake, resting cells were supplied with four different gas mixtures in which ethane was held constant at 10% while oxygen was supplied at 2%, 5%, 10% and 20% concentrations, the balance of each mixture being made up with nitrogen. Endogenous controls were set up for each oxygen concentration in which ethane was omitted. It is apparent from Figure 4-5 that the rate of gas uptake was unaffected by oxygen concentration over the range 5 - 20% and remained linear throughout the experiment. Oxygen did however become limiting at 2%; after an initial period of linear gas uptake at a lowered rate, the rate declined with time. Gaseous alkane consumption by Acremonium 3E/1 resting cells is therefore accompanied by consumption of molecular oxygen.

Figure 4-4. Gas uptake by resting cells of Acremonium 3E/1 in atmospheres of gaseous alkanes in air at 30°C. -■- 10% ethane, 90% air; -▲- 10% propane, 90% air; -●- 10% n-butane, 90% air; -◆- 10% methane, 90% air; each point the mean of three replicates; endogenous oxygen uptake subtracted (21 $\mu l\ hr^{-1}$).

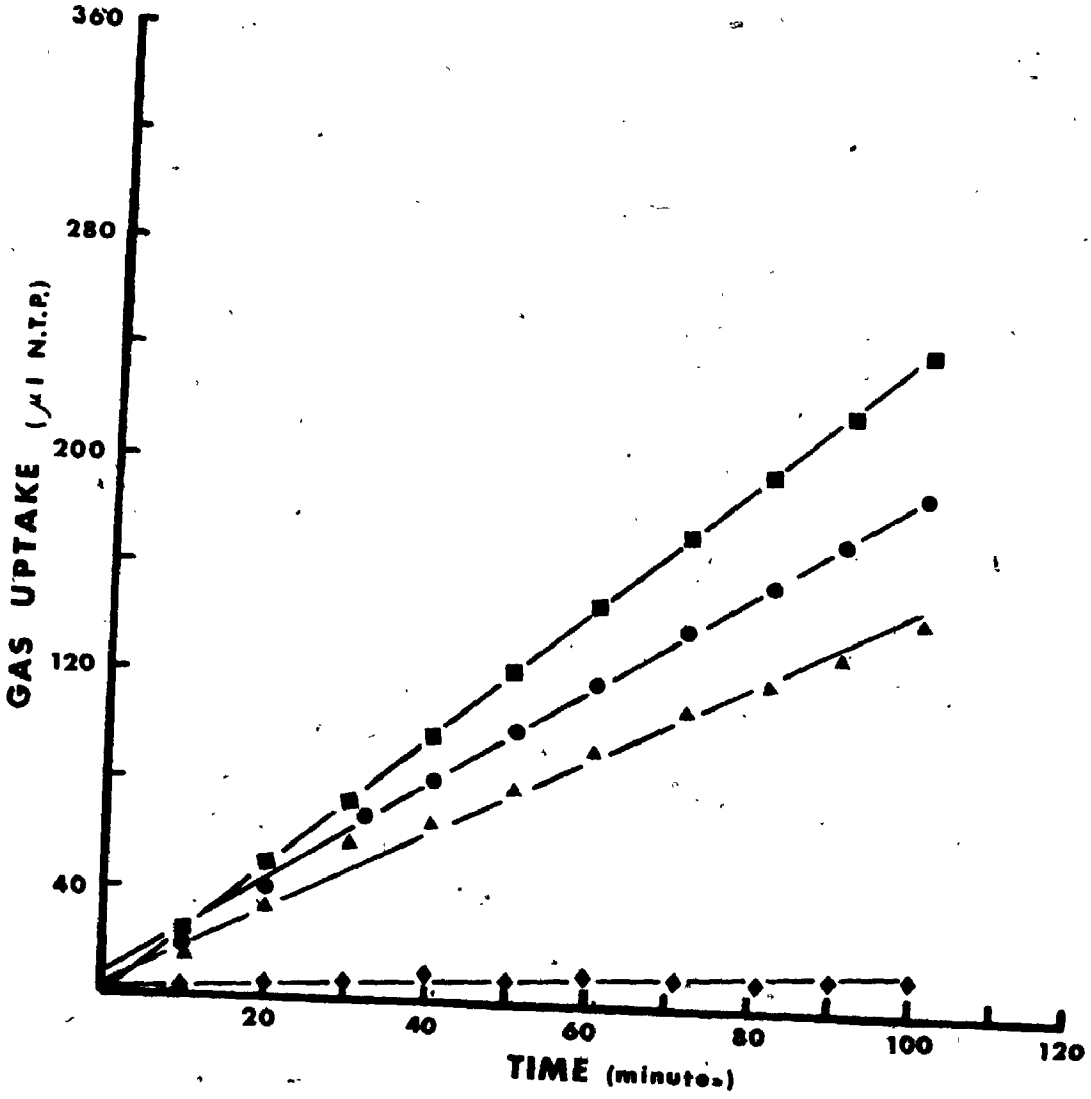
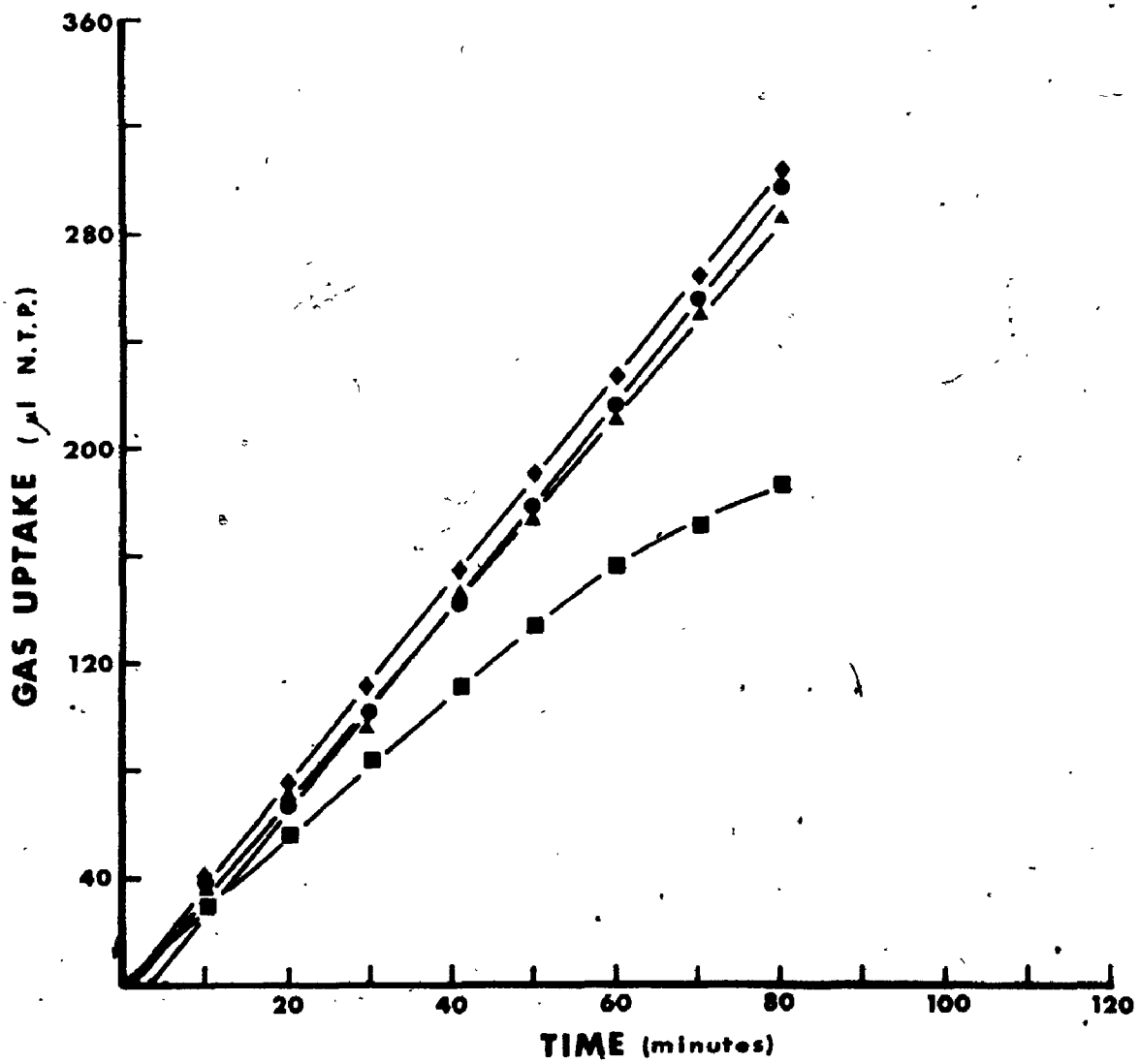

360
280
200
120
40
GAS UPTAKE (μl N.T.P.)
20
40
60
80
100
120
TIME (minutes)

Figure 4-5. Effect of oxygen concentration upon gas uptake in atmospheres of 10% ethane in nitrogen by resting cells of Acremonium 3E/1 at 30°C. -■- 2% O_2; -●- 5% O_2; -◆- 10% O_2; -▲- 20% O_2; each point the mean of three replicates; endogenous oxygen uptake subtracted (2% O_2 - 21 µl hr^{-1}; 5, 10 & 20% O_2 - 12 µl, hr^{-1}).


360
280
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120
40
GAS UPTAKE (µl N.T.P.)
20
40
60
80
100
120
TIME (minutes)

4.3.2. Oxidation of possible metabolic intermediates of gaseous alkanes:

If the initial step in oxidation of gaseous alkanes were dehydrogenation to the corresponding 1-alkene, then the following products would be expected:

$CH_3-CH_3 \longrightarrow CH_2 = CH_2$
ethane — ethylene

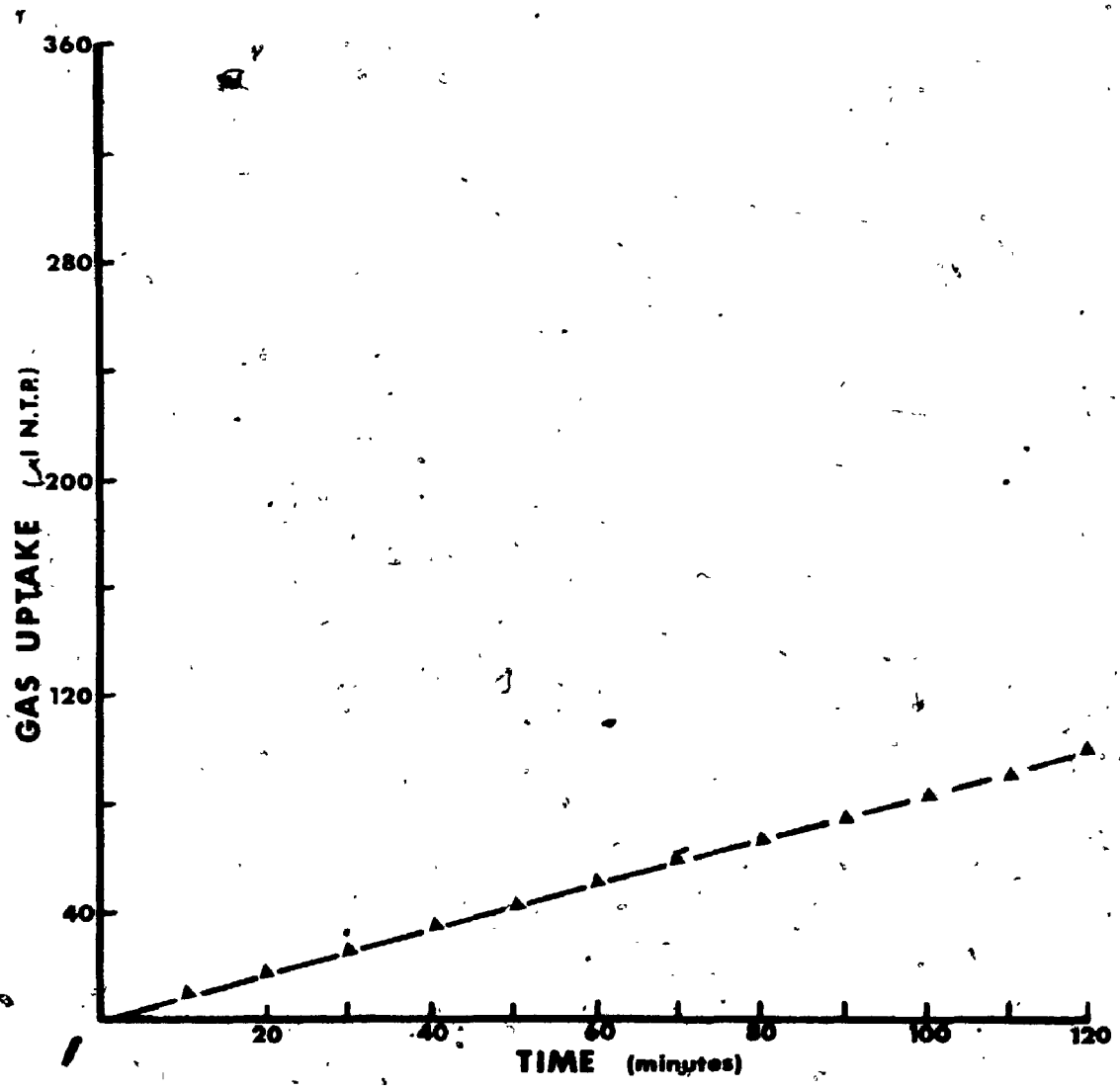
$CH_3CH_2-CH_3 \longrightarrow CH_3CH = CH_2$
propane — propylene

$CH_3CH_2CH_2-CH_3 \longrightarrow CH_3CH_2CH = CH_2$
n-butane — 1-butene

One would expect these compounds to be rapidly metabolized without a lag phase by resting cells, assuming a common enzyme system for all three alkanes.

Ethylene, propylene and 1-butene were supplied to resting cells as 10% mixtures in air in two different experiments. No gas uptake was recorded in either experiment in ethylene in air or 1-butene in air mixtures; data from a representative experiment (Fig. 4-6) do show gas uptake from propylene and air atmospheres however. Greater potential error was introduced into such data because of the difference in solubility between propylene (α = 0.11, 30°C) and oxygen (α = 0.0261, 30°C). However a mean substrate-

Figure 4-6. Gas uptake by resting cells of Acremonium 3E/1 in an atmosphere of 10% propylene in air at 30°C; endogenous oxygen uptake subtracted (16 $\mu l\ hr^{-1}$).


360
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120
40
GAS UPTAKE (µl N.T.P.)
20
40
60
80
100
120
TIME (minutes)

dependent gas uptake rate of 17.2 µl mg^{-1} hr^{-1} was recorded for the two experiments.

If n-alkanes are metabolized via an initial terminal attack then the corresponding primary alcohols, aldehydes and fatty acids should be oxidized rapidly without a lag phase. Such compounds, corresponding to ethane, propane and n-butane were supplied to resting cell suspensions and oxygen uptake measured in two different experiments for each substrate (in the case of fatty acids the sodium salts were used).

Data from a representative experiment (Fig. 4-7) shows that the hypothetical intermediates of ethane were all oxidized rapidly without lag. Oxygen uptake had ceased after 150 minutes with ethanol, acetaldehyde and acetate. The propane series (Fig. 4-8) shows the same pattern for 1-propanol, propionaldehyde and propionate except that oxygen uptake had not ceased even after 200 minutes. The same can be said of the n-butane series (1-butanol, butyraldehyde and butyrate) from Figure 4-9.

The three primary alcohols all showed an initial phase of rapid, linear oxygen uptake at approximately the same rate in each case, 37.6, 35.5 and 34.9 µl mg^{-1} hr^{-1} respectively for ethanol, 1-propanol and 1-butanol oxidation (mean rate of two experiments in each case). This was followed by a second phase at a lesser rate which was initiated at approximately the same point in the case of each alcohol, after consumption of 0.80, 0.70 and 0.71 µmole of

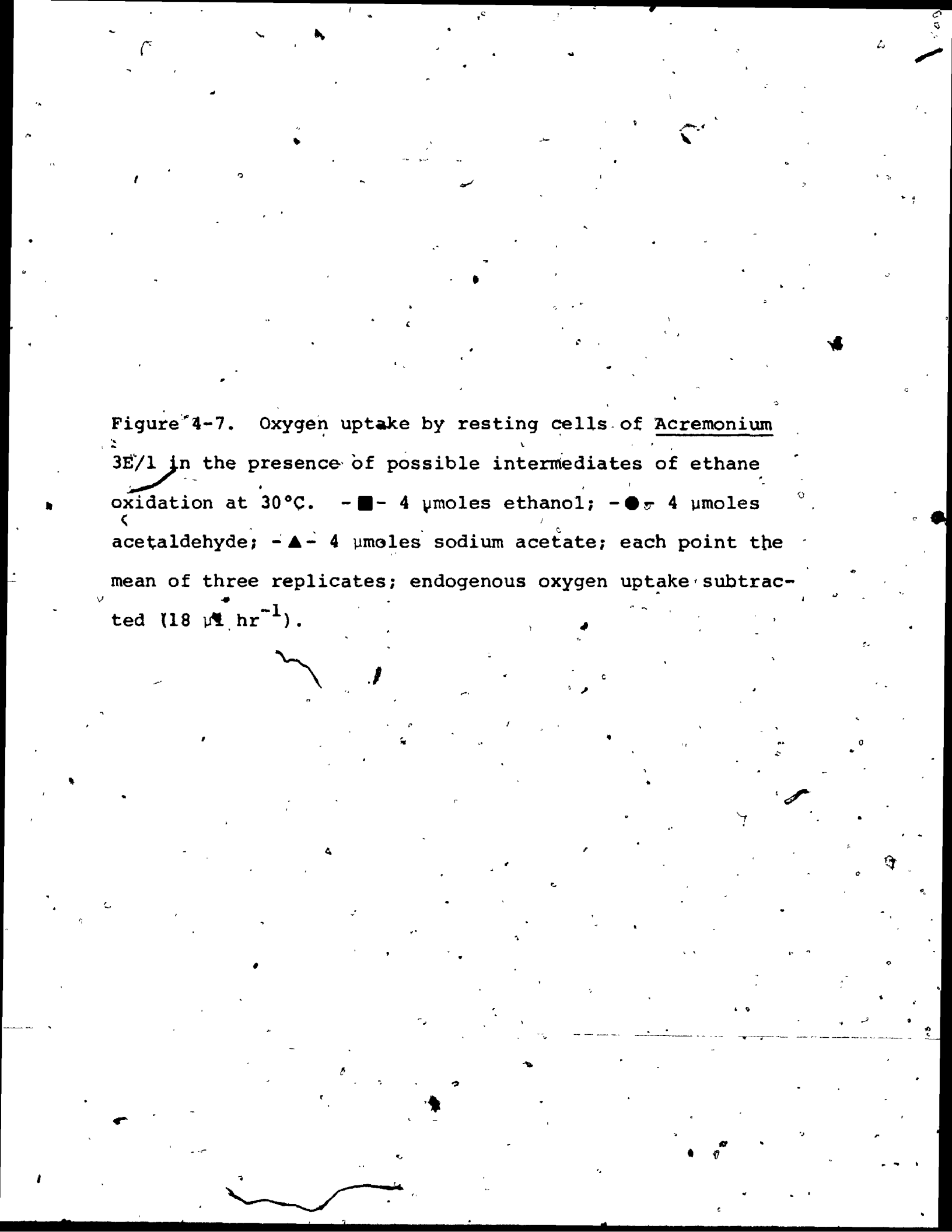


Figure 4-7. Oxygen uptake by resting cells of Acremonium 3E/1 in the presence of possible intermediates of ethane oxidation at 30°C. -■- 4 μmoles ethanol; -●- 4 μmoles acetaldehyde; -▲- 4 μmoles sodium acetate; each point the mean of three replicates; endogenous oxygen uptake subtracted (18 μl hr^{-1}).

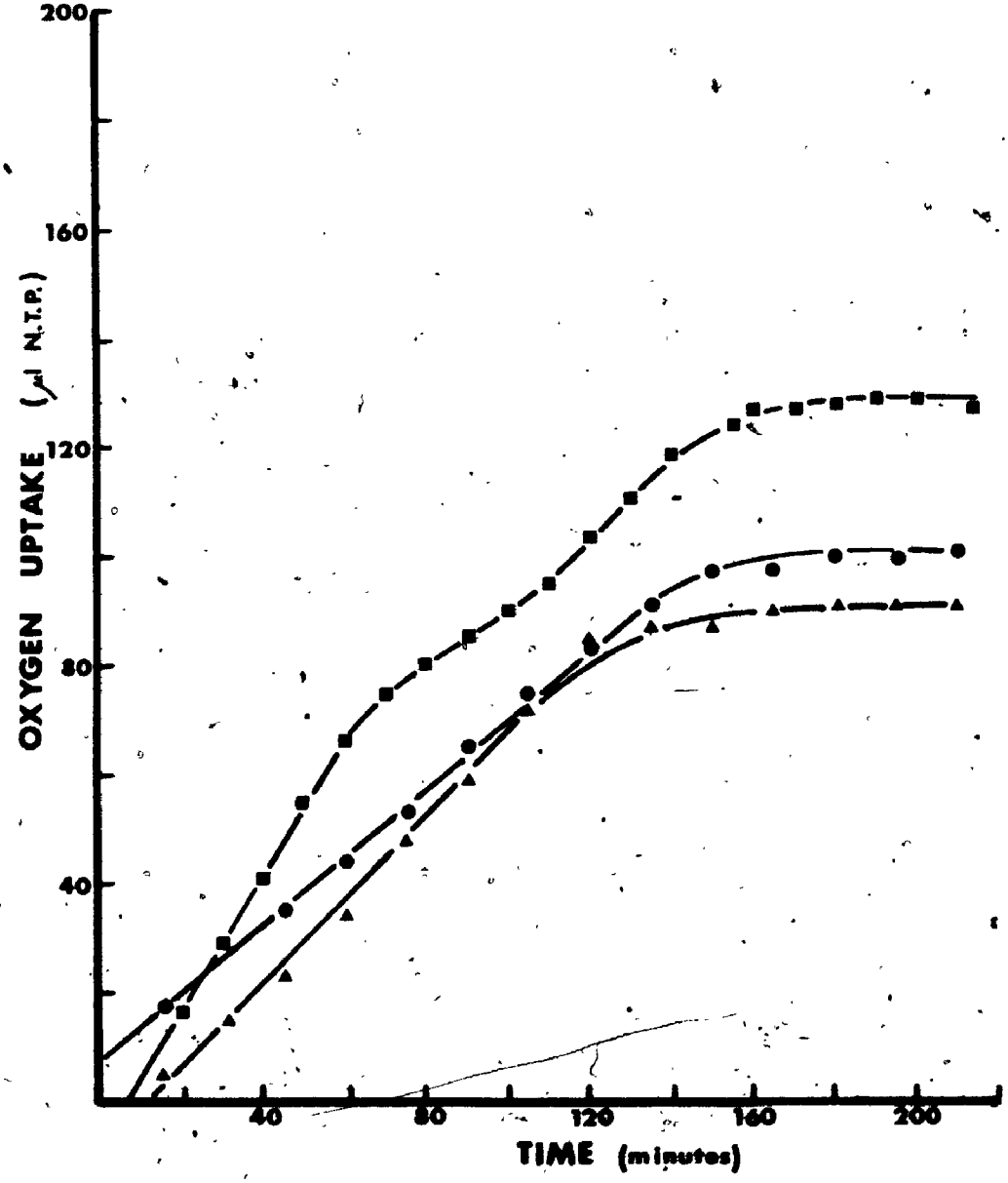

200
160
120
80
40
OXYGEN UPTAKE (μl N.T.P.)
40
80
120
160
200
TIME (minutes)

Figure 4-8. Oxygen uptake by resting cells of Acremonium 3E/1 in the presence of possible intermediates of propane oxidation at 30°C. -■- 4 µmoles 1-propanol; -●- 4 µmoles propionaldehyde; -▲- 4 µmoles sodium propionate; each point the mean of three replicates; endogenous oxygen uptake subtracted (24 µl hr^{-1}).

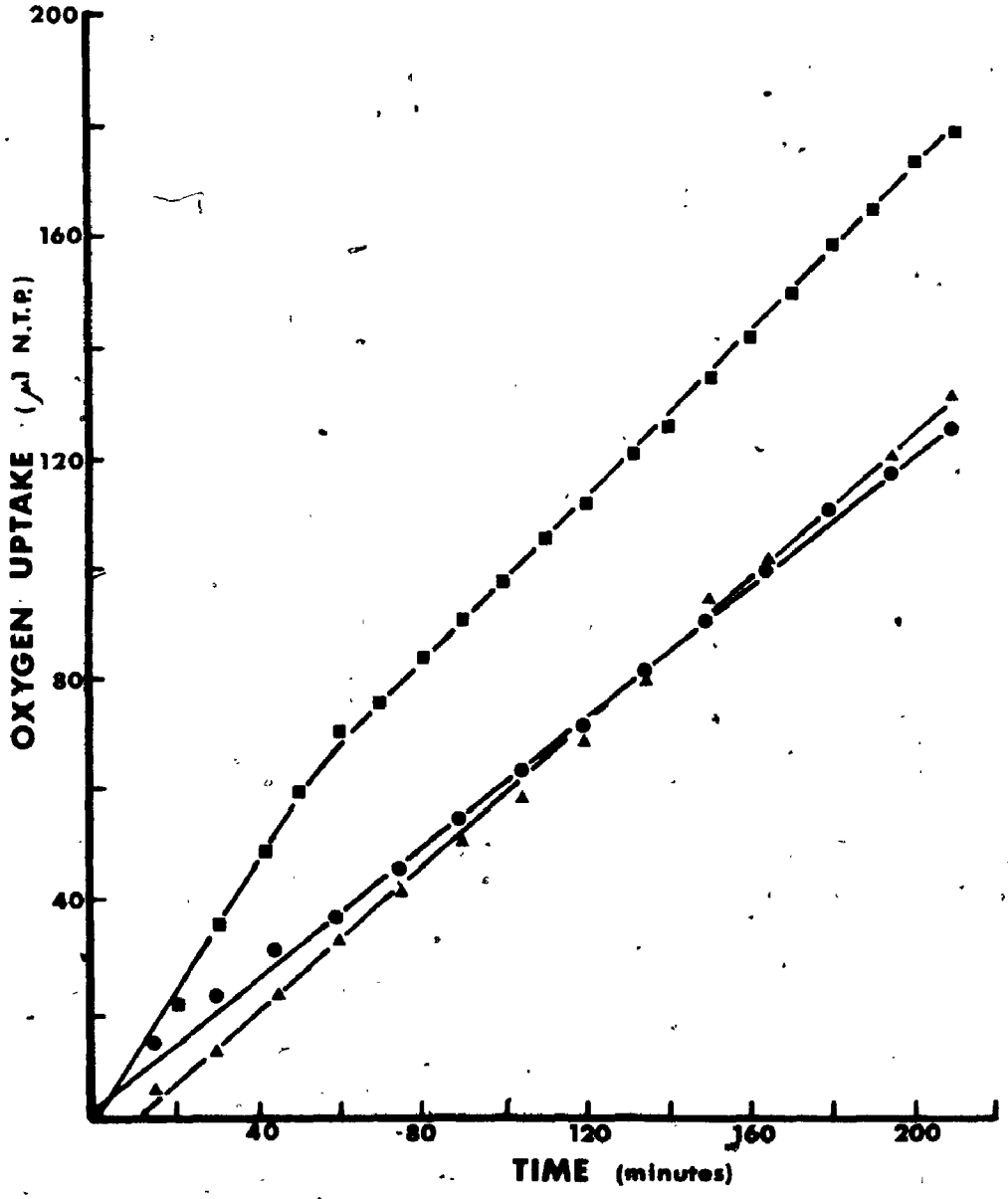
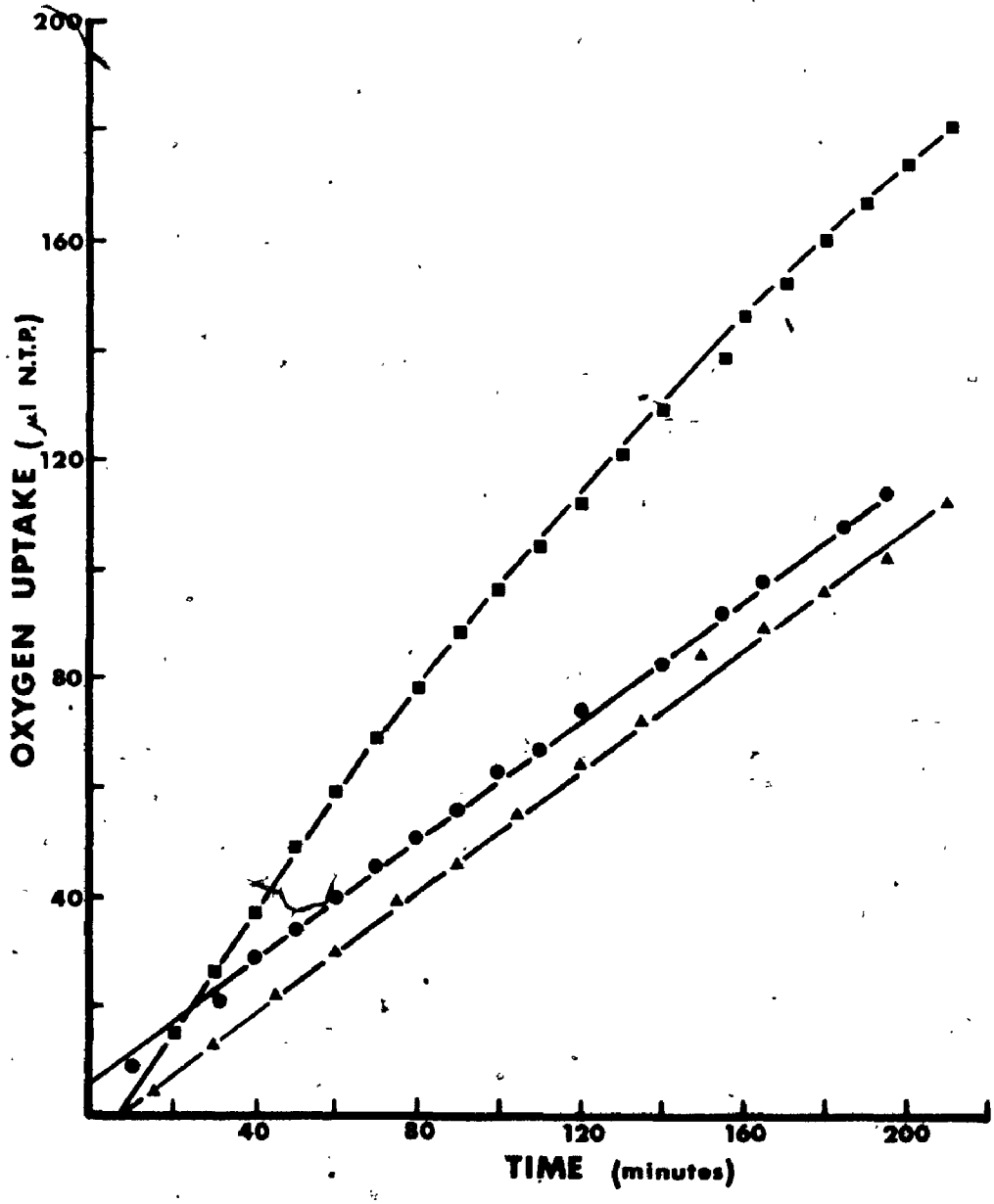

200
160
120
80
40
OXYGEN UPTAKE (µl) N.T.P.
40
80
120
160
200
TIME (minutes)

Figure 4-9. Oxygen uptake by resting cells of Acremonium 3E/1 in the presence of possible intermediates of n-butane oxidation at 30°C. -■- 4 µmoles 1-butanol; -●- 4 µmoles butyraldehyde; -▲- 4 µmoles sodium butyrate; each point the mean of three replicates; endogenous oxygen uptake subtracted (18 µl hr^{-1}).


200
160
120
80
40
OXYGEN UPTAKE (µl N.T.P.)
40
80
120
160
200
TIME (minutes)

oxygen per µmole of alcohol (means) respectively for ethanol, 1-propanol and 1-butanol. With 1-propanol and ethanol, this second phase was also linear. With ethanol, after a short linear phase, uptake ceased. A steadily decreasing second rate of uptake was observed for 1-butanol.

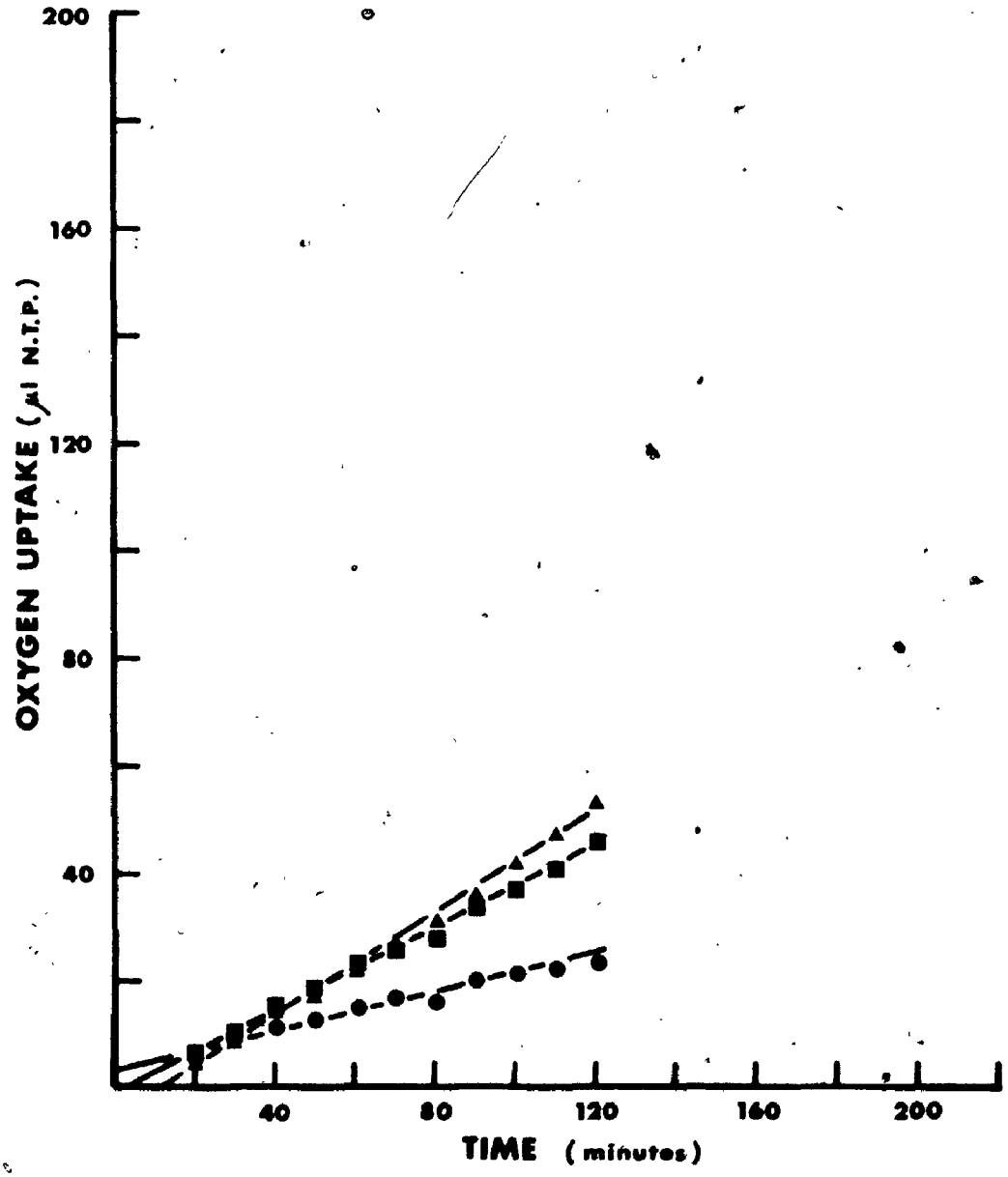
The aldehydes and fatty acids all showed only one phase of linear oxygen uptake. The rates of uptake (means) were all similar,

acetaldehyde	;	17.6 µl mg^{-1} hr^{-1}
acetate	;	24.3 "
propionaldehyde	;	17.6 "
propionate	;	18.9 "
butyraldehyde	;	19.1 "
butyrate	;	17.2 "

but they were markedly lower than the initial rates recorded for the primary alcohols.

Oxygen uptake was measured in the presence of methanol and the three and four-carbon secondary alcohols (there is no two-carbon secondary alcohol) in two separate experiments. Data from a representative experiment are presented in Figure 4-10. The mean substrate-dependent rates of oxygen uptake for the two experiments were: methanol, 5.2 µl mg^{-1} hr^{-1}; 2-propanol, 11.1 µl mg^{-1} hr^{-1}; 2-butanol, 14.4 µl mg^{-1} hr^{-1}. The rate on methanol is 13.8% of the initial

Figure 4-10. Oxygen uptake by resting cells of Acremonium 3E/1 in the presence of alcohols at 30°C. -▲- 4 µmoles 2-butanol; -■- 4 µmoles 2-propanol; -●- 4 µmoles methanol; each point the mean of three replicates; endogenous oxygen uptake subtracted (16 µl hr^{-1}).

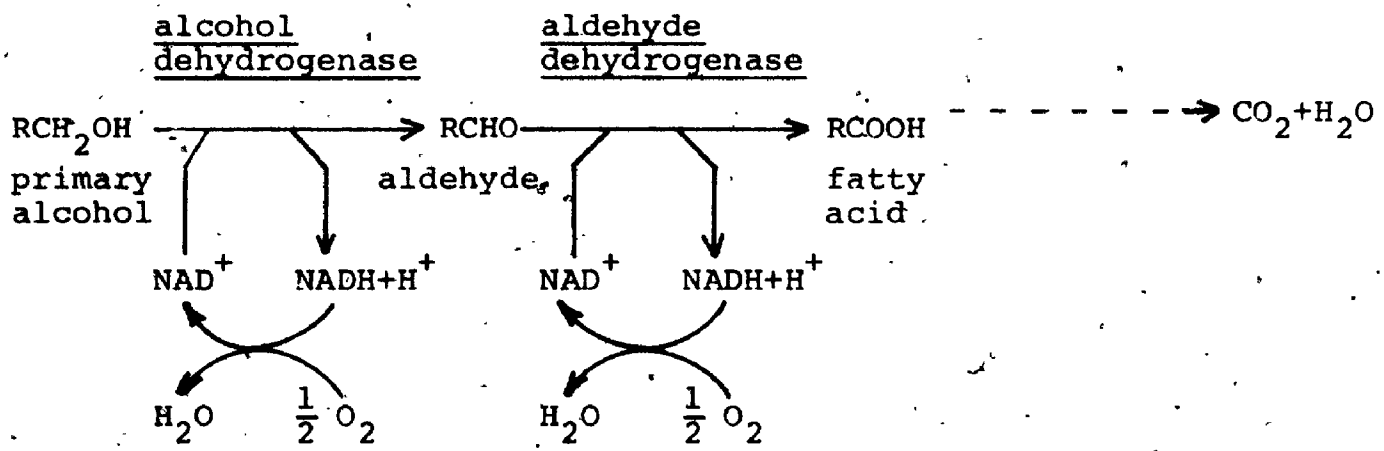

200
160
120
80
40
OXYGEN UPTAKE (µl N.T.P.)
40
80
120
160
200
TIME (minutes)

rate on ethanol. The rates on 2-propanol and 2-butanol are respectively 31.3% and 41.2% of those on their primary isomers.

A marked similarity in both form and rate can be seen between the oxygen uptake curves for two, three and four carbon homologues in the case of each functional group (i.e. the alcohol curves are similar, as are the aldehyde curves, etc.). A common initial pathway can be suggested (Fig. 4-11) for oxidation of all three primary alcohols to the fatty acid level of oxidation involving pyridine nucleotide-linked (probably NAD^+) alcohol and aldehyde dehydrogenases. The three homologues share a common stoichiometry of oxidation up to this point; oxidation of alcohol to aldehyde and aldehyde to fatty acid both require 0.5 µmole of molecular oxygen per µmole of substrate.

All three alcohols show diauxic curves for oxygen uptake by resting cells, indicating that oxygen consumption is dependent upon more than one biochemical reaction. The similarity in uptake rates of all three and the close agreement as to the 'break-point' in uptake rate (after consumption of 0.70 - 0.80 µmole oxygen per µmole of alcohol) suggest a common pathway up to this point.

The stoichiometry of complete enzymatic oxidation of the alcohols to carbon dioxide and water is shown in Figure 4-11. Oxygen consumption proceeded to completion only in the two-carbon substrates. Complete oxidation of ethanol, acetaldehyde and acetate to carbon dioxide and water would



$$CH_3COOH + 2\ O_2 \longrightarrow 2\ CO_2 + 2\ H_2O$$

acetic acid

$$C_2H_5COOH + 3\tfrac{1}{2}\ O_2 \longrightarrow 3\ CO_2 + 3\ H_2O$$

propionic acid

$$C_3H_7COOH + 5\ O_2 \longrightarrow 4\ CO_2 + 4\ H_2O$$

Figure 4-11. Pathway for primary alcohol oxidation.

result in the consumption of respectively 3.0, 2.5 and 2.0 μmoles of oxygen per μmole of substrate. Since the actual figures recorded were 1.43, 1.05 and 0.99 μmoles of oxygen consumed per μmole respectively (means of two experiments) it is apparent that oxidation did not proceed to completion even where oxygen uptake had ceased. It is also apparent that oxidation of ethanol, 1-propanol and 1-butanol proceeded beyond the fatty acid level; oxidation to this point would only require consumption of 1.0 μmole of oxygen per μmole of alcohol; this was exceeded in all three cases.

Corresponding primary alcohols, aldehydes and fatty acids have been implicated as intermediates in the functional oxidation of gaseous alkanes by Acremonium 3E/1. Thus a terminal pathway of alkane oxidation is indicated. These same compounds are intermediates in all proposed terminal alkane oxidative pathways including those beginning with initial dehydrogenation (Fig. 2-1). The apparent inability of Acremonium 3E/1 resting cells to consume ethylene and 1-butene does not support dehydrogenation as the first step in functional oxidation of ethane and n-butane respectively (although the high concentrations used might have been inhibitory). Their inability to consume gaseous alkanes in the absence of molecular oxygen argues for a first step involving oxygen, such as hydroxylation.

Consumption of propylene is difficult to explain. Quite possibly this is an example of non-functional oxidation of the type discussed in sections 2.4 and 2.5.

CHAPTER 5

PRODUCTION OF LABELLED INTERMEDIATES BY RESTING CELLS SUPPLIED WITH [1,2-^{14}C] ETHANE

5.1. Introduction:

Manometric studies indicated that the terminal pathway via primary alcohol, aldehyde and fatty acid was operative in resting cells of Acremonium 3E/1 while oxidizing gaseous alkanes. Proof of such a pathway however depends upon demonstration of the production of biochemical intermediates by cells or enzymes from such cells when supplied with a specific alkane substrate. To this end it was decided to supply resting cells with the major growth substrate in the natural gas supply, ethane, in which the carbon skeleton itself was labelled with carbon - 14 and attempt to follow the initial pathway of labelled carbon in the cell.

5.2. Materials and methods:

5.2.1. Resting cells:

It was not possible, as originally intended, to grow resting cells as previously described (3.2.5.2) upon natural

gas from the domestic line. In October 1972 Acremonium 3E/1 ceased to grow under the previously described conditions, as did the other isolates described in Chapter 3. Tests eliminated the distilled water supply, chemicals and air supply and indicated a problem associated with the natural gas supply.

Quantitative analysis by gas chromatography of natural gas from the line revealed that ethane was still present in adequate concentration for growth, indicating the presence of an inhibitor rather than a lack of growth substrate. Examination of current detailed analysis reports, obtained from Union Gas Ltd., revealed relatively high levels of sulphur compounds for the period when this problem first arose, of the order of 0.09 - 0.22 grains per 100 ft^3 (2.09 - 5.1 $\mu g\ l^{-1}$). Previous analysis-sheets for gas able to support fungal growth contained no reference to detectable sulphur. Quite possibly one or more of the sulphur compounds present was responsible for inhibition of growth. Subsequent correspondence with Union Gas confirmed that natural gas supplies had recently been subject to fluctuation in sulphur content at source (Alberta) for technical reasons. Sulphur levels as high as 0.6 grains per 100 ft^3 (13.92 $\mu g\ l^{-1}$) had been experienced at that time. Moreover, beyond a contractual maximum of 20 grains per 100 ft^3 (464 $\mu g\ l^{-1}$), no assurance could be given that continued elevated sulphur levels would not be experienced in the future.

Monitoring of the gas supply over a period of many

weeks revealed fluctuations in its ability to sustain fungal growth; this ability returned for periods as long as two weeks then reverted once more. The active agent was fungistatic rather than fungitoxic since inoculated cultures, inhibited for three or four weeks, would grow when suitable conditions returned. Attempts to remove inhibitory compounds from natural gas with activated carbon were unsuccessful.

It was decided reluctantly that cell culture for future experiments upon natural gas would have to be abandoned as too unreliable; an alternate gaseous alkane growth substrate was needed. Subsequently resting cells of Acremonium 3E/1 were cultured as before except that a mixture of 20% ethane (C.P. grade from Matheson of Canada Ltd.) in air was passed through the plexi-glas box instead of natural gas. The same high growth rates and cultural characteristics were found with ethane as were previously recorded with natural gas.

A problem of fungistasis also appeared with culture upon ethane; not all cylinders of ethane would support fungal growth. Analysis of different batches of ethane both in our laboratory and in that of Matheson Ltd. (by gas chromatography) revealed that those with relatively high levels of ethylene would not support growth while those with lower levels would. C.P. ethane as supplied by Matheson Ltd. is produced by catalytic reduction of ethylene, hence high residual ethylene levels are not unlikely.

Once the problem was identified, low-ethylene ethane batches were selected for delivery by Matheson and no further trouble was encountered. Cell suspensions for radiotracer experiments were produced as before (sect. 4.2.1).

5.2.2. Exposure of cells to labelled ethane:

[1,2-^{14}C] ethane was supplied by Schwartz/Mann, Orangeburg, New York in breakseal vials containing 500 microcuries at a specified specific activity of 20 millicuries per millimole. This was diluted manometrically in a leak-tight glass vessel with approximately 550 ml of a mixture of 10% unlabelled ethane (Research grade purity, Matheson Ltd.) in air (Fig. 5-1).

The labelled gas mixture was dispensed as required from this storage vessel as shown in Figure 5-1. A 100 ppm mercuric chloride solution (to prevent microbial growth) was introduced into the vessel by syringe, thereby displacing gas out of the sidearm via a fine bore (1/32") Tygon tube into a 'gas pipette'. The latter consisted of a truncated 10 ml graduated pipette equipped with a rubber septum at one end; gas was measured out, as required, by displacement of a measured volume of water from the water-filled pipette.

One millilitre of cell suspension containing 4 mg dry weight of resting cells was exposed to 5 ml of gas mixture in a 10 ml gas-tight syringe (Hamilton Co., Whittier, Calif.) equipped with a detachable valve (Fig. 5-1). A

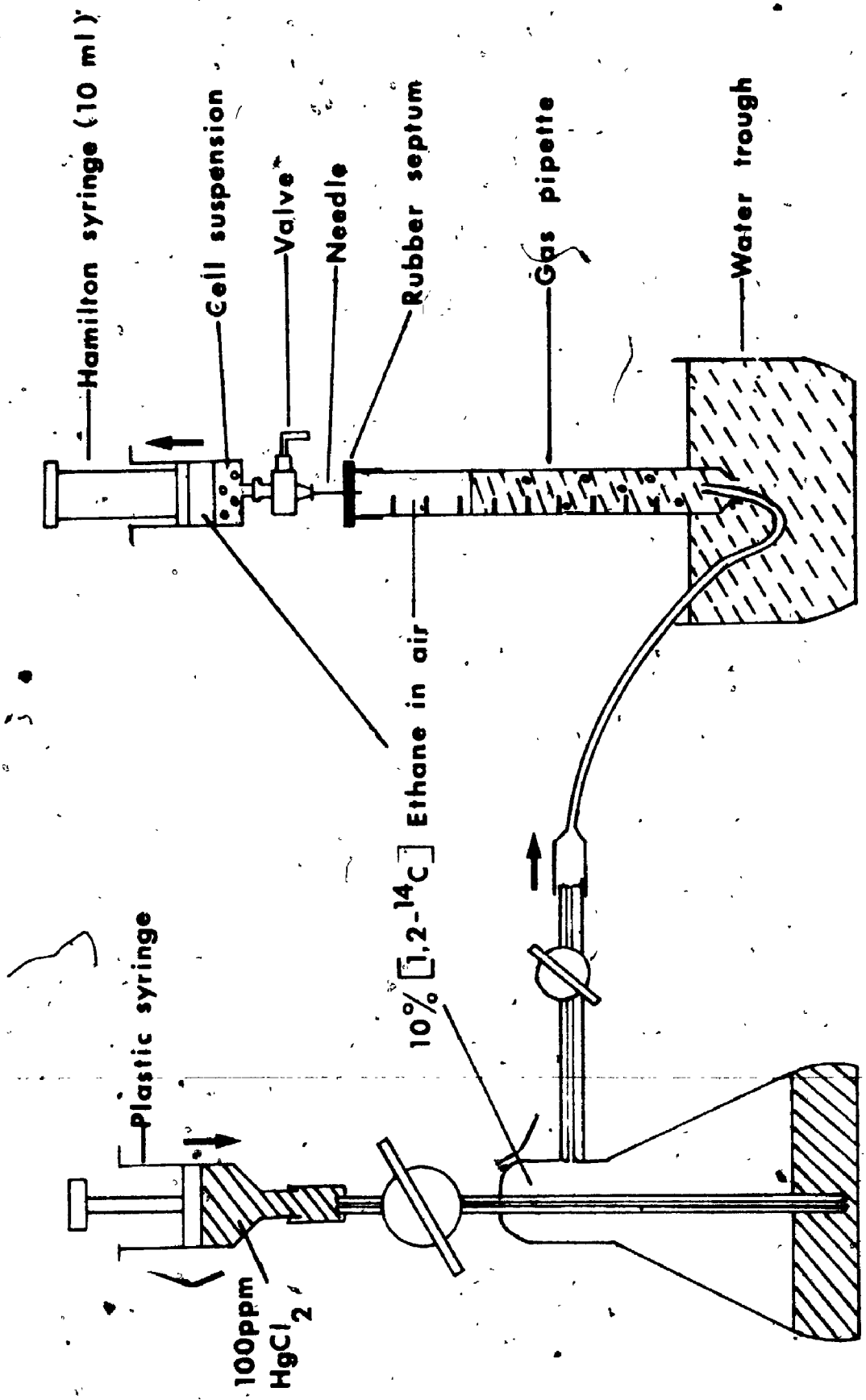


Figure 5-1. Apparatus for storage and transfer of [1,2-^{14}C] ethane and air gas mixture.

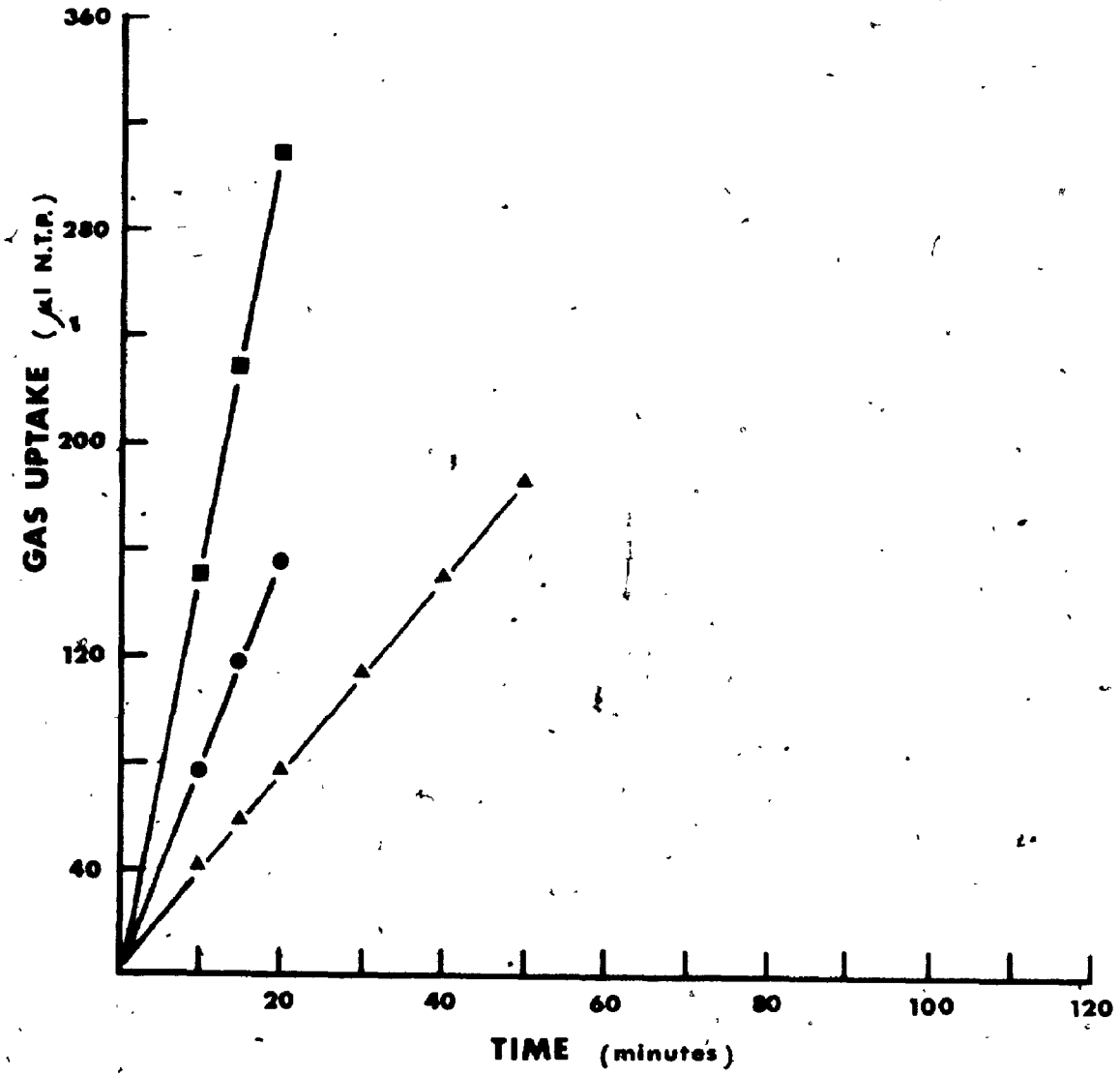
high cell concentration was desirable in order to produce the highest possible rate of uptake of label in a small volume in a short time. A preliminary manometric experiment (Fig. 5-2), with 10% ethane (Research grade) in air, showed that the rate of gas uptake remained linear with time and was not reduced by increasing the cell concentration from one to four milligrams per millilitre.

Charging a syringe was accomplished as follows: a valve was attached to the barrel of a disassembled syringe and closed. One ml of cell suspension was then pipetted into the barrel. The plunger was replaced, depressed a little and the syringe oriented tip up before the valve was opened and all air expelled. A fine bore septum penetration needle was attached to the Luer tip of the open valve. The needle was then forced through the rubber septum of the previously gassed 'gas pipette' and 5 ml of gas mixture was introduced into the syringe by withdrawing the plunger until the gas pipette water level had risen 5 ml. The syringe valve was then closed and the needle detached. The charged syringe was immediately attached to a cradle which was shaken in a thermostatically-controlled water bath shaker (Gilson Medical Electronics Co., Middleton, Wisconsin) at 30°C for a fixed period of time.

5.2.3. Extraction of labelled compounds from cells:

The predicted intermediates in the initial oxidation of ethane were ethanol, acetaldehyde and acetic acid. It

Figure 5-2. Effect of cell concentration upon gas uptake in an atmosphere of 10% ethane, 90% air by resting cells of Acremonium 3E/1 at 30°C. -■- 4 mg ml^{-1}; -●- 2 mg ml^{-1}; -▲- 1 mg ml^{-1}; each point the mean of three replicates; endogenous subtracted (4 mg ml^{-1} - 112 µl hr^{-1}, 2 mg ml^{-1} - 66 µl hr^{-1}, 1 mg ml^{-1} - 24 µl hr^{-1}).


360
280
200
120
40
GAS UPTAKE (µl N.T.P.)
20
40
60
80
100
120
TIME (minutes)

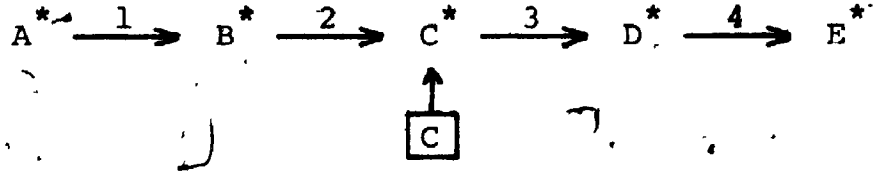
was necessary to identify the presence of carbon - 14 in these compounds in extracts of cells oxidizing [1,2-^{14}C] ethane. (N.B. this is not 'carrier-free' [1,2-^{14}C] ethane but unlabelled ethane enriched with carbon - 14 as outlined in sect. 5.2.2.). The compounds in question are somewhat volatile, having boiling points of 78.5, 20.8 and 118.5°C respectively. The analytical method of choice for identifying such compounds is gas chromatography. The choice of a suitable acid for extraction of the cells had to be made with this in mind. Strong mineral acids such as hydrochloric, sulphuric or perchloric acid were avoided because of the possible corrosive effect of their hot vapours upon the metal parts of the chromatograph and upon the liquid phase of the column itself. Trichloracetic acid is a strong organic acid but its high boiling point (197.5°C) would require using excessive temperatures to remove it from the column. A solution of 10% (wt/vol) formic acid in water was eventually employed. Formic acid is the strongest of the fatty acids (pKa = 3.77), has a boiling point of 100.7°C and is completely undetectable to a flame ionization detector.

At the end of an experiment syringes were removed from the shaker in turn and excess gas expelled; the cell suspension from each syringe was then ejected into a small Pyrex test tube containing 0.25 ml of 60% (wt/vol) formic acid plus 0.25 ml of a solution containing 12% (vol/vol) each of ethanol, acetaldehyde and acetic acid on ice.

This gave a suspension of cells in approximately 10% (wt/vol) formic acid and 2% (vol/vol) of the other three compounds (some loss of cell suspension occurs during ejection from the syringe). The final volume was measured to estimate the loss from ejection. The tubes of suspension, covered with 'Parafilm', were extracted on ice for one hour, at which time the cells were centrifuged down at 10,000 g for 15 minutes and the supernatant (acid extract) pipetted off.

The purpose of the ethanol, acetaldehyde and acetic acid was to effect 'coprecipitation' of labelled compounds. The rationale is that the probably minute amounts of radioactive compounds are more easily detected and collected if large amounts of the 'cold' suspect compounds are added to the extraction medium, the 'hot' compounds being carried along with such 'carrier' compounds.

Use was also made of the technique of 'isotope trapping' the theory of which is outlined as follows. A relatively large amount of an unlabelled suspected intermediate of a particular substrate is provided during metabolism of the labelled substrate.



In the above example the labelled substrate A^* is metabolized via labelled intermediates B^*, C^*, D^* and E^* by en-

zymes 1, 2, 3 and 4 in turn. Normally C^* would be rapidly converted to D^* without accumulating. If however 'cold' C is supplied during metabolism of A^* then C^* will be considerably diluted. Since C predominates over C^*, mainly C molecules are converted to D, allowing C^* to accumulate faster than it is removed. Another possible effect is that added C can bring about an accumulation of B^* by upsetting the equilibrium of the reaction catalysed by enzyme 2. Suppression of the accumulation of labelled intermediates further down the pathway can also occur.

Activity of the acid extracts and percentage activity extracted from the cells was estimated as follows prior to gas chromatographic analysis. The residual pellet of extracted cells was washed by centrifugation and suspended in 1.0 ml of distilled water. The 1.0 ml of cell suspension was pipetted into a scintillation vial. Two 0.1 ml samples of acid extract were each pipetted into separate vials. Each low-background glass vial contained 10 ml of an ice-cold liquid scintillation cocktail, 'Aquasol' (New England Nuclear, Boston, Mass.). This cocktail can accept high concentrations of water without formation of an emulsion and has high quench resistance.

Vials were placed in a Beckman liquid scintillation counter and the activity in counts per minute measured until the two sigma-statistical-counting-error displayed by the instrument fell to 2% of the gross rate. Prior to counting a quench correction was made by adjusting the gain

so that the external standard ratio was close to 1.000 for each vial. The background counting rate was subtracted to give the net sample counts per minute (cpm).

An approximation of the efficiency of extraction of label was made as follows for an extract of a given experiment.

If X = the mean volume of acidified cell suspension of the syringes in ml

and Y = mean net cpm per 0.1 ml acid extract

and Z = net cpm per 1 ml of washed cell suspension then extraction efficiency =

$$\frac{\left[Y \times \frac{X}{0.1}\right] \times 100}{\left[Y \times \frac{X}{0.1}\right] + Z}$$

In any given experiment [1,2-^{14}C] ethane oxidation was followed over a period of one hour. Individual reaction vessels (syringes) were removed from the shaker at the required time intervals. A technical problem was encountered with 'Aquasol' itself; it was found that when exposed to high-colour-temperature-light-sources, such as daylight or fluorescent tubes, extremely high count rates were observed for several hours after such exposure. The problem was overcome by leaving vials so exposed in the dark for 24 hours after which time such activity had subsided. They were subsequently exposed only to lower-

colour-temperature-tungsten-filament-light-sources when necessary. After initial estimation of activity, acid extracts were sealed and stored at -20°C till required for radiochromatographic analysis.

5.2.4. Radiochromatography:

In principle the method used was simple; the gas chromatograph was in effect used as a high resolution distillation apparatus to separate and collect different components of a mixture of volatiles which were later assayed for radioactivity. The actual amounts of individual compounds collected were irrelevant since they consisted almost entirely of added standards which were merely vehicles for isolation of labelled compounds. The important figures were the relative levels of radioactivity in the different components; such figures quantitate the conversion of [1,2-^{14}C] ethane into these component compounds.

A Hewlett-Packard model 7610 high efficiency gas chromatograph, fitted with a flame ionization detector was used for all assays. The instrument was equipped with a 6' x 3/8" copper U-tube column packed with 60/80 mesh Chromosorb WAW coated with 20% (wt/wt) Carbowax 20 M. The detector end of the column was joined to the detector by a Hewlett-Packard model 19034A effluent splitter with a two way 1:11 split ratio. The smaller stream of carrier gas flowed to the detector for detection of components as they emerged from the column; the larger fraction passed out of

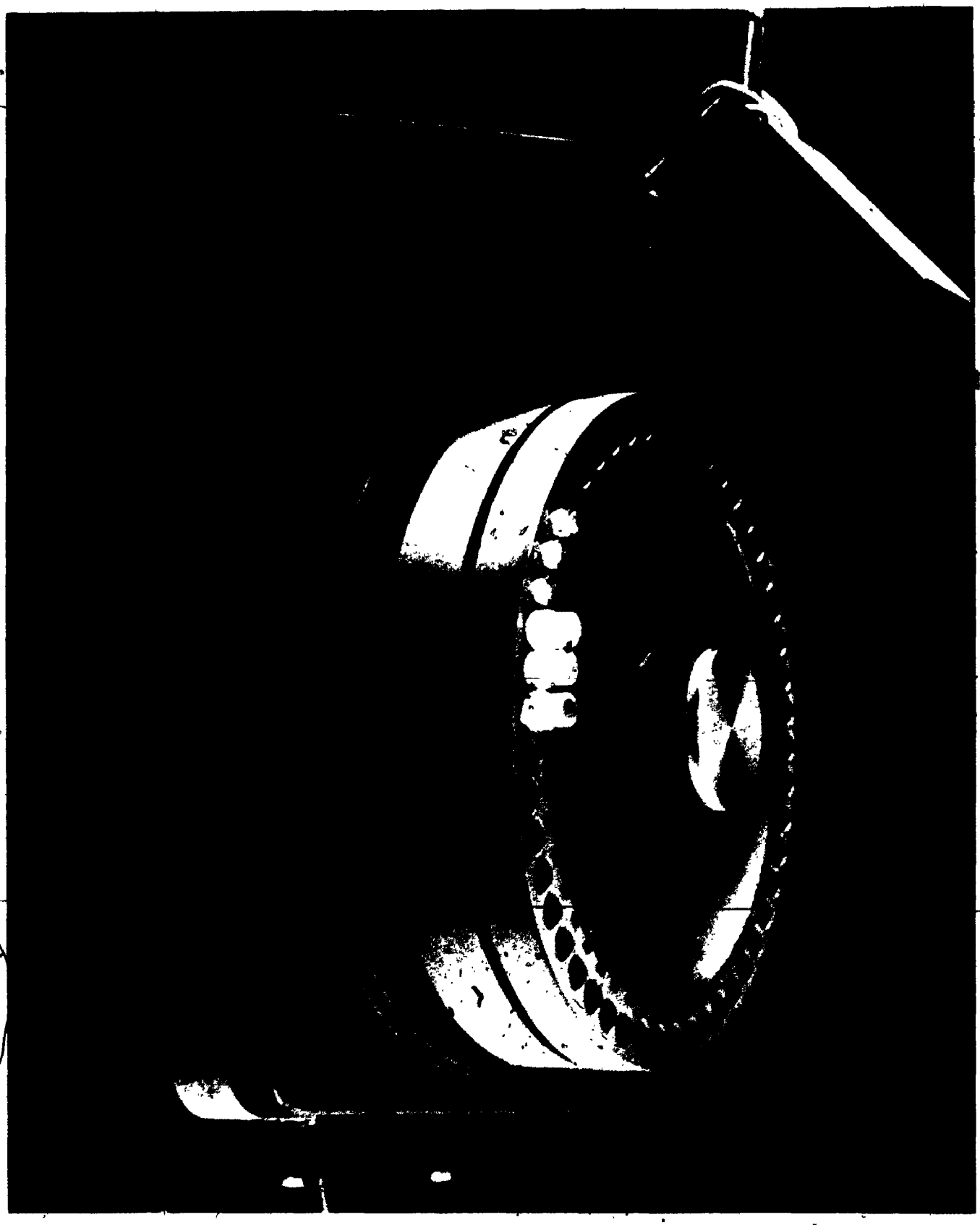
the column oven via a heated stainless steel tube to a Packard model 852 gas fraction collector mounted at one side of the column oven. The fraction collector (Pl. 5-1) consists of a solid aluminum turntable with a central well for coolant and 50 holes to hold cylindrical glass cartridges.

Effluent carrier gas from the splitter is channelled to the heated moveable head of the collector from which it emerges at a nozzle. A gas-tight connection can be made between the outlet nozzle and each cartridge by means of a silicone rubber gasket; the effluent stream can be diverted from one cartridge to the next in sequence by the simultaneous rotation of the turntable and raising and lowering of the head. The device can be activated by a remote switch to direct the effluent containing a particular component (as indicated by the recorder pen) into a particular cartridge. Cartridges contain glass beads upon which vapours in the hot carrier gas stream condense.

Chromatograph parameters were standardized as follows: nitrogen carrier gas, 60 ml per minute; make up gas (nitrogen to replace carrier gas flow to detector lost through the splitter), 60 ml per minute; injection port, 220°C; detector block, 200°C; tube from splitter and collector head, 150°C. Twenty microlitre samples of acid extracts were injected onto a column maintained at 85°C. At the same time a few microlitres of natural gas were injected to give a marker peak, used as a starting point for calcula-

PLATE 5-1

Packard model 852 gas fraction collector.



tion of corrected retention times (corresponds to the air peak produced by a thermal conductivity detector). Immediately after injection of the sample a temperature program was initiated: 8 minutes isothermal at 85°C was sufficient to bring off the low-boiling-point compounds such as ethanol and acetaldehyde; the oven temperature was then raised at 10°C per minute to 140°C and held there till some time after acetic acid had emerged on this temperature plateau.

Initially severe problems of 'tailing' were encountered (asymmetry of peaks with the rear edge trailing behind) with all three components, reducing resolution quite drastically (the trailing edge of one peak merging with the leading edge of the next). The problem was diagnosed as over-interaction of large amounts of very polar sample components with an extremely polar column. Another problem was 'ghosting'. If a sample containing only 10% (wt/vol) formic acid were injected after a sample containing acetic acid had passed through the column then a small peak would appear with the same retention time as acetic acid, a 'ghost peak'. Small amounts of acetic acid stick to the column and are displaced later by a more polar compound, formic acid.

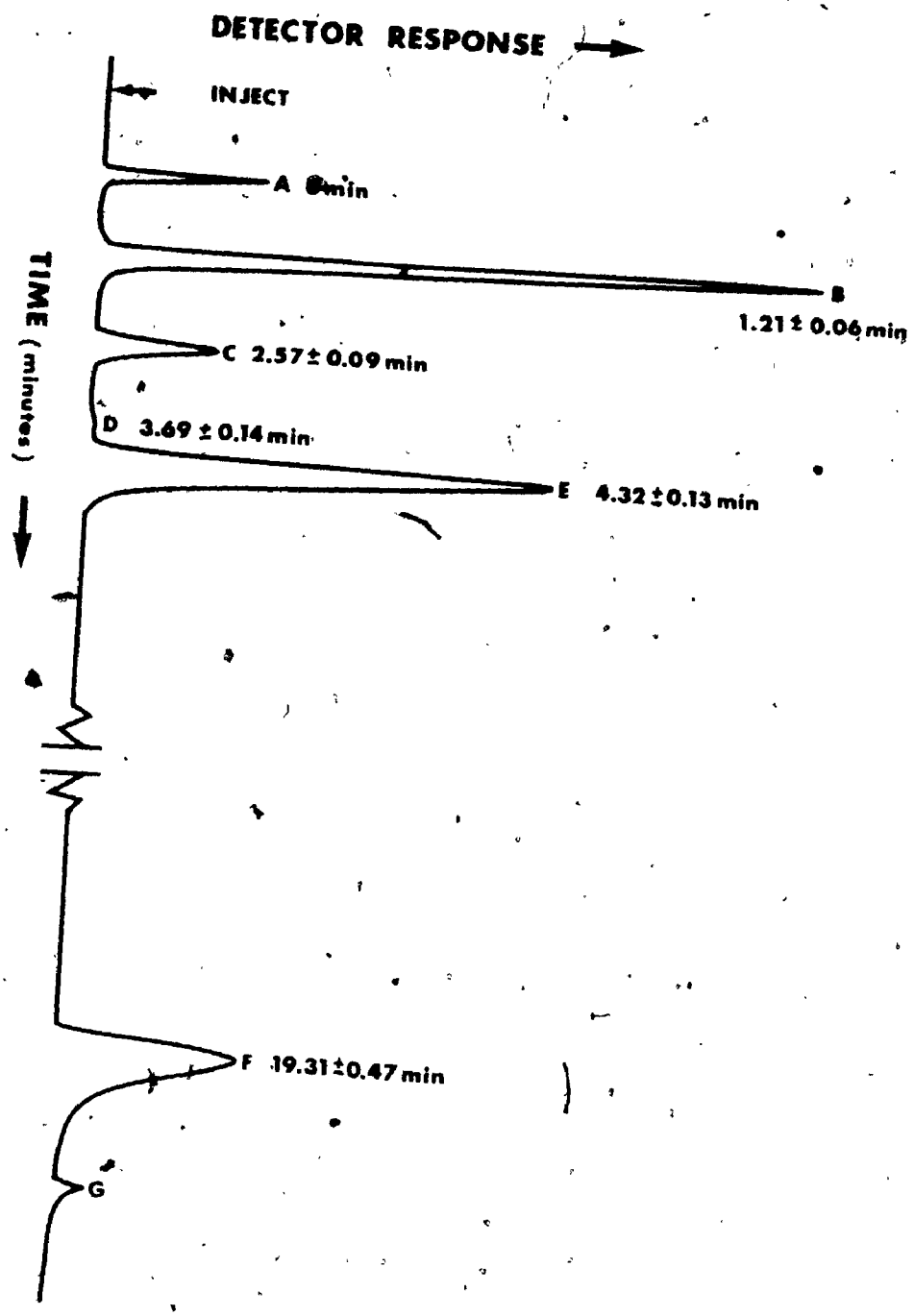
A solution to both these problems was eventually found. Before starting analysis on any given day, three separate 2 µl samples of pure formic acid were injected onto the column at two minute intervals; a temperature

program was then started and run to completion. Active sites on the column which would otherwise interact with sample components causing tailing and ghosting were saturated by formic acid thus allowing normal sample peak formation. It was found that to keep the column 'in condition' two complete programs needed to be run between successive samples each with one injection of 2 µl of pure formic acid.

A typical chromatogram of a 20 µl acid extract injection is seen in Figure 5-3; all chromatograms had essentially the same appearance since all samples had approximately the same chemical composition. Peak A is the natural gas marker injected with the sample; mean corrected retention times of sample peaks were calculated using this peak as time zero. Each value is the mean of six replicates, with Standard Deviation. Such 'corrected' values are more repeatable and reliable than 'true' retention times from the actual time of injection itself. Peaks B, E and F are the principle compounds of interest, acetaldehyde, ethanol and acetic acid respectively; they are easily distinguished from each other by their characteristic shapes and of course their retention times.

Peak C was identified as ethyl formate by comparison with an authentic standard. It is formed by reaction of ethanol and formic acid. Formation of this secondary product was followed for confirmation; when ethanol and formic acid in aqueous solution were freshly mixed it was absent

Figure 5-3. Typical gas chromatogram of an acid extract of resting cells showing corrected retention times of sample components (means of six determinations with Standard Deviations). A-natural gas marker used as time 0 for corrected retention times; B-acetaldehyde; C-ethyl formate; D-ethyl acetate; E-ethanol; F-acetic acid; G-impurity from column.


DETECTOR RESPONSE
INJECT
A 0 min
B
1.21 ± 0.06 min
C 2.57 ± 0.09 min
D 3.69 ± 0.14 min
E 4.32 ± 0.13 min
TIME (minutes)
F 19.31 ± 0.47 min
G

but slowly increased with time, reaching a maximum in a few days after which time it remained stable. Since this secondary reaction could reduce the activity of the ethanol fraction, ethyl formate peaks were also collected and counted. Very slight amounts of ethyl acetate (peak D) were formed in a similar way. Peak G is unidentified but is thought to be a breakdown product of Carbowax 20M, possibly from the action of hot formic acid since it also appears when formic acid alone is injected and increases in size with advancing age of the column regardless of which sample is injected.

Collection of individual peaks was effected as follows. Individual cartridges were plugged at the bottom with half a cigarette filter and filled with #12 Ballotini glass beads (Brinkman Instruments Canada Ltd., Rexdale, Ontario). When a connection was made between a cartridge and the collector head, the carrier gas stream flowed freely through the beads and out of the bottom of the cartridge; volatile materials condensed upon the considerable surface area of these cooled beads. Preliminary trials indicated that dry ice (-78.5°C) was an adequate coolant for trapping ethanol and acetic acid vapours but was totally inadequate for acetaldehyde. For this reason liquid nitrogen (-195.8°C) was used as a coolant as the first volatiles emerged from the column; by the time ethanol had emerged, the liquid nitrogen in the central well of the collector had almost evaporated and was replaced by

ground dry ice for collection of subsequent fractions.

Individual peaks (including C and D) and the intervening base lines were collected in separate cartridges. The following sequence of operations was followed during collection of fractions.

a) Cartridges, capped with plastic closures (to prevent frost accumulation on the beads), were placed in the turntable and the first one aligned next to the collector head.

b) The turntable well was filled with liquid nitrogen from a Dewar bottle until rapid boiling ceased, indicating that the turntable was cooled.

c) The closures were removed from the first half dozen cartridges in line.

d) A 20 µl sample was injected onto the column with natural gas and the temperature program initiated.

e) Upon appearance of the natural gas peak the remote switch was activated bringing the first cartridge under the head.

f) The second cartridge was brought into position as soon as the recorder pen was seen to rise from the baseline for the acetaldehyde peak and was left in position until the pen came down to baseline once more at which time the next cartridge was placed in position etc.

g) As soon as a cartridge moved past the head it was recapped.

h) The final cartridge was left under the head for

some time after collection of the acetic acid peak to collect any slight acetic acid 'tail' or any high boilers which might come off after acetic acid.

i) Cartridges were removed from the turntable and their contents tipped into individual pre-chilled, low-background glass vials, each containing 10 ml of ice-cold 'Aquasol'. Vigourous shaking dissolved the condensate in the cocktail after which the beads were allowed to fall to the bottom of the vial.

No peaks for water or formic acid are apparent in Figure 5-3; this is not because these compounds come off the column later than the others but because both are invisible to the flame ionization detector; this is because water will not burn and formic acid burns so poorly. Such a situation is of course an advantage in this particular application.

A major consideration was to keep the activity of the sample high enough to be able to count individual fractions accurately; 20 µl was found to be the maximum that the column would accept. Injections larger than this gave rise to severe tailing and hence poor resolution.

The effluent gas stream from the detector contained small amounts of labelled compounds, principally $^{14}CO_2$. An extraction fan connected to a flexible, wide hose was mounted above the detector outlet to carry exhaust gases to a nearby fume hood.

5.2.5. Statistical considerations of liquid scintillation counting:

The background count rate of a low background vial containing 10 ml of 'Aquasol' and the beads from one cartridge was recorded as 67.9 cpm. Without the beads the normal background rate would be approximately 50 cpm. Quite possibly the beads were made from a glass with a relatively high potassium content which could give rise to the elevated background rate. Sample vials were routinely counted until the two sigma (2 σ) error displayed by the instrument fell to 2% of the gross count rate.

From Wang and Willis (1965) the best estimate of the standard deviation of the counting rate (σ) is:

$$\sigma = \sqrt{\frac{r}{t}} \qquad \text{(i)}$$

where r = count rate in counts per minute (cpm)

and t = counting time in minutes.

The Standard Deviation of the net sample counting rate (gross rate minus background rate)

$$\sigma_s = \sqrt{\frac{r_g}{t_g} + \frac{r_b}{t_b}} \qquad \text{(ii)}$$

where r_g = gross count rate

t_g = counting time for r_g

r_b = background count rate

t_b = counting time for r_b

In practice it is found that where the ratio of the gross rate to the background rate exceeds 10 the contribution from the background standard deviation is insignificant and the following formula can be used:

$$\sigma_s = \sqrt{\frac{r_g}{t_g}} \qquad \text{(iii)}$$

Where this ratio is less than 10 however formula (ii) should be used.

Due to the small injections made necessary by the constraints of the system many of the gross count rates fell into this latter category. For the counting data presented in the next section (5.3) σ_s was calculated by formula (ii) or (iii), whichever was appropriate. Two Standard Deviations (2 σ) were then expressed as a percentage of the net sample count rate. For example if net sample count rate = 329.6 cpm; σ_s = 4.06; 2 σ = 8.12 = 2.5% of 329.4 therefore 329.4 $\pm$ (2.5%). It can be stated therefore from known statistical relationships that there is 95.45% probability that the true count rate of the sample lies within the limits, measured cpm $\pm$ 2 σ. Gross count rates of less than 100 cpm were rejected.

5.2.6. Collector efficiency:

An estimate of the efficiency of the system was achieved as follows. A standard 2% (vol/vol) solution of ethanol in water was prepared enriched with [1-^{14}C] ethanol

(New England Nuclear, Boston, Mass.). Replicate 20 µl samples were injected onto the column in an 85°C isothermal oven and each ethanol peak collected in the usual way in a single cartridge. Activity recovered from the cartridge could be compared with the activity of replicate 20 µl samples injected directly into vials of 'Aquasol'. In this way loss of sample to the detector via the splitter and from collection and transfer to the vial was taken into account.

Table 5-1 is self explanatory; a mean 82.5% efficiency was achieved in trapping of the label of [1-^{14}C] ethanol. Some variation was encountered due to use of a 100 µl syringe which does not have the same reproducability as a pipette. The efficiency was measured with the effluent splitter maintained at 85°C in the column oven; when acetic acid was collected from acid extracts however the temperature of the oven and hence the splitter had reached 140°C. An efficiency of 82.5% can only be held valid at both temperatures if the split ratio of the splitter remains constant at both temperatures. Calibration of the splitter (by flow measurement using a soap bubble flow meter) revealed that it was in fact constant; a ratio of 1:11 was found at both temperatures.

5.3. Results and discussion:

Data are presented in Tables 5-2 to 5-7 and in Figures 5-4 to 5-9. In the tables the net activity (cpm) per

Table 5-1. Efficiency of collection of [1-^{14}C] ethanol standard.

	Net cpm of [1-^{14}C] ethanol and counting error[a]		
	Collected in cartridge	20 µl of standard solution	% activity collected
Trial 1	2,543 (± 2.1%)	3,145 (± 2.0%)	
Trial 2	2,507 (± 2.1%)	2,976 (± 2.0%)	
Mean	2,525	3,061	82.5%

[a]Two Standard Deviations (2 σ) expressed as a percentage of net sample count rate.

fraction of a 20 µl sample of acid extract is presented together with the counting error calculated as per section 5.2.5. for acetaldehyde, ethanol, ethyl formate and acetic acid (no significant activity was recorded in the ethyl acetate peak nor in any of the baseline fractions between the peaks at any time). In the figures, graphs of ethanol activity are plots of the sum of activities of ethanol and ethyl formate at particular time intervals (any labelling in ethyl formate was derived from ethanol). Acetaldehyde was only plotted when significant activity was recorded. An approximation of the percentage activity extracted, calculated by the formula in section 5.2.3. is also presented for each extract in the tables.

The total activity (cpm) in an acid extract is due to a) volatile labelled intermediates collected and counted individually as outlined, and b) other labelled compounds (these probably consist of non-volatile compounds which are deposited in the column near the injection port when the sample is vapourized). It is useful to be able to compare the activity of individual collected fractions of a 20 µl injection and the sum total of activity present in the injection. That is, how much was recovered?

Rather than correct activity values for individual fractions upwards to compensate for loss of label in collection 20 µl samples of each acid extract were counted directly in a scintillation vial and the activity corrected downwards (x 0.825) to take into account the estimated

efficiency of collection (82.5%). Such values are plotted in the figures for direct comparison. 'Uncollected activity' values were calculated by subtracting the sum total of activities of individual collected fractions from these latter corrected values. 'Uncollected activity' therefore corresponds to the labelled compounds referred to in (b) above.

Data presented are from three experiments each performed twice. Due to the variation in activity experienced between different batches of cells no attempt was made to average data from two replicate experiments; instead they are presented individually.

Data from experiments in which resting cells were supplied with ^{14}C-labelled ethane in air are presented in Tables 5.2 and 5.3 and Figures 5-4 and 5-5. Data from isotope trapping experiments in which cells were supplied with 10 μmoles of cold ethanol per syringe are presented in Tables 5.4 and 5.5 and in Figures 5-6 and 5-7. Similar experiments using 10 μmoles of acetaldehyde yielded the data in Tables 5.6 and 5.7 and Figures 5-8 and 5-9.

It is postulated that ethane is metabolized as follows:

$$\underset{\text{ethane}}{C_2H_6} \longrightarrow \underset{\text{ethanol}}{C_2H_5OH} \longrightarrow \underset{\text{acetaldehyde}}{CH_3CHO}$$

$$\longrightarrow \underset{\substack{\text{acetic}\\\text{acid}}}{CH_3COOH} \begin{cases} \nearrow CO_2 \\ \searrow \underset{\text{constituents}}{\text{cell}} \end{cases}$$

Table 5-2. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane in air at 30°C. Experiment I.

Sample time (minutes)	Net cpm and counting error[a]				% activity extracted[b]
	20 µl acid extract	Fractions from one 20 µl injection			
		Acetaldehyde	Ethanol	Ethyl formate	Acetic acid	
4	1,179 (± 2.1%)	0	193 (± 2.8%)	0	239 (± 2.7%)	99.3
12	3,436 (± 1.5%)	0	199 (± 2.8%)	0	631 (± 2.2%)	98.2
28	6,974 (± 1.5%)	0	575 (± 2.3%)	0	231 (± 2.7%)	95.5
48	11,978 (± 1.5%)	0	650 (± 2.2%)	67 (± 4.7%)	3,830 (± 1.5%)	93.4
60	13,474 (± 1.5%)	0	325 (± 2.5%)	0	4,503 (± 1.6%)	90.7

[a] Two Standard Deviations 2 (σ) expressed as a percentage of net sample count rate.

[b] Calculated by formula in section 5.2.3.

3 4

OF/DE

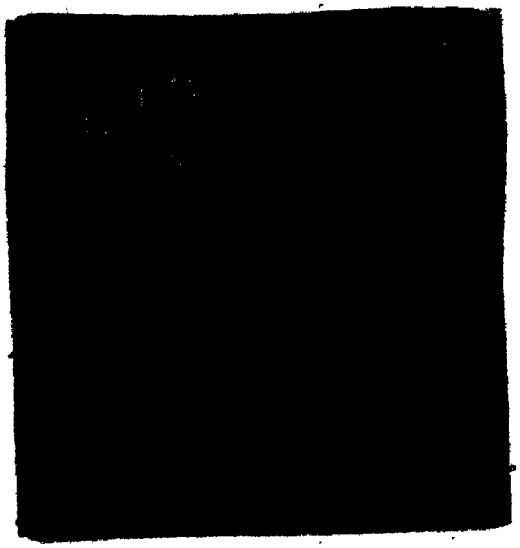


Figure 5-4. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane in air at 30°C. Experiment I. -▲- total activity, 20 µl acid extract (x 0.825); --◆-- 'uncollected activity'; -●- ethanol; -■- acetic acid.

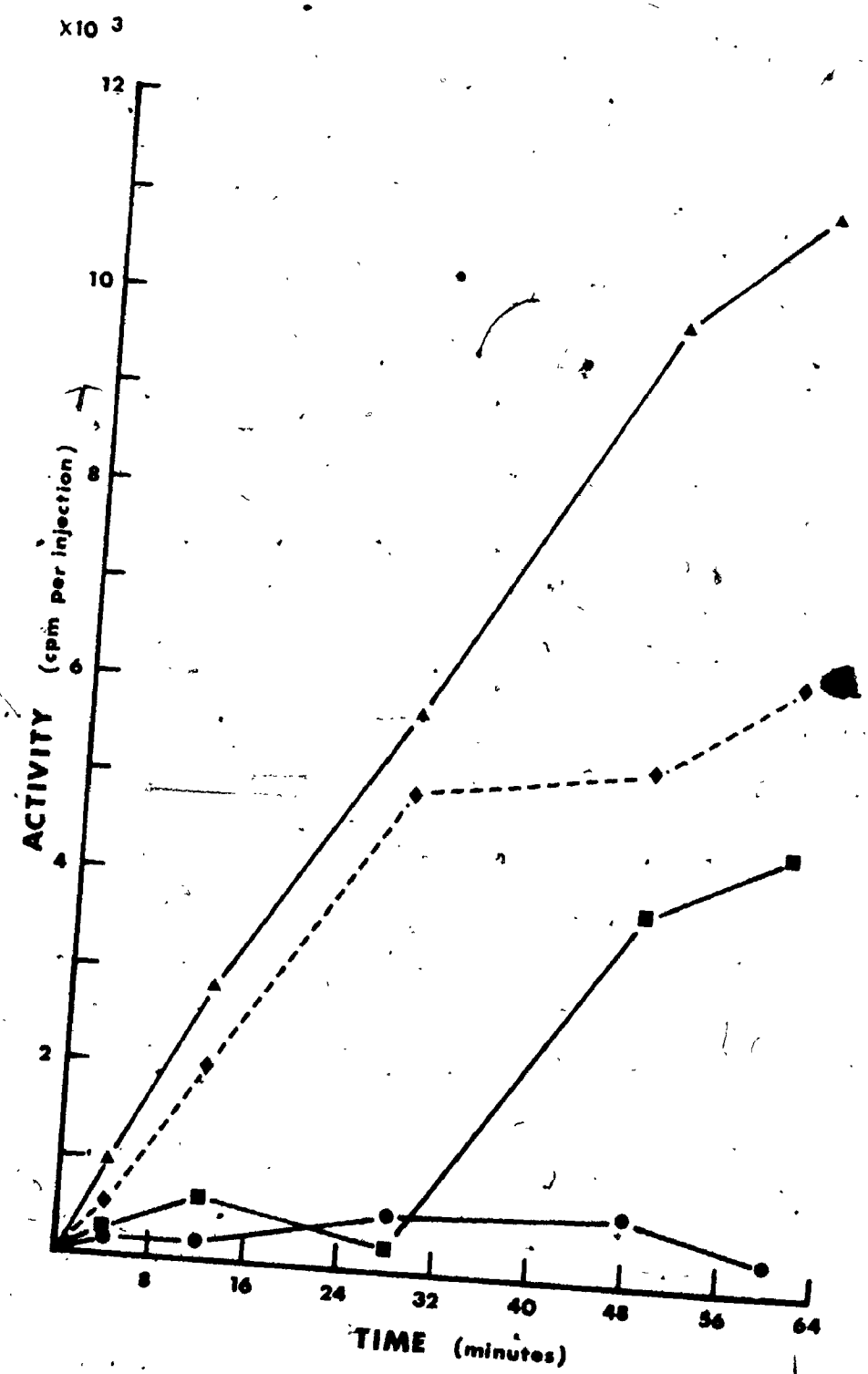

X10 3
12
10
8
6
4
2
ACTIVITY (cpm per injection)
8
16
24
32
40
48
56
64
TIME (minutes)

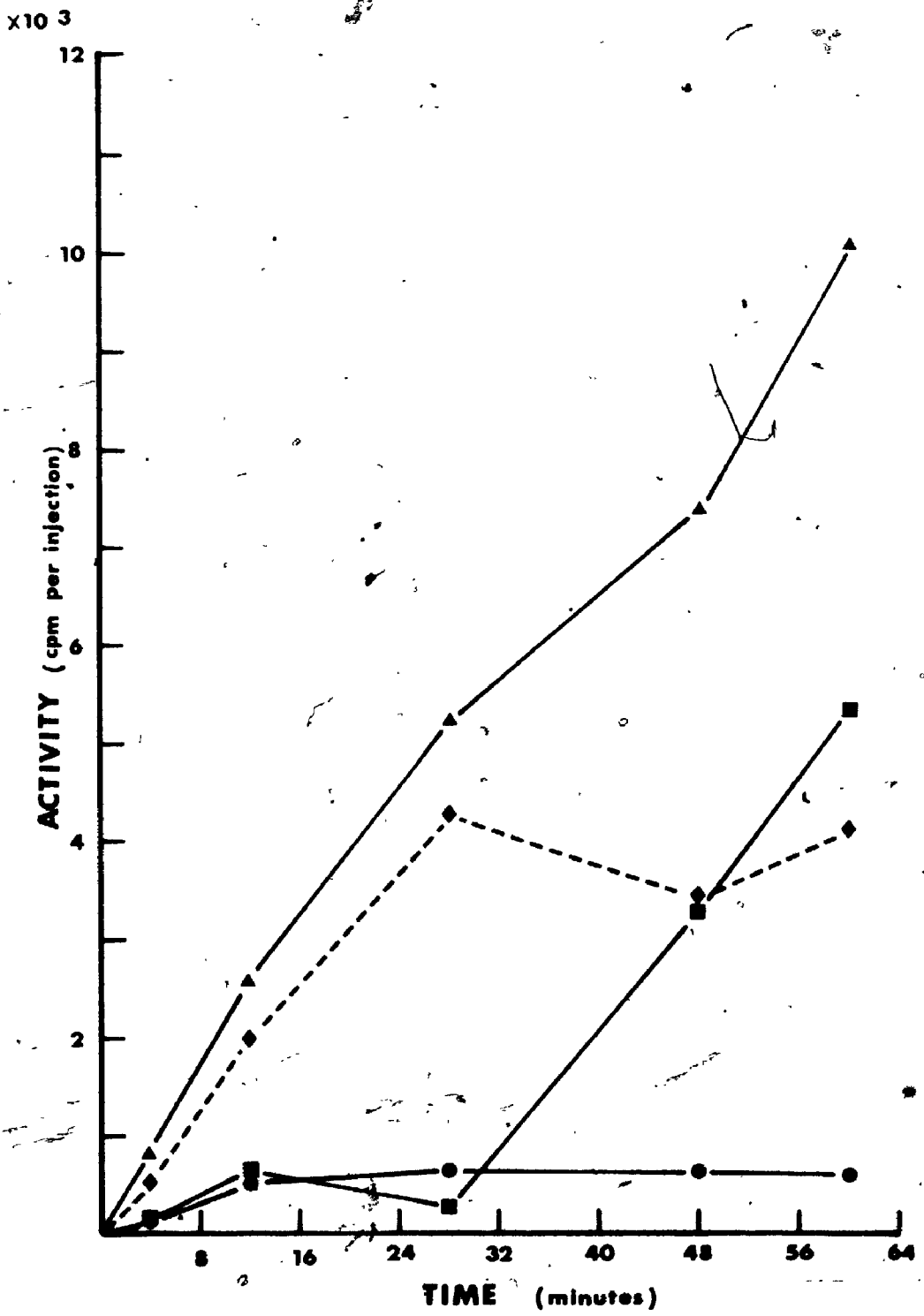
Table 5-3. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane in air at 30°C. Experiment II.

Sample time (minutes)	Net cpm and counting error[a]					% activity extracted[b]
	20 µl acid extract	Fractions from one 20 µl injection				
		Acetaldehyde	Ethanol	Ethyl formate	Acetic acid	
4	1,014 (± 2.1%)	0	128 (± 3.3%)	0	178 (± 2.9%)	99.0
12	3,168 (± 2.0%)	0	455 (± 2.3%)	76 (± 4.3%)	653 (± 2.2%)	98.6
28	6,331 (± 2.0%)	0	642 (± 2.2%)	0	287 (± 2.5%)	95.4
48	8,952 (± 2.0%)	0	572 (± 2.3%)	56 (± 5.3%)	3,309 (± 2.0%)	91.6
60	12,223 (± 2.0%)	0	597 (± 2.2%)	0	5,334 (± 2.0%)	91.4

[a]Two Standard Deviations 2 (σ) expressed as a percentage of net sample count rate.

[b]Calculated by formula in section 5.2.3.

Figure 5-5. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane in air at 30°C. Experiment II. -▲- total activity, 20 µl acid extract (x 0.825); --◆-- 'uncollected activity'; -●- ethanol; -■- acetic acid.


X10 3
12
10
8
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2
ACTIVITY (cpm per injection)
8
16
24
32
40
48
56
64
TIME (minutes)

The 'uncollected activity' under this scheme would have to consist of labelled compounds further down the metabolic pathway than acetic acid. Figures 5-4 and 5-5 indicate that initially (0 to 28 minutes) most of the labelling was in the 'uncollected' fraction while only low levels of labelled ethanol and acetic acid were produced; no labelled acetaldehyde was detected. After 28 minutes a rapid rise in labelled acetic acid was observed while labelled ethanol remained little changed. Beginning at this same point in time a discontinuity was observed in the rise in 'uncollected activity'; its rate of increase slowed down considerably in Figure 5-4 and in Figure 5-5 the activity even declined. Accumulation of label into acid extractable compounds had not ceased when the experiment was terminated after one hour.

Such data indicate an initial flux of labelled carbon flowing from ethane through ethanol, acetaldehyde and acetic acid into unknown later intermediates ('uncollected activity') and probably CO_2. During this early period little accumulation of ^{14}C-labelled carbon occurred as ethanol and acetic acid and none as acetaldehyde (aldehydes being extremely toxic, it would not be of advantage to the cell to allow such a compound to accumulate). After 28 minutes it seems likely that a metabolite or metabolites in the 'uncollected' fraction reached a level sufficient to inhibit metabolism of acetic acid. The total flux of labelled carbon however continued unchecked, resulting

in accumulation of labelled acetic acid.

When 10 µmoles of ethanol was supplied, total uptake of labelling into acid extractable compounds was reduced by 35.8% and 30.1% respectively in Experiments I and II over that in the ethanol-free controls after 60 minutes (Figs. 5-6 and 5-7). As might reasonably be predicted the only fraction to show increased accumulation of label from ethane was ethanol, the trapping compound. Accumulation of labelled acetic acid and 'uncollected activity' was suppressed indicating that such compounds are more highly oxidized intermediates of ethane than ethanol. The 'radiocarbon flux' was apparently trapped in a sink of cold ethanol from which there was a slow conversion to later labelled intermediates. Again no label was detected in acetaldehyde.

After addition of 10 µmoles of acetaldehyde to cells oxidizing [1,2-^{14}C] ethane the total uptake of labelling into acid extractable compounds was reduced by 59.7% and 67.6% respectively in Experiments I and II (Figs. 5-8 and 5-9) over the acetaldehyde-free controls after 60 minutes. Small amounts of labelled acetaldehyde did accumulate, the first direct demonstration that it is in fact an intermediate in the oxidation of ethane. Accumulation of labelled ethanol was also stimulated by added acetaldehyde, providing good evidence that ethanol is a precursor of acetaldehyde and not the reverse. Suppression of the accumulation of both labelled acetic acid and 'uncollected

Table 5-4. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane in air plus 10 µmoles ethanol at 30°C. Experiment I.

Sample time (minutes)	Net cpm and counting error[a]				% activity extracted[b]
	20 µl acid extract	Fractions from one 20 µl injection			
		Acetaldehyde	Ethanol	Ethyl formate	Acetic acid	
4	630 (± 2.2%)	0	382 (± 2.4%)	0	0	99.1
12	1,804 (± 2.1%)	0	908 (± 2.2%)	209 (± 2.9%)	81 (± 4.2%)	99.0
29.5	4,318 (± 1.5%)	0	2,012 (± 2.0%)	237 (± 2.7%)	461 (± 2.3%)	98.6
44	4,931 (± 1.6%)	0	2,416 (± 1.8%)	285 (± 2.5%)	862 (± 2.2%)	99.0
60	9,198 (± 1.5%)	0	3,961 (± 1.6%)	352 (± 2.4%)	936 (± 2.1%)	96.6
60C[c]	14,325 (± 1.5%)	N.M.[d]	N.M.	N.M.	N.M.	92.2

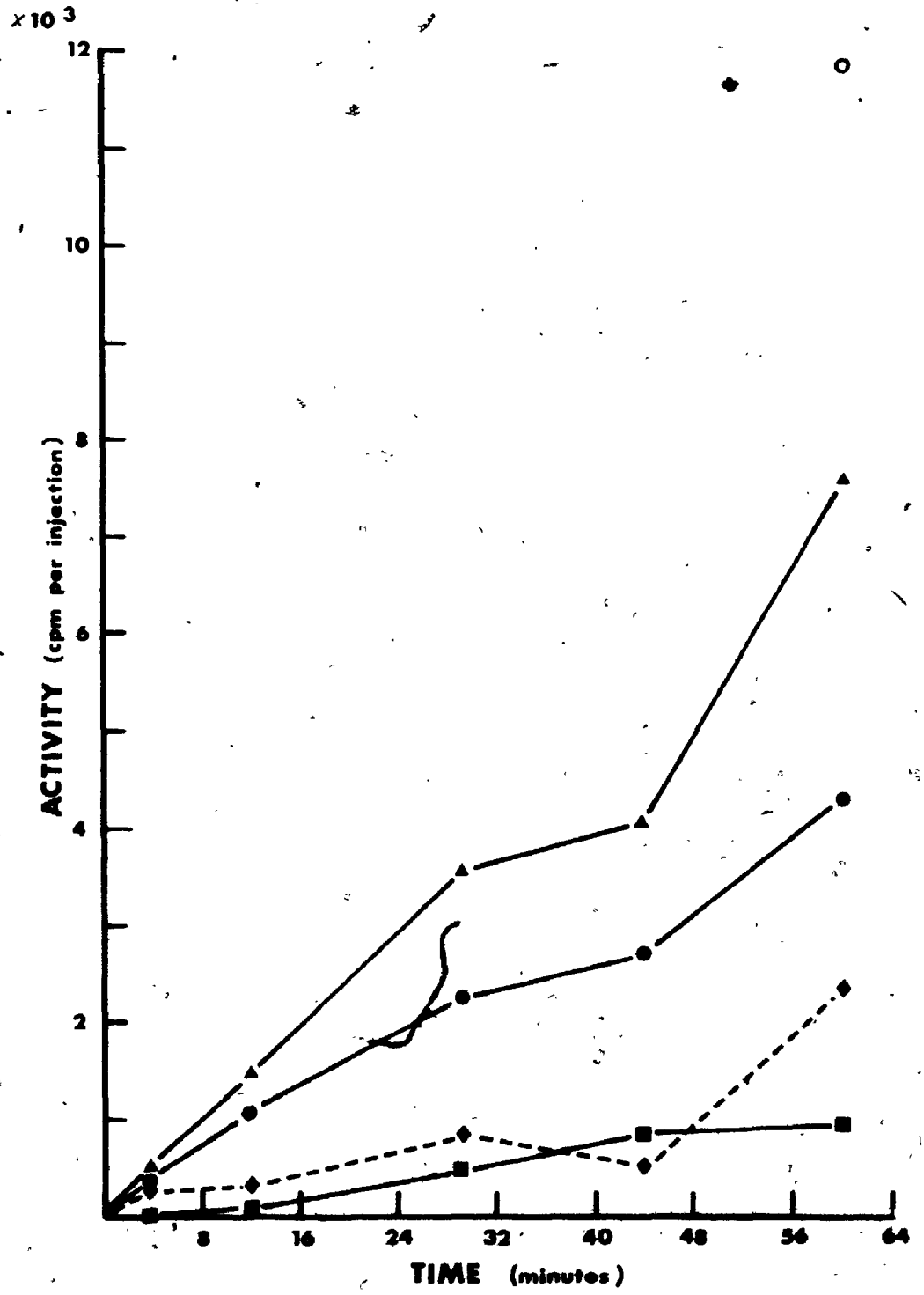
[a] Two Standard Deviations 2 (σ) expressed as a percentage of net sample count rate.

[b] Calculated by formula in section 5.2.3.

[c] 60 minute control without ethanol.

[d] Not measured.

Figure 5-6. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane plus 10 µmoles ethanol in air at 30°C. Experiment I. -▲- total 20 µl activity, acid extract (x 0.825); ○ 20 µl acid extract of 60 min. ethanol-free control (x 0.825); --◆-- 'uncollected activity'; -●- ethanol; -■- acetic acid.


x 10 3
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ACTIVITY (cpm per injection)
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16
24
32
40
48
56
64
TIME (minutes)

Table 5-5. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane in air plus 10 µmoles ethanol at 30°C. Experiment II.

Sample time (minutes)	Net cpm and counting error[a]					% activity extracted[b]
	20 µl acid extract	Fractions from one 20 µl injection				
		Acetaldehyde	Ethanol	Ethyl formate	Acetic acid	
4	478 (± 2.3%)	0	329 (± 2.5%)	34 (± 7.6%)	38 (± 7.1%)	99.6
12	1,409 (± 2.1%)	0	640 (± 2.2%)	61 (± 5.0%)	102 (± 3.7%)	99.5
28	3,320 (± 2.0%)	0	1,881 (± 2.1%)	237 (± 2.7%)	341 (± 2.4%)	99.3
44	3,891 (± 2.0%)	0	2,585 (± 2.1%)	201 (± 2.8%)	697 (± 2.2%)	99.0
60	6,752 (± 2.0%)	0	2,822 (± 2.1%)	321 (± 2.5%)	1,095 (± 2.1%)	98.7
60C[c]	9,646 (± 1.5%)	N.M.[d]	N.M.	N.M.	N.M.	87.2

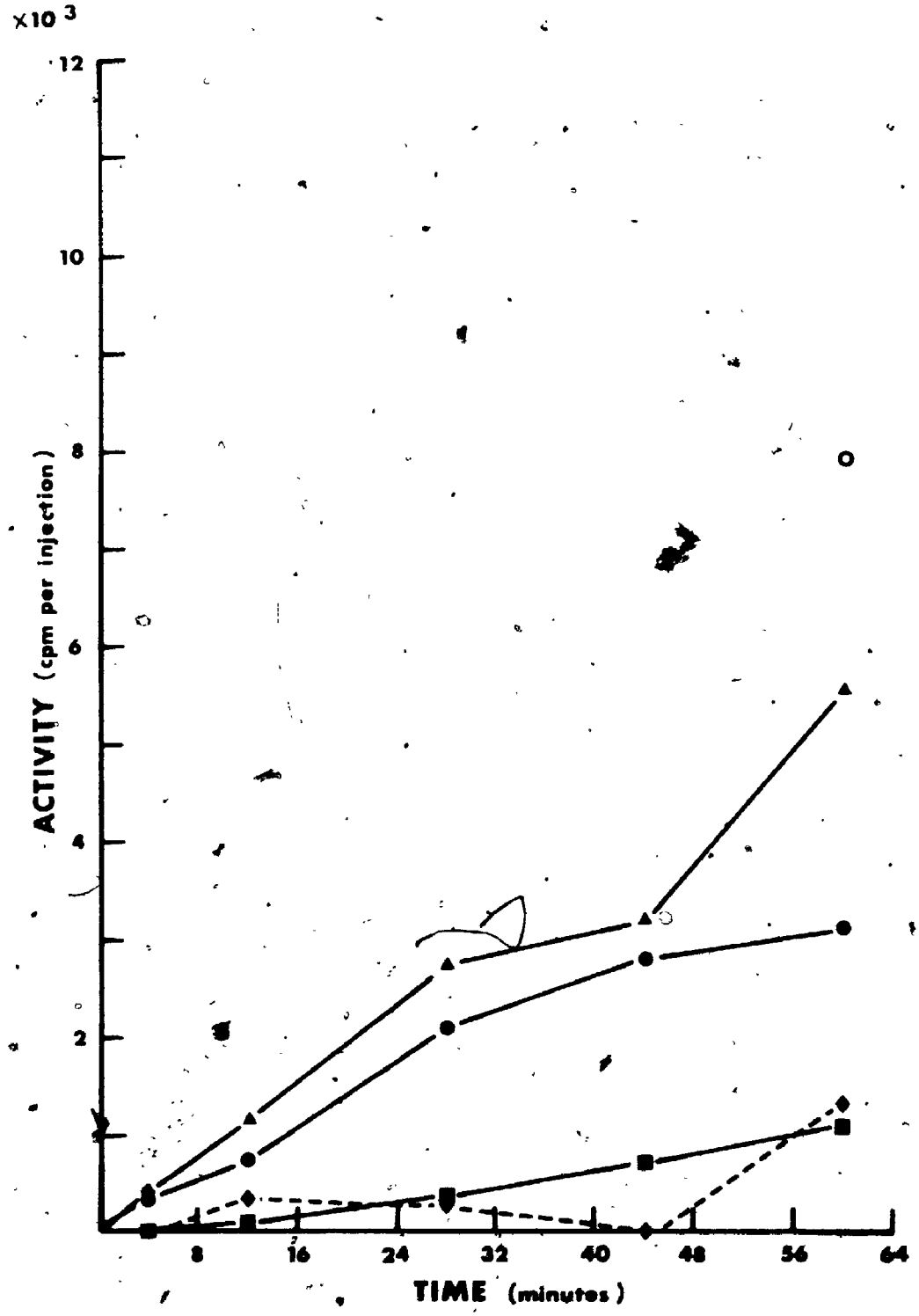
[a] Two Standard Deviations 2 (σ) expressed as a percentage of net sample count rate.

[b] Calculated by formula in section 5.2.3.

[c] 60 minute control without ethanol.

[d] Not measured.

Figure 5-7. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane plus 10 µmoles ethanol in air at 30°C. Experiment II. -▲- total activity, 20 µl acid extract (x 0.825); O 20 µl acid extract of 60 min. ethanol-free control (x 0.825); --◆-- 'uncollected activity' -●- ethanol; -■- acetic acid.


×10 3
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ACTIVITY (cpm per injection)
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24
32
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48
56
64
TIME (minutes)

Table 5-6. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane in air plus 10 µmoles acetaldehyde at 30°C. Experiment I.

Sample time (minutes)	Net cpm and counting error[a]					% activity extracted[b]
	20 µl acid extract	Fractions from one 20 µl injection				
		Acetaldehyde	Ethanol	Ethyl formate	Acetic acid	
4	107 (± 3.6%)	0	59 (± 5.1%)	0	37 (± 7.2%)	100
12	451 (± 2.3%)	205 (± 2.8%)	138 (± 3.2%)	0	54 (± 5.5%)	99.8
29	1,512 (± 2.1%)	367 (± 2.4%)	503 (± 2.3%)	49 (± 5.8%)	207 (± 2.8%)	99.8
46	3,886 (± 2.0%)	33 (± 7.9%)	2,033 (± 2.1%)	62 (± 5.0%)	906 (± 2.2%)	99.8
60	5,719 (± 2.0%)	0	2,631 (± 2.1%)	0	900 (± 2.2%)	99.7
60C[c]	14,198 (± 1.5%)	N.M.[d]	N.M.	N.M.	N.M.	95.9

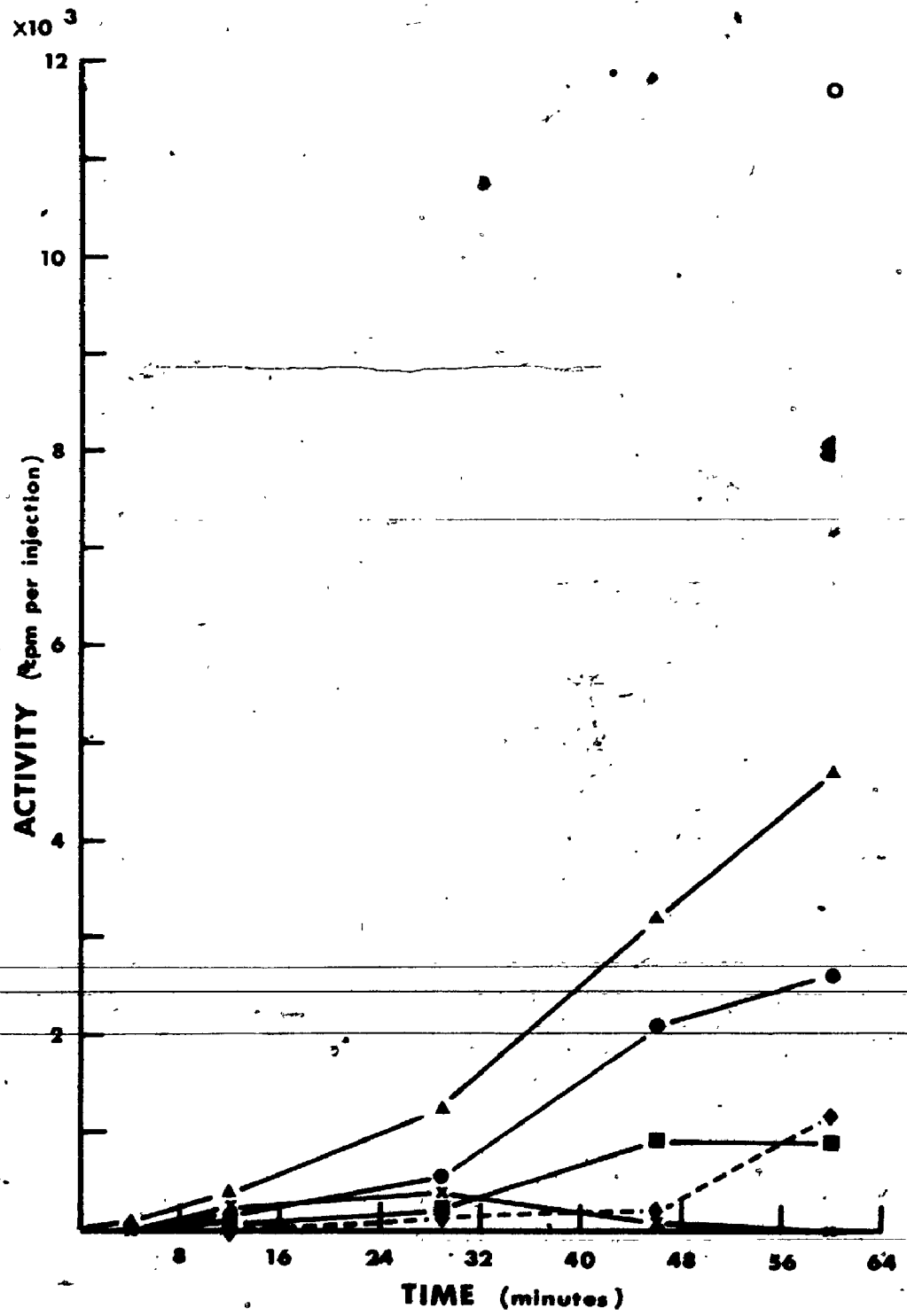
[a] Two Standard Deviations 2 (σ) expressed as a percentage of net sample count rate.

[b] Calculated by formula in section 5.2.3.

[c] 60 minute control without acetaldehyde.

[d] Not measured.

Figure 5-8. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane plus 10 µmoles acetaldehyde in air at 30°C. Experiment I. -▲- total activity, 20 µl acid extract (x 0.825); O 20 µl acid extract of 60 min. acetaldehyde-free control (x 0.825); --◆-- 'uncollected activity'; -●- ethanol; -■- acetic acid; - x - acetaldehyde.


X10 3
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ACTIVITY (cpm per injection)
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48
56
64
TIME (minutes)

Table 5-7. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C-ethane] in air plus 10 µmoles acetaldehyde at 30°C. Experiment II.

Sample time (minutes)	Net cpm and counting error[a]				% activity extracted[b]
	20 µl acid extract	Fractions from one 20 µl injection			
		Acetaldehyde	Ethanol	Ethyl formate	Acetic acid	
4	124 (± 3.4%)	34 (± 7.8%)	0	0	0	99.7
12	435 (± 2.3%)	120 (± 3.4%)	103 (± 3.7%)	0	0	99.6
28	1,220 (± 2.1%)	471 (± 2.5%)	299 (± 2.5%)	0	77 (± 4.3%)	99.6
44	2,220 (± 1.9%)	461 (± 2.3%)	628 (± 2.2%)	69 (± 4.6%)	244 (± 2.6%)	99.5
60	3,728 (± 1.5%)	50 (± 5.8%)	1,526 (± 2.1%)	108 (± 3.6%)	641 (± 2.2%)	99.5
60C[c]	11,506 (± 1.5%)	N.M.[d]	N.M.	N.M.	N.M.	89.1

[a] Two Standard Deviations 2 (σ) expressed as a percentage of net sample count rate.

[b] Calculated by formula in section 5.2.3

[c] 60 minute control without acetaldehyde.

[d] Not measured.

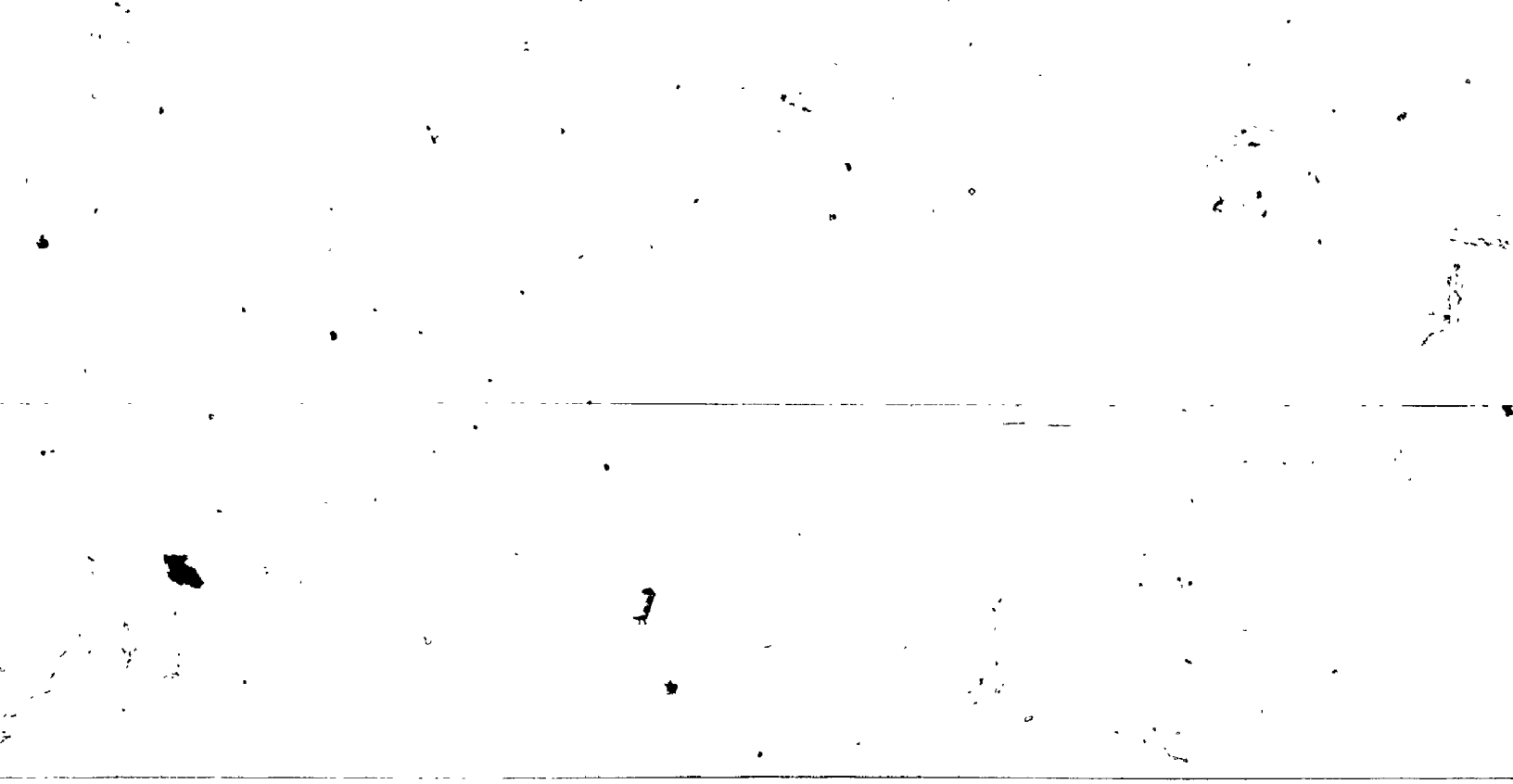
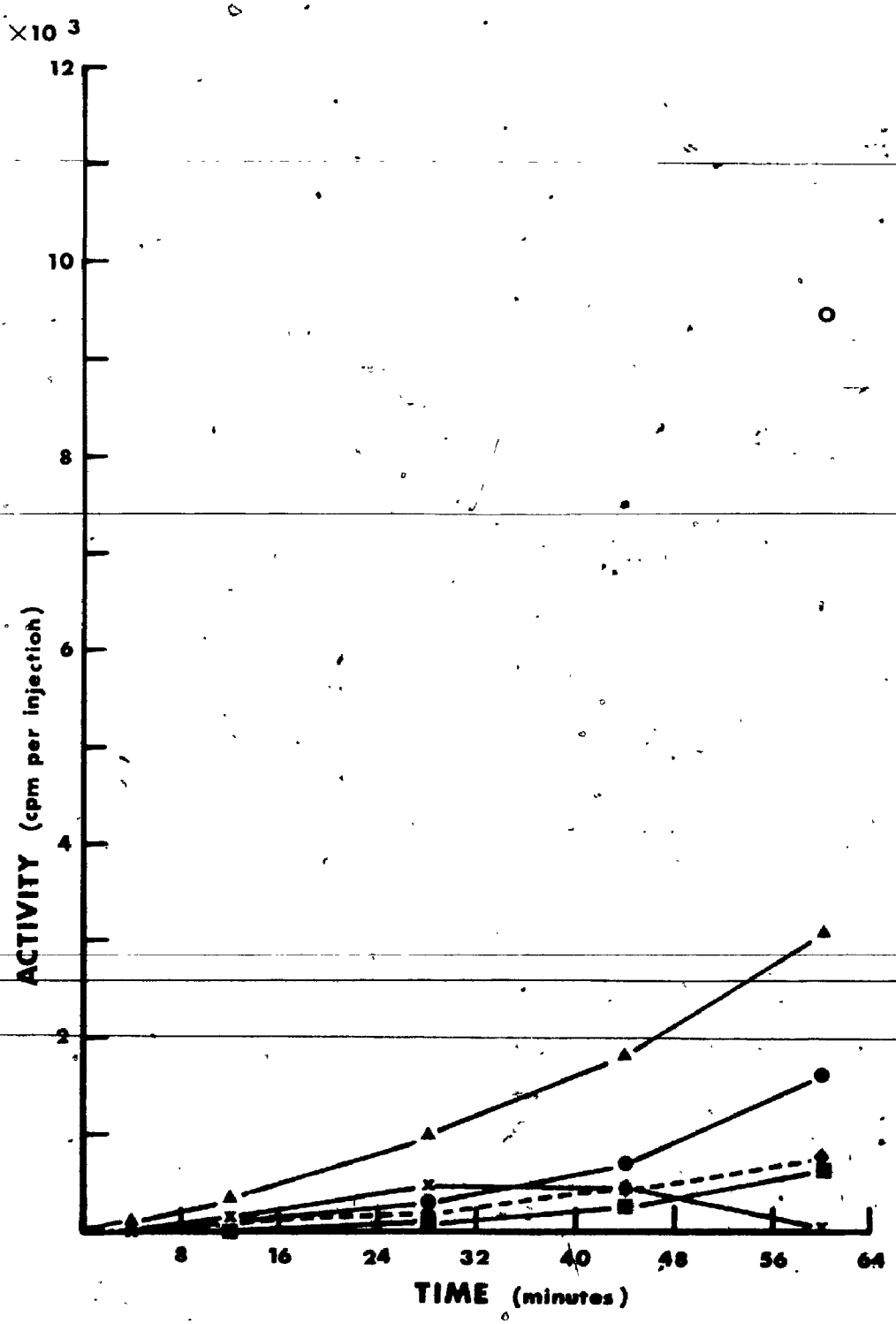


Figure 5-9. Activity of labelled intermediates in acid extracts of Acremonium 3E/1 resting cells supplied with [1,2-^{14}C] ethane plus 10 µmoles acetaldehyde in air at 30°C. Experiment II. -▲- total activity, 20 µl acid extract (x 0.825); O 20 µl acid extract of 60 min. acetaldehyde-free control (x 0.825); --◆-- 'uncollected activity'; -●- ethanol; -■- acetic acid; - x - acetaldehyde.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ACTIVITY (cpm per injection)
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64
TIME (minutes)

activity' implicates acetaldehyde as a precursor of these.

The greatest accumulation of labelling in these latter experiments using acetaldehyde occurs not in the sink of 'cold' acetaldehyde but in ethanol. Conceivably the added acetaldehyde affects the equilibrium of alcohol oxidation to acetaldehyde. The rapid disappearance of labelled acetaldehyde again indicates the cell's ability to rapidly metabolize this potentially toxic compound, helping to explain why it does not normally accumulate in the absence of a trapping agent.

CHAPTER 6

IN VITRO STUDIES ON ETHANE ASSIMILATION

6.1. Introduction:

The evidence presented in the preceding sections supports the alcohol, aldehyde, fatty acid pathway for the early stages of ethane assimilation in Acremonium 3E/1. Such evidence however sheds little light upon the nature of the enzyme or enzymes responsible for catalysing the initial 'fixation' reaction (i.e. the biochemical transformation of the ethane molecule to its first stable intermediate) of the ethane molecule, beyond indicating some substrate specificity and a probable requirement for molecular oxygen.

Studies were initiated on cell-free preparations of ethane-grown cells of Acremonium 3E/1 to learn more about this first step in ethane assimilation. After consideration of reports on related enzyme systems in other organisms (sect. 2.5.) attention was focussed on certain key areas. Of particular interest was the location of initial ethane 'fixation' in the cell and whether the enzyme(s) responsible are soluble or bound to membranous organelles (particulate). Coenzyme requirements and the effect of

specific inhibitors were also considered since these have also been important aspects of previous reports.

6.2. Materials and methods:

6.2.1. Preparation of a cell-free system:

Cells of Acremonium 3E/1 were cultured as described in section 5.2 on ethane. After initial harvest however, resting cell-suspensions were prepared by five washings (centrifugation at 1000 g) in ice cold 0.05M Tris-HCL buffer (pH 7.6). After the final wash, cells were compacted by centrifugation at 7000 g for 15 minutes, after which the supernatant was discarded.

The compacted pellet of washed cells was then resuspended in three volumes of ice cold stabilizing medium which had the following composition: Tris-HCl buffer (pH 7.6) - 0.05M, D-mannitol-0.22M, EDTA (disodium salt) -0.5 mM, dithiothreitol-0.5 mM, bovine serum albumen-0.05%. These reagents were all obtained from Sigma Chemical Co., St. Louis, Mo. It was not known how many of the ingredients listed were actually essential; they were included with the intention of providing as much protection as possible to intracellular enzymes against potentially adverse conditions created when they were released from the organized confines of the intact cell.

The buffer was selected to avoid large pH changes upon release of cell contents, particularly organic acids,

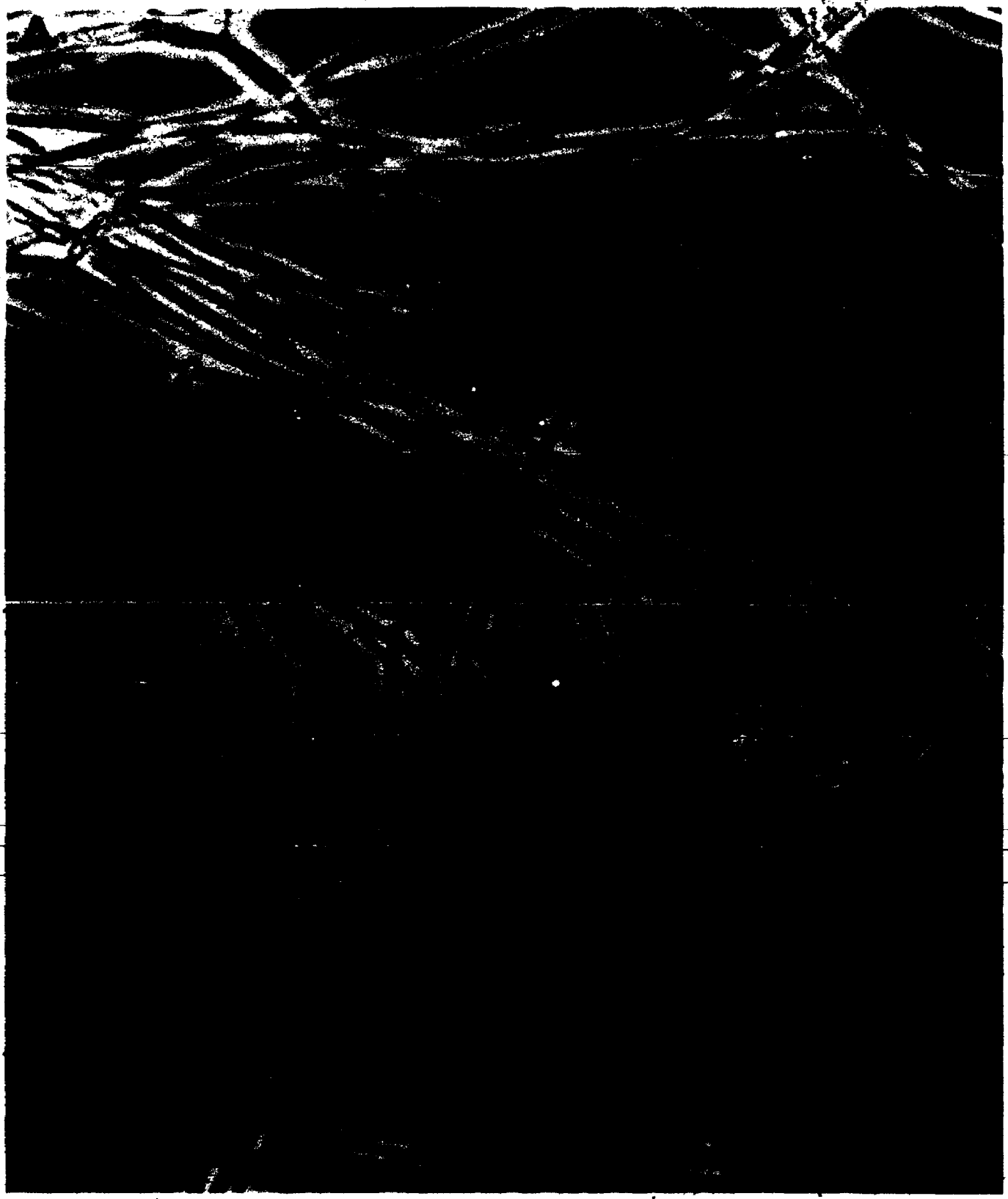
into the medium. D-mannitol was added to maintain a high enough osmotic pressure to prevent damage to potentially osmo-sensitive organelles, such as mitochondria or microsomes. Dithiothreitol is a reducing agent, effective in preventing oxidation of enzyme thiol groups to disulphide bridges. EDTA is a chelating agent which should protect enzymes from possibly harmful heavy metal ions released by cell breakage. Bovine serum albumen is known to stabilize suspensions of mitochondria.

After resuspension twice in the stabilizing medium (to completely replace the wash buffer), a homogenate of broken cells was prepared using a Mickle vibratory cell disruptor (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.). Each of the two glass vials of the instrument received 6 ml of cell suspension and 7.5 g of prechilled #12 Ballotini glass beads (Brinkman Instruments, Canada Ltd., Rexdale, Ont.). Vials were subjected to two 2.5 minute periods of shaking at full power, interspersed by a five minute cooling period on ice; the instrument was pretuned to give maximum amplitude. Such treatment was adequate to break approximately 99% of all cells. The appearance of a cell suspension before and after cell disruption can be seen in Figure 6-1 A and B. Note the short lengths of empty hyphal walls in Figure 6-1 B compared with long intact hyphae in Figure 6-1 A.

The homogenate was decanted off the beads into centrifuge tubes on ice and centrifuged twice at 1000 g for 10

PLATE 6-1

A. Washed suspension of ethane-grown Acremonium 3E/1 cells; phase contrast. B. Homogenate prepared from A with a Mickle vibratory cell disruptor; note empty hyphal wall fragments; phase contrast.



minutes, to remove unbroken cells and cell wall fragments; the supernatant from such treatment will henceforth be referred to as the 'crude homogenate' or just 'crude' and was kept on ice until required.

6.2.2. Centrifugal fractionation of crude homogenates:

A measured volume of crude homogenate was centrifuged at 10,000 g for 20 minutes twice in a refrigerated centrifuge equipped with a swinging bucket rotor. The combined pellets from these runs were resuspended in the stabilizing medium with the aid of a glass homogenizer (hand operated) and designated fraction P_1. The remaining supernatant (S_1) was then centrifuged for 90 minutes at 105,000 g in a fixed-angle rotor. The pellet from this run was resuspended as before and designated fraction P_2; the final clear supernatant was designated fraction S_2. Biological material was maintained as close to 0°C as possible at all times. These procedures are summarized in Figure 6-1.

6.2.3. Protein estimation:

Protein in crude homogenates and fractions was estimated by the method of Lowry et al. (1951) after trichloroacetic acid precipitation. Protein values were corrected for bovine serum albumen added to the stabilizing medium. Bovine serum albumen was also used as a protein standard. During the course of all experiments involving centrifugal fractionation, a balance sheet was maintained of the distri-

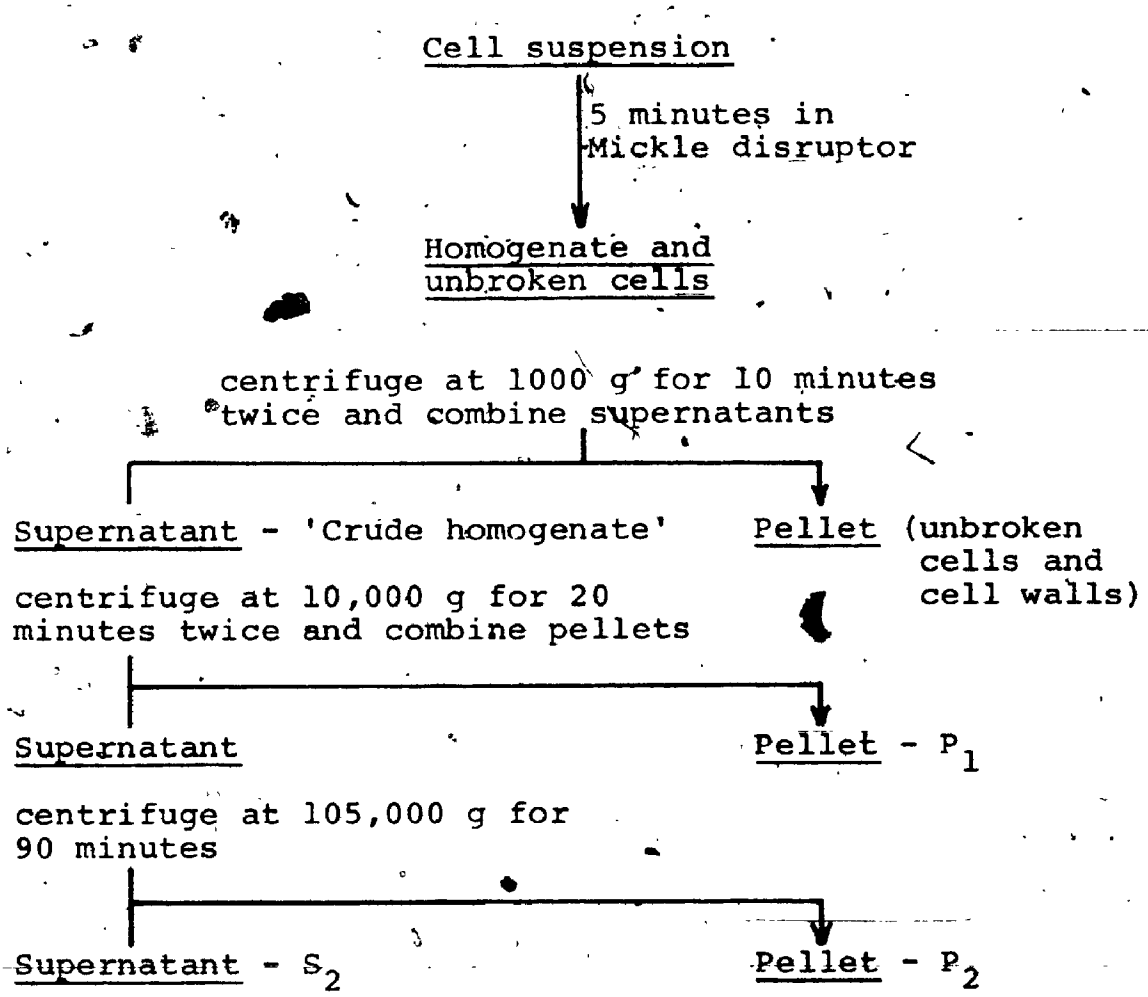


Figure 6-1. Centrifugal fractionation of a crude homogenate of Acremonium 3E/1.

bution of total protein in the three fractions of the crude homogenate.

6.2.4. Radioassay of ethane fixation:

The method used to expose the various enzyme preparations to labelled ethane was essentially the same one described in section 5.2.2. One millilitre of a particular preparation was added to a 10 ml Hamilton syringe which was then charged with 5 ml of labelled ethane in air and placed on the water bath shaker at 30°C as before. After shaking for the desired period of time a syringe was removed from the shaker and after expulsion of the gas phase (into a fume hood) the contents of the syringe were ejected into a small test tube, containing 0.5 ml of 20% (vol/vol) trichloroacetic acid, on ice. After sealing with 'Parafilm' the tube was left for several hours on ice to allow complete protein precipitation before centrifugation at 10,000 g for 15 minutes to remove the protein.

Aliquots of 0.1 ml of the decanted supernatant (acid extract) were pipetted into each of two low-background scintillation vials containing 10 ml of 'Aquasol' (ice cold). After the usual precautions against anomalous counts due to the effect of light the vials were counted as previously outlined (5.2.3.) until the two sigma counting error displayed on the counter fell to 2% of the gross count rate. With the larger sample size employed with these experiments count rates were normally sufficiently high above background

(57 cpm) such that special statistical consideration of low activity count rates (5.2.5.) was not necessary.

With the larger sample size however a problem had to be considered which in whole cell experiments had been safely ignored, that is, contribution to the sample count rate by dissolved [1,2-^{14}C] ethane. Control experiments in which stabilizing medium alone was exposed to the labelled ethane and air mixture in syringes and finally treated as usual with acid were performed and the resulting acid extracts counted. The mean net count rate was 637 cpm per 0.1 ml extract for the labelled gas mixture used for all but a few of the experiments described herein. When this batch was exhausted a new one was prepared which had a much higher specific activity (the yial of [1,2-^{14}C] ethane as supplied must have had a higher specific activity than that specified). The figure for this batch was 2,214 cpm per 0.1 ml acid extract. To avoid confusion data derived from this latter batch are so specified. Values for mean net cpm per 0.1 ml are derived from gross cpm corrected for background and activity due to dissolved [1,2-^{14}C] ethane.

6.2.5. Coenzyme requirements:

NAD^+ (grade III), NADH (grade III), $NADP^+$ (Sigma grade) and NADPH (type II) were supplied by Sigma Chemical Co. In all radioassay experiments using coenzymes, a syringe received 0.95 ml of enzyme preparation plus 0.05 ml coenzyme solution in stabilizing medium containing 0.5 µmole

of pyridine nucleotide giving a final concentration of 0.5 mM coenzyme in the syringe. Coenzyme-free control syringes received 0.05 ml of stabilizing medium.

6.2.6. Inhibitors:

Sodium azide (NaN_3) and potassium cyanide (KCN) were added to syringes to a final concentration of 1.0 mM immediately before gassing.

Carbon monoxide inhibition of susceptible enzymes has usually been shown to be dependent upon the ratio of carbon monoxide to oxygen, rather than just the absolute carbon monoxide concentration, since it usually inhibits competitively by binding at the oxygen binding site. Using the gas pipette previously described (Fig. 5-1), two mixtures of carbon monoxide (Research Purity, Matheson of Canada Ltd.) and the usual labelled 10% ethane, 90% air mixture were prepared, such that, in one the $CO:O_2$ ratio was 1:2 and in the other 2:1. Control gas mixtures were prepared in the same way except that carbon monoxide was replaced by nitrogen (Food grade, Canadian Liquid Air Ltd.). Mixtures were prepared by adding CO to the labelled ethane and air mixture to obtain the desired $CO:O_2$ ratio. Final mixtures contained 9.1% and 27.1% CO respectively for 1:2 and 2:1 mixtures; these were replaced by N_2 in the controls to compensate for the reduction in ethane substrate. Syringes containing enzyme preparations were exposed to 5 ml of such mixtures in the usual way.

6.2.7. Assay of cytochrome oxidase activity:

Cytochrome oxidase is an enzyme characteristically found in mitochondria. It catalyses the following reaction:

$$4 \text{ ferrocytochrome c} + 4H^+ + O_2 \longrightarrow 4 \text{ ferricytochrome c} + 2H_2O$$

In the present studies, cytochrome oxidase activity was used as a marker to locate mitochondria and mitochondrial fragments in crude homogenates and fractions derived from them.

Using a modification of the methods of Smith (1955) and Simon (1957) oxidation of reduced mammalian cytochrome c (horse heart cytochrome c, type III, Sigma Chemical Co.) was followed by the decrease in absorbance at 550 nm. The 3 ml reaction mixture in 0.1M potassium phosphate buffer (pH 7.0) contained 0.045 µmole of reduced cytochrome c; the blank contained buffer alone; the reaction was initiated by addition of 0.1 ml enzyme preparation. The absorbance at 550 nm was measured every 10 seconds for 2 minutes after which time a drop of saturated potassium ferricyanide was added and the absorbance of the completely oxidized cytochrome measured. The original 90 µM stock solution of cytochrome c in buffer (in the oxidized state) was reduced by the addition of a few milligrams of sodium dithionite; ex-

cess dithionite was removed by bubbling with pure oxygen for five minutes. Absorbance measurements were made at 25°C with a 1.0 cm light path in a Unicam SP 1800 spectrophotometer (Pye Unicam, Cambridge, U.K.). All subsequent spectrophotometric work was also performed on this instrument.

The decrease in absorbance is not linear with time but a linear plot can be achieved by plotting log (absorbance minus absorbance of fully oxidized cytochrome) against time. From this plot, k min^{-1}, the first order velocity constant (the reaction is first order with respect to cytochrome c) can be calculated. From k and the initial concentration of cytochrome c, the initial rate of oxidation can be calculated in nanomoles of cytochrome c oxidized per minute at 25°C.

6.2.8. Assay of NADPH - cytochrome c reductase activity:

NADPH - cytochrome c reductase, catalyses the following reaction:

$$NADPH + H^+ + 2 \text{ ferricytochrome c} \longrightarrow NADP^+ + 2 \text{ ferrocytochrome c} + 2H^+$$

Cytochrome c reduction was followed using the method of Masters et al. (1967) by the increase in absorbance at 550 nm. The 3.1 ml reaction mixture in 0.05 M Tris-HCL

buffer (pH 7.6) contained 0.109 µmole oxidized cytochrome c, 0.3 µmole EDTA and 0.1 ml of enzyme preparation; the blank contained buffer alone; the reaction was initiated by the addition of 0.3 µmole NADPH. The increase in absorbance was followed every 10 seconds in the linear portion of the reaction (i.e. for 2 minutes) at 25°C, with a 1.0 cm light path. From the slope of this linear plot, the reaction rate was calculated in nanomoles cytochrome c reduced per minute at 25°C. From Masters et al. (1967) an absorbance change of 1.0 corresponds to the reduction of 47.6 nmoles of cytochrome c per ml with a 1.0 cm light path.

6.2.9. Carbon monoxide difference spectra:

The only method of demonstrating conclusively the presence of cytochrome P-450 in the microsomal fraction is by obtaining a carbon monoxide difference spectrum of a suspension of microsomes (105,000 g sedimentable particles) reduced by dithionite, after the method of Omura and Sato (1967). A broad peak at 450 nm identifies the carbon monoxide complex formed with this cytochrome.

A suspension of P_2 particles in buffer was reduced by the addition of a few milligrams of sodium dithionite, split between two 1.0 ml cuvettes, which were then closed. Carbon monoxide was bubbled through one cuvette for 30 seconds; it was then re-closed. An absorption spectrum was then obtained over the range 410 to 490 nm with the carbon

monoxide-treated cuvette in the sample position and reduced particles in the reference position. Various different particle concentrations and buffers were used.

6.2.10. Assay of alcohol dehydrogenase activity:

Biological oxidation of ethanol to acetaldehyde is normally catalyzed by an NAD^+-linked alcohol dehydrogenase

$$\text{ethanol} + NAD^+ \longrightarrow \text{acetaldehyde} + NADH + H^+$$

The method used was based on that outlined by Lebeault et al. (1970). NAD^+ reduction was followed by the rise in absorbance at 340 nm. A 1.1 ml reaction mixture in quartz semi-micro cuvettes (1.0 cm path length) contained 7.0 µmoles NAD^+ and 18 µmoles ethanol in 0.05 M Tris-HCL buffer (pH 8.4); the blank contained buffer alone; the reaction was initiated by addition of 0.1 ml enzyme preparation. The increase in absorbance was followed every 10 seconds for two minutes at 25°C, during which time it was linear. The reaction rate was calculated from the slope of this plot using $E^{mM}_{340} = 6.2$ (Sigma data sheet) for NADH. Units were nanomoles of NAD^+ reduced (and hence ethanol oxidized) per minute at 25°C.

6.2.11. Assay of endogenous $NADP^+$ reduction:

Endogenous $NADP^+$ reduction in crude homogenates was measured by the method described in 6.2.10. using 7.0 µmoles

$NADP^+$ but no ethanol. The extinction coefficient for NADPH is identical to that for NADH. Units were nanomoles $NADP^+$ reduced per minute at 25°C.

6.2.12. Electron microscopy of ethane-grown Acremonium 3E/1 cells:

Cells cultured as previously described (sect. 6.2.1.) were fixed in Franke's fixative (Franke et al. 1969) in 0.1M cacodylate buffer (pH 7.6) for 90 minutes on an ice bath. They were washed six times with 0.1M cacodylate buffer then treated with 1% osmic acid in the same buffer for a further 90 minutes at room temperature. After two washes in buffer and one in distilled water the cells were prestained with 0.5% aqueous uranyl acetate for 12 hours. In the above operations cells were transferred from one solution to another by low speed (1000 g) centrifugation. The cells were embedded in 1.5% water agar, dehydrated in an ascending acetone series and finally embedded in Epon 812. Sections about 500-1000 Å thick were cut with a diamond knife on a Servall-Porter-Blum MT-1 microtome, stained with lead acetate (Reynolds 1963) for 10 minutes and examined with a Philips EM200 electron microscope at 60kV.

6.3. Results and discussion:

The variation in activity from one batch of cells to the next which has been referred to in chapter 5 was also found in enzyme preparations from different batches of cells.

Such variation could not be explained by the variation in protein content in cell homogenates. Therefore, as in chapter 5, results of duplicate experiments are presented separately. Ethane 'fixation' was assayed by the total accumulation of labelled compounds in acid extract samples. In the tables and figures which follow, the mean net cpm per 0.1 ml acid extract (mean cpm of two replicate vials) are given along with the Standard Deviation (SD). Net cpm were derived from gross cpm by correcting for background and activity due to dissolved [1,2-^{14}C] ethane. Where data are presented graphically, error bars are omitted where they do not extend beyond the confines of the points.

Data presented in Tables 6-1 and 6-2 demonstrate the effect of added coenzymes on ethane 'fixation' by crude homogenates. Data in Table 6-1 are derived from two separate experiments (two different enzyme preparations) due to a temporary shortage of reaction vessels. It should be remembered that the figures presented represent the accumulation of labelled compounds <u>after</u> one hour <u>not</u> the <u>rate</u> of accumulation <u>per</u> hour. The latter figure would of course be more useful, however it was not feasible to handle simultaneously the number of reaction vessels necessary for a time course experiment with each coenzyme.

It is apparent from the control data that comparatively little ethane fixation into stable intermediates took place in the absence of added coenzyme. NAD^+ had no apparent significant effect; slight stimulation was apparent in one

Table 6-1. Effect of coenzymes (0.5 µmole) upon [1,2-^{14}C] ethane 'fixation' by a crude homogenate of Acremonium 3E/1 cells at 30°C. Experiment I.

	Mean net cpm per 0.1 ml extract after one hour and SD[a]	cpm of 0.1 ml as % of control	Protein per syringe (mg)
Part I			
Control	866 (± 28)	100%	3.225
NADPH	9,289 (± 129)	1,072%	"
$NADP^+$	7,204 (± 175)	832%	"
Part II			
Control	1,416 (± 85)	100%	2.826
NADH	1,856 (± 48)	131%	"
NAD^+	1,555 (± 100)	110%	"

[a]Standard Deviation.

Table 6-2. Effect of coenzymes (0.5 µmole) upon [1,2-^{14}C] ethane 'fixation' by a crude homogenate of Acremonium 3E/1 cells at 30°C. Experiment II[a].

	Mean net cpm per 0.1 ml extract after one hour and SD[b]	cpm per 0.1 ml as % of control	Protein per syringe (mg)
Control	1,269 (± 233)	100%	2.834
NADPH	16,481 (± 438)	1,299%	"
$NADP^+$	12,085 (± 63)	952%	"
NADH	3,121 (± 168)	245%	"
NAD^+	1,478 (± 48)	116%	"

[a]High specific activity [1,2-^{14}C] ethane and air mixture used.

[b]Standard Deviation.

experiment with NADH. NADPH was apparently the preferred coenzyme, since it produced a ten to thirteen fold increase in ethane 'fixation' over the control. $NADP^+$ also exerted a marked stimulatory effect, yielding an accumulation of labelled compounds on average of approximately 75% of that with NADPH. Stimulation by a reduced coenzyme of an alkane-oxidizing system is normally indicative of a monooxygenase. Stimulation by the oxidized form has been taken as evidence of a dehydrogenase. Such a pronounced effect by both forms of one coenzyme is something of a paradox. The reduced form however does seem to be the preferred coenzyme and was added to all subsequent reaction mixtures where [1,2-^{14}C] ethane fixation was measured, unless otherwise indicated.

In Figure 6-2 accumulation of radioactivity is plotted against time for a crude homogenate supplied with NADPH. Accumulation continues over the full hour chosen for previous experiments but is not linear; the rate of accumulation decreases with time.

Table 6-3 presents data from an experiment designed to localize 'ethane-fixing' enzyme(s) in the cell. Certain broad generalizations concerning the composition of centrifugal fractions of crude eukaryote cell homogenates can be made. Intact mitochondria can normally be expected in the 10,000 g sedimentable-particulate-fraction (P_1). The endoplasmic reticulum of the cell normally pinches off into small membranous vesicles known as microsomes upon cell disruption; these bodies are normally sedimented by 105,000 g

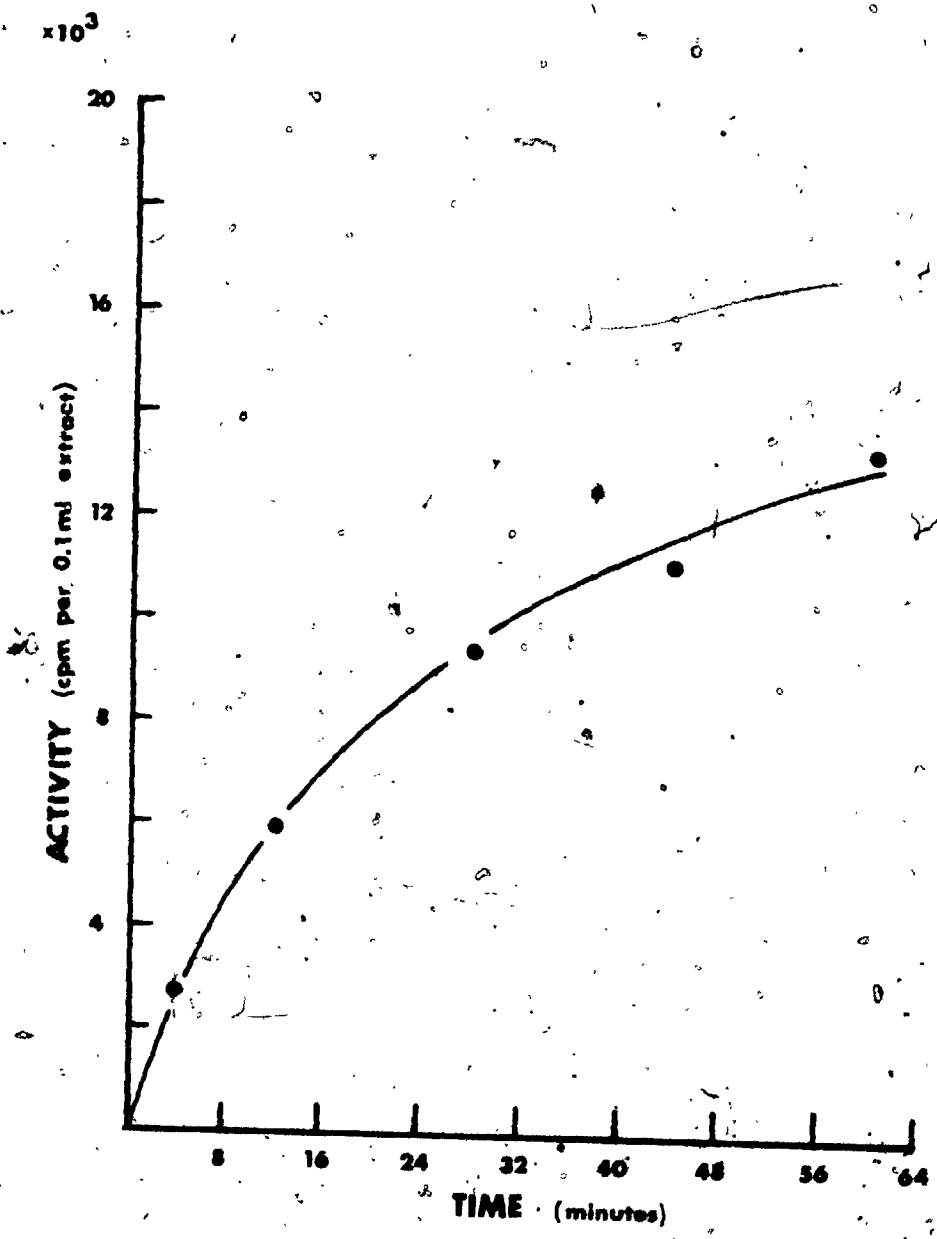
Table 6-3. Distribution of protein and '[1,2-^{14}C] ethane-fixing' activity in fractions of a crude homogenate of Acremonium 3E/1 cells.

	Protein		[1,2-^{14}C] ethane 'fixation'[a]		
	mg per ml of crude	% of total	Mean net cpm per 0.1 ml extract after one hour and SD[b]	cpm per fraction as % of total of P_1 + P_2 + S_2	mg protein per syringe
Crude	3.789	-	9,945 (± 16)	-	3.599
P_1	0.441	11.7%	887 (± 61)	19.0%	0.418
P_2	0.897	23.8%	3,552 (± 63)	76.2%	0.819
S_2	2.429	64.5%	222 (± 35)	4.8%	2.308

[a]In the presence of 0.5 µmole NADPH at 30°C.

[b]Standard Deviation.

Figure 6-2. Fixation of [1,2-^{14}C] ethane by a crude homogenate of Acremonium 3E/1 cells in the presence of 0.5 µmole NADPH at 30°C (3.379 mg protein per syringe).


x10³
20
16
12
8
4
ACTIVITY (cpm per 0.1ml extract)
8
16
24
32
40
48
56
64
TIME (minutes)

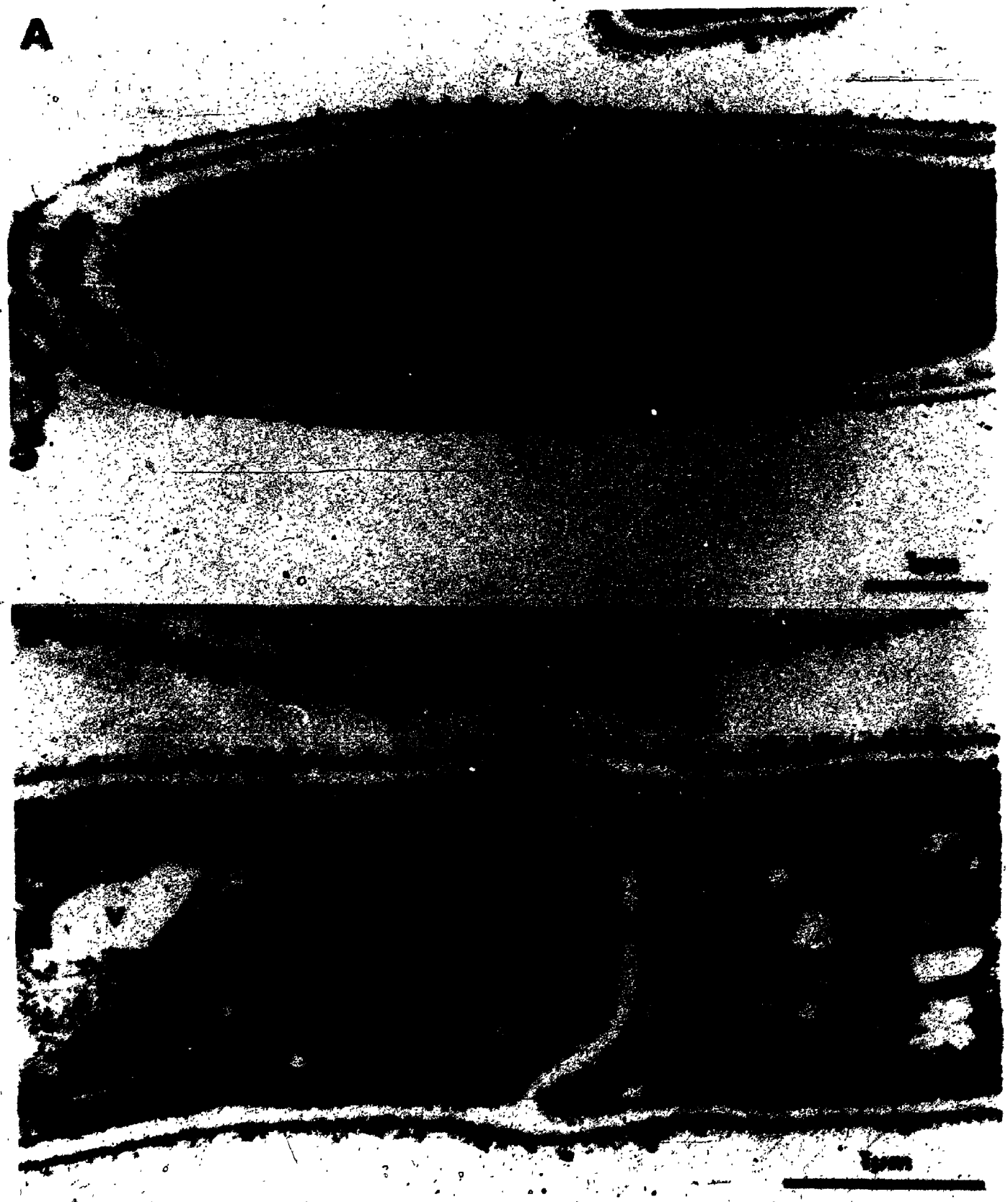
(P_2). Any particles smaller and/or less dense than the microsomes remain in the 105,000 g supernatant (S_2) though this fraction is generally considered to be more or less free of particulates and composed principally of soluble material. The above scheme however is only a broad generalization and cannot be assumed to hold good with all organisms. Zalokar (1965) reviewed the literature upon centrifugal fractionation of fungal organelles.

The electronmicrographs in Plate 6-2 demonstrate that the organization of cell organelles in ethane-grown Acremonium 3E/I cells is similar to that normally found in eukaryotic cells. Therefore the basic assumptions for centrifugal fractionation are likely to apply. Sections reveal the conventional, expected fungal ultrastructural features: cell walls, cytoplasm bound by a plasma membrane, nucleus bound by a nuclear envelope, often elongate mitochondria, endoplasmic reticulum, ribosomes, vacuoles etc. (Bracker 1967). The stacks of membranes surrounding the nucleus in Plate 6-2 A are unusual but not necessarily associated with ethane-utilization.

Ethane fixation was assayed in each of the three fractions obtained by differential centrifugation. An attempt was made to assay the activity of each fraction at approximately the same protein concentration it occupied in the original crude homogenate; in a hypothetical case if the original homogenate (containing 3.050 mg ml^{-1} total protein) was made up of 0.450 mg ml^{-1} P_1 protein, 0.850 mg ml^{-1} P_2

PLATE 6-2 A and B

Electron micrographs of ethane-grown cells of Acremonium 3E/1. CW cell wall; ER endoplasmic reticulum; GL glycogen granules; M mitochondrion; N nucleus; NE nuclear envelope; PM plasma membrane; S septum; SM stack of membranes (four layers around nuclear envelope); V vacuole; WB Woronin body.


A

protein and 1.750 mg ml^{-1} S_2 protein (from Lowry assay, sect. 6.2.3.) then these same concentrations would be approximated when syringes were charged with individual fractions (S_2 was used undiluted, P_1 and P_2 suspensions were diluted accordingly). Hence the values for 'mg protein per syringe' in Table 6-3 should add up to a total close to the value 'mg protein per ml' of the original homogenate (x 0.95).

Activity of individual fractions and a control of unfractionated original crude were assayed for total accumulated labelled compounds after one hour in the presence of added NADPH. No attempt was made to derive specific activities for the protein of different fractions since linearity of accumulation had not been established over the one hour period. A comparison of activity however can be made taking into account distribution of protein between the fractions. Fraction S_2 with 64.5% of the total protein had only 4.8% of the total accumulated labelled compounds of all three fractions. Most of the activity resided in the particulates, primarily in P_2, but P_1 was also quite active, they accounted for 76.2% and 19.0% respectively of accumulation with 23.8% and 11.7% of the total protein. It is apparent that the three fractions separately do not have the same ability to accumulate labelled compounds as they do when combined together in the original crude homogenate; the sum total of accumulated labelled compounds of the three individual fractions is only 46.9% of that of the

intact crude homogenate.

Gallo et al. (1973a) noted that when cells of Candida tropicalis were broken by shaking with glass beads, the mitochondria of the cells suffered considerable damage, such that mitochondrial fragments were produced which would not sediment at 10,000 g but sedimented with the microsomes at a higher g force. It was quite feasible that this could also be true with Acremonium mitochondria; it is difficult to imagine the elongate mitochondria in Plate 6-2 surviving such harsh treatment intact. While verifying the data in Table 6-3 with two further duplicate experiments mitochondria and any fragments derived from them were localized by assay of cytochrome oxidase activity. Data from these experiments are presented in Tables 6-4 and 6-5; values reported are means of two determinations.

Apart from the lower overall activity of the original crude homogenates the same general pattern of distribution of ethane-fixing activity can be seen (especially in Table 6-4) as was noted in Table 6-3. Better recovery of activity was obtained however during fractionation; the sum totals of accumulated labelled compounds of individual fractions were 75.9% and 80.9% of that of the intact crude in Tables 6-4 and 6-5 respectively. This loss might result from damage to particulates during resuspension of pellets in the glass homogenizer, a process which cannot be completely standardized from one experiment to the next. Alternatively, there might be present in fraction S_2 factors which

Table 6-4. Distribution of protein, '[1,2-^{14}C] ethane-fixing' activity and cytochrome oxidase activity in fractions of a crude homogenate of Acremonium 3E/1 cells. Experiment I.

	Protein		[1,2-^{14}C] ethane 'fixation'[a]			Cytochrome oxidase	
	mg per ml of crude	% of total	Mean net cpm per 0.1 ml extract after one hour and SD[b]	cpm per fraction as % of total of $P_1+P_2+S_2$	mg protein per syringe	Mean units[c] per mg protein and SD[b]	Units per fraction as % of total of $P_1+P_2+S_2$
Crude	3.326	-	3,728 (± 18)	-	3.160	519.1 (± 115.2)	-
P_1	0.464	14.4%	501 (± 14)	17.7%	0.441	679.1 (± 21.2)	41.1%
P_2	0.821	25.5%	2,062 (± 19)	72.9%	0.777	506.5 (± 65.9)	54.1%
S_2	1.934	60.1%	267 (± 21)	9.4%	1.837	18.9 (± 5.2)	4.8%

[a] In the presence of 0.5 µmole NADPH at 30°C.

[b] Standard Deviation.

[c] Nanomoles oxidized per minute at 25°C.

Table 6-5. Distribution of protein, '[1,2-^{14}C] ethane-fixing' activity and cytochrome oxidase activity in fractions of a crude homogenate of Acremonium 3E/1 cells. Experiment II.

	Protein		[1,2-^{14}C] ethane 'fixation'[a]			Cytochrome oxidase	
	mg per ml of crude	% of total	Mean net cpm per 0.1 ml extract after one hour and SD[b]	cpm per fraction as % of total of $P_1+P_2+S_2$	mg protein per syringe	Mean units[c] per mg protein and SD[b]	Units per fraction as % of total of $P_1+P_2+S_2$
Crude	2.645	-	7,396 (± 313)	-	2.513	606.8 (± 5.2)	-
P_1	0.291	9.7%	1,144 (± 69)	19.1%	0.276	1,375.2 (± 362.1)	42.3%
P_2	0.808	26.8%	4,624 (± 143)	77.3%	0.768	629.4 (± 49.4)	53.9%
S_2	1.914	63.5%	216 (± 37)	3.6%	1.818	18.5 (± 2.9)	3.7%

[a] In the presence of 0.5 µmole NADPH at 30°C.

[b] Standard Deviation.

[c] Nanomoles oxidized per minute at 25°C.

stimulate ethane 'fixation' or stabilize the particulate enzymes.

As suspected, cytochrome oxidase activity was found not only in fraction P_1, where intact mitochondria were expected, but also in P_2, indicating that breakage of mitochondria was taking place. Again a loss in activity was experienced when the sum total of units of cytochrome oxidase in the individual fractions are compared with the units per millilitre of original crude homogenate; losses of 55,6% and 41.2% were found in Tables 6-4 and 6-5 respectively. The percent distribution of recovered cytochrome oxidase activity remained fairly constant in both experiments. More units of cytochrome oxidase were found in P_2 than in P_1. This does not necessarily mean however that most of the original mitochondria exist as 105,000 g sedimentable fragments. Simon (1957), amongst others, noted that higher cytochrome oxidase activity was often recorded for broken than for whole mitochondrial preparations. The reason given was that cytochrome c, being a relatively large molecule (molecular weight 12,384), was only available to the enzyme on the surface of intact mitochondria; breakage of the organelle exposed any enzyme molecules on the inside to the substrate.

Since most of the ethane-fixing activity was found in fraction P_2, time course experiments were conducted to follow accumulation of labelled compounds in the presence of NADPH with different concentrations of such particles.

These data are presented graphically in Figure 6-3. When particle concentrations were increased to higher levels than that of such particles in the original homogenate (1.576 and 1.895 mg protein per syringe), accumulation of labelled compounds did not follow the pattern observed in the crude homogenate (Fig. 6-2). Fairly rapid accumulation of labelled compounds was observed for the first 15 minutes then a decline in rate until a level of approximately 4-5,000 cpm per 0.1 ml extract was reached, at which point accumulation ceased. With a very low concentration of particles (0.087 mg protein per syringe) the accumulation rate was low but apparently linear for 30 minutes.

The same phenomenon was observed in previous experiments (Tables 6-3, 6-4 and 6-5). P_2 suspensions did not accumulate labelled compounds beyond this 4-5,000 cpm 'plateau', even after incubation for a full hour (higher values were later obtained when ethane of higher specific activity was used).

Tables 6-6 and 6-7 present data from duplicate experiments in which suspensions of P_2 particles were exposed to the labelled ethane mixture in the presence of different coenzymes. Such data should be closely compared with those from the same experiment performed with crude homogenates (Tables 6-1 and 6-2). No significant accumulation and therefore ethane 'fixation' occurred in the absence of added coenzyme or when NAD^+ was supplied. Only slight activity was recorded with added NADH. Again the highest

Figure 6-6. Effect of coenzymes (0.5 µmole) upon [1,2-^{14}C] ethane 'fixation' by fraction P_2 of an Acremonium 3E/1 homogenate at 30°C. Experiment I.

	Mean net cpm per 0.1 ml extract after one hour and SD[a]	cpm per 0.1 ml as % of NADPH	Protein per syringe (mg)
Control	0	0%	0.831
NADPH	3,468 (± 112)	100%	"
$NADP^+$	305 (± 124)	8.8%	"
NADH	187 (± 27)	5.4%	"
NAD^+	0	0%	"

[a]Standard Deviation.

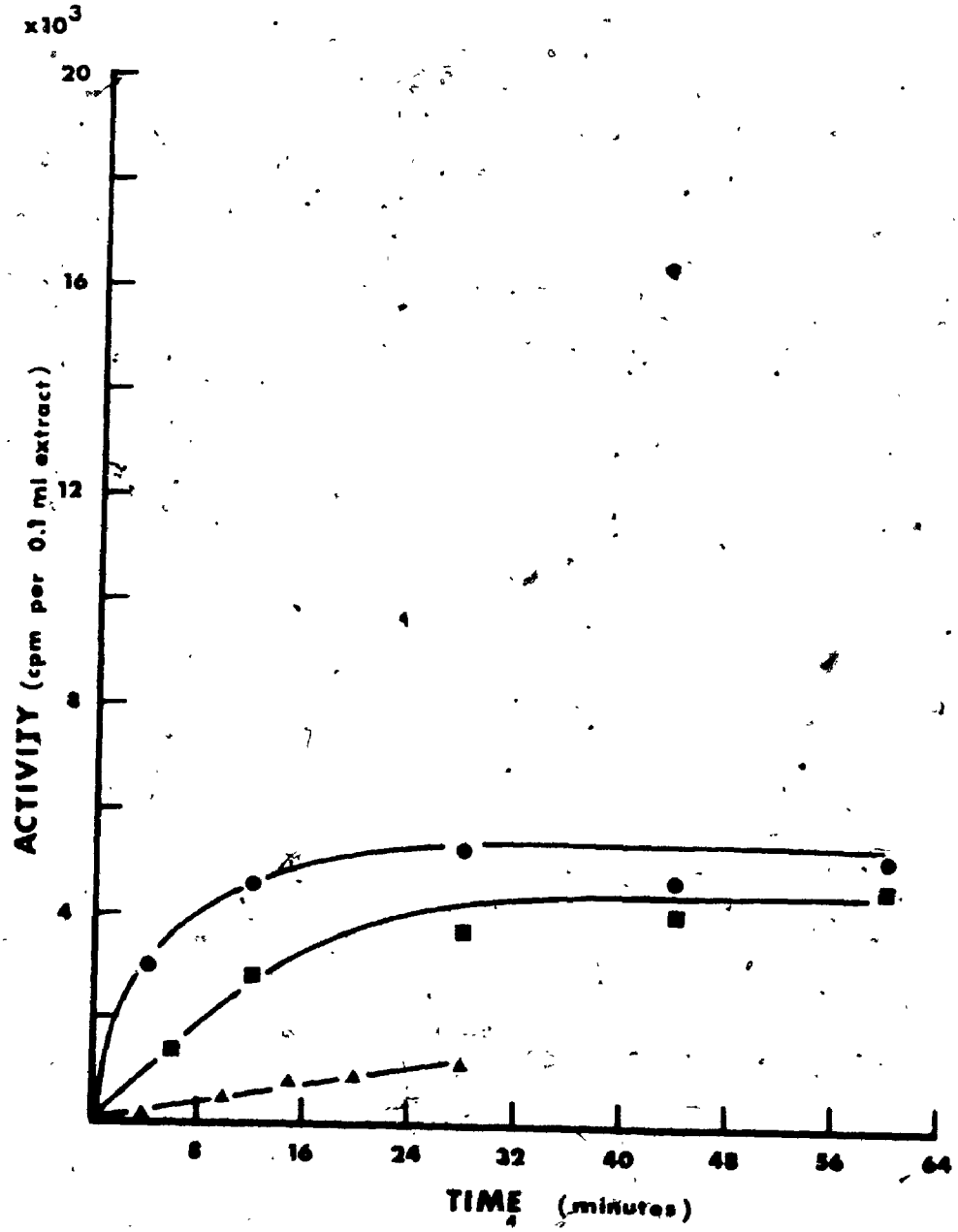
Table 6-7. Effect of coenzymes (0.5 µmole) upon [1,2-^{14}C] ethane 'fixation' by fraction P_2 of an Acremonium 3E/1 homogenate at 30°C. Experiment II[a].

	Mean net cpm per 0.1 ml extract after one hour and SD[b]	cpm per 0.1 ml as % of NADPH	Protein per syringe (mg)
Control	0	0%	1.005
NADPH	12,664 (± 247)	100%	"
$NADP^+$	994 (± 1)	7.8%	"
NADH	590 (± 35)	4.7%	"
NAD^+	0	0%	"

[a]High specific activity [1,2-^{14}C] ethane and air mixture used in this experiment.

[b]Standard Deviation.

Figure 6-3. Fixation of [1,2-^{14}C] ethane by fraction P_2 of an Acremonium 3E/1 homogenate in the presence of 0.5 µmole NADPH at 30°C. -●- 1.895 mg protein per syringe; -■- 1.516 mg protein per syringe; -▲- 0.087 mg protein per syringe.


x10^3
20
16
12
8
4
ACTIVITY (cpm per 0.1 ml extract)
8
16
24
32
40
48
56
64
TIME (minutes)

activity was recorded with NADPH. The greatest contrast with the previous data was found in the effect of $NADP^+$ the addition of which produced less than 10% of the label accumulation recorded for NADPH, compared to approximately 75% in intact crude homogenates. Reference to the work of Gallo et al. (1973a) offers a possible explanation. In their work on Candida tropicalis these authors, in attempting to explain some anomalous past data, suggested and found evidence for the existence in their crude homogenates of a mitochondrial ATP-dependent transhydrogenase, which catalysed the following reaction irreversibly:

$$NADH + H^+ + NADP^+ \xrightarrow{ATP \quad ADP + Pi} NAD^+ + NADPH + H^+$$

Other systems in nature are known by which $NADP^+$ can be reduced by NADH either directly by a reversible transhydrogenase,

$$NADH + H^+ + NADP^+ \rightleftharpoons NAD^+ + NADPH + H^+$$

or indirectly via coupled reactions,

$$A + NADH + H^+ \longrightarrow NAD^+ + AH_2$$

$$AH_2 + NADP^+ \longrightarrow A + NADPH + H^+$$

The latter reaction alone could be the source of NADPH as long as there is an adequate supply of AH_2. A good example is glucose-6-phosphate-dehydrogenase catalyzing the following reaction:

$$\text{glucose 6-phosphate} + \text{NADP}^+ \longrightarrow \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+$$

This is considered to be a major source of NADPH for various reductive functions in many organisms.

It can be postulated that if, as seems likely, NADPH is essential for ethane fixation in Acremonium cells then a system must be present to reduce the $NADP^+$ generated by use of NADPH as a reductant. One would expect to find such a system in crude homogenates of such cells. The fact that $NADP^+$ all but substitutes for NADPH in crude homogenates, yet has little effect upon P_2 alone indicates that the $NADP^+$ reducing system in its entirety is not found in this fraction.

It would seem that the crude homogenate has a considerable capacity to generate reducing equivalents but that its ability to make use of this capacity in NADPH production is limited by a lack of $NADP^+$ unless it is supplied exogenously. Isolation of fraction P_2 effectively separates the 'ethane-fixing' enzyme system from the NADPH-generating system which supports it. This system can be

replaced by exogenous NADPH. Without added co-enzyme (control) or with just NAD^+ no ethane fixation takes place. The marginal effects of $NADP^+$ and to a lesser extent NADH (in effect an input of reducing equivalents) can possibly be attributed to incomplete resolution of fractions; P_2 is still contaminated by traces of its 'support system'. A low but definite rate of endogenous $NADP^+$ reduction by crude homogenates from two different batches of cells is demonstrated in Table 6-8. Endogenous NAD^+ reduction was absent.

The premature termination of ethane fixation in P_2 suspensions (Fig. 6-3) as compared to that in crude homogenates (Fig. 6-2) might be explained by limiting amounts of NADPH. In the former case ethane 'fixation' would cease when all the exogenous NADPH had been converted to $NADP^+$; in crude homogenates NADPH could be re-generated from $NADP^+$, allowing more ethane 'fixation' to take place.

It is apparent from Tables 6-9 and 6-10 that carbon monoxide is a potent inhibitor of ethane fixation. Respectively 81.2% and 69.5% inhibition were found for duplicate experiments where the ratio of $CO:O_2$ was 1:2. Where this ratio was adjusted to 2:1 virtually complete inhibition occurred. Due to the method employed to prepare gas mixtures, the final ethane concentration differed in 1:2 and 2:1 mixtures due to the different amounts of carbon monoxide added to the existing ethane and air mixture to prepare them. This reduction in substrate did not apparently affect accumulation of label, since 1:2 and 2:1 nitrogen

Table 6-8. Endogenous $NADP^+$ reduction by crude homogenates of Acremonium 3E/1 cells.

	Units[a] per mg protein	Units per ml crude
Experiment I	19.6	59.1
Experiment II	30.2	87.1

[a]Nanomoles reduced per minute at 25°C.

Table 6-9. Carbon monoxide inhibition of [1,2-^{14}C] ethane 'fixation[a]' by fraction P_2 of an Acremonium 3E/1 homogenate at 30°C. Experiment I.

	Mean net cpm per 0.1 ml extract after 15 minutes and SD[b]	cpm per 0.1 ml as % of N_2 control	mg protein per syringe
$CO:O_2$ = 1:2	246 ± 35	18.8%	0.940
N_2 control	1,308 ± 9	100.0%	"
$CO:O_2$ = 2:1	0	0%	"
N_2 control	1,220 ± 83	100%	"

[a]In the presence of 0.5 µmole NADPH.

[b]Standard Deviation.

Table 6-10. Carbon monoxide inhibition of [1,2-^{14}C] ethane 'fixation[a]' by fraction P_2 of an Acremonium 3E/1 homogenate at 30°C. Experiment II.

	Mean net cpm per 0.1 ml extract after 15 minutes and SD[b]	cpm per 0.1 ml as % of N_2 control	mg protein per syringe
$CO:O_2$ = 1:2	1,203 (± 23)	30.5%	0.780
N_2 control	3,943 (± 134)	100%	"
$CO:O_2$ = 2:1	127 (± 18)	3.2%	"
N_2 control	3,960 (± 108)	100%	"

[a]In the presence of 0.5 µmole NADPH.

[b]Standard Deviation.

controls did not differ significantly in label accumulation.

Carbon monoxide inhibition is characteristic of metalloporphyrin enzymes involved in direct reaction with molecular oxygen (e.g. haemoglobin or cytochrome oxidase). Such inhibition in an alkane-oxidizing system points to the involvement of cytochrome P-450.

The effects of cyanide and azide are more difficult to interpret. It can be seen from Tables 6-11 and 6-12 that while 1.0 mM potassium cyanide had no effect 1.0 mM sodium azide inhibited label accumulation by 41.9% and 48.3% respectively for duplicate experiments.

McKenna and Coon (1970) reported that the Pseudomonas oleovorans hydroxylase system, which does not contain cytochrome P-450, is sensitive to cyanide but not to carbon monoxide. Heinz et al. (1970) reported that their oleic acid hydroxylating system from a Torulopsis sp. was inhibited by carbon monoxide but not by cyanide or azide, even at 10 mM, in fact cyanide even stimulated hydroxylation: Duppel et al. (1973) however found that the Candida tropicalis P-450-hydrocarbon hydroxylase system was inhibited not only by carbon monoxide but by cyanide and azide also but only after 15 minutes of preincubation at 0°C with the latter two inhibitors. Without such pre-incubation even 10 mM cyanide was ineffective. Cardini and Jurtshuk (1970) reported that their cytochrome P-450 system from a Corynebacterium sp. was inhibited by 13% and 20% respectively by 1.0 mM concentrations of cyanide and azide (without pre-

Table 6-11: Effect of inhibitors upon [1,2-^{14}C] ethane 'fixation[a]' by fraction P_2 of an Acremonium 3E/1 homogenate at 30°C. Experiment I.

	Mean net cpm per 0.1 ml extract after 15 minutes and SD[b]	cpm per 0.1 ml as % of control	mg protein per syringe
Control	3,744 (± 10)	100.0%	1.480
NaN_3 (1mM)	2,174 (± 42)	58.1%	"
KCN (1mM)	3,827 (± 21)	102.2%	"

[a] In the presence of 0.5 µmole NADPH.

[b] Standard Deviation.

Table 6-12. Effect of inhibitors upon [1,2-^{14}C] ethane 'fixation[a]' by fraction P_2 of an Acremonium 3E/1 homogenate at 30°C. Experiment II.

	Mean net cpm per 0.1 ml extract after 15 minutes and SD[b]	cpm per 0.1 ml as % of control	mg protein per syringe
Control	3,904 (± 331)	100.0%	1.047
NaN_3 (1mM)	2,017 (± 22)	51.7%	"
KCN (1mM)	3,625 (± 83)	92.8%	"

[a]In the presence of 0.5 µmole NADPH.

[b]Standard Deviation.

incubation).

If, as seems likely, ethane 'fixation' in Acremonium involves a cytochrome P-450-hydroxylase, significant NADPH-cytochrome c reductase activity should be associated with 'ethane-fixing' activity as found by Gallo et al. (1973a, 1973b) in Candida tropicalis oxidizing n-decane. That this is in fact true can be seen from data in Tables 6-13 and 6-14. Almost 60% of the cytochrome c - NADPH reductase activity was found in the 'ethane-fixing' particulates; over 40% however was found in the soluble fraction. This enzyme is characteristically microsomal; the harsh treatment required for cell disruption and/or re-suspension of pellets after centrifugation could have helped solubilize it in these preparations. Although P_2 is more effective in 'fixing' ethane than P_1, a higher specific activity was recorded for this enzyme in the latter fraction (units per milligram of protein); P_2 however did contain a greater proportion of the total activity (units) of the homogenate. Quite possibly the specific activity in P_2 is lowered by the presence of many other proteins, from broken mitochondria for example.

Despite indications of the involvement of cytochrome P-450 in ethane 'fixation' (CO inhibition, NADPH - cytochrome c reductase activity) its presence could not be demonstrated by a carbon monoxide difference spectrum. Suspensions of P_2 particles ranging in concentration from 1-3 mg protein per ml were tried. Particles were suspended in stabilizing

Table 6-13. Distribution of cytochrome c - NADPH reductase activity in fractions of a crude homogenate of Acremonium 3E/1 cells. Experiment I.

	Protein		Cytochrome c - NADPH reductase	
	mg per ml crude	% of total	Units[a] per mg protein	Units per fraction as % of total of $P_1+P_2+S_2$
Crude	2.943	-	245.9	-
P_1	0.291	10.3%	393.9	22.2%
P_2	0.932	33.1%	171.0	30.8%
S_2	1.595	56.6%	152.5	47.0%

[a]Nanomoles reduced per minute at 25°C.

Table 6-14. Distribution of cytochrome c - NADPH reductase activity in fractions of a crude homogenate of Acremonium 3E/1 cells. Experiment II.

	Protein		Cytochrome c - NADPH reductase	
	mg per ml crude	% of total	Units[a] per mg protein	Units per fraction as % of total of $P_1+P_2+S_2$
Crude	3.120		208.0	
P_1	0.383	12.6%	350.2	22.6%
P_2	0.946	31.2%	227.7	36.3%
S_2	1.706	56.2%	142.7	41.0%

[a]Nanomoles reduced per minute at 25°C.

medium, 0.05 M Tris-HCL buffer (pH 7.6) or 0.1 M potassium phosphate buffer (pH 7.0). No trace was ever found of a peak at 450 nm despite repeated attempts.

The presence of an NAD^+-dependent alcohol dehydrogenase active upon ethanol is demonstrated by data presented in Tables 6-15 and 6-16. This is apparently a soluble enzyme; no activity was found in the particulates, only in S_2.

Table 6-15. Distribution of NAD^+ - dependent alcohol dehydrogenase in fractions of a crude homogenate of Acremonium 3E/1 cells. Experiment I.

	Protein		Alcohol dehydrogenase
	mg per ml crude	% of total	Units[a] per mg protein
Crude	2.712	-	66.4
P_1	0.373	15.1%	0
P_2	0.777	31.6%	0
S_2	1.313	53.3%	126.0

[a]Nanomoles NAD^+ reduced per minute at 25°C.

Figure 6-16. Distribution of NAD^+ - dependent alcohol dehydrogenase in fractions of a crude homogenate of Acremonium 3E/1 cells. Experiment II.

	Protein		Alcohol dehydrogenase
	mg per ml crude	% of total	Units[b] per mg protein
Crude	3.022	-	55.2
P_1	0.488	20.6%	0
P_2	0.696	29.3%	0
S_2	1.190	50.1%	187.9

[a] Nanomoles NAD^+ reduced per minute at 25°C.

CHAPTER 7

GENERAL DISCUSSION

A terminal oxidative pathway for two, three and four-carbon n-alkanes is indicated for Acremonium 3E/1 from studies employing [1,2-^{14}C] ethane and manometric studies on resting cells. The relatively low rates of oxygen uptake with secondary three and four carbon alcohols argue against sub-terminal attack upon propane and n-butane as the major pathway for these substrates. A common pathway is indicated for functional oxidation of ethane propane and n-butane as far as the fatty acid level of oxidation.

From Figure 2-1 it can be seen that implication of the corresponding primary alcohol, aldehyde and fatty acid as intermediates in n-alkane oxidation is not proof of the involvement of a monooxygenase in the initial alkane 'fixation' reaction. The primary alcohol is not necessarily the product of the initial 'fixation' reaction; 1-alkenes and 1-hydroperoxides have also been suggested as the first stable product with later production of primary alcohols.

The approximate standard free energy change ($\Delta G'$) for theoretical ethane dehydrogenation by NAD^+ can be derived

from the free energies of formation of reactants and products (Rossini et al. 1953):

$$C_2H_6 + NAD^+ \longrightarrow C_2H_4 + NADH + H^+ \qquad \Delta G' + 19.8 \text{ Kcal mole}^{-1}$$

ethane — ethylene

The large input of energy required makes this reaction quite unfeasible unless driven by an external source of energy (use of $NADP^+$ instead of NAD^+ would not effect $\Delta G'$ since both have the same redox potential for the half reaction; oxidized form ⟶ reduced form).

A similar calculation for hydroxylation reveals a quite different picture:

$$C_2H_6 + NADPH + H^+ + O_2 \longrightarrow C_2H_5OH + NADP^+ + H_2O \qquad \Delta G' - 86.5 \text{ Kcal mole}^{-1}$$

ethanol

This reaction is strongly exergonic and is therefore much more feasible than dehydrogenation.

The absence of any uptake of gaseous alkanes without molecular oxygen argues for an initial 'fixation' reaction involving oxygen uptake rather than initial dehydrogenation. The absence of gas uptake by resting cells of Acremonium 3E/1 in atmospheres of ethylene or 1-butene in air and the

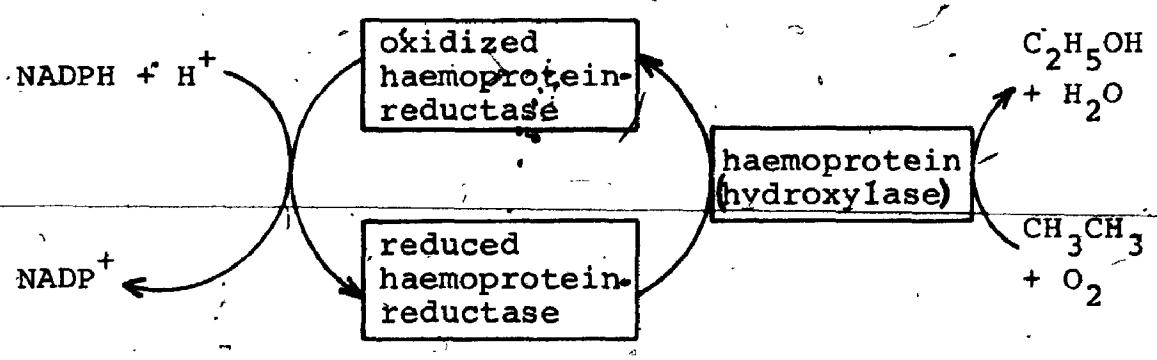
inhibitory effect upon growth of low concentrations of ethylene do not argue for initial dehydrogenation of alkanes.

In the partially purified enzyme system from Acremonium 3E/1 ethane 'fixation' required a reduced coenzyme (NADPH). Oxidized coenzymes ($NADP^+$ or NAD^+) were virtually ineffective. This is good evidence against initial dehydrogenation of ethane; oxidized coenzymes have always been implicated in this mechanism. An explanation was put forward in chapter 6 to account for the stimulation of ethane 'fixation' by $NADP^+$ in crude preparations. Involvement of reduced coenzyme indicates initial hydroxylation (by a monooxygenase system) or 1-hydroperoxide formation and subsequent reduction to a primary alcohol.

Biological 1-hydroperoxidation of n-alkanes has never been demonstrated, only inferred. There are however definite similarities between Acremonium 3E/1 enzyme preparations and known monooxygenase systems from other organisms. Direct incorporation of $^{18}O_2$ into a substrate is the most conclusive proof of monooxygenase activity; this has been demonstrated for comparatively few systems however. Carbon monoxide inhibition of initial alkane 'fixation' is generally taken as good evidence of a monooxygenase system involving a haemoprotein which reacts directly with molecular oxygen. Carbon monoxide is a potent inhibitor of ethane 'fixation' by the Acremonium 3E/1 system. The presence of NADPH - cytochrome c reductase activity associated with 'ethane-fixing' activity in Acremonium 3E/1 prep-

arations again points towards the involvement of a cytochrome-like haemoprotein in ethane 'fixation'.

The most completely resolved and understood eukaryote monooxygenase systems are the mammalian liver microsomal system described by Coon and coworkers (Coon et al. 1972) and the Candida tropicalis microsomal system described by Gallo et al. (1973a, 1973b) and Duppel et al. (1973) which were referred to in section 2.5. The Acremonium 3E/1 system closely resembles these systems in many ways. It is apparently microsomal and dependent upon NADPH rather than NADH. The presence of cytochrome c - NADPH reductase associated with 'ethane-fixing' activity suggests a two protein 'ethane-fixing' system in which electrons are transferred from NADPH to a haemoprotein hydroxylase directly by a reductase without the intervention of a non-haem-iron-protein of the rubredoxin type:



Certain apparent deviations from the pattern of the liver and C. tropicalis systems deserve comment. Despite all the indications to the contrary the presence of cytochrome P-450 could not be proven in Acremonium 3E/1.

Possibly another haemoprotein is involved, similar to cytochrome P-450. It seems more likely however that technical difficulties prevented the spectral demonstration of the cytochrome-CO complex. The presence of such a complex was indicated by CO inhibition of ethane 'fixation'. A possible explanation might be in the use of sodium dithionite as a reductant. Noticeable flocculation of microsomal suspensions occurred after short exposures to this reagent.

Although ethane 'fixation' in Acremonium 3E/1 appears to be predominantly microsomal, as in the liver and C. tropicalis systems, a significant proportion of such activity was found in the 10,000 g 'mitochondrial' fraction. As it was seen in chapter 6, centrifugal fractionation of organelles was not very precise. It seems quite possible that although most of the endoplasmic reticulum is recovered in the 105,000 g pellet as microsomes, larger vesicles and/or clumps of membrane fragments sediment with intact mitochondria at 10,000 g. One might speculate that the membrane stacks seen in Plate 6-1 A could give rise to such clumps upon cell disruption.

Studies on the postulated monooxygenase systems of Candida intermedia (Liu and Johnson 1971) and Cladosporium resinae (Walker and Cooney 1973) have not been pursued as far as those on Candida tropicalis. In Cladosporium alkane-oxidizing activity was localized only to the 10,000 g supernatant, which could contain both soluble and particulate enzymes. Lack of a test for CO inhibition leaves

possible haemoprotein involvement an open question in both C. intermedia and C. resinae.

Apparent differences between the systems of C. intermedia and C. resinae and those of Candida tropicalis and Acremonium 3E/1 warrant some comment. The lack of ethane-oxidizing activity in the 10,000 g supernatant of C. intermedia could be due to the appropriate coenzyme not being supplied. The corresponding fractions of C. tropicalis and Acremonium 3E/1 (105,000 g particulates) were also inactive unless NADPH was supplied.

Alkane-oxidizing activity of the 10,000 g supernatant of Cladosporium resinae was stimulated by both NADPH and NADH equally well. This is somewhat unusual. Almost without exception in eukaryote metabolism the coenzyme used for reductive functions is NADPH rather than NADH. The reverse is true for oxidative energy yielding functions; NAD^+ is usually used rather than $NADP^+$. There is quite possibly a phenomenon here similar to that described by Gallo et al. (1973a) in Candida tropicalis. Crude extracts were stimulated by NADH and NADPH while purified microsomes required NADPH (discussed in section 6.3.). A similar phenomenon was found in Acremonium 3E/1. The 10,000 g supernatant of Cladosporium resinae is more akin to a crude homogenate than a purified microsomal suspension. It seems quite possible from the data of Walker and Cooney (1973), that the alkane monooxygenase of C. resinae is microsomal and NADPH-dependent. Stimulation of alkane oxidation by NADH

might be attributed to a transhydrogenase (possibly in mitochondrial fragments) which transferred reducing equivalents from NADH to endogenous $NADP^+$.

Quite possibly all four fungi discussed have basically similar alkane monooxygenase systems. It would of course be foolish to make broad generalizations about fungal alkane monooxygenases based on such limited data. The fungi are an extremely heterogenous group of organisms, believed to be of polyphyletic origin. The difference between the monooxygenase systems of two eubacteria, Corynebacterium 7EIC and Pseudomonas oleovorans performing essentially the same function serves to illustrate the danger of making hasty generalizations based upon scant evidence (sect. 2.5.).

It seems likely that a single enzyme is responsible for oxidation of primary alcohols to aldehydes and another for oxidation of aldehydes to fatty acids. Data from Gallo et al. (1973a, 1973b) indicated that the alcohol and aldehyde dehydrogenases responsible for the oxidation of 1-decanol and decanal respectively in Candida tropicalis were NAD^+-dependent and microsomal. Similar alcohol and aldehyde dehydrogenases were found by Liu and Johnson (1971) in Candida intermedia, active upon the same substrates. These were again NAD^+-dependent and particulate. They were found together in 10,000 g and 105,000 g particulates. The corresponding enzymes in Cladosporium resinae (Walker and Cooney 1973) were not localized any more precisely than the hydroxylase system of this species. They were found with

it in the 10,000 g supernatant. However stimulation was claimed by both NAD^+ and $NADP^+$. Here again it is pertinent to point out once more that oxidative enzymes, such as alcohol and aldehyde dehydrogenases, are normally NAD^+ rather than $NADP^+$-linked in eukaryotes. In view of the impurity of the preparation however it is possible that $NADP^+$ could have stimulated substrate oxidation by allowing re-oxidation of NADH to NAD^+ by a transhydrogenase as it was suggested before. Aldehyde dehydrogenase activity in the presence of NAD^+ or $NADP^+$ upon acetaldehyde could not be demonstrated in Acremonium 3E/1 extracts. Yet the presence of such an enzyme was inferred by manometric and tracer studies upon whole cells. It is possibly inactivated during cell disruption.

A soluble NAD^+-dependent, alcohol dehydrogenase active upon ethanol was found. Unlike the enzymes described by Liu and Johnson (1971) and Gallo et al. (1973a, 1973b) which were active on long chain substrates, this enzyme was not bound to the alkane-hydroxylating particles.

Once two, three and four carbon fragments have reached the fatty acid level of oxidation in Acremonium 3E/1 their further assimilation can proceed via pathways common to many microbes not only those utilizing hydrocarbons. A likely first step is activation by an acyl coenzyme A synthetase to form the corresponding acyl CoA.

$$RCOOH + CoA + ATP \xrightarrow{Mg^{++}} RCOCoA + AMP + PPi$$

Such an enzyme was found in alkane-grown Candida tropicalis extracts active upon long chain fatty acids (Duvnjak et al. 1970).

As discussed in section 2.4.1. the most probable fate of two and three carbon acyl CoA derivatives is via the glyoxylate and methyl malonate pathways respectively, resulting in net synthesis of succinate, a tricarboxylic acid (TCA) cycle intermediate. Butyryl CoA can be split by one turn of the β-oxidative spiral into two acetyl CoA fragments which can then pass to the glyoxylate cycle.

The adaptive or constitutive nature of alkane oxidation in Acremonium 3E/1 could not be determined due to practical difficulties. Culture on carbohydrate media resulted in production of conidia, not vegetative mycelium. This subject was discussed at length by Van der Linden and Thijsse (1965). Microbial alkane oxidation is predominantly inducible. Van der Linden and Thijsse caution against acceptance of claims of constitutive systems based on oxygen uptake in the presence of alkane substrates by carbohydrate-grown cells. Such data might be due to oxidation of oxygenated impurities in the alkanes.

It is reasonable to assume that C_{2+} n-alkane-utilizing microbes in general and fungi in particular evolved from organisms with more conventional carbon metabolism. The widespread occurrence of this ability in many unrelated taxa suggests this adaptation has occurred independently many times since fossil fuels became available as sub-

strates in the natural environment.

It is apparent that only the initial biochemical steps in n-alkane assimilation are peculiar to alkane utilizers. Biochemical transformations of fatty acids are universal; these are the building blocks of membrane phospholipids. Adaptations from carbohydrate to hydrocarbon utilization would principally be concerned with uptake of the hydrophobic substrate into the microbial cell and the biochemical mechanism needed to reach the fatty acid level of oxidation. In the case of microbes oxidizing n-alkanes via the terminal hydroxylation route, a hydroxylase system and alcohol and aldehyde dehydrogenases would be needed to cope with long chain substrates.

Adaptation to utilization of the gaseous n-alkanes might present less of a problem than is the case with higher homologues. Solubility of these gases in water is high; uptake of such substrates from solution can quite adequately account for growth. The problems of toxicity, found with C_5 to C_{10} homologues are not encountered. Development of a mechanism for hydrocarbon uptake by direct contact is not required. Alcohol and aldehyde dehydrogenases active upon low molecular weight substrates (two, three and four carbon atoms) are widespread amongst many microbes utilizing conventional carbon sources. Adaptation to use of gaseous alkanes could, in theory, be accomplished by evolution of a single enzyme system, the hydroxylase system.

Alkane-utilizing microbes have most frequently been

isolated from environments where a natural enrichment of such organisms is expected. Such an enrichment usually takes place where a potential n-alkane substrate exists in the presence of adequate moisture, oxygen and mineral nutrients at a suitable temperature. A natural gas leak in soil would be such a habitat. Fungi able to grow upon gaseous alkanes were isolated from such environments by McClee et al. (1972) and Adamse et al. (1972). Natural enrichment of organisms utilizing C_2 - C_4 gaseous alkanes would not be expected in a sewage treatment plant the source of the isolates described in chapter 3.

Davis (1967) discussed the use of the natural abundance of ethane and propane-utilizing microbes in soil as an indication of underlying petroleum reserves. Seepage of natural gas overlying such reserves takes place to the surface producing a natural enrichment of such organisms in the soil. An abundance of methane-utilizing microbes is not necessarily positively correlated with underlying natural gas reserves, probably due to widespread methane production by anaerobic bacteria.

It was noted that certain ethane-oxidizing bacteria, notably Mycobacterium paraffinicum, were the best indicators of ethane seepage. Ethane-utilizing moulds and actinomycetes often appeared upon soil plates incubated in ethane and air atmospheres but could not be positively correlated with natural ethane seepage. Davis suggested that with moulds and actinomycetes the isolation procedure had selected

for microbes which normally subsisted upon soil organic matter but were biochemically versatile enough to adapt to ethane-utilization when this substrate was available. The versatility of Phialophora jeanselmei is apparent from the wide variety of habitats it is known to occupy; sewage, paper-mill effluent slimes and even living human tissues.

Initial adaptation of fungi to utilization of C_2 to C_4 n-alkanes may be 'easier' than adaptation to higher homologues. However it can be argued that the shorter the alkane substrate-chain-length, the 'harder' the cell has to 'work' to 'make a living'. Pertinent theoretical data for growth upon ethane and n-hexadecane are presented in Table 7-1.

It can be seen from the data presented that ethane hydroxylation must proceed at eight times the rate of n-hexadecane hydroxylation for 'fixation' of the same amount of alkane carbon per unit time. This can be accomplished by either a higher turnover number per mole of enzyme or a higher concentration of enzyme per cell. Whichever is the case, it is also apparent from Table 7-1 that carbon fixed from ethane is more costly in terms of expended molecular oxygen and reducing equivalents than that from n-hexadecane.

The extra electrons required for ethane hydroxylation represent a loss of potential energy conservation from mitochondrial electron transport. Assuming a P/O ratio of 3, the two electrons required for one hydroxylation are used at the expense of synthesis of three molecules of ATP

Table 7-1. Comparison of ethane and n-hexadecane as microbial carbon and energy sources.

	Ethane	n-Hexadecane
1. Hydroxylations required per gram of carbon 'fixed'[a]	2.51×10^{22}	3.14×10^{21}
2. Reducing equivalents[b] required per gram of carbon 'fixed'	0.0830	0.0104
3. Oxygen (g) required per gram carbon 'fixed'	0.667	0.083
4. Approximate potential net energy conservation per mole of alkane oxidized to $CO_2 + H_2O$ (kilocalories)[c]	112.0	1,064.0
5. $\Delta G'$[d] per mole of alkane for complete combustion (kilocalories)	350.0	2,492.0
6. Efficiency of energy conservation (4 as % of 5)	31.9%	42.7%

[a] As the corresponding primary alcohol.

[b] Two reducing equivalents = one mole [NADPH + H^+].

[c] By the terminal hydroxylation pathway at pH 7.0, 25°C, assuming P/O ratio of 3 and 8.0 kilocalories conserved per mole of ATP formed.

[d] Standard free energy change in kilocalories at 25°C, 1 atmosphere.

which would represent conservation of approximately 24 kilo calories. Despite the highly exergonic nature of the reaction, energy conservation has never been demonstrated for any monooxygenase system. This apparent waste of potential energy is greater with ethane utilization when expressed in terms of loss per gram carbon 'fixed' and contributes to the lower efficiency of energy utilization for this substrate. This lower efficiency means of course that a higher proportion of the 'fixed' alkane carbon must be used for energy production, which lowers the efficiency of conversion of 'fixed' carbon into cell material.

Reference has been made to the possible involvement of transhydrogenases in reducing $NADP^+$ to NADPH to promote hydroxylation by monooxygenases in relatively crude enzyme preparations from broken fungal cells. The data in Table 7-1 further emphasize the need for a highly active $NADP^+$-reducing system in cells hydroxylating ethane. It should not be lightly assumed however that a transhydrogenase serves this function in intact cells. Transhydrogenases are characteristically mitochondrial enzymes. Mitochondria exhibit selective permeability. Pyridine nucleotides do not normally pass freely in and out of <u>intact</u> mitochondria. It cannot be assumed therefore that $NADP^+$, generated by an extra-mitochondrial hydroxylase, can pass freely into the mitochondrion for reduction by a transhydrogenase and that the NADPH produced can freely pass out of the mitochondrial compartment.

In this instance a useful parallel can be drawn between fungal cells hydroxylating short chain alkanes and mammalian liver cells. Both are highly active in reductive biochemical reactions and require an abundant supply of NADPH. Mitochondrial transhydrogenases are found in liver cells but are believed to be important only for producing NADPH for mitochondrial fatty acid synthesis. NADPH for extramitochondrial reductive functions (including microsomal hydroxylations) is believed to be generated by extramitochondrial $NADP^+$-linked dehydrogenases of the following type:

$$XH_2 + NADP^+ \longrightarrow X + NADPH + H^+$$

(many of the exceptions to the rule about NAD^+ involvement in oxidative functions are thought to act in this way). A similar mechanism is likely in fungal cells.

For every molecule of NADPH used in hydroxylation two molecules of NADH are produced by extramitochondrial oxidation of the primary alcohol product to the corresponding fatty acid. Can reducing equivalents be transferred from NADH to $NADP^+$ outside the mitochondria? A 'shunt' mechanism whereby this can be accomplished indirectly has been suggested for liver cells utilizing a TCA cycle intermediate, citrate, which can pass out of the mitochondria:

$$\text{citrate} + \text{ATP} + \text{CoA} \xrightarrow[\text{citrate lyase}]{} \text{oxaloacetate} + \text{acetyl CoA} + \text{ADP} + \text{Pi}$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow[\text{malic acid dehydrogenase}]{} \text{malate} + \text{NAD}^+$$

$$\text{malate} + \text{NADP}^+ \xrightarrow[\text{malic enzyme}]{} \text{pyruvate} + \text{NADPH} + \text{H}^+ + \text{CO}_2$$

This is in effect an indirect transhydrogenation from NADH to $NADP^+$ of the type referred to in chapter 6. Such a mechanism, rather than a direct mitochondrial transhydrogenase, could also account for the unusual effect of coenzymes in Cladosporium resinae extracts.

Progress in the present study can be summarized as follows:

1) Eleven new cultures were isolated by natural gas enrichments; they all made good growth at the expense of ethane, propane or n-butane but not methane or liquid n-alkanes. They were all hyphomycetes, three isolates of Phialophora jeanselmei, four Graphium isolates and five Acremonium isolates.

2) The morphology of representative cultures was described in detail on carbohydrate and gaseous alkane growth substrates.

3) Submerged growth characteristics of several cultures on gaseous alkanes were described.

4) Evidence is provided in support of a terminal oxidative pathway for gaseous n-alkane assimilation in a selected culture; Acremonium 3E/1, via corresponding primary alcohols, aldehydes and fatty acids in turn.

5) In vitro studies on ethane assimilation by Acremonium 3E/1 supported hydroxylation of the terminal methyl group as the first biological 'fixation' reaction of the alkane molecule. The evidence was consistent with involvement of an enzyme system of the monooxygenase type. This system appeared to be microsomal, NADPH-dependent and contained a haemoprotein, possibly cytochrome P-450.

Several future lines of research are indicated by the present findings:

1) More extensive surveys of various habitats for gaseous-alkane-utilizing fungi would be welcome; at present the taxonomic and ecological significance of this ability in fungi is not known.

2) Metabolic studies on Acremonium 3E/1 are far from complete. The enzyme system responsible for initial ethane 'fixation' has yet to be isolated and studied in depth. The involvement of cytochrome P-450 has yet to be proven. The possibility of an alternate sub-terminal pathway in the case of propane and n-butane requires further study as does the unexplained uptake of propylene but not ethylene or 1-butene. An attempt should be made to demonstrate production of alkenes from labelled alkane substrates.

3) Studies of a similar nature on other fungal cultures are desirable. Data from the work on Acremonium 3E/1 at present can only be related to work on fungal assimilation of long-chain liquid n-alkanes.

4) A comparison of electronmicrographs of carbohydrate and gaseous alkane-grown cells might reveal ultrastructural adaptation to this habit. It would be interesting to see whether the membrane stacks found around Acremonium 3E/1 nuclei were related to ethane utilization. Electron microscopy in conjunction with centrifugal fractionation of homogenates might more precisely localize the site of ethane 'fixation' in the cell.

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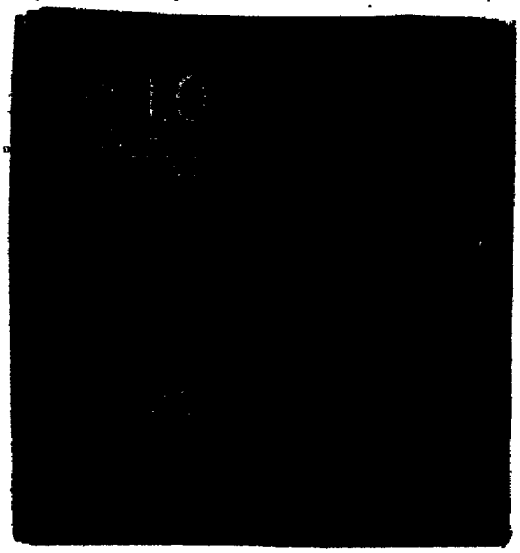

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