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STUDIES ON HYDROCARBON
METABOLISM IN
Acinetobacter lwoffi

Thesis presented before the School of Graduate
Studies, University of Ottawa, in partial
fulfilment of the degree of Master of Science
in Biology

by

VINCENT VACHON

- 1981 -

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I take this opportunity to thank them for the confidence they have shown me in accepting me in their research teams and for their invaluable guidance.

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To all these contributors, I wish to express my most thankful gratitude for their friendly and patient help and encouragement.

ABSTRACT

The lipid composition of Acinetobacter lwoffi was studied during a diauxic growth cycle which occurs when this bacterium is incubated in the presence of ethanol and hexadecane. During this diauxie, ethanol is first utilized, then after a relatively long lag phase a second growth cycle occurs during which hexadecane is used as carbon source.

After an initial increase in the amounts of all lipid components during ethanol utilization, the amount of phospholipid present in the whole culture increased rapidly during hexadecane metabolism, while the amounts of the other lipids changed relatively little after depletion of the ethanol. The average chain length of the phospholipid fatty acids remained relatively constant until growth on hexadecane during which it decreased rapidly, approaching that of palmitic acid which is probably derived directly from the oxidation of hexadecane. During diauxie, the average chain length of the fatty acids of the other lipid components changed only slightly. After the initial increase accompanying the utilization of the ethanol, the amount of every major fatty acid remained nearly constant except for the C_{16} fatty acids which increased rapidly during the utilization of hexadecane. The major fatty acid formed during this period was palmitoleic acid.

It was found impossible to separate completely the cells from the unutilized hydrocarbon so that extracellular lipids could not be

be measured directly. The lipid composition of the cell pellets and of the culture supernatants, which contained an important proportion of the cells, were found to be relatively similar and, thus, no large accumulation of lipid species in the extracellular medium is apparent.

Extracellular lipids were estimated from the amounts of lipids present in the cell pellet and supernatant fractions, as well as from estimates of the proportion of cells present in these two fractions. These calculations suggest that extracellular lipids are present during diauxie at a level similar to that found in cultures grown on ethanol in the absence of hydrocarbon.

RESUME

La composition des lipides d'Acinetobacter lwoffi a été suivie au cours d'une croissance diauxique qui se produit lorsque cette bactérie est incubée en présence d'éthanol et d'hexadécane. Durant cette diauxie, l'éthanol est utilisé en premier, puis, suivant une phase de latence relativement longue, l'hexadécane est utilisé pendant une seconde phase de croissance.

Après une augmentation initiale du montant de tous les lipides au cours de l'utilisation de l'éthanol, le montant de phospholipides présent dans la culture entière a augmenté rapidement au cours du métabolisme de l'hexadécane, tandis que le montant des autres lipides a changé relativement peu après l'utilisation de l'éthanol. Le nombre moyen d'atomes de carbone des acides gras des phospholipides est demeuré relativement constant jusqu'à la phase de croissance sur l'hexadécane pendant laquelle ce nombre a diminué rapidement, se rapprochant de celui de l'acide palmitique qui est probablement formé directement par oxidation de l'hexadecane. Pendant toute la diauxie, le nombre moyen d'atomes de carbone des acides gras des autres lipides n'a changé que légèrement. Après l'augmentation initiale accompagnant l'utilisation de l'éthanol, la quantité de la plupart des principaux acides gras présente dans la culture entière est demeurée à peu près constante. Seul le taux des acides en C_{16} a changé, ayant augmenté rapidement pendant l'utilisation de l'hexadécane. L'acide gras principalement formé au cours de cette période fut l'acide palmitoléique.

Il s'est avéré impossible de séparer complètement les cellules de l'hydrocarbure non-utilisé et les lipides exocellulaires n'ont donc pas pu

être dosés directement. La composition des lipides étant semblable dans les culots et dans les surnageants, qui contenaient une partie importante des cellules de la culture, aucune accumulation de certains lipides n'est apparente dans le milieu exocellulaire.

Les lipides exocellulaires ont été estimés en utilisant les données sur les montants de lipides présents dans les culots et les surnageants de la culture, ainsi que des estimés de la proportion de cellules présente dans ces deux fractions. Ces calculs suggèrent que des lipides exocellulaires sont présents au cours de la diauxie à un niveau semblable à celui que l'on trouve dans les cultures sur éthanol, en l'absence d'hydrocarbures.

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CHAPTER I GENERAL INTRODUCTION

1. Introduction

Interest in the biology of hydrocarbon-oxidizing microorganisms includes the technological, ecological, and fundamental aspects. Of course, these three aspects are interconnected and progress in one is influenced by progress in the others.

Early work in this field developed techniques for the cultivation of petroleum degrading microorganisms and demonstrated their wide taxonomic and ecological distribution (ZoBell, 1946, 1950; Beerstecher, 1954). Attempts were made to use these microorganisms for exploration and for various transformations of crude oil (Beerstecher, 1954; Davis and Updegraff, 1954). A number of methods for stimulating petroleum biodegradation, especially by modification of environmental parameters, have shown great promise as tools for the removal of oil pollutants (Atlas, 1977). These methods find their application in oil tanker operations since the water used to clean the tanks can be treated before being discarded into the sea, thus reducing the impact of this major source of hydrocarbon pollutants (Gutnick and Rosenberg, 1977).

Much attention has been given to the production of single cell protein from petroleum (Johnson, 1964, 1977; Evans, 1968; Champagnat, 1971; Gutcho, 1973; Champagnat and Adrian, 1974; Rockwell, 1976) since microorganisms growing on hydrocarbons are known to contain high levels of protein

(Champagnat et al., 1963). In addition, most organisms used in this process grow on paraffins of relatively long unbranched chains which increase the freezing point of hydrocarbon mixtures. The oil, which remains after growth of the microorganisms, is thus more resistant to freezing in cold climates. The oil is then said to be "dewaxed" (Champagat, 1971).

The use of crude oil raises some problems, however, because of its rising price, and because it contains toxic compounds which are difficult to separate from the cells as are hydrocarbons in general. For these reasons, methane, methanol, and ethanol are replacing crude oil and various hydrocarbon mixtures as the preferred raw materials used for single cell protein production (Wilkinson, 1971; Kharatyan, 1978).

The production of certain oils and fats from hydrocarbons has also stimulated some interest, especially, as will be seen in detail in this dissertation, in regards to the possibility of manipulating the composition of cellular fatty acids by choosing the appropriate hydrocarbon growth substrate (Ratledge, 1970).

Deterioration of oils used in emulsion or lubricant oils, in which a sufficient amount of water is present, has caused some concern because growth in the aqueous phase is accompanied by chemical changes in the oil which lead to modifications of the engineering properties of these materials (Hill, 1968).

Increasing use of petroleum-derived products has led to an increasing frequency of highly polluting crude oil spills. At the same time, foreseable shortages have led to exploration for petroleum in areas such as the ocean floor; here the risks of oil spills are quite high because of the difficulties involved in extraction. Fear of contamination of fragile environments such as the Arctic has stimulated a great deal of research in the ecological aspects of hydrocarbon-degrading microorganisms (Bartha and Atlas, 1977; Colwell and Walker, 1977).

The biodegradability of most hydrocarbons has long been demonstrated in a large variety of microorganisms including bacteria, actinomycetes, yeasts, and filamentous fungi (ZoBell, 1946, 1950; Beerstecher, 1954; Klug and Markovetz, 1971). One characteristic common to all known hycrocarbon-oxidizing microorganisms is that, each organism has a specific range of hydrocarbon melocules it can utilize. There exists a rather great diversity amongst different microorganisms, some being able to utilize only methane, others being only able to utilize aliphatic hydrocarbons of certain chain lengths, and others being only able to utilize certain aromatic compounds (Klug and Markovetz, 1971; Soli and Bens, 1973; Gutnick and Rosenberg, 1977).

Hydrocarbons, being highly reduced organic compounds, provide organisms which can oxidize them with a very rich source of energy. These compounds are also highly insoluble in water, a fact which, in addition to raising some technical problems, poses the question as to how hydrocarbons or their oxidation products are incorporated into the cell.

2. Pathways for the oxidation of aliphatic hydrocarbons

Mechamisms of hydrocarbon oxidation have been frequently reviewed (Foster, 1962; McKenna and Kallio, 1965; van der Linden and Thijsse, 1965; Traxler and Flannery, 1968; Klug and Markovetz, 1971). Work in this field has involved the isolation from cultures of compounds that could logically arise from the oxidation of the parent hydrocarbon molecule, the use of radioactive tracers with whole cells or cell free extracts, and the purification of the enzymes involved.

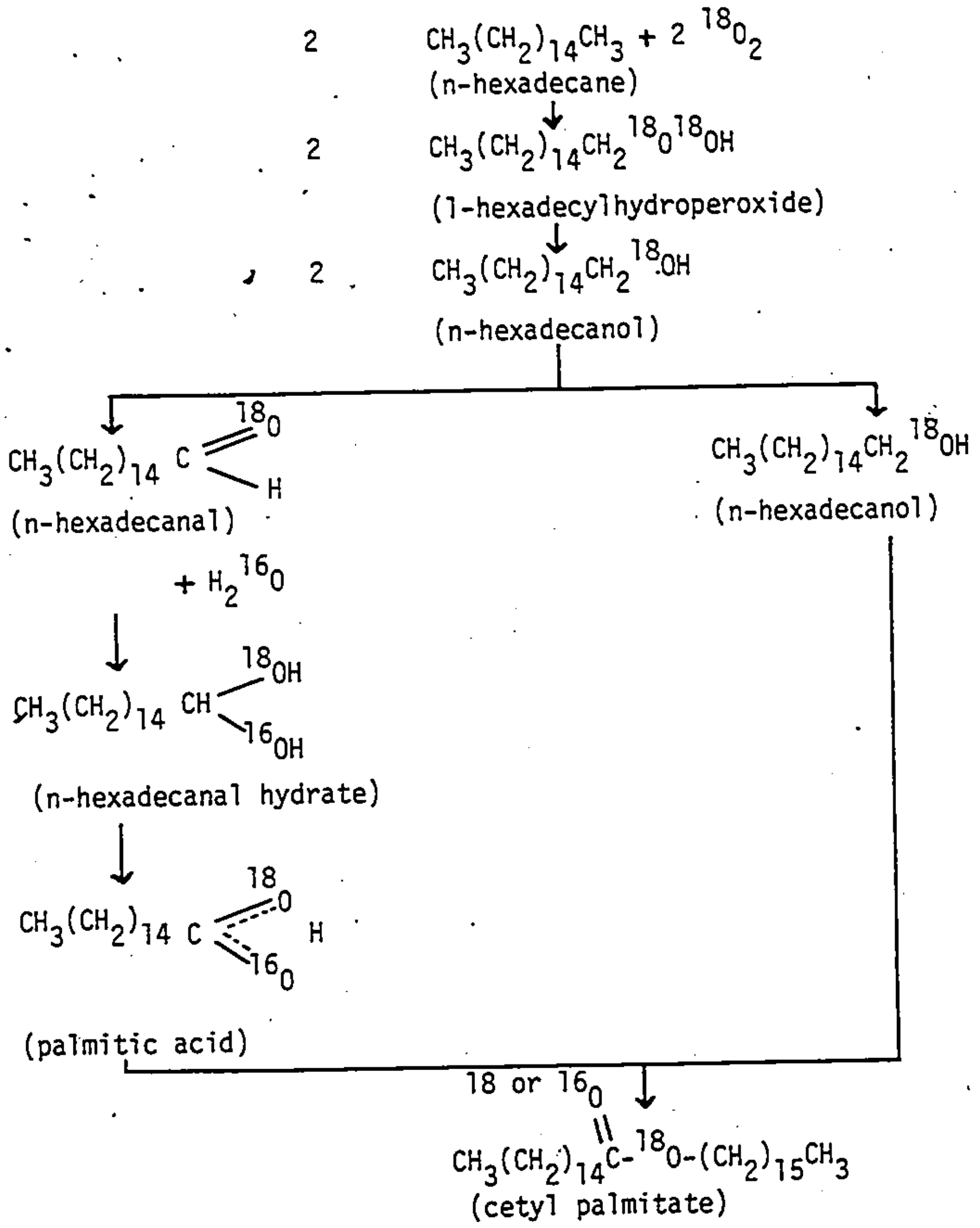
Although aliphatic hydrocarbon oxidation has been shown to proceed through many different pathways in different microorganisms, leading to a variety of alcoholic and ketonic products, the most studied and probably the most common pathways appear to involve the formation of the corresponding fatty acid via the primary alcohol and aldehyde intermediates (Klug and Markovetz, 1971). Three different mechanisms, discussed below, have been proposed for the transformation of aliphatic hydrocarbons to the corresponding fatty alcohol: formation of 1-alkylhydroperoxide intermediates, formation of 1-alkene intermediates, and formation of the primary alcohol directly without additional intermediates.

Early work on the oxidation of hydrocarbons by _Acinetobacter_ sp. H01-N[1] (Finnerty _et al_., 1962 a) has demonstrated the production by this

---

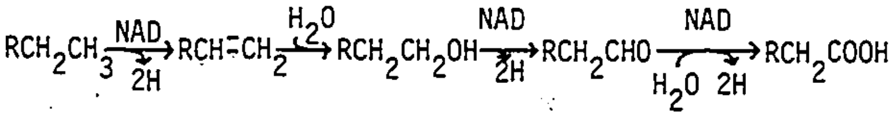
[1] This bacterium has long been designated _Micrococcus cerificans_ but will be referred to, in this dissertation, as _Acinetobacter_ sp H01-N, its now accepted name, to avoid possible confusion since it is one of the most studied hydrocarbon oxidizing bacteria, and since its characteristics are generally close to those of _Acinetobacter lwoffi_.

bacterium of wax esters from the hydrocarbon used as growth substrate (Stewart and Kallio, 1959). Gaseous $^{18}O_2$ was incorporated to the extent of 75% into cetyl palmitate produced from hexadecane (Stewart *et al.*, 1959). This result was interpreted as suggesting the production of 1-hexadecylhydroperoxide as the first intermediate in the following pathway:



This scheme was supported by the finding of Finnerty et al. (1962 b) that Acinetobacter sp. H01-N could utilize 1-alkyl hydroperoxides with wax ester formation at approximately the same rate as when alkanes are used as growth substrate. Support for this pathway was also provided by studies on wax ester formation from 1-alkenes showing a preferential oxidation of the hydrocarbon molecule at the methyl group (Stewart et al., 1960).

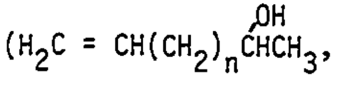
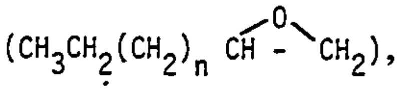
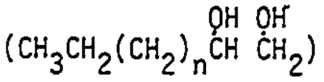
Dehydrogenation of n-alkanes and formation of 1-olefins has, however, also been proposed as the first step in the oxidation of these compounds (Senez and Azoulay, 1961). This proposal was based on the observation that cell-free extracts of Pseudomonas aeruginosa reduced pyocyanin in the presence of n-alkanes under anaerobic conditions (Azoulay and Senez, 1958), and confirmed by infra-red spectrophotometric analysis of the 1-olefin produced (Chouteau et al., 1962). It was also substantiated by the isolation of 1-hexadecene from cultures of different bacteria, including Acinetobacter sp. H01-N, growing on hexadecane (Wagner et al., 1967). The following anaerobic pathway for the oxidation of alkanes was thus proposed (Senez and Azoulay, 1963):



$$RCH_2CH_3 \xrightarrow[2H]{NAD} RCH{=}CH_2 \xrightarrow{H_2O} RCH_2CH_2OH \xrightarrow[2H]{NAD} RCH_2CHO \xrightarrow[H_2O\ \ 2H]{NAD} RCH_2COOH$$

The formation of the primary alcohol from the alkane molecule was found, however, to require atmospheric oxygen in this strain of P. aeruginosa (Azoulay et al., 1963) as well as in a number of other microorganisms (Foster, 1962).

The pathway proposed by Senez and Azoulay (1963) was nevertheless substantiated by the work of Iizuka et al. (1966, 1968) who observed the formation of decanoic acid from n-decane under anaerobic conditions by cell free extracts of Candida rugosa, suggesting that the oxygen of the product could originate from water rather than from air. Accordingly, a Pseudomonas sp. was grown anaerobically on n-alkanes as the only carbon source (Traxler and Bernard, 1969). A NAD-dependent n-alkane dehydrogenase, a NADPH-dependant alkene hydroxylase, and a NAD-, FAD-, and iron-dependent alcohol dehydrogenase were latter purified from this organism (Parekh et al., 1977).

Alpha-olefins may be oxidized at either the methyl group or the double bond. For example, intermediates isolated from cultures of Candida lipolytica growing on 1-hexadecene or 1-heptadecene indicated that three different pathways were probably simultaneously operating in this yeast: methyl group oxidation, leading to the omega-unsaturated primary alcohol ($H_2C = CH(CH_2)_nCH_2CH_2OH$) and fatty acid ($H_2C = CH(CH_2)_nCH_2COOH$), subterminal oxidation, leading to the omega-unsaturated secondary alcohol  $(H_2C = CH(CH_2)_n\overset{OH}{C}HCH_3$, and double bond oxidation, leading to the 1, 2-epoxide  $(CH_3CH_2(CH_2)_n\,\overset{O}{CH - CH_2})$, the 1, 2-diol  $(CH_3CH_2(CH_2)_n\overset{OH}{CH}\,\overset{OH}{CH_2})$ and the 2-hydroxy acid $(CH_3CH_2(CH_2)n\overset{OH}{C}H\ COOH)$. All these intermediates had the same chain length as the parent 1-alkene (Klug and Markovetz, 1968).

Epoxidation of 1-alkenes $(CH_3(CH_2)_nCH = CH_2 \rightarrow CH_3(CH_2)_nCH \overset{O}{-} CH_2)$ was shown to occur readily in Pseudomonas putida[1] (Abbott and Hou, 1973). It was concluded that the epoxide was probably degraded via beta-oxidation after oxidation of the methyl group since 1,7-octadiene $(H_2C = CH(CH_2)_4CH = CH_2)$ was epoxidated to 7,8-epoxy-1-octene $(H_2C \overset{O}{-} CH(CH_2)_4CH = CH_2)$ but could not support growth, nor could 7,8-epoxy-1-octene or 1,2 - 7,8-diepoxyoctane $(H_2C \overset{O}{-} CH(CH_2)_4CH \overset{O}{-} CH_2)$.

Aerobic oxidation of alkanes was studied extensively in P. putida. Cell free extracts of this bacterium where shown to catalyze the oxidation of octane to octanoic acid, and the presence of NAD-dependent dehydrogenases acting on octanol and octaldehyde was demonstrated (Baptist et al., 1963). The initial step in the oxidation of octane was found to involve two enzymes and to require NADH and iron ions (Gholson et al., 1963). Similar results were obtained for the omega-oxidation of fatty acids by cell free extracts of this bacterium (Kusunose et al., 1964 a, b). A third protein component, rubredoxin, was also found to be involved in the omega-oxidation of fatty acids and alkanes (Peterson et al., 1966; Kusunose et al., 1967), and the following pattern was proposed for this reaction (Peterson et al., 1967):

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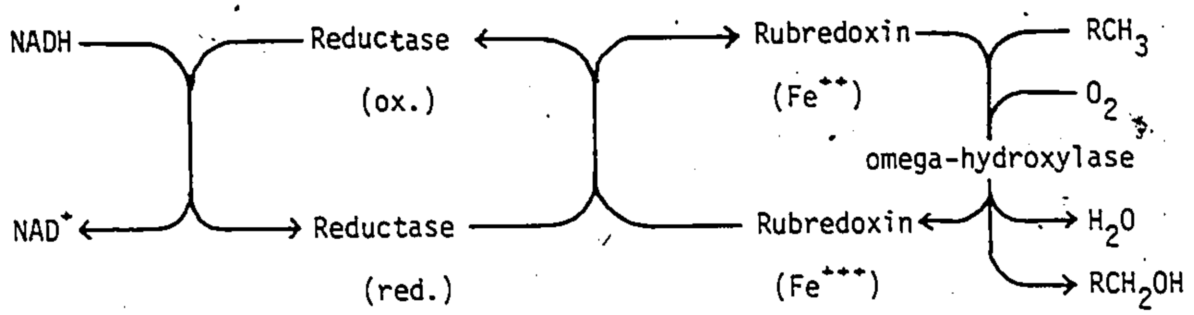
[1] This bacterium has long been designated Pseudomonas oleovorans.



The stoichiometry of this reaction was demonstrated (Peterson et al., 1969), and the components of this system were purified and studied in detail. The rubredoxin reductase (Ueda *et al.*, 1972; Ueda and Coon, 1972) has a molecular weight of about 50,000 and used FAD as a prosthetic group. NADH is the preferred electron donor but NADPH can serve also to a limited extent. Rubredoxin (Peterson and Coon, 1968; Lode and Coon, 1971, 1973) has a molecular weight of 19,500 and binds one or two Fe ions. Both forms of rubredoxin function equally well in the omega-hydroxylase system. The amino acid sequence of rubredoxin was established and compared with that of rubredoxins isolated from different anaerobic bacteria (Benson *et al.*, 1971). The omega-hydroxylase (McKenna and Coon, 1971; Ruettinger *et al.*, 1974, 1977) is another non-heme iron protein containing 1 iron atom bound to the only cysteine residue present in a polypeptide chain of 40,800 molecular weight. This enzyme also requires the presence of phospholipids for maximal activity.

The presence of these three components was confirmed in *P. aeruginosa* and the omega-hydroxylase activity was shown to be mostly associated with membranes while rubredoxin and rubredoxin reductase were found exclusively in the soluble fraction (van Eyk and Bartels, 1970).

Using these purified components, Boyer et al. (1971) demonstrated the reduction of alkylhydroperoxides to the corresponding alcohol in the presence of rubredoxin, rubredoxin reductase and NADH. The omega-hydroxylation of alkanes or fatty acids and the reduction of alkylhydroperoxides all have the same pH optimum (7.4) and are both inhibited by cyanide. It is not yet clear whether an enzyme bound hydroperoxide is formed during the hydroxylation reaction (Lode and Coon, 1973).

Epoxidation of 1-alkenes was also demonstrated using the purified components of the omega-hydroxylase system (May and Abbott, 1972, 1973).

Another enzyme system which catalyzes the oxidation of alkanes to the primary alcohol was studies in Corynebacterium sp. strain 7E1C. This system requires molecular oxygen and NADH and involves cytochrome P-450 (Cardini and Jurtshuk, 1968, 1970). This latter molecule is part of a group of cytochrome P-450 molecules, some of which are also involved in the hydroxylation of camphor in various Pseudomonas species and in a number of hydroxylation reactions in animal tissues (Jurtshuk and Cardini, 1971; Coon et al., 1975).

Lebeault et al. (1970 a) proposed that the anaerobic pathway of Senez and Azoulay (1963) was operating in Candida tropicalis. However, an enzyme system that requires molecular oxygen and cytochrome P-450 was found in this yeast to be active in hydroxylation of alkanes, fatty acids, and certain drugs (Lebeault et al., 1971; Duppel et al., 1973). Phospholipids were required and lysophosphatidylethanolamine isolated from the yeast cells restored maximal activity. Gallo et al., (1973) demonstrated that this system was probably the only one responsible for the formation of the alcohol in C. tropicalis and that reduction of NAD$^+$, previously considered as evidence for an alkane-dehydrogenase activity by Lebeault et al. (1970 a), did not correspond to the formation of the alpha-olefin which could not be detected in the assay system. Since reduction of NAD$^+$ was obtained with only 2 commercial batches of n-decane out of 10, it was concluded that this reaction was due to impurities contained in the hydrocarbon used in earlier experiments.

Cell free extracts of Candida intermedia were also shown to oxidize decane to decanoic acid, the formation of decanol requiring molecular oxygen (Liu and Johnson, 1971).

NAD-dependent long chain alcohol dehydrogenases have been demonstrated in various strains of Pseudomonas (Azoulay and Heydeman, 1963; Baptist et al., 1963; Payne, 1963; Tassin and Vandecasteele, 1971) and yeast (Roche and Azoulay, 1969; Lebeault et al. 1970 a, Liu and Johnson, 1971). In C. tropicalis, two alcohol dehydrogenases were studied, one in the particulate fraction (Lebeault et al., 1970 b) and one in the soluble fraction (Lebeault et al., 1970 c). These two enzymes differ in their substrate specificity but are both induced by alkanes or long chain alcohols.

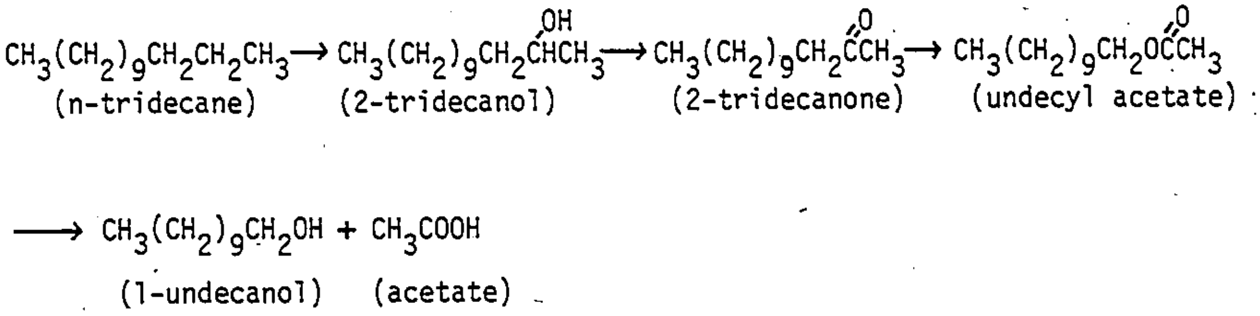
A constitutive NADP-dependent alcohol dehydrogenase and at least two NAD - and NADP- independent alcohol dehydrogenases induced by growth on alkanes, primary alcohols, or alpha-omega-diols were found in P. aeruginosa (van der Linden and Huybregtse, 1969). In fact P. aeruginosa was shown to contain constitutively at least two NADP-dependent and one NAD-dependent alcohol dehydrogenase isoenzymes (Tassin and Vandecasteele, 1972). In addition, a membrane bound NAD- and NADP-independent dehydrogenase, active on long chain alcohols and induced by growth on hydrocarbons, was purified and studied in detail (Tassin _et al_., 1973).

Aldehyde dehydrogenases are also present in hydrocarbon grown yeasts (Lebeault _et al_., 1970 a, b; Liu and Johnson, 1971). In _P. aeruginosa_, a membrane bound (Heydeman and Azoulay, 1963) and a soluble (Bertrand _et al_. 1973) aldehyde dehydrogenase have been described.

The presence of alcohol and aldehyde dehydrogenases, as well as a requirement for molecular oxygen, were also demonstrated in an _Alcaligenes_ sp. (Bertrand _et al_., 1976 a) and in the filamentous fungus _Cladosporium resinae_ (Walker and Cooney, 1973 a) grown on alkanes.

In addition to pathways leading to the corresponding fatty acid from aliphatic hydrocarbons, diterminal oxidation has been observed, leading to the corresponding dicarboxylic acid ($HOOC(CH_2)_nCOOH$) in _Corynebacterium_ 7E1C (Kester and Foster, 1963) and in a _Pseudomonas_ sp. (Ali Khan _et al_., 1963), as well as a variety of subterminal oxidative pathways, leading, for example, to the formation of internal monoalkenes in a _Nocardia_ (Abbott and Cassida, 1968), or various alcohols and ketones in _Mycobacterium rhodochrous_ (Fredricks, 1967).

The subject of subterminal oxidation of aliphatic hydrocarbons was reviewed in detail by Markovetz (1971). Several authors have reported the formation of methyl ketones from alkane molecules. The degradation of methyl ketones was discussed by Forney and Markovetz (1971). The pathway that was studied in greatest detail involves the formation of a primary alcohol, shorter by two carbon atoms than the parent methyl ketone or alkane. Pseudomonas cepacia[1] produced 1-undecanol from 2-tridecanone (Forney et al., 1967). Undecyl acetate was also isolated from cultures of P. cepacia and P. aeruginosa growing on 2-tridecanone and the following pathway was proposed for the oxidation of n-tridecane (Forney and Markovetz, 1968):



$$\underset{\text{(n-tridecane)}}{CH_3(CH_2)_9CH_2CH_2CH_3} \rightarrow \underset{\text{(2-tridecanol)}}{CH_3(CH_2)_9CH_2\overset{OH}{C}HCH_3} \rightarrow \underset{\text{(2-tridecanone)}}{CH_3(CH_2)_9CH_2\overset{O}{C}CH_3} \rightarrow \underset{\text{(undecyl acetate)}}{CH_3(CH_2)_9CH_2O\overset{O}{C}CH_3}$$

$$\longrightarrow \underset{\text{(1-undecanol)}}{CH_3(CH_2)_9CH_2OH} + \underset{\text{(acetate)}}{CH_3COOH}$$

This pathway was supported by the detection of 2-tridecanone, undecyl acetate, and 1-undecanol when 2-tridecanol was supplied instead of 2-tridecanone. Cell free extracts of P. aeruginosa formed labelled undecyl acetate and 1-undecanone-3-^{14}C, when incubated with 2-tridecanone-3-^{14}C, and labelled acetate when incubated in the presence of undecyl acetate-2-^{14}C (Forney and Markovetz, 1969). When n-tridecane was used as substrate, 2-tridecanol and 1-undecanol were detected (Forney and Markovetz, 1970). The formation of undecyl acetate from 2-tridecanone was shown ~~to require~~ molecular oxygen (Britton et al., 1974) and the enzyme catalyzing

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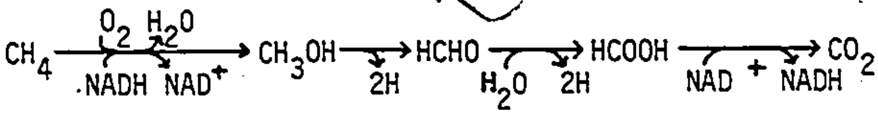
[1] This bacterium has long been designated Pseudomonas multivorans.

this reaction was purified (Britton and Markovetz, 1977). Undecyl acetate esterase, the enzyme catalyzing the last step in this pathway was purified and studied in detail (Shum and Markovetz, 1974 a,b).

Methane oxidation was reviewed by Ribbons et al. (1970), Quayle (1972), and Colby et al. (1979). It is thought to proceed via the following pathway which is similar to those involving molecular oxygen in the first reaction, discussed above for long chain alkanes:



$$CH_4 \xrightarrow[\text{NADH}\ \ \text{NAD}^+]{O_2\ \ H_2O} CH_3OH \xrightarrow[2H]{} HCHO \xrightarrow[H_2O\ \ \ 2H]{} HCOOH \xrightarrow[\text{NAD}\ +\ \text{NADH}]{} CO_2$$

The enzymes involved in this pathway, methane monooxygenase, methanol-, formaldehyde-, and formate-dehydrogenases, have all been studied in some detail (Colby et al., 1979).

Methane is generally considered as being biologically inert under anaerobic conditions. However, a recent report demonstrates the oxidation of this hydrocarbon to CO_2 in the absence of oxygen in samples of lake water (Panganiban et al., 1979). Furthermore, pure cultures of a variety of methanogenic bacteria were able to oxidize small quantities of methane, some of which was incorporated into cellular material (Zehnder and Brock, 1979). These experiments were based on the detection of radioactive material produced from $^{14}CH_4$ under strictly controled anaerobic conditions. The pathway for anaerobic oxidation of methane is completely unknown but must be quite different from those described for aerobic methane oxidation and for anaerobic oxidation of higher paraffins. Some of the first questions requiring clarification are those of the oxidizing agent and of the intermediates involved (Zehnder and Brock, 1980).

Although most studies on hydrocarbon oxidation have involved microorganisms using these substances as growth substrates, a number of microorganisms are known to oxidize hydrocarbons without the ability to assimilate and further metabolize their oxidation products. This ecologically important phenomenon of hydrocarbon cooxidation was reviewed recently by Perry (1979).

3. Regulation of hydrocarbon metabolism.

Hydrocarbon metabolism is generally considered to involve inducible enzymes in most microorganisms studied (van der Linden and Thijsse, 1965; Klug and Markovetz, 1971; Perry, 1979). This conclusion is mostly based on respiration experiments showing a lag in O_2 consumption when cells grown on a non-hydrocarbon substrate are transfered to a medium containing hydrocarbons as the only substrate.

Possible cases of microorganisms capable of oxidizing hydrocarbons constitutively are those of Pseudomonas stutzeri (Hansen and Kallio, 1957) and of Micrococcus paraffinae (Harris, 1957) for which a lag phase was not observed during this type of experiment. Fredricks (1966) maintained also that Pseudomonas fluorescens and a Corynebacterium sp. oxidized hydrocarbons constitutively since the same number of cells was obtained, after 3 days, when cells cultured on glucose were transfered to a medium containing alkanes as the sole carbon source, no matter after how many transfers on glucose.

These results were found to be insufficient evidence for concluding that hydrocarbons are oxidized constitutively by these organisms since Le Petit et al. (1975) isolated some bacteria which, in respiration experiments, did not show a lag phase, but did not consume oxygen in the presence of chloramphenicol. In these organisms, protein synthesis is required for oxidation of hydrocarbon to occur and at least one inducible enzyme is involved. Strains were also isolated which showed a lag phase without chloramphenicol in the system and others which did not, even in the presence of chloramphenicol. This latter group of bacteria is the only one that can be considered as oxidizing hydrocarbons constitutively.

Cells of Pseudomonas aeruginosa grown on n-heptane were found to be simultaneously adapted to utilize 1-heptanol and n-heptanal. The bacteria cultured on the alcohol were adapted to the aldehyde and fatty acid, but not to the alkane, and those grown on the aldehyde were adapted to the acid, but not to the alkane or the alcohol (Azoulay and Senez, 1960). These observations were taken as indicating that these substances are intermediates in the pathway for the oxidation of alkanes.

Some strains were found to be adapted to hydrocarbons if they were induced to utilize a compound which requires an oxygenase for its oxidation. Thus, an Arthrobacter sp. was found to oxidize propane without an induction period, even in the presence of chloramphenicol, after being induced to grow on o-phthalic acid (Perry and Scheld, 1968).

Volfova et al. (1967) found that although cells of Candida lipolytica grown on glucose could adapt to alkane utilization, protoplasts prepared from glucose grown cells could not. Protoplasts prepared from alkane grown cells of Candida tropicalis, however, oxidized alkanes (Lebeault et al., 1969). It was concluded that hydrocarbon oxidation is possible, but that induction of the necessary enzymes is impaired in protoplasts.

In P. aeruginosa, substances such as cyclopropane, 1,2-dimethoxyethane, diethoxymethane, n-butane, dicyclopropylmethane, 1,2-dicyclopropylethane, and dicyclopropylmethanol were effective inducers of n-hexane oxidation although they cannot serve as growth substrates. Malonate and C_5 to C_8 n-alkanes were good inducers as well as good growth substrates (van Eyk and Bartels, 1968). Induction of alkane oxidizing enzymes by 1,6-hexanediol was demonstrated in a strain of P. aeruginosa (van der Linden and Huybregtse, 1967) which is able to metabolize this substance (van der Linden, 1967). This compound, as well as other alpha, omega-diols, did not act as an inducer of alkane oxidation in Pseudomonas putida, but dicyclopropylmethanol, dimethoxyethane, and dicyclopropyl ketone served as gratuitous inducers, while alkanes and alcohols served as metabolizable inducers (Grund et al., 1975). The alkane itself is an effective inducer and does not need to be oxidized, since some of the alkane hydroxylase proteins were induced in mutants which were unable to oxidize the alkane because they lacked one of the three protein components of the hydroxylase system described in the preceding section (Benson and Shapiro, 1975).

A variety of organic compounds, including glucose, were found to repress the oxidation of alkanes in P. aeruginosa (van Eyk and Bartels, 1968). In contrast, n-alkanes were found to inhibit glucose transport and metabolism in the yeast Candida 107 (Gill and Ratledge, 1973 a). Hexane and heptane, but not longer chain alkanes (C_8 to C_{18}), inhibit glucose transport in Cladosporium resinae (Teh, 1975). Hexadecane was shown to inhibit glucose metabolism, but not its transport, in this fungus (Siporin and Cooney, 1976).

Mutants of a few alkane utilizing microorganisms have been isolated and characterized. Some mutants of Pseudomonas denitrificans unable to grow on alkanes were also unable to grow on the corresponding alcohol, aldehyde, or fatty acid (Illarionov, 1972). Mutants of Mycobacterium rhodocrous were found to have lost the ability to use some of the alkanes which the parent strain could metabolize even if all of them could grow on alcohols, aldehydes, and fatty acids (Jenkins et al., 1972). Mutants of P. aeruginosa and P. putida selected for their inability to use n-alkanes were able to grow on the corresponding alcohols, aldehydes, and fatty acids. Complementation studies showed that cell free extracts of two or more of these mutants could oxidize n-alkanes. These different mutant types are thought to lack one or more of the three components of the omega-oxidation system (Macham and Heydeman, 1974; Benson and Shapiro, 1975).

P. putida grows on n-alkanes having six to ten carbon atoms. Revertible point mutants were isolated which had simultaneously lost the ability to grow on any of these alkanes, but were still able to utilize the corresponding alcohols, aldehydes, or fatty acids as sole carbon source. In contrast, P. aeruginosa grows on C_6 to C_{17} n-alkanes, and mutants were isolated which had only lost the ability to utilize C_6 to C_{10} alkanes. Other mutants had lost only the ability to grow on longer chain alkanes. These results were interpreted as indicating that a single hydroxylase is present in P. putida while a second hydroxylase, active on heavier n-alkanes, is present in P. aeruginosa (Neider and Shapiro, 1975). Several mutants of the yeast Saccharomycopsis lipolytica, selected for their inability to grow on alkanes, were also unable to grow on fatty acids (Bassel and Mortimer, 1973). One mutant of P. putida which had lost the ability to grow on even-numbered n-alkanes or fatty acids, was found to lack isocitrate lyase. This enzyme is involved in the glyoxylate cycle without which an organism cannot grow exclusively on carbon sources such as acetate or even-numbered fatty acids. It was concluded that beta-oxidation is the only pathway by which fatty acids are degraded in this bacterium since decarboxylation or alpha-oxidation would yield odd-numbered fatty acids that could support growth (Nieder and Shapiro, 1975).

A mutant strain of _C. lipolytica_ has been studied which utilizes n-tetradecane constitutively (Duvnjak _et al._, 1970 a).

In the genus _Pseudomonas_, enzymes for alkane oxidation are induced by the alkane molecule and are coded by genes borne on a transmissible plasmid (Chakrabarty _et al._, 1973). Transmissible plasmids carrying genes for the degradative pathways of various hydrocarbons and other organic substances such as naphthalene, salicilate, camphor, toluene, and xylene are found in this genus (Wheelis, 1975; Chakrabarty, 1976). Since these compounds represent an important fraction of crude oil and no microorganism found in nature is known to oxidize all of them, much effort has been devoted to the introduction of several of these plasmids into a single bacterial strain (Friello _et al._, 1976).

Problems of this approach stem from the fact that some of these plasmids are incompatible. For example, the OCT plasmid, responsible for alkane oxidation, and the CAM plasmid, which carries the genes for camphor degradation, cannot coexist in the same cell for more than a few generations (Chakrabarty _et al._, 1973). These two plasmids were found to fuse, however, under certain conditions, to give a stable CAM-OCT plasmid specifying the complete set of genes for both pathways (Chakrabarty, 1973; Chou _et al._, 1974). It was concluded that these plasmids possess certain regions of homology that enable them to recombine and form a single plasmid. Although there exists a large body of genetic evidence for these plasmids, it is only recently that they were physically isolated (Palchaudhuri and Chakrabarty, 1976; Palchaudhuri, 1977; Fennewald et al, 1978).

Recombinations between degradative plasmids and other plasmids, readily transfered to other bacterial genera, carrying antibiotic resistance genes (Benedik et al., 1977) have permitted the transfer of hydrocarbon degradation genes to various bacteria such as Escherichia coli, Salmonella typhimurium, Agrobacterium tumefaciens, and Azotobacter vinalandii. In these bacteria, the antibiotic resistance genes were expressed, but the hydrocarbons could not support growth. Hydrocarbon oxidation genes were, however, fully expressed when transfered back to Pseudomonas strains (Benson and Shapiro, 1978; Chakrabarty et al., 1978). In similar experiments, Jacoby et al., (1978) and Nakazawa et al., (1978) succeeded in transfering toluene degradation genes from Pseudomonas to E. coli in which they were expressed at a level dectectable by enzyme assay, but much lower than in the parent strain. The reason for this low expression of hydrocarbon degradation genes in foreign hosts could be that some regulatory or other genes present on the Pseudomonas chromosome are required for growth on hydrocarbons, or that the properties of the membrane of other bacteria are not appropriate for hydrocarbon uptake.

The OCT and CAM-OCT plasmids code for a membrane-bound alcohol dehydrogenase which is NAD and NADP independent (Benson and Shapiro, 1976).

Mutants lacking this enzyme grow on alkanes because the chromosome codes for a number of NAD-dependent alcohol dehydrogenases. Some chromosomal mutants cannot grow on octanol unless they have a functional alcohol dehydrogenase coded by the plasmid. These mutants, however, possess NAD-dependent alcohol dehydrogenase activity. They are thought to lack an alcohol permease or another factor for metabolism of exogenous alcohols (Benson and Shapiro, 1976).

Three genes were identified in the alk regulon of the OCT plasmid: alk A, specifying a soluble fraction of the hydroxylase system, alk B, coding for the membrane bound fraction of the hydroxylase system, and alk C, for the membrane bound alcohol dehydrogenase. Mutants having lost all three of these activities were also isolated as demonstrated by in vitro complementation studies (Benson et al., 1977). Revertants of this latter group of mutants included some constitutive mutants for alkane oxidation, and mutants showing an altered inducer specificity (Fennewald and Shapiro, 1977). An additional regulatory gene (alk D) was found to be required for the synthesis of the membrane bound alkane hydroxylase and alcohol dehydrogenase. This gene is different from the previously mentioned regulatory gene (designated alk R) since it is not required for the normal synthesis of the soluble hydroxylase component. A second gene (alk E) codes for the membrane bound alcohol dehydrogenase in addition to alk C (Fennewald et al., 1979).

Insertion of the Tn7 trimethoprim resistance transposon into or near the alk loci of the CAM-OCT plasmid (Fennewald and Shapiro, 1979) has permitted mapping of these alk genes (Fennewald _et al_., 1979). Polar effects caused by this insertion into alk genes indicated the existence of an alk BAE operon. Linkage studies with Tn7 inserts indicated that the alk BAE operon forms a cluster distinct from alk C and from an alk RD regulatory region (Fennewald _et al_., 1979).

As was previously mentioned, the omega-hydroxylase of the omega-oxidation system of _Pseudomonas_ was found to be associated with membrane components (van Eyk and Bartels, 1970). Illarionov (1976) demonstrated differences in the electrophoretic mobility and amounts of membrane proteins when comparing alkane negative mutants of _P. denitrificans_ with the wild type. The plasmid coded hydroxylase and alcohol dehydrogenase were both found to be exclusively present in the inner membrane of _P. putida_ (Benson _et al_., 1979). Induction of alkane oxidation activity resulted in the appearance of at least three new membrane peptides, coded for by the plasmid, and the disappearance of two membrane peptides coded for by the chromosome. One of the induced peptides was identified as the hydroxylase since the alk B mutation resulted in a modified electrophoretic mobility of this molecule (Benson _et al_., 1979). Induction of alkane oxidation activity appears to involve the membrane since it was blocked by local anesthetics such as procaine and piperocaine which are known to affect membrane processes in mammalian and bacterial cells (Benson, 1979).

4. Influence of the carbon source on the fatty acid composition of hydrocarbon oxidizing microorganisms.

A rather extensive literature has accumulated, over about the last twenty years, showing a direct influence of aliphatic hydrocarbons on the fatty acid composition of hydrocarbon-oxidizing microorganisms. Thus, the lipids of microorganisms growing on hydrocarbons of 13 to 18 carbon atoms were substantially enriched with fatty acids having the same chain length as the parent hydrocarbon molecule. This enrichment was observed in many species of bacteria (Stewart and Kallio, 1959; Stewart et al., 1959; Stevenson et al., 1962; Makula and Finnerty, 1968 a, b, 1972; Edmonds and Cooney, 1969; Finnerty et al, 1973; Patrick and Dugan, 1974; Makula et al. 1975), actinomycetes (Davis, 1964; Dunlap and Perry, 1967, 1968; Fukui and Fukui, 1970; Yano et al., 1971; Yanagawa et al., 1972; King and Perry, 1975 a), yeasts (Mizuno et al., 1966; Klug and Markovetz, 1967; Ratledge, 1968; Thorpe and Ratledge, 1972; Hug and Fiechter, 1973; Mishina et al., 1973; Skipton et al.,1973, 1974; Volfova and Pecka, 1973; Rattray et al., 1975), and fungi (Cooney and Proby, 1971; Cerniglia and Perry, 1974; Gerasimova et al., 1975).

This correlation was not found in microorganisms growing on hydrocarbons shorter than 11 (Dunlap and Perry, 1967, 1968; Cooney and Proby, 1971; Vestal and Perry, 1971; Patrick and Dugan, 1974) or longer than 20 carbon atoms (Dunlap and Perry, 1968; Hankin and Kolattukudy, 1968; Hallas and Vestal, 1978).

With regards to C_{11} and C_{12} hydrocarbons, a slight enrichment for C_{11} or C_{12} fatty acids during growth on n-undecane or n-dodecane was observed in _Acinetobacter_ sp. HO1-N (Makula _et al_., 1968 a), in _Candida lipolytica_ (Mishina _et al_., 1973), and in _Mycobacterium_ sp. OFS (Dunlap and Perry, 1968), but was not apparent in _Cladosporium resinae_ (Cooney and Proby, 1971). Similarly, a slight enrichment for C_{19} or C_{20} fatty acids during growth on nonadecane or eicosane was observed in a _Nocardia_ sp. (Davis, 1964) and in _Arthrobacter simplex_ (Yano _et al_., 1971), but could not be demonstrated in _Mycobacterium_ sp. OFS (Dunlap and Perry, 1968) or in _Mycobacterium convolutum_ (Hallas and Vestal, 1978).

The fatty acid composition of a given organism will thus differ depending on the hydrocarbon growth substrate. It will usually be different than when the organism is grown on a non-hydrocarbon substrate or in complex media. Accordingly, cellular lipids were shown to be enriched considerably with fatty acids having an odd number of carbon atoms when the hydrocarbon substrate had itself an odd number of carbon atoms even with very short chain length hydrocarbons such as propane (Dunlap and Perry, 1967, 1968, Vestal and Perry, 1971; King and Perry, 1975 b).

The fatty acids, formed by oxidation of hydrocarbon molecules, appear to be incorporated directly into complex lipids if their lengths are compatible with the physiological requirements of the organism. Other fatty acid molecules will be elongated, presumably by the fatty acid synthetase system, or shortened, via beta-oxidation, before being incorporated. Of course, a certain proportion of the fatty acids will be degraded further to provide the cell with energy and material for the synthesis of the non-lipid constituents.

This interpretation was supported by studies on the oxidation of 1-alkenes. Wax esters produced by Acinetobacter sp. H01-N growing on these compounds were found to have omega-unsaturated alcohol moieties (Stewart et al., 1960). This bacterium was later shown to have omega-unsaturated fatty acids in its cellular lipids when growing on 1-alkenes (Makula and Finnerty, 1968 b). Omega-unsaturated fatty acids were also found in C. lipolytica (Klug and Markovetz, 1967), in Mycobacterium sp. OFS (Dunlap and Perry, 1968), in A. simplex (Yano et al., 1971), in Corynebacterium simplex (Yanagawa et al., 1972), and in Cunninghamella elegans (Cerniglia and Perry, 1974), when these microorganisms were cultured on 1-alkenes.

The lipids of Mycobacterium vaccae were examined after growth on a variety of hydrocarbons used individually as sole carbon source. When n-alkanes were used, the lipids were enriched with fatty acids of the same chain length as the hydrocarbon. When 1-alkenes were used, high levels of omega-unsaturated fatty acids were detected. Growth on 2-methyl octadecane resulted in the incorporation of iso-fatty acids and growth on 3-methyl octadecane, in the incorporation of anteiso-fatty acids. When the growth substrate was 8-heptadecene, the cellular lipids contained 8- and 9-heptadecenoic acids, 6- and 7-pentadecenoic acids, 9- and 10-methyl heptadecanoic acids, and 7- and 8-methyl pentadecanoic acids. When acetate or n-alkanes were used as growth substrates, double bonds were found only in $\Delta 9$ position and methyl branches were all situated on C_{10} (King and Perry, 1975 a, b). Desaturation of fatty acids thus occurs always at the same distance from the carboxyl group. However, addition of a methyl group can occur on a double bond situated at other positions on the fatty acid molecule derived from an unsaturated hydrocarbon.

Furthermore, Beam and Perry (1974) demonstrated the incorporation into cellular lipids of the omega-oxidation products of long chain alkyl substituted cycloalkanes used as growth substrate by three species of Mycobacterium. Growth of Acinetobacter sp. H01-N on the alkane analogue n-dioctyl ether resulted in the incorporation of 8 n-octoxy-1-octanoic acid into cellular lipids (Modrzakovski et al., 1977).

Since the hydrocarbon used as growth substrate has such a strong effect on the fatty acid composition, changes should occur, with time, after a transfer from one substrate to another as was shown by Mishina et al. (1973) and by Hug et al. (1974).

5. Regulation of lipid metabolism in hydrocarbon utilizing microorganisms.

If fatty acids are derived directly from the oxidation of hydrocarbon molecules rather than from *de novo* synthesis, there should exist a mechanism by which the latter pathway is repressed. This interpretation was substantiated by dual label experiments showing a preferential incorporation into complex lipids of ^{14}C-palmitate in *Acinetobacter* sp. HO1-N cells grown on hexadecane, and preferential ^{3}H-acetate incorporation in acetate grown cells (Sampson and Finnerty, 1974).

A more direct demonstration was provided by Gill and Ratledge (1973 b) who showed reduced levels of acetyl-CoA carboxylase and of the fatty acid synthetase complex in a *Candida* sp. growing on n-alkanes. They pointed out that this repression is more pronounced for acetyl-CoA carboxylase, the fatty acid synthetase complex being involved in the elongation of fatty acids having 10 to 14 carbon atoms. In addition, these authors demonstrated feed-back inhibition of these enzymes by long chain (C_{16} or C_{18}) fatty acyl-CoA esters.

Studies of the acetyl-CoA carboxylase of *Candida lipolytica* (Mishina *et al*., 1976 a) confirmed repression of this enzyme when n-heptadecane or n-octadecane are used as carbon source (Mishina *et al*., 1976 b). Repression and inhibition of acetyl-CoA carboxylase and of the fatty acid synthetase activity were also demonstrated in *Mycobacterium convolutum* (Ascenzi and Vestal, 1979).

The presence of long chain acyl-CoA synthetase activity was demonstrated in the particulate fraction of mitochondria of Candida tropicalis grown on n-alkanes (Duvnjak et al., 1970 b). This enzyme plays an essential role in hydrocarbon-utilizing microorganisms since fatty acids must be activated before they can be incorporated into complex lipids, or elongated by the fatty acid synthetase pathway, or degraded by beta-oxidation. Accordingly, the activity of long chain acyl-CoA synthetase of a Torulopsis sp. was found to be 3 times higher in alkane grown cells than in glucose grown cells (Trust and Millis, 1970).

These results do not, however, exclude the possible interpretation of King and Perry (1975 a) according to which fatty acids longer than the alkane used as growth substrate are synthesized de novo from acetyl-CoA while fatty acids of shorter chain length are derived from beta-oxidation of the oxidized hydrocarbon. This conclusion was based on experiments in which the lipids of Mycobacterium vaccae grown on hexadecane, in the presence of ^{14}C-acetate, were found to contain low levels of radioactivity, proportionally to the amount of each fatty, in C_{16} and shorter fatty acids, but much higher levels in the C_{18} fatty acids. Possibly the radioactivity could, at least in part, be added to longer fatty acids by elongation of the oxidized hydrocarbon.

The results of Yanagawa et al., (1972) suggest that intact incorporation and elongation of the fatty acid formed by oxidation of the hydrocarbon substrate, as well as de novo synthesis occur simultaneously when Corynebacterium simplex is grown on C_{15} hydrocarbons. Growth on 1-pentadecene resulted in the production of 14-pentadecenoic acid (indicative of intact incorporation), palmitic, oleic, and 10-methyl stearic acids (indicating de novo synthesis), and 16-heptadecenoic, 9,16-heptadecenoic, and 10-methyl-16-heptadecenoic acids (probably derived from the elongation of 14-pentadecenoic acid).

6. Ultrastructure of hydrocarbon oxidizing microorganisms.

Most ultrastructural studies of hydrocarbon-oxidizing microorganisms have been concerned with the demonstration of hydrocarbon penetration into the cell. Ludvik _et al_., (1968) and Munk _et al_. (1969) compared the ultrastructure of _Candida lipolytica_ grown on glucose and on hydrocarbons. Using vanadium or nickel naphthenate, compounds soluble in hydrocarbons, they showed an accumulation of these substances on the surface of the cytoplasmic membrane, apparently after penetration through the cell wall. The membrane of hydrocarbon grown cells was also found to be thicker and to contain deep invaginations, some of which were interpreted as possible pinocytic vesicles suggesting an active translocation of the substrate into the cell. Rylkin _et al_. (1974) claim they have demonstrated local transport of n-alkanes through the cell wall of _Candida tropicalis_ using time lapse microphotography. No detail of this experiment is given, however, in their paper.

Yeast cells were also found to contain many more microbodies and a much stronger catalase activity, after being transferred from a malt extract medium to a medium containing n-alkanes as the carbon source (Osumi _et al_., 1974, 1975).

Methane-utilizing bacteria possess, within their cytoplasm, complex membrane structures (Proctor _et al_., 1969; Davies and Whittenbury, 1970; Smith and Ribbons , 1970; Smith _et al_, 1970; Whittenbury _et al_., 1970; de Boer and Hazeu, 1972) which were not found in other bacteria growing on gaseous

(C_2 to C_4) or liquid (C_{11} to C_{18}) n-alkanes (Davies and Whittenbury, 1970).

Most methane-oxidizing bacteria are classified as "obligate methylotrophs" because they can only utilize methane, methanol, formaldehyde, or dimethyl ether as their carbon source. "Facultative methylotrophs" can utilize more complex organic molecules, in addition to C_1 compounds, but not methane (Ribbons et al., 1970; Quayle, 1972; Wilkinson, 1975). Two strains have been described, termed "facultative methane-oxidizing bacteria", which can utilize a variety of organic compounds in addition to methane (Patt et al., 1974; Patel et al., 1978). One of them was shown to have characteristic intracytoplasmic membranes when grown on methane, but not when grown on methanol or glucose (Patt et al., 1976; Patt and Hansen, 1978). Only the ultrastructure of cells grown on methane was reported for the bacterium studied by Patel et al. (1978).

Recently discovered methane-utilizing yeasts (Wolf and Hanson, 1979) did not exhibit the complex arrays of internal membranes characteristic of methane-oxidizing bacteria (Wolf et al., 1980). These yeasts have an ultrastructure which is similar to that of other hydrocarbon-utilizing yeasts. Many more microbodies and an increased level of catalase activity were found in methane grown cells than in glucose grown cells. This result was also obtained with Rhodotorula glutinis strain CY which can grow on hexadecane, in addition to methane or glucose, when grown on either hydrocarbon.

Cells of a Flavobacterium sp. and a Brevibacterium sp. contained large electron-dense inclusions when grown on crude oil (Atlas and Heintz, 1973). These inclusions were probably lipids since they were stained with

Sudan black B. Cells of the Flavobacterium sp. also contained these inclusions when grown on "marine agar" containing no hydrocarbon, so that this feature cannot be attributed only to growth on crude oil. Intracytoplasmic electron-dense bodies were also observed in two Arthrobacter spp. and Candida utilis growing in media containing n-butane or 1-butanol as the only carbon source (Kormendy and Wayman, 1974). These structures were called "oxisomes" and it was suggested that they represent the site of oxidation of alkanes and alcohols, within the cell.

Bertrand et al. (1976 b) described intracellular disc-shaped vesicles observed by transmission and freeze-etching electron microscopy in a marine Alcaligenes sp. grown on n-alkanes. These inclusions are thought to be composed of lipids since, on a dry weight basis, the amount of lipid approximately doubles during a single growth cycle on hexadecane.

Acinetobacter sp. HO1-N was shown to contain electron transparent intracellular vesicles when grown on alkanes, and electron dense osmiophilic vesicles when grown on 1-alkenes, as well as intracellular membranes when grown on either of these substrates (Finnerty et al., 1973; Kennedy and Finnerty, 1975; Kennedy et al., 1975). These intracellular membranes are far less elaborated structures than those, previously mentioned, of methane-oxidizing bacteria. The intracytoplasmic globular inclusions were shown with X-ray diffraction analyses to be composed of the hycrocarbon used as growth substrate (Finnerty et al., 1973; Kennedy et al., 1975). They were purified and shown to be bound by a lipid monolayer and to contain some protein (Scott and Finnerty, 1976 a; Scott *et al*., 1976). Similar inclusions were found in a variety of bacteria and actinomycetes, as well as in two species of *Candida* (Scott and Finnerty, 1976 b) and in a *Penicillium* sp. (Cundell *et al*., 1976).

7. Mechanisms for cellular uptake of hydrocarbons.

Hydrocarbons are characterized by their very low solubility in water. The solubility of n-paraffins in water decreases with increasing chain length. The logarithm of the solubility was found to be a linear function of the molar volume for any homologous series of hydrocarbons (McAuliffe, 1963, 1966). In addition, the solubility of n-paraffins is smaller in sea water than in distilled water (Sutton and Calder, 1974). Hexadecane, for example, was found to have a solubility of 0.9 ppb in distilled water and 0.4 ppb in sea water (Sutton and Calder, 1974).

The generation time of bacteria growing on aromatic hydrocarbons depended on the solubility of the growth substrate, the growth rate increasing with increasing solubility (Wodzinski and Johnson, 1968). Furthermore, the growth rate of a *Pseudomonas* sp. was the same when the growth medium contained just enough substrate for saturation or higher concentrations of naphthalene (solubility = 1.25×10^{-2} g/l) or bibenzyl (solubility = 1.4×10^{-2} g/l) (Wodzinski and Bertolini, 1972). A similar result was obtained for the less soluble phenanthrene (1.6×10^{-3} g/l) (Wodzinski and Coyle, 1974). It was concluded that growth on these aromatic compounds occurs at the expense of the dissolved substrate.

Long chain alkanes are far less soluble than these aromatic hydrocarbons. The uptake of ^{14}C-n-decane and production of $^{14}CO_2$ were extremely small when *Candida lipolytica* was incubated with 10^{-2} ppm of this substrate, a concentration just below saturation, but much higher at higher concentrations

(Miura *et al.*, 1977).

Hydrocarbon biodegradation appears to occur exclusively within the cell as no report of extracellular breakdown of hydrocarbons was found in the literature. Efforts to demonstrate such an activity were unsuccessful (Wagner, F., cited in Hug *et al.*, 1974). As seen in the preceding secti[illegible], hydrocarbons can accumulate within the cell. The site of oxidation of hydrocarbons has been reported to be the cell membrane in *Pseudomonas aeruginosa* (van Eyk and Bartels, 1970), in *Candida intermedia* (Liu and Johnson, 1971), and in *Pseudomonas putida* (Benson *et al.*, 1979), and microsomes in *Candida tropicalis* (Gallo *et al.*, 1971, 1973). Hydrocarbon oxidation was also suggested to occur in intracellular inclusions in *Arthrobacter* spp. and in *Candida utilis* (Kormendy and Wayman, 1974).

Considering the very low solubility of the higher n-alkanes, Johnson (1964) suggested that the more insoluble hydrocarbons are not used in the soluble state, but the cells bind to hydrocarbon globules and the hydrocarbon becomes part of the phospholipid bilayer of the membrane. A hydrophobic pathway would exist through which the hydrocarbon would penetrate the cell.

Several authors have reported that microorganisms grown on hydrocarbon have a greater lipid content than when grown on another carbon source (Raymond and Davis, 1960; Miller *et al.*, 1964; Mizuno *et al.*, 1966; Nyns *et al.*, 1968; Ratledge, 1968; Moo-Young *et al.*, 1971; Vestal and Perry, 1971; Finnerty *et al.*, 1973; Hug *et al.*, 1974; Bertrand *et al.*, 1976 b; Mishina *et al.*, 1977). During continuous culture experiments, Hug *et al.* (1974) have shown an adaptation phase, during which no growth of C. tropicalis occured, following a shift in substrate from glucose to hexadecane. During this phase, the amount of total lipid or phospholipid per cell nearly doubled, before growth resumed at the expense of the hydrocarbon. It was proposed that the lipids provide a hydrophobic environment which allows the diffusion of the alkane substrate to its site of oxidation (Hug et al., 1974). This difference in lipid content is not, however, found in all cases studied (Edmonds and Cooney, 1969; Pelechovǎ et al., 1971; Thorpe and Ratledge, 1972; Mishina et al., 1977) and may depend on growth conditions or strains studied (Thorpe and Ratledge, 1972; Rattray et al., 1975; Mishina et al., 1977).

Microorganisms attached to hydrocarbon globules are easily observed microscopically. McLee and Davis (1972) have shown a series of micrographs of a hydrocarbon globule at different stages over a period of 169 hours with a Torulopsis sp. growing at its surface. Finnerty et al. (1973) published scanning electron micrographs of hexadecane globules with cells of Acinetobacter sp. HO1-N coating their surfaces. A Penicillium sp. was shown to grow as hollow mycelial balls surrounding hydrocarbon droplets (Cundell et al., 1976).

Cells of hydrocarbon-assimilating microorganisms have a stronger binding affinity to hydrocarbon globules than other microorganisms (Mimura et al., 1971 b; Miura et al., 1977; Gutnick and Rosenberg, 1977). Cells grown on hydrocarbons show a higher affinity than cells of the same microorganism grown on other substrates (Käppeli and Fiechter, 1976; Neufeld et al., 1980). All cells in a culture are not attached to hydrocarbon globules, however. The proportion of cells adhering to hexadecane globules fluctuated around

70% of the total number during the entire growth cycle of C. lipolytica (Nakahara et al., 1977). The proportion of cells of Acinetobacter calcoaceticus bound to hexadecane globules was about 100% during the first part of the growth cycle. It decreased rapidly during the second half of the exponential phase to reach about 20% during stationary phase (Neufeld et al., 1980).

The binding affinity of C. tropicalis was found to be unaffected by different pH values, temperature, and chain length of the hydrocarbon substrate (Käppeli and Fiechter, 1976). A polysaccharide-fatty acid complex was isolated from this yeast grown on alkanes. This complex was absent in glucose grown cells and was solubilized by treating the cells with pronase. This treatment also caused a marked decrease in alkane-binding affinity of the cells (Käppeli and Fiechter, 1977). The complex was identified an mannan containing approximately 4% covanently bound fatty acid. It was shown to be localized at the surface of the cell wall, using concanavalin A which binds to the mannan part of the complex. When this substance is added to the cells, the mannan complex is masked and the alkane binding affinity of the cells is much reduced (Käppeli et al., 1978).

Emulsification of hydrocarbons occurs during growth of microorganisms on these compounds. The growth rate during the exponential phase of growth of Candida guilliermondii was found to increase with increasing rotation speed of the fermentor impeller, leveling off at 1000 rpm. The interfacial area between the hydrocarbon and aqueous phases was also related to the agitation rate (Aiba et al., 1969 b). This result was confirmed for other hydrocarbon-degrading yeasts (Moo-Young et al., 1971; Wang and Ochoa, 1972). Growth rate was directly related to interfacial area, not only when n-hexadecane was used as the substrate, but also when the growth substrate was n-hexadecane mixed with hydrocarbons not utilizable by the yeast cells (Katinger, 1973).

Hydrocarbon globules formed in the presence of cells were shown to be much more stable than those formed in cell-free medium (Blanch and Fiechter, 1974). The mean diameter of hydrocarbon globules was dependent on the cell density as well as operation conditions such as the size of the fermentor and agitation speed (Bajpai and Prokop, 1975).

The requirement for strong agitation for maximal growth rates on hydrocarbons could be due to limiting amounts of dissolved oxygen. As pointed out by theoretical considerations (Darlington, 1964) and verified experimentally (Mimura et al., 1971 a), the oxygen demand for growth on hydrocarbons is about 3 times as high as with carbohydrates for the same biomass produced. The rate of transfer of oxygen to the water phase was found to decrease as growth proceeded on hydrocarbons but remained constant when glucose was used. The cell concentration had a strong effect on the rate of oxygen transfer. Micrographs of air bubbles in the culture broth with yeast cells and oil droplets at their surface were presented. Antifoaming agents made air bubbles unstable and prevented their coating with cells, resulting in higher oxygen transfer rates to the water phase. It was concluded that the oxygen uptake directly from these air bubbles cannot be neglected and must be considered in addition to the oxygen dissolved in the aqueous phase (Mimura et al., 1973). Formation of flocs, or aggregates of oil drops, cells, air bubbles, and extracellular material, is thought to have an important influence on the growth kinetics because incomplete mixing can lead to oxygen limitations in cases of high cell density (Einsele et al., 1973).

High agitation rates thus appear to be necessary for proper aeration of the culture and for maximizing the surface area of hydrocarbon globules to which cells can bind. Yoshida et al. (1971), however, compared the growth kinetics of C. tropicalis in shaken flasks, with the substrate C_6 to C_{18} n-paraffins present in the liquid form, with that obtained in a specially designed rotating disks fermenter to which the same substrate is provided in the gas phase. Similar growth rates were obtained for the two types of cultures. Although no oil droplets could be seen, with phase contrast microscopy, in the culture medium from the rotating disks fermenter (Yoshida et al, 1971), the amount of hexadecane present in the water was much higher than the value expected from the solubility in water of this compound (Yoshida and Yamane, 1971). Filtration through 0.8 and 0.1μm pore size membranes reduced the amount of hydrocarbon present in the medium. The alkanes thus appear to be "accomodated" in the form of submicron aggregates and these small vesicles can account for the observed growth kinetics.

A similar conclusion was arrived at by Goma et al. (1971, 1972) since the rate of "pseudo-solubilization" of the hydrocarbon into the aqueous phase of the supernatant was of the same order of magnitude as the rate of hydrocarbon uptake by the cells. When C. lipolytica was grown on a mixture of C_{11} to C_{18} n-alkanes, the more soluble compounds were utilized earlier than the less soluble ones (Goma et al., 1973 a). The "pseudo-solubilization" of the alkanes followed the same order as their uptake by the cells (Goma et al., 1973 b, 1974). Small hexadecane droplets having diameters of 0.1 to 1.0 μm were shown, in electron micrographs, to be attached to the surface of C. tropicalis cells growing on this substrate (Einsele et al., 1975).

Stabilization of the emulsion and formation of submicron vesicles are thought to be due to emulsifying agents released by the cells. Production of such compounds by microorganisms appears to be relatively common and not limited to those growing on hydrocarbons (Zajic and Panchal, 1977).

Many authors have reported the presence of extracellular lipids in the cultures of hydrocarbon oxidizing microorganisms (Klug and Markovetz, 1967; Volfova and Pecka, 1973). Cultures of Cladosporium resinae contained extracellular triglycerides and phospholipids. While the cellular lipid fatty acids reflected the chain length of the alkane used as growth substrate, the extracellular fatty acids did not (Siporin and Cooney, 1975). Acinetobacter sp. HO1-N cultures were reported to contain extracellular mono- and triglycerides, free fatty acids, and wax (Stewart and Kallio, 1959; Finnerty et al., 1973; Makula et al., 1975). These were present in very low concentrations when a non-hydrocarbon substrate was used and they accumulated in the medium throughout growth on hexadecane, reaching a maximum at the end of the growth cycle. This bacterium was recently found to produce also some extracellular phospholipid. Extracellular vesicles composed of hexadecane, phospholipid, lipopolysaccharide, and proteins were isolated from the culture filtrate. These have a polypeptide composition similar to that of the outer membrane of the cell and are thought to provide a mechanism for emulsifying the hydrocarbon in microdroplets which bind to the cells (Käppeli and Finnerty, 1979).

Many workers have isolated different substances having emulsifying properties from cultures of hydrocarbon grown microorganisms. One such substance, produced by Candida petrophilum, was found to be composed of a peptide and fatty acid (Igushi et al., 1969). An emulsifying factor, produced by Endomycopsis lipolytica during growth on hydrocarbons, is thought to be a complex of metal ions with a peptide since it is inactivated by chelating agents (Roy et al., 1979).

Arthrobacter paraffineus and other members of the genera Arthrobacter, Corynebacterium, and Nocardia produce an alpha-branched-beta-hydroxy fatty acid trehalose ester when growing on alkanes (Suzuki et al., 1969). Production of alpha- branched-beta-hydroxy fatty acids and their emulsifying properties were studied more extensively in cultures of Corynebacterium lepus growing on kerosene (Cooper et al., 1979 a, b). A mixture of these fatty acids is procuded ranging from C_{28} to C_{43} with the length of the alkyl branch varying from 6 to 14 carbon atoms. Corynebacterium hydrocarboclastus produced a complex polymer, composed of protein, lipid, and carbohydrate and having emulsifying properties when growing on hydrocarbons (Zajic et al., 1977 a, b). A polymer with emulsifying properties, isolated from cultures of Arthrobacter RAG-1, was found to be a polyssacharide fatty acid ester having an estimated molecular weight of close to 10^6 (Rosenberg et al. 1979 a, b; Zuckerberg et al., 1979).

Rhamnolipids isolated from P. aeruginosa growing on n-hexadecane stimulated growth of this bacterium on n-alkanes, but did not affect its growth on glucose. These lipids have a strong surface activity and emulsifying capacity (Hisatsuka et al., 1971; Itoh et al., 1971). A mutant was isolated which grows very slowly on n-paraffins (Itoh and Suzuki, 1972). Addition to the medium of rhamnolipids, isolated from the parent strain, stimulated the growth on n-alkanes of both the parent and mutant strains. The mutant produced small amounts of rhamnolipid as compared to the parent strain. The trehalose lipid, mentioned above (Suzuki et al., 1969) and isolated from Arthrobacter paraffineus had little effect on the growth of wild type or mutant of P. aeruginosa (Itoh and Suzuki, 1972). The growth stimulating effect of the rhamnolipids is thus not only due to their emulsifying properties. P. aeruginosa also produces a protein having a molecular weight of about 14 300 which stimulates growth on n-alkanes. When the substrate is added to the medium as a fine emulsion prepared by sonification, this protein has no effect on the growth so that it either participates in the emulsification of the hydrocarbons or is necessary for the uptake of these compounds from larger droplets of n-alkanes (Hisatsuka et al., 1975, 1977).

The effect of detergents added to cultures of microorganisms growing on hydrocarbons can be stimulatory or inhibitory depending on the structure and physical properties of these compounds and on the microorganism studied (Tanaka and Fukui, 1971). While a number of synthetic surface-active compounds inhibit growth of hydrocarbon-utilizing yeasts, naturally occuring substances such as phospholipids and agar were found to promote growth of these organisms (Mimura et al., 1971 b). Long chain fatty acids (C_{16} and C_{18}), as well as triglycerides, phospholipids, and certain synthetic detergents, stimulated hexadecane utilization by Acinetobacter lwoffi and P. aeruginosa, but shorter fatty acids (isobutyric, C_8, and C_{12}) were inhibitory (Breuil and Kushner, 1980). Addition of certain surfactants to cultures of C. guilliermondii growing on n-alkanes caused a reduction in the number of cells bound to hydrocarbon globules and a reduction in the growth rate (Aiba et al., 1969 a). Short chain free fatty acids (C_6 to C_{12}) were inhibitory for the growth of C. tropicalis on n-alkanes or glucose (Duvnjak and Azoulay, 1972).

Uptake of hydrocarbons by the cells can thus occur from the dissolved substrate, from large oil globules to which cells are bound, or from small vesicles, formed of the hydrocarbon and extracellular emulsifying agents, that bind to the cells (Erickson and Nakahara, 1975). In a culture, more than one of these mechanisms can probably occur simultaneously (Nakahara et al., 1977; Gutierrez and Erickson, 1977). Only small differences in the rate of uptake of radioactive n-decane were observed when this substrate was added to a suspension of C. intermedia directly or in the form of a fine emulsion. This yeast shows a stronger adhesion force to oil globules than C. tropicalis for which the rate of ^{14}C-n-decane uptake was much higher when the hydrocarbon was added already emulsified (Miura et al., 1977). The preferred uptake mechanism thus appears to depend on the organism studied, the solubility and dispersibility of the hydrocarbon and the growth conditions.

8. The organism studied in this thesis and diauxie.

The bacterium Acinetobacter lwoffi O_{16} (ATCC 29064) was isolated from a sample of sediment taken near the confluent of Brewery's Creek and the Ottawa River, in Hull, Québec. Its psychrophilic and lipolytic properties have been studied in detail in our laboratory (Breuil and Kushner, 1975 a, b). Its biochemical characteristics have been described and its taxonomical position was established (Breuil et al., 1975.). This organism can utilize a variety of carbon sources including crude oil and certain pure alkanes (Breuil et al., 1978 a). Among these, hexadecane gives the most important growth.

In the presence of ethanol and hexadecane, we observe a diauxic growth. The ethanol is used during the first growth phase, while the hydrocarbon is metabolized only after a long lag period that follows the depletion of the first substrate. If hexadecane is labelled with ^{14}C, radioactive CO_2 evolves from the culture only during the second growth cycle (Breuil et al., 1978 a).

Studies on A. lwoffi have shown an important stimulation in the production of lipase activity when this bacterium grows on hydrocarbons (Breuil et al., 1978 a). This phenomenon has also been observed in Pseudomonas aeruginosa, and a correlation seems to exist between the possibility of utilizing hydrocarbons as sole carbon source and the possession of lipase activity (Breuil et al., 1978 a).

Lipase activity, which is negligeable during the first growth phase of diauxie, increases rapidly during growth on hexadecane (Breuil et al., 1978 a). The presence of globular cellular inclusions, similar to those described by

Finnerty et al. (1973), has also been demonstrated in this organism (Shindler et al., 1978). These appear only when the bacterium grows on hydrocarbon and are not observed when a non-hydrocarbon substrate is used. During diauxie, they are present only during the second growth cycle.

This project was initiated to further our knowledge on the physiology of A. lwoffi growing on hydrocarbon, especially as far as its lipids are concerned, in an attempt to clarity the involvement of the lipase in the utilization of hexadecane. The diauxic growth provides a convenient system for the study of the physiological changes accompanying adaptation to hydrocarbon utilization. After the first growth cycle, a number of cells, sufficiently large for chemical analyses, has grown on a non-hydrocarbon substrate. The long lag phase that follows (about 100 hours) is presumably occupied by the transformations that are necessary for the uptake and metabolism of the hydrocarbon, before growth can resume.

Since hydrocarbon metabolism is closely linked with that of lipids, the changes in lipid and fatty acid composition were followed throughout diauxie. Since extracellular lipids may be involved in the uptake of hydrocarbons, an attempt was made to estimate the amount of these compounds present in the extracellular medium.

CHAPTER II MATERIAL AND METHODS

1. Growth conditions.

The basic mineral salts medium (Breuil et al., 1978 a) contained (ing/l): NH_4NO_3 (2.0), KH_2PO_4 (4.0), Na_2HPO_4 (6.0), $MgSO_4$ $7H_2O$ (0.2), $CaCl_2$ $2H_2O$ (0.05), and $FeSO_4$ $7H_2O$ (0.01). The pH was adjusted to 7.5 before sterilization. The carbon sources, sterilized separately and added aseptically to this mineral medium, included 1% (v/v) hexadecane, 0.1 or 1% ethanol, or, for diauxie, 0.1% ethanol and 1.0% hexadecane. The organism was conserved on a semi-solid soil-extract medium at 4°C (Breuil and Gounot, 1972).

Growth was carried out in Erlenmeyer flasks containing 1/5 volume of liquid. The flasks were agitated on a reciprocal shaker at 100 strokes (2cm) per min at 20°C. A strong agitation is required for appropriate aeration and mixing, and care must be taken that the temperature remains above the freezing point of hexadecane (18°C). These two conditions are necessary for the reproducibility of the diauxic growth curve. Growth was monitored by following absorbance at 610 nm, in 18 X 150 mm cuvettes, with a Coleman Junior II spectrophotometer. Protein content was measured using the method of Lowry et al. (1951) with bovine serum albumin as standard. Cell counts were done by plating suitable dilutions on the "Caulobacter" medium of Poindexter (1964) .

The bacterium was first grown on a complex medium, containing 20 g/l peptone and 1.5 g/l yeast extract, for 48 hours. One ml of this culture was then added to 100 ml of the mineral medium containing the carbon source used in the experiment. This served as a preculture and, when turbidity reached an absorbance of 0.8, 1 ml was transfered to 100 ml of medium as the inoculum. For diauxie, ethanol was used as the carbon source for the preculture.

2. Extraction and separation of lipids.

The pellets obtained after centrifugation of the cultures were washed twice with the mineral salts medium used for growth, but without the carbon source. The supernatants and washes were combined and lyophilized. The lipids were extracted from the pellets and supernatants by the method of Bligh and Dyer (1959) as modified by Kates (1972). This method for the extraction of supernatant lipids was preferred to that of Makula *et al.* (1975) who used diethyl ether after acidification of the medium to pH5 with H_2SO_4. Important amounts of lipid were found in the fifth and sixth extractions with the method of Makula *et al.*, while only trace amounts could be detected in the fourth and fifth extractions using the method of Bligh and Dyer, which was also found simpler to perform. These procedures had the added advantages that both pellet and supernatant lipids were extracted with the same method, contrary to Makula *et al.* who used another method for the extraction of the cell pellets (Makula and Finnerty, 1970).

The lipids were separated by thin-layer chromatography on silica gel H (Merck). Hexadecane was separated from the other lipids with petroleum ether (60-70°C). This leaves all the lipids at the origin except hydrocarbons which migrate with the solvent front. When the solvent reached the top of the thin-layer chromatography plate, it was dried under vacuum, in a dessicator. After a few minutes, the plate was transfered into a second jar containing a solvent system composed of petroleum ether/diethyl ether/acetic acid, 80:20:1 (v/v/v) (Mangold, 1969).

This time, the plate was removed when the solvent front had reached to about 4-5 cm from the top so that it was still well under the hexadecane band.

This step yielded three well separated fractions: wax, triglycerides, and a fraction containing the other lipids. A solvent system composed of benzene/diethyl ether/ethanol/acetic acid, 50:40:2:0.2 (v/v/v/v) (Freeman and West, 1966) was used to separate this mixture into the phospholipids, the monoglycerides, the diglycerides, and a band containing free fatty acids and free fatty alcohols.

The major phospholipids were separated using a solvent system composed of chloroform/methanol/30% ammonia, 60:50:2.5 (v/v/v) which gave a band containing phosphatidylethanolamine and another containing phosphatidylglycerol and cardiolipin. This mixture was separated with a solvent system composed of chloroform/methanol/acetic acid/water, 80:26:4:0.3 (v/v/v/v) (Mc Garrity and Armstrong, 1975). Lipid spots were scraped from the plates, with a microscope slide cover glass, into a funnel which had its stem plugged with glass wool, and were eluted from the silica gel with methanol/chloroform, 2:1 (v/v) (McGarrity and Armstrong, 1975).

The wax fraction was saponified in 90% methanolic sodium hydroxide (0.3N), with refluxing, as described by Kates (1972). The alcohol moieties were extracted with petroleum ether and the methanolic phase was acidified before extraction of the fatty acid moieties with the same solvent (Kates, 1972). The same procedure was used, except without refluxing, to separate the free fatty alcohols and free fatty acids.

In order to determine the amount of hexadecane remaining after growth, it was extracted with n-hexane and separated from the extracted lipids by passage through a silica gel column (Kates, 1972). After concentrating the hexane eluate to dryness, the remaining hexadecane was determined by weighing.

3. Visualization and identification of lipids.

Thin-layer chromatography plates were exposed to I_2 vapour or charred, after spraying with H_2SO_4, for non-specific visualization. Dittmer-Lester (1964) reagent was used to detect phosphate in phospholipids, ninhydrin (Skipski et al., 1962), to indicate the presence of a free amino group in phosphatidylethanolamine, and periodate-Schiff reagent (Skipski and Barclay, 1969), with SO_2 treatment, to detect lipids with a viccinal hydroxy group.

Further identification involved co-chromatography of the purified lipids with standards on thin-layer chromatography plates, as well as co-chromatography, on paper, of the deacylated products of phospholipids. After preparation (Kates, 1972), these were run in phenol/water, 100:38 (w/w) (Kates, 1960), and in isopropanol/water/concentrated ammonia, 7:2:1 (v/v/v) (Letters, 1966). The glycol spray of White and Frerman (1967) was used to detect spots of deacylated phospholipids and glycolipids. Wax samples yielded equimolar amounts of alcohol and fatty acid. A separate plate, with standards of the lipids to be separated, accompanied, in the same tank and at the same time, each thin-layer chromatography plate. A separate plate for the standards was necessary because small (7 cm wide) plates were used in this project. Scraping of the gel from the plates was found to be easier with these small plates.

4. Fatty acid and fatty alcohol analyses.

Samples were analyzed in a Hewlett Packard model 402 gas chromatograph equipped with a flame ionization detector. Columns, 183 cm long, were packed with 10% butanediol succinate on chromabsorb W (Chromatographic Specialties, Brockville, Ont.). The hexadecane contaminating the lipid samples after the thin-layer chromatography procedures described above, did not interfere with the analyses with butanediol succinate since the hydrocarbon was present in relatively small amounts and gave a peak very close to that of the solvent. Nitrogen was the carrier gas. Fatty acid methyl esters were prepared using the method described by Kates (1964) and fatty alcohols were analysed directly.

Fatty acids or fatty alcohols were identified by comparison of retention times, relative to palmitate or to cetyl alcohol, with those obtained for standards, as well as with published values (Kates, 1972). Weight percentage was computed according to the peak-height times retention time method (Carroll, 1961). Weight of lipid components was calculated from the fatty acid composition using methyl arachidate as internal standard according to the following equation:

$$W_p = (A_p/A_s) \times W_s$$

where W_p is the weight of the fatty acid, and A_p and A_s are the values obtained for peak-height times retention time of the fatty acid and of the standard, respectively. W_s is the weight of standard added to the sample. Arachidic acid is not produced by <u>A</u>. <u>lwoffi</u> under the conditions studied in this project. Average chain lengths were calculated using the equation:

$$A = \frac{\sum P \times N}{100}$$

where A is the average chain length, and where P is the proportion (in % micromoles) and N, the number of carbon atoms of each fatty acid species. All calculations were done by computer at the University of Ottawa Computing Center.

5. Source of lipid standards.

Free fatty acids, methyl esters of fatty acids, cetyl palmitate, and mono- and diglycerides were purchased from the Sigma Chemical Co. (St. Louis, Mo.), phosphatidylcholine, phosphatidylethanolamine, and cardiolipin, from Calbiochem (San Diego, Calif.), tripalmitin, from the Baker Chemical Co. (Phillipsburg, N.J.), hexadecane, from Humphrey Chemical Co. (North Haven, Conn.), and aliphatic alcohols, from Eastman Kodak Co. (Rochester, N.Y.). Phosphatidylglycerol was a gift of Dr. M. Kates.

CHAPTER III RESULTS

1. Identification of lipids.

Acinetobacter lwoffi cultures were found to contain phospholipids, mono-, di-, and triglycerides, free fatty acids, free fatty alcohols, and wax esters as the major lipids. These were identified by co-chromatography with standards as described in Table 1. The major phospholipids were found to be phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (Table 2). Further identification of the phospholipids involved co-chromatography on paper of their deacylation products with standards, as well as specific stains, as described in Material and Methods (Table 3). No evidence of the presence of glycolipids was found as the only spot stained with periodate-Schiff reagent had chromatographic mobilities corresponding to those of phosphatidylglycerol.

Identification of *A. lwoffi* fatty acids and fatty alcohols was done by gas-liquid chromatography as shown in Table 4 and 5. No attempts were made to determine the position of the double bond in $C_{18:1}$ fatty acid or fatty alcohol and these are referred to as octadecenoate or octadecenol. Chromatographic data obtained were compared with published values whenever these were available.

Table 1. Comparison of Rf values obtained for *A. lwoffi* lipids, standard lipids, and published values[1] with thin-layer chromatography.

Lipids	A[2]			B[3]	
	A. lwoffi lipids	Standard lipids	Kates (1972)[1]	*A. lwoffi* lipids	Standard lipids
Phospholipids	0.00	0.00	[illegible]	0.00	0.00
Monoglycerides	0.02	0.02	0.02	0.31	0.30
Diglycerides	0.18	0.14	0.15-0.21	0.76	0.76
Triglycerides	0.65	0.66	0.60	0.93	0.93
Free fatty acids	0.23	0.21	0.39	0.63	0.65
Free fatty alcohols	0.29	0.27	0.30	0.52	0.50
Wax esters	0.90	0.91	0.88	0.88	0.88
Hydrocarbons	0.98	0.95	0.98	0.84	0.84

1. Values from Kates (1972).
2. Petroleum ether/diethyl ether/acetic acid, 80:20:1 (Mangold, 1969).
3. Benzene/diethyl ether/ethanol/acetic acid, 50:40:2:0.2 (Freeman and West, 1966).
4. —, Not shown.

Table 2. Comparison of Rf values obtained for A. lwoffi phospholipids and standard phospholipids with thin-layer chromatography.

Phospholipids[3]	A[1]		B[2]	
	A. lwoffi lipids	Standard lipids	A. lwoffi lipids	Standard lipids
PE	0.38	0.38	0.28	0.29
PG	0.70	0.70	0.39	0.38
CL	0.70	0.70	0.85	0.85

1. Chloroform/methanol/30% ammonia, 60:50:2.5.
2. Chloroform/methanol/acetic acid/water, 80:26:4:0.3.
   Both solvent systems from Mc Garrity and Armstrong (1975).
3. PE, phosphatidylethanolamine, PG, phosphatidylglycerol, CL, cardiolipin.

Table 3. Comparison of R_f values obtained for A. lwoffi phospholipid deacylation products, standard phospholipid deacylation products, and published values[1] with paper chromatography.

		A[2]			B[3]		
Parent phospholipids[4]	deacylation products	A. lwoffi phospholipids	Standard phospholipids	Kates (1972)[1]	A. lwoffi phospholipids	Standard phospholipids	Kates (1972)[1]
PE	GPE	0.43	0.44	0.45	0.59	0.60	0.62
PG	GPG	0.52	0.54	0.54	0.48	0.48	0.46
CL	GPGPG	0.21	0.24	0.26	0.19	0.20	0.18

1. Values from Kates (1972).
2. Isopropanol / water / concentrated ammonia, 7:2:1 (Letters, 1966).
3. Phenol / water, 100:30 (Kates, 1960).
4. PE, phosphatidylethanolamine, PG, phosphatidylglycerol, CL, cardiolipin.

Table 4. Comparison of Rt values obtained for A. lwoffi fatty acid methyl esters and standard fatty acid methyl esters with gas-liquid chromatography.[1,2]

Fatty acid	A. lwoffi lipids	Standard lipids
laurate ($C_{12:0}$)	0.34	0.34
myristate ($C_{14:0}$)	0.58	0.55
palmitate ($C_{16:0}$)	1.00	1.00
palmitoleate ($C_{16:1}$)	1.17	1.16
stearate ($C_{18:0}$)	1.77	1.75
octadecenoate ($C_{18:1}$)	1.98	1.95

1. Column packed with butanediol succinate and used at 190°C under conditions described in Material and Methods.
2. Values given relative to methyl palmitate.

Table 5. Comparison of Rt values obtained for A. lwoffi fatty alcohols and standard fatty alcohols with gas-liquid chromatography.[1,2]

Fatty alcohols	A. lwoffi lipids	Standard alcohols
dodecanol ($C_{12:0}$)	0.30	0.33
tetradecanol ($C_{14:0}$)	0.50	0.59
hexadecanol ($C_{16:0}$)	1.00	1.00
hexadecenol ($C_{16:1}$)	1.14	—[3]
octadecanol ($C_{18:0}$)	1.83	1.81
octadecenol ($C_{18:1}$)	2.02	1.97

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1. Column packed with butanediol succinate and used at 190°C under conditions described in Material and Methods.
2. Values given relative to hexadecanol.
3. —, Not available.

2. Fatty acid compositions

a) Changes in total amounts of the major fatty acids present in the whole culture during diauxie.

An examination of the total amounts of the major fatty acids present in the whole culture reveals that only the C_{16} fatty acids change considerably (Fig. 1). During the hexadecane phase of diauxie, there is an increase of about 6- fold in the amount of palmitic acid and of about 14- fold in the amount of palmitoleic acid. However, the amounts of the other fatty acids remain nearly constant throughout this period.

b) Changes in the average chain length of fatty acids from lipid components during diauxie.

Growth on hexadecane alone, or during the hexadecane phase of diauxie resulted in a decrease in the average chain length of the fatty acids of A. lwoffi (Fig. 2). The changes are largest for the phospholipid fraction for which a rapid decrease occurs coinciding with the utilization of hexadecane. In the free fatty acid fraction, a rapid decrease occurs at the onset of growth on hexadecane but the average chain length returns to its former level soon afterwards. Changes in any of the three forms of glycerides are small throughout diauxie.

c) Phospholipid fatty acids.

Though considerable proportions of C_{18} fatty acids were present in the phospholipids of cells grown on ethanol, they represent only a very small fraction of the fatty acids of phospholipids of cells grown

Figure 1. Changes in the amounts of the major fatty acids present in the total lipids of the whole culture during diauxie.

Palmitate (○----○), Palmitoleate (○——○), Stearate (●---●), and Octadecenoate (●——●)

Growth curve is represented for reference.

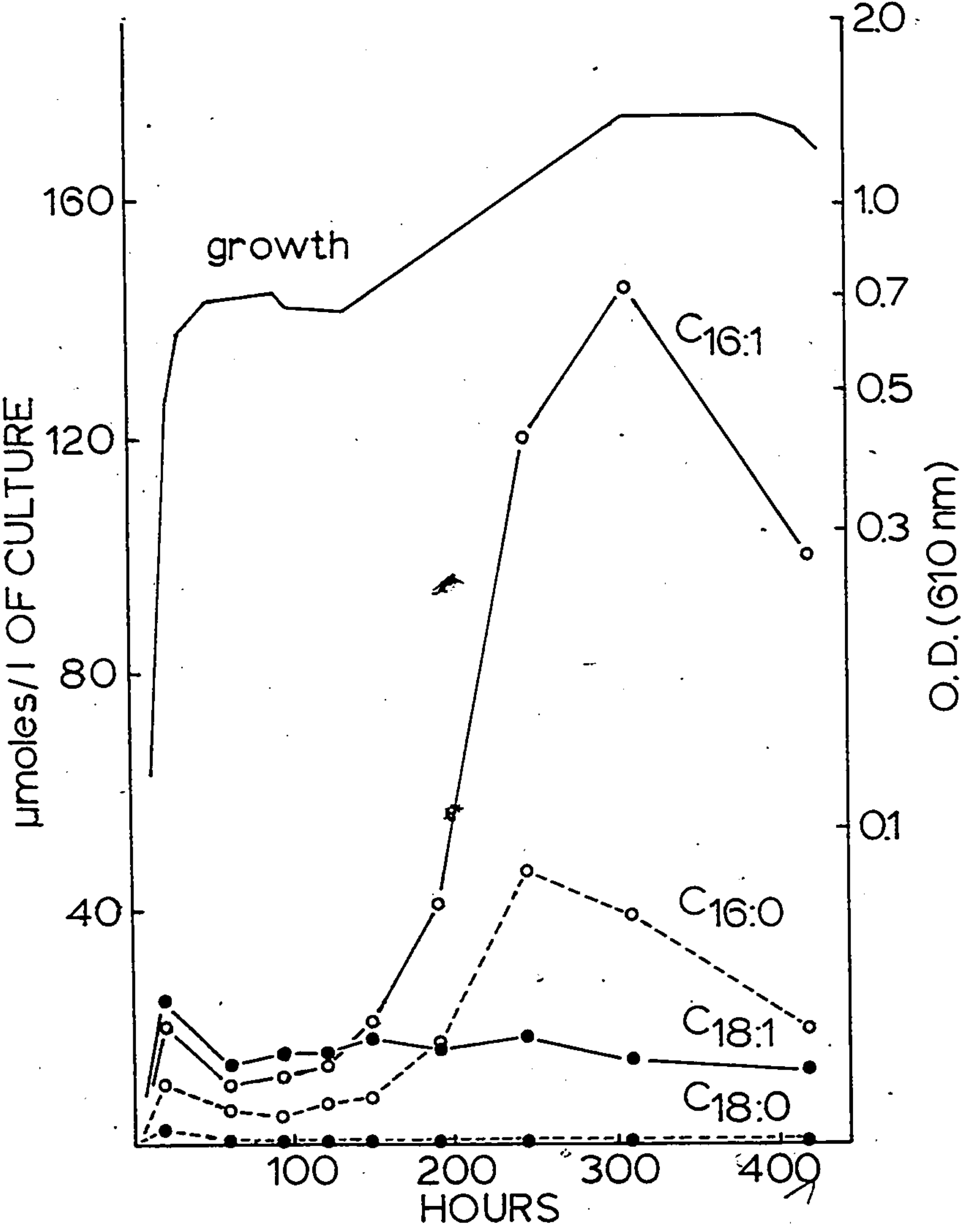
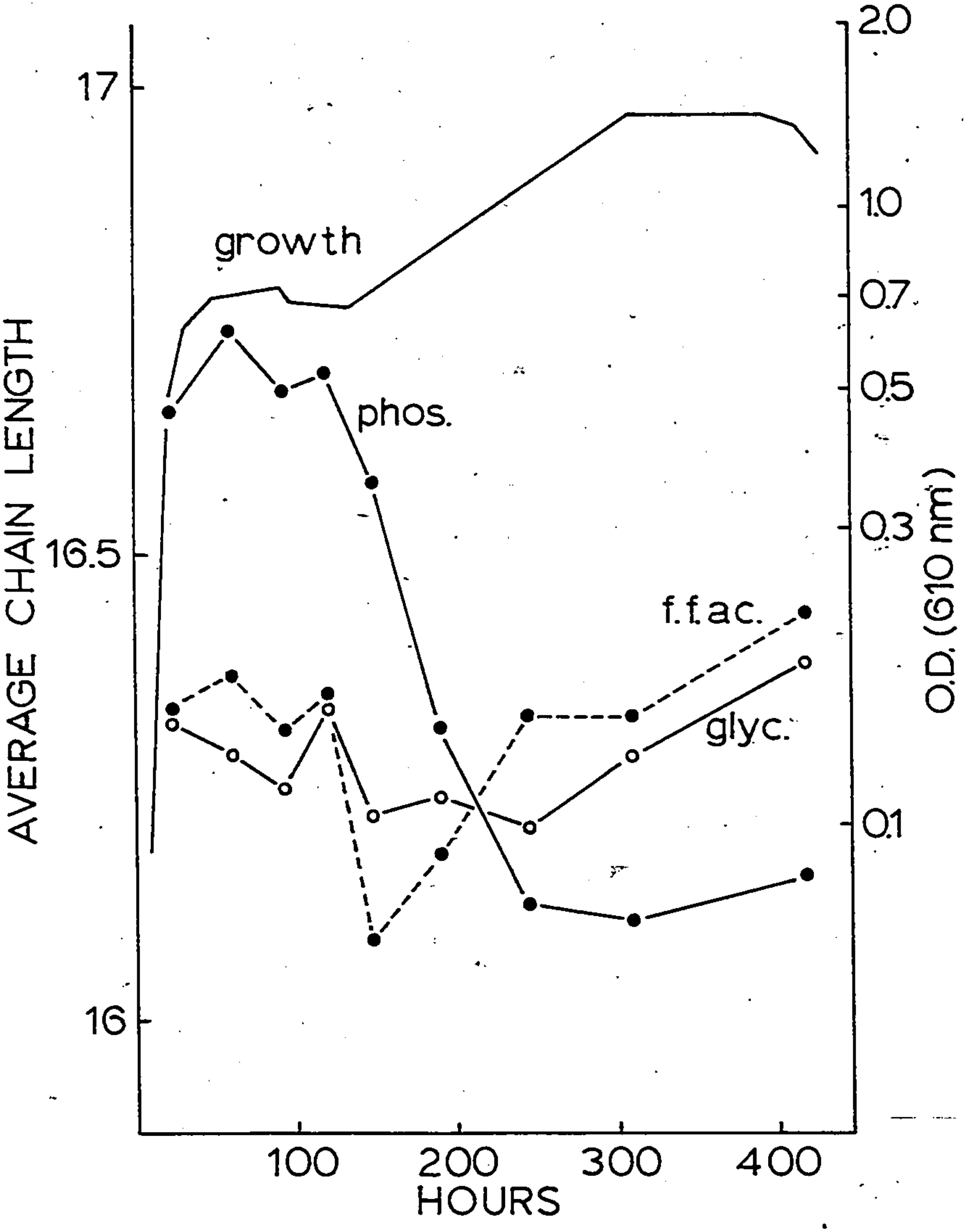

growth
C16:1
C16:0
C18:1
C18:0
160
120
80
40
µmoles/l OF CULTURE
2.0
1.0
0.7
0.5
0.3
0.1
O.D. (610 nm)
100
200
300
400
HOURS

Figure 2. Changes in the average chain length of different lipid components during diauxie.

Phospholipids (●——●), Free fatty acids (●- - - -●), and combined values for glycerides (○——○)

Growth curve represented for reference.


AVERAGE CHAIN LENGTH
17
16.5
16
O.D. (610 nm)
2.0
1.0
0.7
0.5
0.3
0.1
growth
phos.
f.f.ac.
glyc.
100
200
300
400
HOURS

on hexadecane (Tables 6 to 8). After about two generations of growth on hexadecane during diauxie, the relative amount of C_{18} fatty acids in phospholipids had greatly diminished. Coinciding with this, was an increase in the $C_{16:1}$ fatty acid content of all three major phospholipids.

d) <u>Changes in the degree of saturation of the major fatty acids</u>.

The proportion of saturated fatty acids remained relatively constant during diauxie fluctuating between 18 and 27% in the total lipids and between 25 and 36% in the phospholipids (Fig. 3).

Table 6. Fatty acid composition of Phosphatidylethanolamine after growth on ethanol, hexadecane, and during diauxie.[1]

Fatty acids	Ethanol alone (1%)	Diauxie Ethanol phase (.1%)	 Hexadecane phase (1%)	Hexadecane alone (1%)
laurate (C_{12}:0	T	T	0.1	T
myristate (C_{14}:0)	1.4	1.8	1.1	1.0
palmitate (C_{16}:0)	24.1	23.2	19.9	21.9
palmitoleate ($C_{16:1}$)	32.2	30.9	70.0	76.0
stearate (C_{18}:0)	6.1	1.9	0.5	T
octadecenoate (C_{18}:1)	36.2	42.3	8.4	1.1

[1] Figures shown as percentage of fatty acids. All samples taken in late exponential phase of growth.

Table 7. Fatty acid composition of Cardiolipin after growth on ethanol, hexadecane, and during diauxie.[1]

Fatty acids	Ethanol alone (1%)	Diauxie Ethanol phase (.1%)	Diauxie Hexadecane phase (1%)	Hexadecane alone (1%)
laurate C_{12}:0)	0.6	1.3	0.7	T
myristate (C_{14}:0)	2.8	4.4	3.2	1.5
palmitate (C_{16}:o)	31.8	28.5	21.1	22.0
palmitoleate (C_{16}:1)	28.0	28.9	59.0	73.2
stearate (C_{18}:0)	5.8	3.5	2.2	1.0
octadecenoate (C_{18}:1)	31.0	33.4	13.8	2.2

[1] Figures shown as percentage of fatty acids. All samples taken in late exponential phase de growth.

Table 8. Fatty acid composition of Phosphatidylglycerol after growth on ethanol, hexadecane, and during diauxie.[1]

Fatty acids	Ethanol alone (1%)	Diauxie Ethanol phase (.1%)	 Hexadecane phase (1%)	Hexadecane alone (1%)
laurate	T	1.7	0.5	T
myristate	1.5	5.7	2.1	1.5
palmitate	27.2	34.2	26.1	22.0
palmitoleate	28.7	25.0	61.2	73.2
stearate	5.9	4.9	1.4	1.0
octadecenoate	36.8	28.6	8.6	2.2

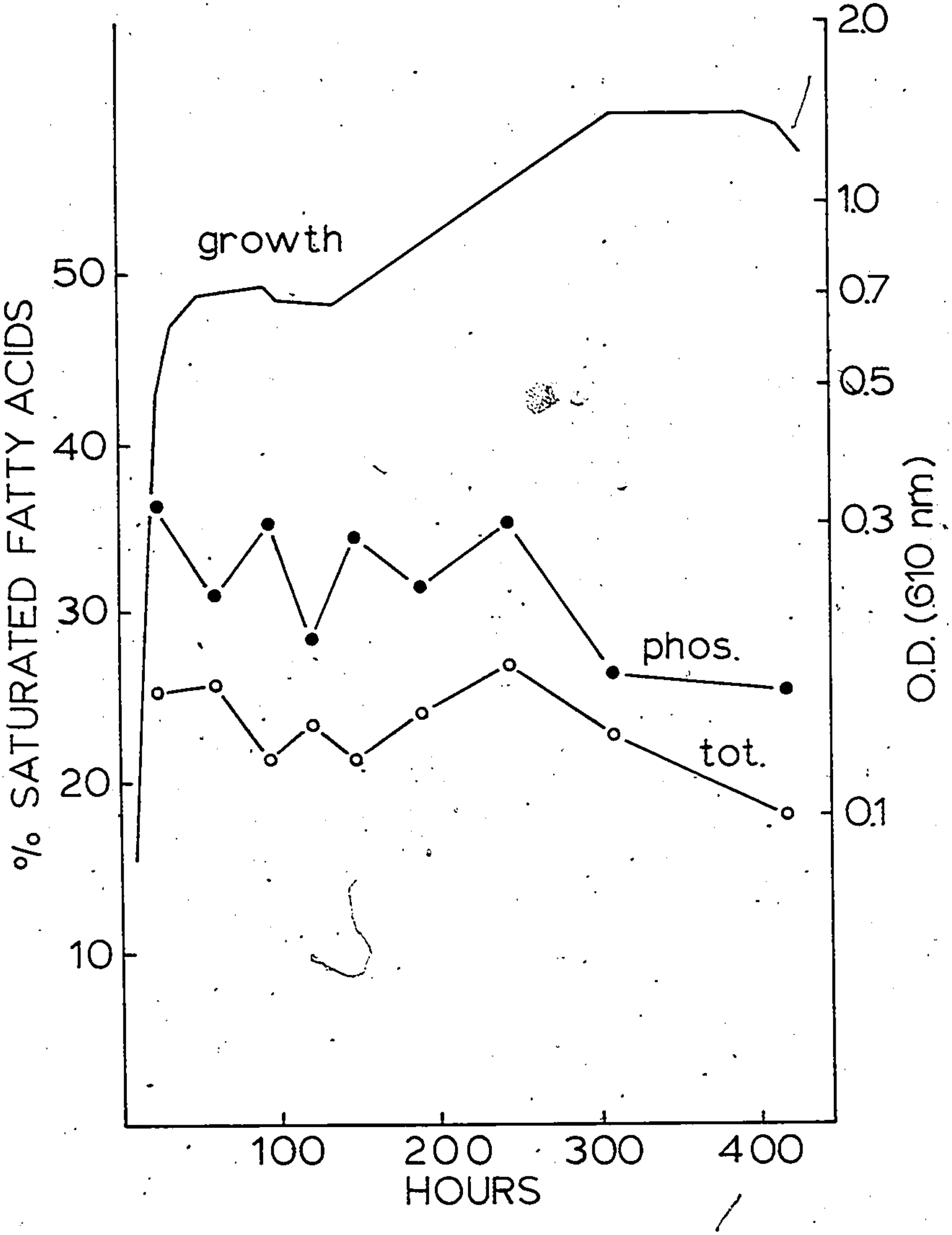
---

1. Figures shown as percentage of fatty acids. All samples taken in late exponential phase of growth.

Figure 3. Changes in the percentage of saturated fatty acids present in the whole culture total lipids and phospholipids during diauxie.

Total lipids (o——o), Phospholipids (●——●)

Growth curve is represented for reference.


growth
phos.
tot.
% SATURATED FATTY ACIDS
10
20
30
40
50
O.D. (610 nm)
2.0
1.0
0.7
0.5
0.3
0.1
100
200
300
400
HOURS

3. Changes in the total amounts of lipid components present in the whole culture during diauxie.

The changes that occur in the total amounts of the different lipid components present in the whole culture at various stages of diauxie were examined (Figures 4 and 5). When the culture enters the lag phase separating the two growth cycles, the total amount of phospholipids decreases to about half the value it has near the end of the exponential phase of growth on ethanol. During this lag period, the level of phospholipids stays nearly constant. During the second growth phase, at the expense of hexadecane, the total amount of phospholipids increases approximately 8- fold and decreases afterwards, at the end of the growth curve, when cells appear to start dying. Throughout diauxie, the total amounts of each of the other lipid components change relatively little. After the initial increase from the amounts present in the inoculum to those present at the end of the ethanol phase of growth, only small fluctuations are observed.

Figure 4. Changes in the amounts of different lipid components present in the whole culture during diauxie.

Phospholipids (●——●), triglycerides (●----●), diglycerides (○——○), and monoglycerides (○----○).

Growth curve represented for reference.

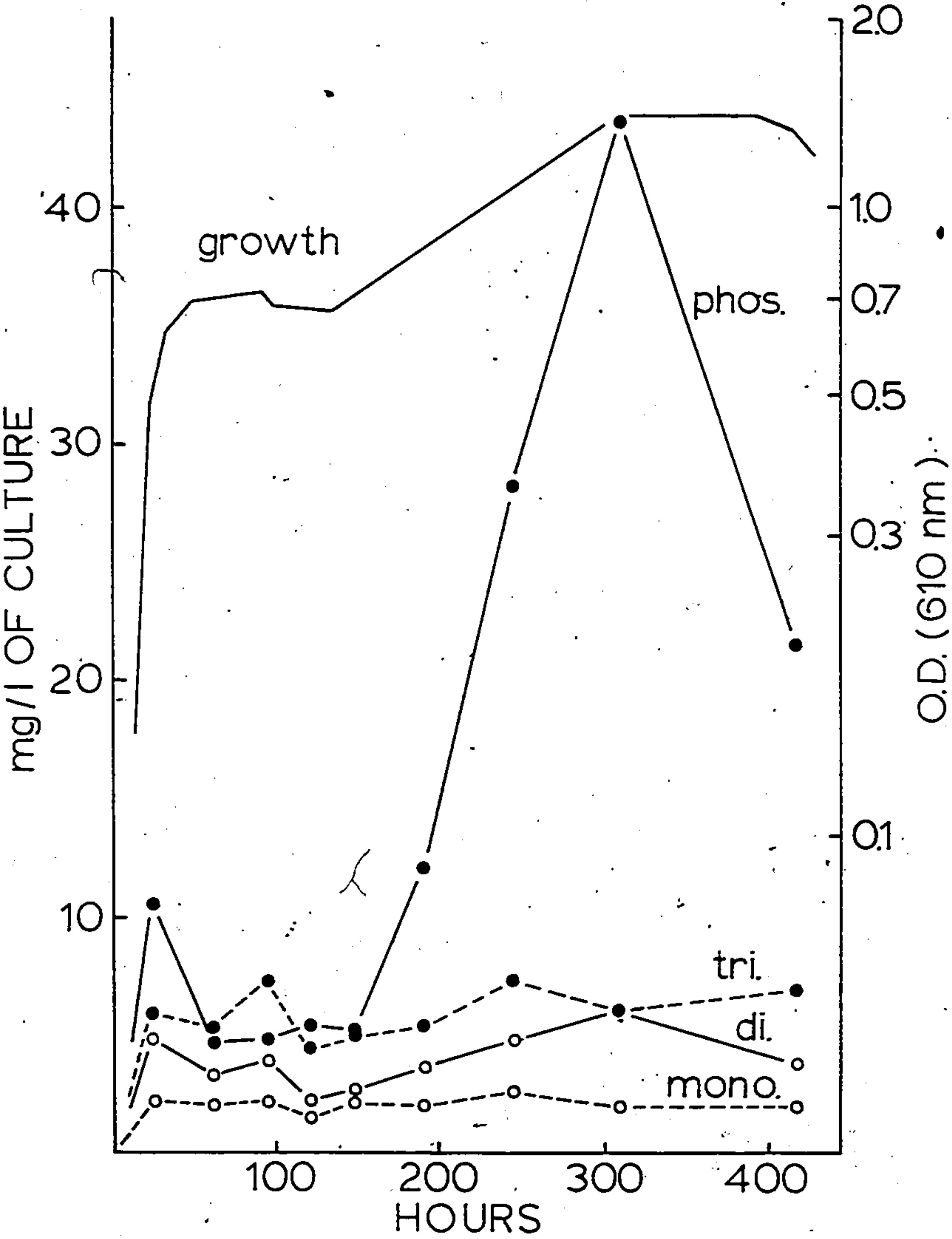
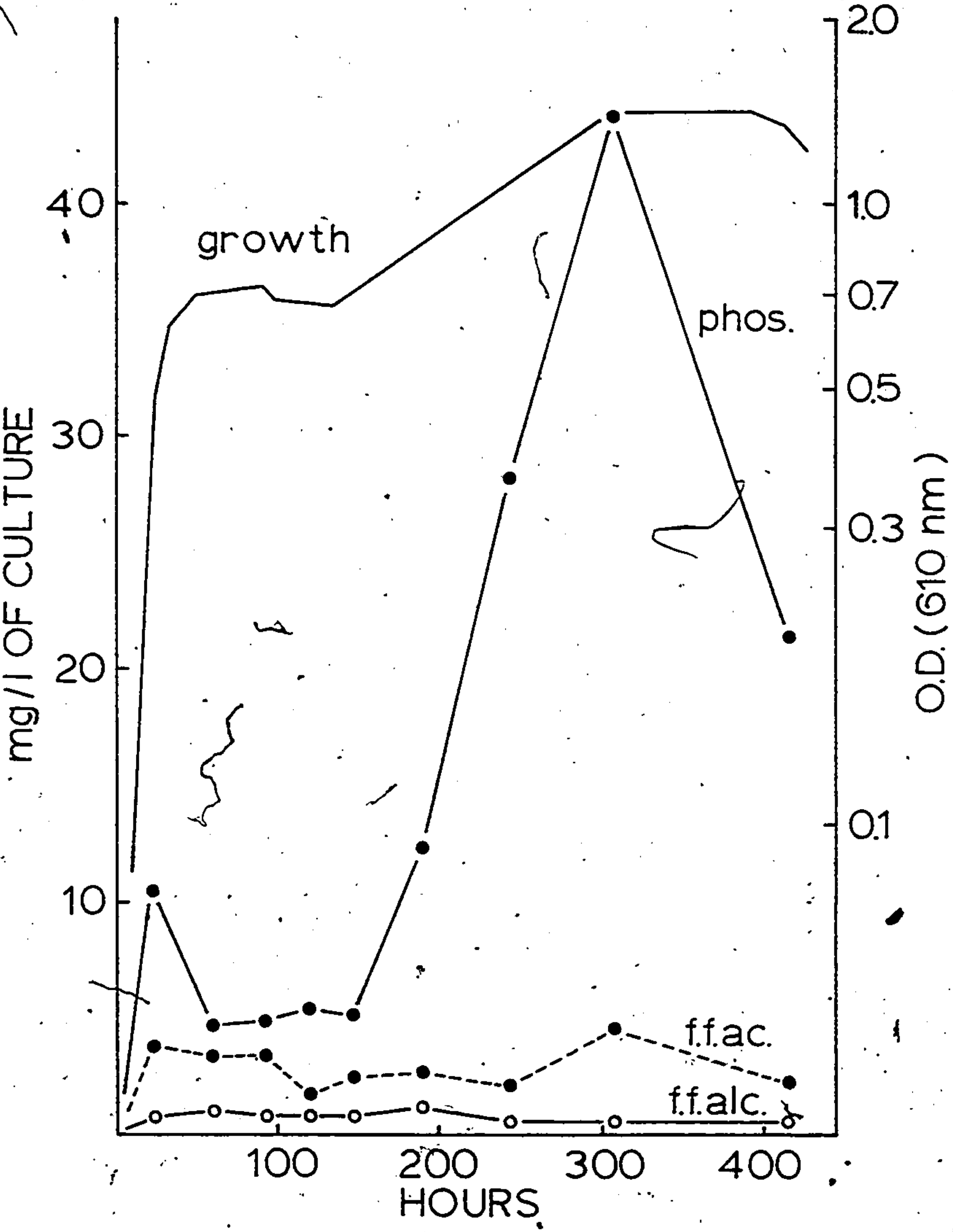

growth
phos.
tri.
di.
mono.
mg/l OF CULTURE
10
20
30
40
O.D. (610 nm)
2.0
1.0
0.7
0.5
0.3
0.1
100
200
300
400
HOURS

Figure 5. Changes in the amounts of different lipid components present in the whole culture during diauxie.

Phospholipids (●——●), free fatty acids (●----●), and free fatty alcohol (○——○).

Growth curve represented for reference.


growth
phos.
f.f.ac.
f.f.alc.
mg/l OF CULTURE
10
20
30
40
O.D. (610 nm)
0.1
0.3
0.5
0.7
1.0
2.0
100
200
300
400
HOURS

4. Problems of defining extracellular lipids for hydrocarbon-degrading microorganisms.

a) Separation of cells from the unutilized hydrocarbon.

When cultures of hydrocarbon-degrading microorganisms are centrifuged, a scum forms at the surface of the supernatant which consists mostly of the unused substrate. The following experiment showed that free lipids, being insoluble in water and miscible in hexadecane, also accumulate in this layer. To 10 ml of the mineral salts medium, containing 0.1 ml of hexadecene, were added 10 mg of tripalmitin and 1.0 mg of phosphatidylcholine. This mixture was sonicated to obtain a fine emulsion, and then centrifuged for 30 min. at 23,300 g at 4°C. At this temperature, hexadecane is frozen and forms a crust which is easily separated from the aqueous phase of the supernatant. Only about 8% of the lipid fatty acids were detected in the aqueous phase while the rest remained associated with the hexadecane.

In addition, cells of oil-degrading microorganisms are known to bind, with considerable affinity, to globules of hydrocarbon in emulsion (Chapter I, part 7). Different types of filters which retain bacteria were found to also retain the hydrocarbon globules, and glass filters that would yield an appreciable fraction of the hexadecane in the filtrate, also permitted cells to go through.

A method used to overcome the problem of separating the cells from the unutilized hydrocarbon is to wash the cells with organic solvents such as hexane (Walker and Cooney, 1973 b; Siporin and Cooney, 1975). This prodecudre was not used in the present study because some lipids could possibly be extracted from the cells. Detergents could also be used, but these can also extract material from the cells. The lipase of *A. lwoffi*, for example, was shown to be released into the extracellular medium by treating the cells with Triton X_{100} (Breuil *et al*. 1978 a).

Centrifugation at relatively high speed and above the freezing point of hexadecane was found, in preliminary experiments, to increase the size of the pellet obtained. Cells were thus harvested by centrifugation at 23,300 x g (the maximum acceleration our Sorvall centrifuge can maintain) for 30 min. at 20°C. This centrifugation procedure was used during this project except when specified otherwise. At 20°C, the supernatant consists of two liquid phases which are difficult to separate. The supernatant as a whole was thus used for the analyses.

Under these conditions most of the hexadecane remained in the supernatant fraction. Washing of the pellets with the mineral salts solution used for growth medium reduced the amount of hexadecane remaining in the pellet fraction. The hexadecane removed by washing was probably in large part adhering to the wall of the centrifuge tube. Only two washings were done for each sample since only trace amounts of hexadecane were removed from the pellet fraction with a third washing. Using this procedure, about 90% of the unutilized hydrocarbon was recuperated in the combined supernatant and washes fraction. The hexadecane remaining associated with the precipitated cells could be present inside the cells in the form of intracellular inclusions (Finnerty *et al*. 1973) or on the cell surface in the form of small hydrocarbon globules (Einsele et al., 1975). Although some extracellular lipid could probably be present in these hydrocarbon globules, most of it probably remains in the supernatant hexadecane phase.

A large proportion (38 to 75%) of the total lipids was present in the whole supernatant fraction after centrifugation, but not all supernatant lipids can be considered as extracellular since as much as 10 to 40% of the cells from the whole culture did not sediment (Table 9).

b) Comparison of pellet and supernatant lipid compositions.

Since the cells could not be separated from the unutilized hexadecane without using techniques which could extract material from the cells, the extracellular lipids could not be measured directly. If some lipid class(es) accumulated in the extracellular medium in appreciable amounts, however, a comparison of the lipid compositions of the pellet and of the whole supernatant (containing both the aqueous and hydrocarbon phases) should reveal an excess of this material in the supernatant since most of the hexadecane remains in this fraction.

The percentage of the amount of total pellet lipids was calculated for each lipid class and compared with the percentage of total supernatant lipids of the same lipid class (Figures 6 to 12). The proportion of the phospholipids (Fig. 6) decreased rapidly in both the pellet and the supernatant as the cells from the exponential phase of growth on ethanol entered stationary phase. During the lag phase separating the two growth cycles, the percentage of the phospholipids appears to have increased slightly with rather large fluctuations

Table 9. Comparison of the amounts of total lipid and numbers of cells found in the pellet and whole supernatant fractions during diauxie.

		Total lipid[1]		Cell counts[2]	
Time (h.)	O.D. (610 nm)	Pellet	Supernatant[3]	Pellet	Supernatant[3]
24	0.530	14.7	13.7	3.1×10^8	1.7×10^8
57	0.680	9.7	10.4	3.0×10^8	1.8×10^8
94	0.650	8.6	13.1	1.8×10^8	1.3×10^8
122	0.620	8.8	7.0	6.4×10^8	7.3×10^7
145	0.690	11.2	6.9	5.4×10^8	6.7×10^7
190	0.820	15.9	10.9	7.6×10^8	1.5×10^8
248	1.100	11.5	33.6	7.5×10^8	4.5×10^8
306	1.390	35.9	26.2	1.4×10^9	3.1×10^8
411	1.300	14.1	22.4	9.4×10^8	5.6×10^8

1. Values shown as total weight of fatty acids and fatty alcohols (in mg) per liter of culture.
2. Counts represent numbers per ml of original whole culture and supernatant. Counts of the supernatant fractions were corrected for dilution by the added washing solution.
3. Including aqueous and hexadecane phases of the supernatant.

Figure 6. Comparison of the proportions of total pellet lipids and total supernatant lipids found in the Phospholipid fraction during diauxie.

Figures shown as % of total fatty acids and fatty alcohols.

(●——●), pellets (○——○), supernatants.

Growth curve represented for reference.

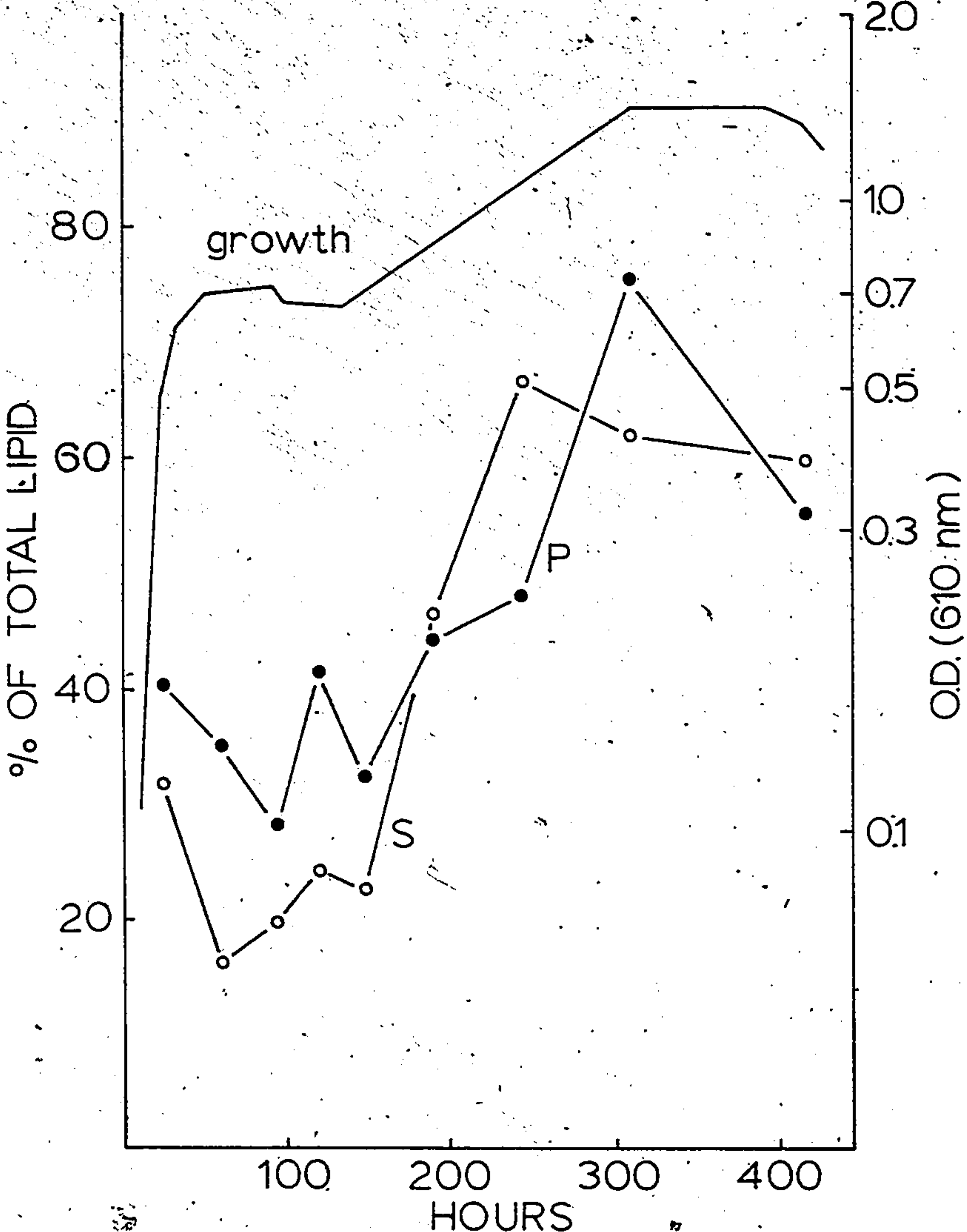

growth
P
S
% OF TOTAL LIPID
80
60
40
20
O.D. (610 nm)
2.0
1.0
0.7
0.5
0.3
0.1
100
200
300
400
HOURS

Figure 7. Comparison of the proportions of total pellet lipids and total supernatant lipids found in the triglyceride fraction during diauxie.

Figures shown as % of total fatty acids and fatty alcohols.

(● —— ●), pellets, (o —— o), supernatants.

Growth curve represented for reference.

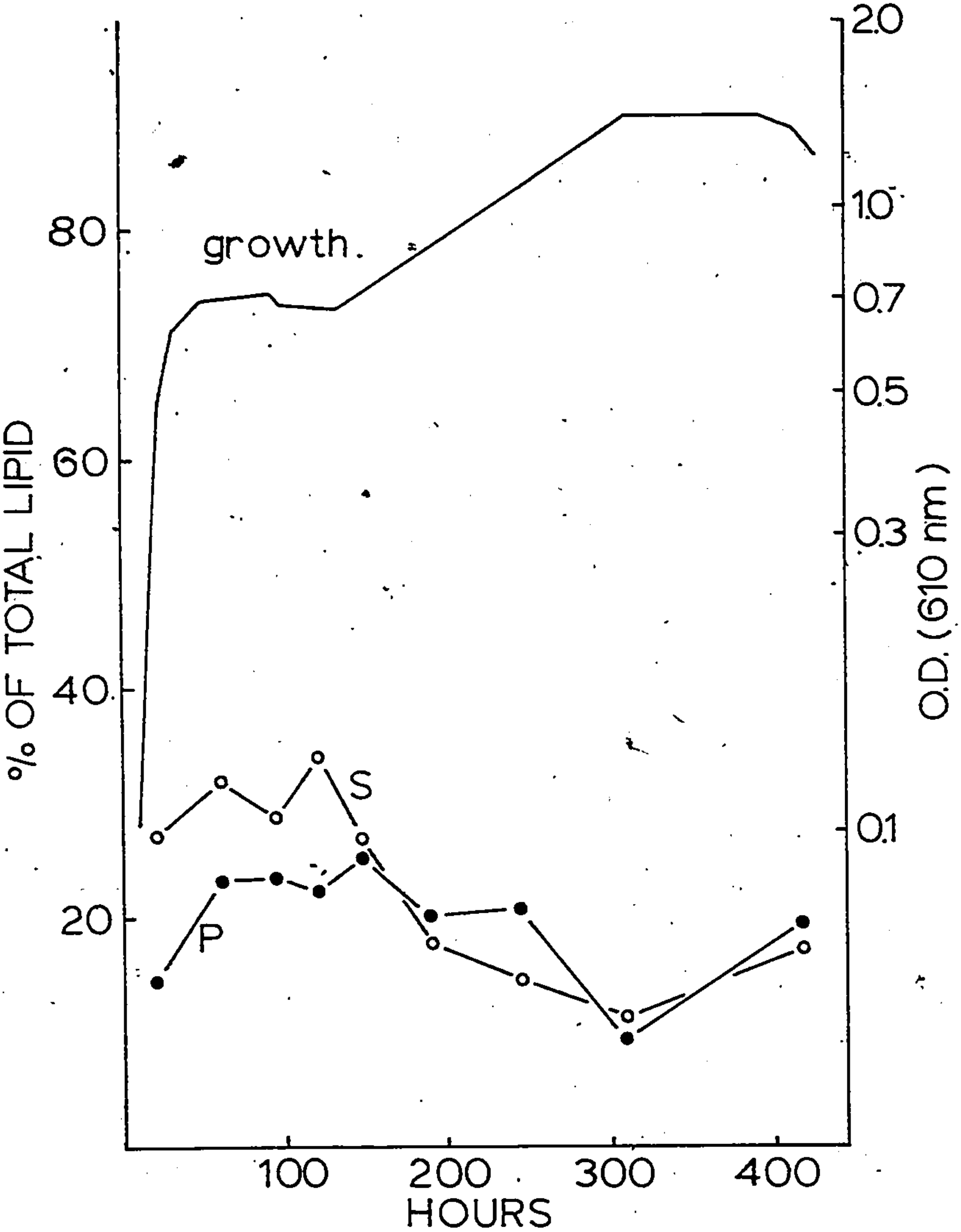

% OF TOTAL LIPID
80
60
40
20
O.D. (610 nm)
2.0
1.0
0.7
0.5
0.3
0.1
growth.
S
P
100
200
300
400
HOURS

Figure 8. Comparison of the proportions of total pellet lipids and total supernatant lipids found in the Diglyceride fraction during diauxie.

Figures shown as % of total fatty acids and fatty alcohols.

(●——●), pellets, (○——○), supernatants.

Growth curve represented for reference.

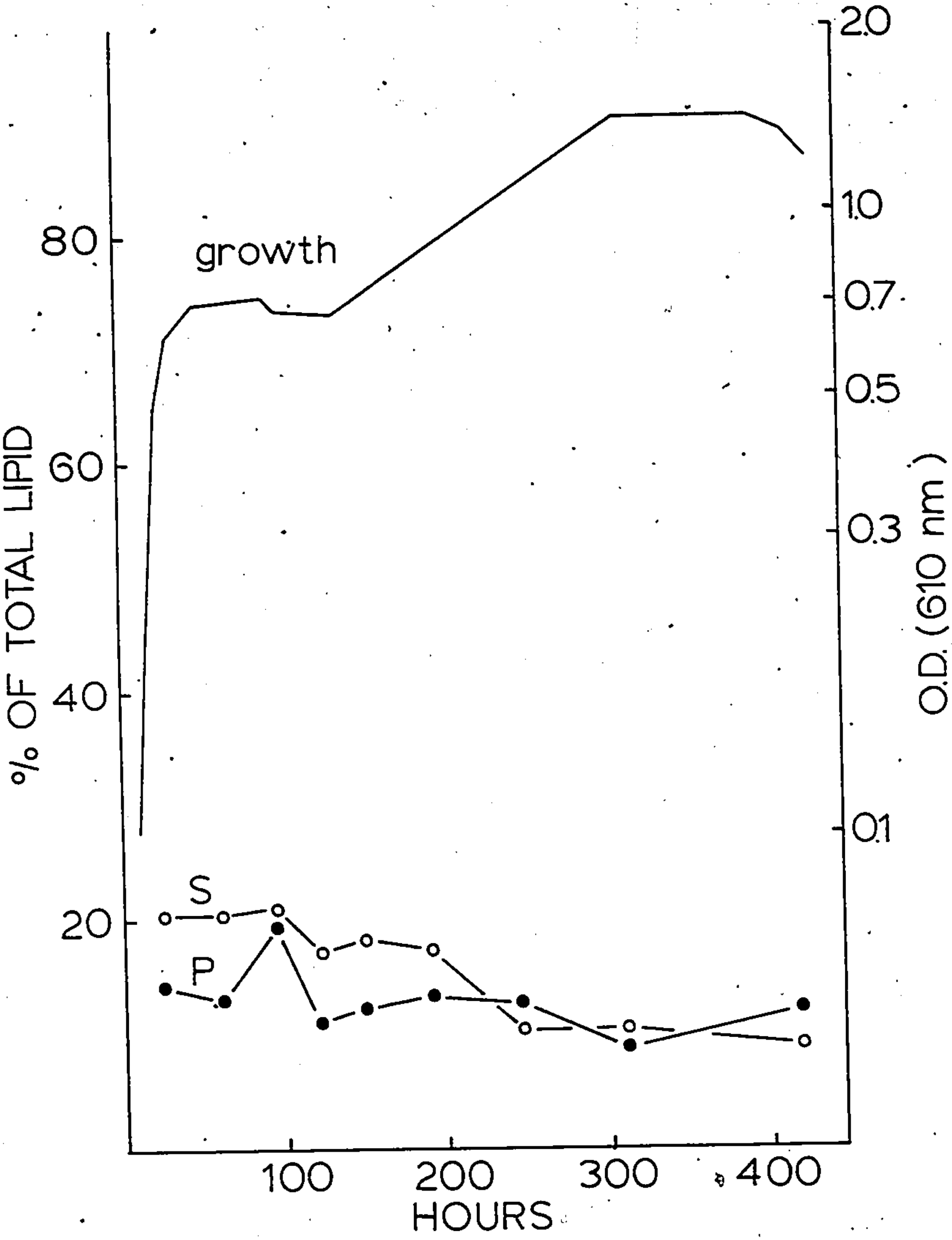
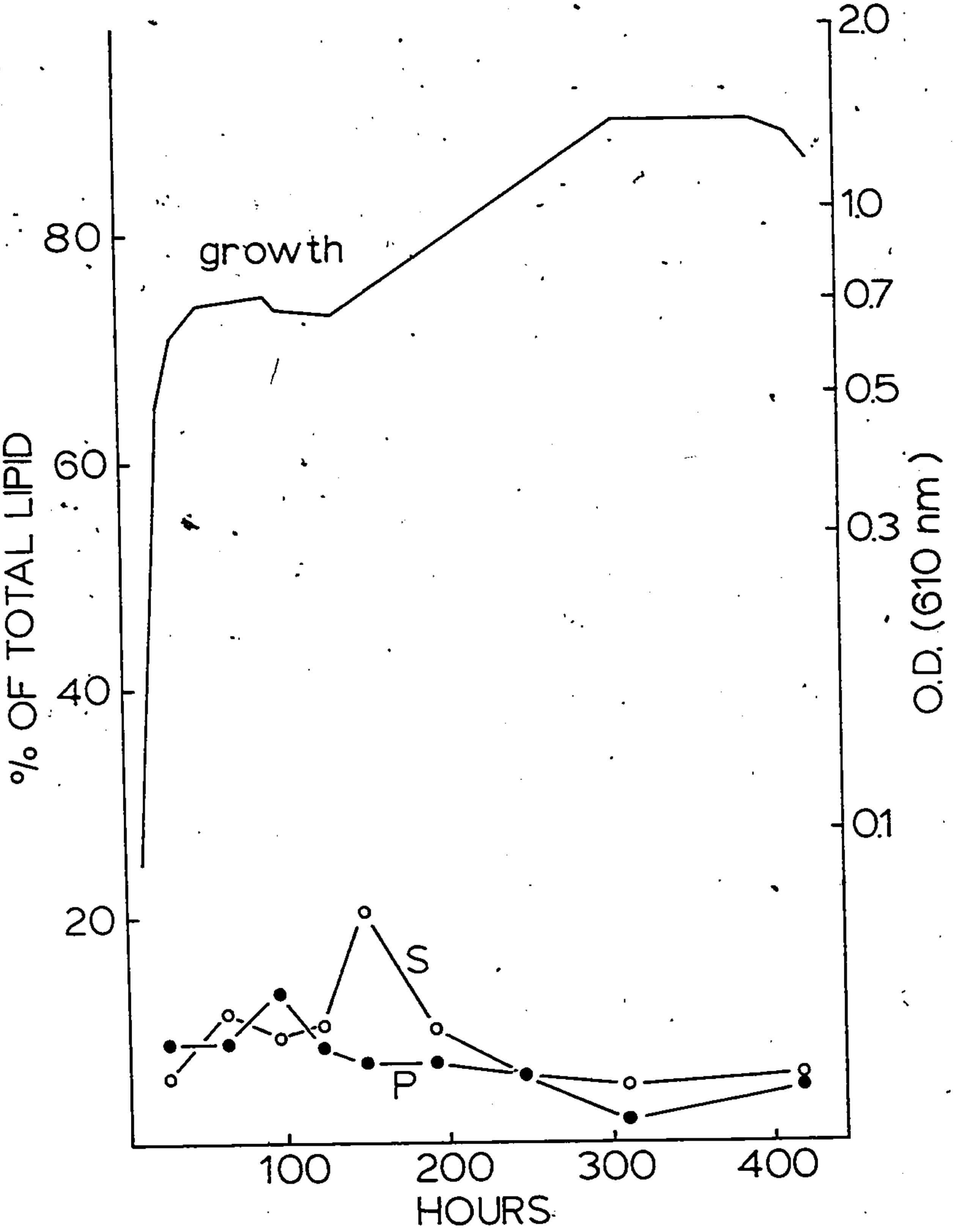

growth
S
P
% OF TOTAL LIPID
80
60
40
20
O.D. (610 nm)
2.0
1.0
0.7
0.5
0.3
0.1
100
200
300
400
HOURS

Figure 9. Comparison of the proportions of total pellet lipids and total supernatant lipids found in the Monoglyceride fraction during diauxie.

Figures shown as % of total fatty acids and fatty alcohols.

(●——●), pellets, (○——○), supernatants.

Growth curve represented for reference.



% OF TOTAL LIPID
80
60
40
20
growth
S
P
100
200
300
400
HOURS
2.0
1.0
0.7
0.5
0.3
0.1
O.D. (610 nm)

Figure 10. Comparison of the proportions of total pellet lipids and total supernatant lipids found in the Free fatty acid fraction during diauxie.

Figures shown as % of total fatty acids and fatty alcohols.

(●——●), pellets, (○——○), supernatants.

Growth curve represented for reference.

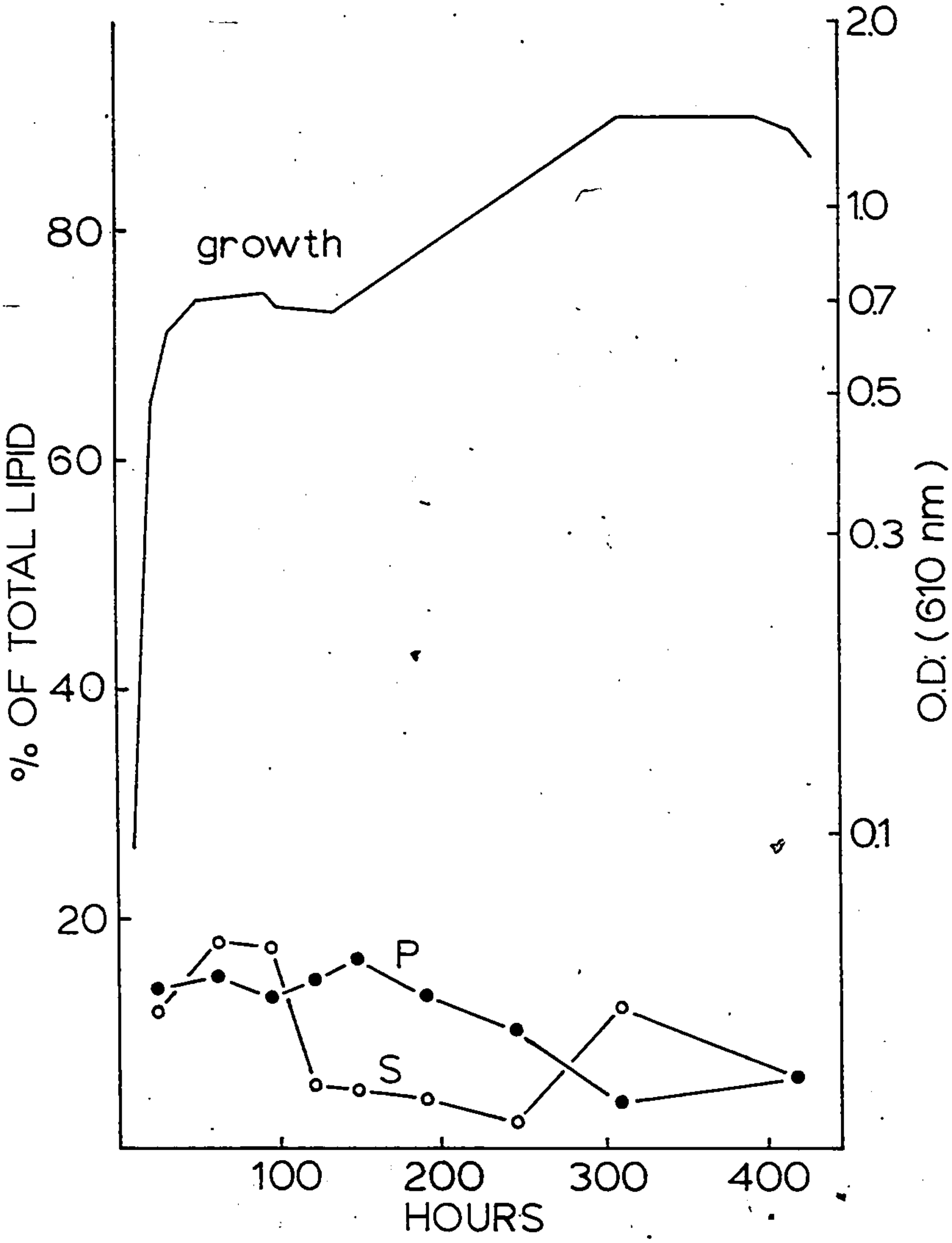

growth
P
S
% OF TOTAL LIPID
80
60
40
20
O.D. (610 nm)
2.0
1.0
0.7
0.5
0.3
0.1
100
200
300
400
HOURS

Figure 11. Comparison of the proportions of total pellet lipids and total supernatant lipids found in the Free fatty alcohol fraction during diauxie.

Figures shown as % of total fatty acids and fatty alcohols.

(●—●), pellets, (○——○), supernatants.

Growth curve represented for reference.

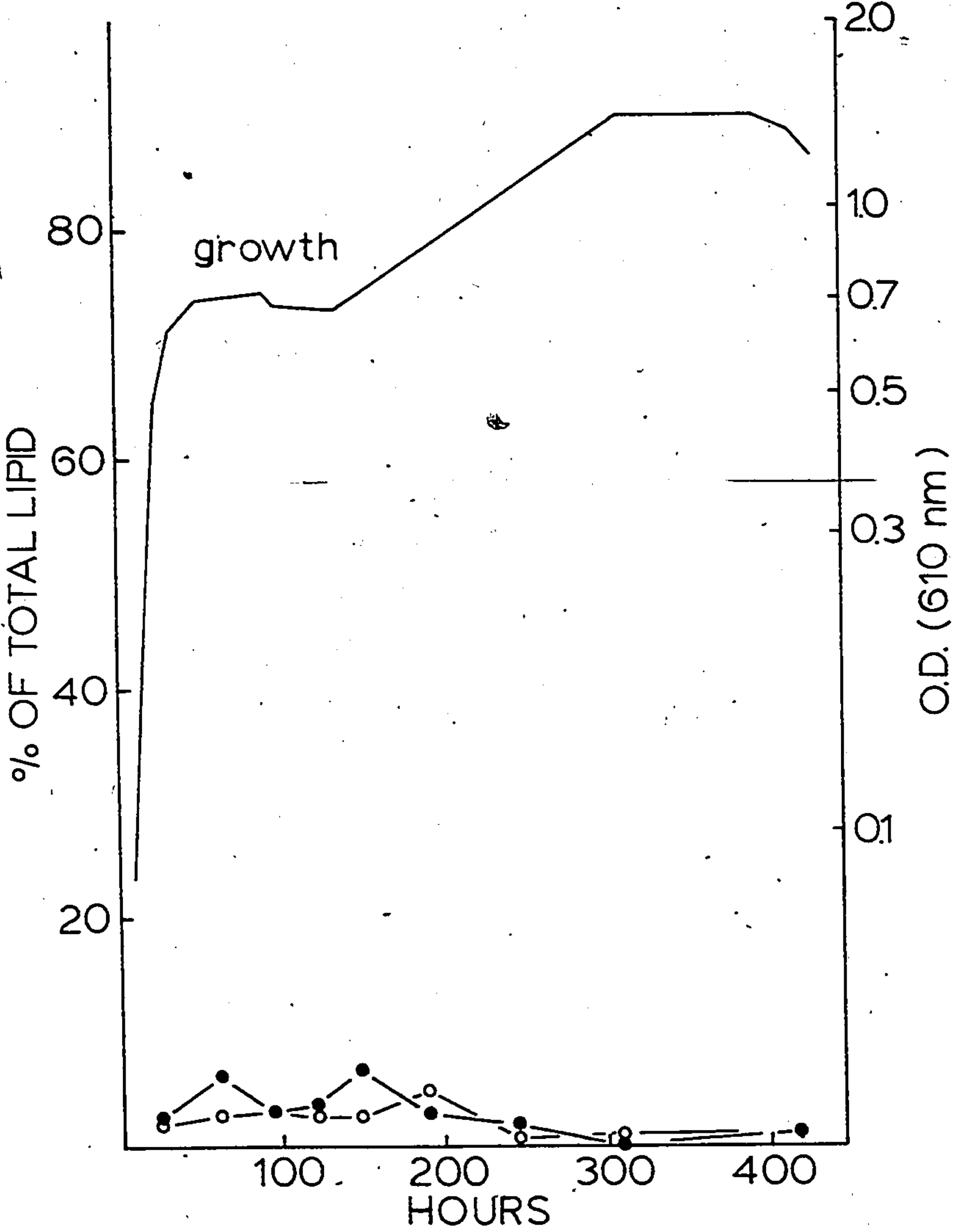
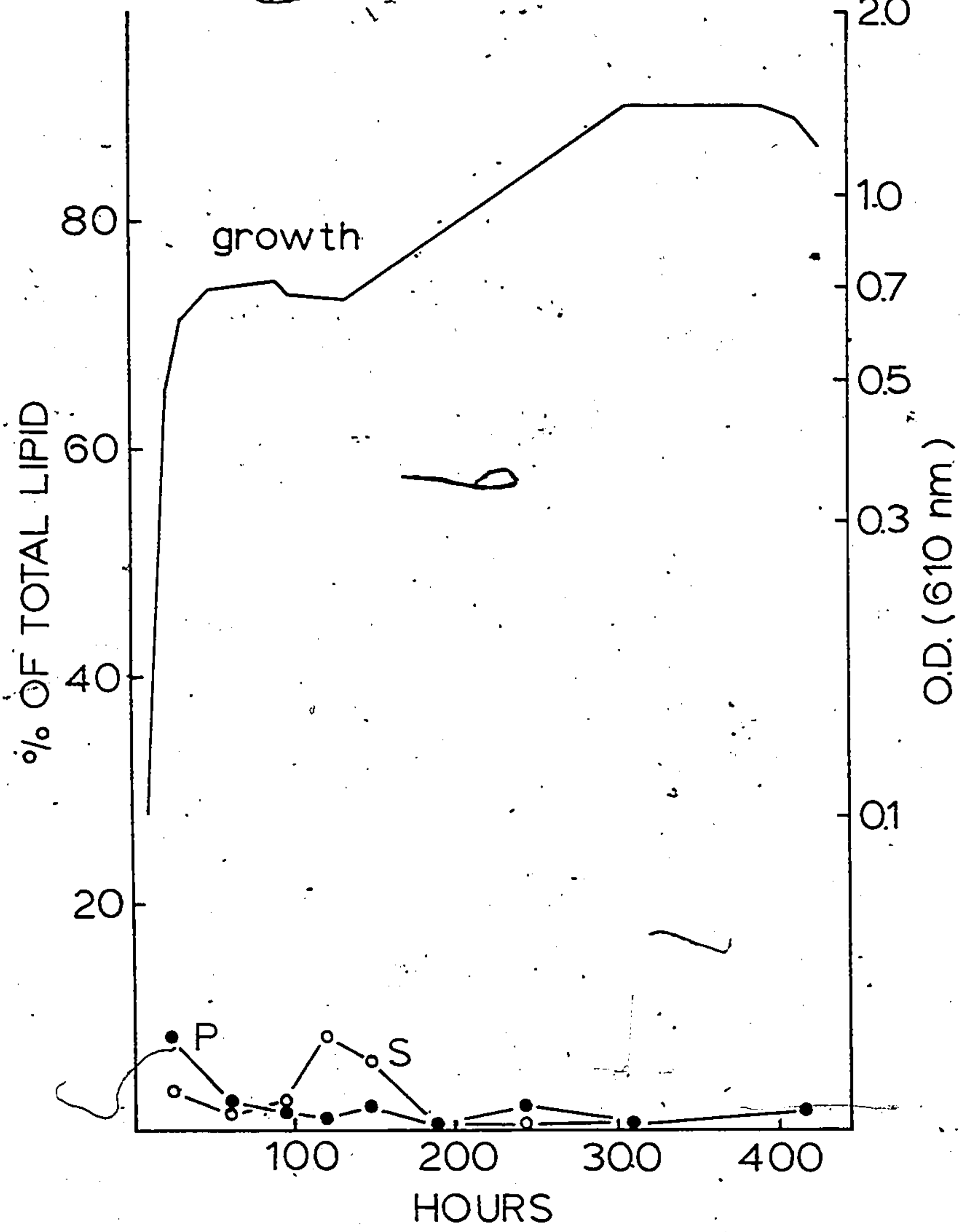

growth
% OF TOTAL LIPID
80
60
40
20
O.D. (610 nm)
2.0
1.0
0.7
0.5
0.3
0.1
100
200
300
400
HOURS

Figure 12. Comparison of the proportions of total pellet lipids and total supernatant lipids found in the Wax ester fraction during diauxie.

Figures shown as % of total fatty acids and alcohols.

(●——●), pellets, (o——o), supernatants.

Growth curve represented for reference.


growth
P
S
% OF TOTAL LIPID
80
60
40
20
O.D. (610 nm)
2.0
1.0
0.7
0.5
0.3
0.1
100
200
300
400
HOURS

in the pellet. When the bacterium metabolized the hexadecane, the proportion of phospholipids increased rapidly. This increase coincided with the rapid increase in the total amount of phospholipids and with the rapid decrease in the average chain length of phospholipid fatty acids noted above. During the stationary phase of the second growth cycle, the proportion of the phospholipids decreased, especially in the pellet fraction. During diauxie, phospholipids represent a higher proportion of total supernatant lipids than of total pellet lipids only near the middle of the exponential phase of growth on hexadecane. In the other stages of the growth curve, similar or smaller proportions of phospholipids were found in the supernatant than in the pellet.

The proportion of triglycerides (Fig. 7) increased in both the pellet and the supernatant at the beginning of stationary phase of growth on ethanol. During the lag phase separating the two growth cycles, triglycerides remained relatively constant in the pellet, but fluctuated somewhat in the supernatant. During growth on hexadecane, the proportion of triglycerides decreased gradually before increasing again during the stationary phase of growth on hexadecane. During diauxie, triglycerides represent a higher proportion of total supernatant lipids than of total pellet lipids during the period preceding growth on hexadecane.

Diglycerides (Fig. 8) represent a higher proportion of the lipids of the supernatant than of those of the pellet until near mid-exponential phase of growth on hexadecane. Monoglycerides (Fig. 9) were found in similar proportions in the pellet and supernatant fractions except during a relatively brief period near the onset of growth on hexadecane when the proportion of this lipid was higher in the supernatant.

The free fatty acids (Fig. 10) were proportionnally more abundant in the pellet fraction, during diauxie, except during part of the lag phase separating the two growth cycles and at the end of the exponential phase of growth on hexadecane. Alcohols (Fig. 11) and wax esters (Fig. 12) represented a small proportion of the total lipids of both the pellet and the supernatant fractions. The proportion of wax was higher in the supernatant than in the pellet during a relatively brief period near the onset of growth on hexadecane.

In general, the lipid compositions of the pellets and supernatants were relatively similar. The differences mentioned above suggest that some lipids which were proportionnally more abundant in the supernatant fraction could be extracellular. This interpretation is based on the assumption that, on the average, the cells that remain trapped in the hexadecane phase of the supernatant during centrifugation have the same lipid composition as the cells that sediment. This is not necessarily true, however, since the proportion of any given class of lipids was often larger in the pellet than in the supernatant fractions.

Extracellular lipids could be present in the cultures in the same proportions as they are in the cells. This could possibly occur if an important number of cells would lyse. The lipids thus liberated would be extracellular since they would no longer be associated with intact and living cells.

Estimations of the protein content of the pellet and supernatant fractions show that a large proportion of the proteins remained in suspension upon centrifugation (Table 10). A good part of these supernatant proteins, however, must be associated with the cells contained in this fraction which were trapped in the hexadecane phase (Table 9). As was mentioned earlier, microbiological filters retained, in addition to the cells, most of the unutilized hydrocarbon. The filtrate consisted then of the aqueous phase of the supernatant and contained the water soluble proteins that would be liberated from the cells if these had lysed. The amount of protein found in the aqueous phase of the supernatant, obtained by filtration, was very small, suggesting that lysis of an important number of cells had not occured (Table 10).

To further test the possibility of cell lysis, an experiment was done in which cultures were centrifuged at 23,000 g for 30 min. at 4°C so that the hexadecane was frozen and the aqueous phase of the supernatant could be recuperated easily. The concentration of protein found in this fraction was compared with the concentration of protein present in the whole supernatant (including both hexadecane and water phases) and in the whole culture (Table 11). Again, the amount of water soluble protein was small during diauxic growth.

It was demonstrated, using bovine serum albumin mixed by sonification with 1% hexadecane in the mineral salts medium, that water soluble proteins are recuperated entirely in the aqueous phase after

Table 10. Comparison of the amounts of protein found in the pellets, supernatants, and in samples of filtered supernatant.[1]

Time (h.)	O.D. (610 nm)	Pellet	Supernatant[1]	Filtered supernatant[2]
24	0.530	210	210	12
57	0.680	222	154	12
94	0.650	230	126	18
122	0.620	250	129	18
145	0.690	276	126	18
190	0.820	412	196	18
248	1.100	375	840	20
306	1.390	875	588	35
411	1.300	700	700	42

[1] Values shown as ng/ml of original culture. Supernatant values were corrected for dilution by the added washing solution.

[2] The supernatant was shaken vigorously and filtered through a 0.45 nm pore size Millipore filter membrane.

Table 11. Comparison between the amounts of protein found in the aqueous phase of the supernatant, in the whole supernatant, and in the whole culture, during diauxie.[1]

Time	Whole culture	Supernatant	
		Aqueous phase[2]	Whole[3]
24	130	26	142
48	372	30	146
97	380	39	146
143	380	40	140
192	695	46	255
241	927	54	560
300	1340	52	690

1. Values shown as ng of protein per ml.
2. Taken directly from the centrifuge tube with a Pasteur pipette after centrifugation at 23,300 g for 30 min. at 4°C.
3. Including aqueous and hexadecane phases of the supernatant.

centrifugation under the conditions used for these experiments.

c) Estimations of extracellular lipids.

The extracellular lipids (L_E) could be calculated from the amounts of lipid present in the pellets (L_P) and in the whole supernatants (L_S) and measurements of the relative number of cells present in the pellet (C_P) and supernatant (C_S) fractions using the following equation:

$$L_E = L_S - \left[L_P \ (C_S/C_P) \right] .$$

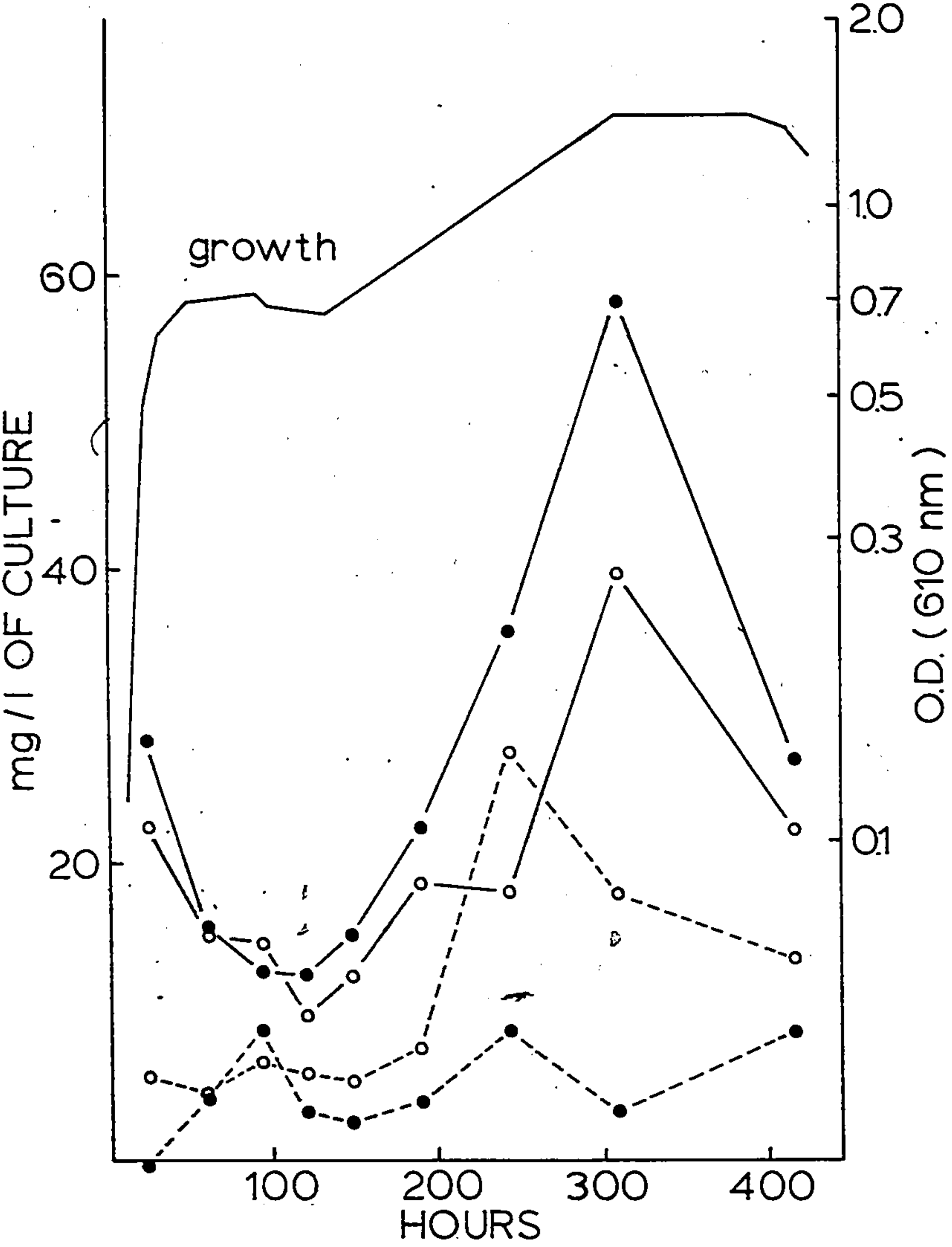
Since the supernatant contained a large number of cells, these calculations give the amount of lipid present in the supernatant which is not accounted for by the cells present in this fraction.

Cell counts (Table 9) or protein estimates (Table 10) were used for the measurements of the amount of cells present in the pellets and supernatants. Since the amount of water soluble proteins was small during diauxie (Table 10), it appears that extracellular proteins are present only in small quantities. The total amounts of lipid calculated as being extracellular during diauxie are shown in Figure 13. These are compared with the total amounts of lipid calculated as being cellular by subtracting the value obtained for extracellular lipids from the amounts present in the whole culture (Fig. 13). The amounts of lipid calculated as extracellular for each lipid class using cell counts are

Figure 13. Total amounts of lipid calculated as being cellular and extracellular during diauxie.

(●——●), cellular lipids calculated using protein content, (●----●), extracellular lipids calculated using protein content, (o——o), cellular lipids calculated using cell-counts, (o---o), extracellular lipids calculated using cell counts.

Growth curve represented for reference.


growth
mg/l OF CULTURE
60
40
20
O.D. (610 nm)
2.0
1.0
0.7
0.5
0.3
0.1
100
200
300
400
HOURS

shown in Table 12; those calculated using protein content are shown in Table 13. The amount of calculated extracellular lipids is generally higher for calculations based on protein content especially during the hexadecane phase of diauxie. In these tables, a number of values were calculated as negative, the amount of supernatant lipids being more than accounted for by the number of cells or the amount of protein present in the supernatant. More negative values are obtained with the calculations based on protein content than with those based on cell counts.

During the first growth cycle of diauxie, hexadecane is not utilized. The lipids produced during growth on ethanol alone should be comparable to those produced during the ethanol phase of diauxie. Since the hydrocarbon is absent, it cannot interfere with the sedimentation of the cells and supernatant lipids are, in this case, truly extracellular. A. lwoffi was grown on 0.1% ethanol until early stationary phase under the conditions used for diauxie experiments except that hexadecane was absent. The cells were centrifuged and total lipids were measured in the pellet and supernatant. The whole culture contained 24.4 mg/l total fatty acids while the supernatant contained 4.2 mg/l or 17.2% of the total fatty acids of the culture. A similar proportion of extracellular lipids was found for midexponential phase cultures of A. lwoffi grown on 1.0% ethanol (30.7 mg/l in the whole culture and 6.3 mg/l or 20.5% of the total, in the supernatant). Ethanol grown cells thus produce

Table 12. Changes in the amounts of lipids calculated as extracellular using cell counts during diauxie.[1]

	Ethanol phase		Lag phase		Hexadecane phase				
Time (h.)	24	57	94	122	145	190	248	306	411
O.D. (610 nm)	.530	.680	.650	.620	.690	.820	1.100	1.390	1.300
phospholipids	1.0	-0.2	0.4	1.3	1.1	3.5	19.0	10.1	8.7
monoglycerides	0.1	0.6	0.2	0.6	1.3	0.8	1.5	1.1	0.9
diglycerides	1.6	1.5	1.1	1.1	1.1	1.4	2.6	2.0	1.0
triglycerides	2.5	1.8	3.6	2.1	1.5	1.6	3.4	1.9	2.1
free fatty acids	0.4	0.9	1.1	0.2	0.1	0.1	1.2	2.8	0.8
alcohols	0.1	-0.1	0.1	0.1	0.1	0.4	0.1	0.3	0.3
wax esters	-0.1	0.0	0.3	0.6	0.4	0.0	0.2	0.1	0.3
Total	5.6	4.5	6.8	6.0	5.6	7.8	28.0	18.3	14.1

[1] Values shown as mg of lipid per 1 of culture.

Table 13. Changes in the amounts of lipids calculated as extracellular using protein content during diauxie.[1]

	Ethanol phase		Lag phase		Hexadecane phase				
Time (h.)	24	57	94	122	145	190	248	306	411
O.D. (610 nm)	.530	.680	.650	.620	.690	.820	1.100	1.390	1.300
phospholipids	-1.3	-0.4	1.1	0.1	0.2	1.8	10.2	-1.0	6.0
monoglycerides	-0.4	0.6	0.5	0.4	1.1	0.6	0.5	0.8	0.6
diglycerides	0.8	1.4	1.5	0.8	0.7	0.9	0.3	0.7	0.4
triglycerides	1.6	1.7	4.1	1.5	0.7	0.9	-0.3	0.5	1.2
free fatty acids	-0.3	0.8	1.4	-0.2	-0.4	-0.4	-0.8	2.2	0.5
alcohols	-0.1	-0.1	0.2	0.0	-0.1	0.3	-0.3	0.2	0.2
wax esters	-0.6	0.0	0.3	0.6	0.3	0.0	-0.2	0.0	0.1
Total	-0.3	4.0	9.1	3.2	2.5	4.1	9.4	3.4	9.0

[1] Values shown as mg of lipid per l of culture.

relatively large amounts of extracellular lipids. These amounts are comparable to those calculated as extracellular for early stationary phase of the ethanol growth cycle of diauxie using either cell counts or protein content.

d) Problems of measuring cell mass in the presence of hydrocarbon.

The results of extracellular lipid estimates, presented in the previous section, depend on the method used for estimating the proportion of cells remaining in the supernatant after centrifugation. The accuracy of extracellular lipid measurements thus depends on the accuracy of cell mass estimates.

Cell counts, especially of the supernatant samples, probably yield underestimates since many cells adhere to a hydrocarbon globule and form a single colony. This problem was recognized by Raymond and Davis (1960). Extensive shaking of the culture probably increases the numbers obtained by breaking the hydrocarbon phase into small globules but can probably not eliminate this source of error. Direct cell counts, under the microscope, are also inaccurate because some small hydrocarbon globules in the emulsion are difficult to distinguish from the cells, even using phase contrast microscopy. Large hydrocarbon globules, also, probably prevent some of the cells from being seen during microscopic observations.

Protein estimates probably give more accurate results since a larger number of cells is taken for the measurements. Furthermore, the samples are not diluted, as for cell count measurements, before the actual assay is performed. The presence of hexadecane in the assay system did not interfere with measurements of known amounts of protein (Breuil et al., 1978 b). A strong correlation between increase of protein and O.D. has been demonstrated for cultures of A. lwoffi with O.D. values above 0.4 (Breuil et al., 1978 a).

These considerations are consistent with the results obtained in the present study. The proportions of total protein and cells from the whole culture that remained in the supernatant upon centrifugation are lower than the proportion of total lipids found in the supernatant (Table 14). While the proportion of proteins remaining in the supernatant is relatively close to that of the lipids, the proportion of cells counted in the supernatant fraction is much lower.

Table 14. Comparison of the proportions of total lipids, total proteins, and cell numbers found in the supernatant fraction during diauxie.[1]

Time (h.)	O.D. (610 nm)	Total lipids	Total protein	Cell numbers
24	0.530	48	50	35
57	0.680	52	41	38
84	0.650	60	35	42
122	0.620	44	34	10
145	0.690	38	31	11
190	0.820	41	32	16
248	1.100	75	69	38
306	1.390	42	40	18
411	1.300	61	50	37

[1] Values shown as percentage of material present in the whole culture, found in the supernatant fraction after centrifugation.

CHAPTER IV GENERAL DISCUSSION

1. Extracellular lipids.

Extracellular emulsifying agents are thought to be involved in the microbial uptake of hydrocarbon molecules (Chapter I, part 7). Most of these compounds that have been characterized are lipids or contain lipid moieties as was described earlier. In most studies, however, these compounds were assumed to be at least in part extracellular and little effort has been devoted to demonstrating their extracellular location or to estimating the amount present in the extracellular medium.

Some authors (Klug and Markovetz, 1967; Volfova and Pecka, 1973) were mainly interested in characterizing possible intermediates in the pathway for the oxidation of hydrocarbons while others were concerned with the demonstration of the emulsifying properties of compounds produced by microorganisms growing on hydrocarbons. Since the emulsifying compounds are most likely associated with the oil phase of the culture, this fraction (Suzuki et al., 1969; Cooper et al., 1979 a, b) or the whole supernatant (Igushi et al., 1969; Rosenberg et al., 1979, a, b; Zuckerberg et al., 1979; Itoh et al., 1971; Zajic et al., 1977 a, b) were taken for the analyses. In other cases, emulsifying compounds were isolated from the cell pellet (Hisatsuka et al., 1971) or from the supernatant obtained after treating the cells with EDTA (Hisatsuka et al., 1975). In all these studies, at least part of the material analyzed was probably extracted from the cells.

In some studies (Kappeli and Finnerty, 1979; Roy _et al_., 1979), the aqueous phase of the supernatant was obtained by filtration and used as a source of the compounds studied. In these cases, the substances analyzed were truly extracellular, but no estimate of the amount present in the extracellular medium could be done since the amounts of emulsifying agents associated with large hydrocarbon globules remaining on the filter were not taken into account.

Because of the difficulty encountered in separating the cells of _Acinetobacter lwoffi_ from the unutilized hydrocarbon of the cultures, it was impossible to measure directly the amount of lipids present in the extracellular medium. It is somewhat surprising that cultures of _Acinetobacter_ sp. H01-N (Finnerty _et al_., 1962 a), a closely related bacterium growing on hexadecane, could apparently be sedimented completely upon centrifugation at 25,000 g for 15 min. at 25°C, since it was reported that no phospholipid was detected in the supernatant fraction (Finnerty _et al_., 1973; Makula _et al_., 1975). In contrast, in this work, hardly more than half of the cells were found in the pellet fraction when cultures of _A. lwoffi_ were centrifuged at 23,000 g for 30 min. at 20°C. In both studies, the supernatant was the culture broth remaining after centrifugation and separation from the pellet and, thus, contained both the aqueous and hydrocarbon phases.

In fact, relatively large amounts of phospholipids were found recently in the filtrate of the supernatant of cultures of _Acinetobacter_ sp.H01-N grown on hexadecane (Kappeli and Finnerty, 1979). Phospholipids thus had to be present in the supernatant and at least part of the lipids reported as extracellular by Finnerty et al., (1973) and by Makula et al. (1975) could have been extracted from cells or have been liberated because of cell lysis.

Hydrocarbon degrading microorganisms are well known to bind quite strongly to hydrocarbon globules in the culture (Mimura et al., 1971 b; Mc Lee and Davis; 1972; Finnerty et al., 1973; Cundell et al., 1976; Miura et al., 1977; Gutnick and Rosenberg, 1977). The proportion of *A. lwoffi* cells bound to large hexadecane globules which do not sediment was estimated to about 40% of the total number present in the whole culture. This value is relatively smaller than that of 70% reported for *Candida lipolytica* cultures (Nakahara et al., 1977). In both of these studies relatively large fluctuations were observed in the proportion of cells remaining in the hydrocarbon phase of the supernatant during any of the growth stages. In contrast, cells of *Acinetobacter calcoaceticus* had binding affinities which were characteristic of different parts of the growth curve. All cells were associated with the hydrocarbon phase until midexponential phase of growth. The proportion of cells bound to the hydrocarbon then decreased rapidly during the second half of exponential growth. During stationary phase, the proportion of bound cells remained constant at about 20% of the total number of cells present in the whole culture (Neufeld et al., 1980). The binding force can thus differ even for two relatively closely related microorganisms, as was observed for *Candida tropicalis* and *Candida intermedia* (Miura et al., 1977).

If lipids were somehow secreted from the cells, the composition of extracellular lipids should probably be different from that of the cellular lipids. Accordingly, the pellets and supernatants of Acinetobacter sp. HO1-N contained different proportions of the various lipid classes (Finnerty et al., 1973; Makula et al., 1975). Hexane extracts of cultures of Cladosporium resinae contained lipids having a much higher proportion of lauric acid than cellular lipids (Siporin and Cooney, 1975). A. lwoffi is known to possess a strong lipase activity when growing on hydrocarbon, and most of this activity can be assayed without breaking the cells (Breuil et al., 1978 a). The composition of extracellular lipids would thus be further modified by this enzyme. The lipid composition of the pellet and supernatant fractions of cultures of A. lwoffi were relatively similar, however, so that no accumulation of large amounts of any single lipid class in the extracellular medium is apparent during diauxie.

The supernatant of cultures grown on ethanol, in the absence of hydrocarbon, was found to contain relatively high amounts of extracellular lipids. The proportion of whole culture lipids found in the supernatant of ethanol grown cultures was similar to that estimated for extracellular lipids during the ethanol phase of diauxie. These estimates were calculated from the amounts of lipid present in the cell pellets and culture supernatants and the number of cells present in these two fractions.

Extracellular lipids were estimated for cultures of Acinetobacter sp. HO1-N during a two growth cycle experiment (Makula et al., 1975). This experiment does not involve a true diauxie since the hexadecane was added only after the cultures growing on acetate had reached stationary phase of growth. Extracellular mono- and triglycerides were present in relatively small amounts during growth on acetate. After addition of hexadecane to the cultures the amount of monoglycerides increased gradually about 11 fold in the supernatant during growth on the hydrocarbon. The amount of supernatant wax esters, which was very small during acetate metabolism, increased greatly during the latter part of the growth on hexadecane. The amount of triglycerides remained relatively constant throughout the experiment. These data are difficult to compare with those of the present study since neither the amount of lipid present in the whole culture nor the total amount of cellular lipids were reported for this experiment. Acinetobacter sp. H01-N grown on hexadecane was also reported to accumulate much higher amounts of supernatant free fatty acids and triglycerides than when it is cultured in a nutrient broth-yeast extract medium. The total amount of supernatant lipids was 60 times greater in hexadecane grown cultures (Finnerty et al., 1973).

In contrast, the results of the present study suggest that, although some extracellular lipids are present during diauxie, their amount does not increase greatly during hexadecane metabolism. Although the precision of the estimates presented here for A. lwoffi extracellular lipids was criticized in the preceding chapter, these are probably more realistic than those reported for Acinetobacter sp H01-N cultures (Finnerty et al., 1973; Makula et al., 1975) since in these latter studies all cells were assumed to be in the pellet fraction. This is not necessarily true, however, as it would contrast with the results of the present and other studies (Miura et al., 1977; Nakahara et al., 1977; Neufeld et al., 1980). In any case, a demonstration of the absence of cells in the supernatant fraction of Acinetobacter sp. H01-N cultures was not published.

Recently published experiments have shown that addition of certain lipids such as long chain fatty acids, triglycerides, phospholopids, and wax esters, or certain synthetic detergents to cultures of A. lwoffi stimulates growth on hexadecane (Breuil and Kushner, 1980). The lipids were added in concentrations ranging from 200 to 500 mg/l of culture and lower concentrations had no detectable effect on the growth kinetics. Although these amounts of lipid support relatively little growth, they are much higher than the amounts of extracellular lipids estimated in the present study.

The stimulatory effect of added lipids was mostly due to a sharp reduction in the lag phase of growth. At least part of this effect can be attributed to the emulsifying properties of the added lipids. These considerations support the hypothesis that extracellular lipids are involved in the uptake of hydrocarbon by microorganisms. The results presented in this dissertation, however, suggest that the amount of extracellular lipids produced by A. lwoffi is too small to stimulate growth significantly. Although this small amount may contribute to the emulsification of the hydrocarbon, binding of the cells to large hexadecane globules appears to play a major role in the uptake of the substrate. This binding also makes it difficult to measure the precise amount of extracellular lipid since these are not easily distinguished from the lipids associated with cells trapped in the hydrocarbon phase of the cultures.

2. Hydrocarbon metabolism.

The results presented in this dissertation confirm the often reported observation (Chapter I, part 4) that a substantial proportion of the fatty acids of microorganisms grown on long chain aliphatic hydrocarbons, have the same chain length as the parent hydrocarbon molecule. There is good evidence that this correlation during hydrocarbon utilization is caused by direct incorporation of the hydrocarbon, after oxidation to the corresponding fatty acid, into complex lipids, coupled with inhibition of de novo fatty acid synthesis (Gill and Ratledge, 1973; Sampson and Finnerty, 1974; Mishina et al., 1976; Ascenzi and Vestal, 1979).

Incorporation of oxidized hexadecane, during the hydrocarbon phase of diauxie of A. lwoffi, was found to take place mostly in the phospholipids. Acinetobacter sp. HO1-N was shown to incorporate radioactive hexadecane at a higher rate into phospholipids than into neutral lipids (Makula and Finnerty, 1971). Most of the hexadecane incorporated into the phospholipids of A. lwoffi or Acinetobacter sp. HO1-N (Makula and Finnerty, 1968) was in the form of palmitoleate rather than palmitate. This suggests the presence of a particularly active fatty acid desaturase in both organisms when growing on hexadecane. This accumulation of C_{16} fatty acids, also greatly reduces the proportion of C_{18} fatty acids during growth on hexadecane. The proportion of unsaturated fatty acids was maintained within relatively narrow limits, thus, apparently maintaining membrane fluidity and function (Mc Elhaney, 1976).

Those metabolic activities that are reflected by changes in lipid composition appear, during diauxic growth, to be limited to the phospholipid component. No accumulation or depletion of reserve forms, such as glycerides or wax esters, was observed during the hexadecane phase. Although the total amount of neutral lipid in the whole culture remained relatively constant, their proportion decreased gradually as phospholipids were synthesized. Higher proportions of neutral lipids were found in hydrocarbon grown cells (compared with cells grown on other carbon sources) of a *Nocardia* sp. (Raymond and Davis, 1960), *Candida petrophilum* (Mizuno *et al*., 1966), *Mycobacterium vaccae* (Vestal and Perry, 1971), and an *Alcaligenes* sp. (Bertrand *et al*., 1976 b). This was not the case for other microorganisms including *Candida* 107 and *C*. *tropicalis* (Thorpe and Ratledge, 1972) and *Acinetobacter* sp. HO1-N (Finnerty *et al*., 1973).

During the lag period of diauxie, following depletion of the ethanol, the amount of all lipid classes present in the whole culture remained essentially constant. In contrast, Hug *et al*.,(1974) observed, during continuous culture experiments in which the substrate was changed suddently from glucose to hexadecane, a transition phase during which the amount of total lipids and phospholipids of *Candida tropicalis* cells nearly doubled before cell growth resumed. This latter result was interpreted as supporting the hypothesis that a high lipid content of the cells is necessary for hydrocarbon uptake (Hug *et al*., 1974). The results of the present study cannot rule out this hypothesis but the lipid content of *A*. *lwoffi* cells grown on ethanol appears to be sufficient for growth on hexadecane.

3. Intracellular inclusions.

As was mentioned earlier, during the hexadecane phase of diauxie, globular electron transparent inclusions, similar to those described by Finnerty *et al*., (1973), appear in the cells of *A. lwoffi* (Shindler *et al*., 1978). In the case of *Acinetobacter* sp. HO1-N, these inclusions were shown, using X-ray diffraction techniques, to be composed of hexadecane, or whatever hydrocarbon the cells had been grown on, which had accumulated inside the cell (Finnerty *et al*., 1973; Kennedy *et al*., 1975). The results obtained for lipid analyses of *A. lwoffi* cells during diauxie are consistent with this conclusion since no accumulation of neutral lipids was observed and phospholipids would tend to form small vesicles and bilaminar structures in the aqueous cytoplasmic medium (Lucy and Glauert, 1964). These inclusions, which appear to have a uniform structure and have diameters of 1/8 to 1/4 of the cell diameter (Shindler *et al*., 1978), are probably too large to be formed by a simple accumulation of phospholipid in the cytoplasm. Phospholipids could, however, as was shown for *Acinetobacter* sp. HO1-N, form the lipid part of the membrane surrounding these inclusions (Scott and Finnerty, 1976). Of course, more direct experiments would be required to fully characterize these inclusion bodies in the case of *A. lwoffi*.

4. Role of the lipase.

No involvement of a lipase activity is apparent from the lipid analyses presented in this dissertation. Although a strong lipase activity is induced during the hexadecane phase of diauxie (Breuil *et al*., 1978), the amount of all three forms of glycerides remained essentially constant during this period. The lipase could have been involved in maintaining these metabolic pools to constant levels, but if glycerides were formed during the period of hexadecane metabolism and degraded at the same rate, one would expect the average chain length of these compounds to decrease and gradually approach that of palmitic acid. This was not observed, however, the average chain length of the glycerides remaining quite constant. These considerations do not rule out an involvement of the lipase, but its metabolic "role" remains difficult to identify.

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