STUDIES OF MICROBIAL HYDROCARBON FERMENTATIONS

by

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in the Department

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Food Science

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September, 1972
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Date Sept. 18, 1972
ABSTRACT

A two part study of hydrocarbon utilization by microorganisms was undertaken. In the first part it was decided to attempt the genetic transformation of Bacillus subtilis with respect to the ability to grow on hydrocarbon carbon source. One hundred forty-four cultures of Bacillus organisms were isolated on a medium with refined kerosene as the sole carbon source. Most of these cultures were found to be B. cereus (92 isolates), B. lentus or B. firmus (15 isolates) and B. megaterium (8 isolates). Neither of the 2 strains identified as B. subtilis were capable of sufficient growth on kerosene medium, even with complex supplements added, to warrant a transformation experiment.

In the second part of this study, a culture which was classified as a member of the genus Arthrobacter was investigated for its ability to grow rapidly on hydrocarbons. Dodecane was shown to be the n-alkane utilized most readily. The acidity produced by this culture was not due to formation of fatty acids, other organic acids or amino acids and was attributed to the acidity generated by utilization of the ammonium nitrogen source. When grown in a fermentor with pH control, the Arthrobacter spp. oxidized ammonia forming nitrate and nitrogen oxide gases when the dodecane carbon source was exhausted. This is the first time ammonia oxidation has been reported in an n-alkane fermentation system.
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PART I. INVESTIGATION OF KEROSENE UTILIZING BACILLUS SUBTILIS STRAINS

INTRODUCTION

Most industrial and food fermentations use traditional carbohydrate based substrates as their carbon source. For example, much of the monosodium glutamate used in food products is produced by Corynebacterium glutamicum (Micrococcus glutamicus) grown on crude starch hydrolysate or molasses (51). The citric acid fermentation uses sucrose as the substrate for Aspergillus niger (81). Because there can be economic advantages in terms of the raw material purchase price and in the ease of product isolation when using a petroleum fraction or a hydrocarbon as the microbial carbon nutrient source, an increased emphasis has been placed on fermentations of the hydrocarbon organisms. One approach to the development of hydrocarbon fermentations is to isolate cultures which can utilize hydrocarbons and which produce or have the potential to produce products when exposed to specific environmental or nutritional conditions. Another approach to fermentation research is to select an organism which forms a useful product from carbohydrate and to convert it through genetic manipulation so that the modified organism can utilize hydrocarbons for growth and product formation.

In this experiment it was decided to use the latter concept. It has been shown that certain bacterial organisms can accept into the cell deoxyribonucleic acid (DNA) from closely related bacterial strains and incorporate this DNA into their own genetic make-up. This process, termed "transformation" in which an inheritable character of a DNA molecule is transferred, has been extensively studied for Bacillus subtilis (97). Because a number of inducible enzyme systems have been
transformed using **B. subtilis** (96), it was felt that the enzyme or enzymes for hydrocarbon oxidation might also be transformable. There are a number of strains of the spore forming rod, **B. subtilis** which are used to make products of commercial interest to the food industry, such as amylase which is used in the baking industry and inosine which is used as a food flavour enhancer.

Since the purpose of this investigation was to attempt to demonstrate the transformation of the genetic character responsible for hydrocarbon utilization, a readily detectable product from the carbohydrate utilizing **B. subtilis** was desirable. Because of the ease of detecting antibiotics quantitatively, a strain of **B. subtilis** (ATCC 14593) which produces the antibiotic bacitracin was selected for the first experiments. Bacitracin is a polypeptide antibiotic active against many Gram positive and a few Gram negative organisms (48). Since bacitracin can be produced by organisms grown on acetate carbon source (47), it is possible that a strain transformed to hydrocarbon utilization would still form antibiotic since acetate is the normal product of the bacterial degradation of alkanes.
Transformation of *Bacillus subtilis*

Genetic transformation in bacteria is the uptake of dissolved deoxyribonucleic acid (DNA) by an organism and the incorporation of this DNA into the genetic material of the organism. The occurrence of transformation in a culture is shown by a change in the inheritable characteristics of that particular strain of organisms. Transformation has been extensively reviewed by Spizizen et al. (97), Schaeffer (86), and Ravin (84). It was first reported in the classic studies of Avery et al. in 1944 (12). They showed that the addition of high molecular weight DNA from a strain of *Diplococcus pneumoniae* which formed smooth Type III capsular polysaccharide to an actively growing culture of rough Type II pneumococci resulted in a small proportion of the latter population being converted to Type III. The proof that this change was heritable and that it resulted from the uptake of purified DNA, free from protein and ribonucleic acid (RNA) was one of the first conclusive demonstrations that DNA is the material involved in genetic inheritance. Since that time transformation has been shown to occur for many characteristics in species in other genera, such as drug resistance and capsular polysaccharides in *Hemophilus influenzae* (4, 5) and *D. pneumoniae* (12, 50), and nutritional independence, inducible enzyme forming ability and antibiotic production in *Bacillus subtilis* (94, 96, 18). Although transformation occurs most readily when both the DNA donor and recipient strains are in the same species, that is, when there is a high degree of homology between the DNA of the two strains, it can also occur between different species in the genera *Hemophilus* (87),...
Bacillus (73), Neisseria, (21) and Rhizobium (15). Transformation between the genera Streptococcus and Diplococcus and between Streptococcus and Staphylococcus indicate how closely these organisms are related (19).

Transformation in the genus Bacillus was first reported by Spizizen in 1958 (94). He showed that three auxotrophic mutant strains of Bacillus subtilis could be converted to nutritional independence by the addition of wild strain DNA under suitable conditions. Strain 168, which required indole or tryptophan had a particularly high rate of transformation when wild type DNA was added. Further investigations by Spizizen and his co-workers (95, 96, 8, 115, 116) using this strain demonstrated the optimum conditions for transformation. It was found that cells display their greatest competence, or ability to take up DNA, at a definite stage in the growth cycle, i.e. in the late logarithmic phase. This competent period is related to changes in the cell wall during presporulation. Competent cells are produced by growing them four hours in a glucose-ammonium-salts semi-starvation medium, then harvesting and resuspending them in transformation medium for 90 minutes. There are certain specific requirements for this transformation medium. As well as a low concentration of glucose, ammonium ions and salts, there is a requirement for divalent cations, in particular barium, strontium, calcium or magnesium. These are thought to stabilize the DNA by neutralizing the charge on the DNA molecules and possibly the net negative charge on the cell surface (116). Because zinc, nickel and especially cupric ions tend to inhibit transformation, a chelating agent such as ethylenediaminetetraacetic acid or histidine is added to prevent the latter divalent metal interference. The optimum temperature and pH
ranges for transformation with *B. subtilis* are 34 - 37°C and 6.9 - 7.4 respectively. The transformation reaction is possible with a DNA concentration as low as $10^{-5} \mu g/ml$ for *B. subtilis*, but it occurs with greatest frequency at concentrations above $1 \mu g/ml$. A medium rich in nutrients, especially with added amino acids, reduces transformation by promoting cell wall synthesis. This is thought to interfere with the binding and uptake of DNA.

As well as intraspecific transformation of *B. subtilis*, interspecific transformation in the genus *Bacillus* has been reported by Marmur et. al. (73). These workers correlated the degree of homology in the DNA base composition between species with the ability to transform. In particular, it was shown that similarity of overall base composition, as indicated by the percent quanine plus cytosine, is a necessary but not sufficient condition for interspecific transformation. *B. natto*, *B. subtilis* var. *aterimus*, *B. niger*, *B. subtilis* var. *niger* and *B. polymyxa* had similar base compositions to *B. subtilis* and were capable of transforming the latter organism. The DNA from 16 other species of *Bacillus* could not transform *B. subtilis*.

It should be pointed out that most of these transformations reported by Marmur et. al. (73) were not truly interspecific. According to a definitive study of the taxonomy of the genus *Bacillus* by Smith et. al. (91), cultures identified previously as *B. natto* were shown to be strains of *B. subtilis* and *B. niger* was reclassified as *B. subtilis* var. *niger*. Marmur's experiments merely confirm that these strains and varieties are indeed very closely related to *B. subtilis*. *B. polymyxa* is the only separate species which transformed with *B. subtilis* and this
was at a very low level (0.04%).

Biswas and Sen (18) have also attempted intra and interspecific transformation of Bacillus organisms, in this case with respect to antibiotic production. Within the species B. subtilis the ability to produce bacitracin was successfully transformed to 3 out of 5 bacteriologically inactive strains. The ability to form bacilysin and unidentified antimicrobial and antifungal substances was successfully transferred to about 40% of the other strains. Attempts to transform 5 other species of Bacillus with DNA from antibiotic producing B. subtilis strains failed, in agreement with Marmur's results (73).

Classification of Bacillus subtilis

The genus Bacillus is made up of the aerobic, endospore forming Gram positive rod shaped organisms. Prior to the work of Smith, Gordon, and Clark (90,91) and Knight and Proom (65), there was considerable confusion regarding the taxonomy of organisms within the genus Bacillus. Smith et. al. (91) developed a systematic key for the classification of the individual species, which is followed in Bergey's Manual (20). They studied 1,134 cultures of which all but 20 were placed in three groups according to their morphology and physiology. About 65 percent of the cultures belonged to the same group as B. subtilis. Smith's scheme was modified in a key prepared by Wolf and Barker (114). In their key, Bacillus organisms are placed in three groups according to spore and cell morphology. B. subtilis is placed in Group I which is made up of organisms whose spores are oval or cylindrical and the sporangia are not definitely swollen. Part of the key is shown in Figure 1.
Figure 1. Partial key for *Bacillus* organisms.
There have been relatively few reports of Bacillus organisms growing on hydrocarbons in the literature. Shah (89) has cited the early papers of Tausson (104) and Sohngen (92) as examples of Bacillus utilizing hydrocarbons. However, as pointed out by Fuhs (37), the organisms isolated in these early studies were not spore formers and many of them were Gram negative. The only culture which fits the present day classification of Bacillus is the B. phenanthrenicus of Tausson (103). This was a strain capable of utilizing the aromatic hydrocarbon phenanthracene.

Many members of the genus Bacillus have been isolated in studies of the microbial deterioration of aircraft fuels. Felix and Cooney (34) reported the response of nine strains of Bacillus isolated from a fuel system. They found that vegetative cells could survive for only a limited time in a basal salts-JP-4 (a kerosene type fuel) medium and that the cells could not grow at all. Spores of these strains, under similar conditions, could survive but not grow; any that did germinate soon died. It was concluded that Bacillus organisms found in fuel systems were present because they formed long-surviving and widely distributed spores, not capable of growth in those systems.

In a study of amino acid producing organisms isolated from soil, Shah et. al. (89) found four strains of Bacillus which grew well using kerosene as the carbon source. These organisms resembled B. cereus var. mycoides (2 strains), B. sphaericus and B. subtilis. The B. subtilis strain did not fit all the criteria of that species given in Bergey's Manual and may have been a B. pumilis.
MATERIALS AND METHODS

Isolation of Bacillus spp. on kerosene carbon source

Forty-eight soil sludge samples were collected in sterile test tubes from an oil refinery's waste dumping area. In order to isolate the aerobic spore forming bacteria which could grow with kerosene as carbon source, 10 to 15 ml of sterile distilled water and one drop of Tween 80 emulsifier were added to each sample. The contents of each tube were mixed on a test tube agitator. Seven millilitres of the aqueous layer were transferred to sterile tubes and heated in a water bath at 80°C. After 15 and 30 minutes incubation, 1.0 ml and 0.1 ml aliquots were withdrawn and spread on Petri plates containing the following hydrocarbon basal medium (70):

\[
\begin{align*}
K_2HPO_4 \text{ (anhyd.)} & \quad 2.50 \, g \\
KH_2PO_4 & \quad 1.75 \, g \\
NH_4Cl & \quad 1.00 \, g \\
MgCl_2 \cdot 6H_2O & \quad 0.10 \, g \\
FeCl_2 \cdot 4H_2O & \quad 0.05 \, g \\
CaCl_2 \cdot 2H_2O & \quad 0.01 \, g \\
MnCl_2 \cdot 4H_2O & \quad 0.002 \, g \\
Na_2SO_4 \text{ (anhyd.)} & \quad 0.05 \, g \\
\text{Yeast extract} & \quad 0.10 \, g \\
\text{Distilled water} & \quad 1,000 \, ml \\
\text{pH} & \quad 6.8 \\
\text{Agar} & \quad 15 \, g
\end{align*}
\]

After the surface of each plate had dried, two drops of Fisher odorless kerosene, lot number 783716, were spread over the surface. The plates
were incubated at 30°C for 48 hours.

After incubation, colonies were examined microscopically, and those containing spore forming rod shaped organisms were transferred to hydrocarbon basal medium slants for further classification.

### Classification of kerosene-utilizing **Bacillus** cultures

**Media:**

i) Nutrient agar, pH 6.0.

Difco nutrient agar was adjusted to pH 6.0 with dilute sulfuric acid.

ii) Glucose broth (91).

\[
\begin{align*}
\text{Tryptose} & : 10 \text{ g} \\
\text{K}_2\text{HPO}_4 & : 5 \text{ g} \\
\text{Beef extract} & : 3 \text{ g} \\
\text{Yeast extract} & : 2 \text{ g} \\
\text{Glucose} & : 10 \text{ g} \\
\text{Distilled water} & : 1,000 \text{ ml}
\end{align*}
\]

Glucose was steam sterilized separately as a 25% (w/v) solution at 15 p.s.i. (121°C) for 15 minutes and added aseptically.

iii) Glucose ammonium salts.

\[
\begin{align*}
\text{(NH}_4\text{)}_2\text{PO}_4 & : 1.0 \text{ g} \\
\text{KCl} & : 0.2 \text{ g} \\
\text{MgSO}_4 & : 0.2 \text{ g} \\
\text{Yeast extract} & : 0.2 \text{ g} \\
\text{Glucose} & : 5.0 \text{ g} \\
\text{Bromcresol purple} & : 20 \text{ ml of a 0.4% solution} \\
\text{Distilled water} & : 1,000 \text{ ml}
\end{align*}
\]
The medium was dispensed in test tubes with Durham tubes added to measure gas production. Glucose was sterilized separately and added aseptically.

iv) Voges-Proskauer medium (for *Bacillus* spp.).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Proteose-peptone</td>
<td>7 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1,000 ml</td>
</tr>
</tbody>
</table>

**Test procedures**

i) Microscopic examination.

Cultures grown on both hydrocarbon basal medium and nutrient agar were examined at medium power (400 x) with a phase contrast microscope. Organisms which had suitable morphology-cylindrical spores in non-swollen sporangia were Gram stained by standard procedures (27).

ii) Growth at 60°C, pH 6.0.

Cultures to be tested were streaked on nutrient agar, pH 6.0, slants and incubated at 60°C. Slants were examined for growth after two and five days.

iii) Anaerobic growth.

Glucose broth tubes were steamed to remove dissolved and gaseous oxygen, inoculated, capped with sterile melted vaspar (50% Vaseline and 50% paraffin) and incubated at 30°C. After 7 and 14 days the tubes were checked for growth.

iv) Effect of glucose on growth.

Glucose-ammonium-salts medium was inoculated with cultures and examined for growth, acid and gas production after 10 days. The inhibitory effect of glucose was determined by comparing growth on nutrient agar
slants to growth on nutrient agar slants with 10 g/l glucose added.

v) Voges-Proskauer test.

Five millilitres of medium was inoculated and incubated five days at 30°C. At that time 3.0 ml of 5% α-naphthol in absolute ethanol was added to each tube, followed by 1.0 ml of 40% potassium hydroxide. The test is positive if a bright pink colour appears in five minutes.

In each classification step a culture of Bacillus subtilis ATCC 14593 was included as a control.

Growth studies of Bacillus subtilis isolates

To improve the growth shown on kerosene basal medium slants by B. subtilis isolates, various supplements were added to the medium to test for stimulatory effects. Growth studies were carried out with 100 ml of medium in 250 ml Erlenmeyer flasks agitated on a New Brunswick Gyrotory Shaker at 200 r.p.m. Growth was measured as cell dry weight after 60 hours incubation at 30°C. Dry weights were determined by centrifuging cells at 10,000 xG for 15 minutes, washing twice in 0.05 M phosphate buffer, pH 6.8, resuspending in distilled water and drying overnight at 100 - 105°C.

The supplements used to stimulate growth were malt extract in the range 0 to 1.0 g/l and soil extract in the range 0 to 100% of the medium. Soil extract was prepared by boiling soil in distilled water in a 1:1 (w/v) ratio for eight minutes, decanting the aqueous layer through cheesecloth and centrifuging at 15,000 xG for 20 minutes. The clear supernatant was used in the medium with 1g/l NH₄Cl and 1.5 g/l KH₂PO₄ added and adjusted to pH 6.8 with dilute hydrochloric acid.
RESULTS AND DISCUSSION

Isolation of *Bacillus* spp. on kerosene

The procedure for isolating spore-forming organisms which would grow on kerosene carbon source resulted in the isolation of 172 cultures. Microscopic examination showed that 28 cultures were non-sporeformers. These were made up of an assortment of rods, cocci and 1 yeast. These organisms appeared in plates which had a large amount of sludge carry over from the soil sample, resulting in protection from the heat treatment. Of the 144 sporeformers, 117 belonged to the Group I morphology class which includes *B. subtilis*.

Classification.

The classification of the Group I cultures following the key of Wolf and Barker (114) gave the results shown in Table I. There were no strains of *B. coagulans* present, as indicated by lack of growth at 60°C, pH 6.0. All of the strains which grew well under anaerobic conditions were large cells, about 2 x 5μ, and were probably *B. cereus* and its variants. The cultures which were inhibited by glucose and could not use ammonium salts as nitrogen source were made up of *B. lentus* and *B. firmus* and were not examined further. Of the organisms which grew well in glucose ammonium salts, eight strains had very large cells (2 x 8μ and longer) and contained many vacuoles. These are strains of *B. megaterium*. Only two cultures showed the reactions and characteristics of *B. subtilis*. Both were Gram positive and had cells about 0.8 x 2.0μ.
<table>
<thead>
<tr>
<th>Test</th>
<th>Number of cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>No growth at 60°C, pH6.0</td>
<td>117</td>
</tr>
<tr>
<td>No anaerobic growth in glucose broth.</td>
<td>25</td>
</tr>
<tr>
<td>Acid in glucose - NH₄⁺ - salts medium. Glucose non-inhibitory.</td>
<td>10</td>
</tr>
<tr>
<td>Voges-Proskauer positive. Small cells, non-vacuolated.</td>
<td>2</td>
</tr>
</tbody>
</table>
Growth studies of *B. subtilis*

The strains of *B. subtilis* isolated showed poor growth on kerosene basal medium slants - there was just a thin film of growth formed. In order to determine if some unknown factor was needed by these strains to promote growth on kerosene, different concentrations of malt and soil extract were added to the medium. The growth curves on these media are shown in Figures 2, 3, 4 and 5. With malt extract, there is not a significant difference between the growth in the control and kerosene flasks. The increased growth at higher malt extract concentration is probably due to the utilization of its content of maltose and dextrins (about 80 - 90% carbohydrate) (93). Similarly, there was no stimulation of growth on kerosene with the addition of soil extract. Increased concentrations of soil extract appear to be slightly inhibitory.

From these results it is concluded the strains of *B. subtilis* isolated in this project were not capable of sufficient growth on kerosene to warrant a transformation experiment. The strains isolated behaved similarly to the *Bacillus* species studied by Felix and Cooney (34), which could survive as spores but not actively grow in a kerosene fuel system.

Some of the *Bacillus* organisms isolated in the initial experiments did grow well on kerosene. In particular, those which resembled *B. cereus* grew rapidly to form large, spreading colonies on agar plates and slants. These large colonies on the initial isolation plates may have contributed metabolites and endproducts which the poorer growing organisms such as *B. subtilis* could use. This would account for *B. subtilis* showing up on the plates but not being able to grow on the kerosene.
medium alone. Such a relationship could also exist in a natural environment.
Figure 2. Effect of malt extract on the growth of *B. subtilis* strain 1 in kerosene medium.

■ = with kerosene  ○ = without kerosene

Figure 3. Effect of soil extract on the growth of *B. subtilis* strain 1 in kerosene medium.

■ = with kerosene  ○ = without kerosene
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**Figure 4.** Effect of malt extract on the growth of *B. subtilis* strain 2 in kerosene medium.

- ■ = with kerosene
- ○ = without kerosene

**Figure 5.** Effect of soil extract on the growth of *B. subtilis* strain 2 in kerosene medium.

- ■ = with kerosene
- ○ = without kerosene
PART II. UTILIZATION OF N-ALKANES BY AN ARTHROBACTER SPP.

INTRODUCTION

The development of hydrocarbon fermentations has accelerated rapidly in recent years. Previously, hydrocarbon microbiology was studied as an unusual occurrence, with application mainly to oil exploration and corrosion associated with oil products (118, 17). In the 1960's the industrial potential of microorganisms growing on hydrocarbons was recognized, and many interesting and valuable products can now be produced by hydrocarbon fermentation. Many of these products are important in the food industry.

The first food application of hydrocarbon fermentations was the suggestion of Champagnat and Llewelyn in 1962 (23) that food and feed yeast could be produced from oil. This group, working for British Petroleum Ltd. (BP) has studied two methods of producing feed yeasts: one, by growth of Candida lipolytica in a continuous fermentation using refined n-paraffins as carbon source, and the other by growth of C. lipolytica on gas oil, which results in simultaneous de-waxing (n-alkane removal) of the gas oil (24, 71). Both processes appear to be successful. The protein concentrate produced from hydrocarbons has been evaluated for nutritional value and toxicological safety. When mixed in animal feeds the yeast concentrate is completely acceptable, and can be used to replace or supplement other protein sources. In the future it is hoped to produce human food grade protein from hydrocarbon yeast that can be used in textured protein products, similar to those now made with vegetable protein. The production of feed yeast is increasing rapidly around the world: BP produces 4,000 tons/year from
n-paraffins at Grangemouth, Scotland, and 16,000 tons per year from
gas oil at Lavera, France (9). As well as these pilot plants, BP has
entered a joint venture to produce up to 350,000 metric tons annually
from n-paraffins at two plants in Italy. Czech workers are completing
research for the production of 100,000 ton/year (31, 32, 82) with
simultaneous de-waxing of gas oil. The Soviet Union is planning to help
alleviate its shortage of cattle feed with the production of one million
tons/year of fodder yeast from n-paraffins by 1975 (11). The production
of an acceptable protein concentrate from single cell protein for
human consumption is still a few years away. There are problems of
palatability and nucleic acid toxicity to be overcome.

There are many food products other than single cell protein which
can be obtained from hydrocarbon grown microorganisms, as indicated in
the extensive review by Abbott and Gledhill (1). Most of these food
products are produced from organisms growing on n-alkanes. Two of the
highest yielding processes are glutamic acid and citric acid fermentations.
Glutamic acid is an important flavour enhancing food additive, and is
produced by *Corynebacterium glutamicum* (*Micrococcus glutamicus*) in
large quantity from carbohydrate, and by *C. hydrocarboclastus* growing
on n-alkanes. Yields as high as 75 g/l have been reported from the
latter. This may be high enough to rival the carbohydrate process
industrially. Lysine, threonine, phenylalanine (106), and tryptophane,
which may have use as dietary supplements, have also been produced in
hydrocarbon fermentations.

Citric acid, a sequestrant and acidulant used in the food industry
has been reported at remarkably high yields. A mutant strain of the
yeast *Candida lipolytica* which was unable to degrade citric acid produced 112 g/l in 3 days. This represents a yield of 138%, considerably higher than on carbohydrate substrates. Although no detailed information is available, the large number of patents and high yields for citric acid from hydrocarbons makes its commercial production very likely in the near future. Numerous other products with food applications are formed by micro-organisms growing on alkanes, such as flavour enhancing nucleotides (guanylic and inosinic acids), vitamins and pigments. Lipase and oxygenase enzymes may also find future uses.

In order to be commercially feasible, a hydrocarbon fermentation must be able to compete economically with the corresponding carbohydrate substrate fermentation. An important factor in this is the lower cost of hydrocarbons: 2 to 4 cents/lb. for refined hydrocarbons versus 4 to 8 cents/lb. for refined carbohydrates, and 0.25 cents/lb. for natural gas versus 1.5 - 2.5 cents/lb. for cereal grains (52). Because hydrocarbons are in a more reduced form and contain no oxygen, a higher yield on a weight basis can be obtained. For example, it requires only half as much hydrocarbon substrate as carbohydrate to give a certain cell yield. Product recovery is easier from a hydrocarbon process. At the end of a fermentation the medium usually contains just inorganic salts, cells, substrate and product, without the complex organic substances found in crude carbohydrates such as molasses. The immiscibility of the oil phase with the aqueous medium makes its removal much simpler and less expensive.

On the other hand, some of these properties add difficulties to hydrocarbon fermentations. Because of the reduced state of paraffin substrates, these processes require about three times as much oxygen as
conventional aerobic processes and result in much greater heat production. Immiscibility of the aqueous and oil phases creates a need for much more mixing. These increased costs of aeration, heat removal and agitation must be balanced against the possible advantages of substrate price, product yield and ease of recovery in a hydrocarbon process in order to evaluate its feasibility.

The purpose of this study is to characterize the growth and possible product formation of an organism growing on n-alkanes. When isolated, this culture showed abundant growth and produced a high level of acidity on hydrocarbon medium. This study is an investigation of which hydrocarbons support the best growth and of the nature of the acidity produced by this microorganism growing on n-alkanes.
LITERATURE REVIEW

Classification

The classification of bacteria resembling the culture used in this project, that is, those organisms which show rod shaped or filamentous growth breaking up into rods and cocci, is very difficult. A number of genera of related organisms show this type of growth at different stages of their life cycle and on different types of media. These include members of the Nocardia, Mycobacteria and Arthrobacter. Much of the early work of Jensen (58), Conn and Dimmick (28) and Clark (26) was concerned with separation and classification of parasitic and saprophytic forms of these organisms. Jensen laid much of the groundwork by defining the soil mycobacteria as the acid-fast, rod to filamentous forms, which divide into cocci, while the corynebacteria were non-acid-fast and showed varied morphology, with irregular shaped rods and filaments, sometimes branched, which broke up into small rods or cocci. Some strains formed large coccoid cells called cystites. Conn and Dimmick added to the study of these forms by broadening the definition of Mycobacterium to include partially acid fast and branched forms. They proposed the name Arthrobacter for a group of common soil organisms previously included in the genus Corynebacterium. These organisms were characterized by a complicated morphological cycle which included rods, cocci, club-forms, branched and unbranched filaments. These cultures were Gram variable and non-acid-fast. This designation of Arthrobacter was supported by Clark and first appeared in Bergey's Manual in 1957 (20). The classification, morphology, and physiology of Arthrobacter has been extensively characterized since then by Stevenson and Lochhead's group.
(25, 72, 98). The life cycle of Arthrobacter starts with coccoid cells which enlarge into pleomorphic rods which later divide into small rods and coccoid forms. On certain media filaments and cystites may be formed. The cycle is usually complete within 24 hours.

The mycobacteria and nocardia have been investigated by Gordon and her co-workers (39, 40, 41). They give the criteria of acid-fastness and non-mycelial filamentous growth, sometimes branching, which later breaks into rods and cocci, for the mycobacteria. The Nocardia are regarded as non-acid fast organisms with a definite mycelial growth. The mycelium is usually branching and may contain aerial hyphae. More rudimentary strains may fragment into rods and coccoid forms. These descriptions differ from Bergey's Manual, in which the mycobacteria can contain organisms which are highly branched and only slightly acid fast, and the nocardia include some partially acid fast forms which have a rudimentary mycelium which fragments rapidly into rods and cocci.

The difficulty in classifying these organisms is summed up in a remark by H.L. Jensen, (42)¹

"It has been customary to talk about Nocardia as representing a transition between Mycobacterium and Streptomyces, but there is another group with which the nocardiae merge, the arthrobacters ...

... Like Dr. Gordon, who knows of no borderline between Nocardia and Streptomyces, I know of no borderline between Arthrobacter and Nocardia."

Microbial hydrocarbon utilization

Microorganisms have been known to grow on hydrocarbons since 1895, when Miyoshi observed the fungus *Botrytis cinerea* growing on paraffin wax (83). The study of microbial hydrocarbon utilization in the first half of this century was mainly concerned with which organisms could grow on hydrocarbons, which ones were attacked, and the use of petroleum organisms in oil prospecting. These early developments are discussed in the reviews by ZoBell (117, 118) and Beerstecher (17).

As summarized by Quayle (83), the straight chain alkanes of intermediate chain length (9 to 16 carbon atoms) are most easily utilized by microorganisms as compared with branched chain alkanes and aromatics. This is a generalization based on reports in the literature and may not hold true for any particular organism. Most of the organisms capable of growth on hydrocarbons are common soil types, including bacteria, yeasts and fungi. The bacteria most often found in isolating hydrocarbon users are in the genera *Brevibacterium*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Streptomyces* and *Pseudomonas*. *Candida* and *Torula* yeasts and *Aspergillus*, *Fusarium* and *Penicillium* fungi frequently can grow on hydrocarbons as well (37, 35, 83). Although the n-alkane hydrocarbons most frequently support growth, many other types can be attacked by some organisms such as branched alkanes, olefins, saturated ring compounds and aromatics, including the multi-ring ones like anthracene. Hydrocarbon containing products such as kerosene fuels, rubber, and asphalt are subject to microbial degradation.

Much work in the last few years has been devoted to determining the pathways and mechanisms of n-alkane oxidation (35, 108, 75, 64). The
principle pathway of microbial attack on n-alkanes starts with the addition of oxygen to the terminal methyl group, as first studied by Senez and Azoulay (66, 14) and Thijssse and van der Linden (105). They showed that species of *Pseudomonas* oxidize an n-alkane, e.g. n-hexane, by the addition of one oxygen atom to the terminal methyl group giving the primary alcohol. This was subsequently dehydrogenated to the corresponding aldehyde and then fatty acid, which was utilized by the common $\beta$-oxidation pathway. These results have been confirmed in many studies since. Leadbetter and Foster (67) and Stewart et.al. (100) showed that molecular oxygen is incorporated directly into the alcohol molecule by using isotopically labelled oxygen with *Pseudomonas* and *Micrococcus* organisms respectively. The enzymes involved have been partially characterized. Hayaishi (45, 46) introduced the concept of mixed-function oxygenase (or hydroxylase or oxidase) enzymes which catalyse the first step of the oxidation. Studies using cell-free extracts have shown that this initial enzymatic reaction requires ferrous ions and molecular oxygen in order to produce the corresponding alcohol. The subsequent conversion of the alcohol to the corresponding acid are carried out by nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) requiring dehydrogenases (16, 13, 70). Recent investigations have been concerned with the mechanism of the initial oxidation step and the nature of the electron transport system involved between molecular oxygen and NADH$_2$ (79, 80, 22). This methyl group oxidation pathway has been demonstrated in *Mycobacterium rhodochrous* (36) and a thermophilic Bacillus (44).

Klug and Markovetz (63) and Lebault (69) have shown that *Candida* yeasts utilize n-alkanes by a similar pathway.

$\beta$-oxidation of the fatty acid is not the only pathway of utilization
of n-alkanes. Stewart et al (100) found that after the terminal methyl group was oxidized by a strain of *Micrococcus cereificans* the alcohol was combined with a fatty acid to form high molecular weight esters. Both the alcohol and acid moieties in the ester were derived from the n-alkane, for example n-hexadecane (*C*$_{16}$) led to formation of cetyl palmitate (*C*$_{32}$). Some bacteria and yeasts form dicarboxylic acids from alkanes (60, 6, 57). It appears that the monocarboxylic acid is formed first, followed by ω-oxidation of the other end group. This is followed by β-oxidation to acetate. Some strains of *Pseudomonas* (36), *Brevibacterium* (109) and *Penicillium* accumulate secondary alcohols and ketones when grown on n-alkanes. In most cases these are products of side reactions and are not metabolized further by the organism.

An interesting aspect of hydrocarbon metabolism is the phenomenon of co-oxidation, in which a non-utilizable hydrocarbon is oxidized by an organism which is growing on an assimilable hydrocarbon. This was first reported by Leadbetter and Foster (68) who found that *Pseudomonas methanica* grown on methane would simultaneously oxidize ethane to ethanol, acetaldehyde and acetate, propane to acetone and propionic acid and butane to 2-butanone and butyric acid. This culture could not grow on ethane, propane or butane as sole carbon source. Davis and Raymond (29) showed that a *Nocardia* species growing on n-hexadecane or n-octadecane could co-oxidize the alkyl side chains of aromatic and cyclic hydrocarbons to give cyclic carboxylic acids. Since then dozens of co-oxidative transformations of aromatic compounds have been reported, most of them using *Nocardia* cultures (85).
Utilization of hydrocarbons by *Arthrobacter*

The first reports of hydrocarbon oxidation by members of the genus *Arthrobacter* were by Klein et al. (61, 62). They found that *Arthrobacter* strains grown on glucose would co-oxidize n-hexadecane to the corresponding 2, 3, and 4 hexadecanones in the ratio of 72:23:4%. The corresponding alcohols were intermediates in the oxidation. The organisms were not able to grow on the n-alkane or ketones as the sole carbon source. Perry and Scheld isolated an *Arthrobacter* organism which oxidized propane constitutively after growth on α-phthalate (78).

A number of workers in the Japanese fermentation industry have isolated *Arthrobacter* strains which grow rapidly on n-alkane carbon sources. The group at the Kyowa Hakko Kogyo Co. Ltd. have developed a number of strains which form interesting products, including trehalose lipid emulsifiers (101), orotic acid and orotidine (59), and derivatives of phenazine (102). These mutant strains of *A. paraffineus* were grown on a basal salts medium with a mixture of C-12 to C-14 n-alkanes added as carbon source. Many of these cultures and strains of *A. simplex* have been patented for the production from hydrocarbons of glutamic acid (up to 39 g/l), lysine (10 g/l), threonine, tryptophan, citric acid (28 g/l yield), inosinic acid, polysaccharides, and many other amino acids and nucleotides (1).

**Oxidation of ammonia**

The oxidation of ammonia is part of the process of nitrification. Nitrification is a general term used in reference to the oxidation of organic forms of reduced nitrogen and ammonia by soil organisms leading
to the formation of nitrites, nitrates and sometimes gaseous oxides (2).
The major groups of organisms responsible for nitrification in the soil are the *Nitrosomonas* and *Nitrobacter* autotrophic bacteria. *Nitrosomonas* are thought to oxidize ammonia to nitrite through the intermediates hydroxylamine and the unstable nitroxyl radical (NOH) (110). Energy in the form of adenosine triphosphate (ATP) is derived by coupling these oxidations with oxidative phosphorylation in the electron transport system. Some of this ATP is used to produce reduced pyridine nucleotides for the fixation of carbon dioxide. Similarly, nitrobacters use the oxidation of nitrite to nitrate to generate energy and reducing power for autotrophic growth.

Some heterotrophic organisms are also capable of nitrification, usually at a much lower rate than the autotrophs. In a survey of heterotrophic nitrifiers, Eylar and Schmidt (33) isolated 26 moderately active cultures. Most of these were fungi identified as *Aspergillus flavus*, with a few *Penicillium* and unidentified bacteria. One pathway which has been found for heterotrophic nitrification involves organic intermediates and occurs in *A. flavus* (74) and *P. atrovenetum* (55). The immediate precursor of nitrate and nitrite is 3-nitropropionic acid which is formed from either β-alanine or aspartic acid. These organisms do not produce nitrite and nitrate until active growth has almost ceased and do not depend on nitrification for energy for growth. The role of this phenomenon has not yet been explained in possible biosynthetic pathways.

An inorganic pathway of nitrification operates in some heterotrophic organisms. Using a cell free extract of *Aspergillus wentii*, Aleem et al.
(3) demonstrated the oxidation of ammonium ions through the intermediates hydroxylamine and nitrite to give nitrate. This is similar to the pathway operating in *Nitrosomonas* and *Nitrobacter*. The enzyme systems of both *A. wentii* and the autotrophs require copper ions and have similar cytochrome systems. Aleem and his co-workers state that this inorganic pathway for ammonia oxidation is the only one in heterotrophs and that 3-nitropropionic acid is not involved. This disagreement over organic or inorganic intermediates has not been resolved.

Heterotrophic nitrification in bacteria has been studied by Gunner (43) working with a strain of *Arthrobacter globiformis* isolated from soil. After growth in a basal salts medium with succinate as carbon source, this culture oxidized ammonium ions when suspended in a buffer solution without carbon source. The products of oxidation were small amounts of hydroxylamine and nitrite, and a large amount of nitrate. Unidentified nitrogen oxide gases were also produced and were postulated to be intermediates in the oxidation. Gaseous oxides have also been reported by Anderson (10) to be formed by an extract of *Nitrosomonas* cells. Nitric oxide and nitrous oxides were released from hydroxylamine under anaerobic conditions. Nitrous oxide was also formed non-enzymatically in boiled extracts.

Another type of heterotrophic ammonia oxidation, which has not been recognized by workers in the soil nitrification area, is the formation of nitrite from ammonia by methane utilizing bacteria. This was first reported by Hutton and ZoBell (53, 54) before Schmidt (88) observed the first heterotrophic nitrification by *Aspergillus flavus*. Hutton and ZoBell showed that some methane grown bacteria produced nitrite from
ammonia at a rate proportional to the rate of methane consumption. These results have been confirmed by Whittenbury et. al. (111), who found that more than 100 cultures of methane utilizing bacteria could all convert ammonia to nitrite. In these organisms, the ammonium ions compete with methane for the methane oxidase enzyme (111). It is postulated that methane oxidase may have developed from a mutation in the ammonia oxidase enzyme of autotrophic bacteria. If this is the case, an examination of the mechanism of methane oxidation may help elucidate the mechanism of ammonia oxidation.
MATERIALS AND METHODS

Characterization of hydrocarbon specificity

Cultural conditions

The Arthrobacter organism was grown in the hydrocarbon basal medium described in Part I for these experiments. Fifty ml of medium in 250 ml Erlenmeyer flasks were steam sterilized at 121°C for 15 minutes before inoculation. The hydrocarbons were sterilized separately and added aseptically to give a concentration of 10 ml/l. A 2% (v/v) inoculum of an actively growing 24 hour shake flask culture was added to each flask. Flasks were incubated on a New Brunswick Gyrotory shaker operating at 200 strokes per minute in a 30°C incubator. Dry weights were determined as in Part I. The pH of the medium was measured with a meter after the cells had been removed by centrifugation. All trials were performed in duplicate.

Analysis of kerosene

The kerosene used in these experiments was Fisher odorless kerosene, lot No. 704351. The major n-alkane components of this kerosene were identified using gas chromatography. These components were identified by comparing their retention times with pure standards and by observing the change in the areas of the n-alkane peaks when pure n-alkanes were added to the kerosene.

The analysis was done using a Becker Model 3810 gas chromatograph equipped with a single column analytical head. The column was HI-EFF-8BP, cyclohexandimethanol succinate. The unit was equipped with a flame
ionization detector and nitrogen carrier gas, flow rate 40 ml/min. The temperature was programmed from 50 to 190°C at 12°C/min., and then held isothermally for 5 minutes. 0.5 μl of sample was used with the injection port temperature set at 220°C. The Vitatron model UR402 recorder was equipped with an electronic integrator to measure peak areas and give quantitative results.

Analysis of kerosene from shake flasks

The organism was grown in shake flasks for 24, 48, 72 and 120 hour periods. On each pair of duplicates, the pH of the medium was measured. The residual kerosene was removed from the surface of each sample after centrifugation, dried over anhydrous sodium sulfate, and analysed gas chromatographically. Uninoculated 5 day control samples were also run.

Respirometry

The oxygen uptake of intact cells on different n-alkanes was measured using standard manometric techniques (107). The apparatus used was a Gilson Differential Respirometer. The flasks contained 1.0 ml of cells suspended in hydrocarbon basal medium, 2.0 ml of basal medium, 0.2 ml of 20% potassium hydroxide in the center well and 50μl of n-alkane in the side arm. The substrate was tipped into the flask after 10 minutes equilibration in the 30°C water bath.

Growth on different n-alkanes

The organism was grown in shake flask culture using the pure n-alkanes present in kerosene as the sole carbon source. After 42 hours the growth (dry weight) and pH drop of the media were measured.
Growth curve in shake flasks

The dry weight and pH of the medium were determined during a 7 day growth period. The organism was grown on the two n-alkanes which supported the most growth, dodecane and tridecane, and kerosene.

Characterization of acid production

Fatty acid analysis

The Arthrobacter culture was grown in shake flask for 48 hours on dodecane, tridecane and kerosene carbon sources. The fatty acids were extracted by adjusting 300 ml of culture fluid to pH 8.5 and centrifuging 20 minutes at 10,000 XG to remove the cells. The supernatant was adjusted to pH 1.5 with 6 N hydrochloric acid and extracted three times with 50 ml of diethyl ether. The combined ether fractions were extracted twice with 40 ml of 5% sodium carbonate. The aqueous carbonate layer was acidified to pH 1.5 with 6N hydrochloric acid and extracted twice with 15 ml of ether. The combined final ether extracts were dried over anhydrous sodium sulfate and evaporated to about 3 ml with a stream of dry nitrogen.

These ether extracts of the acidic lipid fractions from the culture fluid were analysed by thin layer chromatography. The extracts were spotted on Silica Gel G (0.25 mm thick) thin layer plates, and developed with petroleum ether (30 - 60°C B.P): ether: acetic acid, (90:10:1, v/v) solvent system (38). Decanoic acid was run as a fatty acid standard and sebacic acid as a dicarboxylic acid standard. Spots were visualized on the plates with two different spray reagents: 0.04% bromcresol green in ethanol, and charring with 50% sulfuric acid followed heating at 105°C
for 30 minutes.

The extract from the kerosene medium was further analysed using gas chromatography. The acids in this extract were methylated by boiling for 2 minutes on a steam bath with 5 ml of boron trifluoride-methanol (14% w:v) reagent (112). Ten ml of distilled water was added and the total was extracted with 5 ml of ether. The ether layer was dried over anhydrous sodium sulfate, followed by removal of the ether by evaporation under a stream of dry nitrogen with the vessel held in an ice bath. The small amount of oily residue was injected into a Becker Model 3810 gas chromatograph equipped with HI-EFF-8BP column with nitrogen carrier gas. The temperature was programmed for linear increase from 120°C to 230°C at 12°C/min., and then held isothermally. Standard methyl esters of fatty acids were analysed under the same conditions to give standard retention times.

**Organic acid analysis**

Cells were grown on dodecane, tridecane, and kerosene in shake flask culture and removed from the medium by centrifugation. The culture media were tested for the presence of organic acids by thin layer chromatography on Silica Gel G (0.25 mm). Unused medium was run as a control, and adipic and tartaric acids were run as standards for comparison. The plate was developed with ethanol:water:25% ammonia, (100:12:16), dried 10 minutes at 100°C and sprayed with 0.04% bromocresol green in ethanol (adjusted to a slight blue colouration), in order to show acidic spots (yellow).
Amino acid analysis

The culture fluid from cells grown on dodecane, tridecane, and kerosene was analysed for amino acids by descending paper chromatography on Whatman No. 1 filter paper. Unused medium was spotted as a control, and a mixture of glutamic acid and lysine as standards. The chromatogram was run with butanol:acetic acid:water (4:1:2), and spots visualized by spraying with a standard ninhydrin solution.

Materials balance experiments

Cultural conditions

For the materials balance experiments, the Arthrobacter strain was grown in the hydrocarbon basal medium described previously, with the modification that 7 g/l ammonium sulfate was added. Dodecane was used as the carbon source in a concentration of 10 ml/l (7.49 g/l). The cyclone research fermentor of Dawson (30), equipped with pH and temperature control was used in this study. The fermentor was sterilized at 121°C for 30 minutes before filling with 1,500 ml of sterile medium. The pH electrode was sterilized separately in 500 ppm sodium hypochlorite followed by rinsing with sterile distilled water. An actively growing 5% inoculum, grown 24 hours on the shaker, was used. Medium was circulated through the fermentor with a Cole-Parmer magnetic drive pump. The temperature was maintained at 30°C by circulating water through the jacketed side arm of the fermentor.

The pH of the medium was kept at 6.85 ± 0.05 by addition of sterile 0.5 N sodium hydroxide or 0.5 N hydrochloric acid by a Fermentation Design pH control unit connected to an Ingold sterilizable electrode.
The control unit was equipped with peristaltic pumps for both acid and base and a continuous pH recorder. The addition of acid or base was monitored with a Rustrak recording ammeter connected to the control unit. Sterile air was pumped through a port in the side of the fermentor at a rate of 1.8 l/min. The effluent air was bubbled through a collecting vessel in order to trap specific gases. Samples were removed aseptically at suitable times for analyses. The set up of the apparatus can be seen in Figure 6.

Analytical procedures

Growth and acidity

Growth was measured as dry weight. The acidity was calculated over 1 hour intervals from the Rustrak recording ammeter and the volume of sodium hydroxide or hydrochloric acid added from the reservoirs.

Residual dodecane

To a 10 ml sample of the fermentation broth was added 50μl of tridecane as an internal standard. Two ml of n-hexane was added and mixed in to extract the hydrocarbons. The resulting emulsion was centrifuged and the hexane layer was removed and dried over anhydrous sodium sulfate. The extract was analysed using the gas chromatograph and column described previously, with the temperature programmed isothermally at 100°C for 1.5 minutes, and then increased linearly to 172°C at 12°C/min. The dodecane was determined quantitatively by the ratio of its peak area to the area of the tridecane as measured by the integrator, using a calibration curve of peak area ratio versus weight ratio.
Figure 6. Equipment set-up in fermentation experiments.
1. recording ammeter. 2. pH control unit.
3. air sterilizing filter. 4. cyclone fermentor.
5. pH electrode. 6. effluent gas trap.
7. sodium hydroxide reservoir. 8. medium circulating pump. 9. temperature control water jacket. 10. temperature control water bath.
Dissolved bicarbonate

A 3 ml sample of medium was placed in a Warburg flask with 0.5 ml of 3 N sulfuric acid in the side arm. The flask was attached to a Gilson Differential Respirometer, equilibrated at 30°C and the acid tipped in from the side arm. The volume of carbon dioxide gas released was read directly from the manometer. Under these conditions, 24.6 μl carbon dioxide represent 1 millimole.

Carbon dioxide gas

The carbon dioxide in the effluent air was trapped in a vessel containing 3 N potassium hydroxide. The air going into the fermentor was scrubbed free of carbon dioxide by passing through a similar trap. The carbon dioxide retained in the effluent trap was measured by removing a sample, adjusting it to pH 7-8 with 1 N hydrochloric acid and making a suitable dilution (usually 1:25) with CO₂-free distilled water. The carbon dioxide was then determined in the same way as dissolved bicarbonate, given above.

Cell carbon

The amount of carbon in the cells was determined by centrifuging the cells from 10 ml of medium, washing twice in phosphate buffer (0.05 M, pH 7.0), and resuspending in 5 ml of CO₂-free distilled water. The carbon content was measured by injecting the cell suspension, previously homogenized with a Potter homogenizer (77), into a Beckman Model 915 Total Organic Carbon Analyser. Oxalic acid solution was used to construct a calibration curve.
Nitrogen analysis

For cell nitrogen, the cells were centrifuged from the medium, washed twice in phosphate buffer (0.05 M, pH 7.0), and resuspended in distilled water. Total nitrogen was determined on medium directly from the fermentor. Both cell and total nitrogen were determined by the micro-Kjeldahl method given in A.O.A.C. (49). Ammonia nitrogen was determined on the medium after centrifugation by using the micro-Kjeldahl method from the distillation step onwards. Nitrate was determined by the phenoldisulfonic acid method and nitrite by the naphthylamine-sulfanilic acid method (7).

Volatile amines

The effluent air from the fermentor was checked for volatile amines by bubbling the air through a trap containing 3 N sulfuric acid. The air entering the fermentor was passed through a similar trap. The effluent trap was analysed for nitrogenous organic compounds by the micro-Kjeldahl method.

Nitrogen oxide gases

Gaseous nitrogen oxides in the effluent air were collected in a trap containing 175 ml of 5 percent potassium hydroxide and 25 ml of 3 percent hydrogen peroxide. The peroxide was added to convert all the nitrogen oxides collected to nitrate (43). Nitrate in the trap was determined by the phenoldisulfonic acid method (7).
RESULTS AND DISCUSSION

Classification

The culture used in this study was isolated from a water cooling tower, Ioco Refinery, Imperial Oil Ltd., by plating on hydrocarbon basal medium with kerosene as the sole carbon source. This organism was chosen for investigation because it produced a high acidity on the kerosene medium - the colour of an added pH indicator (bromcresol green) was changed within 24 hours. Growth on agar led to small (1 - 2 mm.), circular, convex colonies with the margin entire. Streaks on agar slants resulted in filiform growth. No pigment was produced; the colonies were opaque and white to off-white. Broth media showed a light turbidity and sediment with little surface growth. The optimum growth temperature was near 30°C, with no growth occurring at 37°C.

Microscopic examination showed the cells to be non-motile, without endospores or capsules being formed. They were not acid fast when stained by the Ziehl-Neelson method (27). The culture displayed a high degree of pleomorphism. In young cultures there were numerous long rods and some filaments. Filaments were more common in broth media than on solid media. As the culture aged, rods and filaments were observed to fragment into shorter rods, usually within 24 hours on high nutrient media. These rods slowly transformed into coccoid shaped cells which predominate in old cultures. The coccoid cells were 0.8 - 1.0\(\mu\) in diameter, smaller rods were 1.0 \times 3.0\(\mu\), with longer ones up to 10\(\mu\), and some filaments were longer than 50\(\mu\). The culture was Gram variable-rods and filaments tended to be Gram positive, cocci tended to be Gram negative. The organism had a similar morphology on kerosene medium but
rod and filamentous forms lasted longer, up to 4 or 5 days. Occasional swollen forms were also observed. These morphological features can be seen in Figure 7, which shows filaments, some at different stages of fragmentation, different size rods and coccoid forms. The morphological cycle of this culture fits the descriptions of *Arthrobacter* given by Mulder (76) and Stevenson (99). Most arthrobacters do not grow at 37°C and are Gram variable (20). Mycobacteria and nocardia can have similar morphology, but the mycobacteria are acid fast while the nocardia are branched and usually mycelial. The organisms in both of these genera generally grow well at 37°C, are Gram positive, and fragment much later in the growth cycle. For these reasons, the culture examined in this study was identified as a species of *Arthrobacter*.

**Characterization of hydrocarbon specificity**

In order to determine which components of the kerosene were used by the organism, the main n-alkane fractions in the Fisher's odorless kerosene were identified by comparing their retention times in the gas chromatograph with those of pure n-alkanes. The gas chromatogram which was obtained is shown in Figure 8. The quantitative analysis of each component is listed in Table II. It can be seen that the major n-alkanes in this lot of kerosene are undecane, dodecane and tridecane, with lesser amounts of decane, tetradecane and pentadecane. Since 72.5% of the kerosene is n-alkane, the remainder is probably made up of branched chain alkanes and olefins.

Because microorganisms are known to metabolize normal alkanes much more readily than branched and olefinic hydrocarbons (108) it was decided to measure the disappearance of the different n-alkanes during growth. The composition of the kerosene from a 5 day growth flask, compared to
Figure 7. Cells of *Arthrobacter* spp. grown in glucose-nutrient broth.
1. filamentous forms. 2. swollen rods. 3. fragmented filament. 4. typical rod. 5. typical coccoid forms.
Figure 8. Gas chromatographic analysis of Fisher odorless kerosene. 
$C_9$ = nonane; $C_{10}$ = decane; $C_{11}$ = undecane; 
$C_{12}$ = dodecane; $C_{13}$ = tridecane; $C_{14}$ = tetradecane; 
$C_{15}$ = pentadecane; $C_{16}$ = hexadecane.
Table II. Normal Alkanes in Kerosene

<table>
<thead>
<tr>
<th>n-alkane</th>
<th>Percent in Kerosene</th>
<th>Percent in Control</th>
<th>Percent after Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>nonane</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>decane</td>
<td>5.4</td>
<td>1.2</td>
<td>2.4</td>
</tr>
<tr>
<td>undecane</td>
<td>19.3</td>
<td>14.7</td>
<td>15.3</td>
</tr>
<tr>
<td>dodecane</td>
<td>18.0</td>
<td>19.3</td>
<td>17.7</td>
</tr>
<tr>
<td>tridecane</td>
<td>16.8</td>
<td>18.5</td>
<td>17.6</td>
</tr>
<tr>
<td>tetradecane</td>
<td>9.5</td>
<td>11.5</td>
<td>8.5</td>
</tr>
<tr>
<td>pentadecane</td>
<td>3.1</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>hexadecane</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>72.5</td>
<td>68.6</td>
<td>64.9</td>
</tr>
</tbody>
</table>
a sterile control, is given in Table II. It can be seen that the control flask lost a significant amount of decane and undecane due to evaporation. As well as the evaporation of decane and undecane, the growth flask shows a drop in dodecane, tridecane and tetradecane due to microbial oxidation.

In order to get a more precise measurement of which n-alkanes the organism could use most readily, the oxygen uptake of the culture was measured in the presence of the n-alkanes. The plots in Figures 9 and 10 indicate that dodecane and tridecane are the most rapidly oxidized by this organism.

Because this study was concerned with the acid production of this organism and not just growth, the pH drop and dry weight were determined on the various n-alkane carbon sources. The results in Figures 11 and 12 indicate that as well as supporting the greatest amount of growth, dodecane and tridecane also lead to the greatest acid production. Therefore these hydrocarbons were selected for further investigation.

The growth curves of this *Arthrobacter* strain on dodecane, tridecane and kerosene (Figures 13 & 14) indicate that dodecane is utilized most rapidly for growth and acid production. The growth on tridecane may be slower to initiate because the culture must adapt to an uneven chain length i.e. $\beta$ -oxidation of an odd carbon number substrate leading to propionate formation. The culture probably requires a longer period to synthesize enzymes to metabolize this latter substrate.

From the abrupt halt in growth it can be seen that 4.5 is the limiting pH. The slower growth and acid production observed on kerosene
Figure 9. Oxygen uptake of *Arthrobacter* culture on various *n*-alkanes

\( \nabla \) = endogenous; \( \bullet \) = nonane; \( \Delta \) = decane; \( \square \) = undecane;
\( \triangledown \) = dodecane; \( \circ \) = tridecane; \( \blacksquare \) = tetradecane; \( \triangledown \) = hexadecane.
Figure 10. Oxygen uptake of *Arthrobacter* culture as a function of n-alkane chain length. 3.3 mg cells (dry weight) per flask.
Figure 11. pH drop in shake flask medium as a function of n-alkane chain length. Hydrocarbon numbers indicate n-alkane chain length; K indicates kerosene.

Figure 12. Growth in shake flask culture as a function of n-alkane chain length. Hydrocarbon numbers indicate n-alkane chain length; K indicates kerosene.
Figure 13. Growth curve of *Arthrobacter* culture on different hydrocarbon carbon sources.

- □ = dodecane; ○ = tridecane; △ = kerosene.
Figure 14. pH curve of medium after growth on different hydrocarbons.

□ = dodecane; ○ = tridecane; △ = kerosene.
substrate probably results from the lower concentration of the more readily metabolized alkanes in the mixture. It may also be due to inhibition by other hydrocarbons in the kerosene.

Characterization of acid production

The thin layer chromatograms of the acidic lipid extract from the medium of dodecane, tridecane and kerosene grown cells are shown in Figures 15 and 16. It can be seen that there are no spots corresponding to mono or dicarboxylic acids from the dodecane and tridecane extracts. The kerosene extract contained a small amount of fatty acid, and possibly some dicarboxylic acid. To analyse these further, this extract was methylated and injected into a gas chromatograph, giving the chromatogram shown in Figure 17. Only two peaks could be identified (other than ether) by their retention times - the methyl esters of octanoic and nonanoic acids. Since this is a highly concentrated extract (>200 X) analysed at very high sensitivity (attenuation X50 on the gas chromatograph), none of these compounds is present in a significant concentration. The other peaks found probably represent minor co-oxidation products from the other kerosene components and small amounts of ketones or aldehydes carried through in the extraction.

Since some organisms grown on n-alkane hydrocarbons produce organic acids such as citric and fumaric acids extracellularly (1), the presence of such compounds was tested by thin layer chromatography (Figure 18). The only spots which appeared in the growth media were also present in the unused medium. These are due to components in the yeast extract used in the medium.
Figure 15. Thin layer chromatogram of the acidic lipid extract sprayed with bromcresol green.
1. dodecane medium extract. 2. tridecane medium extract. 3. kerosene medium extract. 4. decanoic acid. 5. sebacic acid. 6. unused medium.
Cross-hatched areas represent strongly acidic spots.
Figure 16. Thin layer chromatogram of the acidic lipid extract, charred with sulfuric acid.
1. sebacic acid. 2. decanoic acid. 3. kerosene medium extract. 4. dodecane medium extract.
5. tridecane medium extract. 6. unused medium.
Cross-hatched areas represent darkly charred spots.
Figure 17. Gas chromatogram of methylated extract from kerosene medium.

$S = \text{solvent (ether)}$; $C_8 = \text{methyl octanoate}$;

$C_9 = \text{methyl nonanoate.}$
Figure 18. Thin layer chromatogram for the separation of organic acids.
1. tartaric (T) and adipic (A) acid standards. 2. dodecane growth medium. 3. tridecane growth medium. 4. kerosene growth medium. 5. unused medium.
After the possibility of fatty acid and organic acid formation had been eliminated, the presence of acidic amino acids in the growth medium was investigated. Paper chromatograms of the growth medium showed there were no ninhydrin positive compounds present. Before spraying, the chromatogram was viewed under ultraviolet light to check for the presence of nucleotides. None were visible.

**Materials balance experiment**

Because the acidity produced by this culture was not due to the formation of acidic organic compounds, it was most likely the result of the "physiological acidity" of the ammonium salts nitrogen source (56). This acidity results from the assimilation of ammonium ions by the organism for amino acid and nucleotide synthesis. As ammonium ions are taken up by the cells hydrogen ions are released to maintain an ionic balance in the medium.

In order to determine if this was the origin of the acidity, the *Arthrobacter* strain was grown in a fermentor with pH control. Higher nitrogen concentration was used in the medium so that it would not be limiting. The changes in the carbon and nitrogen components in the system were followed during the course of the fermentation, along with growth and acidity.

As can be seen in Figure 19, the cell dry weight increases rapidly after a lag of about 5 hours. After 24 hours, the growth stopped abruptly and cell dry weight declined slowly after that. Carbon dioxide gas was produced most rapidly during the active growth stage of the culture, with the highest rate occurring just before the end of growth.
Figure 19. Change in substrate concentration and growth parameters during fermentation. 

Δ = dodecane; ○ = dry weight; □ = acidity; 
■ = carbon dioxide.
The concentration of dissolved bicarbonate did not reach a significantly high level. The dodecane in the medium was utilized rapidly. It was completely depleted after 24 hours. The exhaustion of the carbon and energy source at this time caused the culture to stop growth and metabolism.

The overall carbon balance for the system is plotted in Figure 20. The net loss of carbon during the growth phase was probably due to the inefficiency of the trap for carbon dioxide gas. Most of the carbon dioxide escaped into the atmosphere. More traps would be required to catch this lost carbon dioxide but this could not be done because the aerating pump was not powerful enough to force the air through more than one trap in series.

The changes in cellular nitrogen, ammonia nitrogen in the medium and total nitrogen in the medium are shown in Figure 21. The cell nitrogen and ammonia nitrogen curves follow the growth of the organisms up to about 19 hours. At this time there is a sudden drop in the ammonia nitrogen and in the total nitrogen of the system. This sudden loss of nitrogen coincides with an anomalous increase in acid production. Up till this point the titratable acidity is proportional to nitrogen uptake and growth, but at 19 hours there is a sharp increase in the titratable acidity, which continues until 24 hours. After the 24 hour point, the acidity drops rapidly and loss of nitrogen ceases, which corresponds with the end of growth and exhaustion of the dodecane carbon source. The nitrogen loss amounts to $0.21 \pm 0.02 \text{ g/l}$, or $15 \pm 2 \text{ meq/l}$. The anomalous increase in acidity is about 14 meq/l above the acidity due to growth alone.
Figure 20. Carbon balance during fermentation.

- O = cell carbon;
- • = carbon dioxide carbon;
- □ = dodecane carbon;
- ■ = total carbon.

TIME (hours)
Figure 21. Nitrogen balance during fermentation. 
〇 = cell nitrogen; ◊ = ammonia nitrogen; 
□ = total nitrogen.
In order to account for this loss of nitrogen from the system, the effluent gases were passed through an acid trap to collect volatile nitrogen compounds—amines or ammonia. It was not expected that any would be found because the pH of the medium was below the pK of amines (pK_a = 10-11) and ammonia (pK_a = 10.25) and therefore these compounds would not be volatile. No nitrogen compounds were found in the acid trap.

In view of the studies of Gunner (43) which showed that *Arthrobacter globiformis* could oxidize ammonium ions to nitrogen oxide gases and nitrate and reports of ammonium ion oxidation by methane grown bacteria (54, 111), the possibility of this oxidation occurring during the fermentation was investigated. The production of nitrogen oxide gases was checked by passing the effluent gas from the fermentor through the trapping solution used by Gunner, and analysing it for nitrate. The results showed that the trap contained 66 mg of nitrate nitrogen, equivalent to 44 mg/l from the fermentation medium. This represents about 21% of the nitrogen lost from the medium.

The nitrate nitrogen concentration in the medium was found to be 37 mg/l, about 18% of the decrease in the Kjeldahl nitrogen. This figure for nitrate nitrogen is of questionable accuracy, since there may have been interference in the determination due to chloride ions and organic matter. Because nitrate is not quantitatively measured by the Kjeldahl method, it would be part of the drop in total nitrogen during the fermentation.

These results show that this *Arthrobacter* strain can oxidize ammonium ions in the medium. This oxidation takes place in the rapid log phase of active growth of the culture, when the dodecane carbon source is
becoming depleted. This is similar to the system described by Gunner (43) in which an Arthrobacter globiformis strain, which was grown on succinate carbon source, oxidized ammonium ions when suspended in buffer without carbon nutrient source.

The oxidation of ammonium ions described here is the first demonstration of this phenomenon in an n-alkane carbon source fermentation and raises a number of interesting questions about the pathways and mechanisms involved. The oxidation probably occurs via the inorganic heterotrophic pathway as demonstrated in A. globiformis. This pathway is the only one which has been shown to yield gaseous products. There is the possibility that the culture uses this pathway as a source of energy as the carbon source becomes exhausted. Since the initial oxidation of ammonium to hydroxylamine is endergonic (ΔF' = +3.85 kcal/mole) (2) subsequent oxidation steps, for example the dehydrogenation of hydroxylamine to the postulated nitroxyl (NOH) intermediate would have to be coupled with an electron transport system to produce ATP. It may be that the oxidation is only partially completed due to a lack of the required enzyme systems, leading to the non-enzymatic formation of nitrous oxide as demonstrated by Anderson (10). Even if the culture were capable of deriving some energy from this oxidation, it cannot fix carbon dioxide as occurs in Nitrosomonas and Nitrobacter and therefore growth stops when the carbon source is exhausted. This would occur at a stage of the rapid logarithmic growth phase before energy and carbon storage reserve materials are formed.

Although there are some similarities between the growth of microorganisms on methane and higher n-alkane carbon sources, it is not likely
that the initial ammonia oxidation step of this *Arthrobacter* culture is the same as in the methane utilizing bacteria. In the latter organisms, ammonium ions are oxidized by the methane oxidase enzyme competitively with methane (113). This phenomenon is probably due to the similarity between the geometry of the \( \text{CH}_4 \) molecule and \( \text{NH}_4^+ \) ion. Conversely, there is less similarity between the ammonium ion and the terminal methyl group of an \( \text{n-alkane} \). Also, methane oxidase is now believed to operate by a mechanism which differs from other hydrocarbon oxidase enzymes (113).

The oxidation of ammonium ions can be regarded as a form of the co-oxidation reaction which has been observed in other hydrocarbon systems (68, 29, 85). Hydrocarbon co-oxidations are characterized by the transformation of a non-growth supporting hydrocarbon to an oxygenated product while the culture grows on another substrate, either hydrocarbon or non-hydrocarbon. This oxidation can be a side reaction of the oxygenase enzyme for the growth supporting hydrocarbon, or it can be catalysed by a separate inducible enzyme.

Further research is needed to determine the relation of ammonium ion oxidation to hydrocarbon fermentations. It would be interesting to answer questions concerning how widespread this reaction is in \( \text{n-alkane} \) fermentations, what pathways and mechanisms are involved, and what value this reaction could be in industrial fermentations, for example, could an organism be found which would selectively oxidize a substituted amino group to form a product? These problems require further investigations to provide answers.
CONCLUSIONS

The culture isolated in this study was a member of the genus Arthrobacter. It was capable of rapid growth and acid production on a number of hydrocarbon carbon sources in a nutrient salts medium. While capable of good growth on refined kerosene carbon source, the Arthrobacter spp. grew best on the purified n-alkanes, dodecane and tridecane.

The acidity generated by the culture was not due to the formation of acidic compounds such as fatty acids, organic acids or amino acids. It was due to the physiological acidity of the ammonium ion nitrogen source - as ammonium ions are taken up by the culture, acidic inorganic anions are left in the medium. When grown under conditions of pH control and limited hydrocarbon concentration, this culture oxidized ammonium ions to form nitrate in the medium and nitrogen oxide gases. This is the first time this oxidation has been observed in an n-alkane carbon source system. Oxidation of ammonium ions may be a minor source of energy for the culture when the carbon source is depleted, or may be a co-oxidation reaction not yielding energy.


