# A STUDY OF THE BACTERIAL UTILIZATION OF HYDROCARBONS

by

HERBERT FRANK HAAS

B. S., Kansas State College
of Agriculture and Applied Science, 1938

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A THESIS

submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1940

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# INTRODUCTION

The state of knowledge of the biological utilization of hydrocarbons, both as a source of energy for growth and a source of carbon in the synthesis of protoplasm, is very fragmentary. To the present time, the listerature on this subject has been primarily of a survey nature. Various investigators have described the results which have been obtained when microorganisms were subjected to an environment in which hydrocarbons were their only source of energy. By this means they have proved that a biological oxidation of these compounds can occur.

It is interesting to note that the first record of hydrocarbon-utilizing organisms deals with the simplest homologue of the paraffin series. Sohngen (1906) published a paper on the utilization of methane by a "methane bacillus." Subsequent work revealed the breakdown of solid paraffin by bacteria (Sohngen, 1913; Buttner, 1926; and Haag, 1926). From this, it is evident that the physical state has little effect upon the ability of the organisms to break down the hydrocarbon molecules in order to release the energy and carbon which they contain. Gaseous, liquid, and solid hydrocarbons have all been subjected to bacterial decomposition.

Hydrocarbons, as a class, represent compounds with an excellent store of energy. Some idea of the available energy may be secured by comparing the heat of combustion of some six carbon compounds. For example, glucose has 674,000 calories, as compared to 1,002,400 calories in hexane. The high energy content of hydrocarbons is due to the highly reduced state of the carbon atoms in the moledules.

Since bacterial decomposition of hydrocarbons has been established, the fact must be of significance in considering the carbon cycle. The popular concept of the carbon cycle fails to take into consideration the carbon which is involved in hydrocarbons. This is perhaps due to an oversight, or to a lack of knowledge as to the extent of this type of biological activity. The percentage of carbon in hydrocarbons varies from 80 to 89 per cent; therefore, a significant amount of the world's carbon is combined in this form. Over a billion barrels of crude oil are used each year, and there is evidence for the belief that 20,000,000,000 barrels of oil still remain in the ground in known oil fields. In fact, during 1939, 2,000,000,000 barrels were added to our reserves. It is becoming more firmly established that perhaps the formation of petroleum is still going on today (Egloff, 1940). Investigations have disclosed that there are numerous source beds of petroleum in which organic matter is being

changed by biological action, mainly bacterial. These source beds have been found at the bottom of great bodies of water, such as the Pacific Ocean.

The role of bacteria in the production of hydrocarbons is now widely accepted, although the theory is not substantiated by direct evidence that higher molecular weight hydrocarbons, other than methane, can be produced by bacterial activity (Thayer, 1931).

The decomposition of hydrocarbons by the action of microorganisms is substantiated by some evidence gathered by a few investigators, but there are still many problems regarding this work that remain unsolved.

These problems constituted the primary inducement for this particular undertaking in which we have sought to confirm the biological oxidation of hydrocarbons through respiration studies and, in addition, to conduct a survey of microorganisms capable of utilizing compounds of this nature.

# REVIEW OF LITERATURE

Methane utilizing organisms have been grouped by Orla-Jensen (1909) under the generic designation of Methanomonas. They are monotrichous short rods capable of growing in the absence of organic matter and securing growth energy by the oxidation of methane, forming carbon-dioxide and water.

The first study of this group was by Sohngen (1906) and Kaserer (1906). Sohngen isolated what he called a methane bacillus which was used by Orla-Jensen (1909) as the type culture for the genus Methanomonas and renamed as Methanomonas methanica.

Munz (1915) isolated and studied a methane oxidizing organism which differed somewhat from Sohngen's organism, in that it was smaller, elliptical to cylindrical in shape, and non-motile. He classed it as a facultative autotrope.

Stormer (1907) isolated an organism which he called B. hexcarbovorum, that was able to utilize toluene, xylol, and illuminating gas, in addition to methane, as a source of carbon.

Organisms resembling B. fluorescens liquefaciens, as described by Lehmann and Neuman (1896), were isolated from swamp rice soils by Aiyer (1920). These cultures utilized methane and oxidized it completely to carbon dioxide and

water. The organisms would lose this methane-utilizing ability if cultivated on ordinary culture media. He found it necessary to culture them on silica-jell plates; it was found that a solution of mineral salts and agar would not support growth.

Sohngen (1913) made a very valuable contribution in this field. He reported that gasoline, kerosene, paraffin oil, and paraffin wax could be used by many classes of bacteria as a source of carbon and energy. The hydrocarbon compounds were oxidized to carbon dioxide, water, and a trace of organic acids. These interesting organisms were isolated from garden soil, ditch water, and compost. They were largely distributed between the genera Mycobacterium and Pseudomonas. He particularly emphasized the ability of certain members of the genus Mycobacterium to utilize paraffin wax. The cultures described by him are as follows: Mycobacterium phlei, M. Lacticola, M. album, M. luteum, M. rubrum, and M. hyolinum.

The other organisms which largely belong to the genus Pseudomonas, and have the ability to utilize various hydrocarbons such as kerosene, paraffin oil, and paraffin wax, are named by Sohngen (1913) as follows: B. fluorescens liquefaciens, B. fluorescens non-liquefaciens, B. pyocyaneum, B. punctatum, and B. lipolyticum. Only one member of the

genus Micrococci was described by him. He designated it as Micrococcus paraffinae.

Tausz and Peter (1919) described three new hydrocarbon-utilizing bacteria which they isolated from garden soil by means of a hydrocarbon-inorganic mineral medium, to which a little water-soluble extract from leaves and grass were added for supplemental factors. The following names were assigned to their cultures: Bacterium aliphaticum, Bacterium aliphaticum liquefaciens, and Paraffin bacterium. The hydrocarbons used in the enrichment cultures were hexane, cyclohexane and dimethycyclohexane, and paraffin oil respectively.

The first two species, B. aliphaticum and B. aliphaticum liquefaciens, were inert towards cyclic hydrocarbons and hexylene, but attacked paraffinic compounds such as n-hexane, n-octane, di-methyloctane, n-hexadecane, tricontane, tetratricontane, and other olefinic compounds such as n-caprylene and hexadecylene. The latter organism, Paraffin bacterium, is without effect upon naphthenes, benzoid hydrocarbons, and some paraffins (such as n-hexane and n-octane), but attack higher paraffins (such as nexadecane, tricontane, and tetratricontane).

Since the organisms seem to prefer aliphatic compounds over the cyclic hydrocarbons, these authors have taken advantage of this specificity in purifying naphthenic or cyclic

hydrocarbons. They allowed cultures of the hydrocarbon-utilizing bacteria to act upon mixtures of cyclic and aliphatic hydrocarbons. The bacteria assimilated all of the aliphatic compounds, leaving nothing but naphthenic compounds. They also devised a qualitative test for aliphatic hydrocarbons on this basis. The presence of very small portions of aliphatic compounds in natural oils or artificial mixtures is indicated by clouding, due to bacterial growth in the media containing the hydrocarbon under consideration.

Matthews (1924) worked on the partial sterilization of soil with various hydrocarbons and aromatic compounds. She noted that when these compounds were added to soil, there was not a specific bacterial response for each one, but that a single bacterium appeared to be able to flourish on a variety of hydrocarbons such as pinene, naphthalene, and pseudocumene. Bacillus liquefaciens is given as an example, as there seems to be very few substances that this organism will not attack or tolerate. Therefore, she resorted to total bacterial counts on soil treated with various compounds such as benzene, naphthalene, toluene, phenol, xylene, hexane, pseudocumene, mesitylene, cymene, and pinene. Increased bacterial counts per gram of soil were noticed after the addition of each compound. The increase in counts was was corrolated with increases in molecular weights and heat

of combustion of each compound. These increases were not due to the destruction of protozoa in the soil, since she took precautions to eliminate this factor.

The work of matthews (1924) was continued by Gray and Thorton (1928) at the Rothamsted Experimental Station, England. They isolated various organisms capable of decomposing aromatic compounds such as naphthalene, toluene, cresol, and phenol. Organisms belonging to the following genera were isolated: Micrococcus, Mycobacterium, Bacterium, Bacillus, and Spirillum.

A paraffin wax-mineral salts solution for the isolation of saprophytic members of the genus Mycobacterium was used successfully by Buttner (1926) and advocated by him as an enrichment medium. He isolated twelve strains of Mycobacterium species, most of which resembled M. phlei, and found that several species of Actinomycetes were also capable of this activity.

Haag (1926) noted that the utilization of commercial paraffin wax by mycobacteria was corrolated with the iodine number, indicating that the organisms attack the unsaturated bonds in the molecules of the paraffins. Haag (1927) used paraffin utilization as a basis of separation of Mycobacterium from Corynebacterium species. He found that mycobacteria could utilize paraffin while the corynebacteria could

not.

Jensen (1934) studied saprophytic mycobacteria and corynebacteria and confirmed Haag's (1927) work. He found that some species of Mycobacterium, such as M. lacticola, would use paraffin, but that Corynebacterium species, such as Cory. helvolum and Cory. simplex, could not utilize paraffin.

Oil-bearing regions, such as the great Baku oil field of Russia, was found by Tauson (1929) to be rich in a great variety of microorganisms which utilize hydrocarbons. He found three species of bacteria which he designated as Bacterium naphthalinicus, B. naphthalinicus liquefaciens, and B. naphthalinicus nonliquefaciens, which could utilize naphthalene as a source of carbon. An organism, B. phenanthrenicus, was also isolated by him which could attack phenanthrene easily. An organism, B. benzoli, capable of utilizing benzene, toluene, and xylene, was also reported. He also made a study of the oxidation of benzene hydrocarbon derivities by an organism he designated as B. toluolicum.

A coccus or cocco-bacillus was reported by Lipman and Greenberg (1932) as being isolated from petroleum, obtained at a depth of 8,700 feet. This organism was capable of oxidizing petroleum completely to carbon dioxide.

The action of mixed cultures upon lubricating oils was determined by Tauson and Schapira (1934). They observed an

increase in the refractive index and saponification numbers. The iodine number of the oils decreased as bacterial action progressed.

The effect of leaking natural gas upon soil fertility was noted by Harper (1939). He believed that the increased nitrogen content of soil, permeated by natural gas, was due to nitrogen fixation by various clostridia in the soil, especially since some nitrogen-fixing anaerobes were isolated, one of which was Clostridium butyricus.

Organisms capable of decomposing kerosene into methane and possibly ethane were isolated by Thaysen (1940) from the water at the bottom of a kerosene storage tank, which had ignited spontaneously. One of the organisms isolated was a short, monotricous rod, which was capable of fermenting kerosene to methane, acetaldehyde, lactic and acetic acid. It was suggested by him that organisms could have produced gaseous hydrocarbons which would set up an explosive mixture when combined with the proper amount of air.

Microorganisms that are capable of attacking petroleum products were obtained from soil by Stone, White, and Fenske (1940), by means of a medium containing mineral salts and petroleum. The organisms were not identified, but were described as gram-negative rods which form white, iridescent or yellowish-green colonies on nutrient agar. Various frac-

tions of petroleum were prepared and subjected to bacterial action. They found that the oils high in paraffinic hydrocarbons were more readily assimilated than those containing a high percentage of aromatic compounds. The naphthenic fractions occupied an intermediate position.

# CULTURAL METHODS

## Review of Literature

The most successful method of isolating organisms, which are capable of utilizing hydrocarbons, is the use of a mineral salts-hydrocarbon enrichment medium, in which the hydrocarbon is the only source of carbon and energy for the bacterial cells. This technique was used, with minor modifications, by Sohngen (1913), Tausz and Peters (1919), Tauson (1929), Buttner (1926), Haag (1926), Jensen (1934), and Gray and Thorton (1928).

It is interesting to observe that the media used by various workers in the field was quite simple in composition. Sohngen (1913) used a medium composed of the following constituents:

| | |
|---|---|
| Tap or distilled water | 100 cc. |
| $K_2HPO_4$ | 0.5 g. |
| $NH_4Cl$ | 0.5 g. |
| $CaCO_3$ | Trace |

To this medium was added a few drops of hydrocarbon. If the hydrocarbon was a solid like paraffin wax, he heated the media, and added melted paraffin. By shaking thoroughly and cooling quickly, he obtained the paraffin in a finely

dispersed state which would offer more surface area of exposure to bacterial action.

Buttner (1926) used the same salts as Sohngen (1913) but added $MgSO_4$ (0.5 g.).

Haag (1926) determined the influence of various ions and found that the following medium was best for the growth of Mycobacterium species:

| | |
|---|---|
| Carbon source (variable) | 1.0 % |
| $NH_4Cl$ | 0.5 % |
| $CaHPO_4$ | 0.5 % |
| $MgSO_4$ | 0.02 % |

Tausz and Peter (1919) used different salts. Their medium was composed of the following salts, dissolved in ditch water:

| | |
|---|---|
| $MgNH_4PO_4$ | 0.1 % |
| $K_2HPO_4$ | 0.08 % |
| $CaSO_4$ | 0.01 % |
| NaCl | Trace |
| KI | Trace |
| FeCl | Trace |

The various media described above were made to a semi-solid state by the addition of washed agar or silica-jell.

Aiyer (1920) claimed that the use of agar interferes with the utilization of methane by his culture of B. fluor-

escens liquefaciens. He found it necessary to use silica-jell.

## Cultural Methods Employed

The medium used in this work, which proved to be quite satisfactory, is composed of the following salts dissolved in one liter of distilled water:

| | | |
|---|---|---|
| $MgSO_4$ | 0.2 | g. |
| $CaCl_2$ | 0.02 | g. |
| $KH_2PO_4$ | 1.0 | g. |
| $K_2HPO_4$ | 1.0 | g. |
| $KH_4NO_3$ or $(NH_4)_2SO_4$ | 1.0 | g. |

The medium is neutralized to approximately pH 7.0 to 7.2 with dilute NaOH. Two per cent of washed agar was added whenever a semi-solid medium was needed.

Cultural Method No. 1. Source material was plated on nutrient agar and colonies were picked, thereby securing pure cultures immediately. These cultures were then placed in various media described below, in which the hydrocarbon was the only source of energy and carbon.

Cultural Method No. 2. Plates were made with mineral salts agar and streaked with source material, or pure cul-

tures. If volatile hydrocarbons were used, such as gasoline, the petri dishes were inverted and the hydrocarbon material poured into the lid. This is a modification of Sohngen's (1913) method. The volatile hydrocarbon vapors were sufficient to support bacterial growth.

If relatively non-volatile hydrocarbons were used, such as kerosene and light oils, it was poured over the surface of the inoculated agar without interfering with the growth of the cultures.

Cultural Method No. 3. Hydrocarbon material was added to a mineral salts solution, which could be put up in flasks or test tubes. If solid paraffin was to be added, both the medium and paraffin were sterilized separately, and the paraffin added to the medium while in a melted condition. This tends to give a rough, irregular mass of paraffin, which offers sufficient surface for bacterial action.

If liquid hydrocarbon material was used such as kerosene, it could be layered on the surface of the medium. Only a very small quantity of hydrocarbon material was necessary. Approximately one per cent was the most common amount used; however, if a light, volatile hydrocarbon, such as gasoline, was used, thicker layers of hydrocarbon would not affect the growth appreciably by cutting off the oxygen supply. Liquid hydrocarbons, with the exception of viscous oils, do not

retard the diffusion of oxygen. This is probably due to convection currents within the liquid hydrocarbons which carry the oxygen down to the surface of the mineral salts medium. This phenomena was demonstrated by means of a simple experiment, which will be described later.

Cultural Method No. 4. A new technique for isolating and culturing bacteria was devised by Dr. L. D. Bushnell. It consisted of constricting, by means of a Bunsen burner or a blast lamp, the lower third of a test tube, as illustrated in Fig. 1.

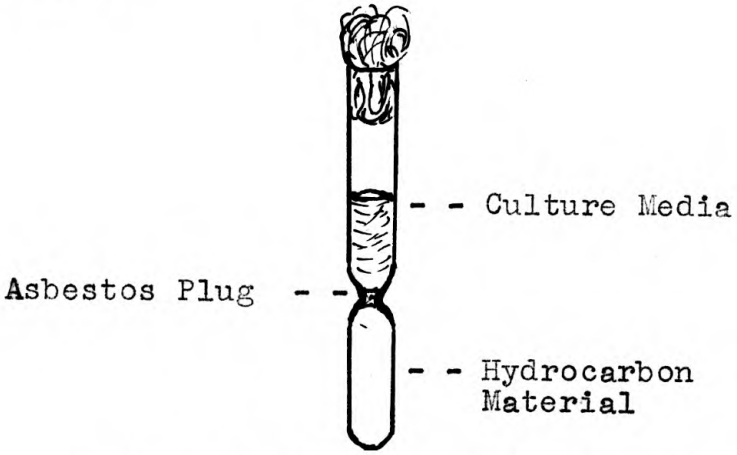


Fig. 1. Constricted Culturing Tube.

If the hydrocarbon material to be used is viscous, such as crude oil, it is placed in the lower portion of the test tube below the constriction. Then the liquid mineral salts medium is added above. If the size of the opening of the constriction is controlled, the upward flow of oil can be regulated. In practice, it was found that crude oil could

be regulated so as to allow about two drops per day to pass upward. This slow translocation of oil permits a larger surface area of the oil to be exposed to the culture medium. This technique was particularly valuable in isolating organisms from crude oil.

If lighter and less viscous hydrocarbons are used, a porus plug of asbestos fiber will retard the flow of the hydrocarbon sufficiently to make the method practical for cultural purposes.

The major disadvantage in this method is that all the components are to be sterilized separately and added to the tubes aseptically.

All the above-described methods were used successfully at one time or another throughout this investigation.

### Experiment to Determine the Effect of Various Hydrocarbons Upon the Oxygen Supply of the Cultures.

Due to the low specific gravity of hydrocarbons, all media containing these products have their surfaces covered by a hydrocarbon layer. This layer might be effective in retarding the diffusion of atmospheric oxygen into the medium; therefore, the following simple experiment was carried out to determine, in a biological manner, whether or not the oxygen supply was being reduced by the presence of

hydrocarbons.

To a series of test tubes containing a washed suspension of bacterial cells and dilute methylene blue solution, was added various quantities of petroleum products. As the bacterial suspension used up the dissolved oxygen in the medium, the methylene blue would be reduced, indicating that the cells were using up the dissolved oxygen more rapidly than it was entering the solution. By comparing these tubes with a control tube, which did not contain hydrocarbon material, the effect on oxygen diffusion could be ascertained.

The results indicated that layers of gasoline, Skellysolve, or kerosene did not affect the diffusion of oxygen into the medium, even though the layers were several centimeters in depth. Light and heavy oils reduced the oxygen supply somewhat, but a blue zone (0.25 cm.), indicating the presence of oxygen, was present for over eight hours.

It is interesting to note that the organisms isolated from the bottom of large storage tanks containing petroleum products were largely of the aerobic or facultative anaerobic type, indicating that aerobic conditions probably exist in the bottom of the storage tanks, due to convection currents.

## Effect of Hydrogen Ion Concentration On Growth of Hydrocarbon-Utilizing Bacteria

The influence of the hydrogen-ion concentration of the media was determined by arranging a series of mineral-salts-agar media, ranging in pH from 6.0 to 9.0, and streaking the plates with various cultures. Three Pseudomonas species were used, one of which was Pseudomonas aeruginosa, and another organism which was a slow, lactose fermenting strain.

The results indicated that the organisms were not extremely sensitive to changes in hydrogen-ion concentration, since they grew well in media that had a pH of 6.5 to 9.0. This is not in harmony with Tauson's (1929) findings, in which he claimed that the medium must be as neutral as possible. He used the same type of organisms, namely members of the Pseudomonas group; however, the organisms are not as fastidious as he indicates.

Table 1. Effect of pH on hydrocarbon utilizing cultures

| | pH Range | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Culture | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 | 8.5 | 9.0 | 9.5 |
| Slow Lactose Fermenting | + | +++ | ++++ | ++ | +++ | ++++ | +++ | ++ |
| Ps. # 8 | + | ++++ | +++++ | ++++ | ++++ | ++++ | +++ | ++ |
| Ps. Pyo. | ++ | ++++ | +++++ | +++++ | ++++ | ++++ | ++++ | +++ |
| Ps. Aerug. | ++ | ++++ | +++++ | ++++ | ++++ | ++++ | ++++ | ++ |

# CULTURAL STUDIES

## Review of Literature

The first cultural studies reported in the literature were by Sohngen (1906) and Kaserer (1906), regarding an organism which was capable of utilizing methane. Methanomonas methanica (Sohngen), Orla-Jensen (1909) is described by Bergey (1939) as follows:

> Short rods: 1.5 to 2.0 by 2.0 to 3.0 microns, motile in young cultures by means of a single flagellum. In older cultures nearly spherical. Can be cultivated in an atmosphere composed of one part $CH_4$ and two parts air on washed agar containing the necessary inorganic salts. The growth is membranous.

Stormer (1907) isolated an organism that he called Bacterium hexcarbovorum, which could use methane and other compounds such as benzoid hydrocarbons. No description was given of the organism.

Munz (1915) reported the isolation of an organism similar to Methanomonas methanica, but differing in that it was smaller and non-motile.

Organisms resembling Pseudomonas fluorescens, which were capable of using methane, were isolated by Aiyer (1920).

Sohngen (1913) reported a variety of non-spore-forming organisms which were capable of utilizing benzin (gasoline),

petroleum (kerosene), paraffin oil, and paraffin wax. Most of these organisms possessed the characteristics of the Pseudomonas group of organisms. These organisms are now listed as a species of the genus Pseudomonas (Bergey, 1939).

Organisms belonging to a group of saprophytic Mycobacteria were also found by Sohngen (1913), Buttner (1926), Haag (1927), and Jensen (1934), to be capable of utilizing paraffin was as the sole source of carbon and energy for cellular metabolism. T he following Mycobacteria were recorded by these authors.

Sohngen (1913): Mycobacterium phlei
Mycobacterium lacticola
Mycobacterium album
Mycobacterium luteum
Mycobacterium rubrum
Mycobacterium hyolinum

Buttner (1926): Mycobacterium lactocola planum
Mycobacterium lactocola fiburgense
Mycobacterium eos.

Haag (1927): Mycobacterium testudinis
Mycobacterium ranicola

Haag was able to divide all the known saprophytic mycobacteria, which were capable of utilizing paraffin, into

four general types. Listed below is an example of each group:

Mycobacterium lacticola

Mycobacterium phlei

Mycobacterium eos.

Mycobacterium leteum

Jensen (1934) reported the following cultures of Mycobacterium species as being capable of utilizing paraffin in their metabolism:

Mycobacterium tuberculosis (avian type)

Mycobacterium lacticola

Mycobacterium phlei

Mycobacterium equi

Mycobacterium coeliacum

Mycobacterium rubropertinctum

Mycobacterium flavum

Species of Actinomycetes, which were capable of utilizing paraffin wax when grown on mineral salts agar, were reported by Buttner (1926). Four species of Actinomycetes successfully cultured in this manner were:

Actinomyces chromogenes albus

Actinomyces bovis

Actinomyces "Eppinger" [1]

1. Species isolated by these workers were not identified.

## Actinomyces "Trautwein"[2]

Haag (1927) reported that species of the genus Corynebacterium would not attack paraffin wax, and used this characteristic to separate organisms of this type from the saprophytic Mycobacterium. This work was confirmed by Jensen (1934). Some of the Corynebacterium species used by these workers were as follows.

Haag (1927): Corynebacterium bruneum
Corynebacterium aureum
Bacterium fulvum
Bacterium turcosum
Bacterium rubrum
Bacterium subflavum
Bacterium helvolum
Bacterium ochraceum
Bacterium erythrogenes

Jensen (1934): Corynebacterium helvolum
Corynebacterium cremoides
Corynebacterium insidiosum
Corynebacterium filamentosum
Corynebacterium simplex
Corynebacterium nubilum
Corynebacterium tumescens
Corynebacterium fimi

2. Species isolated by these workers were not identified.

Jensen (1934): Corynebacterium liquefaciens
Corynebacterium lacticum

As a result of the present work with some species of Corynebacterium, some results contradictory to those of Haag (1927) and Jensen (1934) were obtained. The cultures Corynebacterium simplex, Corynebacterium fimi, and Corynebacterium tumescens, and five unidentified species of Corynebacterium were secured from Dr. F. E. Clark, Department of Microbiology, United States Department of Agriculture.

Corynebacterium simplex and four of the unidentified Corynebacterium cultures grew quite well in the mineral salts medium to which solid paraffin (M.P. 52°), or kerosene had been added. The cultures Corynebacterium fimi and Corynebacterium tumescens would not grow, thus confirming Jensen's (1934) work in regard to these two cultures.

Cultures of B. aliphaticum, B. aliphaticum liquefaciens, and "Paraffin bacterium," as reported by Tausz and Peter (1919) were described as follows.

B. aliphaticum and B. aliphaticum liquefaciens are small, gram negative rods, 2.0 microns by 1.5 microns, possessing peritrichous flagella. They grow well on ordinary nutrient agar, producing beautiful irredescent round colonies, the mother colony becoming surrounded by small daughter colonies as growth progresses. On potato slants, the colon-

ies are first white, and then turn to a brownish color. Glucose and lactose are not fermented. B. aliphaticum liquefaciens differs from B. aliphaticum, in that it is proteolytic and lipolytic, while the latter is not.

The culture designated as "Paraffin bacterium" is described by Tausz as large, gram-positive rod-forms, 4 to 6 microns long and 2.0 microns wide. The organism is characterized by its rapid spore formation. He compared it with Bacillus subtilus, Bacillus liptosporus, Bacillus sessilis, and Bacillus anthracis, morphologically. The organism forms white colonies on nutrient agar, liquefied gelatin within three days, and gives a reddish-brown growth on potato slants. Lipase and catalase are present.

The characteristics of the first two cultures indicate that they may possibly be members of the genus Pseudomonas. This view is also shared by Haag (1926). He also mentioned that perhaps the organisms were members of the fluorescent group of bacteria.

Gray and Thorton (1928) reported that the following organisms were capable of utilizing naphthalene, when grown in its presence on mineral salts medium:

Micrococcus sphaeroides

Mycobacterium crystallophagum

Mycobacterium convolutins

Mycobacterium actinomorphum

Bacterium cycloclastes

Bacterium iophagum

Pseudomonas rathonis

Pseudomonas desmolyticum

Pseudomonas arvilla

Pseudomonas salopum

Pseudomonas boreopolis

Vibrio neocistes

Vibrio cuneata

Tauson (1929) found that B. fluorescens liquefaciens, B. pyocyaneus, and B. stutzeri were able to utilize both hard and soft paraffin, white vaseline, and kerosene. Other organisms were mentioned by him as being able to utilize certain compounds. An organism designated by him as B. benzoli could use benzene, toluene, and xylene. Naphthalene was utilized by three other organisms, which he named as follows:

B. naphthalinicus

B. naphthalinicus liquefaciens

B. naphthalinicus non-liquefaciens

A phenanthrene oxidizing organism was also named by Tauson (1929) as B. phenanthrenicus.

Since no additional description of the organisms could be obtained it is probable, through what information we have, that these organisms may be members of the Pseudomonas group. Tauson (1929) seemed to believe that these organisms had only a specific activity, but he did not say whether the organisms were tried on other carbon compounds.

Thaysen (1940) describes short, monotrichous rods, which are capable of fermenting kerosene.

Stone, White, and Fenske (1940) isolated small, gram-negative rods, from soil which would form white, iridescent or yellowish-green colonies on nutrient agar. The organisms were motile, but not markedly proteolytic or saccharolytic. The characteristics are common to the members of the Pseudomonas group.

In summarizing the literature, it was noticed that the utilization of saturated hydrocarbons is a characteristic common only to the Mycobacterium and Pseudomonas group of microorganisms. The ability to use unsaturated compounds is more generally distributed throughout the various genera.

## Source of Cultures

The possibilities of isolating cultures of microorganisms capable of utilizing hydrocarbons in their metabolism

was considered to be greater in the natural habitats which contained hydrocarbon material. Such habitats could be oil wells, sedimentation ponds, and in water which is commonly found in the bottom of large storage tanks. Samples were secured from these various sources, and a bacteriological analysis made to isolate the hydrocarbon utilizing organisms.

Samples of Crude Oil. Samples of mid-continent crude oil were obtained from an oil well (near Florence, Kansas), and from a pipe line (Sinclair Refining Company, Kansas City, Kansas).

The oil well sample was from a depth of 2,200 feet. The field had been producing for nearly twenty-five years, but was declining. The crude oil was mixed with water, presumably of subterranian origin. The contents of this sample were streaked upon nutrient agar. Within a few days, translucent, smooth, light cream colored convex colonies developed, which secreated a greenish-fluorescent pigment into the surrounding agar. Sub-cultures were made from one of the colonies on to mineral salts agar, and then covered with kerosene. Under these conditions, the culture grew rapidly, but did not produce any pigment. The growth along the streak was dull, flat, and translucent. The organisms were motile, gram-negative rods, producing only an acid reaction

in glucose, and an alkaline reaction in other sugars.

The organism was identified as a member of the genus Pseudomonas, and was designated as Culture No. 1, but no effort was made at this time to determine the species.

Pipeline Crude Oil. A sample of this crude oil was streaked on nutrient agar and three different colony types developed; however, when these were sub-cultured on mineral salts agar plus kerosene no growth took place.

Then a sample of the crude oil was placed in a constricted test tube, as described in Method No. 4. After several weeks, a faint turbidity was noted in the culture media. This was examined microscopically, and it revealed numerous gram-negative, extremely short rods (resembling Brucella abortus in morphology). The culture was purified by streaking on nutrient agar. It gave a thin, transparent, veil-like growth on nutrient agar. It was not saccharolytic. Only on glucose was some acid produced after about ten days. When the organism was cultured on mineral salts medium and covered with kerosene, growth occurred, causing the medium to become slightly turbid. The organism was designated as Culture No. 2, but was not identified at this time.

Micrococci from Sedimentation Pond. A sample was taken of the emulsion of crude oil and water which was dumped in the pond as a waste product, and subjected to bacteriologi-

cal analysis by streaking a little of the material on plates of mineral salts agar, covered with kerosene. Numerous small, discrete, dull-reddish colonies developed along the line of inoculation. The colonies were examined under the microscope and found to be composed of large micrococci. The organisms were predominantly gram-positive, non-motile, and did not have any definite arrangement.

When transferred to mineral salts medium containing kerosene and light and heavy mineral oil, the organism would grow at the interface of the two liquids. It was particularly interesting to note that as the cultures grew older, the hydrocarbon layers would become more turbid, due to the formation of an emulsion and an increase in the number of suspended bacterial cells. The culture medium would remain fairly clear until after several weeks, and then some of the bacterial growth would fall to the bottom. This phenomena is probably due to the high lipoid content of the cells, which cause them to remain suspended at the interface of the two liquids. Because of this behavior, plate counting could not be used successfully. The culture, designated as Culture No. 3, would retain its characteristic dull red color on any of the media.

Culture From Crude Oil Saturated Soil. This culture,

designated as Culture No. 4, was obtained from soil from the vacinity of an oil well which had been saturated with crude oil for years.

The organism was isolated in the same manner as Culture No. 1. The organisms were long, slender, filamentous rods which tended to become irregular in both morphology and size. It was gram-negative and would not ferment glucose. This organism could possibly be a member of one species of Corynebacterium.

Cultures from Sinclair Storage Tanks. The history of Culture No. 5 is unknown, since some labels were destroyed during the incubation of these cultures.

Culture No. 6 was isolated from the water in a tank of "finished re-run cracked gasoline."

Culture No. 7 was isolated from water in a tank of finished gasoline."

Culture No. 8 was obtained from water in a tank of "finished kerosene."

All of the cultures mentioned above were isolated by plating on nutrient agar, and then sub-cultured on mineral salts agar to which kerosene or gasoline had been added. All of these cultures grew best with kerosene, although some growth was obtained with gasoline.

The cultures 5, 6, 7, and 8 had all the characteristics of some species of Pseudomonas. On nutrient agar slants, a diffusible greenish pigment was liberated. Glucose was the only carbohydrate fermented. Acid was produced, but no gas. Cultures 5 and 7 were not proteolytic, but 6 and 8 would liquefy gelatin within one week. No attempt was made to identify the species of these organisms, but they are probably members of the genus Pseudomonas.

Culture From Distillate Tank. Samples were taken from various storage tanks at the Phillips Refinery in Kansas City, Kansas. Plate counts were made on the water samples from each tank. Water from a distillate tank had a bacterial count of 981,000 per cc. Gram-negative rods could be seen in a slide preparation made directly from the water. A culture, designated as Culture No. 9, was secured from the plates used for counting.

This organism is unlike the other organisms described. It is a gram-negative rod resembling E. coli in morphology; it will ferment a variety of carbohydrates such as sucrose, galactose, mannitol, maltose, and glucose, with the production of acid and gas. Lactose is fermented slowly by this organism, giving a slight acid reaction in about six days. After about thirteen days, both acid and gas are produced.

This organism is unique, in that it can utilize the hydrocarbons found in gasoline, "Skelly-solve", and kerosene, but it does not seem to be able to utilize mineral oils and paraffin wax very readily.

## Stock Culture Survey

In considering the work of various investigators, including our own, the role of the "pseudomonas-type" as hydrocarbon-utilizing bacteria seems to be quite universal. In order to establish this fact more firmly, stock cultures of Pseudomonas from various other institutions, and the American Type Culture Collection, were studied on mineral salts agar, which contained kerosene as the sole source of carbon and energy for metabolism. The relative growth of each of the cultures is listed in Table 2.

The results indicate that the ability to utilize hydrocarbons is an outstanding characteristic of this genus, particularly since known cultures, such as the type species Pseudomonas Aeruginosa, was found to possess this ability.

A stock culture of Mycobacterium phlei was also able to grow quite well in mineral salts medium to which kerosene had been added. This organism has been described above as being able to utilize paraffinic compounds such as paraffin oil and paraffin wax.

Thirty-eight stock cultures of the Proteus group, including Proteus vulgaris, could not grow under these conditions.

Other miscellaneous stock cultures which were tried on mineral salts agar with kerosene, but which did not show growth were: Mycoplana bullata, Mycoplana dimorpha, E. coli, and Staphylococcus aureus, Azotobacter chroococcum, Serratia marcescens, Aerobacter aerogenes, Rhizobium trifolium, and Lactobacillus casei.

Table 2. Growth of stock cultures of Pseudomonas on mineral salts agar with kerosene.

| Name of Culture | Origin and History | Relative Amount Growth |
|---|---|---|
| Ps.Aeruginosa | A. T. C. C. - 256 | + |
| " " | A. T. C. C. - 262 | +++ |
| " " | A. T. C. C. - 914 | ++++ |
| " " | U.S.D.A. Food & Drug - 100 | ++ |
| " " | U.S.D.A. Food & Drug (Sour Cream) - 101 | + |
| " " | U.S.D.A. - Coon (Bovine Mastitis) - A 118 | 0 |
| " " | U.S.D.A. - Coon (Bovine Mastitis) - 1441 | ++ |
| " " | U.S.D.A. - Coon (Bovine Mastitis) - 1445 | ++ |
| " " | Univ. of Illinois - 208 | ++++ |
| Ps.Pyocyaneus | Cornell University Burnett - 54 | +++ |
| " " | Cornell University (Chicken) - 55 | ++ |
| " " | Cornell University (Turkey) - 56 | +++ |
| " " | Cornell University (Swine) - 57 | +++ |
| " " | Cornell University (Water) - 58 | ++++ |
| " " | Yale University - C-9 | ++++ |
| Ps.Pyocyaneus | Yale University - C-10 | +++ |
| " " | Yale University (Brigham) - C-11 | +++ |
| " " | University of Colorado No. 10628 | ++++ |
| " " | University of Colorado No. 11257 | +++ |
| " " | University of Colorado No. 11368V | +++ |
| Ps. (Species Unknown) | Oklahoma A. and M. (Water) (B) | 0 |
| " | Oklahoma A. and M. (Water) (C) | +++ |
| " | Harnden (Hotis) | +++ |
| " | Harnden (Tiny) | +++ |
| " | Harnden (Pig Spleen) | ++ |
| " | Harnden (Raw Milk) | +++ |
| " | Harnden (Bovine Mastitis) | +++ |
| " | Harnden (Abcess, Horse) | ++ |
| " | Harnden (Bear Feces) | ++++ |
| " | Harnden (Ice Cream) | ++ |
| " | Harnden (Racoon Feces) | ++ |

\+ - Slight Growth
++ - Moderate Growth
+++ - Good Growth
++++ - Excellent Growth

## Study of a Hydrocarbon-Utilizing Mold

Molds which are capable of utilizing paraffin wax as the only source of carbon and energy in their metabolism have been described by various workers.

Rahn (1906) described a paraffin oxidizing mold which was a Penicillium species.

Sohngen (1913) found that certain species of fat-splitting molds such as Aspergillus niger, Penicillium glaucum, and Oidium lactis, would not grow with paraffin as the only source of carbon. He did, however, isolate a Papulospore species and two Penicillia that grew well on paraffin wax but would not utilize the hydrocarbons found in gasoline or kerosene.

Gainey (1917) noticed that paraffin used for sealing air spaces around soil when kept in wire baskets for vegatative experiments, would result in irregularities in the accumulation of ammonia and nitrates in the soil. Microscopic examination of these baskets revealed much growth of hyphae adjacent to the paraffin wax; therefore, he concluded that the fungus could utilize paraffin as a source of carbon and consequently used the ammonia and nitrates in their growth. He did not attempt to describe or classify the mold.

The hydrocarbon-utilizing mold isolated in this work was first observed as a contaminant on some old mineral salts agar plates to which kerosene had been added. The mold would grow quite well when transferred to mineral salts containing either kerosene or paraffin wax, but not as luxuriously as when cultured on potato dextrose agar.

The growth on paraffin wax was quite abundant, the particles of paraffin wax being completely engulfed by mold mycelium after several weeks incubation at room temperature. No quantitative measurements were made of the amount of paraffin wax used by the organism, but the size of the particles was observed to diminish as the growth of the mold developed.

The growth of this mold on potato dextrose agar was at first white; as the conidia were formed the color changed to a dull greenish-blue. The mold was morphologically similar to Penicillium in its formation of conidia.

The growth of this Penicillium-like species on kerosene is unusual since previous workers, such as Sohngen (1913) reported that their cultures of Penicillium would not utilize the hydrocarbons of kerosene. In our work the growth with kerosene was not as abundant as it was with paraffin wax.

# METHODS OF STUDY

## Respiration

In this study of bacterial respiration of various hydrocarbons, quantitative manometric measurements and chemical determinations were made according to the methods outlined below.

Determinations of the oxygen-uptake were made with the aid of improvised respirometers as diagramed in Fig. 2.

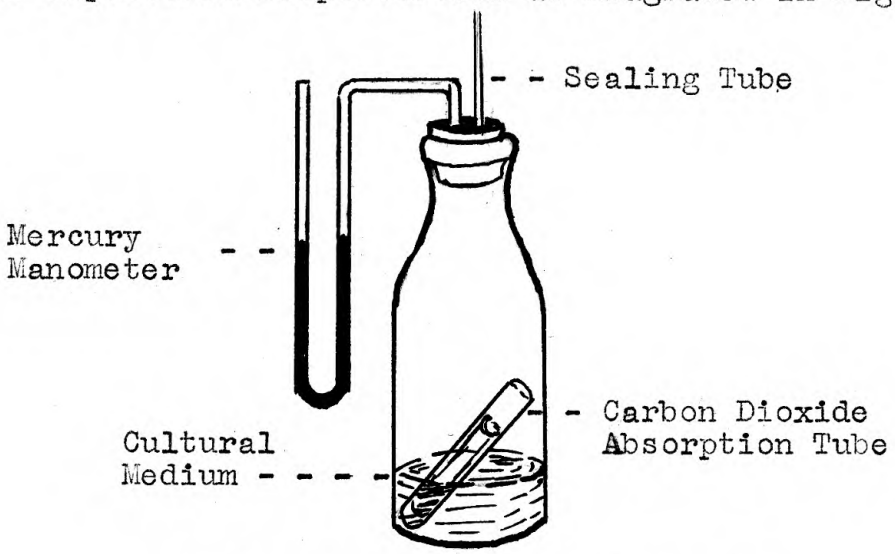


Fig. 2. Devised respirometer.

Quart-sized milk bottles containing the mineral salts medium and a large test tube containing coiled filter paper were stoppered with cotton plugs and sterilized by autoclaving at 15# to 20# for one hour. Then ten cc. of twenty per cent NaOH was placed in the test tube containing the

filter paper for the absorption of $CO_2$. Five cc. of hydrocarbon and one cc. of inoculum were added aseptically. The cotton plugs were removed and two-holed rubber stoppers were inserted into the mouth of the bottles. The manometers were then inserted in one hole of the rubber stoppers and small pieces of narrow tubing were inserted in the other. The stoppers were then wired on tightly. The small pipette tubes remained open until the experiment was to begin, and were then sealed by a glast lamp, thus assuring level mercury columns.

The top of the mercury columns were marked at the beginning of the experiment. Difference in the height of these columns gave readings which could be used to calculate oxygen uptake.

Carbon dioxide was determined in the following manner: A second respirometer which did not contain alkali was arranged in a manner parallel to the one described above. The $CO_2$ would not be absorbed and in this case would exert a positive pressure which could be measured. The differences in the volumes of the duplicate bottles, with and without alkali, represented the carbon dioxide produced. The values obtained in this manner were checked by using the regular Warburg and Barcroft flasks and manometers. Since the results obtained by both methods was about the same, this

would indicate that the method described above was fairly accurate.

The main disadvantage was that light hydrocarbons, such as gasoline and "Skelly-solve", were soluble in rubber and caused the stoppers to swell. In addition, some hydrocarbons were lost, causing variation in pressure inside the bottles. Consequently, we used this method only with the heavier hydrocarbons such as kerosene, mineral oils, and paraffin wax.

Corrections were made for the vapor pressures of the hydrocarbons by using control uninoculated bottles made up exactly in the same manner.

The formula used in calculating the oxygen uptake was based on the ordinary gas laws. By letting X represent the difference in volume or oxygen uptake, the following formula may be applied:

$$X = \frac{P_1 \, p_1}{760} \left[ Vg \cdot \frac{273}{T - 273} + V_{liq} \cdot \alpha \right]$$

$$- \frac{P_2 - h - p_2}{760} \left[ V_f \cdot \frac{273}{T_2 - 273} + V_{liq} \cdot \alpha \right]$$

$P_1$ and $P_2$ = initial and final barometric pressures respectively.

$T_1$ and $T_2$ = initial and final temperature respectively.

h = height of mercury column.

$V_g$ = volume of air in the bottles.

$V_{liq}$ = volume of liquid (medium).

$\alpha$ = solubility of oxygen in water.

## Nitrate Determinations

The determination of nitrates was carried out by the phenyldisulfonic acid method modified as follows:

10.0 cc. to 50.0 cc. of the medium was diluted to 100 cc. by the addition of a dilute solution of $AgSO_4$ and $CuSO_4$. Then the samples were shaken a few minutes and a mixture of $MgCO_3$ and CaO was added. After shaking for about three minutes the samples were filtered and the first 20 to 25 cc. of filtrate was discarded. 10 to 25 cc. of the filtrate (depending on the nitrate content) were placed in evaporating dishes and evaporated to dryness.

The dishes were allowed to cool, and 2 cc. of phenyldisulfonic acid was added to each dish. After rotating the dishes so that the reagent would come in contact with all the residue, the digestion was allowed to continue 15 minutes. Approximately 15.0 cc. of cold water was then added and the contents washed into a 100 cc. graduate. Fifty per cent KOH was added until a yellow color was developed, and the volume made up to 100 cc. The color produced was compared with the color produced by a standard containing

1.0 P.P.M. of nitrate in a colorimeter. The following calculations were used:

$$\text{Nitrates in sample} = \frac{\text{Standard}(NO_3\ \text{P.P.M.}) \times \text{Reading of Std.}}{\text{Reading of Unknown}}$$

Then multiply X by the Dilution of the Sample.

## Ammonia Determinations

Ammonia was determined by the Nessler's method. It was necessary to remove interfering substances by distillation, since a clear solution could not be obtained by other means, such as coagulation with copper sulfate as is commonly recommended.

## Nitrite Determinations

Qualitative nitrite tests were made by the use of Griess' reagent. Most tests were negative, so that quantative nitrite tests were not necessary.

## Sulfate Determinations

Sulfates were determined volumetrically by the benzidine-hydrochloride method. The sulfates react with benzidine hydrochloride in hydrochloric acid solution to form a slightly soluble compound of benzidine and sulfuric acid. This precipitate is filtered, washed free of excess hydro-

chloric acid, and the amount of sulfuric acid is determined by titration with standard sodium hydroxide (.05N).

## pH. Determinations

All pH determinations were made by means of the quinhydrone electrode potentiometer.

## Bacteriological Examinations

Quantitative bacteriological counts were made by the dilution-plate method with nutrient agar as the cultural medium. The counts were all determined under uniform conditions. The cultures were grown in an 125 cc. Erlenmeyer flask containing 50 cc. of mineral salts medium and with 3 cc. of the various hydrocarbon products. Counts were always made on control flasks without hydrocarbons.

## Nature of Hydrocarbon Products Used in This Study.

"Skelly-solve" is a commercial product used in various laboratories as a solvent for extraction. It is a mixture containing low B. P. hydrocarbons such as hexane and heptane. This product was not further purified for this work.

The gasoline used for this work was Sinclair H-C Regular. This gasoline was treated with sulfuric acid, washed several times, then treated with sodium hydroxide and redis-

tilled. This removed all the unsaturated hydrocarbons and any organic acids that might be present.

The kerosene designated as "raw kerosene" is of the ordinary commercial grade, while that designated as "treated kerosene" has been subjected to repeated acid treatment with concentrated $H_2SO_4$, neutralized, and then redistilled.

The light and heavy mineral oils are oils sold by the Parke-Davis Company for medical purposes, and for this reason are highly purified.

The paraffin wax used in this work is a commercial product with a melting point of 52°.

The hydrocarbon products such as mineral oils and paraffin-wax were sterilized by autoclaving at 17# for one hour. The kerosene was steamed for several hours in an Arnold sterilizer. "Skelly-solve" and gasoline were not sterilized but were caught in sterile bottles after redistillation.

## RESULTS OBTAINED

### Quantitative Bacterial Counts

In our work we found that most of the Pseudomonas species give the greatest bacterial counts on petroleum fractions such as kerosene, light and heavy mineral oil, and paraffin wax (see Tables 2, 3, 4, and 5, and Figs. 3, 4, 5, and 6).

Table 3. Plate counts per cc. of some representative cultures grown on mineral salts medium plus kerosene.



| Incubation Time in Days at Room Temp. | Ps. pyocyaneus Culture No. 58 (Cornell Univ.) | | Ps. pyocyaneus Culture No. C10 (Yale Univ.) | | Ps. Strain Culture No. 8 (K.S.A.C.) | | Slow Lactose Fermenting Culture No. 9 (K.S.A.C.) | | Slow Lactose Fermenting Culture No. 9A (K.S.A.C.) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Kerosene | Control | Kerosene | Control | Kerosene | Control | Kerosene | Control | Kerosene | Control |
| 1 Day | 30,000,000 | 2,646,000 | 55,000,000 | 4,599,000 | 88,500,000 | 2,646,000 | 27,000,000 | 4,788,000 | 54,000,000 | 3,213,000 |
| 2 Days | 260,000,000 | 2,580,000 | 268,000,000 | 4,330,000 | 624,000,000 | 6,663,000 | 8,500,000 | 8,750,000 | 12,660,000 | 7,400,000 |
| 4 Days | 352,000,000 | 1,080,000 | 1,130,000,000 | 3,830,000 | 1,204,000,000 | 6,220,000 | 209,500,000 | 9,690,000 | 66,000,000 | 7,700,000 |
| 6 Days | 1,020,000,000 | 3,420,000 | 1,900,000,000 | 2,650,000 | 1,900,000,000 | 5,950,000 | 265,000,000 | 1,505,000 | 539,000,000 | 1,260,000 |
| 10 Days | 3,450,000,000 | 6,300,000 | 2,400,000,000 | 6,450,000 | 1,850,000,000 | 3,300,000 | 350,000,000 | 1,870,000 | 530,000,000 | 1,730,000 |

Table 4. Bacterial counts of Pseudomonas pyocyaneus grown on various petroleum products.
(Initial count: 5,700,000)



| Incubation Time | Control | Gasoline | Skelly-Solve | Treated Kerosene | Raw Kerosene | Light Mineral Oil | Heavy Mineral Oil | Paraffin Wax |
|---|---|---|---|---|---|---|---|---|
| 1 Day | 5,750,000 | 7,750,000 | ------ | 25,300,000 | 20,050,000 | 25,250,000 | 18,550,000 | ----- |
| 2 Days | 18,200,000 | 8,500,000 | 107,500 | 536,500,000 | 412,500,000 | 610,000,000 | 215,000,000 | 79,500,000 |
| 5 Days | 18,000,000 | 9,500,000 | 83,000 | 2,550,000,000 | 1,864,000,000 | 1,962,000,000 | 890,000,000 | 596,000,000 |
| 7 Days | 12,670,000 | 1,400,000 | ------ | 2,575,000,000 | 2,810,000,000 | 1,930,000,000 | 2,010,000,000 | 1,475,000,000 |
| 9 Days | 20,150,000 | 1,780,000 | 630,000 | 2,450,000,000 | 1,710,000,000 | 3,480,000,000 | 2,160,000,000 | 3,880,000,000 |
| 11 Days | 15,100,000 | 45,700,000 | 1,640,000 | 2,175,000,000 | 990,000,000 | 3,670,000,000 | 2,795,000,000 | 2,085,000,000 |

Utilization of ammonia, nitrates, and sulfates by Ps. pyocyaneus on the various hydrocarbon media tabulated above.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ammonia Content After Growth-P.P.M. | 105.00 | 105.00 | 105.00 | 00.00 | 22.60 | 00.00 | 16.60 | 19.00 |
| Per Cent Utilized During Growth | 00.00 | 00.00 | 00.00 | 100.00 | 72.77 | 100.00 | 83.00 | 81.00 |
| Nitrate Content After Growth-P.P.M. | 760.00 | 504.00 | 760.00 | 110.00 | 197.00 | 00.00 | 230.00 | 199.00 |
| Per Cent Utilized During Growth | 00.00 | 33.55 | 00.00 | 85.50 | 74.08 | 100.00 | 69.74 | 73.82 |
| Sulfate Content After Growth-P.P.M. | 210.60 | 203.80 | 210.60 | 65.49 | 134.80 | 88.03 | 81.71 | 84.76 |
| Per Cent Utilized During Growth | 000.00 | 00.97 | 00.00 | 68.91 | 35.61 | 58.20 | 61.20 | 59.75 |

Table 5. Bacterial counts of Pseudomonas culture no. 8 on various petroleum products.
(Initial count: 4,150,000 per cc.)



| Incubation Time | Control | Gasoline | Skelly-Solve | Treated Kerosene | Raw Kerosene | Light Mineral Oil | Heavy Mineral Oil | Paraffin Wax |
|---|---|---|---|---|---|---|---|---|
| 2 Days | 4,900,000 | 3,485,000 | 168,000,000 | 361,500,000 | 362,000,000 | 207,500,000 | 111,500,000 | 51,000,000 |
| 4 Days | 6,100,000 | 1,830,000 | 136,500,000 | 2,615,000,000 | 2,245,000,000 | 1,175,000,000 | 206,000,000 | 745,000,000 |
| 6 Days | 5,250,000 | 2,400,000 | 18,500,000 | 2,130,000,000 | 1,625,000,000 | 1,020,000,000 | 223,000,000 | 1,270,000,000 |
| 11 Days | 5,200,000 | 695,000 | 127,500,000 | 2,625,000,000 | 1,635,000,000 | 1,070,000,000 | 385,000,000 | 1,535,000,000 |

Utilization of ammonia, nitrates, and sulfates by Pseudomonas culture No. 8 on the various hydrocarbon media tabulated above.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ammonia Content After Growth-P.P.M | 262.50 | 262.50 | 189.00 | 47.20 | 52.50 | 78.75 | 210.00 | 218.75 |
| Per Cent Utilized During Growth | 00.00 | 00.00 | 28.00 | 83.02 | 80.00 | 73.81 | 20.00 | 16.67 |
| Nitrate Content After Growth-P.P.M | 615.60 | 581.40 | 610.45 | 329.35 | 356.36 | 339.61 | 615.60 | 531.13 |
| Per Cent Utilized During Growth | 00.00 | 5.56 | 0.82 | 46.50 | 42.10 | 44.83 | 00.00 | 13.70 |
| Sulfate Content After Growth-P.P.M | 240.00 | 240.00 | 236.40 | 55.20 | 146.40 | 134.40 | 124.80 | 127.20 |
| Per Cent Utilized During Growth | 00.00 | 00.00 | 1.50 | 77.00 | 31.00 | 44.00 | 48.00 | 47.00 |

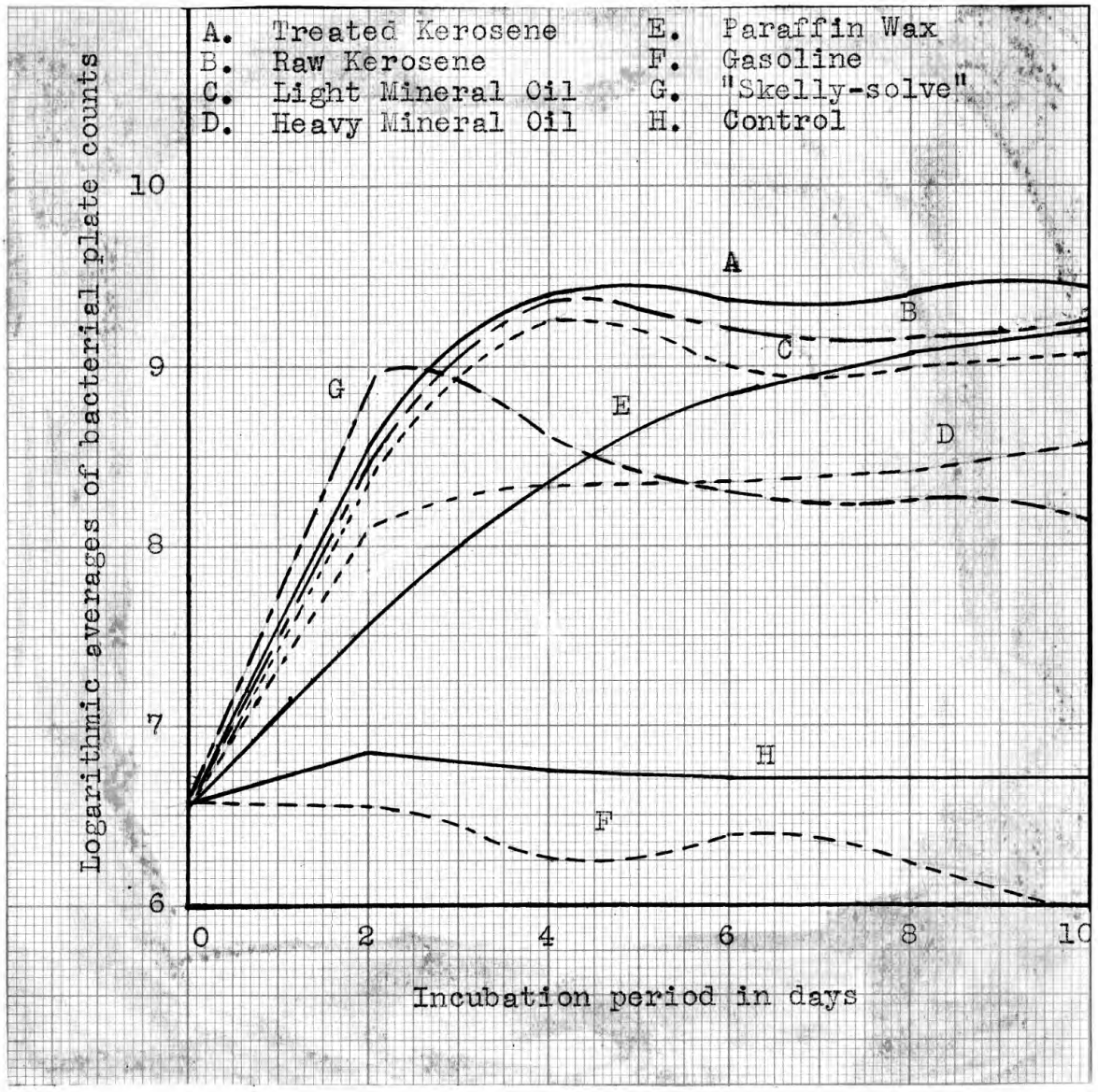


Fig. 3. Growth of Pseudomonas culture No. 8 on various petroleum products.

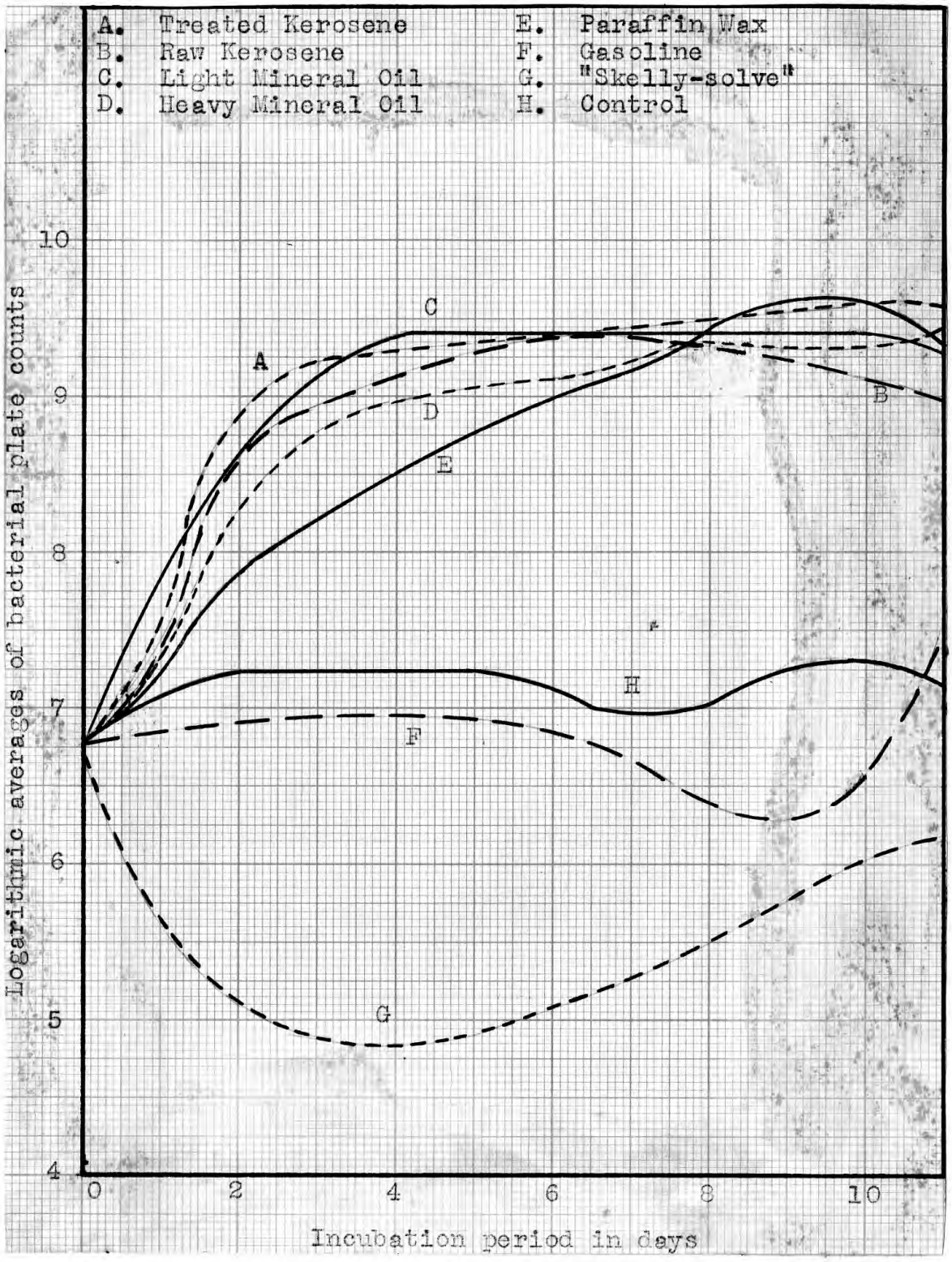


Fig. 4. Growth of Pseudomonas pyocyaneus on various petroleum products.

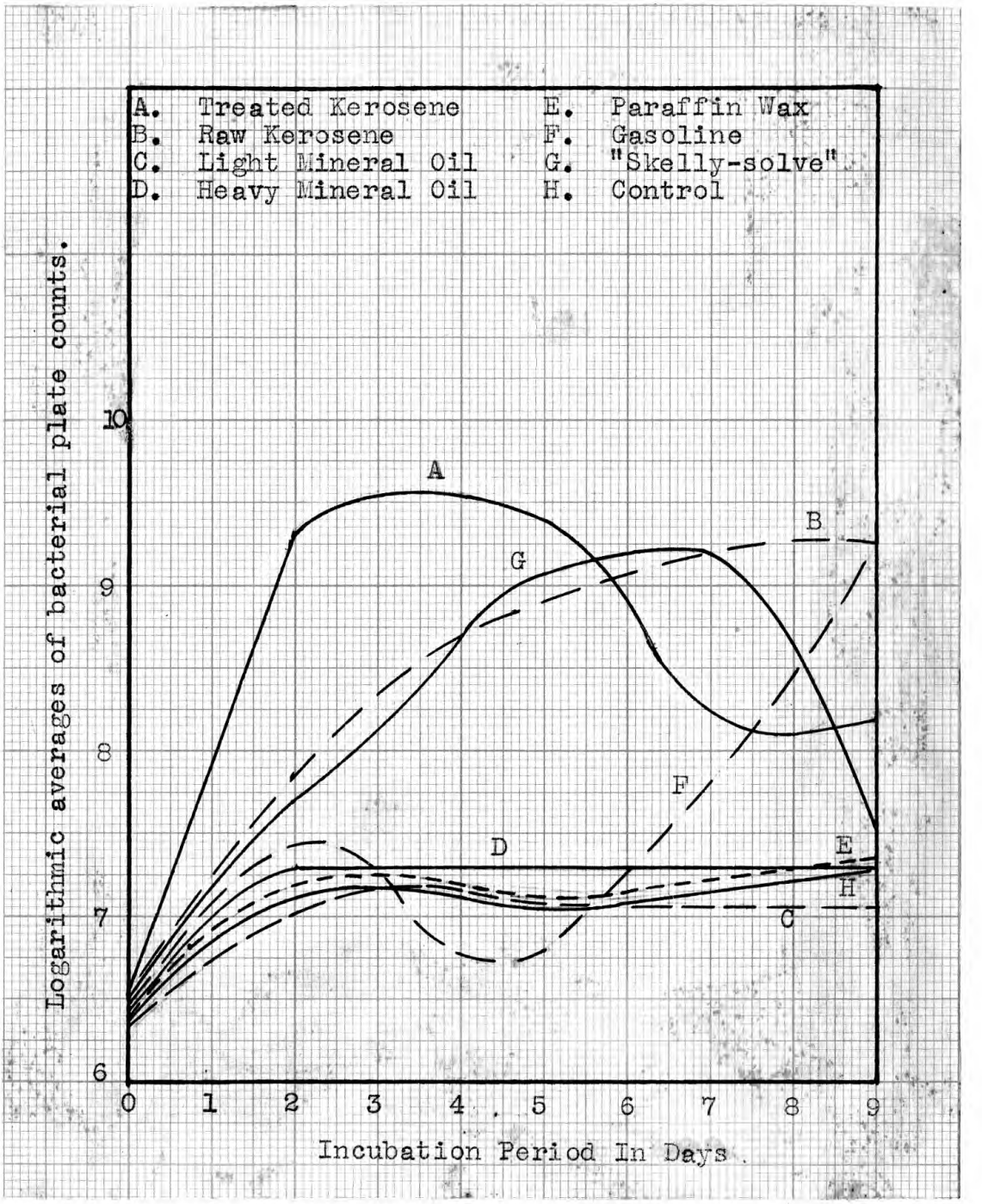


Fig. 5. Growth of "slow-lactose fermenting" culture on various petroleum products.

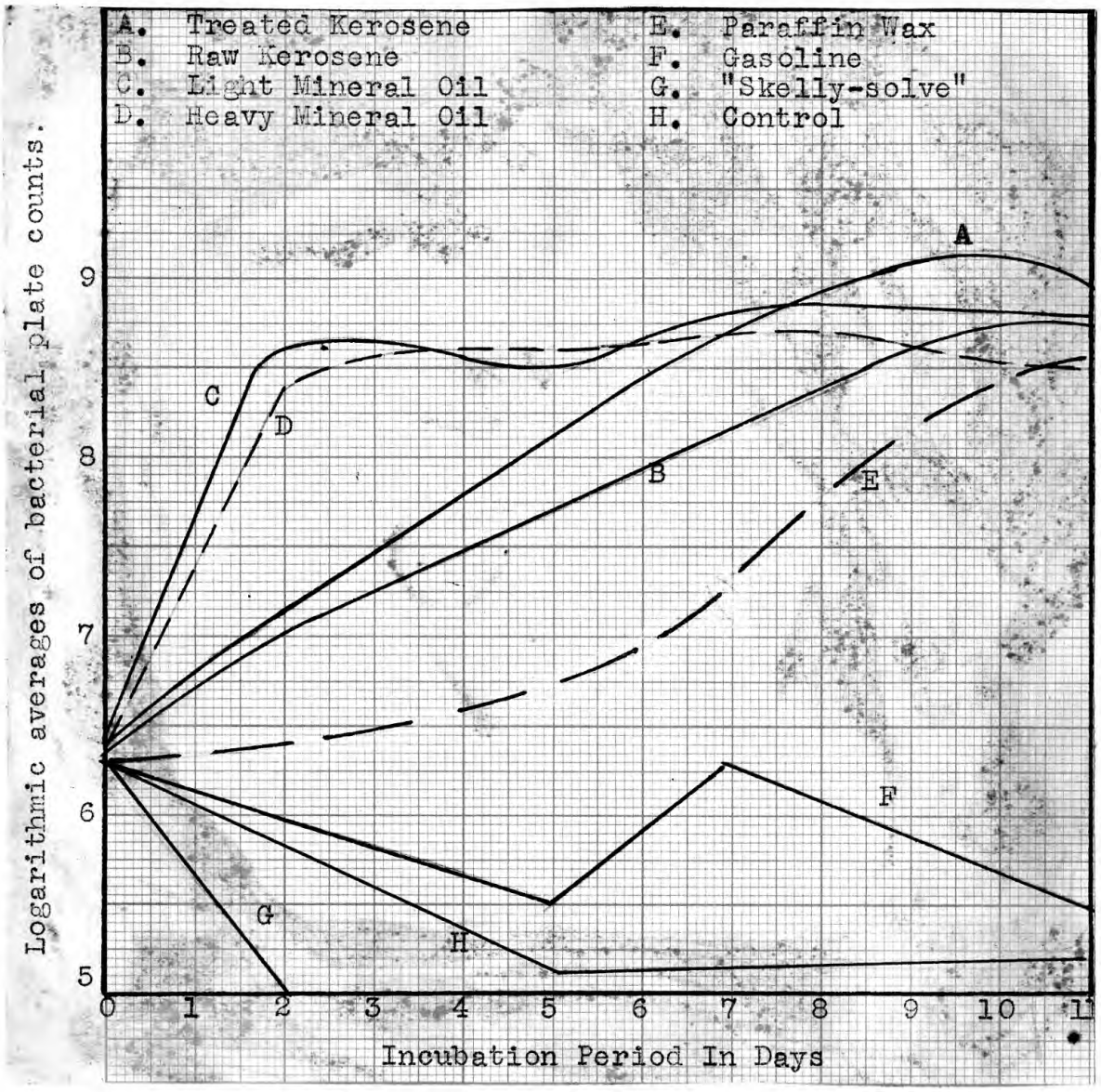


Fig. 6. Growth of Cory. simplex on various petroleum products.

The "slow lactose fermenting" culture is able to use the lighter hydrocarbons quite readily (see Table 6). In addition, it could use kerosene quite well, but not the mineral oils or paraffin wax. This phenomena may be due to the fact that the organisms have a specificity for paraffinic hydrocarbons; therefore, they can utilize petroleum products which have a high content of straight chain molecules. On the other hand, fractions possessing a high percentage of cyclic or naphthenic hydrocarbons would not be as readily assimilated. This organism may be analogous to the cultures described by Tausz (1919) which he claimed would attack only paraffinic compounds.

The ability of the culture to attack aliphatic or cyclic compounds was determined by setting up a series of test tubes containing mineral salts medium and this organism with various pure hydrocarbon compounds added (see Table 8).

It was found that "Skelly-solve", which is a hydrocarbon fraction containing a high percentage of hexane, was readily utilized, but cyclic compounds, such as benzene, toluene, xylene, and light mineral oil, were not utilized. The Pseudomonas strains, which could utilize mineral oils quite readily, were also able to utilize the cyclic compounds. Final proof of this specificity for aliphatic hy-

Table 6. Bacterial counts of "slow lactose fermenting" cultures grown on various petroleum products. (Initial count: 2,360,000)

| Incubation Time | Control | Gasoline | Skelly-Solve | Treated Kerosene | Raw Kerosene | Light Mineral Oil | Heavy Mineral Oil | Paraffin Wax |
|---|---|---|---|---|---|---|---|---|
| 2 Days | 14,600,000 | 2,970,000 | 40,000,000 | 203,500,000 | 59,000,000 | 10,450,000 | 17,050,000 | 20,000,000 |
| 5 Days | 13,400,000 | 6,800,000 | 1,091,000,000 | 235,000,000 | 242,000,000 | 14,400,000 | 13,800,000 | 19,150,000 |
| 7 Days | 15,750,000 | 79,500,000 | 145,000,000 | 164,500,000 | 146,500,000 | 12,050,000 | 17,650,000 | 23,150,000 |
| 9 Days | 18,600,000 | 162,500,000 | 30,000,000 | 165,500,000 | 147,500,000 | 12,450,000 | 18,850,000 | 25,900,000 |

Utilization of ammonia, nitrates, and sulfates by "slow lactose fermenting" culture on the various hydrocarbon media tabulated above

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ammonia Content After Growth-P.P.M. | 300.00 | 150.00 | 00.00 | 200.00 | 180.00 | 300.00 | 300.00 | 300.00 |
| Per Cent Utilized During Growth | 00.00 | 50.00 | 100.00 | 66.66 | 60.00 | 00.00 | 00.00 | 00.00 |
| Nitrate Content After Growth-P.P.M. | 707.94 | 567.72 | 00.00 | 492.48 | 556.09 | 615.60 | 612.52 | 620.04 |
| Per Cent Utilized During Growth | 00.00 | 19.80 | 100.00 | 30.44 | 14.45 | 13.04 | 13.48 | 12.42 |
| Sulfate Content After Growth-P.P.M. | 255.60 | 158.60 | 247.90 | 158.60 | 152.00 | 239.40 | 248.90 | 255.60 |
| Per Cent Utilized During Growth | 00.00 | 37.95 | 2.97 | 37.51 | 40.53 | 6.30 | 2.58 | 00.00 |

drocarbons by this culture must be postponed until pure naphthenic hydrocarbons, such as cyclohexane and cyclododecane are tried in the same manner, in place of the aromatic compounds that were used in this study.

Corynebacterium simplex could assimilate the same hydrocarbons as the Pseudomonas species; however, it could not tolerate the light hydrocarbons, such as "Skelly-solve." In fact, this product acted as a germicide under the conditions of this experiment (observe Table 7).

Table 8. Comparison of the utilization of various hydrocarbons by various bacteria.

| | "Slow Lactose Fermenting" | Ps. pyo. #58 | Cory. Simplex |
|---|---|---|---|
| "Skelly-solve" | +++ | +++ | 0 |
| Light Oil | 0 | ++ | ++ |
| Benzene | 0 | + | 0 |
| Toluene | 0 | + | ++ |
| Xylene | 0 | ++ | +++ |

+++ Vigorous Growth
++ Moderate Growth
+ Slight Growth

Table 7. Bacterial counts of Corynebacterium simplex grown on various petroleum products.
(Initial count: 7,150,000)

| Incubation Time | Control | Gasoline | Skelly-Solve | Treated Kerosene | Raw Kerosene | Light Mineral Oil | Heavy Mineral Oil | Paraffin Wax |
|---|---|---|---|---|---|---|---|---|
| 3 Days | ---- | No Growth | 370,000 | 35,150,000 | 31,400,000 | 1,211,000,000 | 705,000,000 | No Growth |
| 6 Days | 490,000 | Reinoculated | No Growth | 6,000,000 | 9,850,000 | 965,000,000 | 1,195,000,000 | Reinoculated |
| 8 Days | 418,000 | 7,200,000 | No Growth | 1,565,000,000 | 273,500,000 | 2,050,000,000 | 1,685,000,000 | 65,000,000 |
| 11 Days | 500,000 | 1,615,000 | No Growth | 1,635,000,000 | 1,735,000,000 | 1,930,000,000 | 1,040,000,000 | 750,000,000 |
| 14 Days | 495,000 | 480,000 | No Growth | 530,000,000 | 1,515,000,000 | 1,765,000,000 | 925,000,000 | 1,390,000,000 |

Utilization of ammonia, nitrates, and sulfates by Corynebacterium simplex in the various hydrocarbon media tabulated above.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ammonia Content After Growth-P.P.M. | 180.00 | 180.00 | 180.00 | 110.00 | 130.00 | 70.00 | 110.00 | 110.00 |
| Per Cent Utilized During Growth | 00.00 | 00.00 | 00.00 | 38.89 | 27.28 | 61.12 | 38.89 | 38.89 |
| Nitrate Content After Growth-P.P.M. | 636.12 | 617.45 | 617.30 | 572.85 | 488.38 | 629.62 | 536.94 | 616.60 |
| Per Cent Utilized During Growth | 00.00 | 2.96 | 2.96 | 9.95 | 23.22 | 1.01 | 15.59 | 2.96 |
| Sulfate Content After Growth-P.P.M. | 292.80 | 278.40 | 278.40 | 180.00 | 216.00 | 228.00 | 168.00 | 288.00 |
| Per Cent Utilized During Growth | 00.00 | 4.92 | 4.92 | 38.53 | 26.00 | 22.10 | 42.62 | 1.64 |

## Respiration Data

Haag (1926) reported that the ratio of carbon dioxide to the amount of paraffin used by a culture was equal to 0.37. This ratio held true for various organisms, such as M. lacticola, M. phlei, Ps. pyocyaneum, and Actinomycetes chromogenes. He could only recover about 4/5 of the theoretical $CO_2$. The remaining 1/5 was believed by him to be used by the bacteria in the synthesis of protoplasm and other organic compounds, such as fatty acids.

Stone, White, Fenske (1940) determined the respiration quotient of bacteria on various petroleum products, such as neutral oil. They secured a ratio of $CO_2/O_2$, varying from 0.50 to 0.70. The work just described is the only major work of this nature on the study of bacterial respiration of hydrocarbons that has ever been published. It indicates, however, that this type of respiration can be studied in much the same manner used in the study of the respiration of carbohydrates.

The oxygen uptake as determined manometrically is recorded on Figs. 7, 8, 9, and 10, for each of the cultures studied. The carbon dioxide production was also determined as the difference between the volumes secured with and with-

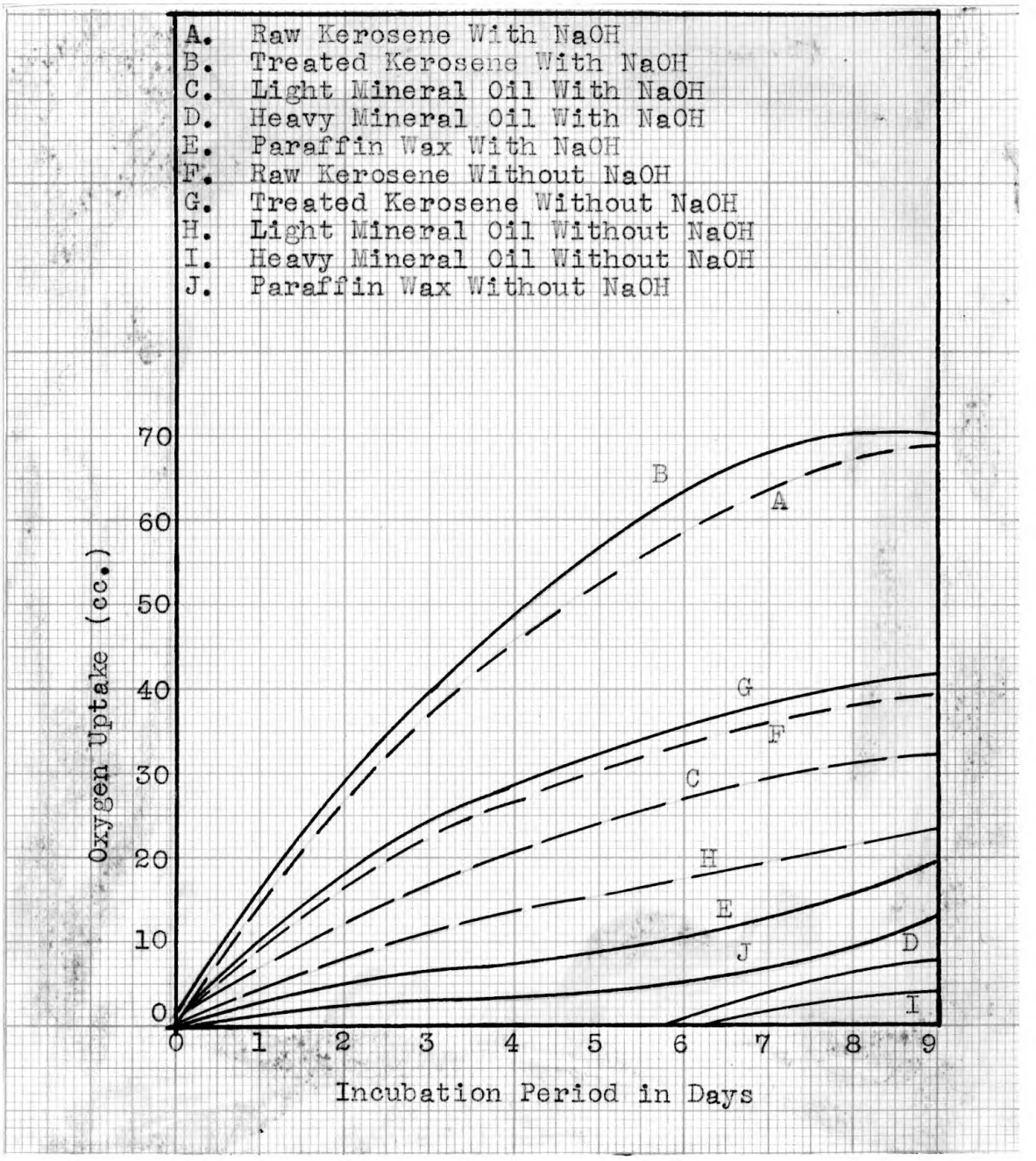


Fig. 7. Oxygen uptake by Pseudomonas Culture No. 8 on various hydrocarbons.

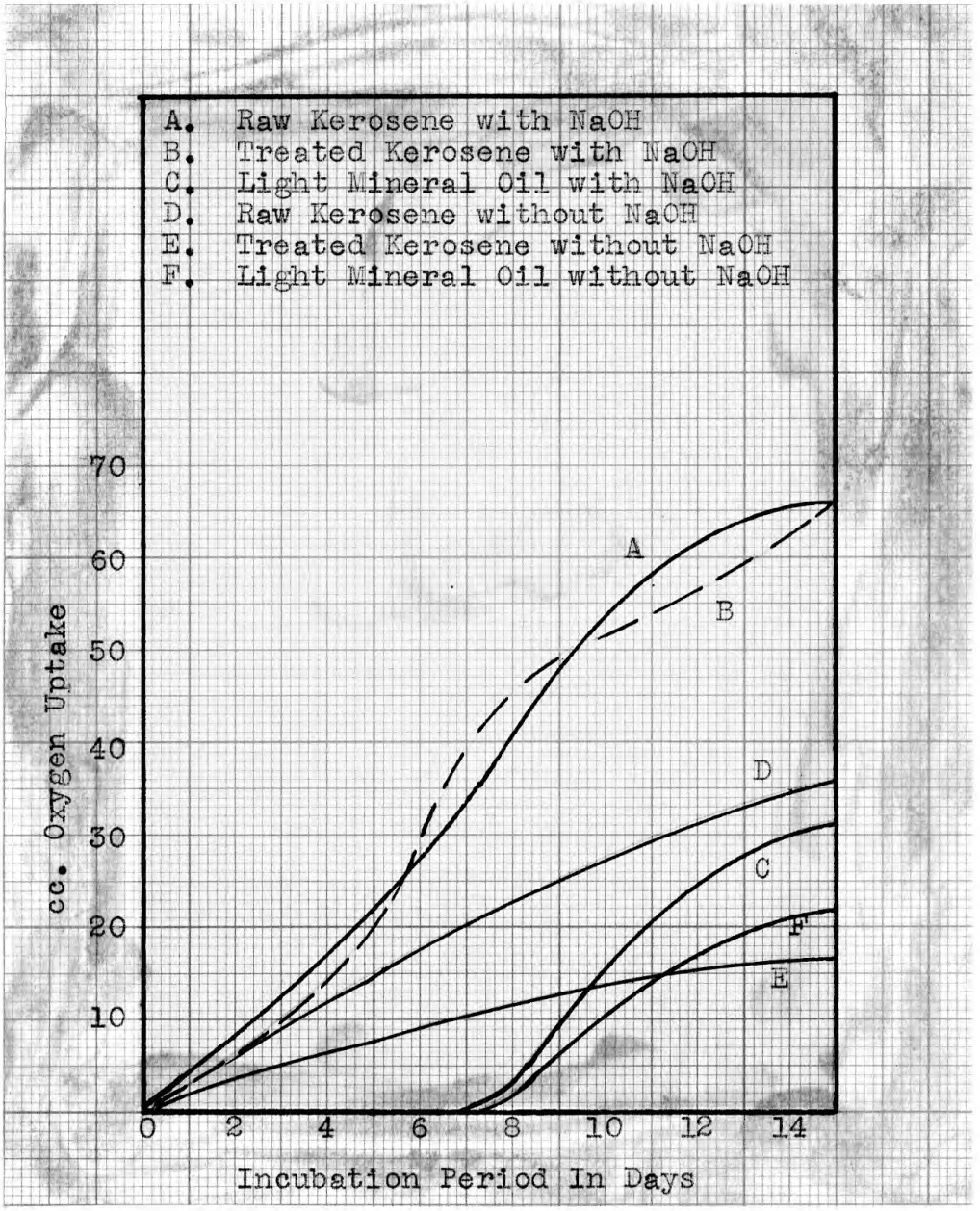


Fig. 8. Oxygen uptake by Ps. pyocyaneus on various hydrocarbons.

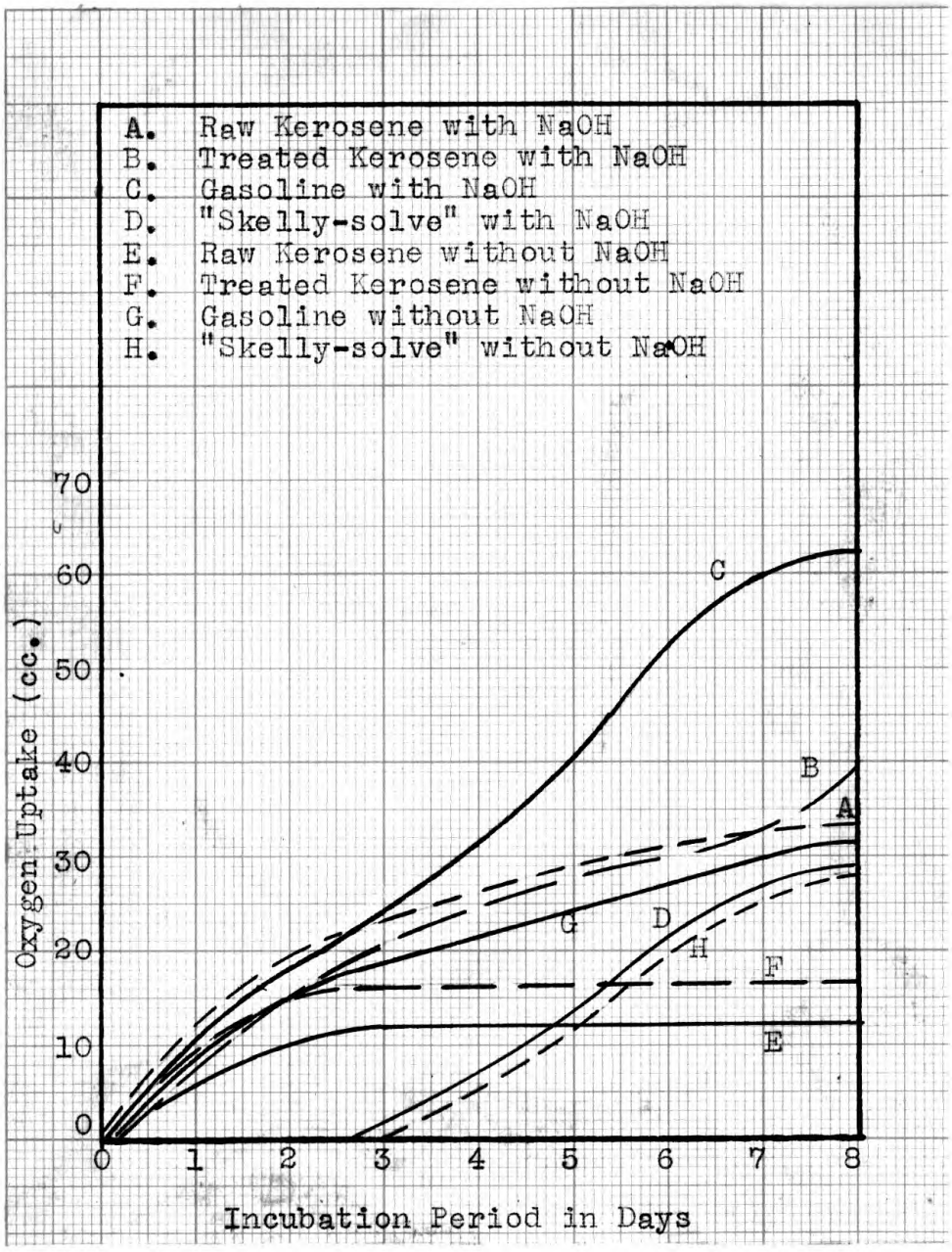


Fig. 9. Oxygen uptake by "slow lactose fermenting" culture on various hydrocarbons.

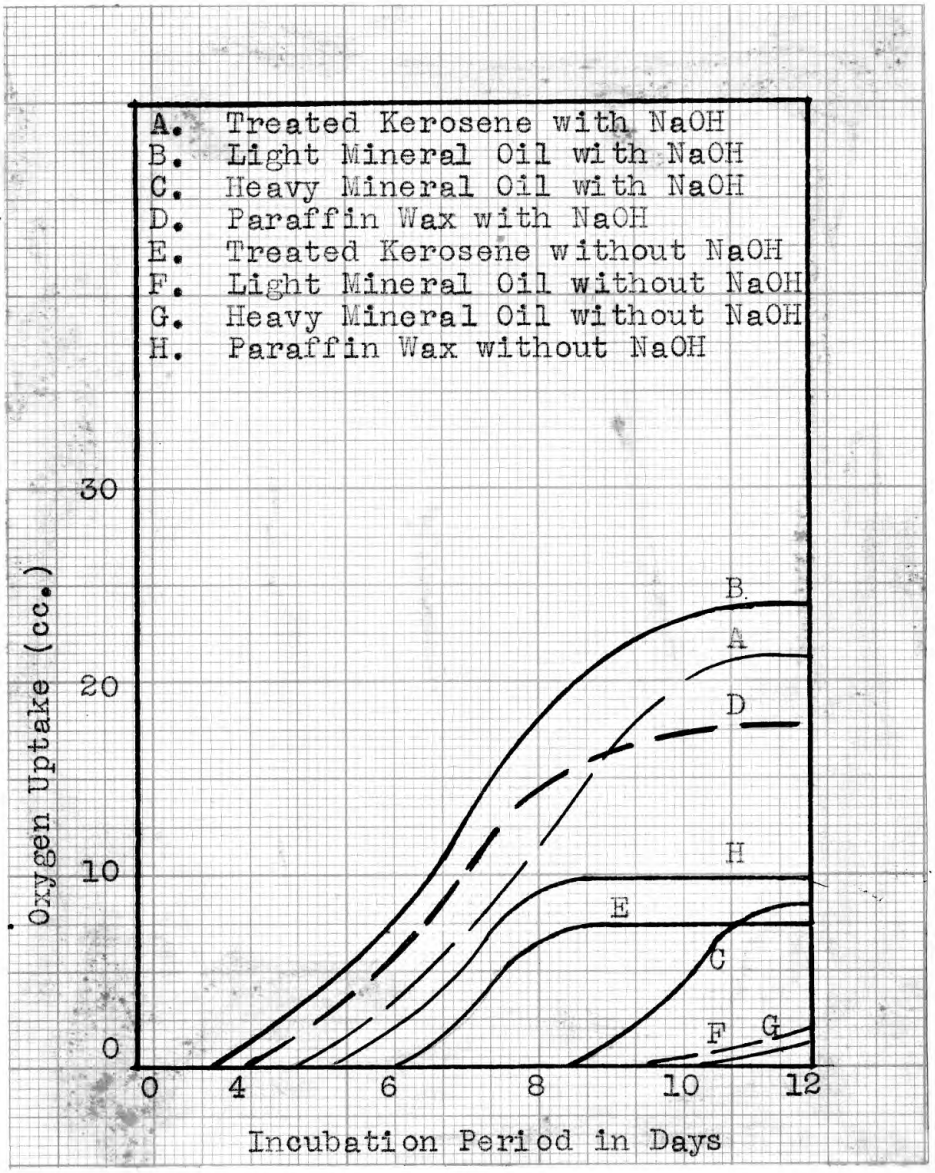


Fig. 10. Oxygen uptake by Cory. simplex on various hydrocarbons.

out alkali. The respiratory quotients $\left(\frac{CO_2}{O_2}\right)$, based on these values vary from 0.30 to 0.70. This is the same as the values recorded by Stone, White, and Fenske (1940) (see Table 9).

Table 9. Respiratory quotients (Average)

| | Paraffin Wax | "Skelly-solve" | Gasoline | Raw Kerosene | Treated Kerosene | Light Mineral Oil | Heavy Mineral Oil |
|---|---|---|---|---|---|---|---|
| Ps. pyo. #58 | N.G. | N.G. | 0.51 | 0.55 | 0.55 | 0.46 | N.G. |
| "Slow lactose fermenting" | N.G. | 0.51 | ---- | 0.54 | 0.51 | N.G. | N.G. |
| Ps.Strain #8 | 0.66 | N.G. | N.G. | 0.58 | 0.58 | 0.72 | 0.67 |
| Cory.Simplex | 0.57 | N.G. | N.G. | N.G. | 0.32 | 0.30 | 0.56 |

No growth = N.G.

It would be unsafe to try to draw conclusions from this data alone, but there does seem to be a trend toward a higher quotient when the higher molecular weight hydrocarbon fractions are used.

## Utilization of Ammonia, Nitrates and Sulfates

The ammonia nitrogen used in cellular metabolism is indicated in Tables 4, 5, 6, and 7. The determination of

the preferance of the organisms for either ammonia or nitrate nitrogen is complicated by the possibility that perhaps some of the nitrates are reduced to ammonia, thus yielding a source of oxygen, but the percentage of ammonia used is consistently higher than that of the nitrates, thus indicating that ammonia is the best source of nitrogen.

The utilization of combined oxygen in the form of nitrates and sulfates is governed by conditions of the experiment. For example, the percentage utilization of these compounds is greater in the respirometers where the oxygen supply is somewhat limited than it is in the flasks exposed to the atmosphere. The Pseudomonas cultures in particular have a higher demand for sulfates (compare Tables 4 and 5 with Tables 10, 11 and 12).

Combined oxygen, however, is not essential in the respiration of hydrocarbons by bacteria. This was demonstrated by arranging a series of tubes of media which lacked one or all of the compounds containing oxygen (see Table 13).

It is interesting to note that phosphates are essential. The tubes had approximately 50 P.P.M. of phosphate; yet, this was insufficient for maximum growth. It may be possible that phosphorlation plays as important a role in hydrocarbon respiration as it does in carbohydrate respiration.

Table 10. Utilization of ammonia by various cultures in the respirometer bottles.

| Medium | Ps.Pyocyaneus | | Ps. Culture No. 8 | | "Slow Lactose Fermenting" | | C. simplex | |
|---|---|---|---|---|---|---|---|---|
| | $NH_4$ (PPM) Content After Growth | Per Cent Used | $NH_4$ (PPM) Content After Growth | Per Cent Used | $NH_4$ (PPM) Content After Growth | Per Cent Used | $NH_4$ (PPM) Content After Growth | Per Cent Used |
| Control | 105.0 | 2.0 | 262.5 | 0.0 | 295.0 | 0.0 | 297.6 | 0.0 |
| "Skelly-solve" | 105.0 | 0.0 | 157.5 | 40.0 | 156.5 | 47.0 | 297.6 | 0.0 |
| Gasoline | 105.0 | 0.0 | 262.5 | 0.0 | 59.0 | 80.0 | 297.6 | 0.0 |
| Raw Kerosene | 70.5 | 22.9 | 131.2 | 50.1 | 254.2 | 13.9 | 297.6 | 0.0 |
| Treated Kerosene | 75.0 | 28.6 | 157.5 | 40.0 | 251.2 | 14.9 | 264.0 | 11.3 |
| Light Oil | 46.5 | 55.7 | 159.5 | 39.2 | 295.0 | 0.0 | 273.6 | 8.1 |
| Heavy Oil | 97.5 | 7.2 | 262.5 | 0.0 | 295.0 | 0.0 | 273.6 | 8.1 |
| Paraffin Wax | 53.3 | 49.3 | 262.5 | 0.0 | 295.0 | 0.0 | 271.2 | 8.9 |

Table 11. Utilization of nitrates by various cultures in the respirometer bottles.

| Medium | Ps. Pyocyaneus | | Ps. Culture No. 8 | | "Slow Lactose Fermenting" | | C. simplex | |
|---|---|---|---|---|---|---|---|---|
| | $NO_3$ (PPM) Content After Growth | Per Cent Used | $NO_3$ (PPM) Content After Growth | Per Cent Used | $NO_3$ (PPM) Content After Growth | Per Cent Used | $NO_3$ (PPM) Content After Growth | Per Cent Used |
| Control | 446.3 | 13.0 | 629.3 | 0.0 | 718.2 | 0.0 | 632.4 | 0.0 |
| "Skelly-solve" | 324.7 | 36.9 | 406.9 | 35.3 | 625.9 | 12.9 | 00.0 | 0.0 |
| Gasoline | 280.1 | 45.4 | 567.3 | 9.8 | 636.1 | 25.4 | 0.0 | 0.0 |
| Raw Kerosene | 277.3 | 46.9 | 333.5 | 47.0 | 631.0 | 12.1 | 0.0 | 0.0 |
| Treated Kerosene | 284.7 | 44.5 | 368.3 | 41.5 | 677.2 | 5.7 | 608.4 | 3.7 |
| Light Oil | 262.4 | 48.9 | 460.0 | 27.0 | 718.2 | 0.0 | 617.9 | 2.3 |
| Heavy Oil | 462.5 | 9.9 | 585.2 | 7.1 | 718.2 | 0.0 | 621.1 | 1.8 |
| Paraffin Wax | 457.6 | 10.8 | 521.2 | 17.2 | 718.2 | 0.0 | 620.0 | 1.9 |

Table 12. Utilization of sulfates by various cultures in the respirometer bottles.

| Medium | Ps. Pyocyaneus | | Ps. Culture No. 8 | | "Slow Lactose Fermenting" | | C. simplex | |
|---|---|---|---|---|---|---|---|---|
| | $SO_4$(PPM) Content After Growth | Per Cent Used | $SO_4$(PPM) Content After Growth | Per Cent Used | $SO_4$(PPM) Content After Growth | Per Cent Used | $SO_4$(PPM) Content After Growth | Per Cent Used |
| Control | 210.6 | 0.0 | 240.2 | 0.0 | 255.0 | 0.0 | 297.6 | 0.0 |
| "Skelly-solve | 210.6 | 0.0 | 236.4 | 13.1 | 162.4 | 36.3 | 297.6 | 0.0 |
| Gasoline | 210.6 | 0.0 | 240.2 | 0.0 | 209.9 | 17.7 | 297.6 | 0.0 |
| Raw Kerosene | 98.5 | 53.2 | 188.6 | 21.5 | 176.7 | 30.8 | 297.6 | 0.0 |
| Treated Kerosene | 94.4 | 55.2 | 193.8 | 13.2 | 128.2 | 49.8 | 264.0 | 11.3 |
| Light oil | 68.5 | 67.5 | 214.1 | 10.9 | 247.1 | 2.7 | 273.6 | 8.1 |
| Heavy Oil | 96.8 | 54.5 | 217.4 | 9.5 | 255.0 | 0.0 | 273.6 | 8.1 |
| Paraffin Wax | 71.4 | 66.1 | 169.8 | 29.3 | 248.0 | 2.8 | 271.2 | 8.9 |

Table 13. Effect of combined oxygen on bacterial growth.

| Hydrocarbon | Medium | Cory. simplex | Cory. Species (17A) | Ps. Strain No. 6 | Ps. Strain No. 8 | "Slow Lactose Ferment" | Ps. pyo. No. 58 |
|---|---|---|---|---|---|---|---|
| Light Oil | Complete | +++ | +++ | +++ | +++ | 0 | +++ |
| Kerosene | Complete | 0 | 0 | +++ | ++++ | +++ | ++++ |
| Light Oil | Minus $NO_3$ | +++ | +++ | +++ | +++ | 0 | +++ |
| Kerosene | Minus $NO_3$ | 0 | 0 | +++ | ++++ | +++ | ++++ |
| Light Oil | Minus $PO_4$* | +++ | +++ | ++ | ++ | 0 | ++ |
| Kerosene | Minus $PO_4$* | 0 | 0 | + | + | + | ++ |
| Light Oil | Minus $SO_4$ | ++++ | +++ | +++ | +++ | 0 | +++ |
| Kerosene | Minus $SO_4$ | 0 | 0 | +++ | +++ | +++ | ++++ |
| Light Oil | Minus All | +++ | +++ | 0 | +++ | 0 | ++ |
| Kerosene | Minus All | 0 | 0 | +++ | +++ | +++ | +++ |

++++ - Excellent Growth
+++ - Good Growth
++ - Moderate Growth
+ - Slight Growth

* It was necessary to add 50 P.P.M. of phosphate as a source of phosphorous.

## Acid Production

The production of organic acids by hydrocarbon-utilizing bacteria was not appreciable, as is quite evident as seen by studying Table 14. This plate is representative of the changes in pH secured by various cultures under these conditions. These particular cultures do not produce appreciable quantities of acid from carbohydrates, so it is not surprising that acids are not produced in abundance from hydrocarbons. However, there is some evidence for acid production. This is revealed by the ease of emulsification of the hydrocarbons following bacterial action. This emulsifying characteristic, due to bacterial action, was noted also by Stone, White, and Fenske (1940). An increase in the saponification number of mineral oil was noted by Tauson and Schapiro (1934). They believed that this was evidence of the presence of fatty acids and naphthenic acids. The evidence indicates that long-chain acids are formed, but that they are extremely weak acids; consequently, no great changes in pH are produced.

## Changes Produced in Certain Hydrocarbons by Bacterial Action

The only previous work pertaining to changes produced in petroleum fractions as a result of bacterial action was

by Tauson and Schapiro (1934). They observed that the refractive index, saponification number, and iodine number increased as bacterial action took place. They also state that the organisms attack the unsaturated hydrocarbons, and that unsaturated hydrocarbons may be produced as intermediate products.

The following procedure was followed in our determination of the changes produced in kerosene, due to bacterial action:

Four one-liter Erlenmeyer flasks were filled with 300 cc. of mineral salt medium and 300 cc. of kerosene. Two of the flasks were inoculated with Ps. pyocyaneus (No. 58), and two were left uninoculated as controls. One of each pair was aerated, and the other pair was allowed to remain unagitated. After one week of incubation at room temperature, the flasks were analyzed for organic acids and alcohols, and a distillation was run on the kerosene to see if any changes in the ratio of volume to boiling point could be detected. The results are listed on Table 15.

If the distillation data of the controls is compared to that of the kerosene acted upon by bacteria it will show that no essential differences can be detected until the distillation volume reaches 80 per cent. Then the kerosene, which has been subjected to bacterial action, showed an in-

Table 14. Change in pH produced by cultures on various hydrocarbons

| | Respirometers | | | | Flasks | |
|---|---|---|---|---|---|---|
| | Ps. pyo. No. 58 | Slow Lactose Ferment. | Ps. Strain No. 8 | Cory. simplex | Ps. Strain No. 8 | Cory. simplex |
| Gasoline | 6.66 | 6.82 | No Growth | No Growth | No Growth | No Growth |
| "Skelly-solve" | No Growth | 6.85 | " " | " " | " " | " " |
| Raw Kerosene | 5.65 | 6.61 | 6.57 | 6.81 | 5.45 | 6.19 |
| Treated Kerosene | 5.81 | 6.78 | 6.57 | 6.65 | 5.45 | 5.79 |
| Light Oil | 5.50 | 6.88 | 6.53 | 6.59 | 5.92 | 5.85 |
| Heavy Oil | 6.52 | No Growth | 6.70 | 6.59 | 6.53 | 6.26 |
| Paraffin Wax | 6.15 | " " | 6.69 | 6.71 | 6.63 | 6.33 |
| Control - No Hydrocarbon | 6.60 | " " | 6.87 | 6.85 | 6.87 | 6.90 |
| Culture Medium Alone | 6.98 | 7.10 | 6.90 | 6.89 | ---- | ---- |

Table 15. Distillation tests on kerosene after bacterial action.
(*Ps. pyocyaneus* culture No. 58)

| | Control (Aerated) | Control (Not Aerated) | No. 58 (Aerated) | No. 58 (Not Aerated) |
|---|---|---|---|---|
| Initial B. P. | 120° | 116° | 120° | 120° |
| Per Cent Distilled | B.P. | B.P. | B.P. | B.P. |
| 5 | 189 | 183 | 181 | 179 |
| 10 | 193 | 190 | 189 | 184 |
| 15 | 196 | 192 | 193 | 194 |
| 20 | 199 | 196 | 195 | 194 |
| 25 | 201 | 197 | 197 | 199 |
| 30 | 202 | 201 | 201 | 202 |
| 35 | 206 | 202 | 194 | 204 |
| 40 | 211 | 205 | 206 | 204 |
| 45 | 215 | 210 | 210 | 209 |
| 50 | 217 | 215 | 213 | 215 |
| 55 | 220 | 219 | 215 | 218 |
| 60 | 223 | 222 | 214 | 221 |
| 65 | 226 | 226 | 226 | 226 |
| 70 | 227 | 228 | 226 | 229 |
| 75 | 226 | 219 | 225 | 231 |
| 80 | 211 | 213 | 232 | 223 |
| 85 | 227 | 211 | 238 | 234 |
| 90 | 225 | 223 | 243 | 243 |
| 95 | 220 | 216 | 251 | 231 |
| End Pt. | 232 | 228 | 257 | 248 |

crease in temperature per volume, whereas the controls did not show such changes. This increase is maintained to the end point of the distillation. An increase of 22° C. in the end point is obtained.

The increase is probably due to the formation of polymers of unsaturated hydrocarbons during the process of distillation. The origin of the unsaturated hydrocarbons is due to bacterial action on the kerosene. This indicates that the formation (dehydrogenation) of unsaturated compounds is probably one of the intermediate products of respiration. This observation confirms that of Tauson and Schapiro (1934).

The evidence of organic acids was the emulsion of kerosene and medium, and a small change in pH from 6.93 to 6.81.

## SUMMARY AND CONCLUSIONS

Bacterial cultures of organisms capable of using petroleum fractions such as "Skelly-solve", gasoline, kerosene, light and heavy mineral oils and paraffin wax as the sole source of carbon and energy for their metabolism were isolated from various sources. Natural habitats of such bacteria such as oil bearing soil, sedimentation ponds, and "water bottoms" of various petroleum storage tanks, were found to be good sources for the isolation of organisms of

this type. Organisms possessing this ability are not necessarily confined to such habitats, since practically all the Pseudomonas cultures are capable of utilizing kerosene, regardless of their origin. This was demonstrated by the fact that cultures isolated from various sources such as abcesses, mastitis, infected udders, water, and fecal matter of animals, were all able to utilize kerosene in their metabolism.

Bacteria of other genera were also found and described as being capable of this activity. Species of Micrococci, Corynebacterium, and a "slow lactose fermenting" organism were isolated and described. The "slow lactose fermenting" culture was outstanding in that it seemed to possess the ability to use paraffinic compounds in preferance to the naphthenic or cyclic hydrocarbons.

It was significant that the cultures were able to withstand as high as 10 to 15 transfers without diminution in growth. This indicates that accessory growth factors are not needed, or that the organisms were able to synthesize these substances from the hydrocarbons.

Studies on the respiration of hydrocarbons by bacteria indicated that the hydrocarbons were oxidized largely to carbon dioxide and water. The respiratory quotients of various bacterial cultures on different hydrocarbons varied

from 0.30 to 0.70. No correlation between the respiratory quotient and the nature of the hydrocarbon could be observed.

Some evidence was obtained to show that long-chain organic acids and unsaturated hydrocarbons were formed during the bacterial decomposition of the hydrocarbon fractions. The organic acids were indicated by small changes in the pH of the medium and also by the formation of emulsions of oil and water.

The production of unsaturated hydrocarbons was revealed indirectly by changes in the distillation temperatures of the kerosene. The boiling points of the last twenty per cent of kerosene to be distilled over were higher, indicating that probably polymers of a higher boiling point were formed during the process of distillation, from the unsaturated hydrocarbons produced by bacterial action.

As a result of this investigation, it has been established that the bacterial utilization of hydrocarbons is quite a common characteristic of some types of organisms and that probably in nature this process occurs to a greater extent than was formerly believed. The oxidation of hydrocarbons was found to function on comparative simple media; in fact, ordinary well water at the bottom of a distillate tank was able to support a bacterial count of approximately 900,000 organisms per cubic centimeter.

The respiration studies indicated that the oxidation of hydrocarbons is very similar to the oxidation of other organic compounds, and that end products, such as carbon dioxide, water, organic acids, and unsaturated hydrocarbons are produced.

ACKNOWLEDGEMENT

Appreciation is expressed to Dr. L. D. Bushnell, Head of the Department of Bacteriology, for guidance and for many helpful suggestions during this study.

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