A MORPHOLOGICAL AND PHYSIOLOGICAL STUDY OF AN UNIDENTIFIED HYDROCARBON OXIDIZING MICROCOCCUS

by

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INTRODUCTION

The capability of bacteria to utilize hydrocarbons as a source of energy and food was historically considered to represent a rather specialized or unique metabolic capability. Citations by Bushnell and Haas (1941) lead one to conclude that the early research workers considered that microorganisms growing on hydrocarbons were a separate physiological group. During recent years this concept has greatly changed. The present idea is that hydrocarbon oxidizing bacteria are common in nature (Kester, 1961).

In contrast to this ecological approach most physiological investigations of hydrocarbon utilizers have been concerned with the uniqueness of the attack on the paraffinic chemical groupings or end product formation. Relatively little research has been aimed at understanding general physiological characteristics of any of these bacteria. This investigation was undertaken to characterize the respiratory activities of an unclassified bacterium capable of luxuriant growth on a variety of liquid and solid aliphatic paraffines. Because the organism has not yet been classified, a comprehensive morphological study by usual compound microscope procedures plus detailed study by means of the electron microscope was undertaken. Furthermore, cultural and fermentation characteristics were included as part of this investigation to learn more of the physiology of this microorganism.

In study of respiration of intact cells comparisons of various types of chemicals known to have specific action were included using cells oxidizing either glucose or dodecane. It was believed that information regarding inhibition would give valuable data for further studies of the metabolism of this bacterium.

REVIEW OF LITERATURE

Historical and General

M. Miyoshi (1895, cited by Beerstecher, 1954) reported that a thin layer of paraffin was penetrated by the fungus Botrytis cinera. Apparently, this is the first report of microbial use of any hydrocarbon. Ability of fungi to attack paraffin was also reported by Rahn (1906) who found that various soil molds, including Penicillium glaucum, decomposed paraffin and utilized it as a sole source of energy. Bacteria were also found developing on paraffin. In the meantime, Söhngen (1906) showed that 17 species of soil bacteria possess the ability to utilize paraffin wax.

The capabilities of soil microorganisms to utilize aromatic compounds were studied by Matthew (1924). Later, Gray and Thornton (1928, cited by Bushnell and Haas, 1941) reported a Micrococcus (with some other bacteria) grew on naphthalene mineral salt medium. Branson (1964) reported various soil microorganisms including molds, actinomycetes and bacteria were capable of utilizing anthracene, benzene, dodecane, phenol, and toluene. Lipman and Greenberg (1932) isolated a coccus or coccobacillus from petroleum obtained at a depth of 8,700 feet which was capable of completely oxidizing petroleum to carbon dioxide.

Stone, Fenske and White (1942) studied bacteria from soil attacking petroleum and oil fraction. They found the breakdown of the oil was an oxidative change.

Bushnell and Haas (1941) found that Pseudomonas strains utilized hydrocarbons more rapidly than did other bacteria, while certain species of micrococci and corynebacteria were also able to assimilate these compounds.

The action of microorganism on hydrocarbon was reviewed by ZoBell (1944, 1950). He noted that microbial multiplication, oxygen consumption, nitrate or sulfate reduction, modification of hydrocarbon and the formation of various metabolic products had been employed as criteria of the utilization of hydrocarbons. He also noted that (1) aliphatic hydrocarbons were more susceptible to microbiological attack than aromatic; (2) long-chain hydrocarbons were more susceptible than short-chain hydrocarbons; (3) unsaturated hydrocarbons were more susceptible than the corresponding saturated compounds; (4) branched-chain hydrocarbons were more susceptible than unbranched.

Beerstecher (1954) gave a list of factors controlling the relative susceptibility of various hydrocarbons to oxidation, namely the inherent chemical stability of the hydrocarbon, the presence of enzyme systems, the ability of the hydrocarbon to dissolve lipoidal cellular constituents, its ability to dissolve or disperse in the medium and to pass through the cell wall, specific toxic effects of dispose of wastes, diverse metabolic pathways and nutritional requirements for use of different hydrocarbons, osmotic and surface tension effects. Webley, Duff, Farmer (1956) had devised an experiment to prove the evidence for β-oxidation in the mechanism of saturated aliphatic hydrocarbons by soil species of Nocardia.

The recent 20 years literature more or less concentrated in the mechanisms of oxidation of the hydrocarbons, and identification of the final products in the cells. Morris (1957) and Stewart et al. (1959) used the Warburg microrespirometer to study the bacterial oxidation of hydrocarbons.

No report concerned the adaptive control of respiration of hydrocarbon users. Also there were no reports about their respiration on various carbohydrates as substrates, or cellular responses to inhibitors in certain substrates.

Leadbetter and Foster (1959) studied a methane-utilizing pseudomonas capable of oxidizing gaseous paraffin to the corresponding alcohol, aldehyde, and acid. They used O^{18} to prove that hydrocarbon utilization by these bacteria was accompanied by incorporation of molecular oxygen. In 1960 they showed that methylketones were one of the terminal products from cultures oxidizing propane and n-butane.

Stewart and Kallio (1959) found cetylpalmitate formed by a gram-negative coccus from the esterification of cetyl alcohol and palmitic acid. Cells were grown on C_{14}-C_{18} n-alkanes and the primary alcohol moiety had the same number of carbon atoms as the substrate hydrocarbon. Heydeman (1960) and Procter (1960) also found primary alcohol as a major metabolic intermediate of oxidation of n-alkane.

Based on the foregoing studies, Kester (1961) summarized the sequence of reaction in the oxidation of n-alkanes.

1. N-alkane is oxidized to a corresponding fatty acid stepwise with a terminal carbon free radical, then a 1-hydroperoxide, then a 1-alcohol, then an aldehyde, and then to acid.

2. N-alkane was oxidized to methylketone by first forming a second carbon free radical compound, then a 2-hydroperoxide, then a 2-alcohol, and finally methylketone.

Kester (1961) used enrichment procedures with propane the common energy source, though ethane and isobutane were also used. Both bacteria and

actinomycetes were isolated. All actinomycetes strains plus many bacteria were able to utilize hydrocarbons.

MATERIALS AND METHODS

A single strain of microorganism used was isolated from the soil-pipeline interface in central Texas. An enrichment of several drops of dodecane on 25 mls of mineral salts solution was used to isolate this microorganism. The enrichments were incubated at room temperature in test tube where 10 g of soil was added. After several days, the interface between dodecane and mineral salts solution became turbid due to the growth of hydrocarbon utilizing bacteria. A transfer at 48 hr intervals was made to test tubes containing fresh mineral salts-dodecane medium. Pure cultures were obtained by streaking on nutrient agar plates and incubating for 48 hr.

To obtain enough cells for the morphological and Warbury respiratory rate studies, a peptone broth with the following composition was prepared.

Yeast extract	3 grams
Peptone	5 grams
K_2HPO_4	2.5 grams
KH_2PO_4	1.0 grams
Water	1,000 mls
10% Glucose	100 mls

To study the respiratory rates in various substrates, the cells free of culture medium must be obtained. The Ashby's solution used to wash the cells had the following ingredients:

KH_2PO_4	0.7 grams	$CaCl_2$	0.02 grams
K_2HPO_4	1.8 grams	$FeCl_3$	0.005 grams
$MgSO_4$	0.2 grams	Microelements*	1.0 ml
NaCl	0.2 grams	Distilled water	1,000 ml

* Solution containing 0.05% each of the salts of Mo, Zn, Mn, Cu, and Bo.

In order to study the effect of dodecane as a growth medium for the cells, the cells were cultivated in dodecane and mineral salts medium. This culture medium consisted of the following:

Dodecane	50 mls
Ashby's solution	100 mls
K_2HPO_4	0.5 gram
KH_2PO_4	0.2 gram
$(NH_4)_2PO_4$	0.4 gram
Distilled water	100 mls

The emulsified substrate for Warburg studies was prepared by heating 100 mls of water to 80 C. To this was added 5 grams of sodium-bentonite, and the proper quantity of hydrocarbon. This suspension was blended until it became homogeneous.

In order to obtain heavy suspensions of cells for respiration studies, peptone broth or dodecane mineral salts medium inoculated from a 24 hr agar slant was incubated on a shaking machine 20 to 24 hrs at 30 C.

The cells were removed by centrifuging and washed by suspending them in the Ashby's solution and centrifuging them 20 min at 5,000 rpm. After three washings, the cells were free of culture medium and waste products.

The respiratory activities of the cells were studied immediately after the cells had been washed, or the cells were refrigerated in double concentration of Ashby's solution at 5 C not more than 48 hrs.

For dodecane utilization studies, the approximate number of cells in the suspension as used was 250 million per ml. When diluted to 25 times, turbidimetric readings on the Bausch and Lomb Spectronic 20 at 500 mμ were O.D. equal 0.2.

To study the respiratory rates in the substrates such as glucose or related compounds, most of the turbidities of the cell suspension (diluted

25 times) were adjusted to give 0.05 optical density at 500 mμ. The approximate number in the suspension used was 50 million per ml.

One milliliter of cell suspension described above was added to the Warburg flask. Then 0.5 ml of substrates with 1.7 ml of double concentration Ashby's solution made the total volume to 3.2 ml. For absorption of carbon dioxide the center well contained a cylinder of filter paper which extended 5 mm above the rim of the well and 0.2 ml of 20 per cent KOH.

Different concentrations of inhibitors were used for the comparison of the effects of inhibitors on the substrate utilization. These were dissolved in distilled water where possible or were prepared as emulsions in bentonite.

In study of inhibitors, one ml of cell suspension was used with 0.5 ml of substrates, 0.5 ml of inhibitors and 1.2 ml of Ashby's solution. To the center well added 0.2 ml of 20 per cent KOH on a cylinder of filter paper as above.

All measurements were made at 30 C. Most of the experiments were of one and a half hour duration, and readings were taken at 15 min intervals.

The dimension of individual cells were measured and calculated from a known magnification of electron micrograph and also measured with a stained smear of bacteria under ordinary microscope with a linear ocular micrometer.

For morphological studies, the technique of electron microscope was used. The electronmicrographs were shadow cast with gold or with platinum-palladium. Electron microscope preparations used 2 drops of collodion dissolved in amyl-acetate (3%) placed on the surface of distilled water to

form a thin film. The concentration and amount of plastic material could be varied as needed to secure proper film thickness. Grids (250 mesh) were placed on the film. A clean glass slide was used to pick up the grids and the films which were then allowed to air dry. A drop of bacterial suspension was placed on the surface of the collodion film. After the suspension dried, shadow casting technique was used to apply a thin film of the metal on the cells. In shadowing the length of metal wire used was approximately 2 inches of 0.004 inch diameter, wound onto a tungsten hairpin inside the Kinney evaporator. The glass slide with the dried grids was placed approximately 10 cm directly below the hairpin. The metal was evaporated by heating it to a brilliant white incandescence in the vacuum. In the absence of oxygen, atomic metallic particles fly in all directions until colliding with some part of the apparatus. Thus, a film was built up on surface of cells.

MORPHOLOGICAL STUDIES

These bacteria stained gram negative. Regardless of age or type of growth medium they stain uniformly gram negative. Likewise, varying the pH of the growth medium from pH 5.0 to pH 9.2 had no effect on the staining reaction.

The morphology of this microorganism will be considered under three general conditions: I. Colony and slant growth on solid media; and II. cells grown on solid medium; and III. cells grown in various peptone broths.

I. When the bacteria were grown on the surface of nutrient agar plate, the colony was well formed. Colonial morphology is one of the

important characteristics used in classifying bacteria. These bacteria were highly aerobic. The colonies which grew on the surface of nutrient agar were large and circular due to an abundant supply of oxygen. The elevation was always flat, and the surface was smooth and glistening. The margins were entire and the consistency and texture of the mass of cells butyrous. No pigment was formed and density was opaque. Those colonies beneath the surface of nutrient agar appeared as a hemispherical form. They were more or less like a football. Those colonies were quite small usually not more than 1.5 mm in length and 0.8 mm in width.

When the bacteria were grown on the surface of nutrient agar stroke, they formed a moderate growth with filiform appearance, butyrous consistency and translucent density. It produced no pigment, but on the edge of the slant one could see, when toward the light, a fluorescence reflected. When the bacteria were grown on the surface of potato slant, an abundant growth formed, glistening with butyrous consistency.

II. The microorganism is sperical and very homogeneous with the respect to size. In general, cells had a diameter of 0.8 to 1.0 micron when grown on solid media.

The microorganisms occur mostly in pairs but single and short chains were also found. A capsule of varying thickness surrounded the spherical bodies. The largest capsules were developed by the bacteria with an abundance of food materials. Usually with cultivation of bacteria in the medium of peptone broth, a fairly large capsule stained by aqueous fuchsin could be seen.

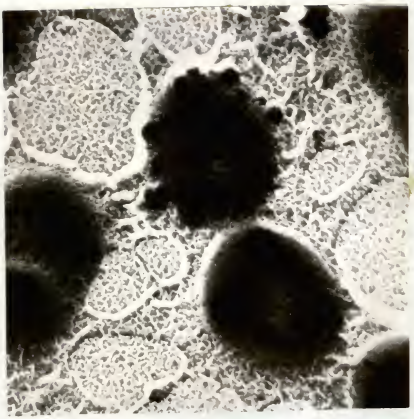

Fig. 1. An electron micrograph of the micro-organism. A fairly large capsule can be seen. The cells were grown on agar stroke for 24 hours and at 30 C. Approximate magnification of this picture is 36,000X.

Fig. 2. An electron micrograph of the micro-organism. The cells were grown on agar stroke for 14 days. A fairly old culture, but apparently it shows no morphological differences from young culture. Approximate magnification is 36,000X.

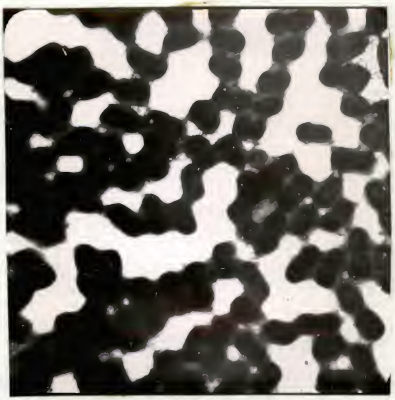

Fig. 3. An electron micrograph of the micro-organism grown on nutrient agar stroke for 24 hours. Approximate magnification is 6,000X.

No flagella have been found, and also not motile in motility agar stab, so that the bacteria should be classed as non-motile. In the electron micrographs, a dense material which might be nucleus or some polymetaphosphorus material was sometimes found near the center of the cells. These granules reflected the electron beam and appeared as dark bodies.

As shown in preceding pages, the cell had no variation in morphology throughout the periods of growth on solid medium. A young cell and an old cell had no difference so far as size and shape are concerned,

III. But when examining the same organism grown in the peptone broth, the microorganism showed marked variation in morphology, existing as short and long rods. The formation of rod-shaped bacteria was probably due to cells rapid growth without separation. It did not seem to be simply the factor of lower surface tension of a medium since cultivating the cells in dodecane and mineral salts solution did not reveal any rod-shaped bacteria. The rod-shaped bacteria were especially long and abundant when the culture was put on a shaking machine and examined when cells were young. Possibly certain components in the peptone broth induces some factors which favor the growth of cells. A further study of this pleomorphism is needed.

The microorganisms change, particularly in peptone broth, was of great interest. When observed under the phase microscope on a hanging drop slide, some flat cells appeared among spherical cells during lag phase. Then longer rods appeared. When the stationary phase was reached, each long rod divided, and relatively short rods appeared.

No branched-shape bacteria were found. The following electron micrographs show the bacterial morphological variation.

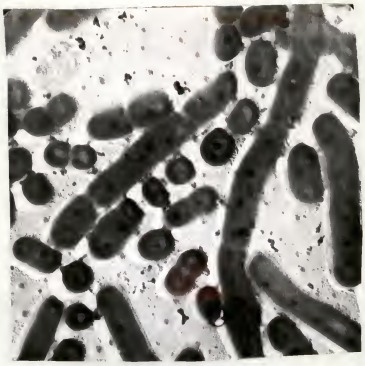

Fig. 4. Typical variation in morphology is shown with this microorganism grown on peptone broth on a shaking machine for 24 hr. The dense inner substance of the cells can be seen here. Approximate magnification is 9,600X.

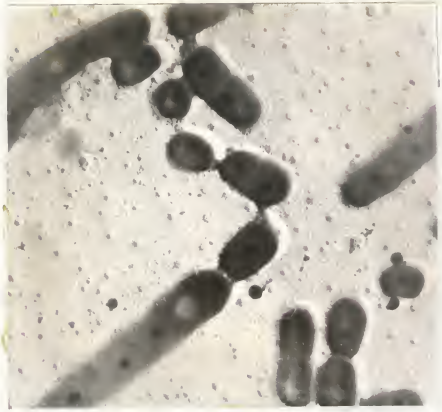

Fig. 5. An electron micrograph of some dividing bacteria. Approximate magnification 9,600X.

The dense material can be seen at the center of the cells in some of those pictures. It seems the inner substance of the cells remains intact, although the cell wall can no longer be seen.

PHYSIOLOGY

The description of certain properties and behavior of the bacterium in physiological terms would serve to increase our understanding of its metabolic activity and also aid in classification. The following list illustrates the behavior of this bacterium in various fermention tests:

Sugar	Reaction*
Arabinose	- Turn alkaline slowly
Dextrose	+
Ducitol	-
Fructose	-
Inulin	-
Lactose	-
Maltose	-
Raffinose	-
Rhamnose	-
Salicin	-
Sorbitol	-
Sucrose	-
Trehalose	-
Xylose	+

* + = acid only
- = no acid and gas

Some other physiological properties of the bacterium list as follows:

Substance	Reaction
Blood agar plate	α-hemolysis
Gelatin	Hydrolyzed
Litmus milk	Acid-curd and reduction
3% of NaCl mineral salts medium	Growth
5% of NaCl mineral salts medium	No growth
1% of Na-hippurate	Benzoic acid formed
Nitrate	Not reduced to nitrite

Substance	Reaction
Peptone water	Indole formed
Starch	Not digested
Triple sugar iron stab	Hydrogen sulfide formed
Urea	Not used as sole source of carbon
Nutrient broth	Turbidity and white precipitation

The cells were able to grow on dodecane and mineral salts medium at pH 5.0 and as high as pH 9.5; growing best at the range of pH 6.5 to 7.5. The following table illustrates the effect of pH on the growth of the bacterium.

pH	Amount of Growth*
4.5	-
5.0	+
5.5	++
6.0	+++
6.5	++++
7.0	++++
7.5	++++
8.0	+++
8.5	+++
9.0	++
9.5	+
10.0	-

* The amount of growth based on the observation of turbidity of growth medium.

EXPERIMENTS ON RESPIRATION

The first series of tests on respiratory rates were made to determine the microorganism response to the presence of different growth substrates. The respiration of microorganism grown on peptone broth with glucose, peptone broth, and dodecane mineral salts medium were studied in the presence of glucose, peptone, and dodecane as a respiratory substrate. Figure 6 shows O_2 uptake rates observed in this series.

Fig. 6. Comparisons of oxygen uptake of the microorganism grown in peptone broth with glucose, and peptone broth without glucose. The washed cells were in the presence of solutions of glucose, peptone, dodecane served as a respiration substrate.

-------- = Cells grown in peptone broth with glucose.

_____ = Cells grown in peptone broth without glucose.

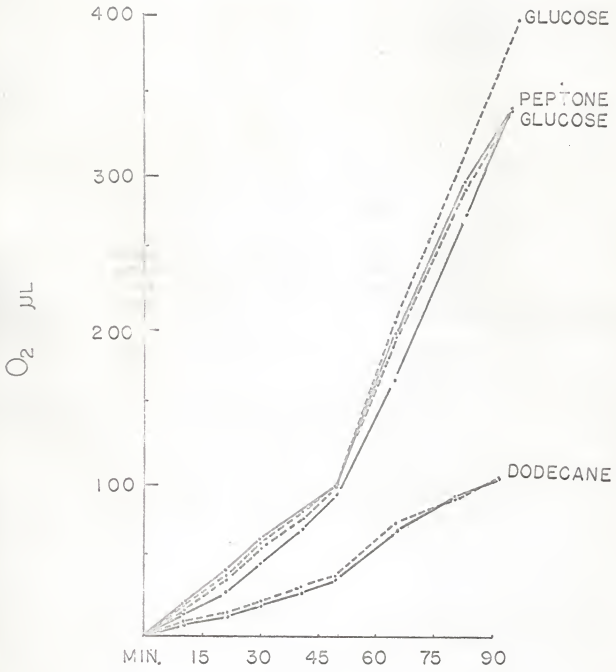
400
300
200
100
O_2 µL
GLUCOSE
PEPTONE
GLUCOSE
DODECANE
MIN. 15 30 45 60 75 90

Fig. 6

It is plainly evident that cells incubated in peptone broth with glucose were more capable of utilizing glucose than those incubated in peptone broth. As to the capability of utilizing peptone and dodecane, the presence or absence of glucose in culture medium did not influence the comparative rates.

An experiment was designed so that the Warburg apparatus was not shaken in the first fifty minutes, in order to see the cells response to the mechanical shaking. The graph shows the oxygen uptake of the cells increased after shaking.

In Fig. 7. the microorganisms grown in dodecane mineral salts medium were more capable of utilizing dodecane than those grown on peptone broth. Cells grown in peptone broth gave more rapid oxygen uptake in glucose or peptone than dodecane. These results indicate a degree of adaptation to the growth substrate but not an absolute lack of activity preceding induction to a new substrate.

The second series of tests on respiratory rates was made to determine the relative ease with which different types of compounds served as respiratory substrates.

Fig. 8 shows the oxygen uptake with glucose and galactose was approximately five hundred microliters within a hundred and five minutes duration. The Na-formate utilization gave a respiratory rate about three hundred microliters within the same time. Sucrose and lactose as well as Na-citrate showed that only 30-40 microliters of oxygen were consumed. Cells used here were grown on peptone broth.

Fig. 9 shows the respiration of cells in the presence of glucose, Na-acetate, Na-succinate, Na-lactate and glycerol. All substrates were at

Fig. 7. Comparisons of oxygen uptakes of the microorganism grown on peptone broth and dodecane mineral salts medium. The washed cells were in the presence of solutions of glucose, peptone broth, dodecane which served as respiration substrates.

_____ = Cells grown on peptone broth.

---------- = Cells grown on dodecane mineral salts.

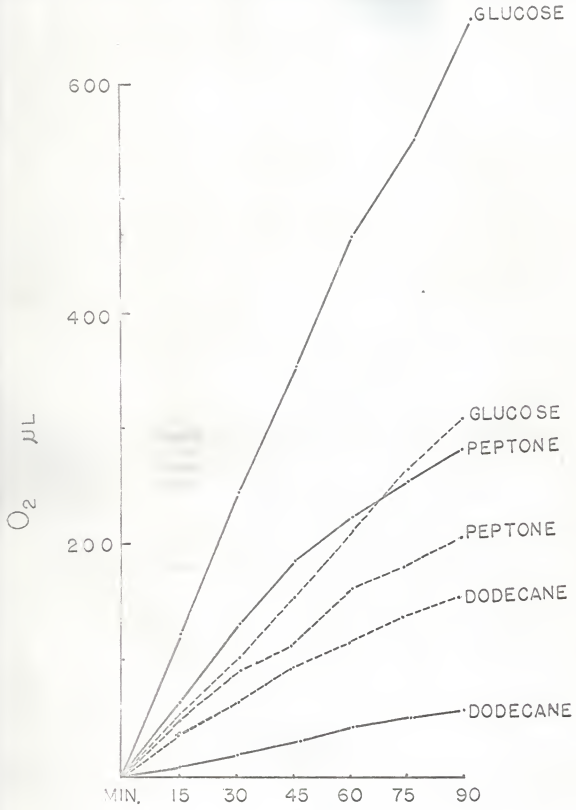
GLUCOSE
600
400
200
O_2 µL
GLUCOSE
PEPTONE
PEPTONE
DODECANE
DODECANE
MIN. 15 30 45 60 75 90

Fig. 7

Fig. 8. Respirations of the microorganism in different kinds of substrates; glucose, galactose, Na-formate, lactose, sucrose, Na-citrate in 0.5 M were used. Cells used here were grown on peptone broth.

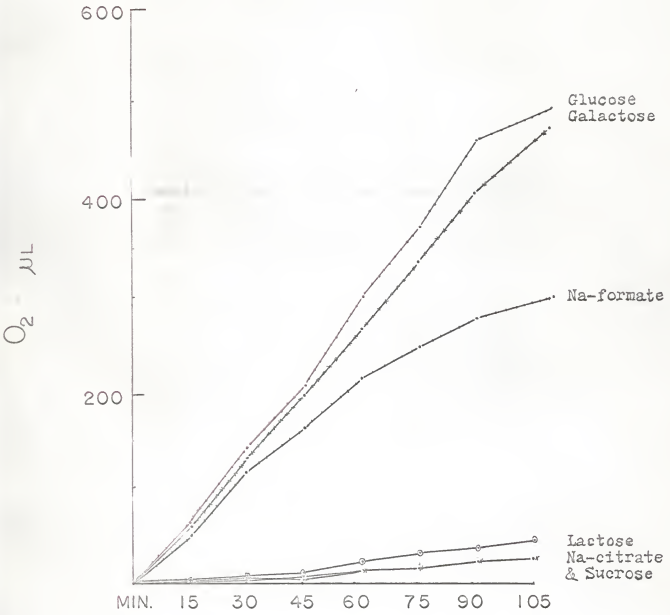
600
400
200
O_2 µL
MIN. 15 30 45 60 75 90 105
Glucose
Galactose
Na-formate
Lactose
Na-citrate
& Sucrose

Fig. 8

Fig. 9. Respiration rates of the microorganism in different kinds of substrates; glucose, Na-acetate, Na-succinate, Na-lactate, glycerol. Cells used here had grown on dodecane mineral salts.

Fig. 10. Respiration rates of the microorganism in different kinds of hydrocarbon; lauric aldehyde, lauric acid, dodecane, dodecene, dodecyl alcohol. Except lauric-aldehyde, all hydrocarbons were made to a form of bentonite emulsion in 0.25 M. Cells used here had grown on peptone broth.

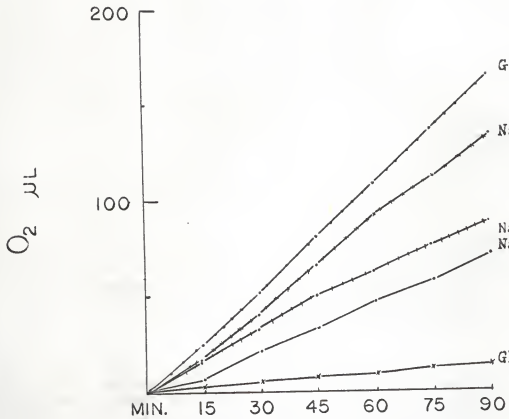
200
100
O_2 µL
Glucose
Na-acetate
Na-succinate
Na-lactate
Glycerol
MIN. 15 30 45 60 75 90

Fig. 9

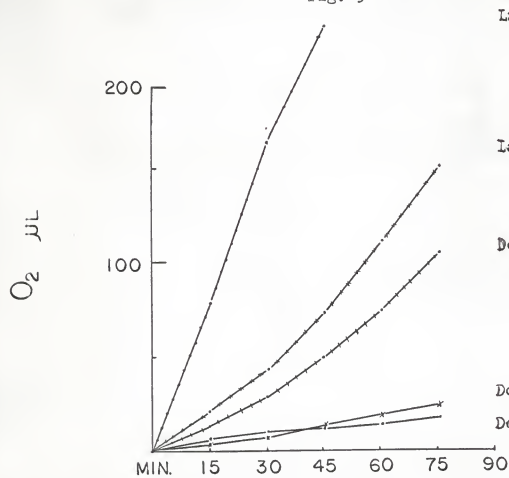
200
100
O_2 µL
Lauric aldehyde
Lauric acid
Dodecane
Dodecene
Dodecyl alcohol
MIN. 15 30 45 60 75 90

Fig. 10

a concentration of 0.25 M. These microorganisms were grown on dodecane mineral salts medium for four days, then washed until it was free of medium.

Among the substrates, glycerol is the least readily oxidized. Glucose was the best substrate for O_2 uptake. Na-acetate was better than Na-succinate, and Na-succinate was better than Na-lactate.

Fig. 10 shows the respiratory rates of the cells in the presence of a number of twelve carbon compounds related to the paraffin dodecane. These were liquid lauric aldehyde, emulsified lauric acid, emulsified dodecane, emulsified dodecene, and emulsified dodecyl alcohol. All substrates were made up to a concentration of 0.25 M. Cells used here were grown on peptone broth.

Among the substrates, dodecyl alcohol was the least readily oxidized, while lauric aldehyde was the best substrate. Lauric acid gave an uptake rate of 156 microliters within 75 min duration, compared with 103 microliter for dodecane. Dodecene was not readily utilized showing only a rate of 23 microliter per 75 min.

The third series of tests on respiratory rates was made to determine the effects of inhibitors on the utilization of glucose and dodecane as a substrate. All cells used here were grown on peptone broth for one day, then washed until free of medium.

The effect of various concentrations of Na-cyanide added to the microorganisms suspension in the presence of glucose is shown in Fig. 11. Increasing the concentration of Na-cyanide made the respiratory rates of the cells decrease. The concentration of 0.01% showed some inhibition, but its inhibitory effect was lower than the concentration of 0.025%,

0.05% and 0.1%, respectively. As the concentration of Na-cyanide reached 0.25%, the microorganism showed no respiration.

The various concentration of Na-cyanide added to the cells suspension in the presence of dodecane as a respiratory substrate is shown in Fig. 12. Not until the concentration of Na-cyanide reached 0.025%, did cells show apparent retardation of respiration. As the concentration increased from 0.05% to 0.25%, the respiration of the cells decreased gradually. As it was in glucose, the microorganism in 0.25% Na-cyanide showed also a minimum respiratory rate.

The various concentrations Na-azide applied to the cells suspension in the presence of glucose is shown in Fig. 13. It seems that Na-azide was incapable of inhibiting the whole respiration of the microorganism. In concentrations of Na-azide as high as 5%, the cells still had 61 µl of oxygen uptake. There may have been slight inhibition as the cells had 95 µl of oxygen uptake in the presence of 0.1% Na-azide which was 87 µl less than the cells which had no inhibitor.

The various concentration of Na-azide applied to the cells suspension in the presence of dodecane is shown in Fig. 14. The respiratory pattern here is quite similar to Fig. 13 except that dodecane respiration seemed to be slightly less sensitive than glucose to concentrations of 0.1% and 0.5% azide.

Respiration in the presence of Na-malonate as an inhibitor to the microorganism in the presence of glucose is shown in Fig. 15. It can be seen that Na-malonate did not serve as an inhibitor. The respiration of the microorganism in the presence of inhibitor was higher than the control, indicating that Na-malonate could be utilized by the microorganism.

Figure 16 shows various concentrations of Na-malonate applied to the microorganisms in the presence of dodecane. Here also Na-malonate was used by the microorganism as an energy source instead of inhibitory factor. The exhaustion of Na-malonate can be seen on the 0.1% and 0.2% curves after 30 and 60 minutes respiration.

The rates of oxidation of glucose in the presence of various concentrations of iodoacetate are shown in Fig. 17. Little inhibition was shown until the concentration of the iodoacetate reached to 1.5%. The cells ceased their respiration, when the concentration of inhibitor reached 2.2%. The concentration of inhibitor lower than 1.0% seemed to have stimulated the respiration of the cells.

Figure 18 shows the oxidation of dodecane in the presence of iodoacetate. Unlike glucose oxidation, cells respiring on dodecane were very sensitive to the inhibitor. Only 0.1% iodoacetate completely eliminated the respiration of the cells. As little as 0.005% iodoacetate added to the cells suspension gave measurable inhibition.

The effect of 2,4-dinitrophenol on the cells in the presence of glucose is shown in Fig. 19, while the effect of 2,4-dinitrophenol on the cells in the presence of dodecane is shown in Fig. 20. The respiration of the microorganism was not slowed down in the presence of inhibitor. There might have been some stimulation to the cells when small amount of 2,4-dinitrophenol was added to the respiratory substrates.

Figure 21 shows the effect of different amounts of n-octanol treated as an inhibitor when added into the cells suspension in the presence of glucose. The curves show that 0.005 ml of n-octanol added to the cells

Fig. 11. Respiration rates of the microorganism in varying concentrations of Na-cyanide in the substrate glucose.

Fig. 12. Respiration rates of the microorganism in varying concentrations of Na-cyanide in the substrate dodecane.

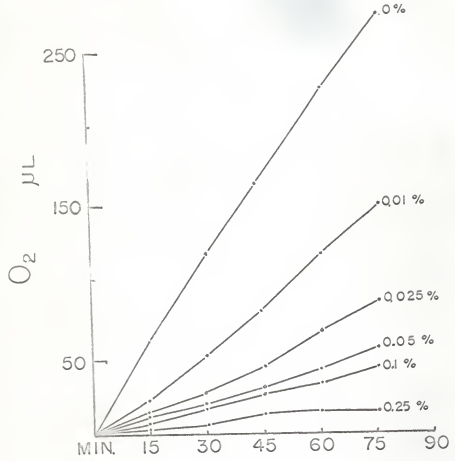
0 %
0.01 %
0.025 %
0.05 %
0.1 %
0.25 %
250
150
50
O_2 µL
MIN. 15 30 45 60 75 90

Fig. 11

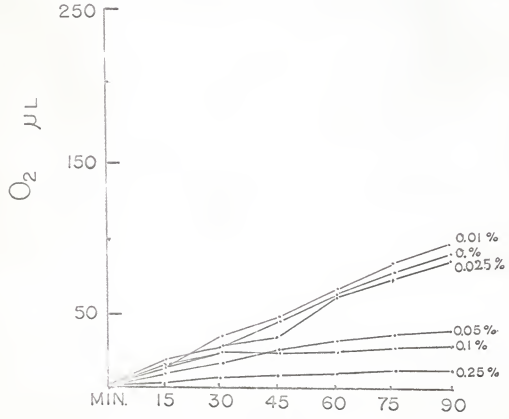
0.01 %
0. %
0.025 %
0.05 %
0.1 %
0.25 %
250
150
50
O_2 µL
MIN. 15 30 45 60 75 90

Fig. 12

Fig. 13. Respiration rates of the micro-organism in varying concentrations of Na-azide in the substrate glucose.

Fig. 14. Respiration rates of the microorganism in varying concentrations of Na-azide in the substrate dodecane.

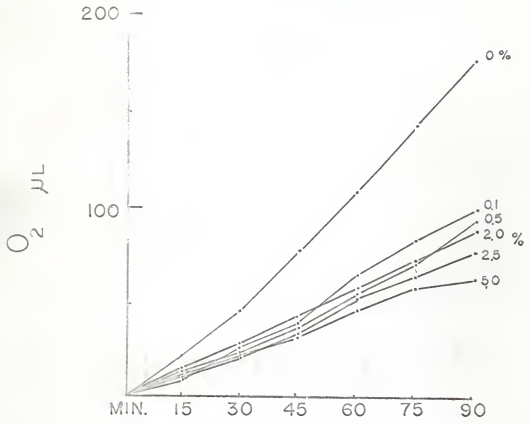
200
100
O_2 µL
0 %
0.1
0.5
2.0 %
2.5
5.0
MIN. 15 30 45 60 75 90

Fig. 13

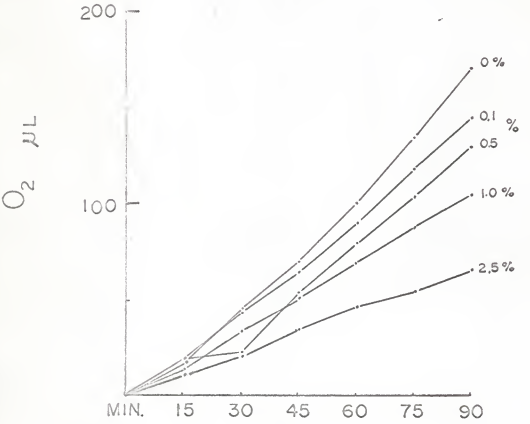
200
100
O_2 µL
0 %
0.1 %
0.5
1.0 %
2.5 %
MIN. 15 30 45 60 75 90

Fig. 14

Fig. 15. Respiration rates of the microorganism in varying concentrations of Na-malonate in the substrate glucose.

Fig. 16. Respiration rates of the microorganism in varying concentrations of Na-malonate in the substrate dodecane.

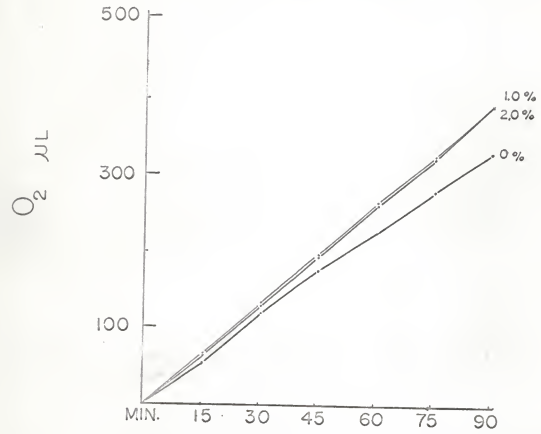
500
300
100
O_2 µL
MIN. 15 30 45 60 75 90
1.0 %
2.0 %
0 %

Fig. 15

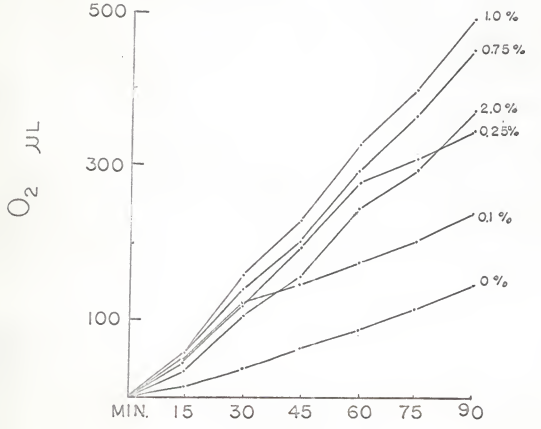
500
300
100
O_2 µL
MIN. 15 30 45 60 75 90
1.0 %
0.75 %
2.0 %
0.25%
0.1 %
0 %

Fig. 16

Fig. 17. Respiration rates of the microorganism in varying concentrations of iodoacetate in the substrate glucose.

Fig. 18. Respiration rates of the microorganism in varying concentrations of iodoacetate in the substrate dodecane.

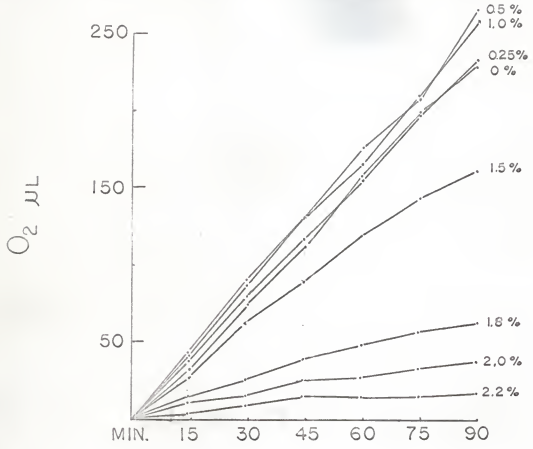
250
150
50
O_2 µL
MIN. 15 30 45 60 75 90
0.5 %
1.0 %
0.25%
0 %
1.5 %
1.8 %
2.0 %
2.2 %

Fig. 17

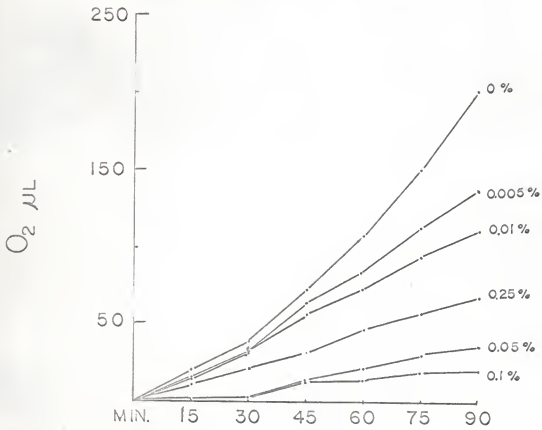
250
150
50
O_2 µL
MIN. 15 30 45 60 75 90
0 %
0.005 %
0.01 %
0.25 %
0.05 %
0.1 %

Fig. 18

Fig. 19. Respiration rates of the microorganism in varying concentrations of 2,4-dinitrophenol in the substrate glucose.

Fig. 20. Respiration rates of the microorganism in varying concentrations of 2,4-dinitrophenol in the substrate dodecane.

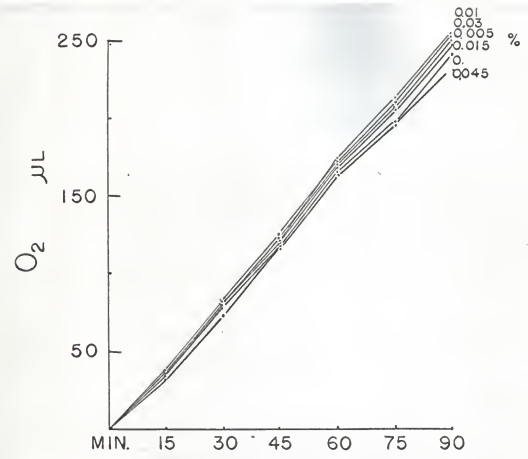
250
150
50
O_2 µL
MIN. 15 30 45 60 75 90
0.01
0.03
0.005
0.015
0.
0.045
%

Fig. 19

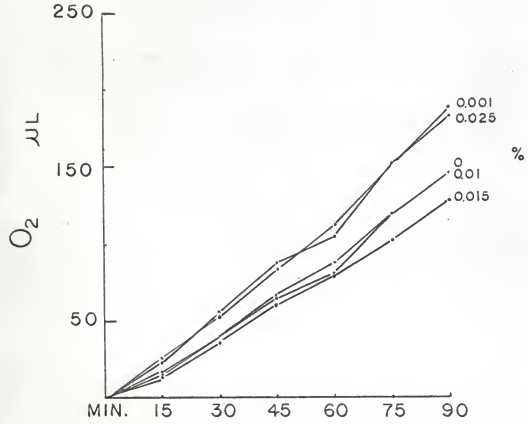
250
150
50
O_2 µL
MIN. 15 30 45 60 75 90
0.001
0.025
0
0.01
0.015
%

Fig. 20

Fig. 21. Respiration rates of the microorganism in varying amounts of n-octanol in the substrate glucose.

Fig. 22. Respiration rates of the microorganism in varying amounts of n-octanol in the substrate dodecane.

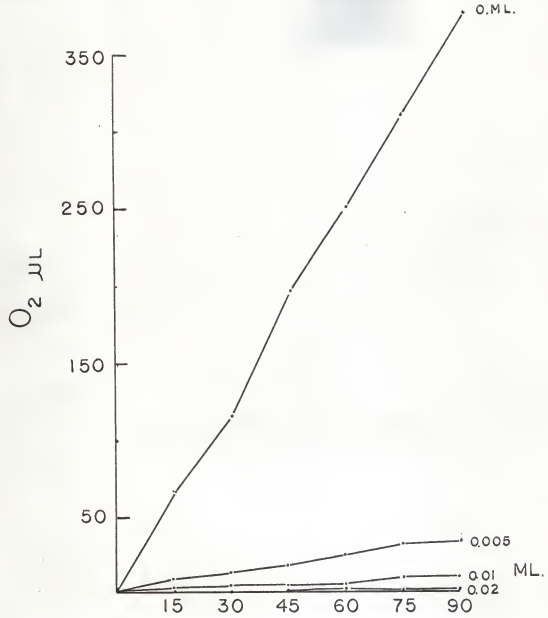
0.ML.
350
250
150
50
O_2 µL
0.005
0.01
0.02
ML.
15
30
45
60
75
90

Fig. 21

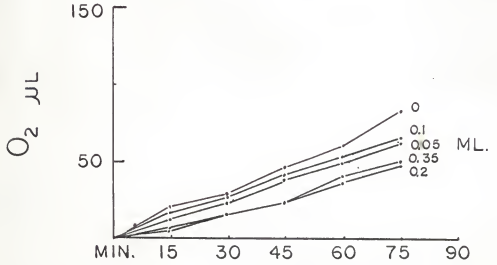
150
50
O_2 µL
0
0.1
0.05
0.35
0.2
ML.
MIN.
15
30
45
60
75
90

Fig. 22

Fig. 23. Respiration rates of the microorganism in 0.15 ml 2-octanol in varying amounts of glucose. Same amounts of glucose but no 2-octanol set as a control.

..... = 0.05 ml
- - - - = 0.1 ml
.-.-.-.- = 0.2 ml
._____. = 0.3 ml
.---.--- = 0.5 ml

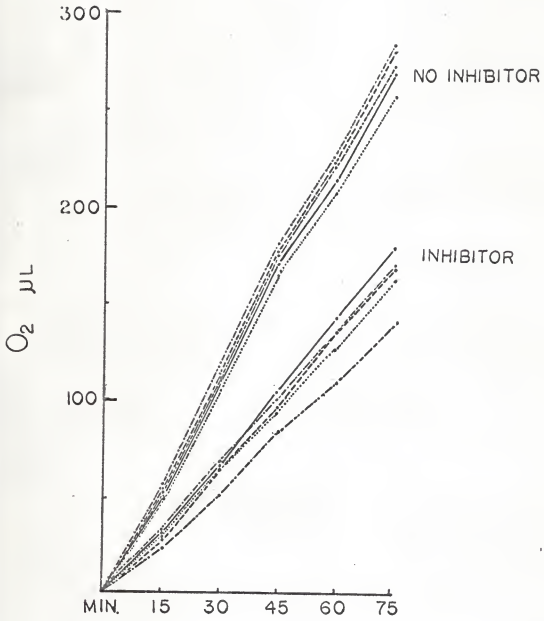

Fig. 23

Fig. 24. Respiration rates of the microorganism in 0.15 ml 2-octanol in varying amounts of dodecane. Same amounts of dodecane but no 2-octanol set as a control.

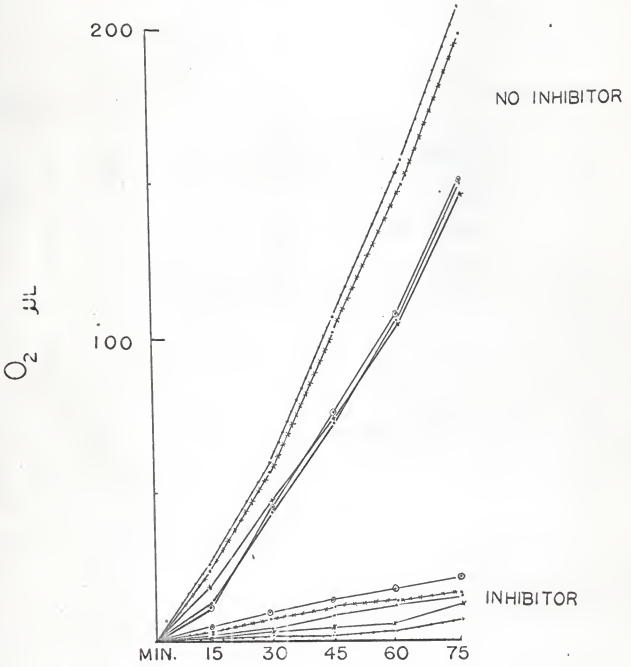

Fig. 24

suspension retards its respiration. The cells ceased their respiration completely when the amount reached 0.02 ml per flask.

Figure 22 shows different amounts of n-octanol added to the cells in the presence of dodecane. Unlike glucose, the n-octanol did not inhibit dodecane oxidation. Even 0.35 ml of n-octanol added to the suspension did not retard the respiration of the cells very much. No increasing inhibition appeared with increased amounts of n-octanol added to the cell suspension.

The 2-octanol served as an inhibitor to cells in the presence of glucose as shown in Fig. 23. In order to determine if 2-octanol was a competitive or non-competitive inhibitor, an experiment was carried out by adding constant concentrations of 2-octanol into varying concentrations of substrate. In the test 0.15 ml of 2-octanol was added to the microorganisms suspension in the presence of 0.5, 0.4, 0.3, 0.2, 0.1 ml of 10% glucose. A series of Warburg flasks which contained the same amounts of glucose but no 2-octanol were run for controls.

The curves show that varying the concentration of glucose does not influence the effect of inhibitor. This indicates non-competitive inhibition.

In contrast to the action of n-octanol on oxygen uptake by cells using dodecane, 2-octanol gave a strong inhibition as shown in Fig. 24. Varying the substrate again gave evidence of non-competitive action.

DISCUSSION

Karstrom (1930) demonstrated that Leuconostoc mesenteroides could not ferment lactose immediately unless the organism had been cultivated in the presence of lactose. When the lactose-fermenting cells were passed in the lactose-free media, they lost the ability to ferment lactose without delay. Karstrom concluded that enzymes such as the lactose-utilizing ones which appear only as a response to the presence of the substrate in the culture medium or cell suspension were termed adaptive enzymes. Karstrom's work was soon confirmed and extended to other organisms.

In many studies on enzymatic adaptative the change may be only a quantitative one. The enzyme may be present in the cell but in only small amounts or with limited activity.

This microorganism was capable of utilizing glucose, even though the culture medium had no glucose present. Glucose utilization thus appears to result from the so-called "constitutive" enzyme system.

The cells grown on dodecane mineral salts medium could oxidize dodecane more readily than cells grown on media without dodecane.

These experiments have demonstrated that the culture medium played an important role to the respiration patterns of the cell.

It is generally agreed that of all the foodstuffs used by the microorganism for the maintenance of the steady state, as well as for the processes of multiplication and growth, carbohydrates show the maximum efficiency in their oxidation.

The microorganism was also capable of utilizing Na-formate. It was suggested that the monosaccharides such as glucose and galactose,

after having diffused into the cells, could be broken down into small fragments like pyruvic acid, acetic acid such as is known to be common in other bacteria.

The respiration rates depend on either the permeability of the substrates or the activities of enzymes inside the cells.

The cells were rather slow to act on the disaccharides, such as lactose and sucrose. Whether this was due to a lack of specific hydrolases or whether it was a problem of permeability into the cell is not known. Since the higher hydrocarbon compounds dodecane were oxidized by the cell, it seems to be illogical that a disaccharide and Na-citrate could not enter the cell. Further studies are needed here.

Acetic acid is reactive and takes part in a large number of synthetic reactions. The respiration rate of this microorganism in the presence of acetate salts was relatively high as would be expected from known action by other bacteria.

High respiration rates of cells in the presence of succinate and lactate indicate that these are intermediates in the oxidative pathways of carbohydrate metabolism in this bacterium as they are in most cells.

The presence of hydroxyl group in certain molecules served as powerful inhibitors to this microorganism. Glycerol as well as other compounds with hydroxyl group could not be utilized by this microorganism.

As shown in Fig. 8 and 9, a comparison of carbohydrates readily oxidized by the cells is made as follows:

1. Monosaccharide > formate > disaccharides or citrate.
2. Monosaccharides > acetate > succinate > lactate > glycerol.

As shown in Fig. 10, the sequence of respiration rates of this microorganism in the presence of hydrocarbons can be arranged as follows:

Lauric aldehyde > lauric acid > dodecane > dodecene > dodecyl alcohol.

It is not known why dodecane is more readily oxidized than dodecene. ZoBell (1944) had pointed out that unsaturated hydrocarbons were more susceptible than the corresponding saturated compounds.

It is also not known why lauric aldehyde is more readily oxidized than lauric acid. The fate of a n-alkane, as summarized by Kester (1961), would be either an acid or a methylketone. Before forming acid, a corresponding aldehyde must be formed. The results with the organism used in our work indicate that pathways other than those proposed by ZoBell and Kester must exist. It is significant that dodecyl alcohol, a compound with a hydroxyl group, could not be used.

As shown in Fig. 11 and 12, different amounts of cyanide added to the cells suspension gave different inhibitory effects. Glucose respiration was more sensitive to cyanide than dodecane respiration. The high sensitivity to cyanide indicated that (1) the cyanide ions are able to diffuse into the cells; (2) the microorganism may possess a cytochrome system; (3) the inhibitory effect of cyanide depends somewhat on the substrate; and (4) more respiratory enzymes of the cells may be required for oxidizing glucose or these may have more heavy metals than dodecane enzymes so that the presence of small amount of cyanide slowed down the total respiratory rate more when the cells were in glucose solution than in dodecane.

The patterns of inhibition of azide shown in Fig. 13 and 14 were different from cyanide. Cell respiration did not cease even though 2.5%

of azide was added into the cell suspension. Was the azide entry into the cell limited? Since azide interferes with phosphorylation this may indicate other oxidative pathways. Further studies are needed.

The addition of malonate into the cell suspension in the presence of glucose or dodecane shown in Fig. 15 and 16, had no inhibitory effect. On the contrary, the respiration of the microorganism increased. It seems that this microorganism is capable of utilizing malonate as a respiratory substrate. Possible products of breaking down or transforming a malonate are formic acid, acetic acid, oxalic acid, glyoxylic acid, or pyruvic acid, etc.

When adding the different amounts of iodoacetate into the microorganisms suspension, two types of inhibitions resulted as shown in Fig. 17 and 18. To microorganism in glucose solution, no inhibitory effect was shown until iodoacetate was more than 1%. To microorganism in dodecane, cells were quite sensitive to the inhibitor. Even adding concentrations of 0.005% into the microorganism suspension gave measurable inhibitory effects.

It indicates that iodoacetate effectively inhibited the enzyme systems oxidizing dodecane but not the enzyme involved in oxidizing glucose.

Furthermore, the oxidized glucose must have "shunted" before the triose phosphate stage, because iodoacetate acts at this stage in the Meyerhof pathway.

The 2,4-dinitrophenol, especially, in low concentration, increased respiration to some extent in this organism as shown in Fig. 19 and 20.

According to Nord (1945), the uncoupling activity of 2,4-dinitrophenol provides a biochemical basis, not only for the decreased utilization of the energy derived from respiration but also for the increased respiration of DNP-treated organism. Thus, the 2,4-dinitrophenol may have decreased synthetic reactions in the microorganism, and allowed increased respirations.

A small amount (0.02 ml) of n-octanol stopped completely the respiration of cells in the substrate glucose but not in dodecane as shown in Figs. 21 and 22. Contrary to iodoacetate, the n-octanol effectively inhibited the enzyme oxidized glucose but not the enzyme oxidized dodecane. It might be the active center of the enzyme oxidized glucose is more hydrophilic than the enzyme oxidized dodecane, so that the hydroxyl group of n-octanol formed a film of adsorbed molecules over the enzyme. Other surface effects are possible. Figs. 23 and 24 showed 2-octanol acted as a noncompetitive inhibitor to substrates. As it can be seen, increasing amounts of substrates to definite volume of 2-octanol in Warburg flasks, the respiration rates of cells were not changed. Unlike the n-octanol, the 2-octanol was not so toxic to microorganism in the presence of glucose solution but relatively toxic in dodecane. The 2-octanol, with a hydroxyl group at second carbon, served as an inhibitor but gave an entirely different inhibitory effect than n-octanol.

SUMMARY

The morphological study by both the compound microscope and the electron microscope showed the bacterium to possess a coccoidal form, a stable shape

when grown on agar media of various composition. However, in peptone glucose broth protoplasmic syntheses apparently exceeded cell wall or division rate so that rods were commonly present. In fermentation tests most tubes became slightly alkaline except glucose and xylose. No gas was formed.

In respiration studies with the Warburg microrespirometer, hydrocarbon oxidation was shown to be more rapid in cells grown on dodecane than when cells were grown on glucose and peptone. This indicates an inducible system of enzymes. These bacteria were able to carry out an active respiration utilizing a number of sugars, organic acids, and hydrocarbons. Inability of cell suspensions to oxidize citrate may have been due to permeability factors.

Comparisons of various inhibitors when cells were oxidizing glucose and dodecane indicated that it may be possible to study the mechanism of hydrocarbon utilization by this method. Cyanide is a highly potent inhibitor of all respiratory activity while azide was not effective in giving complete cessation of oxygen uptake even in relatively high concentrations. Malonate, rather than being an inhibitor, was utilized as a substrate by this organism. Dodecane respiration was much more sensitive to the effects of iodoacetate than was glucose respiration. Usually inhibitory concentrations of 2,4-dinitrophenol did not inhibit oxygen uptake but rather gave a slight stimulation.

Comparison of inhibition by n-octanol and 2-octanol showed glucose respiration to be much more sensitive to the inhibition by n-octanol while the dodecane oxidation was inhibited to a much greater degree by 2-octanol.

Comparisons of various inhibitor-substrate concentration ratios indicate a non-competitive inhibition by 2-octanol.

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A MORPHOLOGICAL AND PHYSIOLOGICAL STUDY OF AN UNIDENTIFIED HYDROCARBON OXIDIZING MICROCOCCUS

by

MIN CHEN

B. S., Taiwan University, 1959

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1966

An unclassified bacterium which is capable of utilizing hydrocarbons as sole carbon source was studied in this thesis. A morphological study by usual compound microscope procedures and detailed study by means of electron microscope was undertaken. Moreover, cultural and fermentation characteristics were included as part of this investigation.

In sugar fermentation most tubes reacted alkaline except glucose and xylose which gave acid with no gas produced.

The bacterium is a coccoidal form when grown on solid media but changes its appearance into short and long rods when grown in peptone broth. It is a gram-negative and highly aerobic bacterium.

A series of experiments made use of Warburg technique to test its physiological characteristics.

It was concluded that:

1. The organism had an adaptive response to its growth medium.

2. The organism could make use of various kinds of substrates and had a high capability to utilize monosaccharides and lauric aldehyde.

3. The respiration of the microorganism was more or less inhibited by cyanide, azide, iodoacetate, n-octanol and 2-octanol, respectively, but stimulated by Na-malonate and 2,4-dinitrophenol.

4. In the presence of dodecane, the respiratory rate of cell was much slower than in the presence of glucose when iodoacetate used as inhibitor.

5. On the contrary, the cells were very sensitive to n-octanol when they were in the substrate glucose than in dodecane.

6. It seems that iodoacetate strongly inhibits the dodecane utilizing enzymes and n-octanol inhibits the glucose utilizing enzyme system. At least two kinds of enzymes involved in glucose and dodecane metabolic pathway can be claimed.

7. 2-octanol served as a non-competitive inhibitor with both dodecane and glucose as substrates.