ACTION OF MICROÖRGANISMS ON HYDROCARBONS[1]

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Many microörganisms possess the ability of utilizing hydrocarbons as a sole source of energy in their metabolism. Gaseous, liquid and solid hydrocarbons in the aliphatic, olefinic and naphthenic series are susceptible to microbial decomposition. Nearly a hundred species of bacteria, yeasts and molds representing thirty genera have been described which attack one or more kinds of hydrocarbons. Such microbes appear to be quite widely and abundantly distributed in nature where they may be of considerable importance in the carbon cycle and to various industries.

Wherever exposed to mineral solutions in which microbial life is possible, petroleum, rubber or other types of hydrocarbons may be slowly decomposed by microörganisms. The microbial oxidation of hydrocarbons may help to account for the rapid disappearance of petroleum which pollutes fields and waterways, for the deterioration of certain rubber products both natural and synthetic, for the spoilage of cooling oils, for the depreciation of oiled or asphalt-surfaced highways and for the modification of petroleum or its products stored in the presence of water. The failure of underground pipe-lines or electrical conduits "protected" from corrosion by paraffin-impregnated materials, elastomers or other hydrocarbon derivatives may be attributed in part to the activities of microorganisms which decompose hydrocarbons.

Obtaining intermediate products of economic value such as fatty acids, for example, from the microbial decomposition of hydrocarbons, or employing micro-

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organisms for the elimination of industrial wastes around refineries are almost unexplored possibilities. In this category, too, are certain methods of prospecting for petroleum which are based upon the ability of bacteria to utilize petroleum hydrocarbons. Likewise very little is known regarding the effect of the growth of hydrocarbon-oxidizing bacteria in pharmaceutical and medicinal preparations containing petrolatum or mineral oil.

These are some of the problems which are discussed on the following pages in summarizing our knowledge on the occurrence, characteristics, biochemical activities and possible economic importance of microörganisms which attack hydrocarbons.

Historical. Although paraffin wax is often considered to be biologically inert, Miyoshi (93) reported fifty years ago that thin layers of paraffin were penetrated by *Botrytis cinerea*. The ability of fungi to attack paraffin was reported in 1906 by Rahn (104), 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 the paraffin. Söhngen (121) showed that paraffin was attacked by 17 different species of soil bacteria, paraffin wax being more readily attacked than petroleum ether, paraffin oil, crude petroleum or caoutchouc. The widespread occurrence of microörganisms which attack paraffin is attested by many observers (cf., (53, 60, 138, 142, 145, 150, 181)).

The utilization of methane by mixed cultures of soil bacteria was reported by Kaserer (83) in 1906. The bacteria also utilized hydrogen as a source of energy. The microbial utilization of methane was studied more thoroughly by Söhngen (119), who noted the disappearance of methane, the production of CO_2 and the accumulation of organic matter (bacterial cell substance) in a mineral salts solution saturated under pressure with methane and oxygen. From enrichment cultures Söhngen isolated a rod-shaped organism which he called *Bacillus methanicus*, now known as *Methanomonas methanica* (12). Subsequently the bacterial oxidation of methane was studied by many others (1, 43, 44, 64, 65, 94, 95, 120, 131, 151, 169, 178).

Störmer (131) was the first to demonstrate the microbial assimilation of aromatic hydrocarbons. He isolated *Bacillus hexacarbovorum*, an organism capable of utilizing toluene and xylene. Wagner (164) described *Bacterium benzoli a* and *b* which utilized toluene, xylene, benzene and various aliphatic hydrocarbons. *B. benzoli a*, which resembled *Mycobacterium phlei*, grew on phenol and pyrocatechol as well as on benzene. *B. benzoli b* was similar to *Mycobacterium lacticola*. Mixed cultures of soil bacteria studied by Wagner quantitatively destroyed samples of crude oil which probably contained naphthenic, aliphatic and aromatic hydrocarbons.

The bacteria studied by Tausz and Peter (152) failed to attack benzene, toluene or xylene, but several different aliphatic, olefinic and naphthenic hydrocarbons were attacked. Caprylene, hexadecene (cetene), cyclohexane, methylcyclohexane and paraffinic hydrocarbons ranging from *n*-hexane to tetratriacontane, $C_{34}H_{70}$, were oxidized by *Bacterium aliphaticum liquefaciens*. After noting a high degree of specificity in the ability of various bacteria to attack different

kinds of hydrocarbons, Tausz and Peter proposed the use of bacteria for the analysis of petroleum and for the purification of hydrocarbons.

Some of the literature on the microbial utilization of petroleum hydrocarbons has been summarized in short reviews by Hessel (67), Tausson (144), Bushnell and Haas (19), Strawinski (132) and ZoBell *et al.* (181). The literature on the microbial utilization of rubber hydrocarbons has been summarized by ZoBell and Beckwith (179).

MINERAL REQUIREMENTS OF HYDROCARBON OXIDIZERS

The microbial oxidation of hydrocarbons takes place in very simple media and throughout a wide range of environmental conditions. When enriched with hydrocarbons the water from lakes, rivers, wells and oceans generally provides for the multiplication and biochemical activities of hydrocarbon oxidizers. The conditions necessary for the microbial decomposition of oils have been summarized by Tausson (141) as follows: (a) a physiologically balanced mineral salts solution; (b) the presence of a nitrogen source, such as ammonium or nitrate ion; (c) free oxygen, and (d) a reaction near neutrality.

Hydrocarbon oxidizers have been cultivated in both solid and liquid media over a wide range of salinity, oxygen tension and temperature. Except for the special techniques required for dissolving or dispersing gaseous, liquid and solid hydrocarbons in nutrient media, hydrocarbon oxidizers are cultivated in the same way, in general, as other types of bacteria, yeasts or molds.

The mineral solution first employed by Söhngen (119) for the cultivation of methane-oxidizing bacteria consisted of 0.05% K_2HPO_4, 0.1% $MgNH_4PO_4 \cdot 6H_2O$ and 0.01% $CaSO_4$ dissolved in distilled water. Wagner's (164) mineral solution contained 0.1% each of K_2HPO_4 and NH_4NO_3, 0.025% $MgSO_4$ and traces of $FeCl_3$ and $CaCl_2$. According to Bushnell and Haas (19), the following mineral solution proved to be quite satisfactory for the cultivation of a large variety of hydrocarbon-oxidizing microörganisms:

Distilled water	1,000.0 ml
$MgSO_4$	0.2 g
$CaCl_2$	0.02 g
KH_2PO_4	1.0 g
K_2HPO_4	1.0 g
NH_4NO_3 or $(NH_4)_2SO_4$	1.0 g
$FeCl_3$ (conc. sol.)	2 drops

A similar mineral solution was used by Stone *et al.* (129) except for the addition of traces of Mn and Zn, which were thought to promote the growth of hydrocarbon oxidizers.

The nitrogen requirements of most hydrocarbon oxidizers are satisfied by ammonium salts. Nitrate nitrogen is utilized by many. Methane-oxidizing bacteria were found (94) to grow almost equally well on any concentration of either NH_4Cl or KNO_3 ranging from 0.00025 to 0.3%. Organic compounds such as leucine, asparagine or peptone also satisfied the nitrogen requirements. Growth

was best in a medium containing from 0.1 to 0.5% K_2HPO_4 and very poor in media containing less than 0.005% K_2HPO_4.

Bushnell and Haas (19) found that the presence of 50 mg/L of phosphate was necessary to secure minimum growth of cultures of *Pseudomonas* in media containing hydrocarbons as the only source of energy, suggesting that under certain conditions phosphorylation may play an important rôle in hydrocarbon metabolism as it does in carbohydrate metabolism. The ability of *Corynebacterium* species to attack hydrocarbons was not affected by the lack of phosphate (19). We (178) have found phosphate to be beneficial for the microbial oxidation of hydrocarbons.

ZoBell *et al.* (181) used sea water fortified with 0.1% $FeNH_4PO_4$ for growing hydrocarbon oxidizers from marine sources. Sea water diluted 1:10 with distilled water and enriched with 0.1% $FeNH_4PO_4$ proved to be an excellent mineral solution for the cultivation of hydrocarbon oxidizers from "fresh-water" sources. Oil-well brines having salinities as great as 300,000 mg/L have been used successfully for the cultivation of hydrocarbon oxidizers found therein. Elazari-Volcani (34) isolated bacteria from the Dead Sea which grew in 25% salt solution enriched with kerosene or petroleum. Hydrocarbon oxidizers have been demonstrated (178) in marine sediments, both ancient and recent, which grew in sea water treated with 10 to 25% NaCl and enriched with paraffin oil. Sea water contains an average of 3 to 3.5% solids, most of which is NaCl.

Since the microbial oxidation of hydrocarbons is usually accompanied by acid production, the presence of carbonate or phosphate in the medium is desirable to buffer it at a favorable hydrogen-ion concentration. The reaction may become more alkaline in hydrocarbon media in which nitrate is being reduced. Tausson (144), like many earlier workers, recommended the use of media which were approximately neutral in reaction. Bushnell and Haas (19), however, observed that hydrocarbon oxidizers are not extremely sensitive to changes in hydrogen-ion concentration within the range of pH 6 to 9.5. This was confirmed by ZoBell *et al.* (181), who observed the luxuriant growth of hydrocarbon oxidizers throughout the range of pH 6 to 10. The assimilation of paraffin wax by *Aspergillus versicolor* was independent of the initial reaction between pH 5.8 and 7.9 (70). The hydrocarbon oxidizers studied by Strawinski (132) were active from pH 5 to 8, but they grew best at pH 7.6 to 8.

EFFECT OF OXYGEN TENSION

Hydrocarbons are attacked by microörganisms growing under both aerobic and anaerobic conditions. The growth of aerobes appears to be equally good at any oxygen content ranging from 0.1 to 20 or 30 mg/L. (When in equilibrium with the atmosphere at 25°, the oxygen content of fresh water is approximately 8.6 mg/L.) ZoBell *et al.* (181) noted that the multiplication of hydrocarbon oxidizers continued unabated until the oxygen content of the medium was reduced to less than 0.1 mg/L. The oxidative metabolism of such organisms, however, is influenced by the presence of oxygen (170). For example, from three to four times as much ether-extractable material was recovered (132) from aerated than

from unaerated cultures growing on naphthalene under otherwise comparable conditions. Others (91, 136, 141) have also found that vigorous aeration promoted the dissimilation of hydrocarbons by microörganisms.

In their pioneer work, Kaserer (83), Söhngen (119) and Störmer (131) used an atmosphere consisting of about equal volumes of oxygen and methane for demonstrating the microbial oxidation of methane. Münz (94), however, found that *Methanomonas methanica* grew best in an atmosphere consisting of a higher concentration of methane than oxygen. In our experience (178) about 40 volumes of oxygen to 60 volumes of other gases is about as much oxygen as can be used advantageously for the cultivation of methane oxidizers. Higher concentrations of oxygen retard the multiplication of *Methanomonas methanica*. Initial growth is equally good in gas mixtures containing much less than 40% oxygen, but when the initial gas mixture contains only a very little oxygen, the early depletion of oxygen arrests further activity unless the oxygen supply is replenished. This may be illustrated by data from a representative experiment (178) in which methane-oxidizing bacteria were grown in a mineral solution in bottles containing 150 ml of different gas mixtures as follows:

INITIAL GAS MIXTURE			CH_4 CONSUMED IN 7 DAYS AT 28°
CH_4	O_2	CO_2	
%	%	%	*ml*
20	80	0	4.7
40	60	0	12.1
60	40	0	18.3
70	30	0	20.5
80	20	0	18.9
50	45	5	32.3
50	40	10	39.4
50	40	10	38.0

The data from this and similar experiments indicate that the presence of from 5 to 10% CO_2 in the gas mixture enhances the activity of methane oxidizers. Neither carbonate nor bicarbonate ion has proved to be as satisfactory as free CO_2 for initiating the growth and activity of methane oxidizers.

While the microbial oxidation of hydrocarbons is primarily an aerobic process, the substances are attacked in the absence of free oxygen. A good many hydrocarbon oxidizers can utilize nitrate or sulfate as hydrogen acceptor. *Bacterium benzoli* (142) oxidized about 8 grams of benzene in 42 days at 28° utilizing nitrate as the hydrogen acceptor. The reduction of nitrate by hydrocarbon-oxidizing microörganisms has been commonly noted (19, 138, 154, 181). The bacteria in the oil-soaked soil studied by Beckman (11) attacked hydrocarbons under either aerobic or anaerobic conditions, but the nature of the hydrogen acceptor was not specified. The oxidation of petroleum by bacteria in oil-well brines under anaerobic conditions has also been reported (92).

Tausson and Aleshina (146) demonstrated the utilization of hydrocarbons by

strictly anaerobic sulfate reducers. The oxidation of phenanthrene, naphthalene and related compounds by anaerobes which utilized sulfate as the hydrogen acceptor was observed by Tausson and Vesselov (149). Sulfate reducers have been observed to oxidize crude oil (89). The action of *Desulfovibrio* species on paraffinic hydrocarbons has been reported by Novelli and ZoBell (99). It is quite possible that the apparently autotrophic sulfate reducers studied by Czurda (25) were actually utilizing the paraffin wax employed to exclude oxygen from the cultures.

EFFECT OF ORGANIC MATTER

The presence of peptone, carbohydrates and other types of readily utilizable organic matter interferes with the assimilation of hydrocarbons by some, but not all, microörganisms. Presumably such organic materials are preferentially attacked, and their oxidation results in depletion of oxygen and lowering of the oxidation-reduction potential (175) to a point where only anaerobes can function. We have been unsuccessful in demonstrating the decomposition of hydrocarbons by sulfate reducers in the presence of an abundance of other kinds of readily utilizable organic matter.

Following prolonged cultivation on nutrient agar or gelatin, *Bacterium aliphaticum* attacked hydrocarbons much less readily than cultures of this organism which had been maintained on mineral salts media enriched with hydrocarbons (152). Similarly, methane oxidizers tended to lose their ability to utilize methane when cultivated in nutrient peptone or similar organic media (1). After noting its ability to grow in either organic media or in exclusively mineral media, *Methanomonas methanica* was characterized by Münz (94) as a facultative autotroph. Neither 0.5% asparagine nor 1.0% peptone affected the oxidation of paraffin wax by soil microörganisms (53). It has been observed that naphthalene actually disappeared more rapidly from soil rich in organic matter than from organic-poor soils (91, 136).

Low concentrations of organic matter (less than 1 mg/L) generally promote the microbial assimilation of hydrocarbons, probably because the readily utilizable organic matter provides for the multiplication of microörganisms which then attack the hydrocarbons. Moreover, the CO_2 which results from the oxidation of organic compounds may have a beneficial effect upon initiating the activity of hydrocarbon oxidizers. Especially beneficial are small quantities of certain growth factors, including yeast extract, nicotinamide, riboflavin, pyridoxine, thiamine and ascorbic acid, used either alone or in combinations.

After transferring their cultures 10 to 15 times on kerosene media without any diminution of growth, Bushnell and Haas (19) concluded that either hydrocarbon oxidizers do not require accessory growth factors or the organisms with which they were working were able to synthesize these substances. Certain yeasts have been found to produce a growth substance resembling "bios" when grown in mineral media with paraffin as the sole source of energy (137).

Bacterium aliphaticum, which utilizes numerous paraffinic hydrocarbons, has been shown (80) to grow readily on peptone media and to utilize a large number of

carbohydrates. Most of our stock cultures of hydrocarbonoclastic bacteria are maintained on peptone agar slants. It has been found (178), however, that upon transferring such cultures to mineral media the bacteria attack hydrocarbons more energetically if they have been maintained on agar slants flooded with paraffin oil. This suggests selective enzyme formation, although a good many stock cultures having no previous history of contact with hydrocarbons readily utilize hydrocarbons in appropriate media (58, 178).

TEMPERATURE REQUIREMENTS

Most of the experimental work on the microbial decomposition of hydrocarbons has been conducted at temperatures ranging from 25° to 37°. In demonstrating methane oxidation by soil bacteria, Kaserer (83) incubated his cultures at 28° to 30°. Söhngen (119) cultivated *Methanomonas methanica* at 30° to 37°. Wagner (164) used an incubation temperature of 25° in studying the decomposition of hydrocarbons by *Bacillus benzoli*. The methane oxidizers studied by Münz (94) were active at temperatures ranging from 18° to 40°, maximum activity occurring at 34°.

Söhngen (121) was able to demonstrate the largest number of hydrocarbon-oxidizing microörganisms in soil when the cultures were incubated at 26° to 30°, but hydrocarbons were decomposed more rapidly at 37°. In one series of experiments there was evidence for the microbial attack of paraffin by cultures in two days when incubated at 28° or 37°, while at 20° a week was required for such evidence. Similarly, Stone *et al.* (129) found hydrocarbon oxidizers to be more active at 30° and 37° than at 20°.

Although an incubation temperature approximating 27° was used for studying the microbial decomposition of hydrocarbons, Haas (58) noted the growth of certain *Pseudomonas* species at 47°. Some of the hydrocarbon oxidizers isolated from marine materials by ZoBell *et al.* (181) decomposed seven times as much paraffin at 55° as at 22°. Others were active at temperatures as low as 0°, although it required several weeks to establish this point because the rate of microbial activity at low temperatures is very slow. Bacteria which destroyed phenol at 60° were studied by Egorova (33).

DISPERSION OF HYDROCARBONS IN CULTURE MEDIA

Hydrocarbons are virtually insoluble in water with the exception of gaseous ones, the solubility of which is largely a function of the partial gas pressure. This presents a problem in dispersing hydrocarbons in mineral solutions so that they will be available to attacking microörganisms. By maintaining an appropriate atmosphere of gaseous hydrocarbons in a closed system, it is possible to provide for an optimum concentration of such hydrocarbons in the mineral solution, but the dispersal of less soluble liquid and solid hydrocarbons requires special techniques in order to obtain the best results. The chief factor which limits the microbial decomposition of hydrocarbons is the dispersion of the latter in such a way that they can be acted on by enzymes.

Using a slight modification of the method employed by Söhngen (121), Bushnell

and Haas (19) noted that the vapors from slightly volatile hydrocarbons, such as petroleum ether or gasoline, poured into the lid of an inverted petri dish were sufficient to support the growth of bacteria on mineral-salts agar streaked with source material. When working with relatively non-volatile hydrocarbons, such as kerosene or light oils, the hydrocarbon was poured over the surface of inoculated agar with good results. Liquid cultures were covered with a thin layer of liquid hydrocarbons. The thickness of the layer of hydrocarbons did not affect the growth of the microörganisms. Paraffin was added to the medium in a melted condition, thereby giving a rough irregular mass of paraffin which offered sufficient surface for bacterial action. Most workers have introduced paraffin wax in this manner, or else cut it into thin shavings in order to expose as much surface as possible to the action of organisms.

The effect of surface area exposed to the culture is illustrated by the following data obtained in an experiment in which identical quantities of paraffin in various forms were introduced into a mineral medium (178). The medium, in 120-ml glass-stoppered bottles, was inoculated with bacteria and, as a criterion of microbial activity, the oxygen content of the medium was determined before and after incubation:

SET NO.	METHOD OF DISPERSION	SURFACE AREA EXPOSED TO MEDIUM	O_2 CONSUMED IN 7 DAYS AT 28°
		cm^2	*ml*
1	One 10-mm cube	6	0.02
2	Eight 5-mm cubes	12	0.01
3	64 2.5-mm cubes	24	0.03
4	102 thin slices	208	1.32
5	Bottle surface coated	148	1.05
6	Bottle + sand surface	830	2.59
7	Bottle + glass wool	1,900	4.11
8	Control (No paraffin)	0	0.02

In the first four sets 0.9 g of paraffin (mp about 55°) was cut into pieces of different sizes and added to each bottle. In the fifth set 0.9 g of paraffin was melted and the bottles manipulated while cooling so that the interior surface of the bottle was completely covered with a thin layer of paraffin. This process was repeated in dispersing the paraffin in the sixth and seventh sets of bottles whose interior surfaces were supplemented by the surface of ignited silica sand and glass wool, respectively. From the data in the table it will be observed that there was a direct relation between the surface area of paraffin exposed to the culture and the consumption of oxygen. ZoBell (172) has shown that bacteria in dilute nutrient solutions benefit by the presence of solid surfaces, and this is particularly true of hydrocarbon-oxidizing bacteria.

Söhngen (121) found the rate of hydrocarbon utilization to be directly proportional to the surface area of culture in contact with oil, crude oil being oxidized at the rate of 15 mg per square decimeter of culture surface in 24 hours at 28°.

The growth of *Micrococcus paraffinae*, *Mycobacterium album* and *Mycobacterium rubrum* on paraffin or other hydrocarbon media was largely a function of the surface of soil particles dispersing hydrocarbons in such media (122). Both silicon dioxide and iron oxide stimulated the microbial oxidation of petroleum (122). Soil microörganisms quite rapidly decomposed films of paraffin on kieselguhr and other particulate substances (53).

Recognizing the importance of the surface factor, Tausson and Shapiro (147) expressed the rate of oil oxidation in terms of surface area of oil exposed to the culture. Crude oils, for example, were found to be oxidized at the rate of 250 g per square meter of free surface in seven months as compared with 100 g of cylinder oil oxidized under similar conditions.

In their studies on hydrocarbon utilization by sulfate reducers, Tausson and and Aleshina (146) mixed melted paraffin with powdered glass to increase the free surface of paraffin. The glass also served as a "sinker" to keep the paraffin at the bottom of the mineral medium. Petroleum was introduced into the cultures in the form of a semi-liquid mass of the following composition:

Calcium sulfate	20 g
Calcium carbonate	10 g
Kaolin	100 g
Petroleum	30 g

A layer of this dough-like mixture was triturated with an equal volume of mineral solution, after which a layer of the resulting homogeneous mass 8 to 10 mm thick was transferred to culture receptacles. The latter were then filled with mineral solution. The mass of dispersed petroleum was sufficiently stable to withstand autoclave sterilization without an appreciable separation of the petroleum from the solids (146).

In order to demonstrate (178) the presence of hydrocarbon-oxidizing sulfate reducers in soil, oil-well brines and other material, varying quantities of the material in question were introduced into test-tube "deeps" of a paste freshly prepared of the following ingredients:

Plaster of Paris ($CaSO_4$)	50.00 g
Calcium carbonate	10.00 g
$FeNH_4PO_4$	0.01 g
Paraffin oil	10.00 g

These ingredients were mixed to a paste with sea water or other mineral solution. Upon setting, the paste hardened into a slightly porous mass. Air was excluded by a layer of paraffin wax or a mixture of paraffin and petrolatum. The reduction of sulfate by hydrocarbon-oxidizing anaerobes resulted in the formation of H_2S which combined with the iron in the medium, thereby changing the color of the medium from white to black.

By dispersing hydrocarbons adsorbed on the surfaces of inert solids, the microbial attack of nearly all kinds of hydrocarbons tested is greatly increased (178). Certain hydrocarbons which seemed to be invulnerable to microbial attack were

quite rapidly decomposed when dispersed throughout the medium on the surfaces of inert solids. Asbestos fibers and glass wool have proved to be better than sand, presumably because the sand settles in a compact mass to the bottom of the culture medium while glass wool and asbestos, which do not pack, distribute the adsorbed hydrocarbons throughout the medium. Solid particles of small dimensions such as bentonite, kaolin or talcum powder are not beneficial because they pack in a solid mass at the bottom of the culture receptable and the adsorbed oil completely fills the interstitial spaces so that relatively little surface is presented for the action of microörganisms in the mineral solution. Solid particles smaller than bacteria may be injurious to them (172).

Small quantities of liquid and solid hydrocarbons can be uniformly distributed on glass wool, asbestos, sand or other inert solids by dissolving the hydrocarbon in petroleum ether or other suitable solvent. The solvent is then evaporated while manipulating the bottle in such a way that all surfaces are covered.

More rapid utilization is permitted by dispersing hydrocarbons by emulsification than by adsorption on solid surfaces (178). Fairly homogeneous emulsions have been prepared by mixing the hydrocarbon with the mineral solution in a Waring blender and then passing this mixture through a homogenizer several times. When its addition is permissible, the use of a little gum arabic or gum acacia improves the stability of the emulsion. Gum arabic is relatively inert, being attacked by very few bacteria and by these only slowly. Emulsifying cetane, cetene, kerosene and paraffin oil in mineral media increased their utilization by sulfate-reducing bacteria more than tenfold as compared with their utilization when adsorbed on asbestos or glass wool.

Johnson *et al.* (79) isolated hydrocarbon-oxidizing bacteria from soil on plates of dilute soil-extract agar which had been emulsified with petroleum ether just before cooling.

CRITERIA OF HYDROCARBON UTILIZATION

Several criteria have been used as evidence of the microbial utilization of hydrocarbons. Commonest among these are the disappearance or modification of the hydrocarbon, the production of CO_2, acid formation, multiplication of microorganisms or the consumption of oxygen in media consisting of mineral solutions enriched with hydrocarbons as the only source of energy.

Manometric methods have been extensively used for following hydrocarbon oxidation in a closed system, a method which is particularly applicable to quantitative experiments with gaseous hydrocarbons such as methane, ethane, propane, butane, ethylene, propylene, butylene, butadiene, acetylene and cyclopropane. The culture receptacle can be fitted with a manometer to register changes in internal gas pressure, or the culture receptacle can be connected with another bottle of sterile mineral solution in such a way that the sterile solution is sucked into the culture receptacle as the hydrocarbon and oxygen are consumed. The volume of oxygen consumed by hydrocarbon oxidizers always exceeds the volume of CO_2 produced.

In a typical experiment Söhngen (119) observed the following changes in gas composition as a result of the activity of *Methanomonas methanica* in 102 ml of mineral medium for 14 days at 30° to 34°:

	O_2	CO_2	CH_4
	ml	*ml*	*ml*
Initially present	320	0	225
Present after 14 days	172	78	0

The data indicate that part of the methane had been oxidized to CO_2. Some of the methane was converted into bacterial cell substance as indicated by the accumulation of enough organic matter in the culture medium to reduce 48.3 ml of N/10 $KMnO_4$. The accumulation of organic materials in mineral medium in which *Methanomonas methanica* was growing was also observed by Giglioli and Masoni (43).

Stone *et al.* (129) used a Warburg manometric apparatus for determining the oxygen required for the dissimilation of several oils by mixed cultures. It was found that the amount of oxygen consumed and CO_2 liberated varied according to the rate of shaking, the temperature of incubation and the age of the inoculum. The respiratory quotients of light oils were found to be in the neighborhood of 0.63, indicating that a large percentage of the molecules attacked was completely oxidized to CO_2. The theoretical respiratory quotient for complete oxidation of a long-chain paraffin hydrocarbon with the formula C_nH_{2n+2} is approximately 0.67 (129). The fermentation of heavy oils containing longer molecules than the lighter oils did not yield as much CO_2, and the respiratory quotients were lower than for lighter oils.

Bushnell and Haas (19) found the respiratory quotients of various bacterial cultures on different hydrocarbons ranged from 0.30 to 0.70, there being no direct correlation between the R.Q. and the nature of the hydrocarbon. The R.Q. of washed cells of *Bacterium aliphaticum* averaged 0.47 for heptane, 0.48 for octane, 0.63 for nonane, 0.63 for dodecane and 0.88 for glucose (79).

Oxygen consumption in 60-ml glass-stoppered bottles of mineral solutions enriched with hydrocarbons as the only source of energy has been used for studying the occurrence and behavior of hydrocarbon oxidizers in marine materials (181). The same procedure has been used for studying the microbial assimilation of rubber hydrocarbons (179).

The decoloration of methylene blue by cultures of *Bacterium aliphaticum liquefaciens* growing in mineral solutions enriched with hexane signified to Tausz and Donath (151) that the dye was utilized as an hydrogen acceptor. We (178) have found that while certain bacteria reduce methylene blue in the presence of certain hydrocarbons, methylene blue reduction is not a reliable criterion of hydrocarbon utilization because some bacteria which utilize hydrocarbons do not decolorize the dye.

Stone *et al.* (129) stated that the breakdown of oil is an oxidative process

characterized by a high bacterial count, emulsification and sometimes a decrease in pH. They observed plate counts ranging from 12,000,000 to 1,470,000,000 per ml in inoculated media enriched with various crude oils or fractions thereof after four days' incubation at room temperature. In the majority of cases the maximum plate counts were obtained after three to six days of incubation. Thousandfold or greater increases in the bacterial population of mineral media enriched with various petroleum fractions have been observed (19) after 7 to 14 days' incubation at 27°. An increase in the plate counts of soil treated with benzene and other aromatic hydrocarbons has been reported (91). Mineral media inoculated with soil and enriched with cetane, naphthalene and biphenyl have been found (133) to contain bacterial populations exceeding a billion per ml after a few days of incubation.

While the appearance of turbidity in hydrocarbon media has been very commonly used as a criterion of growth of microörganisms therein, extensive multiplication may occur without rendering the medium turbid. This is because some organisms are intimately associated with solid surfaces (172) or with the hydrocarbon. Reed and Rice (105), for example, found that only 5 to 30% of the acid-fast organisms examined remained in the water; 70 to 95% migrated to the oil phase. Tausz and Peter (152) related that, although the medium showed no turbidity, bacteria attacking the overlying oil perforated and rendered inhomogeneous layers 1 to 2 mm in thickness. The undersurface of the oil layer in contact with the medium became stringy and small droplets, pitch-like in color and consistency, broke off and sank to the bottom of the culture flask. In other experiments (152) the medium overlayed with oil became turbid in three days.

The emulsification of various kinds of oil by microörganisms has been observed by numerous investigators (11, 19, 130, 147, 152, 178). The microbial emulsification of oil is due in part to the production of organic acids and detergents and in part to the penetration of the oil by myriads of microörganisms which cause either a mechanical or chemical disintegration of the oil. Tausz (150) observed the physical disintegration of oil by motile bacteria dragging microscopic droplets of oil with them as they dart in and out of the oil overlaying the mineral solutions. The production of detergents or surface-active substances by bacteria associated with oil-bearing sediments has been reported by ZoBell (173). The formation of CO_2 by bacteria tends to emulsify oil.

Acid production by active cultures of hydrocarbon oxidizers is definite though usually inappreciable. Decreases of from 0.1 to 1.5 units have been observed in the pH of media in which hydrocarbons are undergoing microbial oxidation. Carbonic and organic acids produced during the dissimilation of hydrocarbons are responsible for the pH decreases.

OXIDATION PRODUCTS

CO_2 always results from the microbial dissimilation of hydrocarbons. Büttner (20) found that from 80 to 90% of the carbon in the paraffin decomposed by soil organisms in 21 to 31 days could be accounted for as CO_2. The remainder was

believed to be converted into cell substances, fatty acids and possibly other intermediate products. Some of his (20) data are summarized below:

MICROÖRGANISM	PARAFFIN DECOMPOSED	CO_2 PRODUCED	C IN PARAFFIN	C IN CO_2	C OXIDIZED TO CO_2
	mg	*mg*	*mg*	*mg*	%
Mycobacterium lacticola	224	585	192	162	84.4
Mycobacterium phlei	182	504	156	137	87.9
Mycobacterium eos (rubrum)	170	460	146	125	85.6
Pseudomonas aeruginosa	90	251	77	69	89.5
Actinomyces chromogenes	81	234	70	64	91.3

Haag (56) pointed out that the amount of paraffin oxidized which could be accounted for as CO_2 in Büttner's experiments may be somewhat too high, because the amount of paraffin decomposed was determined by difference in the amounts extractable by carbon tetrachloride, and the latter would dissolve not only the residual paraffin but also certain lipids that occur particularly abundantly in acid-fast bacteria.

From data given by Söhngen (120), it is estimated that about half of the methane assimilated by *Methanomonas methanica* was oxidized to CO_2 and the other half was converted into bacterial cell substance and other products of metabolism. From 20 to 25% of the hexane, C_6H_{14}, utilized by marine bacteria (181) was converted into bacterial protoplasm. Similar results were obtained with tetratriacontane, $C_{34}H_{70}$.

An appreciable portion of the paraffin oxidized by *Aspergillus flavus* was converted into mold mycelium (142). During the first two weeks of incubation the "economic coefficient," defined by Tausson (142) as the per cent of consumed paraffin accounted for as mold mycelium, was about 90%. After six or seven weeks the "economic coefficient" ranged from 50 to 60%. Assuming that the carbon content of the paraffin and mold mycelium was 85 and 45% respectively, from 25 to 45% of the carbon of the paraffin consumed was converted into mold mycelium. Esters of fatty acids and higher alcohols were detected as intermediate products of metabolism. An appreciable portion of the energy resulting from the oxidation of paraffin by *Aspergillus* and *Penicillium* species is lost as heat (148).

Besides producing CO_2 and cell substance, most of the 17 species of hydrocarbon-oxidizing bacteria studied by Söhngen (121) produced organic acids as intermediate products of metabolism. The formation of fatty acids was believed by Tausson and Shapiro (147) to account for the marked increase in the saponification number and the emulsification of crude oil samples undergoing microbial decomposition. The same view was expressed by Stone *et al.* (130), who state that the drop in pH, the emulsification of unfermented oil and the appearance of a residue indicated that some long-chain organic acids were produced by microorganisms attacking petroleum. Similarly, Bushnell and Haas (19) interpreted changes in pH and emulsification of oil in water as evidence that long-chain organic acids were formed during the microbial decomposition of hydrocarbons.

Bushnell and Haas (19) claimed that there was also some evidence to suggest that unsaturated hydrocarbons were formed by bacteria growing on mineral oil. Traces of unidentified unsaturated hydrocarbons were detected by Tausz and Donath (151) in cultures of *Bacterium aliphaticum liquefaciens* growing on hexane. *Mycobacterium* species growing on paraffin wax were observed by Haag (56) to cause a decrease and then an increase in the iodine number, which was believed to indicate that the bacteria preferentially attacked double bonds and then produced unsaturated compounds.

Indirect evidence was obtained by Hopkins and Chibnall (70) which suggested that ketones may be primary products resulting from the oxidation of higher paraffins by *Aspergillus versicolor*. Further oxidation of the ketones resulted in the production of shorter fatty acids, although CO_2 and mold mycelium were the principal end products of metabolism.

From a culture of mold resembling *Aspergillus flavus* which had been growing for 12 weeks in a medium initially containing 3.65 g of paraffin, Tausson (138) recovered 1.24 g of mold mycelium, 1.29 g of residual paraffin and 0.234 g of non-acidic products which were assumed to consist in part of esters of the higher aliphatic alcohols. Fatty acids and CO_2 were produced during the oxidation of paraffin by certain yeasts (137).

The formation of a compound containing the oxyphenol group resulted from the action of sulfate reducers on phenanthrene and retene (149). Orthosalicylic acid was detected as one of the main products resulting from the oxidation of naphthalene by *Pseudomonas aeruginosa* (134). Jacobs (73) identified phthalic acid in cultures of soil bacteria dissimilating naphthalene. Significant quantities of salicylic acid resulted from the dissimilation of naphthalene by the *Pseudomonas* species studied by Strawinski (132). There was some evidence that the dissimilation of naphthalene passed through the aldehyde stage, and the formation of a complex substance of higher molecular weight than naphthalene was noted. The oxidation of naphthalene and cetane gave the medium an acid reaction (132).

Besides traces of acetic and lactic acids, Thaysen (155) reported the formation of methane, ethane and acetaldehyde from the microbial decomposition of kerosene.

Methods for the bacterial conversion of gaseous paraffinic hydrocarbons to oxygenated organic compounds ranging from low boiling point alcohols to waxy acids, esters and alcohols have been described in the patent literature (135a). It has been claimed that under certain conditions heavier hydrocarbons such as butane are susceptible to conversion to unsaturated compounds capable of undergoing polymerization to produce heavy hydrocarbon molecules. Allegedly these conversions are catalyzed by *Bacillus methanicus* and *B. ethanicus*.

From bacterial cultures growing on mineral oil, Haas *et al.* (60) isolated small quantities of organic acids, the melting points of which ranged from 25° to 30°. No sterols were detected in bacterial cultures acting on oil, but molds growing on hydrocarbons were found to produce small amounts of ergosterol and cholesterol. β-Carotene was detected as an end product resulting from the growth of *Coryne-*

bacterium species on paraffin oil. It was reported (59) further that two other carotenoid pigments besides β-carotene and astacin were produced by a species of *Mycobacterium* growing on a substrate composed of mineral salts and paraffin oil.

A volatile alcohol, probably isopropanol, was found in a mixed culture of yeasts and molds growing on paraffin oil (58). Glycol and glycerol were produced from paraffin oil by a *Mycobacterium* species. Several of the bacteria produced small quantities of oil-soluble organic acids from the oxidation of hydrocarbons. The oxidation of cetane by *Pseudomonas aeruginosa* resulted in no change of pH, according to Schuman *et al.* (114), but the pH was materially lowered during the oxidation of naphthalene by this organism.

Acid formation by *Bacterium benzoli* growing in mineral media enriched with benzene was reported by Wagner (164). CO_2 was the principal product resulting from the oxidation of benzene, although there was indirect evidence for the formation of hydroquinone and various organic acids. *Bacterium benzoli* also produced acid during the oxidation of toluene.

About 15% of the isotopic 7,8,9,10-tetradeuterio-*n*-hexadecane absorbed by rats was found by Stetten (127) to be oxidized to fatty acids, apparently in the liver. The catabolic route of the hydrocarbon was indicated by the appearance of a significant though low concentration of D_2O in the body water and a notably higher isotope concentration in fatty acids of the carcass. The hexadecane was believed to be absorbed prior to alteration of the molecule by bacteria in the intestine and to be converted into palmitic acid by the oxidative attack on a terminal methyl group. El Mahdi and Channon (36), working with rats, and Channon and Devine (22), working with cats, concluded that *n*-hexadecane was absorbed and catabolized by these animals, after finding much less of the hydrocarbon in the body and excrements than the amount fed. The recovery of muconic acid containing 7.2 atom% deuterium from the urine of rabbits injected with deuterio-benzene, C_6D_6, was regarded as evidence that rabbits can oxidize benzene (62a). The oxidation of *p*-cymene to cumic acid by sheep and the partial oxidation of two carcinogenic hydrocarbons, 3,4-benzopyrene and 1,2,5,6-dibenzoanthracene, by rats and mice has also been reported (62a). The modification of carcinogenic hydrocarbons by microbial enzymes is a possibility which merits consideration.

KINDS OF HYDROCARBONS ATTACKED

Crude oils, illuminating gases, petroleum ethers, gasolines, kerosenes, fuel oils, paraffin or mineral oils, petrolatums, asphalts, paraffin waxes, and rubber both natural and synthetic, besides numerous chemically pure hydrocarbons have been shown to be oxidized by a great variety of microörganisms. Although numerous papers on this subject have appeared during the last forty years, our knowledge is still so fragmentary that any generalizations may be premature. The interpretation of experimental results is complicated by the complexity and highly variable composition of crude oils, asphalts, rubbers, etc. In the face of inadequate information on the chemical composition of such substances, it is indeterminate whether observed results are attributable to particular hydro-

carbons, other types of organic compounds or inorganic constituents in the products, or to peculiar experimental conditions. Even work with pure hydrocarbons is complicated by vast differences in physical properties including solubility or miscibility in water, which make it virtually impossible to test their utilizability under strictly comparable conditions.

In the aliphatic series it appears that, in general and within certain limits, long-chain hydrocarbons are attacked more readily than compounds having only a few carbon atoms per molecule. Substantiating this generalization is the fact that methane, CH_4, ethane, C_2H_6, and propane, C_3H_8, are oxidized by relatively few organisms and by these only slowly, while paraffin waxes consisting mainly of compounds ranging from $C_{20}H_{42}$ to $C_{40}H_{82}$ are utilized readily by a large number of microörganisms. A partial list of the microörganisms which have been reported to assimilate paraffin wax is given in table 1.

Söhngen (121) reported that the growth of certain cultures in 200 ml of mineral medium enriched with 2 g of finely divided paraffin wax resulted in the oxidation of the following quantities of paraffin in a month at 28°:

CULTURE	PARAFFIN OXIDIZED
	mg
Mycobacterium album	300
Mycobacterium rubrum	330
Micrococcus paraffinae	180
Bacterium fluorescens liq.	180
Raw culture	540

None of these pure cultures could assimilate methane, and the bacteria grew less rapidly on pentane, hexane, heptane and octane than on higher paraffinic hydrocarbons.

According to Tausz and Donath (151), *Bacterium aliphaticum liquefaciens* utilized all aliphatic hydrocarbons ranging from pentane to decane, but this culture attacked no hydrocarbon lower than pentane. Their "Methane bacterium" oxidized not only methane but also hydrogen, ethane, propane, butane, pentane, hexane, heptane and higher hydrocarbons in the aliphatic series with increasing ease. It was concluded that when a given member of the series is attacked by a given organism, it may be assumed that all higher members of the series will also be attacked by the same organism. The "Paraffin bacterium" described by Tausz and Peter (152) attacked none of the paraffin series lower than hexadecane, $C_{16}H_{34}$, but it readily oxidized triacontane, $C_{30}H_{62}$, tetratriacontane, $C_{34}H_{70}$, and paraffin oil. However, the methane oxidizer studied by Münz (94) utilized neither ethane nor ethylene.

From theoretical consideration based upon thermodynamic reactions, Tausson and Aleshina (146) postulated that sulfate-reducing bacteria could utilize no aliphatic hydrocarbon of chain length shorter than decane, $C_{10}H_{22}$. Supporting this view is the experimental work of Novelli and ZoBell (99), who found that decane was feebly attacked by *Desulfovibrio* species which failed to attack lower

hydrocarbons but utilized with increasing ease tetradecane, $C_{14}H_{30}$, eicosane, $C_{20}H_{42}$, docosane, $C_{22}H_{46}$, hentriacontane, $C_{31}H_{64}$, paraffin oil and paraffin wax.

TABLE 1

List of microörganisms reported in chronological order by various authors to assimilate paraffin wax

AUTHOR	MICROÖRGANISMS
Miyoshi, 1895 (93)	*Botrytis cinerea*
Rahn, 1906 (104)	*Penicillium* species and other soil fungi
Söhngen, 1913a (121)	*Bacterium fluorescens liquefaciens*, *B. pyocyaneum*, *B. stutzeri*, *B. lipolyticum*, *B. punctatum*, *Micrococcus paraffinae*, *Mycobacterium phlei*, *M. album*, *M. luteum*, *M. rubrum*, *M. lacticola*, *M. hyalinum*
Greig-Smith, 1914 (53)	*Bacterium prodigiosus*; soil microflora
Gainey, 1917 (42)	Soil microflora, especially mold fungi
Tausz, 1919 (150)	Microörganisms in soil and canal water
Tausson, 1925a (138)	Mold resembling *Aspergillus flavus* found in soil
Büttner, 1926 (20)	3 species of *Mycobacterium* (probably *phlei*, *lacticola* and *rubrum*), *Actinomyces chromogenes albus*, *A. bovis*, *A. eppinger*, *A. trautwein*; one mold fungus
Haag, 1926, 1927 (56, 57)	*Mycobacterium* and *Actinomyces* species
Fleming, 1927 (38)	Soil molds and bacteria
Tausson, 1928a (141)	*Penicillium*, *Aspergillus* and *Pseudomonas* species
Tausson, 1928b (142)	Soil microflora
Jensen, 1931 (75)	*Actinomyces albus*, *Proactinomyces paraffinae*, *P. agrestis*, *P. polychromogenes*, *P. actinomorphus*, *P. minimus*
Jensen, 1932 (76)	*Proactinomyces corallinus*, *P. salmonicolor*, *P. opacus*, *P. erythropolis*, *P. paraffinae*
Hopkins and Chibnall, 1932 (70)	*Aspergillus versicolor*, *A. flavus*, *A. effusus*, *A. tumari*, *A. parasiticus*, *A. oryzae*
Tausson and Aleshina, 1932 (146)	Sulfate-reducing bacteria from soil
Tausson and Tausson, 1933 (148)	*Aspergillus flavus* and *Penicillum* species
Jensen, 1934 (77)	*Mycobacterium* species
Wackenhut, 1936 (165)	Unidentified bacterium from crude oil
Sturm and Orlova, 1937 (135)	Microörganisms from Ala-Kule Lake
Umbreit, 1939 (100)	*Actinomyces asteroides*, *A. farcinica*, *A. gypsoides* and several *Proactinomyces* species
Tausson, 1939 (137)	*Debaromyces*, *Hansenula*, *Endomyces*, *Torulopsis* and *Monilia* species
Erikson, 1941 (37)	10 strains of *Micromonospora* from lakes
Bushnell and Haas, 1941 (19)	*Mycobacterium phlei*, *M. leprae*, *M. smegmatis*, *Corynebacterium simplex*, *C. fimi*, *C. tumescens*; *Penicillium* species and *Pseudomonas* species
Rogers, 1943 (108)	*Micrococcus paraffinae* and microflora of water
ZoBell, *et al.* 1943 (181)	Microflora from marine sediments and sea water
Novelli and ZoBell, 1944 (99)	*Desulfovibrio* species from soil and marine sediments

Strawinski and Stone (133) found that hydrocarbons in the range of $C_{10}H_{22}$ to $C_{16}H_{34}$ were oxidized by soil bacteria more readily than those of smaller molecular

weight. This observation was confirmed by Stone *et al.* (129), who reported further, however, that heavy viscous oils are not utilized as readily as lighter oils. This was attributed partly to the fact that the more viscous oils are harder to disperse in liquid media and hence there is less surface exposed to microbial enzymes, but it was believed to be due partly to the difficulty with which the larger molecules in heavy oils are assimilated.

The inability of *Aspergillus verscicolor* to assimilate larger molecules of paraffin than $C_{34}H_{70}$ was demonstrated by the experiments of Hopkins and Chibnall (70), who obtained results as follows in mineral media enriched with various paraffins finely divided with a microtome:

HYDROCARBON	FORMULA	GROWTH
n-Tricosane	$C_{23}H_{48}$	Good
n-Heptacosane	$C_{27}H_{56}$	Fair
n-Nonacosane	$C_{29}H_{60}$	Fair
n-Triacontane	$C_{30}H_{62}$	Fair
n-Tetratriacontane	$C_{34}H_{70}$	Slight
n-Pentatriacontane	$C_{35}H_{72}$	None
Paraffin wax		Good

Paraffin wax having a melting point of 45° was found to provide for the growth of soil fungi and bacteria better than paraffin wax melting at 56° (104).

Strawinski (132) concluded that the higher the molecular weight, the longer the chain, and the more saturated the compound, the more susceptible aliphatic hydrocarbons up to C_{16} are to dissimilation by mixed cultures of soil bacteria. This conclusion was based upon plate counts on mineral media enriched with various hydrocarbons. The results of one of his (132) experiments follows:

HYDROCARBON	FORMULA	PLATE COUNT PER ML
Cetane	$C_{16}H_{34}$	1,890,000,000
n-Pentene	$CH_2{=}CHCH_2CH_2CH_3$	181,000,000
Octene	$CH_3CH{=}CH(CH_2)_4CH_3$	30,000,000
Diisobutylene	$(CH_3)_3CCH_2C(CH_3){=}CH_2$	13,000,000
Trimethylethylene	$(CH_3)_2C{=}CHCH_3$	5,100,000
Iso-octane	$(CH_3)_2CH(CH_2)_4CH_3$	5,000,000
Pentene-2	$CH_3CH{=}CHCH_2CH_3$	700,000
n-Octane	$CH_3(CH_2)_6CH_3$	10,000

Pseudomonas species and other cultures with which Bushnell and Haas (19) worked grew better on paraffin wax and mineral oils than on kerosene, and better on kerosene than on gasoline or petroleum ether. *Mycobacterium* species studied by Haas *et al.* (60) grew well on paraffin wax but not on gasoline and only poorly on kerosene. Conversely, a *Pseudomonas* species was observed which would grow on petroleum ether, gasoline and kerosene but not as readily on mineral oils or paraffin wax (60).

Using oxygen consumption as a criterion of activity, ZoBell *et al.* (181) found

petrolatum and lubricating oils to be oxidized more rapidly by mixed cultures of marine bacteria than kerosene, and kerosene was oxidized more rapidly than gasoline. This is in agreement with the observations of Johnson *et al.* (79) that *Bacterium aliphaticum* and *Pseudomonas fluorescens* grew with increasing ease on gasoline, kerosene, lubricating oil and paraffin oil. *Pseudomonas fluorescens* grew on no hydrocarbon of shorter chain length than dodecane, $C_{12}H_{26}$, but *Bacterium aliphaticum* grew less profusely on dodecane than it did on hexane, heptane, octane or nonane. Johnson *et al.* (79) speculated that hydrocarbons which are powerful fat solvents may be less readily tolerated or assimilated than those which are less likely to dissolve cell lipids. As a case in point, it was related that neither benzene, toluene nor xylene was assimilated by *P. fluorescens* or *B. aliphaticum.*

Whether the common observation that aliphatic hydrocarbons are attacked more readily and by more organisms than aromatic hydrocarbons is attributable to the physical properties or the chemical configuration of the compounds is indeterminate, but a good many microörganisms are known to attack benzene and its derivatives. Störmer's (131) *Bacillus hexacarbovorum* utilized toluene and xylene, these benzene derivatives being injurious only when added to mineral media in concentrations exceeding 1:10,000. Wagner's (164) two organisms, *Bacterium benzoli a* and *b*, utilized benzene, toluene and xylene.

The ability of soil bacteria to assimilate benzene, toluene, naphthalene and related aromatic compounds generally regarded as antiseptics has been established (52, 73, 91, 116, 136). Wackenhut (165) isolated an organism from Russian crude oil which developed slowly on benzene.

Among the numerous hydrocarbon-oxidizing bacteria inhabiting soil in the Baku oil fields of Russia, Tausson (140) found *Bacterium naphthalinicus*, *Bacillus naphthalinicus liquefaciens* and *Bacillus naphthalinicus non-liquefaciens*, which oxidized naphthalene. From oil soaked soil Tausson (143) also isolated *Bacillus phenanthrenicus bakiensis* and *Bacillus phenanthrenicus guricus*, both of which utilized phenanthrene besides other hydrocarbons. In his review of the literature on the subject Tausson (144) named additionally *Bacillus benzoli* and *Bacillus toluolicum*, which oxidized toluene, xylene and benzene. Sulfate reducers have been found to attack slowly naphthalene, phenanthrene and retene (149). The sulfate reducers studied by Novelli and ZoBell (99) were unable to attack naphthalene, anthracene, benzene, xylene or cyclohexane.

Most of the *Micromonospora* species studied by Erikson (37) oxidized toluene and naphthalene. The oxidation of naphthalene, anthracene, xylene, toluene and benzene by mixed cultures of marine aerobes has been reported (181).

Enrichment cultures of soil bacteria have been found to attack naphthalene, diphenyl, methylnaphthalene, tetralin, butylbenzene and decalin (133). Cetane or *n*-hexadecane was the most rapidly attacked of any of the 18 compounds studied. Naphthalene and diphenyl supported bacterial populations only slightly smaller than did cetane.

Neither benzene nor xylene were attacked by *Actinomyces oligocarbophilus* (86), which assimilated higher aliphatic hydrocarbons but not the lower ones. None

of the pure cultures of hydrocarbon oxidizers studied by Tausz and Peter (152) was able to utilize benzene, toluene or xylene. After noting that their *Bacterium aliphaticum* quantitatively decomposed aliphatic hydrocarbons ranging from hexane to tetratriacontane but attacked neither aromatic nor naphthenic hydrocarbons, Tausz and Peter proposed the use of this and related organisms for freeing aromatic and naphthenic hydrocarbons from aliphatic compounds. When grown in mixtures of hydrocarbons, *Bacterium aliphaticum liquefaciens* quantitatively utilized aliphatic and naphthenic hydrocarbons, leaving aromatic compounds unaltered. This latter organism grew profusely on cyclohexane, methylcyclohexane, 1,3-dimethylcyclohexane and 1,3,4-trimethylcyclohexane.

Both *Bacterium aliphaticum* and *B. aliphaticum liquefaciens* grew better on caprylene or octene, C_8H_{16}, than on octane, C_8H_{18} (152). This suggests that unsaturated compounds may be more susceptible to microbial attack than saturated ones. Haag (56) concluded that unsaturated bonds in molecules are attacked preferentially, after noting that the higher the iodine number of paraffin waxes the more readily they were utilized by *Mycobacterium* species as shown by CO_2 production:

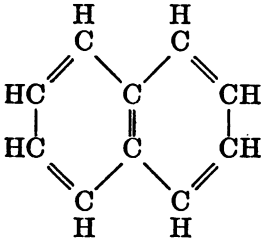
PARAFFIN NUMBER	MELTING POINT	IODINE NUMBER	CO_2 PRODUCED IN 21 DAYS
			mg.
1	36°	4.6	309.0
2	40–42°	4.5	290.8
3	44°	4.5	280.6
4	44–46°	4.1	268.3
5	50–52°	3.8	214.4
6	56–58°	2.8	125.1
7	58°	2.8	117.0
8	59°	2.8	122.8
9	60–62°	2.4	96.5
Ceresin	62°	1.8	252.5
Ozocerite	67–69°	1.2	161.3

Ceresin and ozocerite are complex mixtures of hydrocarbons and other compounds. Treating paraffins to remove double bonds rendered the resulting compounds less susceptible to attack by bacteria. The *Mycobacterium* species studied by Haag developed slightly on amylene or pentene, C_5H_{10}, but not at all on pentane, C_5H_{12}, methane, ethane or ethylene (56).

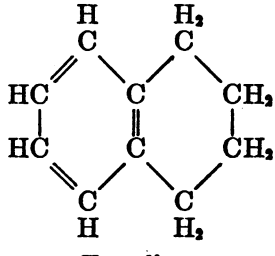
In his studies on the disappearance of ethylene, $H_2C=CH_2$, from ripe apples and bananas, Nelson (96) overlooked the possibility of the microbial consumption of ethylene. The susceptibility to microbial attack of natural and synthetic rubbers, which are essentially polymers of unsaturated hydrocarbons, indicates that at least certain kinds of unsaturated hydrocarbons are easily oxidized by microörganisms (179). In recent experiments, we (178) have observed that cetene, $C_{16}H_{32}$, was oxidized from 13 to 30% faster by *Desulfovibrio* species than was cetane, $C_{16}H_{34}$.

After noting that naphthalene provided for a better growth of soil bacteria than tetralin and that tetralin was utilized more readily than decalin, Strawinski

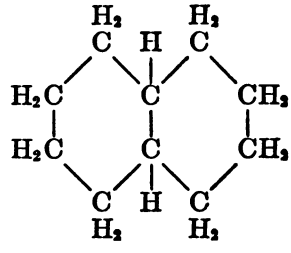
(132) concluded that the saturation of naphthalene reduced the availability of the compound to microbial action:



Naphthalene

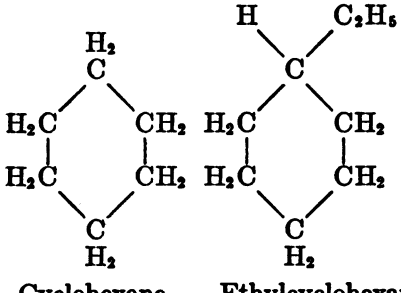


Tetralin

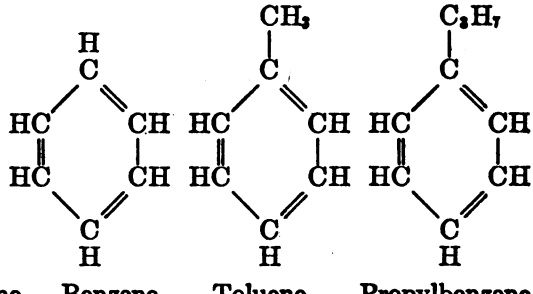


Decalin

Conversely, both cyclohexane and ethylcyclohexane were utilized more readily than either benzene, toluene or propylbenzene:



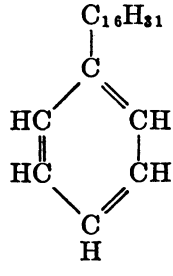
Cyclohexane Ethylcyclohexane



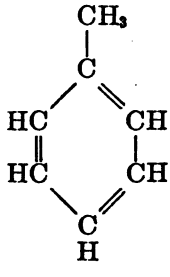
Benzene Toluene Propylbenzene

The bacteria grew better on cyclohexane than on ethylcyclohexane, but growth was much better on either propylbenzene or butylbenzene than on benzene.

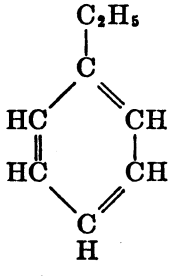
The fragmentary data on the effect of side-chains or branching on the microbial oxidizability of hydrocarbons are contradictory. Strawinski and Stone (133) and Johnson *et al.* (79) have presented evidence which indicates that certain organisms utilize *n*-octane more readily than iso-octane (2,2,4-trimethylpentane), but the reverse has been reported for organisms studied by ZoBell (173, 176). Tausz and Peter (152) found that dimethylcyclohexane and trimethylcyclohexane were utilized somewhat more rapidly by *Bacterium aliphaticum liquefaciens* than was cyclohexane. On the other hand Tausz and Donath (151) reported that this organism attacked cetylbenzene but not benzene, toluene, ethylbenzene or butylbenzene:



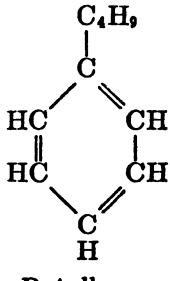
Cetylbenzene



Toluene



Ethylbenzene



Butylbenzene

Three of the four strains of *Bacillus toluolicum* studied by Tausson (143) utilized benzene more readily than toluene or ethylbenzene.

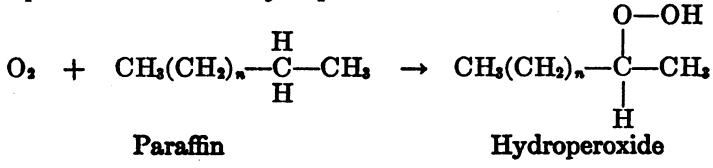
It has been noted that soil bacteria grew fairly well on tertiary butylbenzene, *n*-butylbenzene and isobutylbenzene but not at all on benzene or toluene (133). Either toluene, xylene or phenol was oxidized much more readily by Wagner's (164) *Bacterium benzoli* than was benzene. Under comparable experimental conditions 10 g of toluene were destroyed by this organism in 8 days while only 7 g of benzene were destroyed in 38 days. The addition of a methyl group to naphthalene has been reported (132) to decrease the availability of the compound to microbial action.

According to Matthews (91), the introduction of the methyl group into the benzene ring renders the resulting compound more susceptible to bacterial oxidation. She arranged the compounds as follows upon a basis of their tendency to increase the bacterial population of soil, benzene causing the least rise and pinene the greatest:

POSITION IN RISING SERIES	NAME OF COMPOUND	EMPIRICAL FORMULA	HEAT OF COMBUSTION
			Kg cal/mol
1	Benzene	C_6H_6	800
2	Toluene	$C_6H_5 \cdot CH_3$	935
3	Xylene	$C_6H_4(CH_3)_2$	1,085
4	Pseudocumene	$C_6H_3(CH_3)_3$	1,241
5	Mesitylene	$C_6H_3(CH_3)_3$	1,252
6	Naphthalene	$C_{10}H_8$	1,235
7	Cymene	$C_{10}H_{14}$	1,414
8	Pinene	$C_{10}H_{16}$	1,489

In interpreting these results Matthews proposed the following explanation, which may be subject to question until more experimental data are available, that "In the assimilation of aromatic compounds the most difficult step is probably breaking of the ring . . . It has been found by experiments . . . that cyclohexane is much less readily attacked than hexane. Now for each ring broken benzene gives up 800 units of energy, pinene 1489 . . . Pinene is therefore expected to provide energy more easily than benzene."

The oxidation of a paraffin, under conditions comparable to those encountered by lubricating oils in service in modern engines at 100 to 200°, tends to initiate at a beta carbon atom; that of an alkylnaphthalene at a carbon in the ring to which an alkyl group is attached; and that of an alkyl aromatic at a carbon in an alkyl group adjacent to the ring (182). The initial oxidation product is in every case postulated to be a hydroperoxide:



Paraffin Hydroperoxide

Aldehydes, ketones and organic acids are among the principal products formed from the hydroperoxides. Mixtures of hydrocarbons do not in general oxidize as might be expected, i. e., with the least stable component reacting to the greatest extent. On the contrary, the naphthalene derivatives, which by themselves are the most stable, seem to be oxidized preferentially in mixtures. Paraffins and naphthenes oxidize at comparable rates when chemically catalyzed at temperatures ranging from 30 to 100° (182). The introduction of a benzene ring at the end of a paraffin molecule causes an increase in rate, as does the introduction of olefinic unsaturation. Oxidation rates may be markedly increased by the addition of compounds of copper, lead and iron. Similar studies on the factors which influence the oxidizability of various hydrocarbons, the mechanism of the reactions and the end products resulting from microbially catalyzed oxidations are indicated.

OCCURRENCE OF HYDROCARBON OXIDIZERS IN NATURE

One of the best sources of hydrocarbon-oxidizing microörganisms is oil-soaked soil or water taken from the bottom of storage tanks containing crude oil or petroleum products. Such microörganisms also flourish in the water of petroleum separation tanks and sedimentation ponds. Nearly all of the million or so bacteria per ml of water taken from sedimentation ponds in the East Texas field were able to utilize the crude oil emulsified in the brine (178). Equally large populations of hydrocarbon-oxidizing aerobes were found in production waters from separation tanks in the Bradford, Pa., region. In a brine sample taken from a sedimentation pond at El Segundo, Calif., an average of 86,000 hydrocarbon oxidizers per ml were found (178).

Bacterial populations as follows were found by Haas *et al.* (60) in various source materials:

SOURCE MATERIAL	BACTERIA PER ML
Crude oil sediment pond water (Texas)	168,000
Crude oil sediment pond sediment (Texas)	740,000
Waste oil pond (Texas)	480,000
Crude oil-soaked soil (Texas)	3,800,000
Tank battery oil-soaked soil (Texas)	49,000,000
Kerosene storage tank water (Louisiana)	310,000
Gas oil storage tank water (Louisiana)	1,200,000
Distillate storage tank water (Kansas)	981,000

Other sources from which Haas (58) isolated hydrocarbon oxidizers include water from storage tanks containing gasoline, water from separator pits containing waste petroleum products, sludge from sedimentation ponds, crude oil from pipe lines, crude oil direct from Texas oil wells, water from underground gasoline tanks, water from Supply and Sulfur Springs in Yellowstone National Park, fresh-water wells and ordinary garden soil. Approximately 66% of the hydrocarbon-oxidizing cultures were *Pseudomonas* species. Among those identified were several strains of *P. aeruginosa*, *P. boreopolis*, *P. fluorescens* and *P. striata*. Next in order of abundance were species of *Mycobacterium*, *Proactinomyces*, *Actinomyces*, yeast-like organisms and molds (58).

Approximately 95% of the stock cultures of *Psuedomonas* obtained by Haas (58) from various laboratories and the American Type Culture Collection grew in kerosene media, indicating that the ability to use hydrocarbons is a rather general characteristic of this genus. *Mycobacterium leprae*, *M. phlei* and *M. smegmatis* utilized both light and heavy oils and paraffin wax. Neither the human nor bovine varieties of *M. tuberculosis* developed in hydrocarbon media, but the avian species grew slowly on paraffin.

After noting that most *Mycobacterium* species utilize paraffin, while *Corynebacterium* species were unable to do so, Haag (57) proposed paraffin utilization as a characteristic for differentiating these organisms. However, several strains of *Mycobacterium* examined by Jensen (75, 76, 77) seemed unable to attack paraffin, although most *Mycobacterium* species did attack paraffin. Both Haag (56) and Jensen (77) reported the inability of *Corynebacterium* species to utilize paraffin, but Haas (58) found that *C. simplex* and four other unidentified species of *Corynebacterium* grew in media containing either mineral oil or paraffin as the only source of energy.

Seventeen strains of acid-fast organisms including *Actinomyces asteroides*, *A. farcinica*, *A. gypsoides* and 14 strains classified by Umbreit (160) as *Proactinomyces* grew in Czapek's mineral medium enriched with melted paraffin. None of the closely related non-acid-fast cultures grew in paraffin media.

Lipman and Greenberg (88) reported finding a cocco-bacillus which decomposed petroleum in reservoir fluids coming from an oil well 8,700 feet deep. Sulfate reducers, many of which are known to attack hydrocarbons, have been demonstrated in crude oil and oil-well brines (9, 10, 41, 45, 46, 47, 89, 90, 178). From Russian crude oil, a microörganism was isolated which utilized kerosene, benzene, paraffin wax and paraffin oil (165).

The literature summarized in table 1 is indicative of the widespread and general occurrence in soil of organisms which utilize paraffin. Söhngen (121) found from 50,000 to 200,000 paraffin-oxidizing bacteria per gram of garden soil and up to 3,000 per ml of ditch water. A marked increase in the abundance of microorganisms in soil treated with paraffin was observed by Jensen (75), who noted that nearly all species of *Proactinomyces* found in soil could utilize paraffin, but out of 20 *Actinomyces* tested only *A. albus* and *Actinomyces* species 218W and 6S were able to grow on paraffin as a sole source of energy.

Species of *Mycobacterium*, *Actinomyces*, *Penicillium*, *Aspergillus*, *Bacillus* and *Bacterium* which attack paraffin were found in nearly all samples of soil, hay, leaves, manure and peat examined by Büttner (20). Microörganisms which attack aromatic hydrocarbons appear to abound in soil as adjudged from the increased microbial populations resulting from the addition of benzene, toluene, naphthalene, etc. (52, 73, 91, 116, 136).

By applying crude oil to soil, Baldwin (4) induced the growth of hydrocarbon oxidizers, one of which resembled *Mycobacterium hyalinum*. *Bacterium aliphaticum*, originally isolated form European soil (152), was found in New Jersey soil around a gasoline pump (79). Other hydrocarbon oxidizers have been isolated from oil-soaked soil (11, 140, 141, 145).

The widespread occurrence of methane oxidizers in soil is attested by many observations (1, 64, 65, 94, 95, 119, 120, 131, 151, 178). Large numbers of meth-

ane oxidizers have been found in sewage, manure and river mud (43, 44). Kusnetzow (85) credits methane oxidizers in lake water with playing an important rôle in depleting dissolved oxygen.

All samples of mud which Erikson (37) collected from Wisconsin lakes contained species of *Micromonospora* which utilized paraffin wax, paraffin oil and various aromatic hydrocarbons. Paraffin-decomposing bacteria have been found in Ala-Kule Lake, Russia (135). Bottom deposits from the Dead Sea were found (34) to contain bacteria which utilized crude oil and kerosene. In medicinal mud from Tambookansk and Petrosk, Russia, Goobin (49) found, among other hydrocarbon oxidizers, *Bacterium hidium* which attacked ethane, petroleum ether and kerosene.

All 0.1 g samples of recent marine sediments and all 60 ml samples of sea water examined by Grant and ZoBell (50) and Novelli (98) contained microörganisms which grew on paraffin oil Species of *Proactinomyces*, *Actinomyces*, *Pseudomonas*, *Micromonospora* and *Mycobacterium* which assimilated hydrocarbons were found (181) to be widely distributed in sea water and marine bottom deposits. Microorganisms which can attack rubber hydrocarbons are widely distributed in the sea and in garden soil (179, 180). The hydrocarbon-oxidizing *Desulfovibrio* species studied by Novelli and ZoBell (99) were isolated from marine sediments and garden soil.

After noting the general presence of hydrocarbon-oxidizing bacteria in fresh water from various sources, Isjurova (71) cautioned that neither petrolatum nor paraffin oil could be used as sealing agents to exclude oxygen in biochemical oxygen demand tests because these substances are subject to microbial oxidation. Furthermore, paraffin oil to a depth of 0.5 to 1.5 cm was found to have little effect on the diffusion of oxygen into underlying fluids.

MICROBIAL MODIFICATION OF PETROLEUM

There is ample evidence from field and laboratory observations that crude oil is attacked by soil microörganisms. The rapid disappearance of oil from waterways, from soil around refineries, leaking pipe-lines or oil wells, and from polluted beaches is believed to be due largely to the activity of hydrocarbon-oxidizing microörganisms. This has been described (173) as Nature's way of "pulling the chain" for the disposal of oil which otherwise would pollute fields and waterways. In controlled laboratory experiments the gradual disappearance of oil added to normal soil and marine sediments has been observed (178), while the oil persists almost indefinitely in sterilized soils or sediments.

The oxidation of American and Russian crude oils by soil bacteria was observed by Söhngen (121). Under favorable conditions as much as 7.5 mg of oil per square decimeter of oil surface exposed to the culture was oxidized per day at 28°. Wagner (164) noted the destruction of 1 g of crude oil in 8 days by *Bacterium benzoli* growing in 100 ml of mineral solution overlayed with crude oil.

In 2-liter flasks half-filled with mineral salts solution inoculated with mixed cultures of soil bacteria, Tausz and Peter (152) noted changes in layers of crude oil 1 to 2 mm thick after two or three days' incubation at 25°. Within 7 to 14 days the oil layer became a perforated network with holes the size of a pin head.

The threads of the network continued to become finer until they gradually disappeared. Part of the oil, pitch-like in color and consistency, sank to the bottom of the flask. Layers of crude oil appreciably thicker than 2 mm were not perforated, presumably because bacterial action was retarded by a lack of oxygen. Even thin layers of oil remained intact in sterile controls.

In an experiment with Mendoza crude oil which originally contained 0.3% of an asphaltic residue insoluble in petroleum ether, Tausz (150) found 5.2% of asphaltic residue after the oil had been acted on by bacteria. The view was expressed (150) that besides modifying petroleum in various ways, bacteria contribute to the sinking of oils to the bottom of the ocean. Sinking of oil droplets was caused by the increased density resulting from the presence of so many bacteria having a density greater than that of water.

After observing the rapid disappearance of crude oil from the waters and beaches of San Francisco Bay following the wrecking of a tanker, Beckman (11) demonstrated the presence of hydrocarbon-oxidizing microörganisms along the beaches in oil-soaked soil, slime and sewage. Bacteria and molds caused significant changes in the specific gravity and viscosity of oils in a month at 40°. Crude oil emulsions were broken by proteolytic bacteria, suggesting (11) that in these cases the emulsifying agent was of protein nature. Allegedly the changes occurred under anaerobic as well as aerobic conditions.

The slow destruction of petroleum by several species of soil *Actinomyces*, three species of *Mycobacterium* and a mold was observed by Büttner (20). Tausson (141) expressed the belief that crude oils in nature undergo progressive oxidative modification and destruction until microbial activity is arrested by conditions in oil pools. His belief was based upon the general abundance of hydrocarbon oxidizers in oil-soaked soil and upon observed changes in samples of petroleum acted on by microörganisms. Thermodynamic considerations and experimental results with sulfate reducers suggested to Tausson and Aleshina (146) that, under anaerobic conditions, sulfate reducers tend to convert paraffinic hydrocarbons containing 10 or more carbon atoms per molecule into naphthenic compounds. Conclusive proof is still lacking. Rogers (107) expressed the belief that paraffinic hydrocarbons are oxidized and polymerized to yield naphthene and asphalt base compounds and that sulfate in oil-field water is reduced to sulfide, reactions which might be catalyzed by sulfate reducers.

Microörganisms oxidized Emba crude oil at an average rate of 250 g per square meter of surface exposed to the culture in seven months (147). It is pointed out that, since the oils under investigation consisted mainly of naphthene and polynaphthene hydrocarbons, the disappearance of 45% of the amount initially present established that microörganisms could utilize such hydrocarbons. Microbial activity was found to cause changes in the following properties of Emba crude and cylinder oil prepared therefrom: index of refraction, iodine number, saponification number, density and physical appearance. The middle fractions were oxidized more rapidly than the heavy ones, resulting in an accumulation of the latter (147). In the beginning of the development of bacteria in oil, an intensive consumption of unsaturated compounds took place as indicated by the

decrease in the iodine number. With further development, the iodine number increased slowly, indicating a relative accumulation of unsaturated hydrocarbons and speaking for the view of Tausz (150) on the formation of unsaturated hydrocarbons as intermediate products in the oxidation of saturated hydrocarbons. A sharp and great increase in the saponification number with the development of bacteria pointed to the formation of higher fatty acids and naphthenic acids, and this was regarded (147) as the cause of the formation of water emulsions of oil products invariably observed in such cultures.

The utilization and modification of 31° Iles Dome crude oil by both aerobic and anaerobic bacteria has been reported (92). Ordovician oil-well water, Dakota oil-well water and water in which Frontier shale had been leached for several days was enriched with crude oil and inoculated with mixed cultures of soil, lake-slime or ditch-water microörganisms. Every combination except the sterile controls resulted in visible changes in the oil. The opinion was expressed (92) that over a considerable period of time, drops of oil of super-capillary size can be oxidized by microörganisms to CO_2 and water under the conditions which have prevailed in the reservoir rocks.

Pronounced changes in a variety of Russian crude oils as a result of the activity of the sulfate reducer, *Desulfovibrio aestuari* have been observed (89). Sulfur compounds were preferentially attacked and the crude oil itself was slowly oxidized.

The widespread distribution and great diversity of hydrocarbon-oxidizing microörganisms in soil, water and recent sediments and their demonstrated ability to function throughout a wide range of environmental conditions quite definitely establish that, in the absence of inhibiting agents, petroleum hydrocarbons can be expected to be modified or completely destroyed. Indications are that these changes take place far more rapidly and extensively in aerobic than in anaerobic environments. The activities of hydrocarbon-oxidizing microörganisms may help to explain why Trask (158) and others have failed to find petroleum hydrocarbons in recent sediments.

Conditions inimical to the activity of microörganisms appear to be prerequisite to the accumulation of petroleum hydrocarbons in recent sediments. It has been found (178) that a good many samples of petroleum and more samples of oil-well brines have bacteriostatic properties, the exact cause of which is still unknown. Experiments now in progress at the Scripps Institution (178) indicate that certain heavy metals, the presence of H_2S, low redox potentials (175) and specific oxidase inhibitors prevent the microbial oxidation of hydrocarbons. This suggests that in the search for source sediments of petroleum and the origin of oil, particular attention should be given to substances or conditions in sediments which are inimical to the activities of hydrocarbon-oxidizing microorganisms. As academic examples of inhibitory substances may be mentioned cyanide and urethane, low concentrations of which were found (79) to inhibit the oxidation of hydrocarbons by *Bacterium aliphaticum*.

Little is known regarding the occurrence or activity of bacteria in petroleum deposits. Sulfate-reducing bacteria, some of which attack hydrocarbons, have

been found in oil-well brines from various depths (9, 10, 45, 46, 47, 89, 90, 178). In petroleum from a well 8700 feet deep, Lipman and Greenberg (88) found bacteria which completely decomposed samples of petroleum with the formation of CO_2. Sulfur bacteria have been found in oil from a depth of 6000 feet (72). It is a moot question, however, whether bacteria in reservoir fluids coming from oil wells are species indigenous to subterranean reservoirs or if they are adventitious species introduced during drilling or production operations. The continued abundance of bacteria in reservoir fluids for prolonged periods after anything has been introduced into the well, the peculiar types of organisms found, evidence of activity such as a depletion of sulfates (118) and demonstrated ability of the bacteria to function under similar environmental conditions all speak in favor of the occurrence and activity of bacteria in petroleum reservoirs.

Living bacteria are most abundant in Tertiary formations and gradually disappear in older Cretaceous, Jurassic, Permo-Triassic and Permian formations according to Ginsburg-Karagitscheva (46). This worker, like Rogers (106), interpreted the absence of sulfates from certain oil-well waters as evidence of earlier bacterial activity.

The demonstrated ability of anaerobic sulfate reducers to attack hydrocarbons (99, 146, 149, 178) lends considerable weight to the argument that bacteria may modify petroleum after its formation. The activities of hydrocarbon-oxidizing microörganisms could account for many observed and anomalous conditions. For example, the preferential microbial attack of paraffinic hydrocarbons in the middle range could explain the occurrence of deposits of tars associated with gas typified by extensive deposits in Venezuela and Mesopotamia. The preferential attack of unsaturated hydrocarbons could account for the general absence of olefines in crude oil. Environmental conditions conducive to the activity of bacteria which completely destroy oil could account for certain areas being barren of oil fields which possess all the known geological characteristics of areas containing prolific oil fields. The reasonableness of this latter possibility is vouchsafed by geological conditions which led Thom (156) to conclude that it is much more reasonable to regard the metamorphic destruction of oil pools as having occurred progressively and gradually rather than suddenly.

In summarizing the rôle of bacteria in the formation of petroleum, ZoBell (173, 177) concluded that the release of oil from sediments is one of the most important functions of bacteria in the accumulation of oil in subterranean deposits. Bacteria release oil from sediments and promote its migration and accumulation by dissolving carbonates, producing CO_2 and detergents and by the physical displacement of oil from solids by thigmotaxis. The accumulation of oil tends to preserve it from microbial attack because the microörganisms and their enzymes are active only in the presence of water. Baier (3) envisioned sulfate-reducing bacteria growing at the oil-water interface in petroleum deposits where paraffinic constituents might be converted into oxybitumens or asphaltenes and gas. Although certain constituents of petroleum were shown to be germicidal, Baier (3) concluded that at some little distance from the oil-water interface the concentration of toxic constituents would be low enough to permit the

activity of hydrocarbon-oxidizing microörganisms. Sanders (110) expressed the belief that petroleum deposits provide an aseptic environment for the prolonged preservation or embalmment of the organized remains of certain biological materials.

It is an anomalous situation that the petroleum industry, praiseworthy for its many outstanding scientific and technological achievements, has devoted so little attention to the effects of microörganisms on petroleum or its products. Intensive and extensive microbiological studies are needed to elucidate the theories of the origin and occurrence of crude oil. Besides being of academic interest, information gained from such studies may find important application in the discovery, recovery, refining, modification and exploitation of petroleum or its products. Urgently needed is reliable information on the relative susceptibility of various kinds of hydrocarbons to microbial attack and the nature of the resultant end products under different environmental conditions.

MODIFICATION OF PETROLEUM PRODUCTS

The microbial oxidation of gasoline, kerosene, lubricating oil or similar refined petroleum products has been quite commonly observed (19, 20, 49, 79, 121, 144, 147, 151, 155, 165, 181). Even heavy residues such as asphalt are susceptible to microbial attack (129, 178). The observations suggest that wherever petroleum products are stored in the presence of water for prolonged periods, the possibility of microbial modification exists.

In the water bottoms of kerosene and gasoline storage tanks, Thaysen (154) found nitrate reducers which fermented kerosene with the formation of methane and ethane. Besides causing the corrosion of metal tanks, sulfate reducers in the water bottoms contaminated the gasoline with undesirable H_2S. A spontaneous explosion in a kerosene storage tank was attributed by Thaysen to the microbial formation of an explosive mixture of gases.

Microbial activity may be a factor in gum formation in high test gasolines and the production of "off colors" in water-white distillates (58). Diesel fuels and mineral oil are probably more susceptible to microbial modification than gasoline or kerosene. Haas (58) stressed the possible significance of so many workers reporting the production of oil-soluble acids in petroleum or its products as a result of microbial activity.

Upon examining the lubricating oil and diesel motor fuel from marine engines which had been out of commission for several months, large numbers of hydrocarbon-oxidizing bacteria were found (178) which could have been responsible for the emulsified and corroded condition of the water-contaminated engine oils. Similar circumstantial evidence has been obtained for the deterioration of transformer oils. Such oils stored in the presence of water for prolonged periods are often emulsified, presumably through bacterial activity.

Mycobacterium No. 24 studied by Haas (58) produced 2.14 mg of carotenoid pigments in 500 ml of oil within two weeks, imparting an orange color to the oil. A highly chromogenic strain of *Proactinomyces rubropertinctus* was also isolated from water in oil storage tanks (60). Among the hydrocarbon oxidizers studied

by Söhngen (121) red, orange and yellow pigments were produced by *Mycobacterium rubrum*, *M. phlei* and *M. luteum*, respectively. Some of the hydrocarbon-oxidizing species of *Actinomyces* and *Mycobacterium* observed by Haag (56) were pigmented. The formation of a brown pigment soluble in alcohol indicated to Rahn (104) that paraffin was being used by *Penicillium* species. The growth of *Aspergillus versicolor* (70) on paraffin was accompanied by the production of first yellow and then red pigment. An olive-green pigment was produced in cetane cultures, reddish-orange in naphthalene cultures and yellow-brown pigment in tetralin, decalin and diphenyl cultures studied by Strawinski (132). These examples of pigment production by microörganisms growing on petroleum products should suffice to indicate the possibilities of discoloration.

Observed decreases in the octane rating of aviation gasoline stored over water were attributed (173), at least in part, to bacteria which either (a) preferentially attack branched-chain hydrocarbons which have the highest anti-knock characteristic, (b) produce sulfides which precipitate lead tetraethyl or (c) produce peroxides which catalyze the deterioration of lead tetraethyl.

Though not definitely establishing that the hydrocarbons were oxidized as the source of energy, Kegel (84) found that the bacterial reduction of sulfates in natural gas being cooled by direct contact with water in trickling gas coolers resulted in an undesirable increase in the H_2S content of the gas. By the strategic placement of medium inoculated with *Methanomonas methanica*, Yurovskii *et al.* (169) claimed that 96% of the methane in coal mines was destroyed under experimental conditions.

Much trouble is caused by the growth of bacteria in mineral oil emulsions used as cooling agents in the cutting and grinding of metals in machine shops. The emulsions may be broken, sour objectionable odors sometimes develop, and machinists may become afflicted with dermatitis. Lee and Chandler (87) found 15,000,000 to 50,000,000 bacteria per ml of cutting compound. Predominating was *Pseudomonas oleovorans*, which, like other bacteria isolated from the cutting compound, readily utilized the emulsified oil. Naphthenic acids, used as emulsifying agents in the cutting compound, were also utilized by the bacteria as a source of food. Coal tar disinfectants were ineffective, but the addition of 15 pounds of resorcinol per month to a 500-gallon tank prevented the spoilage of the cutting compound.

Duffett *et al.* (31) found from a million to 350 million bacteria per ml of cutting oil emulsions, including six new species of *Pseudomonas*, *P. oleovorans*, two species of *Achromobacter*, *Bacillus alvei*, yeasts and molds. Bacteria growing in mineral oils have been credited as being the causative agents in industrial dermatitis (30). Cutting oils give rise to a great deal of annoyance and increased labor turnover in machine shops because of epidemics of boils and furuncles (128). The cutting oils become heavily contaminated with suppurative organisms, the most serious of which is *Staphylococcus aureus*. The paraffin oil used in cutting oils is the one forming the most favorable culture medium for these organisms (128). The rancidity and allergenic properties of ointments and other pharmaceutical preparations containing mineral oil or petrolatum have been attributed to the action of hydrocarbon-oxidizing microörganisms.

Hydrocarbon-oxidizing bacteria may be responsible for the fairly rapid disappearance of oils sprayed on various kinds of foliage as a carrier of insecticides. Second and third applications of spray oils generally disappear more rapidly than the first.

The finding in soil underlying defective spots in old asphalt-paved highways of exceptionally large populations of bacteria, many of which are endowed with the ability to oxidize asphalt in the laboratory, suggests that microbial activity may contribute to the deterioration of such highways. There is similar circumstantial evidence to incriminate bacteria as being responsible for the disappearance of asphalt in contact with soil under concrete foundations and other structures under which a layer of asphalt had been applied as a waterproofing agent. When mixed with normal soil, asphalt is slowly decomposed (178), a change which does not occur in sterile soil.

ACTION ON RUBBER HYDROCARBONS

The tacit assumption that rubber stoppers, tubing, gaskets, etc., are biologically inert has led to anomalous experimental results. For example, Hatfield and Morkert (66) observed that rubber stoppers materially increased the biochemical oxygen demand of water samples. This observation was confirmed by ZoBell and Grant (180), who noted further that exposure to pure gum rubber, duprene or neoprene increased the oxygen consumption by bacteria in sea water. In light of the information summarized in the following paragraphs, it is believed that the distilled water in the experiments of Bigger and Nelson (14) was rendered growth-promoting for certain coliform bacteria because the bacteria in question assimilated rubber hydrocarbons. The growth-promoting properties of rubber tubing and stoppers were attributed (14) to the talc dressing, which was believed to adsorb nutrients from the atmosphere.

The utilization of caoutchouc by *Mycobacterium* species was reported by Söhngen (121) more than thirty years ago. From garden soil and canal water, Söhngen and Fol (123) isolated strains of *Actinomyces fuscus*, *A. alba*, *A. chromogenes*, *A. elastica* and other *Actinomyces* species which grew well on purified rubber treated with mineral solution. In such media there was a fair growth of *Mycobacterium rubrum*, *M. lacticola*, *Bacillus mesentericus* and *Pseudomonas fluorescens*.

Arens (2) attributed the red spots which appeared on unpreserved sheet rubber to the growth of *Serratia marcescens*. This organism, along with other chromogenic bacteria, was isolated from damaged crepe rubber (28). Further work (17, 32) established that the "spot disease" of sheet or crepe rubber was due to microörganisms. Unlike mildews and molds which grow primarily on the surface of moist crude rubber at the expense of nitrogenous impurities therein, the "spot disease" bacteria decompcsed the rubber.

In soil and on rubber plants, Novogrudski (100) found actinomyces and bacteria which decomposed caoutchouc. One of these organisms was described as an orange-yellow coccus which decomposed caoutchouc readily in mineral solutions at pH 7 to 8.5 but not at pH 6.3. From fermenting hevea latex, Corbet (24) isolated *Gaffkya verneti*, *Alcaligenes denicri*, *Micrococcus eatoni*, *M. ridleyi*, *M.*

epimetheus, *M. chersonesia*, *Bacillus pandora* and *Torulae heveae*, each of which oxidized rubber hydrocarbons. The gradual loss in weight and decreased elasticity of moist sheets of plantation crepe rubber have been attributed (23, 27) to the activities of *Penicillium* and *Aspergillus* species.

Unexpectedly low yields of rubber from guayule latex under certain conditions of storage have been caused by the activities of soil microörganisms, some of which destroyed as much as 15%, and adversely affected all, of the rubber hydrocarbon in six weeks at 37° (125). Spence (124) described four species of *Actinomyces* and several strains of anaerobic bacteria which decomposed the rubber hydrocarbons in guayule latex. Spence and van Niel (126) characterized the decomposition of hevea latex hydrocarbons as being both rapid and profound after noting the disappearance of 20% of the rubber in purified latex under the influence of soil microflora for six weeks at 20°.

From 20 to 40% of the rubber from latex dispersed in mineral solution was destroyed in a month at 28° by *Actinomyces aurantiacus*, *A. longisporus ruber*, *Aspergillus oryzae*, *Penicillium* species and various raw soil cultures (82). Neither *Azotobacter chroococcum*, *Pseudomonas fluorescens*, *Bacillus mycoides*, *Sarcina ureae* nor *Proteus vulgaris* was able to attack rubber hydrocarbons. By sprinkling finely divided soil on plates of mineral agar coated with a thin film of rubber, Kalinenko (82) showed that rubber-oxidizing bacteria are widely distributed in garden soil.

ZoBell and Beckwith (179) found purified rubber to be oxidized by the following pure cultures: *Bacterium aliphaticum*, *Pseudomonas fluorescens*, *P. neritica*, *Vibrio marinofulvus*, *Serratia marcescens* and certain strains of *Escherichia coli*. Negative results were obtained with *Proteus vulgaris*, *Alcaligenes faecalis*, *Sarcina lutea* and several strains of *E. coli*. Mixed cultures of soil bacteria attacked highly purified caoutchouc, neoprene, duprene, butaprene, chemigum, ameripol, hycar, thiocol RD, butyl rubber and various experimental elastomers synthesized from butadiene, $CH_2{=}CH{-}CH{=}CH_2$, isoprene, $CH_3{-}CH{=}CH{-}CH{=}CH_2$, isobutylene, $(CH_3)_2C{=}CH_2$, acrylonitrile, $CH_2{=}CHCN$, or styrene, $C_6H_5{-}CH{=}CH_2$. In one experiment 78% of the rubber initially present was oxidized to CO_2 and water and from 10 to 20% of it could be accounted for as bacterial protoplasm.

As a general rule, pure rubber is oxidized by bacteria more rapidly than compounded or vulcanized rubber products. The rate of oxidation depends to a large extent upon the surface exposed to the culture. In order to retard or prevent the microbial deterioration of rubber products, Dimond and Horsfall (29) advocated the use of anti-microbial agents in compounding rubber, particularly rubber which is to be used in an aqueous environment. It is not a simple problem because chemicals which are ordinarily effective fungicides may be inactivated by the rubber hydrocarbons, accelerators of vulcanization, antioxidants or fillers used in compounding rubber. Tetramethylthiuram disulfide, an accelerator known as Tuads, rendered rubber resistant to attack by *Macrosporium sarcinaeforme*.

ACTIVITIES OF HYDROCARBON OXIDIZERS IN SOIL

The deduction, that hydrocarbonoclastic microörganisms are active in most normal soils, is based upon the general abundance of hydrocarbon oxidizers in soil (to which reference has been made on the preceding pages) and the apparent destruction of hydrocarbons known to be produced by plants. A great variety of aliphatic hydrocarbons ranging from C_7H_{16} to $C_{35}H_{72}$ are known to occur in plant and insect waxes (23, 117). Extensive search for rubber-producing plants during the War years has shown that most plants contain some hydrocarbons, and certain plants such as guayule, milkweeds and dandelions, for example, contain appreciable quantities of hydrocarbons. It has been estimated that an average of 0.02% of the solids produced by plants consist of hydrocarbons. There is also evidence that soil bacteria produce several hydrocarbons besides methane (74, 173, 177, 178). Inasmuch as these hydrocarbons ordinarily do not accumulate in the soil, it is inferred that they must be attacked by microörganisms. If the hydrocarbons produced by plants since Cambrian times had been preserved, a goodly portion of the earth's carbon would have long ago been tied up. In certain environments where conditions have not been conducive to the activities of hydrocarbon oxidizers, petroleum is believed to have accumulated in structural or stratigraphic traps.

Methane production (5, 6) commonly occurs in soil, and, under favorable conditions, much methane may be oxidized by bacteria. After observing an increase in the organic content of mineral solutions in which methane oxidizers were growing, Giglioli and Masoni (43) suggested that biological methane oxidation in nature continually contributes to the organic fertility of soil. The effect of methane oxidizers on the organic content of soil has been studied by Harrison and Aiyer (64) and Aiyer (1).

Two- to threefold increases have been observed (63) in the nitrogen and organic content of soil exposed for several years to natural gas from buried conduits. After establishing that the increases were not due to the presence of ammonia or nitrogen oxides in the gas, the increases were attributed to the effect of methane on the growth of microörganisms. Increases in the nitrogen and organic content of soil around leaking gas mains, attributable to biological activity, have also been observed (113).

A few days after the addition of paraffin wax to soil, Gainey (42) noted a decrease in the content of nitrate and ammonia. The amount of paraffin oxidized was limited primarily by the supply of available nitrogen. It is entirely possible that had the experiments been conducted over a period of weeks or months until the dead cells of paraffin oxidizers had undergone decomposition, he would have found, like others (63, 113), that treating soil with hydrocarbons had a beneficial effect on its organic and nitrogenous fertility.

The application of crude petroleum to soil was found (4) to increase the total bacterial population, but the number of bacterial types was greatly reduced. The multiplication of aerobes was stimulated more than of anaerobes. The petroleum appeared to be broken down into simpler products and gradually disap-

peared. Up to 50% of the petroleum had disappeared in 56 days from 4000 g of soil treated with 200 ml of crude petroleum having a density of 0.8370. During this period Baldwin (4) observed that both ammonia production and nitrate formation were inhibited. In field experiments it was found that the largest and best ears of corn were produced in hills treated with petroleum. Similarly, Carr (21) found that applications of crude petroleum to soil improved the growth of soybeans. Up to 30 ml of petroleum per gallon of soil, or 0.75% , was beneficial, and petroleum was not injurious until five times this concentration was added to soil. The damage to plants resulting from the application of 4% or more of petroleum was explained (21) as being due to the inability of plants to obtain water fast enough to meet their requirements.

It has been commonly observed that vegetation is killed by the intentional or accidental application of relatively large quantities of oil on soil. The lethal concentration is a function of the kind of oil, soil conditions, method of application, season, kind of vegetation, humidity, and other factors. However, it is only a matter of a few weeks until the arability of the soil has been restored, presumably due to the microbial oxidation of the oil; and there are many reports of the fertility of the soil having been improved by the oil. The systematic application of sublethal concentrations of oil may prove to be beneficial under certain circumstances. For controlling weeds in crops of carrots, parsnips and celery, the application of 65 to 85 gallons of stove oil per acre is required and more than one such application may be necessary during the growing season. From 100 to 300 gallons of oil per year has been applied to the soil for the control of weeds in citrus orchards. Hildebrand (69) gives several references to the recent literature on the use of petroleum oils for controlling weeds.

Truffault and Bezssonoff (159) found a *Bacterium B*, which could utilize either paraffin oil, cyclohexane or methylcyclohexane as a source of energy, growing symbiotically with the anaerobic nitrogen fixer, *Clostridium pastorianum*. The hydrocarbon-oxidizing symbiont closely resembled *Bacterium aliphaticum* of Tausz and Peter (152).

After noting the rapid microbial oxidation of paraffin wax in the soil, Gainey (42) emphasized the often overlooked fact that data obtained by methods which call for the use of wire baskets, pots, stakes or other experimental apparatus rendered "inert" by coating with paraffin prior to burial in the soil may be invalidated by the action of microörganisms on paraffin. Similarly, Rogers (108) has pointed out that coating experimental apparatus with paraffin is a source of error in corrosion tests in water because of the susceptibility of paraffin to microbial oxidation.

Until he learned that the hydrocarbons were themselves rapidly attacked by soil organisms, Greig-Smith (53) was surprised to find that coating dried blood or casein particles with petrolatum or paraffin did not prevent such particles from being attacked by soil bacteria. According to Fleming (38), paraffin could not be used as a coating to decrease the injurious action of lead arsenate on plant roots because the paraffin was rapidly decomposed by soil microflora. The growth of molds was stimulated by the addition of paraffin to soil much more than was the growth of bacteria.

Jensen (75) reported that the addition of paraffin to soil caused a marked increase in the microbial population, the abundance of *Proactinomyces* species being particularly increased. The following organisms were described as being able to oxidize paraffin: *Proactinomyces actinomorphus, P. agrestis, P. minimus, P. paraffinae* and *Actinomyces albus.*

Treating soil with toluene resulted in a temporary decrease followed by a great increase in the bacterial population, according to Russell and Hutchinson (109). Following the application of toluene to soil, the plate count rose from an initial count of 5 to 9 million per gram to 40 million or more. Toluene also caused an increase in ammonia production. An increase in the content of soluble organic matter in soil was observed by Pickering (103) to result from treating soil with benzene or paraffin oil. These workers like Buddin (18), who noted a great increase in the bacterial population and ammonia production in soil treated with M/200 to M/2 concentrations[2] of toluene, benzene, cyclohexane, pentane, hexane, phenol, cresol, quinone, hydroquinone, pyridine, alcohol, ether or acetone, attributed the beneficial effects on soil fertility to the destruction of protozoa or some other inimical factor. Evidence was obtained by Sen Gupta (116), however, which indicated that such aromatic antiseptics were oxidized by soil bacteria. Phenol and cresol were observed to disappear from normal soil but not from sterilized soil, and the second and third applications of these substances disappeared much faster than the first application.

It was finally established by Matthews (91) that while the "antiseptic" hydrocarbons or derivatives promoted the growth of bacteria by destroying predatory protozoa, such substances as toluene, benzene, xylene, pseudocumene, mesitylene, naphthalene, cymene, pinene, hexane, chlorobenzenes, nitrobenzenes and cresols provided energy for the multiplication of certain soil bacteria independent of any effects on protozoa. Tenfold or greater increases in the bacterial population followed the application of M/10 concentrations[2] of benzene. *Bacillus liquefaciens* could tolerate and attack nearly any of the aforementioned compounds, but most species of soil bacteria exhibited a high degree of specificity in their ability to assimilate various kinds of hydrocarbons. Aeration promoted the bacterial oxidation of the compounds.

The addition of naphthalene to soil was found by Tattersfield (136) to cause decreases followed by large increases in the number of bacteria. Microbial multiplication and the decomposition of naphthalene were promoted by aeration. About 50 days were required for the disappearance of 50 mg of naphthalene from 100 g of soil initially treated, but the second 50 mg of naphthalene disappeared in only 20 days and the third 50 mg disappeared in 10 days.

Jacobs (73) described experiments in which the aerobic bacterial population of soil increased from an initial count of a few millions per gram to more than three billion per gram in two or three days following the application of M/10 naphthalene to soil. Within a week or two the bacterial population dropped to around 400,000,000 per gram. Several strains of bacteria were isolated which were capable of using naphthalene as a sole source of energy. The naphthalene oxidizers preferred ammonium to nitrate as a source of nitrogen. The ammonia

[2] The stated concentrations represent moles of hydrocarbon per kg of soil.

content of the soil treated with naphthalene at first decreased and then increased. It was explained (73) that the naphthalene-decomposing bacteria utilize the available nitrogen in the soil; then upon the exhaustion of the naphthalene the bacteria die and undergo decomposition. Potential energy added to the soil in the form of naphthalene provided for the activities of nitrogen fixers, thus resulting in an overall increase in the nitrogen content of the soil.

The observed increases in the bacterial populations of soils treated with certain aromatic compounds have been attributed by Gray and Thornton (52) to the bacterial utilization of the aromatic compounds. Out of 245 soil samples examined, 146 yielded bacteria which could oxidize either naphthalene, phenol or cresol. Some of the 208 strains of such bacteria, representing 7 genera and 25 species, could oxidize toluene, phloroglucinol or resorcinol. Described as new species which could utilize either naphthalene, phenol or cresol were organisms now listed in the Bergey (12) Manual as *Achromobacter cycloclastes*, *A. iophagum*, *Actinomyces convolutus*, *Bacillus closteroides*, *B. platychoma*, *Micrococcus piltonensis*, *M. sphaeroides*, *Mycoplana bullata*, *M. dimorpha*, *Proactinomyces actinomorphus*, *P. agrestis*, *P. coeliacus*, *P. crystallophagus*, *P. erythropolis*, *Pseudomonas arvilla*, *P. boreopolis*, *P. cruciviae*, *P. dacunhae*, *P. desmolyticum*, *P. pictorum*, *P. rathionis*, *P. salopium*, *Vibrio cuneatus*, *V. cyclosites* and *V. neocystes*. *Proactinomyces globerulus* has been described as a phenol oxidizer (51).

MICROÖRGANISMS AS INDICATORS OF OIL DEPOSITS

Anomalies in the appearance of vegetation over and surrounding oil fields are often noticeable, particularly from a scouting plane. While unquestionably these anomalies are in many cases due to the underlying rock formation, in some cases differences in vegetation are believed to be due to the effect of hydrocarbon-oxidizing microörganisms upon the organic fertility, nitrogen content, redox potential, water-holding capacity, particle aggregation or other properties of the soil. Wherever careful tests have been made, it has been found that hydrocarbons are slowly escaping from subterranean deposits. Conspicuous surface seeps of gas, oil, asphalt or tars have provided clues to the discovery of some oil fields, but hydrocarbons have been detected in the soil overlying other oil pools only by the most meticulous analytical procedures. The sensitivity of hydrocarbon-oxidizing microörganisms as indicators of traces of hydrocarbons in soil is the basis of so-called "geomicrobiological prospecting" methods.

In the geomicrobiological prospecting method of Sanderson (111), patented receptacles, containing mineral media inoculated with bacteria which utilize volatile hydrocarbons, are introduced in the soil in holes dug to a depth at which hydrocarbonogenetic bacteria such as methane producers are not active. The growth of bacteria on the media is indicative of the presence of volatile hydrocarbons in the sub-soil. Sanderson also proposed analyzing soil for its content of bacteria which oxidize volatile hydrocarbons, believing that the abundance of such bacteria would bear some relationship to the presence of petroleum hydrocarbons escaping from subterranean deposits. Blau (16) has patented a method of geomicrobiological prospecting in which certain chemical agents are alleged to

produce characteristic color changes when applied to soil in which hydrocarbon-consuming bacteria have been active. He named *Bacillus ethanicus* as an organism which utilizes ethane in soil.

Nearly any method of prospecting by chemical assay or "soil analysis" which ignores the effect of bacteria on the hydrocarbon content or other properties of soil can be expected to give anomalous results under certain conditions. Hydrocarbons may be decomposed or altered by microörganisms in the soil almost as fast as the hydrocarbons enter the biosphere from subterranean deposits. The rapidity with which hydrocarbons are decomposed in soil may vary seasonally with the water content of the soil, its oxygen content, abundance of organic matter, nitrogen content, temperature, growth phase of the organisms and other factors which influence the abundance, kinds and activity of microörganisms in soil. The rapidity with which various kinds of gaseous, paraffinic and aromatic hydrocarbons are destroyed in soil samples is attested by results summarized in the foregoing paragraphs.

Hydrocarbon-oxidizing microörganisms are believed to have been instrumental in the formation of "paraffin dirt", a kind of waxy soil sometimes found overlying subterranean deposits of petroleum. Samples of "paraffin dirt" have been found (178) to contain large numbers of bacteria (predominantly species of *Proactinomyces*, *Mycobacterium* and *Desulfovibrio*) and complex waxes. Apparently the decomposition of the material has been arrested by the accumulation of toxic metabolic products of microörganisms or antibiotic substances and protection from contact with water or air by an outer coating of waxes and heavy hydrocarbons. The latter are probably carried to the soil surface dissolved in lighter hydrocarbons escaping from subterranean deposits. Some of the hydrocarbons may be oxidized by microörganisms, the cell substance of which is added to the mass of the partially preserved material.

BACTERIOSTATIC HYDROCARBONS AND DERIVATIVES

When mixtures of colon-typhoid organisms were treated with petroleum ether at room temperature for 15 minutes, Bierast (13) observed that typical strains of *Escherichia coli* were selectively killed while typhoid and paratyphoid organisms were not injured by this treatment. Gaertner's bacillus responded virtually the same as typhoid organisms. After confirming these observations with a large number of stool specimens from typhoid patients, Hall (61) proposed the use of petroleum ether for demonstrating typhoid organisms in clinical material. The organisms or stool specimens were placed in a tube of broth, treated with an excess of petroleum ether and shaken for half an hour. After standing for two hours at room temperature cells of *E. coli* were almost quantitatively killed while typhoid organisms survived, provided the boiling point of the reagent was less than 50°. Petroleum ethers having higher boiling points such as benzine (b.p. 80°–90°), ligroin (b.p. 100°–110°), pure heptane (b.p. 98°) and octane (b.p. 124°) were not specific in their effects on colon-typhoid organisms. Pentane (b.p. 38°) added to nutrient broth completely inhibited *E. coli* and stimulated the multiplication of typhoid organisms. Irregular results were obtained with dysentery and para-

dysentery bacilli. The cholera vibrio and members of the *Proteus* group were inhibited by petroleum ether of low boiling point in the same manner as *E. coli*.

Schuscha (115) was successful in detecting typhoid organisms in polluted water when the ratio of typhoid organisms to *E. coli* was as high as 1:5000 by precipitating the organisms with iron salts and then treating the precipitate with petroleum ether. The latter inhibited *E. coli* while permitting the free multiplication of typhoid organisms in nutrient solutions. These observations were confirmed by Heyn (68) who found further that twelve hours agitation with petroleum ether rendered *E. coli* cultures sterile. After such treatment cells of *E. coli* as well as diphtheria bacilli were almost quantitatively transferred from the aqueous medium to the petroleum ether.

Walbum (166) failed to find a marked difference in the susceptibility of enteric organisms to petroleum ether, although the typhoid bacillus was somewhat more resistant than *E. coli*. For example, in one experiment 24% of the typhoid organisms survived treatment with petroleum ether as compared with a 12% survival of *E. coli*. Walbum concluded that the germicidal effect of petroleum fractions is related to their boiling point. The following percentage survival of typhoid bacilli was found (166) after exposure in broth cultures for two hours to various pure hydrocarbons:

HYDROCARBON	% SURVIVAL	HYDROCARBON	% SURVIVAL	HYDROCARBON	% SURVIVAL
Pentane	13.4	Cyclohexane	2.1	Butylbenzene	50.8
Hexane	32.4	Menthene	0	Cymene	42.5
Heptane	74	Benzene	0	Isoamylbenzene	35.4
Octane	96	Toluene	0	Cetylbenzene	100.6
Decane	102	Ethylbenzene	0	Styrene	0
Pentene	0	*o*-Xylene	0	Phenylacetylene	0
Hexene	0	Propylbenzene	0	Methylnaphthalene	29.3
Heptene	0	Cumene	0	Pinene	28.2
Cetene	56.4	Pseudocumene	0	Limonene	14.2
Heptyne	0	Mesitylene	0.6	Terpinene	0

After noting that bacterial cells dried on filter paper were killed more readily by petroleum ether than moist cells of the same species, Baier (3) concluded that the germicidal and bacteriostatic effectiveness of petroleum fractions is a function of their solubility in water. Heavy fractions such as paraffin oil were not injurious to bacteria. *E. coli* was more susceptible to low boiling point fractions than were raw cultures of bacteria in mud or pure cultures of various spore formers. Bacteria in nutrient media not killed by exposure to petroleum or its products tended to become increasingly more tolerant, probably through adaptation or the destruction of the bactericidal components (3).

According to Jentsch (78), cyclic hydrocarbons are the only constituents of natural gas which are harmful to bacteria. Facultative anaerobes were found to grow in an atmosphere of illuminating gas but not as well as in an atmosphere of nitrogen or hydrogen. From his studies on the antiseptic and growth-retarding

actions of cyclic hydrocarbons on bacteria, Van de Velde (161) found that cyclohexane, benzene and toluene had relatively little effect. Mesitylene and xylenes were much more effective antiseptics, *o*-xylene being more active than *m*-xylene. The antiseptic property of such compounds was found to be roughly parallel to the ease with which they can reduce $KMnO_4$. The microbial oxidation of low concentrations of benzene, toluene and xylene has been reported by many workers (37, 52, 75, 91, 116, 131, 144, 164, 165, 181).

Species of *Penicillium*, *Mucor*, *Torula*, yeasts and bacteria which attacked benzoic acid were found in humus by Perrier (102), who described four varieties of *Bacillus benzoicus*. The latter organisms grew in a mineral salts solution enriched with 2% potassium benzoate. Phenol and salicylic acid were decomposed more slowly than benzoic acid. Observations on the selective action and microbial utilization of phenol, cresol, naphthalene and other aromatic compounds have been discussed in a preceding section (18, 52, 73, 91, 136).

Fowler *et al.* (39) isolated from sewage an organism, *Flavobacterium helvolus*, which destroyed 0.1 per cent phenol in mineral salts solution. Gray and Thornton (52) noted that phenol in concentrations as high as 0.1% was utilized by *Bacillus closteroides*, *Pseudomonas rathonis* and *Proactinomyces agrestis*, but 0.15% phenol was inhibitory and 0.2% was lethal for these organisms. Certain sewage bacteria studied by Kalabina and Rogovskaya (81) utilized phenol as a source of energy in concentrations as high as 0.3%. The optimum concentration of phenol for phenol-decomposing organisms was 0.05 to 0.1%, under which conditions sewage bacteria destroyed an average of 57 mg of phenol per day.

The following new species of soil organisms which could tolerate or grow in the presence of 0.38% phenol have been described (8): *Bacillus asterosporus*, *B. balcanis*, *B. catenulatus*, *B. exilis*, *B. globifer*, *B. jubatus*, *B. nigrescens*, *B. oehensis* and *B. phenolphilos*. The tolerance of 96 other species of soil bacteria was reported, of which 46 could grow in the presence of 0.1% phenol. According to Bartels (8), many soil bacteria produce phenol during the decomposition of proteins.

In all one-gram samples of soil examined by Vigier (163), microörganisms were found which tolerated 0.2% phenol in mineral solutions. A yeast-fungus was isolated which utilized phenol as a sole source of energy at concentrations as high as 0.085%. Optimum growth occurred in mineral media containing from 0.0265 to 0.053% phenol. Certain thermophilic bacteria readily oxidized 0.1% phenol in sewage from coke-benzene plants (33). These bacteria, which grew well at 60°, were less active in waste waters containing 0.2% phenol; and 0.4% phenol stopped their growth.

Most of the ten lake-mud strains of *Micromonospora* studied by Erikson (37) grew in mineral solutions containing as the only source of energy 0.1% of phenol, toluene, naphthalene, paradichlorobenzene, resorcinol, *m*-cresol, β-naphthol, picric acid, trinitroresorcinol or cholesterol. Concentrations of toluene or xylene exceeding 0.01% were injurious to *Bacillus hexacarbovorum* (131). The work of Reed and Rice (105) suggests that the germicidal effect of hydrocarbons may be a function of the lipid content and polarity or wettability of the particular bacterium under consideration.

Ethylene has been found to stimulate the respiration of molds, but to retard their growth or multiplication (162).

Insecticidal emulsions containing more than 1% of petroleum oils were found by Young (168) to be injurious to certain plants, although there was considerable difference in the toxicity of different oils and kerosenes used for controlling insect pests on growing fruit and vegetables. The relatively rapid disappearance of hydrocarbons from the leaves of plants was attributed partly to evaporation and partly to absorption. The lighter fractions of kerosenes penetrated potato leaves within 0.5 to 10 seconds, but mostly evaporated from the leaves within 1 to 24 hours. In contrast, lubricating oils penetrated potato leaves within 1 second to 50 minutes and evaporated very slowly or not at all. Sections showed that the oils passed from the potato leaves through the stems and into the tubers. Petroleum oils were also shown to be absorbed by turnip, rutabaga, cucumber, squash and onion leaves. It is an unexplored possibility that much of the oil left on the leaves of plants undergoes microbial decomposition.

Young (167) proposed the use of fungi and bacteria to predict the effect of petroleum oils on apple leaves after noting that the toxicity of oils for apple leaves was about the same as for *Rhizopus nigricans*, *Mucor glomerula*, *Helminthosporium sativum*, *Alternaria tenuis*, *Fusarium sp.*, *Aspergillus sp.*, *Chromobacterium violaceum*, *Serratia marcescens*, *Sarcina aurantiaca* and *Bacillus subtilis*. Mold mycelia and bacterial colonies continued to grow on nutrient agar under layers of non-toxic oils 5 μm in thickness. Oils containing more than 11% of sulphonatable residue were more toxic than the less sulphonatable oils. Hyaline, nearly unsulphonatable petroleum oils were found to be useful for preserving the appearance of many kinds of fungi and bacteria in cultures. Confirming the observations of Parish (101), Birkhaug (15) found that cultures of delicate bacteria remained viable in media under layers of liquid paraffin for from 10 to 24 weeks. The beneficial effects of the oil were attributed to protection against drying and the harmful action of oxygen.

SUMMARY AND CONCLUSIONS

All kinds of gaseous, liquid and solid hydrocarbons in the aliphatic, olefinic, aromatic or naphthenic series appear to be susceptible to oxidation by microorganisms, provided the hydrocarbons are properly dispersed.

Nearly a hundred species of bacteria, yeasts and molds have been shown to be endowed with the ability to attack hydrocarbons. Such organisms grow in simple mineral media enriched with hydrocarbons as the sole source of energy. The presence of free oxygen is generally essential, although nitrate or sulfate serve as hydrogen acceptors for some hydrocarbon oxidizers.

Microbial multiplication, oxygen consumption, nitrate or sulfate reduction, modification of hydrocarbons and the formation of various metabolic products have been employed as criteria of the utilization of hydrocarbons. The microbial utilization of hydrocarbons has been observed at temperatures ranging from 0° to 60°. Being relatively insoluble and immiscible in water, most hydrocarbons are rendered more susceptible to attack when dispersed throughout mineral media by adsorption on inert solids or by emulsification.

CO_2 is the principal product resulting from the microbial dissimilation of hydrocarbons. Cell substance, organic acids, alcohols, unsaturated compounds and other substances have also been reported.

In general, aliphatic hydrocarbons are oxidized more readily than aromatic or naphthenic compounds. Within certain limits, long-chain hydrocarbons are attacked more readily than similar compounds of small molecular weight. The addition of aliphatic side-chains increases the susceptibility of cyclic compounds to microbial attack.

Hydrocarbon-oxidizing microörganisms are widely distributed in soil, water and recent marine sediments. They are particularly abundant in oil-soaked soil and water over which petroleum products are stored. Characterized as being able to assimilate one or more kinds of hydrocarbons are 14 species of *Actinomyces*, 13 *Pseudomonas*, 10 *Proactinomyces*, 10 *Mycobacterium*, 9 *Bacillus*, 7 *Bacterium*, 7 *Micrococcus*, 6 *Aspergillus*, 5 *Corynebacterium*, 3 *Vibrio*, 2 *Achromobacterium*, several unidentified strains of *Micromonospora* and *Penicillium* and one or more strains of *Botrytis*, *Debaromyces*, *Desulfovibrio*, *Endomyces*, *Escherichia*, *Gaffkya*, *Hansenula*, *Macromonospora*, *Methanomonas*, *Mycoplana*, *Serratia*, *Spirillum*, *Torula* and *Torulopsis*.

Although certain crude oils are bacteriostatic, samples of others are destroyed or otherwise modified by microörganisms. Microbial activity is believed to have played an important rôle in determining the properties of petroleum deposits, a problem which merits much more attention.

Petroleum products stored in contact with water for prolonged periods may be discolored, emulsified or otherwise altered by hydrocarbon-oxidizing microorganisms. Coolants and certain kinds of pharmaceuticals containing hydrocarbons and moisture may be adversely affected by microbial activity. Natural and most kinds of synthetic rubber are susceptible to microbial oxidation.

Microörganisms in soil oxidize methane and other hydrocarbons of biological origin. Applications of crude oil to soil result in increased microbial populations and, in many cases, improve soil fertility. Even "antiseptic" hydrocarbons such as benzene, toluene, xylene, naphthalene and related compounds are destroyed by microörganisms in soil. Phenol, cresols, naphthols, chlorobenzenes, nitrobenzenes, benzoic acid, salicylic acid and resorcinol in low concentrations are attacked by soil organisms. The microbial assimilation of phenol in concentrations as high as 0.3% has been demonstrated.

Bacteria which oxidize volatile hydrocarbons may provide clues to the location of subterranean deposits of petroleum.

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