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HYDROCARBONS AS SUBSTRATES FOR MICROORGANISMS

by

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No student of the *logos* of the *microbia* [1]) could set foot in Delft without an awareness of the profound and unique role this Laboratory has played in the evolution of microbiological science. He would be singularly phlegmatic if he did not experience a gratification at being on the scene of so much microbiological history. He would be peculiarly stolid if he did not conjure, on the scene in his own way, images of the figures and events in the history of this place.

From my own reactions, I am none of these. My longstanding aspiration to visit this Mecca of Microbiology would have turned into an exhilarating fulfillment if I had come here, as I had originally intended, merely as a pilgrim after a long trek from Texas. Instead, I find myself, unexpectedly, here under the most auspicious circumstances imaginable for me. The presentation of the A. J. KLUYVER Memorial Lecture is a privilege that any microbiologist would sincerely covet. By an inscrutable decision, the Netherlands Microbiological Society confers upon me a distinction which easily could have been accorded, with greater justification, to a number of other individuals. Mindful of this, I am deeply grateful to the Society.

My being here is something like the closing of the last arc of a circle. First, my microbiological lineage may be traced directly to

[1]) KLUYVER's expression (1955, p. 503).

Delft and to Professor KLUYVER; I consider myself a second generation product of his. Second, in a number of respects my topic, in its nature, its varied aspects and its scope, embodies the kind of research that KLUYVER himself was especially partial to. And third, Delft is the ancestral home of hydrocarbon microbiology; it was born here over a half century ago.

Superficially, the microbial utilization of hydrocarbons may seem a pedestrian subject. These microbes cause no disease, they have not to do with nucleic acids nor the solution of genetic codes, and they produce no miracle drug. I intend to show, however, that this field is literally a microcosm replete with fascinating, basic problems and novel findings. It is, moreover, one with numerous actual and potential applications. . .

It is not my intention to emphasize practical aspects here. Yet, the implications of that kind are so obvious that some are worth enumerating before we pass to a consideration of more fundamental issues. KLUYVER always had a keen appreciation of the practical applications of microorganisms, and in this case they are not only numerous and diverse, – they impart character to the field. But my real reason for including this feature is somewhat philosophical. It is almost axiomatic that industrial applications exploit basic, pioneering research. I think that in microbiology an excellent case could be made for the reverse of that thesis: a tremendous impetus is imparted to basic science by prior practical discoveries made through expediency, empiricism, or serendipity. Numerous examples could be given of the salutary effects of commercial applications on academic research. Fundamental information pertinent to the practical developments is eagerly sought, students are attracted to the profession, university research gains vigor, and science and the community profit. This is one of the greatest single influences on the course of microbiological science. It is a microbiological truth that needs explicit declaration: a field rich in practical problems is rapidly invaded and taken over by the fundamental scientist.

It is natural that problems of hydrocarbon microbiology should be closely bound up with the petroleum industry. We may mention the long-standing difficulties of bacterial involvement in the corrosion of iron and the attempts to control it; the problems of water pollution and waste disposal from refineries; bacterial release of oil from underground formations; the remarkable claims apropos bacterial methods of prospecting for petroleum deposits; the de-

composition of additives in gasolines; the formation at water-oil interphases of microbial masses which occlude the orifices in jet engines; and the microbial corrosion of aluminum wing fuel tanks in airplanes.

And then, perhaps the most intriguing and exciting prospect of all, the use of pure or mixed hydrocarbons as fermentable substrates. One may, in this connection, visualize three classes of microbial products: (a) conversion products consisting of oxygenated hydrocarbons with no change in the carbon skeletons, (b) oxygenated products with fewer carbon atoms than the substrate, produced by oxidative degradation of a hydrocarbon, (c) biosynthetic products such as amino acids, vitamins, and lipids.

In terms of sheer magnitude, which is the special forte and hence a golden apple tantalizing the petroleum industry, nothing in the fermentation industry would match the potential scale of a process for the conversion of natural gas, or even fuel grade oil, to animal and/or human feed in the form of microbial cells. Microbiological research and bioengineering may yet succeed in making such a process practical, at least under certain conditions in certain parts of the world.

As raw materials for microbial technology, hydrocarbons have some intrinsic advantages: (1) Excepting carbon dioxide and coal, certain hydrocarbons are by far the cheapest fermentable substrates economically available in bulk supply. (Sewage is not considered useful for this purpose). (2) Any change in the hydrocarbon molecule constitutes a weight yield bonus to the extent to which oxygen appears in the molecule. (3) The relative insolubility in water makes concentration influences of hydrocarbons not so critical, and they are ideal candidates for continuous fermentations employing recycling of the substrate.

With these enviable attributes, all that stands in the way of new industrial fermentation processes based on hydrocarbon substrates, is the obtaining of the right organisms to perform the desired tasks economically. Essentially, this is a selection process – known as "screening" – which employs in variable proportions rational and irrational ideas and techniques. It is generally held that screening by itself is insufficient to achieve the desired end, and examples, such as its less than spectacular success in the search for new, useful antibiotics, come readily to hand. Unrewarding experiences of this kind notwithstanding, I have an abiding faith that the principle

of screening is the most powerful – and indispensable – tool that the industrial microbiologist has in his power.

Its limitations lie not in the tool itself but in the skill and imagination with which the microbiologist wields it, and the lengths to which he is willing to go in quest of an elusive quarry. Considering the primacy of screening for industry, the conclusion seems inescapable that basic research on the principles and practices of screening, as contrasted to interminable application of the existing, more or less standard, techniques, would be well justified. It might lead to revolutionary tactics. Combined with modern technology of automation and with microbiological miniaturization, a real breakthrough in the most formidable single obstruction to a vast, new era of industrial fermentations will have been achieved. Give me how to test, and I will produce any organism![1])

I now leave these considerations to the man in industry, and I turn to some fundamental investigations. It is clear that it is essential to restrict this discussion to some selected aspects. I should like to dwell on some aspects revealing the current "flavor" of the subject and which are also indicative of the major trends. I shall not labor you unnecessarily with the historical background. It has been competently compiled, notably by ZoBELL (1950), BEERSTECHER (1954) and FUHS (1961).

ORGANISMS.

Thanks to FUHS (1961) we have a quite complete recent listing of the organisms known to grow at the expense of hydrocarbons. The list comprises over 100 bacteria, yeasts, actinomycetes and fungi, indicating that the capacity to utilize hydrocarbons is widely distributed in those major groups of microorganisms. There are two points which are pertinent here. First, the great majority of the organisms listed had originally been selected for that property: they have been identified *a posteriori*, usually having emerged from hydrocarbon enrichment or selective cultures. Second, having been compiled from numerous widely scattered publications, the list might give an impression that this rich variety of microorganisms is typically obtained in routine isolation experiments. Examination

[1]) A microbiological parody inspired by ARCHIMEDES' famous declaration with reference to the lever: "Give me where to stand, and I will move the earth" (BARTLETT's "Familiar Quotations", p. 29, 1956).

of the original literature reveals that a great many different hydrocarbons were used as isolation substrates for this assemblage As we shall see presently, the range of individual hydrocarbons which any particular organism can utilize for growth is fairly restricted. For this reason, as well as limitations inherent in selective culture procedures, one should not be surprised, therefore, if the variety of organisms obtained in any particular isolation experiment is quite limited. From a rather lengthy experience in Austin there has been in this regard, little danger that "our cup runneth over".

A. S. KESTER in Austin has made an extensive study of the kinds of organisms obtained from soils in experiments in which he employed a considerable number of individual hydrocarbons as sole organic carbon sources; he also varied the environmental conditions within the conventional ranges. From agar selection plates à la WINOGRADSKY the variety was, as expected, greater than that obtained in liquid enrichments but, notwithstanding, it was unexpectedly restricted and, furthermore, always had the same complexion. The organisms typically obtained were more or less taxonomically related, belonging to the genera *Streptomyces*, *Nocardia*, *Mycobacterium*, *Corynebacterium*, and *Brevibacterium*. The predilection of *Mycobacterium* for hydrocarbons has been appreciated since SÖHNGEN's work in 1913, and paraffin is commonly employed for the selective isolation of *Mycobacterium* from soil (BÜTTNER, 1926; HAAG, 1927; GORDON and HAGAN, 1937). Thus, if we were to generalize, we would have to conclude that the above-named groups are those which in nature do the lion's share of the attack on hydrocarbons.

Other organisms, such as those belonging to the genera *Pseudomonas*, *Flavobacterium*, *Achromobacter*, and a few different filamentous fungi, came up as a minority, but so persistently that it provoked us to wonder just how widespread in the microbial world is the capacity to utilize hydrocarbons; and are there organisms in the soil which fail to assert themselves in isolation procedures? For a true assessment of the hydrocarbonoclastic capacities of soil microorganisms, it is essential to test organisms that have been isolated for reasons other than their ability to compete in hydrocarbon enrichment cultures. In other words, how would organisms obtained on non-hydrocarbon media react when confronted with a hydrocarbon as the sole source of organic nutrition? A study of this kind (BUSHNELL and HAAS, 1941; SOLARI *et al.*, 1959) had already shown

that a variety of stock strains of *Pseudomonas* could attack hydrocarbons. This fact, together with a rapid generation time, helps to understand why *Pseudomonas aeruginosa* commonly emerges in hydrocarbon enrichments.

The well known ability of organisms isolated on hydrocarbons to grow at the expense of non-hydrocarbon substrates was likewise found in all of KESTER's isolates; it suggested that the reverse might also be true. Of 110 actinomycetes isolated from soil on a modified CZAPEK's medium and then tested for their ability to grow in a medium composed of *n*-tridecane and mineral salts, seven were clearly positive. *n*-Tridecane was chosen since previous studies indicated that it is, perhaps, one of the most frequently utilized hydrocarbons. Seventy bacterial species in 30 genera, and 28 fungi in 25 genera, all in stock culture collections, were tested in a similar manner. Seventeen per cent of the bacteria and 21 per cent of the fungi tested grew in *n*-tridecane. The bacteria which grew included species of *Alcaligenes, Bacillus, Brevibacterium, Corynebacterium, Flavobacterium, Micrococcus*, and *Pseudomonas*. It is interesting to note that bacteria in those genera are occasionally encountered during isolations from soils, but with the exception of *Pseudomonas fluorescens* and *P. aeruginosa*, the particular species tested by KESTER have not hitherto been suspected of utilizing hydrocarbons.

The fungi which grew were: *Acremonium potronii, Aspergillus alliaceus, Cephalosporium roseum, Colletotrichum atramentarium, Fusarium bulbigenum* and *Monilia bonordenii*. Soil strains of *Fusarium* and *Acremonium* obtained in ethane and propane enrichments have been shown to grow at the expense of the gaseous alkanes, excepting methane (DWORKIN and FOSTER, 1958; KESTER, 1961). Here in the fungi, likewise, an unsuspected variety and frequency of hydrocarbon utilization is revealed.

Even in the genus *Mycobacterium* the capacity of organisms which had been isolated on non-hydrocarbon substrates to develop at the expense of a hydrocarbon is surprisingly high. Of 59 so-called "atypical" mycobacteria freshly isolated from human sputum or gastric specimens on LOWENSTEIN's medium, 87 per cent grew in a *n*-tetradecane-mineral salts medium (LUKINS, 1962).

These experiments can hardly be considered as anything but pilot studies, for they have dealt with a relatively small number of organisms and a single hydrocarbon. But they permit us to conclude that the utilization of hydrocarbons by microorganisms is a property

no longer to be regarded as the province of a few specialist kinds of organisms. Indeed, hydrocarbons might well be added to the list of compounds routinely tested as substrates in classification studies.

A question of interest to the microbial ecologist is the extent to which the organisms recovered by conventional isolation procedures on hydrocarbon media represent the active hydrocarbon decomposers in nature. Or is their appearance merely an indication of the ability of a few to emerge competitively under the particular selective conditions of the culture procedures employed?

Substrate Specificity.

It is hazardous to generalize about hydrocarbon utilization by any particular organism. There is a specificity at least as great with respect to utilization of individual hydrocarbons as exists, for example, in the case of other organisms, for carbohydrates, organic acids, etc. This may be deduced from the more or less haphazard testing which has been reported by numerous authors; it is impressive in the few studies in which a large variety of essentially pure individual hydrocarbons have been tested for utilizability by pure cultures of bacteria. Where sufficient data have been obtained, it is clear that any one organism is capable of utilizing for growth only a small proportion of the hydrocarbons which are available in pure enough form to test, and which are not prohibitively expensive. Despite the restricted extent of the testing done with any one organism, it would be a fair conclusion that an organism will grow at the expense of a group of hydrocarbons closely related to the one hydrocarbon employed to isolate the culture originally.

Furthermore, in general, the normal alkanes containing 10 to 18 carbons are attacked most frequently and rapidly, and they support abundant growth. This is borne out by the data of Lukins (1962) for 21 strains of *Mycobacterium* representing 9 species (Fig. 1). All except strain T were stock cultures, most of which were kindly furnished by Dr. Ruth Gordon of the Rutgers Institute of Microbiology. The preference of all of these cultures for the long-chain compounds is clear. A few strains utilized all of the *n*-alkanes from hexadecane to propane; none utilized ethane or methane. The "T" strain was isolated from isobutane enrichments and it attacked three of the 11 branched-chain alkanes tested whereas the other organisms were ineffectual against these compounds. Only an oc-

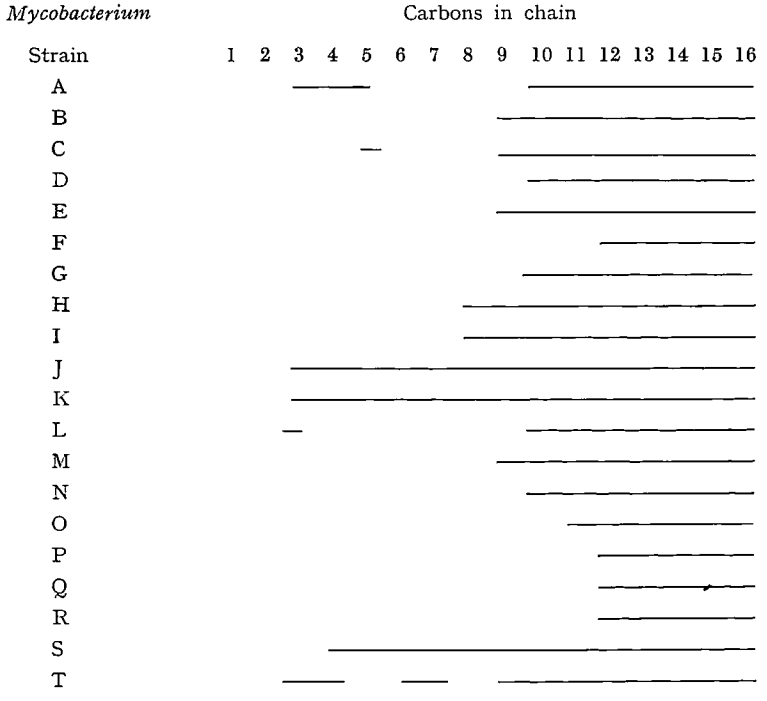

Fig. 1. Growth of stock strains of *Mycobacterium* on *n*-alkanes.

casional positive was recorded for the eight olefins, four cyclics and five benzylsubstituted alkanes also included in the test series.

Gaseous hydrocarbons are not always included, unfortunately, in surveys of this kind and it is difficult to say if organisms isolated on longer chain hydrocarbons are, as a rule, capable of utilizing the gaseous hydrocarbons. As LUKINS (1962) has shown, the propensity of stock cultures of *Mycobacterium* extends in only a few cases to hydrocarbons as small as propane. TAUSZ and DONATH's (1930) isolation from hexane enrichments of a bacterium capable of utilizing all of the gaseous alkanes seems to be exceptional. In any event, only when an organism is isolated in gaseous enrichments can one be certain it will utilize one or more gases. Even then one may find that methane or ethane are not utilized by organisms isolated on propane or ethane (DOSTÁLEK, 1954; BOKOVA, 1954; NECHAEVA, 1949; DAVIS, CHASE and RAYMOND, 1956; KESTER, 1961). Of all

the *n*-alkanes, methane seems to be utilized least frequently by the species that have been tested. In view of the virtually universal bacterial production of methane, this seemed paradoxical. Methane-utilizing specialists are, however, commonly encountered in soils and in muds (SÖHNGEN, 1906; LEADBETTER and FOSTER, 1958; NECHAEVA, 1949). The capacity to assimilate methane carbon implies the possession of more mechanisms than the ability to attack hydrocarbons (KANEDA and ROXBURGH, 1959; LARGE, PEEL and QUAYLE, 1962), a fact which helps explain the limited number of types of methane-utilizing organisms.

Next to organisms belonging to the genus *Mycobacterium*, species of *Pseudomonas* are the best studied organisms in respect to hydrocarbon specificity and, in general, a pattern not unlike that for mycobacteria seems to apply (*e.g.*, KONOVALTSCHIKOFF-MAZOYER and SENEZ, 1956). The behavior of branched-chain hydrocarbons is obviously of great interest in any discussion of substrate specificity. THIJSSE and ZWILLING-DE VRIES (1959) have made the most extensive study of iso-alkane oxidation using a strain of *Pseudomonas aeruginosa* which grew on each of the straight-chain alkanes from C_5 through C_{16}. However, none of four branched hexanes was utilized, only one of eight branched heptanes, and only three of 17 branched octanes. Only compounds containing a single methyl substituent were attacked, and these had to have an unbranched chain at least three or four carbons long. Comparable results were obtained for a second strain of *Pseudomonas* with a series of branched hexadecanes.

Caution must be employed in generalizing from these results, since the organisms studied had been selected on the basis of their ability to attack straight chain alkanes. It would be extremely useful to have as extensive a testing of organisms that have been selected from nature for the primary property of attacking branched alkanes, and particularly to describe the relationship between substrate specificity and isolation substrate. It is obvious that one of the oldest and yet most elementary of the microbiologist's tools – systematic testing of substrate – when applied with imagination is capable of yielding important principles and generalizations.

Before leaving the subject of substrate specificity, a few statistics gleaned from KESTER (1961) are instructive. Twelve different propane isolates were tested for growth in a total of 50 individual hydrocarbons. The relative utilizability of the different groups of hy-

drocarbons is illustrated by the arbitrary scoring summarized in Table 1. The percentages of positives in the last two groups are deceptively high. Most of the positives in column 2 were registered for only four compounds, and one-third of the positives in column 3 were registered for only two compounds.

TABLE 1.

Utilization of individual hydrocarbons arranged in arbitrary groups. (Twelve propane-utilizing organisms).

	15 straight chain alkanes	14 straight chain alkenes (1 double bond)	21 branched alkanes and monocyclics
Number of tests	165	132	240
% Tests positive for growth	80	32	16

As a final point in conjunction with substrate versatility may be mentioned KESTER's (1961) finding that two of the propane isolates, *Corynebacterium* strain 7E1C and *Nocardia* strain 7X8a, made good growth in a series of chloro-alkanes: 1-chloropropane, 1-chlorobutane, 1-chloropentane, 1-chlorohexane, 1-chlorooctane, 1-chlorodecane, 1-chlorohexadecane, and 1-chlorooctadecane. Several 2-chloro-substituted alkanes were not attacked, nor were 1-bromo- or 1-iodopropane. The pathway of breakdown of these compounds and the fate of the chlorine will eventually make an interesting story.

OXIDATION OF NON-GROWTH HYDROCARBONS.

A homologous series of *n*-alkanes consists of very similar compounds, and *a priori* one might not be too surprised if an organism could not tell one of these compounds from another. Nevertheless, as already shown, a striking specificity does exist, and there is posed the problem of how a difference of a single $-CH_2-$group determines utilizability versus non-utilizability of an alkane. An investigation revealed that growth tests may not be a reliable indicator. The first clue to a highly significant finding came from an attempt to understand the remarkable specificity for methane exhibited by a group of bacteria collectively named *Pseudomonas methanica*. These or-

ganisms were unable to grow at the expense of ethane, propane, or butane, but if these gases individually were present, while the organisms were growing at the expense of methane, each of the co-substrates was oxidized (LEADBETTER and FOSTER 1959a; 1960). This technique, which we call "co-oxidation" (FOSTER, 1962), may well prove to be a useful tool for the microbial physiologist concerned with other kinds of substrates. Our discovery has, for example, already been confirmed for the oxidation of alkylbenzenes by *Nocardia* (DAVIS and RAYMOND, 1961) and, in my opinion, co-oxidation provides an important new technique for industrial fermentations. The commercial appeal of the oxidative conversion of a large variety of non-growth hydrocarbons as co-substrates for organisms growing at the expense of cheap substrates, such as methane or natural gas, is obvious. C. E. LOWERY (1962) has shown that co-oxidation very likely takes place during the growth of organisms in complex mixtures of hydrocarbons. For example, growth is made at the expense of certain components of fuel oil, other components being converted to oxidation products.

Our results with *Pseudomonas methanica* naturally led us to re-examine the validity of the specificity charts of the type recorded in Table 1. We concluded that the specificity results do not necessarily imply what they seem to. LUKINS (1962), showed that *Mycobacterium smegmatis* strain 422 is able to grow at the expense of propane as the sole source of carbon, but not methane, ethane, ethylene or propylene. But cells harvested from a propane medium and suspended in buffer were able to oxidize each of the last three of these gases (Fig. 2). In fact, ethane was oxidized at a considerably faster rate than the growth substrate itself. Observations of this kind were made with different organisms having different growth substrate specificities (LUKINS, 1962; KESTER, 1961); there can be little doubt that the capacity to oxidize non-growth substrates is widespread among hydrocarbon-utilizers, at least, and perhaps also in connection with other classes of substrates.

Co-oxidation and oxidation experiments of the foregoing kinds have some rather significant implications for the physiologist. Above all, they mean that the inability to grow at the expense of a particular hydrocarbon is not a consequence only of an organism's inability to attack the substrate; obviously, failure to grow may be due, then, to its inability to assimilate the oxidation products. We will return to this later.

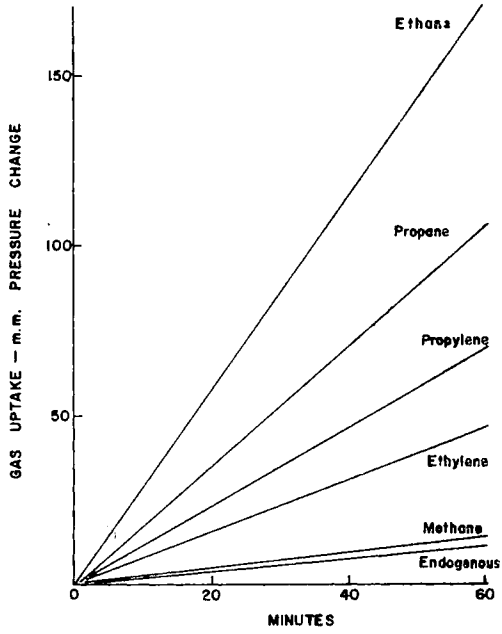

Fig. 2. Oxidation of non-growth substrates by *Mycobacterium smegmatis* 422 (LUKINS, 1962).

Still another explanation exists for the "gaps" in the growth specificity tables, this one being especially useful in rationalizing the refractoriness of alkanes of intermediate carbon chain lengths, *i.e.* C_5 to C_9. Without going into details, I shall simply state that LUKINS (1962) found that *n*-hexane, for example, was decidedly toxic to the growth which *Mycobacterium smegmatis* normally makes on other alkane substrates. Other strains were, on the other hand, able to grow at the expense of *n*-hexane (Fig. 1; see also KONOVALTSCHIKOFF-MAZOYER and SENEZ, 1956).

T. ISHIKURA (1961), in an extensive survey, has found that the intermediate chain length alkanes and alkenes are inhibitory to a number of "garden-variety" bacteria, yeasts, and fungi growing on non-hydrocarbon media. Here, certainly, is an important fallacy in our initial presumption of a physiological equivalence of all the normal alkanes. Other hydrocarbons are also believed to be highly toxic. The mechanism of this toxicity has not been elucidated,

though there are reasons for suspecting an effect on the cytoplasmic membrane.

ISHIKURA's findings may be summarized as follows: (1) olefins were more toxic than the corresponding alkanes, (2) gram-positive bacteria, yeasts and fungi were sensitive to inhibition by olefins of medium carbon chain length, whereas gram-negative bacteria were not, (3) heptene-2 was the most toxic olefin of the eleven tested, (4) the toxicity for species of *Bacillus* (*B. cereus* and *B. megaterium*) was reversed by the presence of 0.3% yeast extract. Among the common individual amino acids, B vitamins and purine-pyrimidine compounds tested, a mixture of L-histidine and DL-methionine proved to be the only compounds with decided capacity to counteract the inhibiting action of heptene-1 for *B. megaterium*.

Earlier we described how failure to grow at the expense of a particular hydrocarbon which is, however, oxidizable had to be interpreted as an inability of the organism to assimilate the oxidation intermediates. This seemed logical since suspensions of *P. methanica*, when furnished primary alcohols as substrates, convert the alcohols almost stoichiometrically to the corresponding fatty acids; further metabolism of these substrates seemed to be blocked. But the answer apparently is not so simple because *P. methanica* can oxidatively metabolize a number of non-growth compounds, non-hydrocarbon and hydrocarbon alike, and use them for the biosynthesis of numerous amino acid components of the cell (LEADBETTER and FOSTER, 1958; 1960). Thus, the question of permeability is eliminated, only to pose the enigma of extensive assimilation and biosynthesis at the expense of compounds which cannot be utilized for total growth. This curious, if not unique, phenomenon should when clarified constitute an intriguing aspect of our knowledge of the factors involved in the orderly, integrated complex of reactions essential for "total growth" as opposed to "partial growth" of the cell.

AN ECOLOGICAL POINT.

We have time here to touch on only one aspect of this topic. Our understanding of the distribution and activities of organisms in nature is, of course, facilitated by knowledge of the physiological potentials of the particular groups. It would seem that in some way or other the occurrence of particular groups in nature would be a

reflection of those physiological potentials. One of the cardinal principles of microbiology is that an increased concentration of food evokes a preferential development of organisms that can use that food. In principle, the presence of abnormal amounts of hydrocarbons will elicit a growth of abnormal numbers of hydrocarbon-utilizing organisms, provided other ordinary requirements for growth are satisfied. Hence, anomalous numbers of hydrocarbon-utilizing organisms theoretically may be used as indicators of anomalous sources of hydrocarbons in nature. This principle provides the basis of microbiological procedures for the exploration of land areas for petroleum deposits (BLAU, 1942; HASSLER, 1943; TAGGART 1946; STRAWINSKI and TORTORICH, 1955). MOGILEVSKII (1959) in Russia has claimed remarkable success for bacterial methods in the discovery of new oil fields. Other studies (DAVIS, RAYMOND and STANLEY, 1959) indicate that this method may be applicable only in special situations. Should employment of this method enable even a small fractional decrease in the number of "dry holes" drilled, this procedure would be of enormous importance.

One would have almost the ideal method for prospecting for petroleum if hydrocarbon-utilizing organisms had a specific requirement for hydrocarbons; but we have already seen that these organisms are remarkably versatile in their substrate ranges, being capable of utilizing a large variety of non-hydrocarbon substrates (FUHS, 1961). In our experience this has been true not only of complex nitrogenous media and carbohydrates, but more particularly for primary alcohols and fatty acids (KONOVALTSCHIKOFF-MAZOYER and SENEZ, 1956; LADD, 1956; KESTER, 1961; LUKINS, 1962). The latter not unexpectedly are found to be utilized by hydrocarbon-oxidizing organisms since, as we shall see presently, they lie on the pathway of oxidation of the hydrocarbons. Even the most "fastidious" hydrocarbon-utilizer known, *P. methanica*, is able to utilize methanol for growth (DWORKIN and FOSTER, 1956). Wherever it has been examined in detail, the capacity to oxidize hydrocarbons has proved to be adaptive (Fig. 3; LUKINS, 1962; also AZOULAY and SENEZ, 1960) and it seems probable that this is universally true. The dual properties of substrate versatility and adaptability to hydrocarbons indicate that hydrocarbon-utilizing organisms could be present in any given location for reasons other than their capacity to utilize hydrocarbons. Long- and short-chain aliphatic primary alcohols and fatty acids are common constituents of plant, animal, and

microbial carcasses. The distribution in nature of hydrocarbon-utilizing organisms which are also omnivorous would, therefore, be influenced by this fact. Even the highly specialized *Pseudomonas methanica* can secure methanol, which occurs as methoxyl groups in one of the most common plant constituents, pectin.

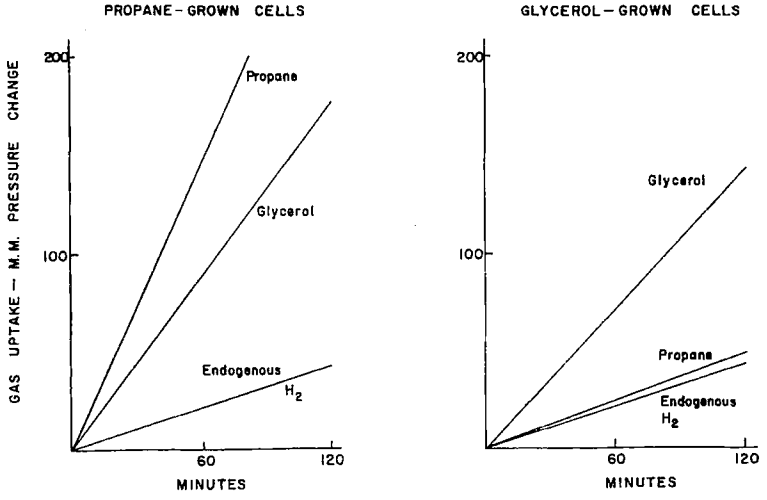

Fig. 3. Adaptive oxidation of propane.

To the foregoing uncertain situation must be added the overriding influence of soil environmental conditions on the qualitative and quantitative population of the soil. Except in very special circumstances, methods of prospecting for petroleum which are based on anomalies in numbers or metabolic activities of hydrocarbon-utilizing microorganisms living in the soil seem to have serious theoretical limitations. Fortunately, however, the microbiologist appears to be just as adaptable as his quarry, and a microbial tool for oil prospecting based on the growth of hydrocarbon-utilizing bacteria in culture vessels placed in bore holes (SANDERSON, 1942) seems to be theoretically sound. Measurable growth of pure cultures of bacteria develops in a mineral salts solution in atmospheres containing minute concentrations of gaseous alkanes. In essence, the bacteria are sensitive indicators of the presence of gaseous hydrocarbons. DOSTÁLEK and SPURNY (1962) have shown that ethane-

and propane-utilizing bacteria can multiply in concentrations of these gases as low as 10^{-5} per cent (v/v), and they have "mapped" regions in Czechoslovakia by this method. Methane-utilizing bacteria were not useful, because of the practically universal formation of this gas by the anaerobic methane-producing bacteria (BARKER, 1955). As DOSTÁLEK and SPURNY have pointed out, the bacteria may detect hydrocarbons in the soil gas phase, but they cannot tell where the gas seeps originate. In fact, in their particular survey, regions of maximal multiplication of the indicator bacteria lay beyond the petroleum deposits, and bacterial minima lay above the deposits. This condition probably is a consequence of the underlying geology, and of migration of the gases from the points of origin in the formation to the surface along open faults. ". . . The interpretation of results of geomicrobiological superficial prospection work would be unreliable without a knowledge of the geological structure of the area" (DOSTÁLEK and SPURNY, 1962, p. 147).

HYDROGEN AUTOTROPHY IN HYDROCARBON-UTILIZING BACTERIA.

TAUSZ and DONATH in 1930 isolated two ethane-utilizing bacteria which they found were capable of existing autotrophically on hydrogen and carbon dioxide. Subsequently, several hydrocarbon-utilizing mycobacteria and one gram-negative coccus also proved to be hydrogen autotrophs (BELYAEVA, 1954; DWORKIN and FOSTER, 1956) and the assumption, first made by TAUSZ and DONATH, of a possible relation between the dual capacities of hydrogen and hydrocarbon utilization, did not seem unreasonable. An analogy to the established relations between hydrogenase and nitrogenase in nitrogen-fixing bacteria could be imagined. LUKINS (1962) has studied this problem in considerable detail employing growth, respiration, and the deuterium exchange reaction in various experiments. In contrast to the previous experiments with organisms originally isolated from soil as hydrocarbon-utilizers, LUKINS tested a series of mycobacteria from stock cultures. If one eliminates the strains of *M. tuberculosis* (which were negative), four (maybe five) out of the total of sixteen mycobacteria, all hydrocarbon-utilizing saprophytes, proved to be hydrogen autotrophs (Table 2). This is a surprisingly high incidence of hydrogen autotrophy; it also exemplifies a rare instance of chemo-autotrophy in organisms not previously selected for that property.

TABLE 2.

Hydrogen autotrophic mycobacteria.

M.fortuitum	strain	389
M.marinum	,,	905
*M.*sp.	,,	"tap-water"
*M.*sp.	,,	8A
M.smegmatis	,,	446 (probable)
Hydrogenase only:		
M.smegmatis	strain	422

A search of the literature has uncovered but one precedent, whose salient characteristic is eloquently epitomized in the following passage that might just as well apply to LUKINS' mycobacteria:

> "How strongly the potentiality to produce these enzymes is fixed in the genetic apparatus of the organism is demonstrated in a most convincing way by the behavior of BEIJERINCK's original culture, which, maintained for forty years – that is, for thousands of generations – on peptone agar, on being transferred to an environment where molecular hydrogen is the only energy source available, answers nature's challenge by the brave device: Here I am, I can also act differently!"

The organism: *Micrococcus denitrificans*; the place of its original isolation: Delft; the place of its recent recognition as an autotroph: Delft; the literature source: Life's Flexibility, in "The Microbe's Contribution to Biology" (page 107); the year: 1956; the author: A. J. KLUYVER.

The incidence of hydrogen autotrophy in hydrocarbon-utilizing bacteria is much greater than one would expect from a random sampling of bacteria, and a putative relation between these two properties in bacteria possessing both seems real enough, at least as far as the testing has gone. It is probable that the incidence already found is minimal; for example, some hydrocarbon-utilizers which fail to grow autotrophically may nevertheless, possess hydrogenase. Two of the four obligately heterotrophic strains which LUKINS (1962) tested in manometric and deuterium exchange experiments did in fact support this contention; furthermore, had the experiments been done with cells pre-exposed (adapted) to hydrogen, hydrogenase might have been detected in a still larger fraction of the strains.

Now there remained to do the reverse type of experiment. If the relation between hydrocarbon- and hydrogen-utilization is indeed a biochemical one, bacteria selected originally for their possession of hydrogenase would attack hydrocarbons. LUKINS isolated ten different hydrogen autotrophic soil bacteria divisible into five different groups according to cultural and morphological characteristics. To these were added an authentic strain of *Hydrogenomonas ruhlandii* (obtained from Dr. W. VISHNIAC) and four other strains of *Hydrogenomonas* (obtained from Dr. C. BOVELL, Jr.). The fifteen cultures were tested for their ability to grow at the expense of eight different individual hydrocarbons. Only two of the soil isolates grew in this experiment, both in decane and tridecane, and both organisms proved to be mycobacteria. None of the typical gram-negative species of *Hydrogenomonas* grew in any of the hydrocarbons, and serious efforts to adapt several of the strains and to detect hydrocarbon oxidation manometrically, were fruitless. Also, several strains of *Azotobacter*, known for its high hydrogenase activity, failed to give any positive indication of utilization of hydrocarbons when tested in suitable experiments.

The negative results with *Hydrogenomonas* put an end to the idea of a biochemical relation between hydrogenase and "hydrocarbonase", but we are still left with the intriguing question of the significance of hydrogen oxidation in hydrocarbon-utilizing bacteria. Although this relation has been found most commonly in the mycobacteria, it has been observed in enough non-mycobacterial forms to imply that the relation is more one associated with hydrocarbon metabolism rather than with mycobacteria as such. Undoubtedly, an interesting evolutionary history lies therein, speculation on which is not fitting here.

OXIDATIVE PATHWAYS.

The oxidation of hydrocarbons has mushroomed into such a comprehensive subject that in the time available I am compelled to dwell on selected issues. I intend to discuss here only the oxidation of aliphatic hydrocarbons, since most of our experience in Austin deals with these compounds. In all probability principles revealed for them are applicable to various kinds of cyclic hydrocarbons. Recent summaries of information on the latter compounds are available (DAGLEY, EVANS and RIBBONS, 1960; FOSTER, 1962).

Whereas the term "oxidation of hydrocarbons" connotes a special, characteristic mode of metabolism, this idea is a considerable exaggeration, for the only thing typical of oxidation of a hydrocarbon is the breaching of the molecule with oxygen, an action involving the participation of a metal, DPNH, and probably only two enzymes (GHOLSON and COON, 1960; COON, 1962). Once oxygenated, the molecule undergoes subsequent oxidative pathways that are, strictly speaking, no longer unique to hydrocarbons and which actually behave more or less as expected according to conventional comparative biochemistry.

In spite of this, some interesting, unexpected pathways have emerged from a study of the utilization of these substrates – more likely the consequence of selection of new and different types of bacteria through their ability to grow on hydrocarbons, than a uniqueness of hydrocarbon pathways themselves.

It now seems conclusively established that some alkanes and alkenes are oxidized by reactions whereby molecular oxygen is incorporated into the substrate molecules. In appropriate experiments isotopic oxygen has been found both in the oxidative conversion products (STEWART *et al.*, 1959; KESTER, 1961; ISHIKURA and FOSTER, 1961) and in the cells of bacteria growing at the expense of hydrocarbons as compared to non-hydrocarbon substrates (LEADBETTER and FOSTER, 1959b). At the moment, very little is known of the details of the incorporation mechanism, and for our purpose it is sufficient merely to illustrate the types of reactions that have figured prominently in the speculations on this subject (Fig. 4).

Hydroperoxide (KALLIO)

$$R \cdot CH_2 \cdot CH_3 \xrightarrow{O_2} R \cdot CH_2 \cdot \overset{O\vdots OH}{\underset{|}{CH_2}} \xrightarrow[-HOH]{+2H} R \cdot CH_2 \cdot \overset{OH}{\underset{|}{CH_2}}$$

Mixed function oxidase (MASON; COON; FOSTER)

$$R \cdot CH_2 \cdot CH_3 + O_2 + DPNH \xrightarrow{+H^+} R \cdot CH_2 \cdot \overset{OH}{\underset{|}{CH_2}} + DPN + H_2O$$

Dehydrogenation (SENEZ)

$$R \cdot CH_2 \cdot CH_3 + DPN \longrightarrow RCH = CH_2 + DPNH + H^+$$

Fig. 4. Proposed oxidation mechanisms.

Less conclusive at this time is the evidence for a second mode of attack on hydrocarbons, a non-oxygenative, dehydrogenative attack on the paraffin molecule, presumably yielding the corresponding olefin with a double bond in the 1,2-position. SENEZ in Marseilles currently is the most active protagonist of this view; his laboratory has amassed a considerable amount of supportive evidence for the anaerobic dehydrogenation of *n*-heptane by a strain of *Pseudomonas aeruginosa* (SENEZ and AZOULAY, 1961), and credulity for this mechanism only awaits a clinching identification of the olefin formed in the reaction. [1])

The establishment of the dehydrogenative reaction would ordain a second new reaction. Whereas a primary alcohol is the most common first, stable product of the oxidation of alkanes (AZOULAY and SENEZ, 1960; FOSTER, 1962), the only instances in which the biochemical attack of the olefinic double bond have been recognizable, namely, in hexadecene-1 and octadecene-1, saturation by dihydroxylation took place (Fig. 5; BRUYN, 1954; STEWART *et al.*, 1960; ISHIKURA and FOSTER, 1961). Thus, if an olefin is produced as an intermediate in the bacterial oxidation of heptane we may confidently predict a new reaction(s) for its further conversion to the corresponding primary alcohol. In appropriate isotopic experiments, attempts to demonstrate a dehydrogenative attack of ethane and propane by other bacteria have been unsuccessful (LEADBETTER and FOSTER, 1960; LUKINS, 1962).

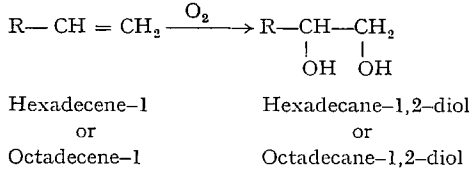

Fig. 5. Dihydroxylation of the olefinic double bond.

[1]) Soon after this manuscript was submitted, SENEZ' laboratory published infrared absorption spectra of carbon tetrachloride extracts of suspensions of *Pseudomonas aeruginosa* which had been exposed to *n*-heptane under anaerobic conditions. Two absorption maxima were noted similar to those in *n*-heptene-1 (J. CHOUTEAU, E. AZOULAY and J. C. SENEZ, Nature **194**, 577, 1962).

Monoterminal Oxidation.

Several investigators (Webley, Duff, and Farmer, 1956; Heringa, Huybregtse and van der Linden, 1961; Stewart *et al.*, 1959; Leadbetter and Foster, 1960; Proctor, 1960; Heydeman, 1960; Gholson and Coon, 1960; Azoulay and Senez, 1960) have conclusively established, even for mammals (Berhard, Bloor and Scheitlin, 1952), that the most common, if not the exclusive, pathway of oxidation of the alkane chain is in the sequence depicted, in its simplest form, in Fig. 6. The attack on a terminal methyl group we refer to as "monoterminal" oxidation.

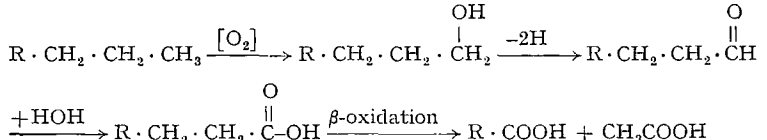

Fig. 6. Monoterminal and β-oxidation of alkane chains.

The pathways which have cropped up in different bacteria prove to be deviations of the unitary scheme in Fig. 6. I wish to discuss the three most important ones.

(1) Ester formation. An extraordinary gram-negative coccus grows at the expense of individual long-chain paraffins and synthesizes appreciable quantities (approximately 25% w/w) of long-chain esters (Stewart and Kallio, 1959). The alcohol moiety of these waxes has the same carbon skeleton as the substrate paraffin, while the acid moiety is typically palmitic acid. Based on Stewart and Kallio's findings, Fig. 7 illustrates the distinctive pathways of this system.

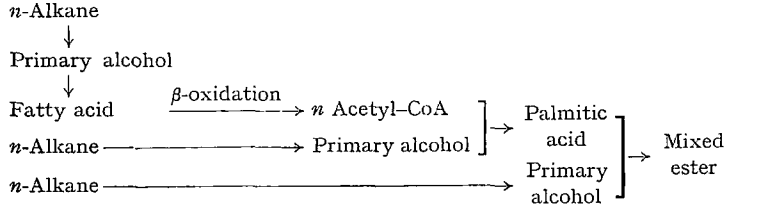

Fig. 7. Wax synthesis (Stewart and Kallio, 1959).

(2) Diterminal oxidation. In the case of long-chain aliphatics there is clear evidence that a substitution confers a character to these compounds enabling an organism to attack selectively a particular end of the molecule. The best example of this is the 1-olefins. The selective attack at the double bond by the yeast *Candida lipolytica*, yielding the corresponding 1,2-diol (BRUYN, 1954), has been described above (Fig. 5). ISHIKURA (1961) obtained evidence that a similar attack takes place in a bacterium he isolated from soil (see Fig. 11). KALLIO's wax bacterium, on the other hand, selectively oxidizes, initially at least, the saturated end of the molecule (STEWART *et al.*, 1960).

The situation where all of the terminal carbons are methyl groups *e.g.*, the branched-chain alkanes, raises the interesting question as to how keenly an organism can distinguish one methyl group from the others. THIJSSE and VAN DER LINDEN have shown that *Pseudomonas aeruginosa* (KSLA strain 473) can, indeed, selectively attack different ends of 2-methylhexane, but only one end per individual molecule. The principal pathway employed by the bacterium was the terminal oxidation of the long alkyl chain, yielding 5-methylhexanoic acid. Another fraction of the 2-methylhexane was terminally oxidized at the shortest alkyl branch, a methyl group, so that the culture filtrates contained a mixture of 5-methyl- and 2-methylhexanoic acids (Fig. 8). One may say, therefore, that this organism cannot make an absolute discrimination between one end of the isoalkane molecule and the other, except that after it attacks one end, it obviously has no taste for the other.

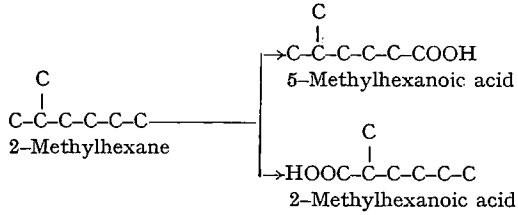

Fig. 8. Oxidation of 2-methylhexane (THIJSSE and VAN DER LINDEN, 1961).

KESTER has now discovered bacteria having either a short memory or an indiscriminate palate – they cannot tell one end of certain *n*-alkanes from the other (1961; KESTER and FOSTER, 1960). These soil isolates oxidize both ends of the same molecule (diterminal oxi-

dation), but sequentially, so that basically the mechanism is consistent with the monoterminal oxidation found in all of the previously described instances of alkane oxidation.

One gram-positive, rod-shaped bacterium (strain 7E1C) was studied in detail. This organism, tentatively identified as belonging to the genus *Corynebacterium*, grows well on all of the individual *n*-alkanes from propane to hexadecane. From the decane through the tetradecane cultures inclusive, KESTER isolated in crystalline form and identified the corresponding dioic acids (Table 3).

TABLE 3.

Dioic acids from *n*-alkanes (KESTER, 1961).

Substrate	Acid product	Formula
Decane (C_{10})	1,10–Decanedioic	$HOOC(CH_2)_8$ COOH
Undecane (C_{11})	1,11–Undecanedioic	$HOOC(CH_2)_9$ COOH
Dodecane (C_{12})	1,12–Dodecanedioic	$HOOC(CH_2)_{10}COOH$
Tridecane (C_{13})	1,13–Tridecanedioic	$HOOC(CH_2)_{11}COOH$
Tetradecane (C_{14})	1,14–Tetradecanedioic	$HOOC(CH_2)_{12}COOH$

Alkanes exceeding 14 carbons in chain length were not tested; it is probable, though, that at least the next higher members of this series would yield the expected diacids. On the other hand, KESTER was unsuccessful in his attempts to demonstrate diterminal oxidation products in cultures in which the organism has grown at the expense of individual *n*-alkanes from propane through nonane, respectively. This may only mean that the diacid did not accumulate in amounts detectable by the methods used (paper chromatography). Credence may, however, be given to diterminal oxidation of smaller chain length alkanes; Dr. T. S. ROBINSON in Manchester has recently obtained preliminary evidence (column and paper chromatography, melting point and mixed melting point) that suberic acid (1,8-octanedioic acid) accumulates in cultures of a soil bacterium growing at the expense of *n*-octane as the sole source of carbon and energy (ROBINSON and HALL, 1962).

KESTER was also able to isolate several other interesting compounds from long-chain alkane cultures (Table 4). The identity of these compounds suggests a probable course of events in the dodecane cultures (Fig. 9).

Support for this postulated sequence was provided by KESTER. He added C^{14}-labeled decanoic acid to a decane grown culture in

TABLE 4.

Other acids from long-chain alkanes (KESTER).

Substrate	Product identified	Formula
n-Decane	10–Hydroxydecanoic acid	$CH_2(OH)\cdot(CH_2)_8\cdot COOH$
n-Dodecane	11–Formylundecanoic acid	$CH(=O)\cdot(CH_2)_{10}\cdot COOH$
	12–Hydroxydodecanoic acid	$CH_2(OH)\cdot(CH_2)_{10}\cdot COOH$
	3–Hydroxydodecanoic acid	$CH_3\cdot(CH_2)_8\cdot C(=O)\cdot CH_2\cdot COOH$

its early stages; after further incubation, unlabeled carrier 10-hydroxydecanoic and 1,10-decanedioic acids were added. Following appropriate purification procedures both products contained significant radioactivity. These experiments constitute important evidence for ω-oxidation of fatty acids. Whereas this has been well established for mammals (DEUEL, 1957; ROBBINS, 1961), we are unaware of any previous example of microbial ω-oxidation.

Alkane → Primary Alcohol → Fatty acid →
12–Hydroxydodecanoic acid → 11–Formylundecanoic acid →
1,12–Dodecanedioic acid.

Fig. 9. Probable sequence in dodecane cultures.

KESTER also addressed himself to the question of the importance of the ω-oxidation (diacid) pathway in *Corynebacterium* strain 7E1C. A limited qualitative survey of a number of pure cultures of other bacteria indicated that although a small proportion of the organisms tested produced diacids, such organisms apparently are not rare or even uncommon. More sensitive methods may enlarge the incidence. Furthermore, inasmuch as the diacids can be used as sources of carbon and energy for the growth of many of these bacteria, it is at least possible that they may be formed and transformed as transient intermediates in the oxidation of hydrocarbons, without accumulating in isolatable amounts.

(3) Carbon chain degradation. In addition to the long-chain acids with the unbroken carbon chains, *Corynebacterium* strain 7E1C also produces considerable acetic acid, evidence of β-oxidation. Since the organism makes abundant growth at the expense of the individual long-chain alkanes, it obviously possesses means of oxidative degradation of the carbon chains. It would be worth-while to know if β-oxidation takes place at the fatty acid stage (decanoic acid) or at the diacid stage (1,10-decanedioic acid) or both. Probably some of both occurs, but there is one indication that the bulk of the β-oxidation and chain breakdown takes place at the fatty acid stage, before the latter undergoes ω-oxidation. This deduction is based on the formation of acetic acid as the only volatile acid when strain 7E1C utilizes alkanes with even-numbered carbon chains, and a mixture of acetic and propionic acids when the organism utilizes alkanes with odd-numbered carbon chains. These data are what one would expect from β-oxidation at the fatty acid stage (Fig. 10). One cannot, however, rule out the formation of short-chain dicarboxylic acids (oxalic, succinic, malonic), which would be expected, along with acetic acid, from the oxidation of even- and odd-numbered long-chain diacids. The point awaits further evidence.

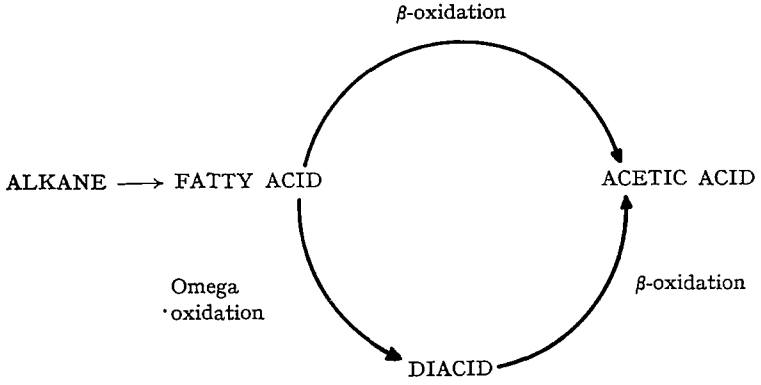

Fig. 10. β-Oxidation at the fatty acid stage.

In any event, there seems to be general agreement regarding β-oxidative breakdown of the alkane chain (WEBLEY, DUFF and FARMER, 1956; AZOULAY and SENEZ, 1960; HERINGA, HUYBREGTSE and VAN DER LINDEN, 1961; THIJSSE and VAN DER LINDEN, 1961).

An interesting suggestion (TRECANNI, CANONICA and DE GIROLAMO, 1955; SENEZ and KONOVALTSCHIKOFF-MAZOYER, 1956) that the fatty acids formed in alkane oxidation may in some cases be degraded by α-oxidation, requires more definitive evidence. This mechanism, which involves successive oxidative decarboxylations of a continuous series of saturated fatty acids, is known to occur in plant tissues (STUMPF, 1956). ISHIKURA (1961) suggests that the oxidation of 1-olefins by his soil bacterium involves an oxidative decarboxylation as the first stage only, followed by degradation of the remaining part of the fatty acid chain by β-oxidation. From the oxidation of octadecene-1, ISHIKURA isolated three odd carbon-numbered fatty acids, propionic, valeric, and heptanoic acids, whose origins are explained in Fig. 11.

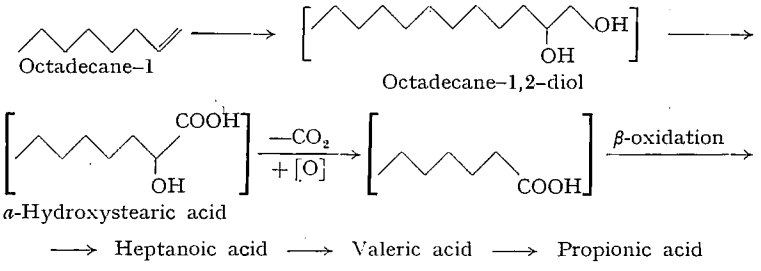

Fig. 11. Olefin degradation (ISHIKURA, 1961).

It should be noted that, strictly speaking, this would not be a case of β-oxidation; the presumed key α-hydroxyacid intermediate is formed from a dihydroxylation of the double bond of the substrate olefin. Likewise, the α-ketoacid probably formed via initial methyl ketone formation and oxidation (described later), while yielding an α-keto acid, involves reactions different from those connoted by α-oxidation of a saturated fatty acid. In both instances, further carbon chain breakdown presumably involves β-oxidation. Thus, α-oxidation certainly occurs in hydrocarbon oxidation, but the evidence is best for its occurring as an initial "preparatory" reaction; α-oxidation of successively formed fatty acids as a mode of degradation of long-chain alkyl groups is a basically different thing, and it will be interesting to see if it actually does occur in some hydrocarbon-oxidizing bacteria.

(4) Methyl Ketones. We have seen that the usual site of

initial attack of the aliphatic, paraffinic molecule is at one of the terminal methyl carbons, yielding consecutively a primary alcohol, aldehyde and fatty acid. Consequently, of considerable interest was the discovery of products oxygenated by *Pseudomonas methanica* in the 2 (or α-) position, *i.e.*, methyl ketones, along with products oxidized in the terminal (1-) position, both with no disruption of the carbon chains (LEADBETTER and FOSTER, 1960). At that time, the idea of both products being manifestations of one free radical mechanism of oxidation of hydrocarbon molecules seemed preferable to invoking two independent, simultaneously functioning mechanisms; since then there has been no reason for shifting that view.

LUKINS (1962) has made an extensive study of methyl ketone metabolism in hydrocarbon-utilizing bacteria, mostly mycobacteria. Each of a considerable number of bacteria, actinomycetes, and filamentous fungi (LOWERY, 1962) capable of utilizing propane or *n*-butane have, when carefully examined, been found to produce 2-propanone (acetone) or 2-butanone (methylethyl ketone), respectively. 2-Pentanone and 2-hexanone were produced by *Mycobacterium smegmatis* 422 from *n*-pentane and *n*-hexane, respectively. Despite assiduous attempts to isolate methyl ketones from the bacterial utilization of the *n*-alkanes with seven or more carbon atoms, the results have been consistently negative. Are the methyl ketones produced only from the gaseous hydrocarbons (propane through hexane), or are they also produced as intermediates from the longer chain hydrocarbons (heptane and up) but elude detection? A clue may lie in the detection of significant radioactivity trapped by carrier (unlabeled) 2-undecanone present during the oxidation of *n*-undecane-1-C^{14} by *M. smegmatis* 422. Purification of the 2:4-dinitrophenylhydrazone derivative by partition column chromatography and repeated paper chromatography in different solvent systems failed to separate the radioactivity from the yellow hydrazone. Pending absolute identification, we therefore incline to the view that methyl ketones can be formed from long-chain alkanes.

With this background, it came as no surprise to us that various species of mycobacteria known to utilize gaseous hydrocarbons will also utilize methyl ketones as sole sources of carbon and energy. The specificity pattern was reminiscent of the hydrocarbons: the short- and the long-chain methyl ketones were attacked, but not those of intermediate chain length. The extent to which toxicity may be a factor here was not examined. A noteworthy observation

may be made about 2-pentanone, which was readily used for growth by all three organisms tested. Shift of the carbonyl group to the 3-position or introduction of a second carbonyl group (2:4-pentadione) made these compounds useless as growth substrates.

Just as in the case of hydrocarbons, however, substrate specificity conclusions derived from growth experiments proved to be misleading. Washed cell suspensions of *M. smegmatis* grown on propane readily oxidized all eight methyl ketones which could not serve as solitary growth substrates (Table 5). Once again we see evidence of the fundamental importance of distinguishing between the capacity of an organism to make the primary attack on a substrate and to assimilate for growth the products of the oxidation. Interesting questions arise relative to the enzyme(s) involved in these oxidations, but they are not germane to this discussion.

TABLE 5.

Non-growth ketones oxidized by *Mycobacterium smegmatis* 422 (LUKINS, 1962).

3–Pentanone
2:4–Pentadione
2–Hexanone
3–Heptanone
4–Heptanone
2–Octanone
Acetophenone

LUKINS also studied the response to hydrocarbon substrates of bacteria that had been isolated from soil on methyl ketone substrates. All four strains isolated on a long-chain ketone (2-undecanone) grew with long-chain hydrocarbon substrates, but not the short-chain alkanes, whereas four other strains isolated on short-chain ketones (2-butanone) grew on short- or long-chain hydrocarbons. A parallel to the relation between isolation substrate and substrate specificity for growth described on page 247 may be noted.

The foregoing results shed no light on the role of methyl ketones formed during the oxidation of gaseous alkanes. Are they intermediates in the oxidation and assimilation of alkanes, or are they the useless products of side reactions? LUKINS tackled this question by means of the technique of simultaneous adaptation. If the methyl ketone were an intermediate, cells adapted to the oxidation of a

particular alkane should also be capable of oxidizing the corresponding methyl ketone. Conversely, cells unadapted to alkane oxidation should be ineffectual on the methyl ketones. The data precisely fit these premises (Fig. 12). The cells grown on propane were simultaneously adapted to the attack of propane, acetone, and *n*-propanol (the homologous non-hydrocarbon substrate). Results essentially no different were obtained with acetone-grown cells. Propanol-grown cells were, on the other hand, devoid of activity towards propane or acetone. The endogenous O_2 uptake was negligible.

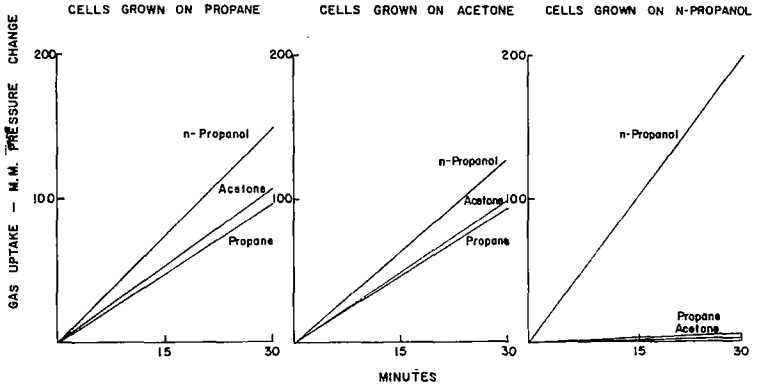

Fig. 12. Adaptation patterns in acetone oxidation.

The above results support the idea that methyl ketones are intermediates in (gaseous) alkane oxidation, but by the same token they could be construed to mean that propane is an intermediate in the oxidation of acetone! This conclusion is patently intolerable, and yet it is clear from this and other experiments that methyl ketone-grown cells are invariably back-adapted to hydrocarbons. Formulating his concept of simultaneous adaptation, STANIER (1947) anticipated the existence of situations of this kind which he interpreted as the consequence of reversibility of the reactions by which the intermediates are formed. Another, simpler explanation may apply in some cases: the attack of different substrates by the same enzyme(s). As we shall see presently, the primary attack on both the alkane and the methyl ketone is in reality the attack of a terminal methyl group.

Only enzymological experiments can clarify the foregoing point,

and we need not belabor it further, except to mention the additional coincidence that the primary oxidation of methyl ketones, like the alkanes, involves an oxygenase-type reaction with the incorporation of molecular oxygen. A further correspondence is noted by LUKINS' isolation of the primary alcohol derivative of a methyl ketone after oxidation of the latter by *M. smegmatis, i.e.*, propane-α-ol-β-one (acetol) from acetone (Fig. 13).

$$\underset{\text{Acetone}}{CH_3COCH_3} \xrightarrow{O_2} \underset{\text{Acetol}}{CH_3COCH_2OH}$$

Fig. 13. Formation of acetol from acetone (LUKINS, 1962).

Acetol had previously been suggested as a bacterial oxidation product of acetone (LEVINE and KRAMPITZ, 1952).

The metabolism of methyl ketones adds a new dimension to hydrocarbon metabolism. Unfortunately, LUKINS was not able to identify the intermediate product in the oxidation of methyl ethyl ketone, or of any longer chain methyl ketones. We are, thus, left with the most interesting question: Is the methyl or the alkyl end of an asymmetrical methyl ketone attacked first? Also requiring study is the extent to which methyl ketones are produced from the long-chain hydrocarbons, and the fraction of the substrate passing through the methyl ketone pathway during the oxidation of short-chain alkanes.

Before our experiments with oxygen-18, it seemed unlikely that the enzymatic attack of methyl ketones would be of an oxygenase type. Whereas oxygenation is not an unexpected mode of initiating the attack on a hydrocarbon molecule, with consequent enhancement of water solubility, the further oxygenation of a molecule already oxygenated was unexpected. We thus concluded that in LUKINS' system the carbonyl group was not the locus of the primary attack on acetone. That leaves the alkyl groups as the only remaining possibility, and the matter is one of attack of a terminal methyl group, just as it is with alkanes. Seen in this light, the parallelism of the oxygenase reactions is quite comprehensible. Until careful work is done with isolated enzyme systems, we shall not know whether the terminal methyl groups of methyl ketones and *n*-alkanes are, in fact, attacked by the same enzyme system(s) or whether the two classes of substrates require enzymes of different

specificities. These compounds may be considered as analogues (Fig. 14). Regardless of which alternative applies, a corollary seems to emerge: saturated compounds whose terminal carbons are methyl groups are enzymatically oxygenated in one of the methyl groups.

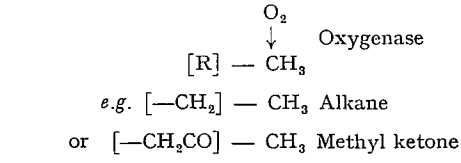

Fig. 14. Analogy of terminal methyl carbon oxidation of alkanes and methyl ketones.

The entire matter of specificity of the enzymes attacking hydrocarbons is a beguiling one, apart from the water-insoluble, hydrophobic natures of such substrates. The large number of individual hydrocarbons and methyl ketones which any one organism can attack, the ubiquity and abundance of hydrocarbon-utilizing organisms in nature, and the common occurrence of the capacity to attack hydrocarbons in stock cultures of organisms presumably out of contact with the hydrocarbon for innumerable microbial generations, lead one to wonder if enzyme specificity for substrates is as great here as it is generally for other enzymes, and if hydrocarbon oxygenases are enzymes normally acting on non-hydrocarbon substrates in the intermediary metabolism of the cell.

Epilogue.

From this cursory and admittedly incomplete survey of hydrocarbon utilization by microorganisms, I think it is obvious, as mentioned at the beginning of this lecture, that the subject does indeed embody elements of general microbiology which Kluyver espoused and enjoyed so much. Where it is possible to codify information on the subject, that task has been made simple by the teachings of Kluyver. And that the seemingly diverse types of attack of hydrocarbon alkyl groups should assort themselves into an intelligible, unitary mode of hydrocarbon attack and degradation, is a superb fulfillment of the Kluyverian concept of metabolism.

> "Hence I want to express the hope that you will grant the microbiologist his fairy tale; for such tales derive their value from

their nature as working hypotheses, and as such they inspire the investigations that will increase man's comprehension of nature" (KLUYVER, 1955).

Acknowledgments.

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