EXPERIMENTS WITH SOME MICROORGANISMS WHICH UTILIZE ETHANE AND HYDROGEN[1]

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An interesting case of extreme specificity of enrichment and selective culture procedures has recently been described for methane-utilizing bacteria (Leadbetter and Foster, 1958). Obligate methane-utilizing pseudomonads (*Pseudomonas methanica*) invariably were obtained from inocula from a great many natural sources. A further degree of specificity within the group was encountered; it was found that emergence of particular pigmented varieties, to the exclusion of others, can be predicted if the enrichment and isolation procedures follow a set pattern. Almost as surprising was the ineffectuality of those procedures in evoking the appearance of nonexacting methane-utilizing bacteria of the type that can also grow at the expense of other hydrocarbon and nonhydrocarbon substrates (Aiyer, 1920; Bokova *et al.*, 1947; Brown and Strawinski, 1957; Hutton and ZoBell, 1949; Kaprálek, 1954; Münz, 1915; Nechaeva, 1949; Slavnina, 1948; Tausz and Donath, 1930).

This paper documents an additional kind of unusual specificity in connection with gaseous hydrocarbon-utilizing bacteria. Three significant findings reported are (1) the use of natural gas (in which methane is by far the major component) as a substrate in enrichment cultures led to the isolation of many different ethane-utilizing organisms, but none capable of utilizing methane as the sole hydrocarbon for growth; (2) a majority of the ethane-utilizing bacteria proved to be facultative autotrophs capable of developing at the expense of hydrogen gas and carbon dioxide as the respective sole sources of energy and carbon. A preliminary note on this subject has already been published (Dworkin and Foster, 1957); and (3) a mold capable of growing at the expense of ethane as the sole organic nutrient is described. At the time this mold was first reported (Foster, 1955) it was apparently the first instance of utilization of gaseous paraffinic hydrocarbons in the true fungi, but at least one other case is now known (Davis *et al.*, 1956).

Finally, problems in the taxonomy of hydrocarbon- and hydrogen-utilizing bacteria are discussed.

MATERIALS AND METHODS

Natural materials used as sources of inoculum included raw sewage and soils from cultivated garden beds and from the proximity of oil deposits. The basal medium employed routinely had the following composition: $(NH_4)_2SO_4$, 2.0 g; KH_2PO_4, 4.0 g; Na_2HPO_4, 6.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 1.0 mg; B (as H_3BO_3), 10 μg; Mn (as $MnSO_4$), 10 μg; Zn (as $ZnSO_4$), 70 μg; Cu (as $CuSO_4$), 50 μg; Mo (as MoO_3), 10 μg; distilled water, 1 L. Solid media were obtained by the addition of 20 g of water-washed agar to the above salts solution.

Isolation of methane-utilizing bacteria was our original goal. Toward that end, and because pure methane was not at hand at the time, we elected to use commercial natural gas as the methane source. As seen from the composition given below,[3] methane was by far the preponderant hydrocarbon in the particular natural gas, representing a molar concentration 15.4 times greater than ethane, the next most abundant component. On a weight basis the relative abundance of methane and ethane was 8.25.

All physiological studies on pure cultures of the hydrocarbon-utilizing organisms were conducted with purified gases. "Pure Grade" methane,

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[3] We are indebted to the United Gas Company of Texas for the following "typical" analysis: volume per cent, CO_2, 2.07; N_2, 1.05; methane, 86.92; ethane, 5.63; propane, 2.61; *iso*-butane, 0.38; *n*-butane, 0.59; *iso*-pentane, 0.23; *n*-pentane, 0.17; hexanes, 0.35.

ethane and ethylene obtained from Phillips Petroleum Company, Bartlesville, Oklahoma, contained at least 99 mole per cent of the respective gases. Hydrogen gas, 99.85 mole per cent, was obtained from Champion Paper Co., Houston, Texas, and the carbon dioxide was "Bone-dry" grade, obtained from Matheson Company, Joliet, Illinois. Henceforth, any reference to methane or ethane gas used will, unless otherwise designated, imply the respective "Pure Grade."

Isolation procedures. One-tenth ml of raw sewage, or 0.1 g of air-dried soil was added to 20 ml of liquid salts medium in 125-ml Erlenmeyer flasks, which then were incubated at 31 C in a 10-L vacuum desiccator. The air in the desiccator was removed and replaced with a gas mixture consisting of 65 per cent air and 35 per cent of the gaseous hydrocarbon desired, except where otherwise designated. Upon the appearance of definite turbidity, a loopful of the culture was transferred both to liquid and to agar media. Following incubation in the appropriate atmosphere, colonies were picked from the plates and repeatedly restreaked until only one kind of colony was present on the plates. The secondary liquid cultures were treated in the same way.

The "direct" method of selective isolation was also employed, whereby 0.1 ml of sewage was spread or 0.1 g of soil sprinkled on surface-dry agar plates. Since spreading a limited amount of inoculum on the plates resulted in the formation of discrete colonies, this method was useful in facilitating the isolation of organisms which, due to their lower rates of growth, would have been overgrown in the liquid cultures.

Manometric procedures. Warburg respirometer techniques were conventional (Umbreit *et al.*, 1954). The experiments were conducted at 31 C. Suspensions of cells were prepared from agar or liquid cultures. The cells were washed by centrifugation and resuspended in distilled water. Liquid cultures were grown in desiccators or in closed suction flasks as described elsewhere (Dworkin and Foster, 1956; Leadbetter and Foster, 1958). Occasionally, cells were grown in a column of medium through which a mixture of natural gas and air was continuously sparged.

Chemical procedures. Gas analyses on a microscale were made as described previously (Dworkin and Foster, 1956; Leadbetter and Foster, 1958). Radioactive carbon was measured with a thin window Geiger-Müller tube using a Tracerlab "64" scaler.

EXPERIMENTAL RESULTS

Isolation of organisms. There was no difficulty in isolating a diversity of organisms from natural gas or ethane cultures. Noticeable turbidity appeared after 5 to 7 days in the primary enrichment cultures, and after 2 to 4 days in pure culture. The cultures were identified according to the criteria presented in *Bergey's Manual* (Breed *et al.*, 1948) and in Gilman (1945). A fairly wide spectrum of organisms was obtained (table 1). Apart from the typical morphology and colony characteristics, the mycobacteria were verified as such by the characteristic acid fast staining properties. In this respect, they behaved identically with an authentic acid fast mycobacterium tested at the same time as a positive stain control. Hydrocarbon-utilizing mycobacteria are well-known (ZoBell, 1950; Beerstecher, 1954; Nechaeva, 1949; Bokova, 1954; Davis *et al.*, 1956), as are representatives of the other bacterial genera isolated in the present work. As noted earlier, the isolation from the "direct" plates of a filamentous fungus tentatively assigned to the genus *Acremonium* was of special interest.

Contrary to expectations based on the 15 to 1 molar superiority of methane over ethane in the natural gas used in their isolation, none of the isolates was able to grow at the expense of purified methane. Each, however, grew within 2 days, and abundantly, with purified ethane as the sole organic nutrient. The basis for the selection of organisms utilizing a minor component of the natural gas, to the exclusion of organisms utilizing the main component, will be reported elsewhere (Leadbetter and Foster, 1958 *unpublished data*). The bacteria will be called "ethane utilizers."

The 5 mycobacteria were isolated on the basis of relatively minor differences in colony characteristics. It will be shown presently that some important physiological differences also exist.

After periodic serial transfer on salts-ethane medium for a period of about 2 years, the stock cultures of Mycobacterium no. 9, Flavobacterium no. 10, Alcaligenes no. 11 and the coccus no. 7 would no longer grow when transferred to fresh salts-ethane medium. They did, however, retain their viability on complex organic media, such as nutrient agar. As a result, the majority of the physiological experimentation was done with

TABLE 1

Some ethane-utilizing organisms isolated from hydrocarbon enrichment cultures

Organism	Source of Inoculum	Substrate
Acremonium no. 15 (a fungus)	Raw sewage	Natural gas
Mycobacterium no 5	Raw sewage	Natural gas
Mycobacterium no. 6	Garden soil	Ethane
Coccus no. 7	Raw sewage	Natural gas
Mycobacterium no. 8	Garden soil	Natural gas
Mycobacterium no. 9	Raw sewage	Natural gas
Flavobacterium no. 10	Oil field soil	Natural gas
Alcaligenes no. 11	Oil field soil	Natural gas
Mycobacterium no. 12	Raw sewage	Ethane

Mycobacteria nos. 5, 6, 8, 12, and the fungus Acremonium no. 15.

Environmental conditions. (a) CO_2 tension. A slight acceleration of the growth rates of the various bacteria was observed in an atmosphere consisting of air 50, ethane 40, and CO_2 10 per cent, respectively. However, the mold responded noteworthily to CO_2. Thus, a moderate amount of growth had developed by the 6th day in the presence of 10 per cent CO_2, whereas no growth had occurred when the atmosphere was not enriched with CO_2. No attempt was made to secure a CO_2-free control. After a period of about 3 weeks, growth in the non-CO_2 cultures was equal to that in the CO_2-enriched cultures.

(b) Temperature. Each of the isolates grew at 25, 31, and 37 C, but not at 45 C. The optimum for most strains was 31 C, i. e., the temperature of isolation.

(c) Phosphate concentration. All of the strains except one grew well over a phosphate range from 0.01 to 1.0 per cent in the salts medium. Mycobacterium no. 5 failed to grow in 1 per cent phosphate medium.

(d) Nitrogen source. Nitrate was a better source of N than ammonium ion, for all the strains. Only the mold was capable of using nitrite-N.

Utilization of other substrates. Table 2 indicates the behavior of the 4 mycobacteria and the mold toward a variety of substrates. It is clear that the mycobacteria are of two types. One type, consisting of nos. 5 and 8, was able to utilize a variety of conventional carbon sources, and the other, consisting of nos. 6 and 12, was unable, under the conditions employed, to utilize any of the substrates tested with the exception of *n*-hexadecane. The hexadecane was technical grade (Eastman Kodak) and is known to contain nonhydrocarbon impurities (Rittenberg and Andreoli, 1956), so the response of strains 6 and 12 to this substrate does not necessarily imply utilization of hexadecane. None of the organisms could grow in salts media with methane or ethylene as the sole carbon source.

Strains no. 5 and 8 resemble the hydrocarbon-utilizing mycobacteria of Nechaeva (1949) and of Bokova (1954) with respect to their versatility in utilizing a large variety of conventional bacteriological substrates. The organisms of those authors differed among themselves in their hydrocarbon specificities, but strains no. 5 and 8 are closest to Bokova's *Mycobacterium perrugosum* var. *ethanicum*, which utilized ethane and higher paraffinic gaseous hydrocarbons, but not methane. Although higher gaseous hydrocarbons were not available for testing at the time, strains 5 and 8 will in all probability utilize the higher homologous paraffinic gaseous hydrocarbons. Of the species described in *Bergey's Manual* (Breed *et al.*, 1948), which does not include hydrocarbon utilization characteristics, *Mycobacterium phlei* is the one most nearly corresponding to the description of Mycobacterium no. 5.

Strains no. 6 and 12 probably should be regarded as varieties of the *Mycobacterium paraffinicum* of Davis *et al.* (1956). Here, likewise, on the assumption that higher gaseous hydrocarbon would be utilized, the resemblance is based on a striking inability to utilize common nonhydrocarbon organic substrates during incubation periods up to 2 weeks. Mycobacteria of this type are not listed in *Bergey's Manual.*

In addition to the substrate tests reported in table 2, the following cultural descriptions apply to these bacteria that were studied in detail.

TABLE 2

*Ability of ethane utilizers to grow in various substrates**

	Mycobacterium No. 5	Mycobacterium No. 6	Mycobacterium No. 8	Mycobacterium No. 12	Acremonium No. 15
Glucose	++++	−	+++	−	+++
Galactose	+++	−	++	−	++
Fructose	++++	−	++++	−	+++
Sucrose	±	−	−	−	±
Lactose	−	−	−	−	−
Xylose	+++	−	++	−	+
Arabinose	+++	−	+	−	++
Ribose	+++	−	+	−	+
Mannitol	+++	−	+++	−	±
Salicin	+++	−	+++	−	+
Methanol	+	−	±	−	−
Ethanol	++++	−	+++	−	−
Acetaldehyde	−	−	−	−	−
Formaldehyde	−	−	−	−	−
Na formate	−	±	−	−	−
Na acetate	−	−	−	−	−
Na pyruvate	+	−	+	−	±
Glycerol	++	−	+	−	±
Ethylene glycol	−	−	−	−	−
Ethylene	−	−	−	−	−
n-Hexadecane	++	++	++	++	−
Nutrient agar	++++	−	++++	−	++

* All carbohydrates were autoclaved separately and added to a final concentration of 0.5 per cent. Volatile compounds were filter sterilized and added to a final concentration of 0.5 per cent, except the aldehydes which were used at 0.05 and 0.1 per cent.

Ethylene was tested at concentrations of 10 and 30 volume per cent, in air.

Mycobacterium no. 5. Thick rod, occurs as single cells in liquid medium, 0.6 by 1.3 μ. Gram-positive. Strongly acid fast. Nonmotile. On salts-ethane plates the colonies are mucoid, about 2 mm in diameter, opaque and convex, with a tendency to become orange pigmented in old cultures. The appearance is the same on nutrient agar. On salts-ethane slants old cultures assume a wrinkled waxy appearance. Growth in liquid media is flaky, the flakes having a tendency to climb the walls of the vessel. Indole negative. Litmus milk alkaline. Gelatin not liquefied after 3.5 weeks.

Mycobacterium no. 6. Short thick rod. Occurs as single cells, 0.5 by 1.0 μ. Gram-positive. Weakly acid fast in young and in old cultures. Nonmotile. On salts-ethane plates the colonies are flat, dry, smooth, and circular with a bright yellow pigment developing after 2 to 3 days. This pigment turns yellowish brown in older cultures. Colonies are 2 to 3 mm in diameter. Growth in liquid medium is yellow and flaky, the flakes having a tendency to climb the walls of the vessel. No growth in litmus milk or in other conventional media after 2 weeks of incubation.

Mycobacterium no. 8. Thick rod. Occurs as single cells, 0.6 by 1.6 μ. Gram-positive. Weakly acid fast in young cultures. On salts-ethane agar the colonies are dry, white, rough, powdery, opaque, and irregular. Colonies are 1 to 2 mm in diameter. On nutrient agar, the colonies are raised, mucoid, opaque, whole, circular, and about 2 mm in diameter. Growth in liquid is flaky, the flakes having a tendency to climb the walls of the vessel. Indole negative. Litmus milk alkaline. Gelatin not liquefied after 3 to 5 weeks.

Mycobacterium no. 12. Long slender rods. Occur as single cells. 0.4 by 2.0 μ. Gram-positive. Weakly acid fast in young cultures. Nonmotile. On salts-ethane agar the colonies are white, flat, dry, smooth, and 1 to 2 mm in diameter. In liquid media growth is flaky, the flakes having a tendency to climb the walls of the vessel. No

growth in litmus milk or in other conventional media after 2 weeks of incubation.

Acremonium species. The mold was identified as a member of the family *Moniliales*. Characteristics of the organism were determined by microscopic examination of a Henrici slide culture containing Waksman's glucose peptone acid-agar. The hyphae were pigmentless and formed a mat of branched, septate mycelia, possessing side branches which were erect and served as conidiophores. Conidia were single or double on the conidiophores, terminal, ovate, and 4 to 5 μ along the long axis. Conidiophores were awl-shaped and unbranched.

Verification of ethane utilization. Theoretically, a small amount of impurity in the ethane could support substantial growth if it and not the ethane were being utilized. This is particularly true under the cultivation conditions employed in this work, namely, relatively high (50 volume per cent) concentrations of ethane and relatively large gas volumes per volume of medium.

(1) Stoichiometric manometry:—If a known quantity of ethane is introduced to a Warburg respirometer, the amount of impurities can be calculated from the manufacturer's statement that the ethane contained not more than 1 per cent impurity. The cells can then be allowed to metabolize until more gas is taken up than can be accounted for by the impurities. A suspension of Mycobacterium no. 6 was used in the illustrative experiment summarized in table 3. This experiment indicated that ethane was utilized, since the gas uptake greatly exceeded that possible from the impurities.

TABLE 3

Gas uptake during oxidation of ethane by a washed suspension of cells of Mycobacterium no. 6

	μL	μmoles
Ethane added	9345	
Maximum impurity in the ethane, as propane	93	4.1
O_2 theoretically required for oxidation of the propane to CO_2 and H_2O	459	20.5
Theoretical gas uptake for oxidation of all the propane (O_2 + propane)	552	
Observed gas uptake*	1755	

* Gas uptake occurred at a steady rate and it was at that rate when the experiment was interrupted for analysis. A solution of KOH was present in the center well for CO_2 absorption. Gas mixture was 50 per cent ethane-50 per cent air.

(2) Comparison of weight of impurity with weight of cells obtained in a growth experiment:—A total of 37 slants of mineral-(washed) agar were lightly inoculated with Mycobacterium no. 5 and incubated in a 10-L desiccator containing 15 per cent ethane and 85 per cent air. Total gas space in the desiccator was 8900 ml. After incubation for 9 days, the cells were washed off the slants, recentrifuged in distilled water, dried, and weighed. Table 4 shows that the amount of cellular carbon obtained was about twice that possible from the impurities, again indicating consumption of ethane.

(3) Determination of carbon dioxide produced:—In this experiment the amount of CO_2 produced was used as an index of the amount of substrate carbon converted. Mycobacterium no. 5 and no. 12 and the mold Acremonium were used. Half-liter suction flasks each containing 100 ml liquid mineral medium were inoculated in duplicate with the respective organisms. The side arms were sealed by rubber tubing and clamps. One flask of each pair was evacuated and gassed with a mixture of 10 per cent ethane and 90 per cent air. The flasks were closed with one-hole rubber stoppers containing a glass tube that was sealed with a clamped rubber tubing. The flasks, with the exception of the mold culture which was incubated under stationary conditions, were incubated on a reciprocal shaking machine until abundant growth had developed. No growth occurred in the duplicate flasks without ethane. A stream of CO_2-free air was then passed through each flask and into a solution of CO_2-free KOH. The ab-

TABLE 4

Analysis of growth culture of Mycobacterium no. 5 in ethane

Ethane present, ml	1335
Maximum impurity in the ethane, as propane, ml	13.4
Cellular carbon theoretically derivable from propane,* mg	14.8
Cellular carbon actually obtained,† mg	26.7

* Assuming 65 per cent conversion of propane carbon to cellular carbon.

† Calculated as 50 per cent of the 53.3 mg dry weight of cells obtained.

TABLE 5

Carbon dioxide production and cell synthesis by ethane utilizers

	Mycobacterium no. 5	Mycobacterium no. 12	Acremonium no. 15
$BaCO_3$ from inoculated control,* mg	3.5	3.9	2.4
$BaCO_3$ from ethane flasks	24.5	39.0	23.3
Dry weight of cells in ethane flask, mg	8.2	8.3	5.8
Theoretical maximum $BaCO_3$ from impurity,† mg	3.5	3.5	3.5
Theoretical maximum dry weight of cells from impurity,† mg	0.35	0.35	0.35

* No ethane present. No growth occurred in these flasks.

† Assuming the impurity to be 1 per cent propane, with ⅔ assimilation of the carbon.

sorbed CO_2 was precipitated as $BaCO_3$, collected, dried, and weighed. Since the cultures were not acidified prior to CO_2 removal, the values for CO_2 represent minimal amounts. The cells from each culture were then collected, washed twice, dried, and weighed. The results (table 5) clearly show that much more CO_2 and cell material were produced than could be accounted for by the impurities.

The experiments in (1), (2), and (3) conclusively establish that the organisms studied were capable of consuming ethane as the sole source of carbon and energy.

Oxidation of hypothetical intermediates. Some preliminary attempts were made to study the intermediate steps in the oxidation of ethane. The results were essentially unrewarding due to the lack of success in obtaining enzymatically active cell-free extracts. The constitutive nature of ethane oxidation precluded the use of the technique of simultaneous adaptation (Stanier, 1947).

A number of methods were tried for obtaining enzymatically active preparations, including disruption by sonic vibration, grinding with alumina, dehydration of cells with acetone, and by storage *in vacuo* over P_2O_5. Addition of di- or triphosphopyridine nucleotides, yeast extract, casamino acids, the ash of ignited ethane-grown bacterial cells, methylene blue and cysteine did not activate any of the preparations with respect to ethane utilization. Organisms no. 5, 6, 8, and 12, were tested.

Suspensions of washed ethane-grown cells of Mycobacterium nos. 5, 6 ,and 12 were tested for their ability to oxidize a number of hypothetical intermediates in the oxidation of ethane. Each oxidized ethanol and ethylene glycol, but not ethylene oxide or Na propionate. Acetaldehyde and Na acetate were slowly oxidized, and ethylene was rapidly oxidized by Mycobacterium no. 6, but not by the others. These results, especially those with Mycobacterium no. 6, are comparable to those obtained by Davis *et al.* (1956) with the constitutive ethane-oxidizing *M. paraffinicum.* However, they neither establish nor eliminate involvement of any of these compounds in the oxidation of ethane. The study of the mechanism of ethane utilization is being continued.

Hydrogen utilization. Tausz and Donath (1930) found that two bacteria with different hydrocarbon specificities, namely, one that could attack certain liquid hydrocarbons but not gaseous and one that could attack both liquid and gaseous hydrocarbons, were able to grow at the expense of hydrogen and carbon dioxide as the sole energy and carbon source, respectively. The organisms listed in table 1 were tested for this property in salts medium in a closed atmosphere consisting of hydrogen 50 per cent, CO_2 10 per cent, and air 40 per cent. Clear-cut evidence of autotrophic metabolism was seen in the abundance of growth made under these conditions by Mycobacterium no. 5, 6, 8, and 12 and the gram-negative coccus no. 7. Three of these novel hydrogen autotrophs are pictured in figure 1. The other organisms listed in table 1 failed to grow in hydrogen.

Quantitative data on the gas transformations were obtained by a manometric experiment employing a washed cell suspension of Mycobacterium no. 8. Two Warburg vessels containing equal amounts of the cellular suspension were gassed through a manifold with a mixture of hydrogen, oxygen, and carbon dioxide in the approximate volumetric ratio of 76:14:10. The manometer fluid was mercury. Vessel no. 1 contained the salts medium, but no cells, and was used as the zero-time control for the precise determination of the composition of the gas mixture at the start of the experiment. Vessel no. 2 contained the salts medium plus cells which were allowed to metabolize until a significant amount of gas uptake had occurred (2906 μL, using K_{O_2} as

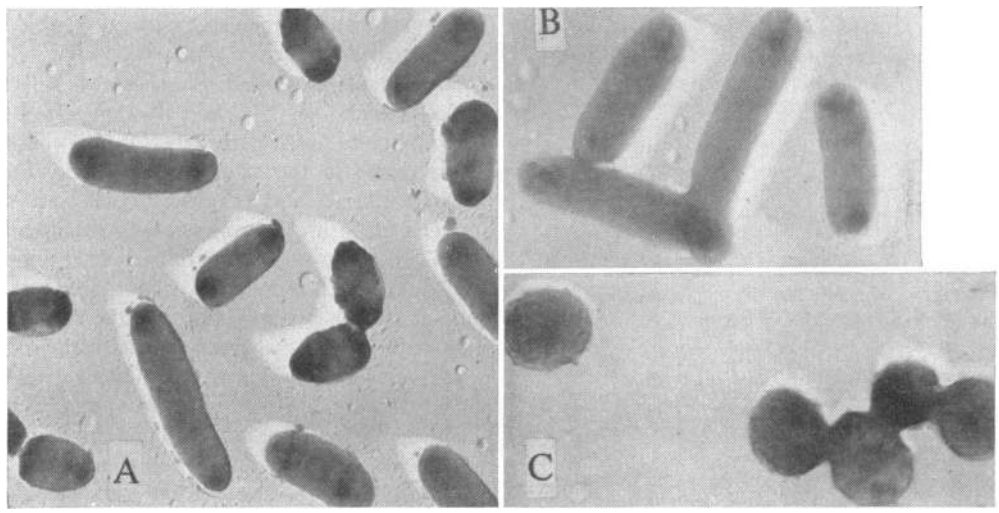

Figure 1. Electron photomicrographs of hydrogen autotrophs. (*A*) Mycobacterium no. 5 (× 14,000) (*B*) Mycobacterium no. 8 (× 17,800), and (*C*) gram-negative coccus no. 7 (× 18,700).

the vessel constant). The difference in the composition of the gas in the two vessels was, then, an indication of the nature of the reaction. Procedures similar to those of Schatz (1952) were employed for determinations of the composition of the gas mixtures. In the experiment described in table 6, the gases were consumed in the ratios of 1:3.8:7.2 for CO_2, O_2, and H_2, respectively.

The amount of H_2 oxidized per unit of CO_2 fixed (7.2:1), e. g., an indication of the efficiency of the chemosynthetic assimilation of CO_2, is about 15 per cent larger than reported for *Hydrogenomonas facilis*, (Schatz, 1952); for *Hydrogenomonas ruhlandii*, (Packer and Vishniac, 1955); and green algae; (Gaffron, 1942). However, reports usually deal with gas balances obtained with suspensions of resting cells, whereas our data were obtained with cells growing in a complete medium. One would expect a less efficient utilization of hydrogen under growth conditions. This was borne out by the data of Ruhland (1924) for *Bacillus pycnoticus*. Finally, it has been demonstrated, with *H. ruhlandii* and with *H. facilis*, that the efficiency of CO_2 fixation in relation to the oxidation of hydrogen varies considerably according to the physiological conditions of the experiment (Packer and Vishniac, 1955; Marino and Clifton, 1955).

A similar hydrogen gas experiment was performed with the gram-negative coccus (no. 7) but this time $C^{14}O_2$ was used. The great majority of the radioactivity of the CO_2 assimilated in this experiment was recovered in the bacterial cells at the end of the experiment. The radioactivity measurements were made on $BaCO_3$ prepared by combustion of the cells.

TABLE 6

Gas data for Mycobacterium no. 8 in a hydrogen experiment

Total gas uptake (O_2 + CO_2 + H_2)	128.0 mm*
O_2 at beginning 2505 μL	
O_2 at end 1567 μL	
O_2 uptake 928 μL = 41.2 mm	
CO_2 at beginning 1509 μL	
CO_2 at end 1265 μL	
CO_2 uptake 244 μL = 10.0 mm	
Gas uptake due to O_2 and CO_2	51.2 mm
Gas uptake due to H_2	76.8 mm (=1742 μL)

* mm Denotes change in manometric pressure expressed in millimeters of mercury.

Thus, by four independent criteria, the abovementioned five bacterial cultures were shown unequivocably to be hydrogen autotrophs: (1) abundance of growth in salts medium in the presence of hydrogen, carbon dioxide, and oxygen, (2) absence of growth in controls without hy-

drogen, (3) direct gas analysis of growth cultures, and (4) assimilation of radioactive CO_2 during growth.

Substrate conditioning of the ability to oxidize hydrogen. The capacity of washed suspensions of these bacteria to oxidize molecular hydrogen was conditioned by the particular substrate on which the cells were grown. Thus, cells of the gram-negative coccus would oxidize hydrogen constitutively only when they had been grown on hydrogen. When they had been grown on either glucose or ethane as sole sources of energy and carbon, cells of this organism would oxidize hydrogen only after a period of adaptation. The adaptation was much slower for ethane-grown cells, than for glucose-grown cells.

In contrast, cells of Mycobacterium no. 5 oxidized hydrogen constitutively, i. e., without any adaptation, irrespective of the substrate on which they had grown, namely, hydrogen, glucose, or ethane.

Cells of Mycobacterium no. 8 represented an intermediate status; hydrogen oxidation was constitutive for hydrogen- and ethane-grown cells, and very rapidly adaptive for glucose-grown cells.

Although hydrogen oxidation by glucose-grown cells of the coccus and Mycobacterium no. 8 undoubtedly was adaptive, the data suggested that the cells may have possessed a small but definite hydrogenase activity at zero time, i. e., before exposure to hydrogen. This situation might be similar to the possession of hydrogenase by pyruvate-grown cells of *H. ruhlandii* (Packer and Vishniac, 1955). The inference is that the metabolism of glucose and pyruvate by the respective bacteria may at some stage involve hydrogenase.

DISCUSSION

Hydrocarbon utilization and hydrogen autotrophy. That such a large proportion of bacteria isolated as hydrocarbon utilizers proved to be hydrogen bacteria was unexpected. Recent similar results with a variety of bacteria isolated with natural gas (described in footnote 3) as the substrate (Shankel and Foster, 1957, *unpublished data*), with propane as the substrate (Kester and Foster, 1957, *unpublished data*) and with ethane as the substrate (Davis and Raymond, *personal communication*) suggest that possession of the dual capacities of hydrocarbon and hydrogen utilization is more than coincidental.

Not all of the hydrocarbon utilizers were able to grow autotrophically with hydrogen, so the parallelism is not complete. However, it is possible that some of the organisms not capable of hydrogen autotrophy may, nevertheless, possess hydrogenase. Although the great majority of previously described hydrocarbon-utilizing bacteria have not been tested for their ability to grow at the expense of hydrogen and carbon dioxide, Tausz and Donath's (1930) two hydrocarbon utilizers were also hydrogen autotrophs. In the only other cross-testing of this kind that we have been able to discover, neither the methane oxidizer of Münz (1915) nor the four varieties of the obligate methane utilizer *P. methanica* (Leadbetter and Foster, 1958) could develop autotrophically with hydrogen and carbon dioxide. The hydrogen autotrophic potentials of Bokova's (1954) *M. perrugosum* var. *ethanicum* and *Mycobacterium rubrum* var. *propanicum* are of special interest. Although reported as negative in this property, the organisms were tested under conditions that would hardly allow expression of this potentiality, i. e., ". . . in an atmosphere consisting of hydrogen (30 per cent) and air" (Bokova, 1954).

However fragmentary, the evidence we had assembled led us to assume that a correlation exists between enzymes involved in hydrocarbon utilization and hydrogenase in the bacteria containing both. We were, therefore, surprised to discover that Tausz and Donath had expressed essentially the same idea[4] 28 years ago, with much less evidence.

The situation is reminiscent of the presumptive relation between hydrogenase and the "nitrogenase" system in nitrogen-fixing bacteria (Shug *et al.*, 1956; Wilson and Burris, 1953; Gest, 1954). It is expected that studies under way will permit a clarification of the question of a possible

[4] "Wir wollen diesen Befund in dem Sinne deuten, dass bei der bakteriellen Oxydation der Kohlenwasserstoffe die Dehydrierung eine Rolle spielt und wollen unsere Bakterien in die Gruppe der Wasserstoff aktivierenden Katalysatoren einreihen, denn sie machen sowohl den molekularen als auch den in den Kohlenwasserstoffen gebundenen Wasserstoff mobil. In dem einen Falle lockern sie die H—H—Bindung in dem anderen Falle die C—H—Bindung. Es soll aber damit nicht etwa gesagt sein, dass bei der Oxydation eine Aktivierung des molekularen Sauerstoffes an Schwermetallsystemen nicht erfolgt." (Tausz and Donath, 1930, p. 147–148.)

relation between hydrogenase and the "hydrocarbonase" complex.

Problems of taxonomy of hydrogen autotrophs. It is of interest that organisms isolated for a property other than hydrogen autotrophy turn out to be hydrogen chemolithotrophs. This sequence applies to methane-producing bacteria (Schnellen, 1947; Barker, 1943; Kluyver and Schnellen, 1947) and to sulfate-reducing bacteria (Starkey, 1947; Butlin and Adams, 1947) among the anaerobic bacteria. Among the aerobes it includes the carbon monoxide bacterium of Kistner (1953) and now the hydrocarbon-oxidizing bacteria described here and by Tausz and Donath (1930).

On the other hand, one cannot ignore the fact that previous deliberate efforts to obtain hydrogen autotrophs directly by specific hydrogen enrichment culture procedures have almost always yielded one type of bacterium, namely pseudomonads.[5] They have been traditionally classified in the genus *Hydrogenomonas*. It now appears that the impression that hydrogen autotrophy among the aerobes is confined to pseudomonads is a fallacy stemming from the rigid selectivity of conventional enrichment culture techniques. Liquid enrichment cultures consisting of a mineral salts solution in a gas atmosphere containing hydrogen, carbon dioxide, and oxygen almost invariably yield pseudomonads, and perhaps only pseudomonads. As Van Niel (1955) has pointed out, this is the most common result in liquid enrichments under aerobic conditions with any kind of substrate.

It should now be possible, by the use of enrichment and selective techniques, to extend greatly the number and variety of bacterial types capable of hydrogen chemolithotrophy. Furthermore, it is very likely that, in the manner described here, many additional hydrogen autotrophs will be found among organisms isolated without regard to that property—if they are subsequently tested for it under suitable conditions (Wilson *et al.*, 1953; Packer and Vishniac, 1955). It is not improbable that many stock cultures of bacteria would be found to possess this character, particularly if they were tested soon after their isolation and before the autotrophic genome could be selected against by serial cultivation in complex laboratory media. Thus, it seems safe to conclude that hydrogen autotrophy will prove to be a property distributed fairly generally throughout various groups of aerobic bacteria. At the moment, we can include in this list the mycobacteria and the gram-negative coccus described here, and the strains of actinomycetes (Takamiya and Tubaki, 1956) described after the experimental work reported here was completed.

It is to be expected, of course, that a greater variety of hydrogen bacteria will be obtained by the use of nonspecific substrates, especially simple ones, than by any direct hydrogen selective techniques. Nevertheless, by the simple expedient of Winogradsky's "direct" plating technique whereby crumbs of soil are sprinkled on the surface of mineral salts agar plates which then are incubated in an appropriate gas mixture, it is a fairly easy matter to obtain hydrogen mycobacteria. This observation has been confirmed by Davis and Raymond (*personal communication*). The extent to which this direct selective technique will reveal additional types of aerobic hydrogen bacteria is being explored.

Recognition of the autotrophic capacity in several different morphological groups is not without consequences for bacterial classification. It raises, in particular, a serious doubt as to the feasibility of retaining one group of organisms, e. g., the genus *Hydrogenomonas*, segregated at the generic level on the basis of the ability to utilize hydrogen as the sole energy source. The case against recognizing physiological genera based on utilization of particular substrates has been presented elsewhere (Dworkin and Foster, 1956). The genus *Hydrogenomonas* is a legacy from the time pseudomonads were the only (nonphotosynthetic) hydrogen bacteria. It is obvious that the uniqueness no longer applies. In none of the other above-mentioned bacterial groups (*Clostridium aceticum* (Wieringa, 1940) may also be included) does hydrogen utilization or autotrophy supersede the preexisting morphologically based genus description. And, certainly, one could not seriously consider the creation of such artificial genera as *Hydrogenomycobacterium*, *Hydrogenococcus*, and *Hydrogenostreptomyces* to accommodate the recently discovered autotrophs!

Two qualities any classification system should supply are consistency and an accurate means of referring to a taxonomic group. Elevation of the nonobligate character of hydrogen autotrophy to

[5] The aerobic spore-forming hydrogen autotroph (*Bacillus pycnoticus*) of Grohmann (1924) and the coccus of Lebedeff (1908) apparently have never been reisolated.

genus rank in the pseudomonads is clearly out of line with the practice in the several other morphological groups of bacteria.[6] In the interests of uniformity and in the avoidance of excessive artificiality, the reasonable approach is to drop the generic name *Hydrogenomonas* in favor of *Pseudomonas*. By taking this step no sacrifice ensues in the reference quality of the classification.

The autotrophic character, not warranting genus distinction because of nonobligateness, can be properly recognized at the species level, as Takamiya and Tubaki (1956) have done in naming their new actinomycete *Streptomyces autotrophicus*. Consistency and accuracy in reference thereby would be achieved. An argument that might be leveled against the practicability of this move deals with the handling of the several supposed species of the genus *Hydrogenomonas*. The distinguishing characteristics (obtained in diverse studies) of most of these have recently been summarized by Packer and Vishniac (1955). The features cited are arbitrary and in many other well-studied groups they would not be regarded as sufficiently distinctive and meaningful as to warrant individuality as species.

Like many comparable situations in bacterial classification, the status of hydrogen autotrophic pseudomonads focuses attention on the indispensability of ascertaining the extent of phenotypic and genotypic variation of each organism being described. In the pseudomonad hydrogen autotrophs, even differences such as oxygen sensitivity and pigmentation may well be a consequence of strain history. The practice of establishing limits of variability of an organism would lead automatically to a wholesome step in bacterial taxonomy: it would necessitate accepting as mere varieties a great many alleged species currently distinguished by trivial and often unreproducible differences.

The proposal to place hydrogen autotrophic pseudomonads in the genus *Pseudomonas* and to recognize hydrogen utilization at the species level achieves the ideals of classification by introducing consistency, by providing an accurate means of identification and by taking cognizance of biological variability. Adherence to these principles will hasten the inevitable day when the most pressing need of bacterial taxonomy will be fulfilled, namely, recognition that the fundamental unit of bacterial taxonomy is what is known as the "form species," "species group," or "biotype" and that its employment will be mandatory.

[6] As a matter of fact, there is a conspicuous inconsistency within the pseudomonads. For example, the well-known *Pseudomonas saccharophila* was isolated as a hydrogen autotroph (Doudoroff, 1940).

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SUMMARY

From enrichment and selective cultures in which natural gas or ethane was the substrate, several bacteria and one filamentous fungus were isolated in pure culture. Despite a 15 to 1 molar preponderance of methane over ethane in the natural gas employed in these experiments, none of the isolates could grow with methane (99 per cent pure) as the sole carbon source. Each did grow with ethane (99 per cent pure) as the sole carbon source. Among the ethane utilizers, four different mycobacteria and a gram-negative coccus grew autotrophically with hydrogen as the sole source of energy. The ability of suspensions of washed cells to oxidize hydrogen was conditioned by the particular substrate on which the organisms were grown. Each of the hydrogen bacteria is a facultative autotroph. A possible relation between the ability of bacteria to utilize hydrocarbons and the ability to use hydrogen is pointed out. Problems in the taxonomy of hydrogen autotrophs are discussed and the untenability of the genus *Hydrogenomonas* outlined. A proposal to drop *Hydrogenomonas* in favor of *Pseudomonas* is made, together with the suggestion that most existing "species" of *Hydrogenomonas* are varieties of one or two species whose autotrophic nature should be designated in the species epithet.

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