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Invalidity of the Acetylene Reduction Assay in Alkane-Utilizing, Nitrogen-Fixing Bacteria

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The cause of the failure of the C_2H_2-C_2H_4 assay for nitrogen-fixing bacteria growing on lower alkanes was studied. Acetylene was a strong competitive inhibitor of methane oxidation for methane-utilizing bacteria, as well as for the oxidation of lower alkanes by other bacteria, so that energy and reducing power were no longer available for the reduction of acetylene by nitrogenase. Nitrogen-fixing bacteria grown on alkanes may reduce acetylene when intermediates of alkane-breakdown or other substrates oxidizable in the presence of acetylene are supplied. Ethylene co-oxidation is not responsible for the failure of the test, because acetylene also inhibits this co-oxidation along with methane oxidation.

Because of its sensitivity and simplicity, the acetylene reduction assay is generally applied to measure biological nitrogen fixation (7). In using the C_2H_2-C_2H_4 assay, it is assumed that (i) acetylene does not interfere with other metabolic activities of the system under study and (ii) ethylene is stable during the investigation. These two factors have not been studied thoroughly as yet, although reports have appeared suggesting that they may influence the results of the test. Brouzes and Knowles (1) reported the prevention of the normal growth pattern of a nitrogenase-repressed culture of *Clostridium pasteurianum* in a medium supplemented with $(NH_4)_2SO_4$ with 0.1 atmosphere of acetylene in the gas phase. The acetylene inhibited cell proliferation and prevented an increase in the rate of carbon dioxide production, normally associated with growth.

The C_2H_2-C_2H_4 test failed to show nitrogenase activity in nitrogen-fixing, methane-oxidizing bacteria of strain 41 of the *Methylosinus* type (2). Nitrogenase activity could not be assayed by the test when the bacterium was growing on methane, although $^{15}N_2$ was fixed. Bacteria growing on methane co-oxidized ethylene, but cells growing on methanol did not. This co-oxidation of ethylene by methane-grown cells might have caused the test to fail. Alternatively, acetylene might have blocked methane oxidation, thus preventing a supply of energy and reducing power to the nitrogenase.

This paper gives the results of a more detailed study of acetylene inhibition of cell metabolism in the C_2H_2-C_2H_4 assay in alkane-utilizing bacteria.

MATERIALS AND METHODS

Organisms. *Mycobacterium vaccae*, originally isolated by Ooyama and Foster (11), was provided by J. J. Perry, North Carolina State University, Raleigh. Strains 41 and E20 have been described previously (2, 3).

Strain Et32 was isolated by incubating 10 g of soil with 25 ml of mineral salts (MS) medium (see below) under 10% ethane in air in a 1-liter Erlenmeyer flask. After 2 weeks of incubation, the enrichment was streaked on plates of MS medium. The bacterium was isolated after incubating under ethane in air, followed by restreaking on the same medium. Strain Et32 is a gram-negative, yellow-pigmented rod.

Strain H2 was similarly isolated from soil, except that hexane replaced ethane. Strain H2 is a gram-positive pleomorphic bacterium.

Strain 3b was isolated from garden soil incubated in an Erlenmeyer flask containing 10% ethane in air. Every 3 weeks the flask was flushed with air, and the ethane concentration was restored. After 4 months, a macroscopically visible, yellowish colony had appeared on the surface of the soil sample. Material from this colony was streaked on plates of nitrate-free MS medium. Colonies had grown on these plates after 6 weeks of incubation in an ethane-containing desiccator at reduced oxygen pressure. A pure culture was obtained after restreaking on the same medium.

Strain H12 was isolated by incubating a mixture of different soil and water samples of approximately 10 g with 25 ml of nitrate-free MS medium in a sealed 2-liter Erlenmeyer flask. Hexane (2 ml) was added, and the oxygen pressure was reduced by flushing with nitrogen. Material from the enrichment was streaked on plates of nitrate-free MS medium after 40 days of incubation. After 4 weeks of incubation in a desiccator containing 5% oxygen and hexane, colonies on these plates were well devel-

oped. The pure culture was obtained after restreaking on the same medium.

Medium. The MS medium, used throughout the investigation, contained the following salts in 1 liter of deionized water: $NaNO_3$, 2.0 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.015 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; $CuSO_4 \cdot 5H_2O$, 5 μg; H_3BO_3, 10 μg; $ZnSO_4 \cdot 7H_2O$, 70 μg; $MnSO_4 \cdot 5H_2O$, 10 μg; $Na_2MoO_4 \cdot 2H_2O$, 100 μg; final pH, 6.8. For solid media, 1.5% agar was added to this nutrient solution.

Chemicals. Methane, ultrapure (99.97%), and other gaseous substrates (commercial purity) were obtained from the Matheson Co.

Culture conditions. Weekly subcultures on slants of MS medium or nitrate-free MS medium were kept in desiccators at reduced oxygen pressure (5% or less) with approximately 5% of the desired gas and N_2 present at 30 C. Growth experiments were also carried out at this temperature.

Oxygen uptake. Dissolved oxygen concentrations were measured at 30 C with a Yellow Springs Instruments model 53 oxygen monitor equipped with a polarographic sensor. This electrode fits into a 15-ml cell; it has an access slot for removal of overflowing liquid. The slot can also be used for adding or withdrawing small samples from the cell.

Gas chromatographic analyses. Analyses were carried out with a flame-ionization detector with a Becker type 409 or type 417 chromatograph. A Porapak R column (110 cm by 4 mm) with nitrogen as carrier gas was used for assaying methane (50 or 150 C when in solution), acetylene reduction (50 C), ethylene and ethane (80 C), and methanol (120 C). Acetylene was assayed with a Porapak T column (120 cm by 4 mm) at 90 C.

RESULTS

Methane-oxidizing bacterium strain 41 of the *Methylosinus* type. The methane-oxidizing, nitrogen-fixing strain 41 did not accumulate ethylene when exposed to methane in the presence of acetylene. However, ethylene was produced linearly by methane-grown cells when methanol or formate was added during the assay (Fig. 1). Also, compounds other than methanol or formate, which are both intermediates of methane oxidation, supported the reduction of acetylene, e.g, ethanol and butanol.

If co-oxidation of ethylene by methane-grown cells caused the failure of the test, ethylene should not be accumulated by such cells in the presence of methanol or formate unless these compounds specifically suppress the co-oxidation. Addition of methanol to a cell suspension reduced the rate of ethylene co-oxidation as well as the rate of methane oxidation, but the co-oxidation was not completely stopped by methanol (Fig. 2), suggesting that co-oxidation was not responsible for the failure of the test with methane-grown cells.

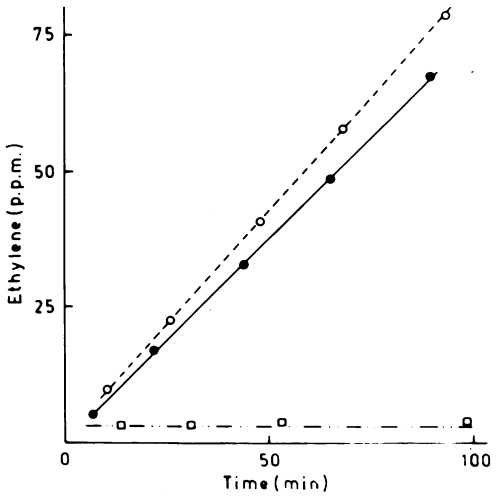

FIG. 1. *Effect of methanol and formate on acetylene reduction by strain 41 grown on methane in nitrate-free MS medium. Portions (9 ml) of a 100-ml culture, growing logarithmically in a 1,000-ml Erlenmeyer flask, were injected into 100-ml Erlenmeyer flasks containing 10% CH_4, 5% O_2, 10% C_2H_2, and 75% N_2 with 1 ml of nitrate-free MS medium containing 0.1% of the compounds indicated. Symbols: (●) Methanol; (○) sodium formate; (□) no supplementary compound added.*

Acetylene, always present during the assay, suppressed the oxidation of methane as well as the co-oxidation of ethylene (Fig. 3). This observation definitely rules out co-oxidation of ethylene as the possible cause of the failure of the C_2H_2-C_2H_4 assay.

Mechanism of inhibition of methane oxidation by acetylene. When strain 41 was cultivated on methane in MS medium, the growth of the bacterium was completely inhibited in the presence of 10% acetylene, whereas growth on methanol in MS medium was not affected. Apparently, the first step in the degradation route of methane, the oxidation of methane to methanol, was blocked by acetylene. This was also shown by the course of the oxygen uptake of whole-cell suspensions of strain 41 grown on methane (Fig. 4). Acetylene suppressed methane-dependent oxygen uptake, whereas it did not influence methanol-dependent oxygen uptake.

The first step in the breakdown of methane by strain 41 is performed by an adaptive enzyme system. Methane did not increase oxygen uptake of whole-cell suspensions of methanol-grown bacteria (Table 1). The enzyme system involved in methane oxidation is presumably analogous to the methane hydroxylase described for *Methylococcus capsulatus* (12) and

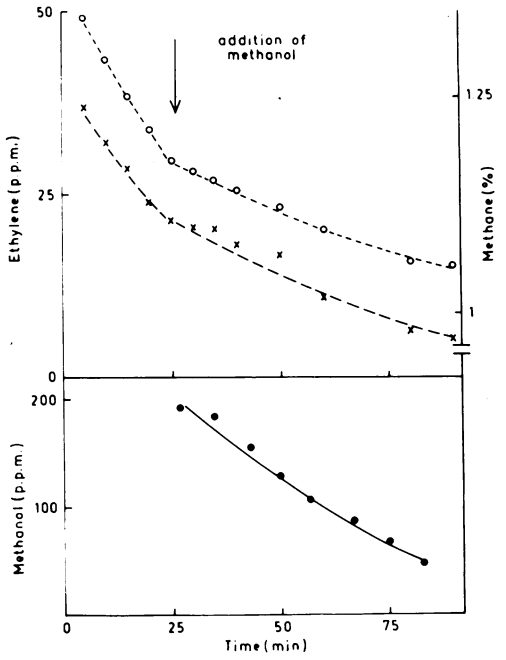

FIG. 2. *Inhibition by methanol of methane oxidation and of ethylene co-oxidation. A 100-ml culture of strain 41, grown on methane in MS medium, was centrifuged and suspended in 10 ml of 0.03 M sodium phosphate buffer, pH 6.8. This suspension was injected into a 100-ml Erlenmeyer flask, containing methane and ethylene in air. Methanol was injected at the time indicated. Symbols: (●) Methanol (liquid phase); (○) ethylene (gas phase); (×) methane (gas phase).*

for *Pseudomonas methanica* (5). Methane hydroxylase is also capable of oxidizing ethane (12) and carbon monoxide (5), explaining the adaptive nature of the co-oxidation of these compounds by strain 41. The co-oxidation of ethylene was also adaptive, demonstrating that methane hydroxylase probably oxidized this compound. Oxidation of methane and co-oxidation of methane hydroxylase-dependent cosubstrates were inhibited by acetylene. Oxidation of intermediates of the methane breakdown (e.g., methanol), as well as that of cosubstrates not dependent upon the hydroxylase for their breakdown (e.g., ethanol), was not influenced by acetylene (Table 1). Acetylene itself was apparently not co-oxidized by the hydroxylase, as judged by the oxygen uptake data. However, by applying lower concentrations of acetylene, its disappearance from the gas phase could be directly measured, and then the effect of methane-grown cells on acetylene was assessed. Methanol-grown cells did not show uptake of acetylene, indicating that methane hydroxylase was involved in the removal of acetylene from the gas phase (Fig. 5).

Activity of methane hydroxylase is obtained only with difficulty in cell-free extracts and, moreover, it does not remain stable for long (5, 12). Therefore, experiments with whole-cell suspensions were undertaken to study the inhibition of methane hydroxylase activity by acetylene. Uptake of methane and of dissolved oxygen, both dependent on methane concentration, by whole-cell suspensions of strain 41 was measured with an oxygen-electrode cell.

The rate of oxygen uptake, after correcting for endogenous respiration, was directly proportional to the rate of methane uptake but independent of the actual concentration of methane. An apparent K_m of approximately 1.7% for methane in the gas phase at 30 C was obtained both by measuring the uptake of methane and by recording the rate of oxygen uptake as a function of the actual methane concentration (Fig. 6a, c, and e). The rate of

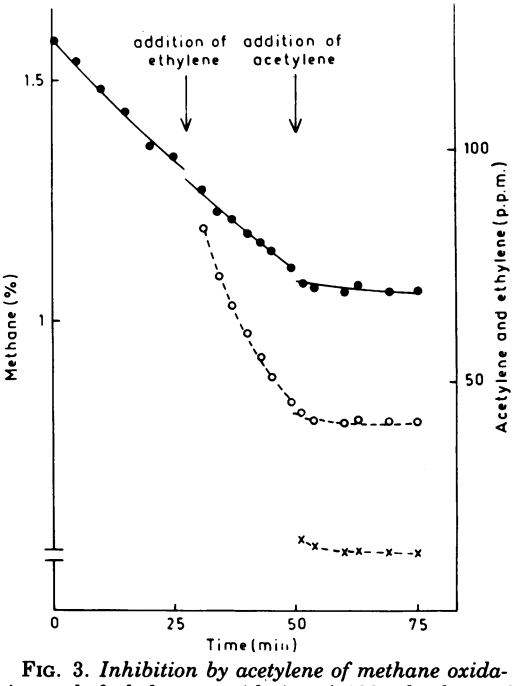

FIG. 3. *Inhibition by acetylene of methane oxidation and of ethylene co-oxidation. A 100-ml culture of strain 41, grown on methane in MS medium, was centrifuged and suspended in 10 ml of 0.03 M sodium phosphate buffer, pH 6.8. This suspension was injected into a 100-ml Erlenmeyer flask, containing methane in air. At the times indicated, 1.5 ml of approximately 5,000 μl of C_2H_4 per liter in air and 2 ml of approximately 750 μl of C_2H_2 per liter in air were injected into the flask. Symbols: (●) Methane; (○) ethylene; (×) acetylene.*

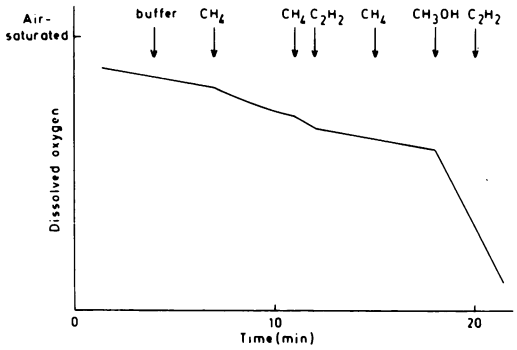

FIG. 4. *Recorder trace of the uptake of dissolved oxygen by a whole-cell suspension of strain 41. A culture growing logarithmically on CH_4 in MS medium was centrifuged and suspended in an equal volume of 0.03 M sodium phosphate buffer, pH 6.8. At the times indicated by the arrows, 0.05 ml of the same buffer, 0.05 ml of buffer saturated with CH_4 or with C_2H_2, or 0.05 ml of buffer with 0.1% CH_3OH was injected into the suspension.*

TABLE 1. *Rates of oxygen uptake by cell suspensions of strain 41*[a]

Substrate	CH_4 grown		CH_3OH grown	
	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$
Methane	100	0	0	
Ethane	100	0	0	
Ethylene	100	0	0	
Acetylene		0		0
Carbon monoxide	50	0	0	
Methanol	1,300	1,300	500	500
Ethanol	800	800	400	400
Formate	250	250	100	100
Formaldehyde	600	600	500	500

[a] Cultures of strain 41 pregrown on methane (CH_4 grown) or methanol (CH_3OH grown) were centrifuged and suspended in 0.03 M sodium phosphate buffer, pH 6.8. The course of the oxygen uptake was recorded for 5 min with an oxygen electrode with ($+C_2H_2$) and without ($-C_2H_2$) acetylene at a final concentration of 1%. Substrates were added by injection into the suspension phosphate buffer with 1% of substrate dissolved (liquid and solid substrates) or saturated with 100% of the gases. Rates of oxygen uptake are expressed as percentages of increase in oxygen uptake over the endogenous rate.

oxygen uptake was independent of the oxygen concentration (Fig. 6d). The inhibition of oxygen uptake after the addition of acetylene was taken as a measure of the inhibition of methane oxidation. Due to the uptake of acetylene, only the deviation during the first 3 min after acetylene addition could be used for the purpose of this experiment (Fig. 6f). The result of eight measurements of inhibition of methane-dependent oxygen uptake by two different concentrations of acetylene at varying methane concentrations is shown in Fig. 6g. The graph obtained is consistent with a competitive pattern of inhibition. An apparent K_m of 0.5 μl/liter for acetylene in the gas phase at 30 C can be deduced from Fig. 6h.

Other alkane-utilizing bacteria. Inhibition of growth on methane by acetylene was not restricted to strain 41. Fifteen other strains of methane-oxidizing bacteria isolated from soil and water, including *Methylosinus* as well as *Methylomonas* types, were cultivated on slants of MS medium with 10% methane in desiccators with and without 50 μl of acetylene per liter. The strains grew only in the absence of acetylene.

The study of the inhibition of cell metabolism by acetylene has been extended to bacteria utilizing straight-chain hydrocarbons. Five such bacteria were isolated from soil. Two of them (strain 3b and H12) were capable of fixing atmospheric nitrogen. Except strain H12, growth at the expense of lower hydrocarbons was prevented by acetylene. With hexadecane and decane, or with nonhydrocarbon substrates, acetylene did not influence growth (Table 2).

Because the oxidation of ethane by strain 3b was inhibited by acetylene, no appreciable ethylene formation from acetylene was found with ethane as substrate. After the addition of acetaldehyde, presumably an intermediary product in the breakdown of ethane, ethylene was formed (Fig. 7). Strain H12 actively reduced acetylene with butane as its energy source, as

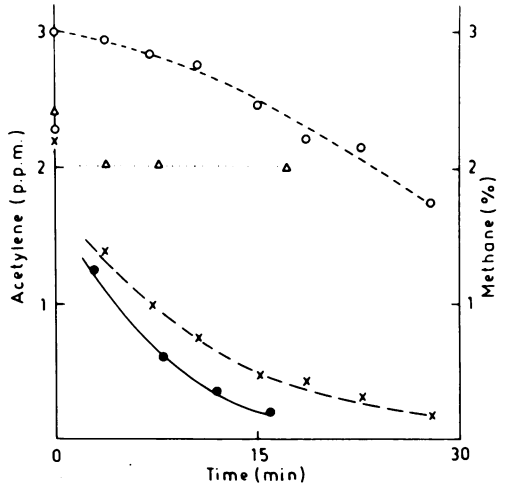

FIG. 5. *Uptake of acetylene by strain 41 grown on CH_4 or on CH_3OH. Cultures of 250 ml were centrifuged and suspended in 10 ml of 0.03 M sodium phosphate buffer, pH 6.8. The suspensions were injected into 100-ml Erlenmeyer flasks containing C_2H_2 in air. Methane-grown cells in the presence (×) or absence (●) of CH_4; concentration of CH_4 (○); methanol-grown cells (△).*

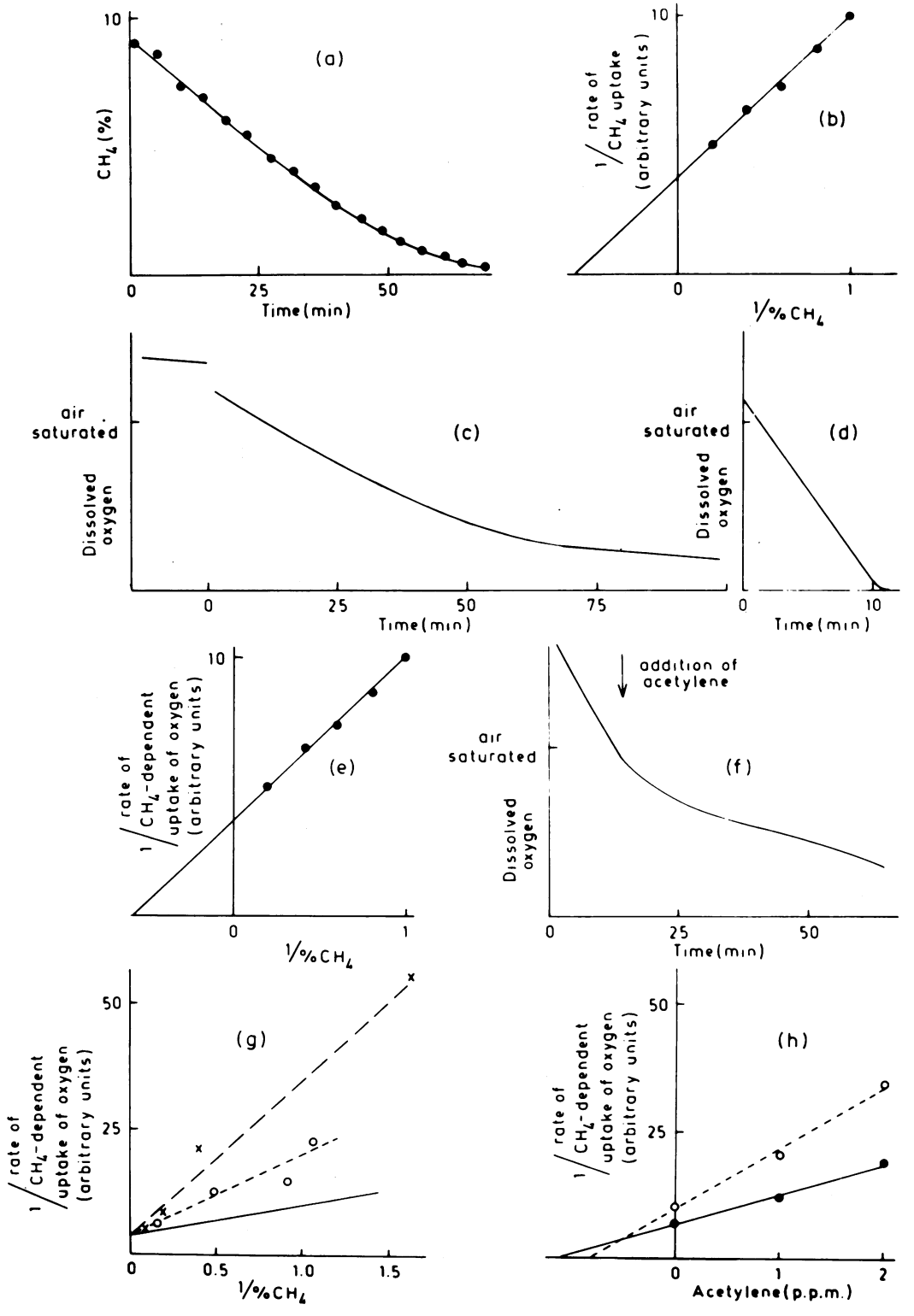
(a)
CH_4 (%)
Time (min)
(b)
1/ rate of CH_4 uptake (arbitrary units)
1/% CH_4
(c)
air saturated
Dissolved oxygen
Time (min)
(d)
air saturated
Dissolved oxygen
Time (min)
(e)
1/ rate of CH_4-dependent uptake of oxygen (arbitrary units)
1/% CH_4
addition of acetylene
air saturated
(f)
Dissolved oxygen
Time (min)
(g)
1/ rate of CH_4-dependent uptake of oxygen (arbitrary units)
1/% CH_4
(h)
1/ rate of CH_4-dependent uptake of oxygen (arbitrary units)
Acetylene (p.p.m.)

TABLE 2. *Inhibition of growth of hydrocarbon-utilizing bacteria by acetylene*[a]

Organism	Growth substrates																			
	Methane		Ethane		Propane		Butane		Hexane		Decane		Hexadecane		Methanol		Ethanol		YEG	
	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$	$-C_2H_2$	$+C_2H_2$
41	+	−	−												+	+			−	
M. vaccae	−		+	−	+	−			+	−	+	+	+	+						
Et32			+	−			+	−	+	−			+	+			+	+	+	+
H2			−		−				+	−	+	+	+	+			−		+	+
E20			+	−	+	−	+	−	+	−	+	+	+	+			+	+	+	+
3b	−		+	−			+	−	−				−				+	+	+	+
H12			−				+	+	+	+	+	+	−						+	+

[a] Organisms were grown on the respective substrates and streaked onto slants of MS medium and slants of a yeast extract-glucose medium (YEG). The slants were placed in sealed 1-liter Erlenmeyer flasks in the absence ($+C_2H_2$) or presence ($-C_2H_2$) of 10% acetylene. Substrates were given to the MS medium slants at concentrations of 10% (gases), as 10 ml of a 1% solution in water in the flasks (alcohols), or by putting a drop of the substrate onto the slants (higher alkanes). Growth (+) or no growth (−) was recorded after 3 weeks of incubation.

could be anticipated from its capacity to grow on this substrate in the presence of acetylene.

DISCUSSION

Co-oxidation of ethylene does not cause the failure of the C_2H_2-C_2H_4 test with the nitrogen-fixing strain 41 of the *Methylosinus* type when acetylene is supplied with methane as the only carbon and energy source. Co-oxidation of ethylene is completely prevented in the presence of acetylene. Acetylene not only completely prevents the co-oxidation of ethylene, but it also inhibits very strongly the oxidation of methane. Thus the supply of energy and reducing power to the nitrogenase, needed for the reduction of acetylene, is impeded. Moreover, if no substrate is available to the bacterium, the nitrogenase presumably is insufficiently protected from oxygen. The bacterium may overcome these obstacles when it is given methanol or other substrates that are oxidized in the presence of acetylene. Results supporting these conclusions have recently been reported by Whittenbury et al. (14) who also found that the use of methane as an electron donor in the C_2H_2-C_2H_4 test led to negative results.

Experiments with whole-cell suspensions showed that acetylene is a competitive inhibitor for methane. However, results of experiments of this type should be treated with caution. The physiological response of the bacterium towards different levels of methane and acetylene was recorded. This response does not need to coincide with the action of methane hydroxylase since, apart from methane, the enzyme depends upon oxygen and presumably nicotinamide adenine dinucleotide, reduced form (NADH), for its catalytic activity. The level of oxygen does not interfere with the physiological response to methane oxidation. The actual NADH level in the bacterial cell during the experiments is unknown but will probably vary with varying methane concentrations or after the addition of acetylene. It is uncertain

FIG. 6. *Inhibition of methane oxidation by acetylene. A culture of strain 41 pregrown on methane in MS medium was centrifuged and suspended in 0.03 M sodium phosphate buffer, pH 6.8. Approximately 0.5 ml of the suspension was placed in the oxygen-electrode cell together with appropriate amounts of buffer solution saturated with CH_4 or O_2 to give the desired concentrations of the gases in the solution. The final volume was brought to 10 ml with buffer solution. Concentrations of CH_4 and C_2H_2 in solution are expressed as concentrations in the atmosphere in equilibrium with the solution. Methane concentration in the liquid phase was followed by periodically taking 2.5-μl samples from the cell for gas chromatographic analyses (a). From this progress curve, tangents at different CH_4 concentrations were used to obtain a Lineweaver-Burk reciprocal plot (b). The same procedure was used to find the apparent K_m for methane using the oxygen-electrode trace after correcting for endogenous respiration (c). The actual CH_4 concentrations needed for the reciprocal plot (e) were known from the methane progress curve (a). Independence of CH_4 oxidation of O_2 concentration was demonstrated with a suspension that was not limited in methane (d). Inhibition by C_2H_2 of CH_4-dependent O_2 uptake was found by injecting buffer solution in equilibrium with 1,000 μl of C_2H_2 per liter into the cell. (f) shows the oxygen trace as affected by 30 μl of C_2H_2-containing buffer; the arrow indicates the addition of C_2H_2. The solid line in (g) represents the result obtained in (e). Taking this line as a standard, points have been calculated by measuring the slope of the oxygen trace before and after the addition of 10 μl (○) or 20 μl (×) of C_2H_2-containing buffer at different CH_4 concentrations. Replotting values of (g) for 1% CH_4 (○) and 2% CH_4 (●) produces (h).*

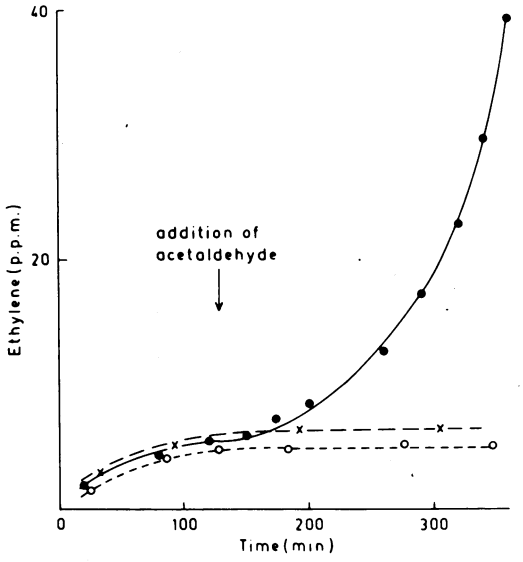

FIG. 7. *Acetylene reduction by strain 3b as affected by ethane and acetaldehyde. Portions (10 ml) of a culture growing on ethane in nitrate-free MS medium were injected into 100-ml Erlenmeyer flasks containing 10% C_2H_2, 2% O_2, 10% C_2H_6, and 78% N_2 (○); (×) C_2H_6 omitted, 88% N_2; (●) 5 μl of acetaldehyde injected as indicated by the arrow.*

what influence this varying NADH level exerts on methane hydroxylase activity, but the straight line in the Lineweaver-Burk plot, obtained from the progress curve for methane oxidation, is encouraging. Oxygen uptake, dependent on the concentration of the substrate, has been employed previously for establishing the apparent K_m for methane (8). The value obtained was approximately 1%, which is of the same order as the apparent K_m of 1.7% found in the present investigation. If this method of assessing an apparent K_m value is acceptable, then the measurement of an apparent K_i performed in this way should be possible.

Acetylene is an extremely effective inhibitor of methane oxidation. The K_i value of 0.5 μl/liter in the gas phase represents a concentration as low as approximately 0.02 μM C_2H_2 in solution (6). Specific inhibitors of methane hydroxylase have been searched for by Hubley et al. (9), but acetylene or substrates co-oxidizable by the hydroxylase (except carbon monoxide) were not considered as likely candidates. These co-oxidizable substrates would presumably also act as competitive inhibitors for methane, albeit with a much higher K_i than acetylene. Ethane, for instance, may inhibit the growth of methane-oxidizing bacteria (13). The fate of acetylene was not studied in the present investigation. The situation with strain 41 and the other methane-oxidizing bacteria tested was also met with the bacteria growing on lower hydrocarbons. Only the enzyme system involved in the first attack on the hydrocarbon was inhibited by acetylene. Growth upon long-chain hydrocarbons was not inhibited by acetylene, suggesting that the inhibition of the alkane hydroxylase by acetylene depends on the size of the substrate molecule. This indicates the existence of a direct relation between substrate and inhibitor with respect to the hydroxylase. The type of inhibition is probably the same as that found for the methane-oxidizing bacteria. Strain H12 was an exception, because it was not inhibited by acetylene, even when it was growing on butane.

The experiments show that the C_2H_2-C_2H_4 test cannot be employed for measuring nitrogenase activity in methane-utilizing bacteria or in bacteria utilizing lower hydrocarbons when alkanes are the sole energy source. Nitrogenase activity can be measured by growing these bacteria on carbon sources other than the alkanes. However, this may raise a problem with methane-oxidizing bacteria since methanol, the only alternative carbon source, is toxic to some strains when supplied at substrate-level concentrations (13). Nitrogenase activity of methane-grown cells can only be measured by supplying the organism during the test with a different energy source, but the linear rate of ethylene production was not always reproducible. Furthermore, in this way it is impossible to measure nitrogenase activity quantitatively.

In natural habitats, the contribution to the fixation of atmospheric nitrogen by methane-oxidizing bacteria and bacteria using lower hydrocarbons is overlooked when using the C_2H_2-C_2H_4 test. Preliminary experiments in which small amounts of methanol or formate were added to samples from various habitats to reveal possible nitrogen fixation by methane-oxidizing bacteria were not successful. Scaling up the activity of these organisms by incubating water samples under methane and then applying the test with methanol or formate added has given inconsistent results. As a consequence, nitrogenase activity should not be measured with the C_2H_2-C_2H_4 test in natural habitats where bacteria utilizing methane or lower hydrocarbons are present. Nitrogenase activity could be measured with $^{15}N_2$.

The strong effect of acetylene upon the metabolic activities of the bacteria studied calls for a careful approach when employing the C_2H_2-C_2H_4 test. Other metabolic activities already found to be affected by acetylene include the production of methane (4, 10) and the growth of *C. pasteurianum* (1).

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