Acetylene as an Inhibitor of Methanogenic Bacteria

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Growth of six pure cultures of methanogens was inhibited by low concentrations of dissolved acetylene (C_2H_2); other archaebacteria (three *Halobacterium* species) and several eubacteria were not similarly affected. The minimum concentration of dissolved C_2H_2 required to inhibit growth of *Methanospirillum hungatei* completely was about 8 µM; dissolved ethylene at 20 µM had little effect on growth. Dissolved acetylene (33 µM) did not alter the E_h of the medium, or result in a loss in viability of *M. hungatei* after 16 h exposures. In anaerobic cell extracts of *M. hungatei*, activities of hydrogenase, NADP reductase, methyl-coenzyme M reductase and ATP hydrolase were not inhibited by C_2H_2 concentrations several times higher than those required for growth inhibition. The intracellular ATP content of all of the methanogens dropped dramatically on exposure to C_2H_2. Moreover, cells of *M. hungatei* and *Methanobacterium bryantii* on exposure to C_2H_2 lost their ability to maintain a transmembrane pH gradient. We suggest that exposure to C_2H_2 results in a decline in methanogen functions which require a H^+-flux, including ATP synthesis, Ni^{2+} uptake and methanogenesis.

INTRODUCTION

Use of the acetylene (C_2H_2) reduction assay as a valid quantitative measure of N_2-fixation depends on C_2H_2 serving solely as an inhibitor of nitrogenase activity. Often this criterion is not met by the test system, since C_2H_2 can interact in some cases at other sites. For example, growth of nitrogenase-repressed *Clostridium pasteurianum* proved sensitive to C_2H_2 (Brouzes & Knowles, 1971). Also, C_2H_2 served as a competitive inhibitor of CH_4 oxidation by methylotrophs and inhibited the growth of several bacteria normally capable of growth on lower molecular weight hydrocarbons (deBont & Mulder, 1976). Furthermore, the nitrous oxide reductase of several denitrifying bacteria (Balderston *et al.*, 1976; Yoshinari & Knowles, 1976), and ammonia oxidation to nitrite in *Nitrosomonas europaea* (Hynes & Knowles, 1978) were sensitive to C_2H_2. At high C_2H_2 concentrations of 4 to 30% (v/v) in the gas phase, inhibition of hydrogen uptake was observed in *Azospirillum brasiliense* (Chan *et al.*, 1980), *Azotobacter chroococcum* (Smith *et al.*, 1976) and several cyanobacteria (Bothe *et al.*, 1977; Lambert & Smith, 1980). Finally, the reversible hydrogenase of *Klebsiella pneumoniae* was also sensitive to inhibition by acetylene (Smith *et al.*, 1976).

In view of the observed inhibition of hydrogenase activity described above, and the fact that, with few exceptions, methanogenic bacteria require H_2 for growth (Zehnder *et al.*, 1980), it seemed reasonable that methanogens should be sensitive to C_2H_2. Indeed, methanogenesis was inhibited by C_2H_2 in anaerobic sediments (Macgregor & Keeney, 1973; Oremland & Taylor, 1975), anaerobic paddy soils (Raimbault, 1975) and rumen fluid (Elleway *et al.*, 1971). We now show for several pure cultures of methanogens that C_2H_2 does not interfere with hydrogenase activity, but with membrane events linked to proton movement and ATP synthesis.

METHODS

Micro-organisms and growth. Cultures of methanogens were obtained from sources already described (Sprott *et al.*, 1979). Synthetic anaerobic media were prepared and dispensed in 10 ml or 100 ml amounts in vessels closed

with butyl rubber stoppers (Sprott & Jarrell, 1981). Bacteria were grown with a CO_2/H_2 gas phase in the S medium of Breuil & Patel (1980), with the exceptions that *Methanospirillum* cultures required acetate (25 mM) and *Methanobacterium bryantii* medium contained additional phosphate, nickel, molybdenum, and NH_4-N (Jarrell *et al.*, 1982). Daily replenishment of the CO_2/H_2 gas atmosphere and the other growth conditions were as described by Sprott & Jarrell (1981). Other cultures were obtained from the culture collection of the National Research Council of Canada. *Halobacterium cutirubrum* (NRCC 34001), *H. halobium* (NRC 34003) and *H. salinarium* (NRC 34002) were grown in the medium of Seghal & Gibbons (1960); *Escherichia coli* SP10 in medium 63 (Prasad & Schaefler, 1974) containing glycerol (0·4%, v/v) (Cohen & Rickenberg, 1956) and Casamino acids (0·02% w/v); *Clostridium pasteurianum* (NRC 2485) in Reinforced Clostridial Medium (Oxoid) containing 340 μg N ml^{-1} as $(NH_4)_2SO_4$; *Desulfovibrio desulfuricans* (NRC 49001, ATCC 7757) in medium N of Saunders *et al.* (1964) reduced with 1 mM-sodium sulphide plus 1·6 mM-cysteine; and *Arthrobacter globiformis* (NRC 32001) in Nutrient Broth (Difco). Anaerobic media were boiled under a CO_2/H_2 gas atmosphere (1:4, v/v; Union Carbide Canada) for *C. pasteurianum* or N_2 for *D. desulfuricans*, and 10 ml samples dispensed into 120 ml serum bottles (closed with butyl rubber stoppers) containing the respective gas phase. All cultures were grown at 35 °C with shaking, except for *Methanobacterium thermoautotrophicum* which was grown at 62 °C. Before use, the CO_2/H_2 gas mixture was passed through copper filings (350 °C) to remove possible traces of oxygen.

Gas chromatography. Methane, C_2H_2 and C_2H_4 were separated on a Porapak T column using the conditions described by Jarrell *et al.* (1982).

Intracellular ATP concentrations. Methanogens grown in 100 ml cultures (analysed when half-way through the exponential phase, as determined by A_{600}) were dispensed in 10 ml samples into 120 ml serum bottles containing CO_2/H_2 (1:4, v/v). Various amounts of acetylene (Union Carbide Canada; 99·5% purity) were injected with an air-tight Hamilton syringe through the serum stoppers and incubation continued with shaking at the growth temperature for the test organism. Periodically, 1·0 ml samples were withdrawn anaerobically by syringe and injected into 1·0 ml ice-cold 12% (v/v) $HClO_4$. After 30 min the solution was carefully neutralized with 0·72 M-KOH and 0·16 M-$KHCO_3$. ATP was measured in 1·0 ml reaction mixtures containing a luciferin–luciferase preparation (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and 10 μl cell extract. Quenching of about 18% was corrected for by including 10 μl of an extract of growth medium in reaction mixtures containing known amounts of ATP. Intracellular ATP was calculated on the basis of cell dry weight. For *Methanospirillum hungatei* the cytoplasmic volume was determined as 2·01 μl (mg dry wt cells)$^{-1}$ (Sprott & Jarrell, 1981), thus allowing calculation of the internal concentration of ATP.

Penetration of labels. Penetration of [^{14}C]sucrose and [^{14}C]glucose into anaerobic methanogen suspensions was as described by Sprott & Jarrell (1981). Similarly, methods to measure the accumulation of the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO) for calculation of intracellular pH (pH_i) of methanogens have been reported (Jarrell & Sprott, 1981). Briefly, for accumulation of DMO, the bacteria were centrifuged anaerobically, the pellets resuspended in 17 mM-citrate/44 mM-sodium phosphate buffer (pH 6·0) prereduced with cysteine (1·27 mM) plus sodium sulphide (0·83 mM), and 5 ml samples of the bacterial suspension were dispensed into 120 ml serum bottles under a CO_2/H_2 gas phase (1:4, v/v). After incubation with or without C_2H_2 for 4 h at 35 °C with shaking, [^{14}C]DMO was injected. Following a 20 min incubation, the contents of each bottle were transferred anaerobically by syringe into an anaerobic glass centrifuge tube and immediately centrifuged (10000 *g*, 10 min, 25 °C). The distribution of DMO between the cell pellet and supernatant was determined and pH_i calculated (Jarrell & Sprott, 1981).

Uptake of ^{63}Ni. Samples (20 ml) of exponential phase *Methanospirillum hungatei* GP1, grown in 100 ml culture to 0·35 mg dry weight ml^{-1}, were dispensed with plastic disposable syringes (flushed with CO_2/H_2) into 120 ml serum vials under CO_2/H_2 (1:4, v/v). The experiment was started by addition of C_2H_2 (0·2 ml) to the vials which were incubated at 35 °C, with shaking. Samples were removed anaerobically to determine the ATP concentration and for ^{63}Ni uptake measurements. For ^{63}Ni uptake, 25 μl $^{63}NiCl_2$ [1·6 μM, 629 μCi μmol^{-1} (23·27 MBq μmol^{-1}); New England Nuclear Canada, Quebec] was added to 2·5 ml cell suspension in a 60 ml serum bottle under a CO_2/H_2 gas atmosphere (1:4, v/v). Uptake, which was linear for at least 15 min, was terminated by filtering 1·0 ml samples through 0·45 μm HA filters (Millipore). Filters were washed with 5 ml 10 mM-potassium phosphate buffer (pH 7·0), and counted in Aquasol (New England Nuclear). ^{63}Ni uptake was very similar when the filter assay was compared to anaerobic centrifugation with correction for extracellular ^{63}Ni present in the cell pellet (Sprott & Jarrell, 1981). Dry weights were determined by washing the cells with water and drying the cell pellet at 62 °C to a constant weight.

Cell viability of Methanospirillum hungatei. The number of methanogen cells which remained viable after exposure to C_2H_2 was determined by colony counts. The appropriate liquid medium containing 1·5% (w/v) agar was prereduced and, while still liquid, 10 ml samples were dispensed into 120 ml serum bottles as for liquid medium preparation. Bacterial culture dilutions were made in serum bottles which contained prereduced liquid medium at 23 °C. Volumes (0·4 ml) of the appropriate dilutions were mixed with prereduced growth medium containing 0·6% (w/v) agar (3 ml, 46 °C) and 0·6 ml was added anaerobically to the solidified medium as an overlay. Colonies were counted after 14 d incubation at 35 °C.

Oxidation–reduction potential. The E_h of methanogen media was determined with a platinum electrode as described by Patel & Agnew (1981).

Cofactor preparation. F_{420} is a fluorescent compound synthesized by methanogens and apparently serving *in vivo* as the coenzyme for several oxidation–reduction reactions (Gunsalus & Wolfe, 1980). F_{420} was isolated from *Methanospirillum hungatei* (McKellar *et al.*, 1981). Extracts of *Methanospirillum hungatei* were used to prepare component B as described by Gunsalus & Wolfe (1980). Methyl-coenzyme M was prepared from CH_3I and 2-mercaptoethanesulphonic acid (Pierce Chemical Co., Rockford, Ill., U.S.A.) according to Gunsalus *et al.* (1978).

Preparation of bacterial extracts. Hydrogenase, $NADP^+$ reductase and methyl-coenzyme M reductase were measured in anaerobic spheroplast lysates of *Methanospirillum hungatei* (Sprott *et al.*, 1979). *Methanospirillum hungatei* GP1 grown to late-exponential phase as a 100 ml culture was harvested anaerobically under N_2 (Sprott & Jarrell, 1981). Cells were washed once with anaerobic 50 mM-potassium phosphate buffer (pH 6·8) (prepared by boiling under N_2, adding cysteine/sodium sulphide and dispensing in serum bottles as for growth medium preparation) and were resuspended in 5 ml of this buffer containing 10 mg dithiothreitol; sufficient NH_4OH was added to raise the pH to 9·0. As judged by phase-contrast microscopy, more than 90% of the cells lysed within 30 min. The pH was readjusted to 7·0 by injecting HCl through the serum stopper and 1 mg DNAase-I was added (Worthington Biochemical Corp.). Extracts were stored in serum bottles under H_2 or N_2 by immersion in liquid nitrogen, or used within several hours of preparation. Protein was estimated with the Bio-Rad dye binding assay reagent, using bovine serum albumin as standard.

Hydrogenase. The reaction mixture (2 ml), in spectrophotometer cuvettes flushed with H_2, consisted of 100 μmol phosphate buffer (pH 6·8) reduced with cysteine/sulphide, and either F_{420} sufficient to give an A_{420} (1 cm path length) of 0·7 or benzylviologen (1 μmol). Anaerobic extract was injected through the rubber stopper to start the reaction. Reaction rates were recorded at 23 °C at 420 nm (F_{420}) or 578 nm (benzylviologen). To determine C_2H_2 effects, the enzyme preparation was preincubated with C_2H_2 for 1 h followed by injection into the assay mixture, which contained the same C_2H_2 concentration as used in the preincubation.

Methyl-coenzyme M reductase. H_2-dependent formation of CH_4 was measured in small (2·1 ml) serum bottles (Gunsalus & Wolfe, 1980). The reaction mixture (0·5 ml) consisted of 17 μmol potassium phosphate buffer (pH 6·8) prereduced with cysteine/sulphide, 10 μmol Mg^{2+}, 2 μmol ATP, 20 nmol F_{420}, 10 μl component B preparation, 2 μmol methyl-coenzyme M and 0·5 mg (protein) spheroplast lysate. The reaction components under N_2 were incubated at 35 °C for 10 min to consume endogenous C_1 units (Ellefson & Wolfe, 1980), methyl-coenzyme M was added (and in some cases C_2H_2) and incubation continued under H_2. Sampling of the gas phase (100 μl) established that the rate of CH_4 synthesis remained linear between the 0·5 and 1 h tests.

F_{420}-NADP$^+$-reductase. An anaerobic assay (2 ml) similar to that of Yamazaki *et al.* (1980) was used. The assay (N_2) consisted of 95 μmol prereduced potassium phosphate buffer (pH 6·8), 10 μmol Mg^{2+}, 20 nmol F_{420} and 60 μg spheroplast lysate protein. When required, C_2H_2 was added anaerobically as previously described. The reaction was initiated by injection of 1 μmol $NADP^+$. Reduction of $NADP^+$ was measured at 340 nm at 23 °C.

ATP hydrolase. A membrane fraction of *Methanospirillum hungatei* was prepared as described by Doddema *et al.* (1978). The reaction mixture (1·0 ml) in 10 ml serum bottles closed with butyl rubber seals contained 100 μmol glycine/NaOH buffer (pH 8·0), 8 μmol $MgCl_2$, extract (1·0 mg protein) and various amounts of C_2H_2. After 1 h at 35 °C, 0·02% (w/v) Triton X-100 and 5 μmol $[\gamma\text{-}^{32}P]ATP$ (New England Nuclear) were added. Incubation continued for 20 min, after which the reaction was stopped with cold $HClO_4$ and unreacted ATP removed by adsorption on Norite A charcoal (Crane & Lipmann, 1953). $^{32}PO_4$ released from ATP was counted in Aquasol. Boiled extract served as a blank.

C_2H_2 concentrations. Concentrations of C_2H_2 dissolved in the liquid phase were calculated according to Flett *et al.* (1976).

Cell growth. Growth was measured as A_{600} in cuvettes of 1 cm path length. Resazurin in samples of culture media was reduced with dithionite before measurements were made.

RESULTS

Rates of growth and CH_4 synthesis were similarly inhibited in cultures of *Methanospirillum hungatei* incubated with C_2H_2. Complete inhibition of growth and CH_4 synthesis was found at 8 μM-C_2H_2 in the liquid phase (Fig. 1*a*). No loss of C_2H_2 from the gas phase or formation of ethylene (C_2H_4) was detected. The inhibition is specific to C_2H_2, since C_2H_4 at concentrations up to 20 μM (0·45%, v/v, in the gas phase) had no effect on growth and only a small effect on CH_4 synthesis (Fig. 1*b*). Ethylene was similarly less inhibitory than C_2H_2 for methanogenesis in sediments, but did inhibit at high concentrations of 5 to 20% (v/v) in the gas phase (Oremland & Taylor, 1975).

Since oxygen is an inhibitor of the growth of methanogens, it was important to exclude it as a possible contaminant in the C_2H_2. Measurements of oxidation–reduction potential of the
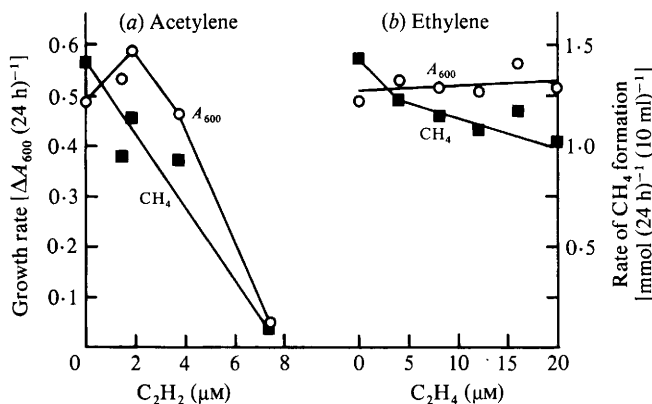
Fig. 1. Concentration dependence and specificity for acetylene inhibition of growth rate (○), and methanogenesis (■). Results are shown for intact cells of *Methanospirillum hungatei* suspended in synthetic acetate medium (E_h −250 mV). Rates were determined by taking readings during growth, with slopes taken from the linear portion of the curves.

medium (E_h of −250 mV) showed no effects of C_2H_2 at concentrations up to 650 μM (2·23 ml added to a 120 ml bottle containing 10 ml of medium). Since, in separate experiments, at least 35 μl O_2 was required to lower the E_h of the medium, the maximum O_2 concentration present in the C_2H_2 could be 1·5% (v/v). With C_2H_2 additions of 8 μM this corresponds to a maximum amount of O_2 of 0·4 μl, or between 25- and 50-fold less than was required to inhibit the growth of *Methanospirillum hungatei*. As a second approach, it was found that the oxygen-sensitive enzyme hydrogenase was not inhibited by large amounts of C_2H_2 (see below).

Exponential growth and maximum rates of CH_4 synthesis occurred in control cultures between 24 and 48 h after inoculation. Since rates of growth and CH_4 synthesis are reported from cultures in the exponential phase (Fig. 1), it follows that the inoculum had been exposed for at least 24 h to C_2H_2. Under these conditions the CH_4 synthesis rate declined to 50% of the maximum when the dissolved C_2H_2 concentration was 4 μM (Fig. 1a). Cell suspensions of *Methanospirillum hungatei* exposed for shorter times of 1 h required a higher concentration of dissolved C_2H_2 (24 μM) to inhibit the CH_4 synthesis rate by 50% (data not shown). Exposure of *Methanospirillum hungatei* to 65 μM-C_2H_2 for 16 h, followed by colony counts in a C_2H_2-free medium, resulted in no loss of cell viability.

Methanospirillum hungatei cells exposed to C_2H_2 showed a decline in intracellular ATP concentration, and this depended on the concentration of C_2H_2 (Fig. 2). An exposure time of 8 h was required before the concentration of intracellular ATP approached very low values at the lower concentrations of dissolved C_2H_2 (15–20 μM). The decline in ATP content could thus explain inhibition in growth, because under these growth conditions *Methanospirillum hungatei* cultures undergo a lag phase of about 24 h (during which time C_2H_2 is present if required) before exponential growth proceeds, with a generation time in this experiment of 13 h.

The decline in intracellular ATP concentration to less than 10% of that present in cultures of *Methanospirillum hungatei* not exposed to C_2H_2 suggested inhibition of ATP synthesis. To test whether C_2H_2 could interfere with the ability of the methanogen to maintain a transmembrane H^+-gradient, the cells were harvested anaerobically and resuspended in anaerobic buffer of pH 6·0. This external pH drop is necessary, since at pH 6·8 there is no ΔpH detectable in *Methanospirillum hungatei* and *Methanobacterium thermoautotrophicum* (Jarrell & Sprott, 1981). This unusual property applies to *Methanobacterium bryantii* strain MoH as well, since DMO was equally distributed between the inside and outside of bacterial cells in growth medium of pH 6·95 (data not shown). *Methanospirillum hungatei* incubated in acidic buffer (CO_2/H_2; 1:4, v/v) maintains only a small pH gradient (Jarrell & Sprott, 1981), which is abolished during a 4 h exposure to 65 μM-C_2H_2 (Table 1). Also shown in Table 1 is the loss in ability of *Methanobacterium bryantii* to maintain a ΔpH during exposure to acetylene.
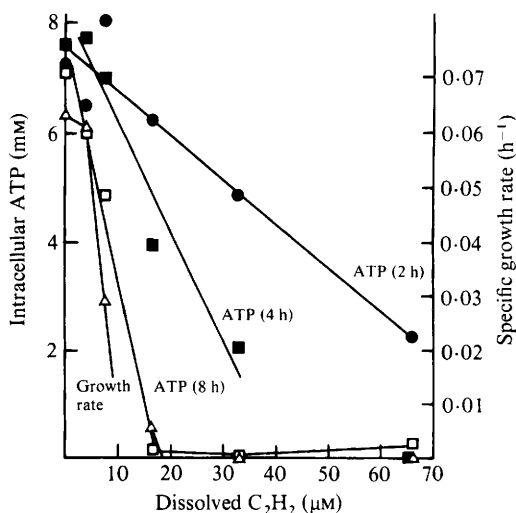
Fig. 2. Decline of intracellular ATP content of *Methanospirillum hungatei*, compared to the decline in growth rate. Intracellular ATP concentrations are shown following exposures to C_2H_2 of 2 h ($\bullet$), 4 h ($\blacksquare$), and 8 h ($\square$). Specific growth rate ($\triangle$) is defined as ln 2.(mean doubling time)$^{-1}$. No ATP was detected in the culture supernatants.

Table 1. *Loss of ability of methanogens to maintain a pH gradient after exposure to acetylene*

Methanospirillum hungatei or *Methanobacterium bryantii* were exposed to C_2H_2 for 4 h or 2 h, respectively, in prereduced buffer at pH 6·0. Uptake of the weak acid [^{14}C]DMO was then determined. Mean values ± S.E. are shown for duplicate (*M. hungatei*) and triplicate (*M. bryantii*) samples. pH_i, internal pH; ΔpH, transmembrane pH difference.

Dissolved C_2H_2 concn (μM)	M. hungatei		M. bryantii	
	pH_i	ΔpH	pH_i	ΔpH
0	6·29	0·25 ±0·01	6·60	0·58 ± 0·05
15	6·28	0·28 ±0·02	ND	ND
30	6·23	0·21 ±0·01	ND	ND
50	6·17	0·17 ±0·01	ND	ND
65	6·00	0·00 ±0·05	6·13	0·11 ± 0·04

ND, not done.

The effect of C_2H_2 on various enzyme activities is shown in Table 2. Cell extracts were exposed for 1 h to concentrations of C_2H_2 at least 10-fold higher than those giving a dramatic decline in intracellular ATP content after 2 h exposures (Fig. 2). None of the enzyme activities tested were inhibited significantly. As reported by Doddema *et al.* (1978) air did not inhibit ATP hydrolysis, since similar rates (and dependence on Triton X-100) were seen in anaerobic spheroplast lysates (data not shown).

Glucose and sucrose penetrated *Methanospirillum hungatei* cells to different degrees: this is thought to reflect barriers at the cytoplasmic membrane and inner cell wall (Sprott & Jarrell, 1981). Exposure of *Methanospirillum hungatei* to C_2H_2 sufficient to abolish maintenance of ΔpH did not render the cytoplasmic membrane permeable to glucose or sucrose (Table 3).

Methanospirillum hungatei cells take up ^{63}Ni, which is incorporated into F_{430} (not shown) as described for *Methanobacterium thermoautotrophicum* (Diekert *et al.*, 1980). ^{63}Ni uptake was only partially inhibited by C_2H_2, and inhibition occurred at a slower rate than did the decline in intracellular ATP concentration (Fig. 3).

The correlation between inhibition by C_2H_2 of growth of *Methanospirillum hungatei* and decline in cellular ATP content can be generalized to include other methanogens (Table 4). No loss of ATP to the medium occurred. Of the methanogens tested, the *Methanobacterium* species
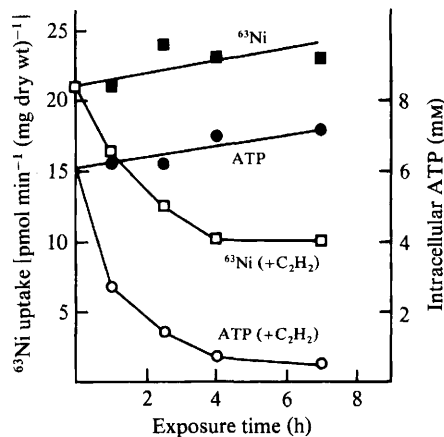
Fig. 3. Effect of dissolved acetylene (65 μM) on ^{63}Ni uptake (□) into cultures of *Methanospirillum hungatei*. Intracellular ATP concentration (○) is shown for comparison. ^{63}Ni uptake (■) and ATP concentration (●) for control cells, which were incubated without C_2H_2, are shown also.

Table 2. *Effect of acetylene on selected enzymes of Methanospirillum hungatei*

Enzymes other than ATP hydrolase were measured in anaerobic spheroplast lysates. ATP hydrolase was assayed in aerobically prepared French pressure cell extracts. Exposures to C_2H_2 were for 1 h at 35 °C.

Enzyme activity*	Dissolved C_2H_2 concn (μM)	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]
Hydrogenase (H$_2$→F$_{420}$)	0	141
	140	106
	710	271
NADP$^+$ reductase	0	22
	710	25
Methyl-coenzyme M reductase	0	2·1
	950	3·6
	1900	3·4
ATP-hydrolase	0	16·6
	900	14·9
	3600	14·7

* Hydrogenase activity was shown to be dependent on H_2. Maximum activity of methyl-coenzyme M reductase required methyl-coenzyme M, H_2, F_{420} and cell-free extract as source of component B. Triton X-100 was necessary in the assay mixture for detection of ATP hydrolase activity.

Table 3. *Penetration of sucrose and glucose into Methanospirillum hungatei after exposure to acetylene*

Labelled compounds were added after 4 h incubation with (or without) 130 μM-C_2H_2. Equilibration with the cytoplasm, without accumulation, corresponds to 100% penetration (gravimetric value).

Dissolved C_2H_2 concn (μM)	Penetration of label (%)	
	Sucrose	Glucose
0	72·3	80·0
130	73·1	80·7

were most sensitive, *Methanospirillum hungatei* intermediate, and *Methanosarcina barkeri* the least sensitive. Eubacteria and the archaebacteria represented by the extreme halophiles were resistant to dissolved C_2H_2 concentrations fivefold higher than those which inhibited the methanogens. However, higher concentrations of C_2H_2 may be inhibitory for growth as was shown for nitrogenase-repressed *C. pasteurianum* (Brouzes & Knowles, 1971).

Table 4. *Effect of acetylene on various bacteria*

The dissolved C_2H_2 concentration was 65 μM for methanogens and 325 μM for extreme halophiles and eubacteria.

Bacterium	Growth rate (% inhibition)	ATP content† (% decline)		
		1 h	3 h	5 h
Archaebacteria				
Methanobacterium bryantii MoH	100	100	100	100
Methanobacterium thermoautotrophicum	100	90	98	100
Methanobacterium strain G2R	100	96	100	100
Methanosarcina barkeri	82	55	67	75
Methanospirillum hungatei JF1	100	54	58	71
Methanospirillum hungatei GP1	100	42	66	87
Halobacterium cutirubrum	0	ND		
H. halobium	1	ND		
H. salinarium	0	ND		
Eubacteria				
Arthrobacter globiformis	0	ND		
Escherichia coli	2	ND		
*Clostridium pasteurianum**	0	ND		
*Desulfovibrio desulfuricans**	0	ND		

ND, Not determined.

* Repressed for nitrogenase by growth with NH_4-N.

† The ATP contents of methanogens not receiving C_2H_2 remained constant during the experiment and, in nmol ATP (mg protein)$^{-1}$, were: *Methanobacterium bryantii*, 3·3; *Methanobacterium thermoautotrophicum*, 7·0; *Methanobacterium* strain G2R, 4·5; *Methanosarcina barkeri*, 3·2; *Methanospirillum hungatei* JF1, 22·6; and *M. hungatei* GP1, 20·0. Under these conditions of assay, the detection limit was about 0·2 nmol ATP (mg protein)$^{-1}$.

DISCUSSION

Acetylene inhibits several functions in methanogens. Inhibition of growth and CH_4 synthesis, and the decline in the intracellular ATP concentration, all show similar kinetics of inhibition dependent on the exposure time and concentration of C_2H_2 used. Inhibitions which require exposures of several hours to the low inhibitor concentrations suggest that penetration to the sensitive site(s) may be a rate-determining step, or that the rate of reaction between C_2H_2 and the site of inhibition is slow. In attempts to investigate the mechanism of inhibition further, we found that the ability of methanogens to maintain a small proton gradient at an acidic external pH of 6·0 (Jarrell & Sprott, 1981) was greatly impaired by C_2H_2. Gross membrane damage did not occur, since the lack of penetration of glucose and sucrose past the cytoplasmic membrane of *Methanospirillum hungatei* remained unaltered after exposure to C_2H_2. This mode of inhibition by C_2H_2 seems limited so far to the methanogens. The growth of several extremely halophilic bacteria, which are taxonomically related to the methanogens (Woese *et al.*, 1978), was not inhibited (Table 4).

The effect of C_2H_2 on intracellular ATP concentrations appears to result from interference in proton movements. A chemiosmotic mechanism appears to account for all of the ATP synthesized by methanogens. As evidence, synthesis of ATP was demonstrated following a rapid pH shift in *Methanosarcina barkeri* (Mountfort, 1978) and *Methanobacterium thermoautotrophicum* (Doddema *et al.*, 1978). This synthesis was shown, in the case of *Methanosarcina barkeri*, to be inhibited by an ATPase inhibitor and by uncouplers (proton conductors). ATP is normally synthesized by methanogens in growth media of pH 6·8–7·0, where the membrane potential is the principal component of the electrochemical proton gradient (Jarrell & Sprott, 1981). This is possible, since Wilson *et al.* (1976) have shown net synthesis of ATP in *E. coli* in response to a membrane potential in the absence of a ΔpH. Acetylene caused a decline in intracellular ATP from methanogens with no loss of ATP into the growth media, and prevented the maintenance of a ΔpH in cells suspended in anaerobic buffer of pH 6·0. These results suggest that ATP

synthesis is inhibited through the effect of C_2H_2 on the flux of protons across a membrane.

An inability to maintain a transmembrane pH difference could be caused either by induced membrane leakiness to protons, or to inhibition of active proton extrusion from the cell. Although the proton pump of methanogens has not been identified, likely candidates are the hydrogenase (F_{420}-linked), where the proton gradient may exist between the cytosol and intracellular vesicles (Spencer *et al.*, 1980), or through ATP hydrolysis (Mitchell, 1975). No evidence was found for inhibition of these theoretical proton pumps. However, more direct evidence is required to determine unequivocally whether proton extrusion from the cell or loss of membrane permeability to protons occurs. Because C_2H_2 stimulates hydrogenase activity (Table 2), some interaction or conformational change of the enzyme may have occurred to give a more active enzyme which no longer pumps protons.

Evidence is increasing to support the suggestion that CO_2 reduction to CH_4 occurs at the site of intracytoplasmic membranes, with energy input supplied by an energized membrane state (Spencer *et al.*, 1980; Kell *et al.*, 1981*a*; Sauer *et al.*, 1981). We believe the uptake of DMO which occurs into intact methanogen cells under appropriate conditions (Jarrell & Sprott, 1981) reflects an H^+-gradient across the cytoplasmic membrane (and not across the intracellular membranes). Firstly, at the pH of the growth medium where DMO uptake does not occur (Jarrell & Sprott, 1981) the cells are still able to synthesize CH_4 and ATP. Clearly, if these processes depend on a pH gradient across the intracytoplasmic membranes, this gradient is not detected by DMO uptake into intact cells. Secondly, if the intracytoplasmic membranes are acidic inside with respect to the cytoplasm, as suggested by Sauer *et al.* (1981), the weak acid DMO would not accumulate inside these vesicles. Finally, the inside volume of any intracytoplasmic vesicles would be relatively small compared to the cytoplasmic volume, making detection of this intravesicular pH gradient difficult. It is possible, however, that C_2H_2 can also abolish a ΔpH across internal vesicles and thus account for inhibition of CH_4 synthesis.

Uptake of Ni^{2+} by *Methanospirillum hungatei* was identified as an additional membrane event affected by C_2H_2. Although the energetics of Ni^{2+} transport in methanogens is unknown, the ion is essential to the growth of *Methanobacterium thermoautotrophicum* (Schönheit *et al.*, 1979) and *Methanobacterium bryantii* (Jarrell *et al.*, 1982).

Use of C_2H_2 to assess N_2-fixing activity of a complex system, such as a water-logged soil, could result in inhibition of methanogens. This would be especially acute at the high concentrations of C_2H_2 (10–20%, v/v) in the gas phase normally employed in the assay. As a result, products of metabolism normally utilized by methanogens (i.e. H_2, CO_2, acetate or formate) may possibly accumulate to levels inhibitory to N_2-fixing microbes. It is known that nitrogenase is inhibited competitively by H_2, showing 50% inhibition at 11% (v/v) H_2 (Hwang *et al.*, 1973), and that weak acids such as acetate may uncouple energy conservation (Kell *et al.*, 1981*b*).

Most of the reports dealing with C_2H_2 inhibition (see Introduction) have employed C_2H_2 concentrations of about 10% (v/v) to assess the validity of the acetylene reduction method. Some of these inhibitions require high C_2H_2 concentrations. For example, growth of *C. pasteurianum*, repressed for nitrogenase activity, was inhibited by 10% (v/v) C_2H_2 (Brouzes & Knowles, 1971), but not by 0·91% (this study). Methanogens (Raimbault, 1975; this study) and *Nitrosomonas europaea* (Hynes & Knowles, 1978) appear unusual in their susceptibility to low concentrations of C_2H_2. Growth of *Methanosarcina barkeri* is inhibited by C_2H_2 (Raimbault, 1975), although this organism was the least sensitive (Table 4) of several methanogens tested.

REFERENCES

BALDERSTON, W. L., SHERR, B. & PAYNE, W. J. (1976). Blockage by acetylene of nitrous oxide reduction in *Pseudomonas perfectomarinus*. *Applied and Environmental Microbiology* **31**, 504–508.

DE BONT, J. A. M. & MULDER, E. G. (1976). Invalidity of the acetylene reduction assay in alkane-utilizing, nitrogen-fixing bacteria. *Applied and Environmental Microbiology* **31**, 640–647.

BOTHE, H., TENNIGKEIT, J. & EISBRENNER, G. (1977). The utilization of molecular hydrogen by the blue-

green alga *Anabaena cylindrica. Archives of Microbiology* **114**, 43–49.

BREUIL, C. & PATEL, G. B. (1980). Composition of *Methanospirillum hungatei* GP1 during growth on different media. *Canadian Journal of Microbiology* **26**, 577–582.

BROUZES, R. & KNOWLES, R. (1971). Inhibition of growth of *Clostridium pasteurianum* by acetylene: implication for nitrogen fixation assay. *Canadian Journal of Microbiology* **17**, 1483–1489.

CHAN, Y. K., NELSON, L. M. & KNOWLES, R. (1980). Hydrogen metabolism of *Azospirillum brasiliense* in nitrogen-free medium. *Canadian Journal of Microbiology* **26**, 1126–1131.

COHEN, G. N. & RICKENBERG, H. V. (1956). Concentration spécifique réversible des amino acides chez *Escherichia coli. Annales de l'Institut Pasteur* **91**, 693–720.

CRANE, R. K. & LIPMANN, F. (1953). The effect of arsenate on aerobic phosphorylation. *Journal of Biological Chemistry* **201**, 235–243.

DIEKERT, G., WEBER, B. & THAUER, R. K. (1980). Nickel dependence of factor F_{430} content in *Methanobacterium thermoautotrophicum. Archives of Microbiology* **127**, 273–278.

DODDEMA, H. J., HUTTEN, T. J., VAN DER DRIFT, C. & VOGELS, G. D. (1978). ATP hydrolysis and synthesis by the membrane-bound ATP synthetase complex of *Methanobacterium thermoautotrophicum. Journal of Bacteriology* **136**, 19–23.

ELLEFSON, W. L. & WOLFE, R. S. (1980). Role of component C in the methylreductase system of *Methanobacterium. Journal of Biological Chemistry* **255**, 8388–8389.

ELLEWAY, R. F., SABINE, J. R. & NICHOLAS, D. J. D. (1971). Acetylene reduction by rumen microflora. *Archives of Microbiology* **76**, 277–291.

FLETT, R. J., HAMILTON, R. D. & CAMPBELL, N. E. R. (1976). Aquatic acetylene-reduction techniques: solutions to several problems. *Canadian Journal of Microbiology* **22**, 43–51.

GUNSALUS, R. P. & WOLFE, R. S. (1980). Methyl coenzyme M reductase from *Methanobacterium thermoautotrophicum. Journal of Biological Chemistry* **255**, 1891–1895.

GUNSALUS, R. P., ROMESSER, J. A. & WOLFE, R. S. (1978). Preparation of coenzyme M analogues and their activity in the methyl coenzyme M reductase system of *Methanobacterium thermoautotrophicum. Biochemistry* **17**, 2374–2377.

HWANG, J. C., CHEN, C. H. & BURRIS, R. H. (1973). Inhibition of nitrogenase-catalyzed reductions. *Biochimica et biophysica acta* **292**, 256–270.

HYNES, R. K. & KNOWLES, R. (1978). Inhibition by acetylene of ammonia oxidation in *Nitrosomonas europaea. FEMS Microbiology Letters* **4**, 319–321.

JARRELL, K. F. & SPROTT, G. D. (1981). The transmembrane electrical potential and intracellular pH in methanogenic bacteria. *Canadian Journal of Microbiology* **27**, 720–728.

JARRELL, K. F., COLVIN, J. R. & SPROTT, G. D. (1982). Spontaneous protoplast formation in *Methanobacterium bryantii. Journal of Bacteriology* **149**, 346–353.

KELL, D. B., DODDEMA, H. J., MORRIS, J. G. & VOGELS, G. D. (1981a). Energy coupling in methanogens. In *Proceedings of the 3rd International Symposium on Microbial Growth on C-1 Compounds*, pp. 159–170. Edited by H. Dalton. London: Heyden & Sons.

KELL, D. B., PECK, M. W., RODGER, G. & MORRIS, J. G. (1981b). On the permeability to weak acids and bases of the cytoplasmic membrane of *Clostridium pasteurianum. Biochemical and Biophysical Research Communications* **99**, 81–88.

LAMBERT, G. R. & SMITH, G. D. (1980). Hydrogen metabolism by filamentous cyanobacteria. *Archives of Biochemistry and Biophysics* **205**, 36–50.

MACGREGOR, A. N. & KEENEY, D. R. (1973). Methane formation by lake sediments during *in vitro* incubation. *Water Resource Bulletin* **9**, 1153–1158.

MCKELLAR, R. C., SHAW, K. M. & SPROTT, G. D. (1981). Isolation and characterization of a FAD-dependent NADH diaphorase from *Methanospirillum hungatei* strain GP1. *Canadian Journal of Biochemistry* **59**, 83–91.

MITCHELL, P. (1975). Proton translocation mechanisms and energy transduction by adenosine triphosphatases: an answer to criticisms. *FEBS Letters* **50**, 95–97.

MOUNTFORT, D. O. (1978). Evidence for ATP synthesis driven by a proton gradient in *Methanosarcina barkeri. Biochemical and Biophysical Research Communications* **85**, 1346–1351.

OREMLAND, R. S. & TAYLOR, B. F. (1975). Inhibition of methanogenesis in marine sediments by acetylene and ethylene: validity of the acetylene reduction assay for anaerobic microcosms. *Applied Microbiology* **30**, 707–709.

PATEL, G. B. & AGNEW, B. J. (1981). A simple apparatus for measuring the E_h of anaerobic media. *Canadian Journal of Microbiology* **27**, 853–855.

PRASAD, I. & SCHAEFLER, S. (1974). Regulation of the β-glucoside system in *Escherichia coli* K-12. *Journal of Bacteriology* **120**, 638–650.

RAIMBAULT, M. (1975). Étude de l'influence inhibitrice de l'acétylène sur la formation biologique de méthane dans un sol de rizière. *Annals Microbiologique (Institute Pasteur)* **126A**, 247–258.

SAUER, F. D., ERFLE, J. D. & MAHADEVAN, S. (1981). Evidence for an internal electrochemical proton gradient in *Methanobacterium thermoautotrophicum. Journal of Biological Chemistry* **256**, 9843–9848.

SAUNDERS, G. F., CAMPBELL, L. L. & POSTGATE, J. R. (1964). Base composition of deoxyribonucleic acid of sulfate-reducing bacteria deduced from buoyant density measurements in cesium chloride. *Journal of Bacteriology* **87**, 1073–1078.

SCHÖNHEIT, P., MOLL, J. & THAUER, R. K. (1979). Nickel, cobalt, and molybdenum requirement for growth of *Methanobacterium thermoautotrophicum. Archives of Microbiology* **123**, 105–107.

SEGHAL, S. N. & GIBBONS, N. E. (1960). Effect of some metal ions on the growth of *Halobacterium cutirubrum. Canadian Journal of Microbiology* **6**, 165–169.

SMITH, L. A., HILL, S. & YATES, M. G. (1976). Inhibition by acetylene of conventional hydrogenase in nitrogen-fixing bacteria. *Nature, London* **262**, 209–210.

SPENCER, R. W., DANIELS, L. & ORME-JOHNSON, W. H. (1980). Product isotope effects on *in vivo* methanogenesis by *Methanobacterium thermoautotrophicum. Biochemistry* **19**, 3678–3683.

SPROTT, G. D., COLVIN, J. R. & MCKELLAR, R. C.

(1979). Spheroplasts of *Methanospirillum hungatii* formed upon treatment with dithiothreitol. *Canadian Journal of Microbiology* **25**, 730–738.

SPROTT, G. D. & JARRELL, K. F. (1981). K⁺, Na⁺, and Mg²⁺ content and permeability of *Methanospirillum hungatei* and *Methanobacterium thermoautotrophicum*. *Canadian Journal of Microbiology* **27**, 444–451.

WILSON, D. M., ALDERETE, J. F., MALONEY, P. C. & WILSON, T. H. (1976). Protonmotive force as the source of energy for adenosine 5′-triphosphate synthesis in *Escherichia coli*. *Journal of Bacteriology* **126**, 327–337.

WOESE, C. R., MAGRUM, L. J. & FOX, G. E. (1978). Archaebacteria. *Journal of Molecular Evolution* **11**, 245–252.

YAMAZAKI, S., TSAI, L. & STADTMAN, T. C. (1980). Stereochemical studies of 8-hydroxy-5-deazaflavin-dependent NADP⁺ reductase from *Methanococcus vannielii*. *Journal of Biological Chemistry* **255**, 9025–9027.

YOSHINARI, T. & KNOWLES, R. (1976). Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. *Biochemical and Biophysical Research Communications* **69**, 705–710.

ZEHNDER, A. J. B., HUSER, B. A., BROCK, T. D. & WUHRMANN, K. (1980). Characterization of an acetate-decarboxylating, non-hydrogen-oxidizing methane bacterium. *Archives of Microbiology* **124**, 1–11.