

## Anaerobic Oxidation of Acetylene by Estuarine Sediments and Enrichment Cultures

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Acetylene disappeared from the gas phase of anaerobically incubated estuarine sediment slurries, and loss was accompanied by increased levels of carbon dioxide. Acetylene loss was inhibited by chloramphenicol, air, and autoclaving. Addition of <sup>14</sup>C<sub>2</sub>H<sub>2</sub> to slurries resulted in the formation of <sup>14</sup>CO<sub>2</sub> and the transient appearance of <sup>14</sup>C-soluble intermediates, of which acetate was a major component. Acetylene oxidation stimulated sulfate reduction; however, sulfate reduction was not required for the loss of C<sub>2</sub>H<sub>2</sub> to occur. Enrichment cultures were obtained which grew anaerobically at the expense of C<sub>2</sub>H<sub>2</sub>.

Acetylene inhibits several microbial processes, including methanogenesis (13), methane oxidation (5), nitrification (23), nitrogen fixation (20), and denitrification (3, 6, 29). Enzyme assays have been devised which exploit the ability of acetylene to be reduced preferentially to C<sub>2</sub>H<sub>4</sub> by nitrogenase (nitrogen fixation) and to block N<sub>2</sub>O reductase (denitrification), thereby allowing quantification of these microbial processes (9, 28). During the course of a denitrification experiment with estuarine sediment slurries, we observed the total disappearance of added N<sub>2</sub>O and C<sub>2</sub>H<sub>2</sub> from the gas phase of control flasks incubated anaerobically for 4 days. Although acetylene can be oxidized by aerobic microorganisms (10), there were no reports at the time of our observation concerning the metabolism of acetylene in the absence of oxygen. However, Watanabe and de Guzman (24) recently reported the disappearance of C<sub>2</sub>H<sub>2</sub> from anaerobically incubated rice paddy soils. We now report that acetylene is oxidized to carbon dioxide by anaerobic bacteria.

### MATERIALS AND METHODS

**Preparation of sediment slurries.** Sediments were taken through short suction cores from the upper 5 cm of an intertidal mud flat in San Francisco Bay at Palo Alto, Calif. The sediments were brown to light gray and covered a deeper, more reduced black layer which was not sampled. Denitrification has been reported to occur in the upper layers of similar coastal sediments (18). The samples were transported immediately to the laboratory, and the experiments were started within 1 h of collection. Sediments were pooled (300 ml) and homogenized in a Waring blender for 5 min under a flow of N<sub>2</sub> with an equal volume of bay water (salinity = 18 to 22‰). The resulting homoge-

nate was pipetted (25 ml) into 250-ml Erlenmeyer flasks which contained 50 ml of bay water under a flow of N<sub>2</sub>. The flasks were sealed under N<sub>2</sub> with recessed black rubber stoppers and were then flushed with a N<sub>2</sub> gassing syringe for 5 min to remove O<sub>2</sub>. Next C<sub>2</sub>H<sub>2</sub> (20 ml), N<sub>2</sub>O (6 ml), or both were added to the gas phase by syringe. Acetylene was generated by reaction of CaC<sub>2</sub> with water, and N<sub>2</sub>O was obtained commercially (Linde Gas Co.). Substrates and inhibitors were added to the Erlenmeyer flasks before inoculation with homogenates at the following concentrations: Na<sub>2</sub>MoO<sub>4</sub> (85 mg/75 ml of slurry), NaClO<sub>3</sub> (42 mg/75 ml of slurry), NaNO<sub>3</sub> (8.5 mg/75 ml of slurry), and chloramphenicol (100 mg/75 ml of slurry). Heat-sterilized sediment slurries were first autoclaved (15 lb/in<sup>2</sup> for 30 min), cooled, and then sealed under N<sub>2</sub>. Slurries incubated under air developed negative pressures upon incubation due to consumption of O<sub>2</sub>. O<sub>2</sub> was added to these flasks daily by allowing the flask to draw up O<sub>2</sub> from a syringe. In experiments requiring larger volumes of sediment, a 1-liter Erlenmeyer flask was used with proportionate volumes of homogenate, bay water, and gas phase. Freshwater sediments were taken from San Francisquito Creek, Palo Alto, during April after the stream had had about 4 months of active seasonal flow. Stream sediments were treated as described above. All sediments were incubated at room temperature (20°C) with constant shaking (150 rpm). Slurries were shaken for 30 min before initial gas chromatograph (GC) determinations to allow for gas equilibration (8).

**Analysis of gases.** A Hewlett-Packard model 5730A GC equipped with both flame ionization (FID) and thermal conductivity (TCD) detectors was used to measure CH<sub>4</sub>, C<sub>2</sub>H<sub>2</sub>, C<sub>2</sub>H<sub>4</sub>, C<sub>2</sub>H<sub>6</sub>, CO<sub>2</sub>, and N<sub>2</sub>O. FID separations were made with Porapak Q (183 by 0.64 cm) and Porapak S (244 by 0.64 cm) columns attached in series and connected to the FID system. A Porapak Q (366 by 0.64 cm) column was used for the TCD system. The oven temperature was 50°C and the carrier gas was helium (ultrahigh purity; flow rate, 30 ml/min for FID and 20 ml/min for TCD). Determination of low levels of N<sub>2</sub>O was made on a Perkin-Elmer model 3920 GC equipped with a <sup>63</sup>Ni electron capture

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detector (ECD), a Porapak Q column (366 by 0.64 cm), and N<sub>2</sub> as a carrier gas (ultrahigh purity; flow rate, 40 ml/min; detector temperature, 250°C). Gases in the headspaces of flasks were sampled by separate syringes for each flask. Injection volumes were 250  $\mu$ l (FID and TCD) or 100  $\mu$ l (ECD).

<sup>14</sup>C tracer experiments. Uniformly labeled <sup>14</sup>C<sub>2</sub>H<sub>2</sub> (specific activity, 59 mCi/mmol; New England Nuclear Corp., Boston, Mass.) was used in tracer experiments. The vial (volume of 10 ml) was sealed with a rubber septum, the internal glassbreak seal was broken, and <sup>14</sup>C<sub>2</sub>H<sub>2</sub> was drawn up by syringe (250  $\mu$ l, corresponding to 12.5  $\mu$ Ci) while the gas was replaced simultaneously by injecting an equal volume of saturated NaCl solution (to avoid pressure differentials). <sup>14</sup>C<sub>2</sub>H<sub>2</sub> was then injected into the gas phase of the sealed experimental flasks (250-ml Erlenmeyer) containing the previously described sediment slurries. Radioactivity in the gas phase was measured with a Packard model 800 gas proportional counter (GPC) attached to the TCD (11). The GPC was modified by bypassing the oxidation step. It gave a linear response over its entire attenuation range when peak areas were compared with standards made by diluting acidified [<sup>14</sup>C]HCO<sub>3</sub><sup>-</sup> in test tubes (limit of detection,  $\approx$ 0.03 nCi/0.25 ml).

Radioactivity in the liquid phase of sediment slurries was measured by filtering 6 ml of a slurry subsample through a 0.45- $\mu$ m Millex filter (Millipore Corp., New Bedford, Mass.). The clear fluid (about 4 ml) was divided equally between two test tubes. To either trap or drive off dissolved CO<sub>2</sub>, a drop of 6 N NaOH or 6 N HCl, respectively, was added to each tube followed by bubbling with air for 7 to 8 min to remove <sup>14</sup>C<sub>2</sub>H<sub>2</sub>. After returning the tubes to neutral pH by adding a corresponding drop of acid or base, 0.5 ml per tube was removed and added to a scintillation vial containing 8 ml of Aquasol (Packard Instrument Co., Inc., Rockville, Md.). Counts of <sup>14</sup>C were measured on a Packard model 2425 Tri-Carb liquid scintillation counter and converted to disintegrations per minute by the channels-ratio method.

**Identification of [<sup>14</sup>C]acetate.** (i) **HPLC method.** The filtered slurry samples from which CO<sub>2</sub> had been removed by acidification were stored frozen at -20°C until analysis. A Spectra-Physics model 740 high-performance liquid chromatograph (HPLC) equipped with a spectrophotometric detector set at 210 nm and a Spherisorb ODS column (22.9 by 0.64 cm; Spectra-Physics Co., Mountainview, Calif.) were used to separate a mixture of formic, acetic, and propionic acids. Perchloric acid (0.01 N) was the carrier (flow rate, 1 ml/min; 37 atm). Then 100  $\mu$ l of the <sup>14</sup>C-labeled filtrate was combined with a 100- $\mu$ l portion of a fatty acid mixture (formic, acetic, and propionic acids, 1 g/liter each) and 10  $\mu$ l of 0.1 N perchloric acid (final pH of mixture,  $\leq$ 2). Thirty microliters of this mixture was injected into the HPLC, and fractions corresponding to each fatty acid peak and to the regions between peaks were collected. Each collected fraction was added to 8 ml of Aquasol and counted on a Packard Tri-Carb model 3320 liquid scintillation counter. Counts were converted to disintegrations per minute by the channels-ratio method.

(ii) **Microbiological assay.** A highly enriched culture of *Methanobacterium soehngenii* was used to

confirm the presence of [<sup>14</sup>C]acetate. This methanogen grows only on acetate, from which it produces equimolar amounts of CH<sub>4</sub> and CO<sub>2</sub> (32). A 100- $\mu$ l amount of the filtered, CO<sub>2</sub>-free <sup>14</sup>C-labeled intermediate was added to serum vials containing 4 ml of the *M. soehngenii* culture (gas phase, 4 ml) sealed under N<sub>2</sub>/CO<sub>2</sub> (4:1) with a recessed butyl rubber stopper. After 1 week of incubation, cultures were acidified by injecting 0.3 ml of 6 N HCl, and radioactive gases were counted by GC-GPC as described previously. Pressure in the vials was measured by syringe deflection and used as a correction factor for total radioactivity (accuracy,  $\sim$ 0.1 atm).

**Determination of sulfide and dissolved CO<sub>2</sub> in slurries.** S<sup>2-</sup> was measured by trapping with Cd<sup>2+</sup> and atomic absorption spectroscopy as described elsewhere (12, 14). Dissolved CO<sub>2</sub> was measured by injecting 0.5 ml of slurry into a 2-ml Vacutainer tube (Becton-Dickinson & Co., Rutherford, N.J.) containing 0.2 ml of 6 N HCl. After vigorous shaking, the septum was briefly (1 to 2 s) penetrated with a 22-gauge needle to achieve ambient pressure by allowing an influx of air into the Vacutainer. The amount of atmospheric CO<sub>2</sub> introduced into the tube was negligible compared with the amount already present. A 0.25-ml sample of the gas phase was withdrawn for quantification of the TCD. Dissolved CO<sub>2</sub> measurements were added to gas-phase CO<sub>2</sub> to give values of total CO<sub>2</sub> ( $\Sigma$ CO<sub>2</sub>).

**Enrichment cultures.** The enrichment medium consisted of the following constituents: distilled water, 980 ml; NaCl, 20 g; K<sub>2</sub>HPO<sub>4</sub>, 0.25 g; KH<sub>2</sub>PO<sub>4</sub>, 0.25 g; NH<sub>4</sub>Cl, 1.0 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.2 g; MgCl<sub>2</sub>·6H<sub>2</sub>O, 4 g; Na<sub>2</sub>SO<sub>4</sub>, 2.2 g; vitamin solution (26), 10 ml; and trace mineral solution (26), 10 ml. The final pH was adjusted to 7.3. The medium was dispensed into 250-ml Erlenmeyer flasks (75 ml) and autoclaved (15 lb/in<sup>2</sup> for 15 min); after cooling, filter-sterilized vitamins were added. The flasks were then sealed with sterile, recessed, black rubber stoppers and flushed with N<sub>2</sub> for 15 min, using a sterile gassing syringe attached to a tank of N<sub>2</sub> (flow rate, 100 ml/min). Acetylene (20 ml) was then injected into the flasks through a sterile 0.22- $\mu$ m Millex filter (Millipore Corp.). Then 5 ml of sediment slurry was removed from experimental flasks with demonstrated C<sub>2</sub>H<sub>2</sub> consumption and injected into the enrichment flasks to serve as inocula. Enrichment flasks were incubated at room temperature with shaking. Hydrocarbons and CO<sub>2</sub> were monitored by FID or TCD gas chromatography, and transfers to subcultures (5 ml) were performed when C<sub>2</sub>H<sub>2</sub> disappeared from the enrichment flasks (5 to 6 days of incubation).

## RESULTS

**Sediment slurry experiments.** N<sub>2</sub>O reductase activity was present in estuarine sediment slurries (Fig. 1). As compared with consumption in unamended flasks, N<sub>2</sub>O consumption was accelerated by chlorate ions, slowed by nitrate ions, and blocked by either autoclaving or C<sub>2</sub>H<sub>2</sub>. After 110 h of incubation, N<sub>2</sub>O was absent from the C<sub>2</sub>H<sub>2</sub> controls. ECD and FID analysis of these flasks at 110 h revealed the presence of only traces of N<sub>2</sub>O and C<sub>2</sub>H<sub>2</sub>, both of which

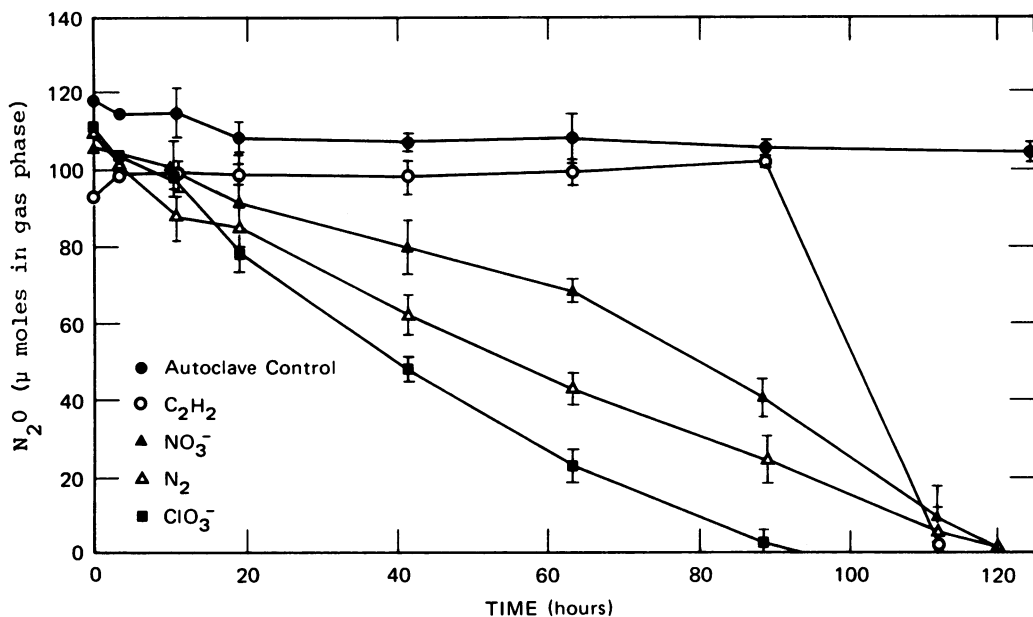


FIG. 1.  $N_2O$  reductase activity in sediment slurries incubated under  $N_2$  plus  $N_2O$  ( $240 \mu\text{mol}$ ). Initial measurements were taken after 30 min of shaking to allow for equilibration. Points represent the mean  $\pm$  standard deviation of three experimental flasks. Standard deviations were not drawn for first two determinations due to overlap.

subsequently continued to decrease with accompanied increases in  $CO_2$  (Fig. 2). The loss of acetylene ( $800 \mu\text{mol}$ ) could not be accounted for by reduction to  $C_2H_4$  or to  $C_2H_6$ , since both of these gases were present only in trace quantities (at 124 h,  $C_2H_4$  was 0.03 to  $0.10 \mu\text{mol}/\text{flask}$  and  $C_2H_6$  was  $<0.001 \mu\text{mol}/\text{flask}$ ). Sediment in flasks from which  $C_2H_2$  and  $N_2O$  disappeared turned progressively blacker after 120 h, indicating the formation of  $FeS$  from enhanced sulfate reduction. By contrast, slurries incubated without  $C_2H_2$  remained gray.

In a long-term (42-day) incubation,  $C_2H_2$  and  $N_2O$  disappeared by 6 days (Fig. 3). This was followed over the next few weeks by progressive increases of  $CO_2$  and acid-labile sulfide. Free sulfide in solution was not observed. The final level of  $CO_2$  produced ( $\sim 4.5 \text{ mmol}$ ) was comparable to the quantity of  $C_2H_2$  lost (4 mmol).

$N_2O$  was not required for  $C_2H_2$  disappearance. Flasks incubated with  $N_2$  plus  $C_2H_2$  in the gas phase lost  $C_2H_2$  after 5 days of incubation. As compared with controls under  $N_2$ ,  $C_2H_2$  flasks produced twice the  $CO_2$  and over 10 times the sulfide after 2 weeks of incubation (Table 1). Molybdate ions, however, failed to block  $C_2H_2$  loss, even though the concentrations used inhibited sulfate reduction (12). Air, autoclaving, and chloramphenicol, however, effectively blocked  $C_2H_2$  loss. The freshwater stream sediments examined were found to be incapable of

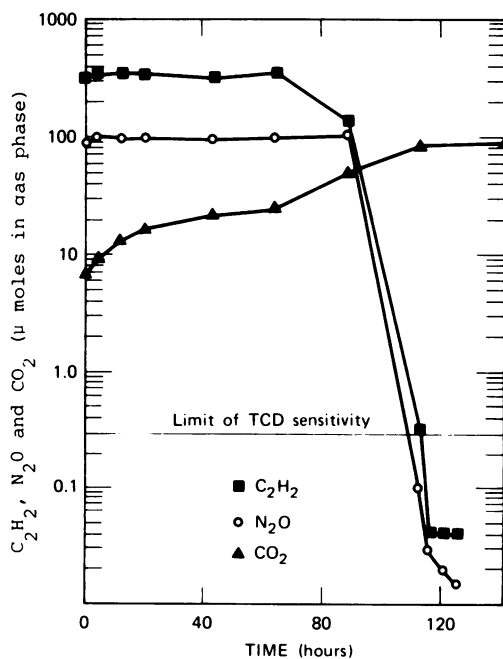


FIG. 2. Disappearance of  $C_2H_2$  and  $N_2O$  and production of  $CO_2$  in the gas phase of an experimental flask. Values above the dotted line were determined on the TCD. Values below the dotted line were not detectable on the TCD and were determined on the FID ( $C_2H_2$ ) or ECD ( $N_2O$ ).

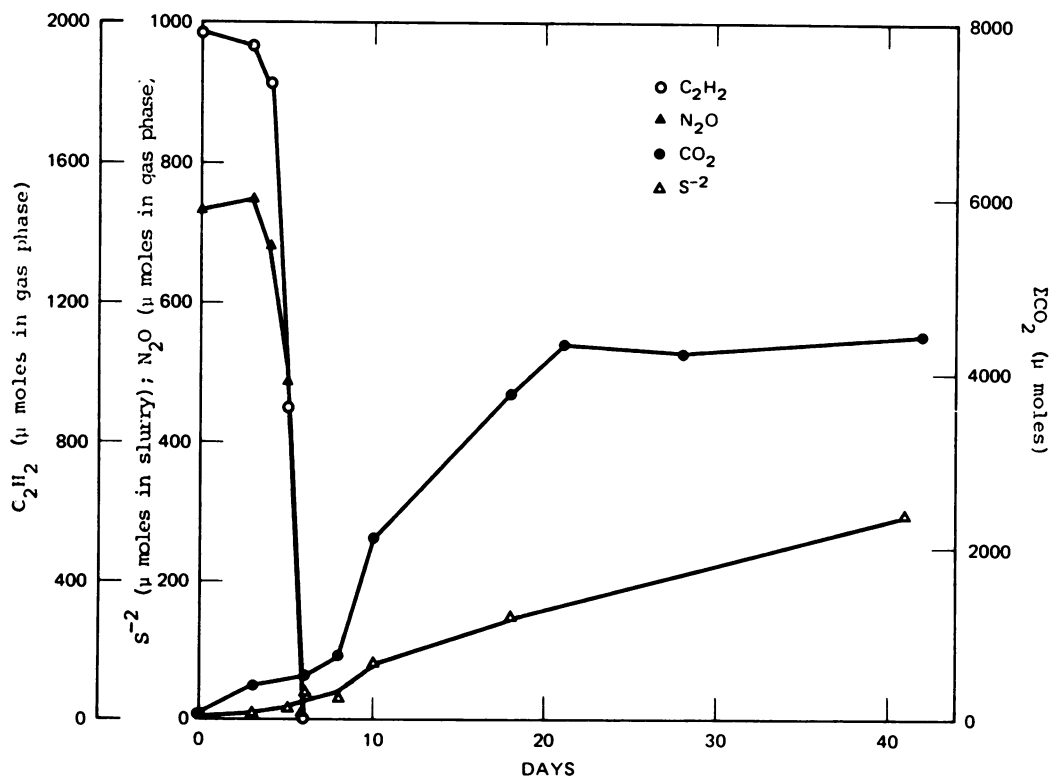


FIG. 3. Loss of N<sub>2</sub>O and C<sub>2</sub>H<sub>2</sub> and formation of ΣCO<sub>2</sub> and acid-labile S<sup>2-</sup> in a long-term incubation experiment. A 1-liter flask was used (volume of slurry, 300 ml).

TABLE 1. Stimulation of S<sup>2-</sup> and CO<sub>2</sub> production by replicate estuarine sediment slurries incubated with C<sub>2</sub>H<sub>2</sub> (10%, vol/vol)<sup>a</sup>

Atmosphere	T1 <sup>b</sup> (μmol/flask)			T2 <sup>c</sup> (μmol/flask)		
	CO <sub>2</sub>	DCO <sub>2</sub>	S <sup>2-</sup>	CO <sub>2</sub>	DCO <sub>2</sub>	S <sup>2-</sup>
N <sub>2</sub>	49	ND <sup>d</sup>	0.8	117	395	4.1
N <sub>2</sub>	49	ND	0.7	110	407	6.4
N <sub>2</sub> + C <sub>2</sub> H <sub>2</sub>	47	ND	0.5	242	912	71
N <sub>2</sub> + C <sub>2</sub> H <sub>2</sub>	41	ND	1.3	238	1,074	104

<sup>a</sup> C<sub>2</sub>H<sub>2</sub> disappeared after 5 days of incubation. CO<sub>2</sub> was sampled in gas phase; DCO<sub>2</sub> and S<sup>2-</sup> were sampled in slurry phase.

<sup>b</sup> First sampling for CO<sub>2</sub> taken after 1 day of incubation; first sampling for S<sup>2-</sup> taken after 2 days of incubation.

<sup>c</sup> S<sup>2-</sup> sampled after 15 days of incubation; CO<sub>2</sub> sampled after 16 days of incubation.

<sup>d</sup> ND, Not determined.

acting upon acetylene, even when supplemented with sulfate ions.

**<sup>14</sup>C tracer experiments.** Tracer experiments established that <sup>14</sup>C<sub>2</sub>H<sub>2</sub> was converted to <sup>14</sup>CO<sub>2</sub> (Fig. 4a). In addition, <sup>14</sup>C-soluble intermediate(s) was present in the acidified fraction and accounted for about 70% of the <sup>14</sup>C<sub>2</sub>H<sub>2</sub> lost after 11 days of incubation. The <sup>14</sup>C-soluble intermedi-

ate(s) decreased to insignificant levels by 41 days, at which time dissolved <sup>14</sup>CO<sub>2</sub> was present (Table 2). A flask incubated without N<sub>2</sub>O gave nearly identical results as a N<sub>2</sub>O plus C<sub>2</sub>H<sub>2</sub> flask (Table 2), and the entire process was inhibited by chloramphenicol and autoclaving. The disappearance of N<sub>2</sub>O and C<sub>2</sub>H<sub>2</sub>, both of which inhibit methanogenesis (2, 13), was followed by increases of CH<sub>4</sub> in the gas phase (Fig. 4b).

**Identification of [<sup>14</sup>C]acetate.** The HPLC procedure separated the mixture of formic, acetic, and propionic acids (Fig. 5a), and counts were recovered in the acetate fraction (no. 3) with a slight tailing off into the next fraction (Fig. 5b). Similar counts were recovered from flasks incubated with C<sub>2</sub>H<sub>2</sub> or C<sub>2</sub>H<sub>2</sub> plus N<sub>2</sub>O, but no counts were obtained for the chloramphenicol or autoclaved controls. About 50% of the counts were recovered as acetate, indicating the presence of other <sup>14</sup>C-soluble intermediates not resolvable by this method (Fig. 5b).

Addition of <sup>14</sup>C-labeled filtrate to *M. soehngenii* enrichments resulted in the appearance of <sup>14</sup>CH<sub>4</sub> and <sup>14</sup>CO<sub>2</sub> in the gas phase of experimental vials (N<sub>2</sub> plus C<sub>2</sub>H<sub>2</sub> filtrate: <sup>14</sup>CH<sub>4</sub> = 4,808 dpm, <sup>14</sup>CO<sub>2</sub> = 2,219 dpm, total injected in filtrate =

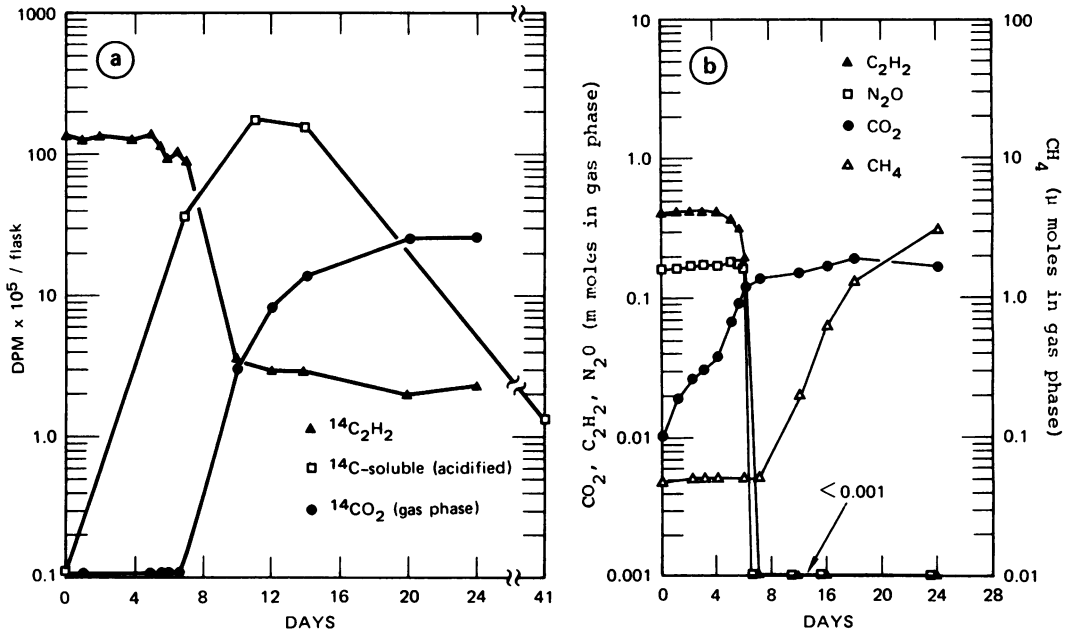


FIG. 4. Results of the  $^{14}\text{C}_2\text{H}_2$  tracer experiment for a flask incubated under  $\text{N}_2$  plus  $\text{C}_2\text{H}_2$  plus  $\text{N}_2\text{O}$  plus  $^{14}\text{C}_2\text{H}_2$ . Total  $^{14}\text{C}_2\text{H}_2$  added at the start was  $\sim 278 \times 10^5$  dpm. Total  $\text{C}_2\text{H}_2$  and  $\text{N}_2\text{O}$  added at the start were 800 and 240  $\mu\text{mol}$ , respectively. Flasks were shaken for >30 min before first determinations. (a)  $^{14}\text{C}_2\text{H}_2$ ,  $^{14}\text{C}$ -soluble (acidified) intermediates and  $^{14}\text{CO}_2$ . (b) Nonradioactive gases.

TABLE 2. Formation of  $^{14}\text{C}$ -labeled intermediates by estuarine sediment slurries incubated with  $^{14}\text{C}_2\text{H}_2$

Days of incubation	Base phase	Treatment	dpm/0.5 ml	
			Acid <sup>a</sup>	Base <sup>b</sup>
14	$\text{N}_2 + \text{C}_2\text{H}_2 + \text{N}_2\text{O}$	None	103,208	109,623
	$\text{N}_2 + \text{C}_2\text{H}_2$	None	128,762	121,969
	$\text{N}_2 + \text{C}_2\text{H}_2 + \text{N}_2\text{O}_2$	Autoclaved	227	220
	$\text{N}_2 + \text{C}_2\text{H}_2 + \text{N}_2\text{O}$	Chloramphenicol	403	325
41	$\text{N}_2 + \text{C}_2\text{H}_2 + \text{N}_2\text{O}$	None	64	21,275
	$\text{N}_2 + \text{C}_2\text{H}_2$	None	791	35,400
	$\text{N}_2 + \text{C}_2\text{H}_2 + \text{N}_2\text{O}$	Autoclaved	358	325
	$\text{N}_2 + \text{C}_2\text{H}_2 + \text{N}_2\text{O}$	Chloramphenicol	451	325

<sup>a</sup> Disintegrations by soluble intermediates.

<sup>b</sup>  $^{14}\text{C}$ -soluble plus  $^{14}\text{CO}_2$  disintegrations.

22,933 dpm;  $\text{N}_2$  plus  $\text{C}_2\text{H}_2$  plus  $\text{N}_2\text{O}$  filtrate:  $^{14}\text{CH}_4 = 2,717$  dpm,  $^{14}\text{CO}_2 = 1,321$  dpm, total injected in filtrate = 19,153 dpm). Since *M. soehngenii* grows only on acetate (32), the microbiological assay confirms the presence of acetate as a soluble intermediate.

**Enrichment cultures.** Incubation of enrichment cultures under an atmosphere of  $\text{N}_2$  plus  $\text{C}_2\text{H}_2$  (10%) resulted in the disappearance of  $\text{C}_2\text{H}_2$  after 5 to 6 days.  $\text{C}_2\text{H}_2$  loss was accompanied by increases of gas-phase  $\text{CO}_2$  (Fig. 6). Filter sterilization of the inocula (Millex, 0.22  $\mu\text{m}$ ) totally inhibited  $\text{C}_2\text{H}_2$  loss and  $\text{CO}_2$  production. The enrichment culture has been taken through

over 14 successful transfers, and sediment particles are no longer present in the media. The cultures turn slightly turbid after a few days of incubation, and microscopic examination (phase contrast,  $\times 1,000$ ; American Optical Corp., Buffalo, N.Y.) has revealed the predominance of numerous highly motile, gram-negative rods.

## DISCUSSION

Whether anaerobic oxidation of gaseous hydrocarbons can take place in nature is a subject of debate among microbiologists and geochemists. In the case of methane, the activity has been reported for both freshwater and marine

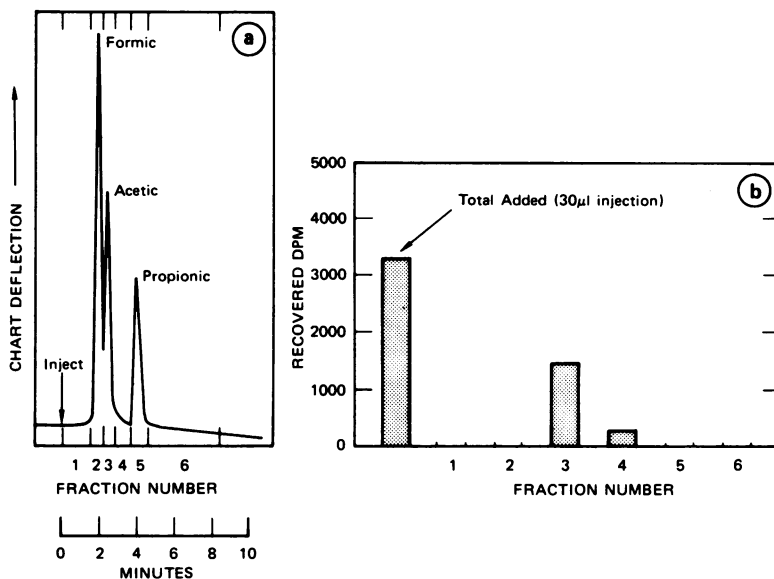


FIG. 5. HPLC identification of  $[^{14}\text{C}]$ acetate as a major constituent of the  $^{14}\text{C}$ -soluble intermediates. Samples were taken after 14 days of incubation. Samples were filtered ( $0.45\ \mu\text{m}$ ), acidified, air purged, returned to neutral pH, and frozen. (a) HPLC chromatogram and collected fractions; (b) radioactivity in collected fractions. Sample shown was for the flask incubated initially with  $\text{N}_2$  plus  $\text{C}_2\text{H}_2$  plus  $^{14}\text{C}_2\text{H}_2$ . Similar results were obtained for the flask incubated with  $\text{N}_2$  plus  $\text{C}_2\text{H}_2$  plus  $\text{N}_2\text{O}$  plus  $^{14}\text{C}_2\text{H}_2$ . No counts were recovered for either the autoclaved or chloramphenicol controls.

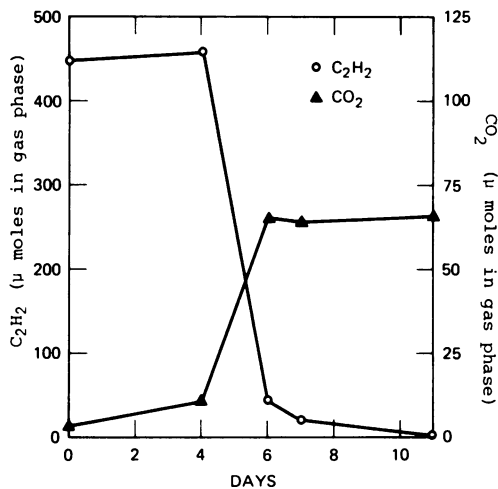


FIG. 6. Loss of  $\text{C}_2\text{H}_2$  and production of  $\text{CO}_2$  by an enrichment culture obtained from estuarine sediment slurries. Results are for the seventh transfer.

environments (15, 16, 31); however, supporting work with bacterial cultures is generally lacking. Davis and Yarbrough (4) reported a small cooxidation of methane and ethane by *Desulfovibrio desulfuricans*. Recently, Zehnder and Brock (30) demonstrated that methanogens can convert  $^{14}\text{CH}_4$  to  $^{14}\text{CO}_2$ ; however, the metabolism of

the methanogens effects an overwhelming net production, rather than consumption of  $\text{CH}_4$ . In the case of  $\text{C}_2\text{H}_2$ , Wake et al. (22) calculated that sulfate-linked  $\text{C}_2\text{H}_2$  oxidation is thermodynamically favorable ( $\Delta G = -188\ \text{kJ/mol}$  of  $\text{SO}_4^{2-}$ ; pH 7), but their attempts to demonstrate this with pure cultures of sulfate reducers were unsuccessful.

Our results clearly show that acetylene is oxidized anaerobically to  $\text{CO}_2$  (Fig. 4a) and that sulfate reduction is stimulated by this process (Table 1). Sulfate reduction, however, is not required for  $\text{C}_2\text{H}_2$  to disappear from the gas phase, since  $\text{MoO}_4^{2-}$  did not prevent  $\text{C}_2\text{H}_2$  loss. Furthermore, the transient appearance of  $^{14}\text{C}$ -soluble intermediates, of which acetate was a major constituent, was followed by  $^{14}\text{CO}_2$  formation (Fig. 4a, Table 2). Sulfate-reducing bacteria have been isolated which can grow solely on acetate (25) or on mixtures of acetate,  $\text{CO}_2$ , and  $\text{H}_2$  (1, 19), and therefore sulfate-reducing bacteria may constitute the terminal organisms involved in  $\text{C}_2\text{H}_2$  oxidation, a role resembling that played by methanogens in benzoate decomposition (7). This conclusion is reinforced by the lack of stoichiometric balance between  $\text{C}_2\text{H}_2$  lost (4 mmol) and sulfide form (300  $\mu\text{mol}$ ) in the 42-day incubation experiment (Fig. 3) since  $\text{C}_2\text{H}_2 + \text{SO}_4^{2-} \rightarrow 2\text{CO}_2 + \text{H}_2\text{S}$ . If direct oxidation of  $\text{C}_2\text{H}_2$  by sulfate-reducing bacteria were taking

place, an equivalent amount of  $S^{2-}$  formed, relative to  $C_2H_2$  lost, should have occurred. We believe, therefore, that the sulfate-reducing bacteria oxidized the acetate intermediate rather than  $C_2H_2$ . Thus, the lack of stoichiometric balance between  $C_2H_2$  lost and  $S^{2-}$  produced can be explained by the uptake of intermediates (e.g., acetate) by bacteria other than sulfate reducers.

The simultaneous disappearance of  $N_2O$  (240  $\mu$ mol) and  $C_2H_2$  (800  $\mu$ mol) was initially thought to be due to the action of  $N_2O$  as the oxidant of  $C_2H_2$ . However, since  $4N_2O + C_2H_2 \rightarrow 4N_2 + 2CO_2 + 2H^+$ , total  $N_2O$ -linked acetylene oxidation would require  $\sim 3.2$  mmol of  $N_2O$ . Our investigations revealed that  $N_2O$  was not required for  $C_2H_2$  oxidation to occur in either estuarine sediments (Table 1) or enrichment cultures (Fig. 6).  $N_2O$  loss in the presence of  $C_2H_2$  has been reported in soils (27) and marine sediments (D. G. Capone, and B. F. Taylor, Abstr. Annu. Meet. Am. Soc. Limnol. Oceanogr., 1979, p. 13). Tam and Knowles (21) attributed  $N_2O$  loss to the relief of  $C_2H_2$  blockage by free sulfide ions. Interestingly enough, free sulfide ions have been reported to inhibit  $N_2O$  reductase in *Pseudomonas fluorescens* (17). In our experiments, however, free sulfide was never observed, and  $N_2O$  disappearance was probably caused by oxidation of organic compounds (possibly including some of the soluble intermediates of  $C_2H_2$  oxidation) once the p  $C_2H_2$  had become low enough to relieve inhibition of  $N_2O$  reductase.

The use of  $C_2H_2$  in estuarine sediments or rice paddy soils (24) for assaying either denitrification or  $N_2$  fixation appears, therefore, to encounter problems after prolonged incubations (e.g., 4 days). Interestingly, the problem posed by anaerobic  $C_2H_2$  oxidation does not appear to occur in the freshwater stream sediments we studied. The organism(s) responsible for the initial conversion of acetylene to a soluble intermediate, therefore, may not have been present in the stream sediments we examined. The pathway of anaerobic acetylene oxidation by pure and mixed cultures needs further elucidation and is currently being pursued in this laboratory.

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#### LITERATURE CITED

1. Badziong, W., R. K. Thauer, and J. G. Zeikus. 1978. Isolation and characterization of *Desulfovibrio* growing on hydrogen plus sulfate as the sole energy source. Arch. Microbiol. 116:41-49.
2. Balderston, W. L., and W. J. Payne. 1976. Inhibition of methanogenesis in salt marsh sediments and whole-cell suspensions of methanogenic bacteria by nitrogen oxides. Appl. Environ. Microbiol. 32:264-269.
3. Balderston, W. L., B. Sherr, and W. J. Payne. 1976. Blockage by acetylene of nitrous oxide reductase in *Pseudomonas perfetomarinus*. Appl. Environ. Microbiol. 31:504-508.
4. Davis, J. B., and H. F. Yarbrough. 1966. Anaerobic oxidation of hydrocarbons by *Desulfovibrio desulfuricans*. Chem. Geol. 1:137-144.
5. deBont, J. A. M., and E. G. Mulder. 1976. Invalidity of the acetylene reduction assay in alkane-utilizing, nitrogen fixing bacteria. Appl. Environ. Microbiol. 31:640-647.
6. Federova, R. I., E. I. Milekhina, and N. I. Il'yukhina. 1973. Possibility of using the "gas-exchange" method to detect extraterrestrial life. Identification of nitrogen-fixing microorganism. Izv. Akad. Nauk SSSR Ser. Biol. 6: 797-806.
7. Ferry, J. G., and R. S. Wolfe. 1976. Anaerobic degradation of benzoate to methane by a microbial consortium. Arch. Microbiol. 107:33-40.
8. Flett, R. J., R. D. Hamilton, and N. E. R. Campbell. 1976. Aquatic acetylene-reduction techniques; solutions to several problems. Can. J. Microbiol. 22:43-51.
9. Hardy, R. W., R. D. Holsten, E. K. Jackson, and R. C. Burns. 1968. The acetylene-ethylene assay for  $N_2$  fixation: laboratory and field evaluation. Plant Physiol. 43:1158-1207.
10. Kanner, D., and R. Bartha. 1979. Growth of *Nocardia rhodochrous* on acetylene gas. J. Bacteriol. 139:225-230.
11. Nelson, D. R., and J. G. Zeikus. 1974. Rapid method for the radioisotope analysis of gaseous end products of anaerobic metabolism. Appl. Microbiol. 28:258-261.
12. Oremland, R. S., and M. P. Silverman. 1979. Microbial sulfate reduction measured by an automated electrical impedance technique. Geomicrobiol. J. 1:355-372.
13. Oremland, R. S., and B. F. Taylor. 1975. Inhibition of methanogenesis in marine sediments by acetylene and ethylene: validity of the acetylene reduction assay for anaerobic microcosms. Appl. Microbiol. 30:707-709.
14. Oremland, R. S., and B. F. Taylor. 1978. Sulfate reduction and methanogenesis in marine sediments. Geochim. Cosmochim. Acta 42:209-214.
15. Panganiban, A. T., Jr., T. E. Patt, W. Hart, and R. S. Hanson. 1979. Oxidation of methane in the absence of oxygen in lake water samples. Appl. Environ. Microbiol. 37:303-309.
16. Reeburgh, W. S. 1979. Methane consumption in Cariaco Trench waters and sediments. Earth Planet. Sci. Lett. 28:337-344.
17. Sørensen, J., J. M. Tiedje, and R. B. Firestone. 1980. Inhibition of sulfide of nitric and nitrous oxide reduction by denitrifying *Pseudomonas fluorescens*. Appl. Environ. Microbiol. 39:105-108.
18. Sørensen, J. B., B. B. Jørgensen, and N. P. Revsbech. 1979. A comparison of oxygen, nitrate and sulfate-respiration in coastal marine sediments. Microb. Ecol. 5: 105-115.
19. Sorokin, Yu. I. 1966. Role of carbon dioxide and acetate in the biosynthesis by sulfate-reducing bacteria. Nature (London) 210:551-552.
20. Stewart, W. D., G. P. Fitzgerald, and R. H. Burris. 1967. *In situ* studies on  $N_2$ -fixation using the acetylene reduction technique. Proc. Natl. Acad. Sci. U.S.A. 58: 2071-2078.
21. Tam, T. Y., and R. Knowles. 1979. Effects of sulfide and acetylene on nitrous oxide reduction by soil and by *Pseudomonas aeruginosa*. Can. J. Microbiol. 25:1133-1138.
22. Wake, L. V., R. K. Christopher, P. A. D. Rickard, J. E. Andersen, and B. J. Ralph. 1977. A thermodynamic assessment of possible substrates for sulfate-reducing bacteria. Aust. J. Biol. Sci. 30:155-172.
23. Walter, H. M., D. R. Keeney, and I. R. Fillery. 1979.

- Inhibition of nitrification by acetylene. *Soil Sci. Soc. Am. J.* **43**:195-196.
24. **Watanabe, I., and M. R. de Guzman.** 1980. Effect of nitrate on acetylene disappearance from anaerobic soil. *Soil Biol. Biochem.* **12**:193-194.
25. **Widdell, F., and N. Pfennig.** 1977. A new anaerobic, sporing, acetate-oxidizing sulfate-reducing bacterium, *Desulfotomaculum* (amend) *acetoxidans*. *Arch. Microbiol.* **112**:119-122.
26. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. *J. Biol. Chem.* **238**:2882-2886.
27. **Yeomans, J. C., and E. G. Beauchamp.** 1978. Limited inhibition of nitrous oxide reduction in soil in the presence of acetylene. *Soil Biol. Biochem.* **10**:517-519.
28. **Yoshinari, T., R. Hynes, and R. Knowles.** 1977. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. *Soil Biol. Biochem.* **9**:177-183.
29. **Yoshinari, T., and R. Knowles.** 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. *Biochem. Biophys. Res. Commun.* **69**:705-710.
30. **Zehnder, A. J. B., and T. D. Brock.** 1979. Methane formation and methane oxidation by methanogenic bacteria. *J. Bacteriol.* **137**:420-432.
31. **Zehnder, A. J. B., and T. D. Brock.** 1980. Anaerobic methane oxidation: occurrence and ecology. *Appl. Environ. Microbiol.* **39**:194-204.
32. **Zehnder, A. J. B., B. A. Huser, T. D. Brock, and K. Wuhrmann.** 1980. Characterization of an acetate-decarboxylating, non-hydrogen-oxidizing methane bacterium. *Arch. Microbiol.* **124**:1-11.