Evidence for Isotopic Exchange during Metabolism of Stable-Isotope-Labeled Formate in a Methanogenic Sediment†

WIM DE GRAAF¹ AND THOMAS E. CAPPENBERG^{2*}

Centre for Limnology, Netherlands Institute of Ecology, 3631 AC Nieuwersluis,¹ and Centre for Estuarine and Coastal Ecology, Netherlands Institute of Ecology, 4401 EA Yerseke,² The Netherlands

Received 24 January 1996/Accepted 10 July 1996

The disappearance of stable-isotope-labeled formate in freshwater sediment from Lake Vechten was studied by gas chromatography-mass spectrometry. Both deuterium- and ¹³C-labeled formate were removed from pore water at a high rate (400 ± 35 [standard deviation] and $1,700 \pm 600 \mu\text{M h}^{-1}$, respectively), with concomitant formation of nonlabeled formate. The removal rate of labeled and nonlabeled formate amounted to 240 ± 15 and $400 \pm 40 \mu\text{M h}^{-1}$, respectively. The formation of nonlabeled formate is explained by isotopic exchange due to the activity of formate dehydrogenase rather than by the turnover of formate.

Volatile fatty acids are important intermediates in the anaerobic degradation of organic matter, as the end products of fermentation and as substrates for terminal carbon mineralization (4, 5, 11). The first members of the fatty acid series, formic and acetic acid, are particularly important with respect to the hydrogen and carbon flow through the indigenous microbial community. They are finally mineralized to water, carbon dioxide, and methane. Acetate is the principal substrate for methanogenesis in freshwater sediments (3, 4, 10) and sulfate reduction in marine sediments (12, 13). Just as the turnover of acetate gives a reasonable estimate of the carbon flow (1, 5, 6, 9), the turnover of formate might give an estimate of the hydrogen flow, as it is considered to be an important hydrogen transfer agent, the solubility of hydrogen being too low to account for the rapid interspecies diffusion (2). However, if formate and hydrogen are interconvertible, overestimation of the turnover of formate due to the exchange of labeled formate with protons of water or carbon of the carbonate pool can be expected. Here we report results of the study of the metabolism of D- and ¹³C-labeled formate in methanogenic sediment from Lake Vechten, The Netherlands.

Sampling and sites. Freshwater sediment samples were taken with a modified Jenkin mud sampler on 18 October 1993, at a 10.5-m depth, from the eastern depression of Lake Vechten, a freshwater mesotrophic monomictic lake (7, 9), located in the center of The Netherlands. The lake was stratified at the sampling date, and the oxygen in the hypolimnion was depleted. The 0- to 5-cm depth horizon of a core from Lake Vechten was rapidly transferred into a 300-ml sealed bottle, and the headspace was flushed with nitrogen for 5 min. The contents of the bottle were shaken thoroughly to homogenize the sediment section. Replicate subsamples (50 ml) of the sediment slurry were transferred with a nitrogen-flushed syringe and needle (2-mm inner diameter) into nitrogen-filled serum vials (100 ml) capped with butyl rubber septa. The headspaces were then flushed with nitrogen before incubation overnight at in situ temperature (8°C). Stable-isotope-labeled formate from a 5 mM solution of the sodium salt (Aldrich,

Beerse, Belgium) was introduced to a final concentration of ca. 100 μM . The sediment was mixed thoroughly, and incubation was started. Subsamples (1.5 ml) were removed by syringe at selected intervals, transferred to Eppendorf vials, and centrifuged with minimal delay (5 min at $4,000 \times g$, 7°C). The Eppendorf vials were stored on ice before and after centrifugation. Pore water was collected and stored frozen at -20°C until analysis.

Analysis of formate by gas chromatography-mass spectrometry and high-performance liquid chromatography (HPLC). Determination of stable-isotope-labeled formate was carried out by gas chromatography (Hewlett-Packard HP 5890A apparatus) with an HP-FFAP capillary column (length, 15 m; inner diameter, 0.32 mm; film thickness, 0.53 μm) and an HP 5970 mass spectrometer (see reference 9). Helium was used as the carrier gas at a flow rate of 4 ml min⁻¹. Pore water samples were acidified before injection with a 4% (vol/vol) solution of hydrofluoric acid (Suprapur; Merck) to a final concentration of 0.1% (vol/vol). Subsamples (1 μl) were introduced with a cooled injection system (CIS 3; Gerstell Analytical Systems, Middelburg, The Netherlands) held at 35°C, and the liner was heated at a rate of 10°C s⁻¹ to 100°C and then at a rate of 5°C min⁻¹ to 200°C. Injection was performed with the oven temperature held at 50°C and then allowed to rise at 10°C min⁻¹ to 120°C, at which temperature it was held for 1 min. The end of the column was linked through a transfer line (150°C) to the ionization chamber of the mass spectrometer (ionization energy, 70 eV). Spectral data were processed with an HP-300 computer. Analysis of labeled formate was performed in the single-ion monitoring mode. Formic acid and its stable isotopes were monitored at m/z 46 and m/z 47. Concentrations were calculated by comparing the peaks of m/z 47 (corrected for a 1.1% contribution of ¹³C from the blank formic acid peak at m/z 46) with those of gravimetric standards: sodium 1-D-formate (Pro Analyse; Merck) acidified with hydrofluoric acid (Ultrapur; Merck) to 0.1% (vol/vol). Good linear calibration was achieved ($r^2 = 0.997$), and there was no significant difference in the sensitivity of the molar response factors for deuterium- and ¹³C-labeled formates. Quantification of the nonlabeled formic acid at m/z 46 was not possible because of the high level and irreproducibility of blanks.

The analysis of (labeled and nonlabeled) formate was carried out by HPLC using an ion exclusion column (IEC-AS1; Dionex) and an anion micromembrane suppressor (AMMS 037107; Dionex). The eluant was a solution of 3 mM octane-

* Corresponding author. Mailing address: Centre for Estuarine and Coastal Ecology, Netherlands Institute of Ecology, Vierstraat 28, 4401 EA Yerseke, The Netherlands. Phone: 31-113571920. Fax: 31-113573631. Electronic mail address: cappenberg@cemo.nioo.knaw.nl.

† Netherlands Institute of Ecology publication number 2160.

FIG. 1. (A) Uptake of DCOO(H) in a mixed-sediment section from Lake Vechten. ○, DCOOH; ●, total formate; *, HCOOH (determined by difference). (B) Uptake of H¹³COO(H) in a replicate mixed-sediment section from Lake Vechten. ○, H¹³COOH; ●, total formate; *, HCOOH (determined by difference).

sulfonic acid (Dionex) and was pumped at a flow rate of 0.9 ml min⁻¹ by an LDC/Milton Roy Constametric 3000 apparatus. Frozen samples were thawed, shaken, and centrifuged before introduction into the eluant stream with a 300-μl loop (Reodyne). After separation, the eluant was neutralized over the membrane suppressor with 15 mM tetrabutylammonium hydroxide used as the regenerant at a flow rate of 0.7 ml min⁻¹. Conductivity detection was performed with an LDC/Milton Roy Conductomonitor III apparatus. Concentrations were calculated by comparison of the peaks with those of gravimetric standards of anhydrous sodium formate in water (Milli-Q).

Disappearance of DCOO⁻. A small amount of DCOONa was injected into a mixed section of Lake Vechten sediment to a calculated final concentration of ca. 100 μM, and the rapid disappearance of *m/z* 47 over time was monitored by gas chromatography-mass spectrometry. Since DCOO(H) does not disappear in an autoclaved sediment sample (data not shown), the disappearance of DCOOH is regarded as being due to its uptake by formate-utilizing microorganisms (Fig. 1A). The concentration profile of labeled formate showed a logarithmic shape over time. The formate concentration as revealed by HPLC decreased at a lower rate (240 ± 15 [standard deviation] μM h⁻¹; 0 order), and in 20 min formate was removed to levels below the limit of detection (2 μM). The difference plot of total formate minus DCOO⁻ clearly shows the appearance and disappearance of HCOO⁻.

Disappearance of H¹³COO⁻. A small amount of H¹³COONa was injected into a replicate sample of mixed sediment to a calculated final concentration of ca. 100 μM, and the disappearance was monitored at *m/z* 47 in a manner similar to that for DCOONa. Labeled formate decreased so rapidly that only 30 μM H¹³COO(H) was detected during the first minute (Fig. 1B), and the small amounts at 3 and 5 min indicate a logarithmic shape for the uptake curve. As revealed by HPLC, the formate concentration decreased much more slowly, though it decreased a little more quickly ($400 \mu\text{M} \pm 40 \text{ h}^{-1}$; 0 order) than in the experiment with DCOO⁻.

Formation of formate from molecular hydrogen. The headspace of a third replicate sediment mix was flushed with hydrogen gas, and the contents of the bottle were vigorously shaken for 1 min. The formate concentration increased in time, with an initial rate of 60 μM h⁻¹ in the first hour of incubation, and increased further to ca. 100 μM after 2 h (data not shown).

TABLE 1. Conversion rates of formate in replicates of a mixed-sediment section from Lake Vechten

Parameter	Rate ^a (μM h ⁻¹) of conversion of:	
	DCOOH	H ¹³ COOH
Uptake of labeled formate	400 ± 35	1,700 ± 600
Formation of HCOOH	160 ± 35	1,300 ± 600
Total uptake (0 order)	240 ± 15	400 ± 40

^a Values are means ± standard deviations.

With nitrogen in the headspace, formate concentrations are generally near the detection limit of ca. 2 μM.

Discussion. There is a large difference between the removal rates of D- and ¹³C-labeled formate, although the removal rates of total formate are comparable (Table 1). This observation points to the rapid interconversion of formate to hydrogen and carbonate rather than the turnover of formate and is supported by the observed formation of formate in H₂-amended sediment; similar observations were made with a pure culture of *Methanobacterium formicum* (15). A comparison of the formate conversion rates with the turnover of acetate in a replicate experiment, amounting to $33 \pm 9 \mu\text{M h}^{-1}$ when ¹³CD₃COONa is used (data not shown), provides more evidence. Whereas reasonable values for acetate turnover can be estimated (6), formate turnover should be in the same range, assuming that methane formation from formate-bicarbonate is ca. 25% and that four formates are needed for one methane (8). Therefore, it must be concluded that isotopic exchange occurs during the utilization of both deuterium- and ¹³C-labeled formate, whereas the exchange rate of ¹³C[carbonate] from H¹³COO⁻ is apparently much faster than that of deuterium from DCOO⁻. The faster disappearance of ¹³C-labeled formate was also observed in an earlier experiment, on 16 September 1993, in which formate disappearance was not monitored by HPLC: reciprocal turnover times were 3.1 ± 0.2 and $9.0 \pm 1.0 \text{ h}^{-1}$ for DCOO⁻ and H¹³COO⁻, respectively, whereas the reciprocal turnover times on 18 October 1993 amounted to 15 ± 0.7 and $40 \pm 14 \text{ h}^{-1}$, respectively.

Exchange might be enhanced by the high-level label addition (to 0.1 mM), which is a disturbance of the natural situation. This suggests that diffusion away from or to the cell surface limits the production and consumption of formate and/or hydrogen. The exchange phenomenon is in agreement with the view that formate, because of its better solubility, is the main hydrogen carrier among the bacteria of a syntrophic consortium (2, 14). The enzyme formate dehydrogenase most likely performs this conversion (15).

This work was supported by EC Environment and Climate contract EV5V-CT92-0065.

We thank S. E. Buykx for analytical assistance.

REFERENCES

1. Ansbaek, J., and T. H. Blackburn. 1980. A method for the analysis of acetate turnover in a coastal marine sediment. *FEMS Microbiol. Ecol.* 5:253-264.
2. Boone, D. R., R. L. Johnson, and Y. Liu. 1989. Diffusion of the interspecies electron carriers H₂ and formate in methanogenic ecosystems and its implications in the measurement of *K_m* for H₂ or formate uptake. *Appl. Environ. Microbiol.* 55:1735-1741.
3. Cappenberg, T. E., and E. Jongejans. 1978. Microenvironments for sulphate reduction and methane production in freshwater sediments, p. 129-138. In W. E. Krumbein (ed.), *Environmental biochemistry and geomicrobiology*, vol. 1. Ann Arbor Science, Ann Arbor, Mich.
4. Cappenberg, T. E., and R. A. Prins. 1974. Interrelation between sulfate-reducing and methane-producing bacteria in bottom deposits of a freshwater lake. III. Experiments with ¹⁴C-labeled substrates. *Antonie van Leeuwenhoek* 40:457-469.
5. Christiansen, D., and T. H. Blackburn. 1982. Turnover of ¹⁴C-labelled ac-

- etate in marine sediments. *Mar. Biol.* **71**:113–119.
6. **de Graaf, W., P. Wellsbury, R. J. Parkes, and T. E. Cappenberg.** 1996. Comparison of acetate turnover in methanogenic and sulfate-reducing sediments by radiolabeling and stable isotope labeling and specific inhibitors: evidence for isotopic exchange. *Appl. Environ. Microbiol.* **62**:772–777.
 7. **Gulati, R. D., and S. Parma.** 1982. Studies on Lake Vechten and Tjeukemeer, The Netherlands. *Hydrobiologia* **95**:1–383.
 8. **Guyot, J.-P., and A. Brauman.** 1986. Methane production from formate by syntrophic association of *Methanobacterium bryantii* and *Desulfovibrio vulgaris* JJ. *Appl. Environ. Microbiol.* **52**:1436–1437.
 9. **Hordijk, C. A., H. Kamminga, and T. E. Cappenberg.** 1994. Kinetic studies of acetate in freshwater sediment: use of stable isotope tracers. *Geochim. Cosmochim. Acta* **58**:683–694.
 10. **Lovley, D. R., and M. J. Klug.** 1982. Intermediary metabolism of organic matter in the sediments of a eutrophic lake. *Appl. Environ. Microbiol.* **43**:552–560.
 11. **Nedwell, D. B.** 1983. The input and mineralization of organic carbon in anaerobic aquatic sediments. *Adv. Microb. Ecol.* **7**:93–131.
 12. **Parkes, R. J., G. R. Gibson, I. Mueller-Harvey, W. J. Buckingham, and R. A. Herbert.** 1989. Determination of the substrates for sulphate-reducing bacteria within marine and estuarine sediments with different rates of sulphate reduction. *J. Gen. Microbiol.* **135**:175–187.
 13. **Sørensen, J., D. Christensen, and B. B. Jørgensen.** 1981. Volatile fatty acids and hydrogen as substrates for sulfate-reducing bacteria in anaerobic marine sediment. *Appl. Environ. Microbiol.* **42**:5–11.
 14. **Stams, A.** 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie Leeuwenhoek* **66**:271–294.
 15. **Wu, W.-M., R. F. Hickley, M. K. Jain, and J. G. Zeikus.** 1993. Energetics of formate and hydrogen metabolism by *Methanobacterium formicum*. *Arch. Microbiol.* **159**:57–65.