

## Rapid Method for the Radioisotopic Analysis of Gaseous End Products of Anaerobic Metabolism

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A gas chromatographic procedure for the simultaneous analysis of  $^{14}\text{C}$ -labeled and unlabeled metabolic gases from microbial methanogenic systems is described.  $\text{H}_2$ ,  $\text{CH}_4$ , and  $\text{CO}_2$  were separated within 2.5 min on a Carbosieve B column and were detected by thermal conductivity. Detector effluents were channeled into a gas proportional counter for measurement of radioactivity. This method was more rapid, sensitive, and convenient than gas chromatography-liquid scintillation techniques. The gas chromatography-gas proportional counting procedure was used to characterize the microbial decomposition of organic matter in anaerobic lake sediments and to monitor  $^{14}\text{CH}_4$  formation from  $\text{H}_2$  and  $^{14}\text{CO}_2$  by *Methanosarcina barkeri*.

During the course of physiological and ecological investigations of anaerobic microbial processes, it is often necessary to monitor metabolic gases utilized or produced. Methane, hydrogen, and carbon dioxide are the major gaseous end products in anaerobic environments where organic matter is being vigorously decomposed. The energy-yielding metabolism of methane-producing bacteria involves the oxidation of hydrogen with the concomitant reduction of carbon dioxide. It was discovered that following the uptake and release of  $\text{H}_2$ ,  $\text{CO}_2$ , and  $\text{CH}_4$  by radioactive tracer studies necessitated the development of a new gas chromatographic technique.

Gas chromatography has been extremely useful for the detection of microbial fermentation end products. Recently, Rogosa and Love (13) and Carlsson (2) have used flame ionization gas chromatography to analyze for various organic compounds in aqueous fermentation media. However, flame ionization detection is limited to the analysis of  $-\text{CH}$ -containing molecules, rendering this method of detection unsuitable for the study of certain gaseous end products of anaerobic metabolism. Gas chromatographic techniques using thermal conductivity detectors have the ability to detect both organic and inorganic gaseous compounds, and have been used to separate and detect  $\text{H}_2$ ,  $\text{CH}_4$ ,  $\text{CO}_2$ , and other major constituents of fuel gases (3). The use of a flow-through method, such as gas proportional counting, for the measurement of radioactivity in gas chromatographic effluents has been reported (6, 10, 12). However, thermal

conductivity detection coupled with gas proportional counting has not been previously used for analysis in biological systems. We present here a procedure for the separation and quantification of labeled and unlabeled metabolic gases present during radioisotopic tracer studies of microbial methanogenesis.

### MATERIALS AND METHODS

**Equipment.** A Packard model 419 Becker dual column gas chromatograph (Packard Instrument Co., Inc., Downers Grove, Ill.) equipped with flame ionization and thermal conductivity detectors was used. The effluent gases from the thermal conductivity detector were fed directly to a Packard model 894 gas proportional counter where the gases were combusted to  $\text{CO}_2$  and water and radioactivity was quantified by a multiplicative ion collection device. The counting efficiency for  $^{14}\text{CO}_2$  and  $^{14}\text{CH}_4$  was 80% when a time constant of 5 s was used. The outputs of the thermal conductivity detector and the gas proportional counter were recorded on a Honeywell Elektronik 19 dual pen recorder (Honeywell Inc., Fort Washington, Pa.) with a 1-mV full-scale sensitivity at an attenuation of 1.

**Column and packing.** Samples were separated on a coiled stainless-steel column (9 ft by  $\frac{1}{8}$  in, about 2.7 m by 0.3 cm outer diameter). The packing material was Carbosieve B (120/140 mesh, Supelco, Inc., Bellefonte, Pa.). The column was conditioned at 200 C overnight before use.

**Gases and gas flows.** Helium (industrial and laboratory grade, Chemetron Corp., Chicago, Ill.) and nitrogen (prepurified, Matheson Gas Products, Joliet, Ill.) were used as carrier gases. Trace  $\text{O}_2$  was scrubbed out of the gases with an Oxy-trap (Regis Chemical Co., Morton Grove, Ill.) placed in the gas line. Either carrier gas could be used when the gas proportional

counter was not operated; helium was the carrier when the proportional counter was used. Propane (CP grade, Matheson Gas Products) was the quench gas for the proportional counter. The flow rate for carrier gas was 50 cm<sup>3</sup>/min and 5 cm<sup>3</sup>/min for quench gas.

**Operation.** After numerous trials, the following parameters were chosen: gas flows as described; inlet temperature of 200 C; oven temperature of 190 C; detector temperature of 200 C; <sup>14</sup>C oxidation furnace temperature of 750 C; current through the thermal conductivity detector of 250 mA for He carrier gas and 150 mA for N<sub>2</sub> carrier gas.

**Culture methods.** Cultures of *Methanosarcina barkeri* were kindly provided by M. P. Bryant. *M. barkeri* was grown under strict anaerobic conditions at pH 7.2 in 18-mm culture tubes that contained 10 ml of filter-sterilized phosphate-buffered basal media (PBBM). The anaerobic culture technique described by Hungate (5) and modified by Bryant and Robinson (1) was used. PBBM contained per liter of distilled water: 0.45 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.9 g of NaCl; 0.18 g of MgSO<sub>4</sub> · 7 H<sub>2</sub>O; 0.1 g of CaCl<sub>2</sub> · 2H<sub>2</sub>O; 0.5 g of NH<sub>4</sub>Cl; 1.5 g of KH<sub>2</sub>PO<sub>4</sub>; 2.19 g of K<sub>2</sub>HPO<sub>4</sub>; 9 ml of trace mineral solution (15); 5 ml of vitamin solution (15); 0.3 ml of 5% FeSO<sub>4</sub>; 1 ml of 0.2% resazurin (Eastman Kodak Co., Rochester, N.Y.) as redox indicator; and 1 g of Na<sub>2</sub>S added after filter sterilization as reducing agent. Cultures were gassed with a mixture of 80% hydrogen and 20% carbon dioxide and incubated at 30 C on a reciprocating shaker.

**Preparation of gas samples.** No special preparative methods were used prior to the injection of gas samples into the gas chromatograph. The procedure for sampling was to withdraw 0.4 cm<sup>3</sup> of atmosphere from a reaction vial and to inject this into the gas chromatograph-gas proportional counting system. The preparation of radioactive gas samples for liquid scintillation was to withdraw 0.4 cm<sup>3</sup> of atmosphere from a reaction vial and trap any <sup>14</sup>CO<sub>2</sub> by injection of the gas into a vial of ethanol-ethanolamine (1:2) solution. The remaining gas, methane, was withdrawn from the trapping vial and injected into a vial filled with 20 ml of scintillation fluid, as previously

described by McBride and Wolfe (10). The trapped <sup>14</sup>CO<sub>2</sub>, in ethanol-ethanolamine, was added to 10 ml of Triton X-100-toluene counting solution. Samples were counted on a Packard model 3375 Tri-Carb scintillation spectrometer.

**Ecological studies.** Sediment was collected anaerobically from a depth of 10 m in Lake Mendota. A sediment slurry was prepared from 100 g of sediment by the addition of 20 ml of PBBM. Five milliliters of the sediment slurry was dispensed into 20-ml gas-tight serum vials. All vials were incubated at 37 C and sampled once a day for H<sub>2</sub>, <sup>14</sup>CO<sub>2</sub>, and <sup>14</sup>CH<sub>4</sub>. This incubation temperature was optimal for methane formation in Lake Mendota sediments.

**Physiological studies.** From an actively growing culture of *M. barkeri*, 1 ml of cell suspension was withdrawn and added to tubes with 10 ml of PBBM. The culture contained 5.2 mg (dry weight) of cells per ml. All tubes were incubated at 30 C and sampled every 8 h for H<sub>2</sub>, <sup>14</sup>CH<sub>4</sub>, and <sup>14</sup>CO<sub>2</sub>.

**Radioactive compounds.** The following radioactive compounds at the concentrations listed were used in ecological and physiological studies: [<sup>14</sup>C]cellulose, 75 μg, 0.2 μCi (ICN, Irvine, Calif.); H<sup>14</sup>COONa, 0.05 M, 5 μCi (New England Nuclear, Boston, Mass.); and HaH<sup>14</sup>CO<sub>2</sub>, 1 μCi (New England Nuclear). The following radioactive compounds were used as standards: <sup>14</sup>CH<sub>4</sub> (Amersham/Searle), specific activity of 7.1 mCi/mmol; and <sup>14</sup>CO<sub>2</sub>, obtained from NaH<sup>14</sup>CO<sub>3</sub> (Amersham/Searle), specific activity of 59.1 mCi/mmol.

## RESULTS AND DISCUSSION

A typical chromatogram (Fig. 1) illustrates the separation of a gas mixture that contained H<sub>2</sub>, CH<sub>4</sub>, and CO<sub>2</sub> at equimolar concentrations. The retention times were 37, 103, and 150 s for H<sub>2</sub>, CH<sub>4</sub>, and CO<sub>2</sub>, respectively, when either helium or nitrogen was used as the carrier. These retention times were much shorter than values reported for columns of comparable

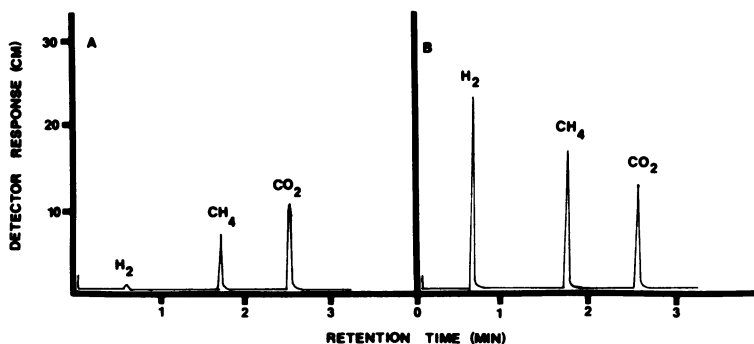


FIG. 1. Separation and detection of 25 nmol each of H<sub>2</sub>, CH<sub>4</sub>, and CO<sub>2</sub> on a 9-ft (about 2.7 m) Carbosieve B column by thermal conductivity gas chromatography. (A) Helium was used as the carrier gas; (B) N<sub>2</sub> was used as the carrier gas. The signal was attenuated at 1x. Peak height and area are proportional to the amount of gas analyzed.

length packed with silica gel or other molecular sieves (8).

The responses of the thermal conductivity detector and the column stability were excellent. Hundreds of samples were run and the separation and detection characteristics remained the same as when the column was freshly packed. Detection of radioactivity by the gas proportional counter also showed reproducible performance. The radioactivity of a gas was measured 20 to 30 s after the gas had been detected by the thermal conductivity detector.

The sensitivity of the gas proportional measurements of radioactivity compared to liquid scintillation are noted in Table 1. The improved sensitivity of counting  $^{14}\text{CH}_4$  by gas proportional methods over liquid scintillation reflects the low solubility of methane in scintillation fluids (4).  $\text{CO}_2$  is easily trapped from gas mixtures and is highly soluble in scintillation fluids. The data for  $^{14}\text{CO}_2$  counting (Table 1) illustrates the similar efficiencies for counting by gas proportional and liquid scintillation techniques.

This technique lends itself well to the study of the anaerobic decomposition of organic matter in lake sediments. The addition of formate to sediments resulted in an immediate increase in the rate of methane formation over the endogenous level (Fig. 2). The rapid appearance of  $^{14}\text{CH}_4$  from  $\text{H}^{14}\text{COONa}$  suggests that formate can be directly metabolized by methanogenic organisms. Cellulose stimulated methane production only after a long lag. The rapid build-up of  $^{14}\text{CO}_2$  prior to  $^{14}\text{CH}_4$  formation indicates that cellulose is not directly utilized by methane bacteria. Cellulose must first be decomposed by other organisms into compounds readily metabolized by methanogenic bacteria (14), such as formate, acetate,  $\text{H}_2$ ,  $\text{CO}_2$ , etc.

The applicability of this method to physiolog-

TABLE 1. Comparison between gas proportional and liquid scintillation counting

Gas	Sample	Counts/min	
		Proportional counter	Liquid scintillation
$^{14}\text{CH}_4^a$	1	1,220	217
$^{14}\text{CH}_4^b$	2	1,217	172
$^{14}\text{CO}_2^a$	1	560	500
$^{14}\text{CO}_2^b$	2	570	425

<sup>a</sup> Sample size was 50 nmol of  $^{14}\text{CH}_4$  or 25 nmol of  $^{14}\text{CO}_2$ .

<sup>b</sup> Gas samples were from a mixture that contained 50 nmol of  $^{14}\text{CH}_4$  and 25 nmol of  $^{14}\text{CO}_2$ .

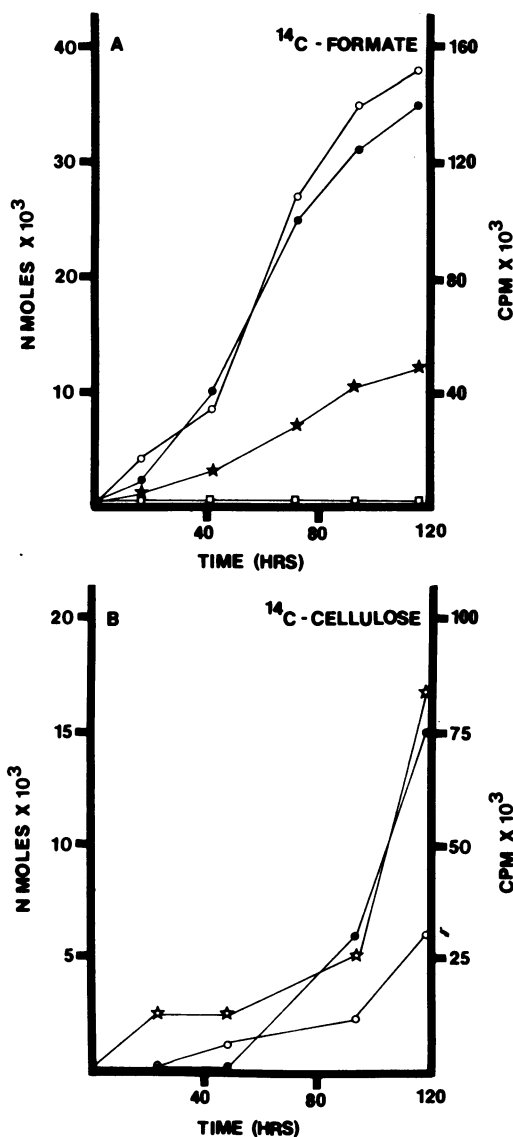


FIG. 2. Gas production from radioactive formate and cellulose added to lake sediment samples. (A) Total nanomoles of  $\text{CH}_4$  (●) and counts per minute of  $^{14}\text{CH}_4$  (○) produced from a slurry sample that contained  $\text{H}^{14}\text{COONa}$  is compared with total  $\text{CH}_4$  formation in a slurry sample without additions (★) and to total  $\text{CH}_4$  formation in a control killed with 5% formaldehyde (□). (B) Total nanomoles of  $\text{CH}_4$  (●), counts per minute of  $^{14}\text{CH}_4$  (○), and counts per minute of  $^{14}\text{CO}_2$  (★) produced from a sediment slurry sample with [ $^{14}\text{C}$ ]cellulose added.

ical studies is shown by Fig. 3. The formation of  $^{14}\text{CH}_4$  from  $\text{H}_2$  and  $\text{NaH}^{14}\text{CO}_3$  by *M. barkeri* was followed with minimal disturbance to the

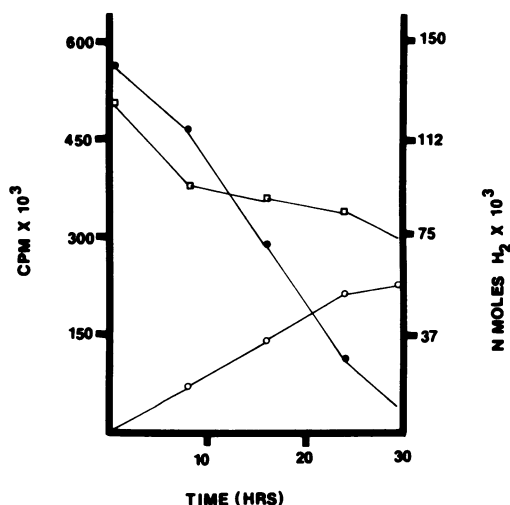


FIG. 3. Production of  $^{14}\text{CH}_4$  from  $\text{H}_2$  and  $\text{NaH}^{14}\text{CO}_3$  by *M. barkeri*. Total  $^{14}\text{CH}_4$  produced, O; total  $\text{H}_2$ , ●; and total  $^{14}\text{CO}_2$ , □.

system. In this way, precise stoichiometric relationships may be determined for the utilization of substrates as energy and carbon sources.

The gas chromatography-gas proportional counting system described here provides a rapid, accurate tool for the simultaneous analysis of gaseous compounds and radioactivity. Such a system has great advantages over gas chromatography and liquid scintillation counting in ease of preparation and handling of samples and in greatly increased accuracy of analysis. These data demonstrate the usefulness of this technique when applied to ecological and physiological investigations. Implementation of this new method for the study of carbon turnover in various ecosystems and for physiological and biochemical analysis of gas-producing and -utilizing microorganisms should be of great value to scientists.

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