APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1991, p. 1866–1869
0099-2240/91/061866-04$02.00/0

Vol. 57, No. 6

Unexpected Errors in Gas Chromatographic Analysis of Methane Production by Thermophilic Bacteria

BYOUNG-KWAN KIM AND LACY DANIELS*

Department of Microbiology, University of Iowa, Iowa City, Iowa 52242

Received 4 January 1991/Accepted 9 April 1991

Unexpected errors in methane measurement by gas chromatography occurred when samples at thermophilic temperatures were analyzed. With a standard curve prepared at room temperature (25°C), stoppered bottles incubated and sampled at 37 to 85°C showed more methane upon analysis than bottles incubated at 25°C: values at 50, 63, and 85°C were 109, 126, and 125%, respectively, of the 25°C value. All variation between 4 and 50°C can be explained by the temperature difference between culture bottle and sampling syringe, and the variation of methane concentration can be predicted by the gas law. Between 50 and 63°C, there was a more dramatic rise than predicted by theory. These variations are important to consider if thermophilic methane production is to be measured accurately. Methods to avoid errors are discussed.

Methane produced by methanogenic bacteria or enzyme systems is most commonly measured by gas chromatography (GC) (1, 3, 15, 16, 21). Most laboratories use a glass syringe (with a rubber or Teflon plunger) with a locking device (e.g., a Mininert valve or a Pressure-Lok syringe) and needle to take samples from a vessel through a rubber stopper or septum. Since cell growth vessel pressure varies greatly with time, the use of a syringe lock is essential (for example, an H_2-CO_2 culture may vary from 0.6 to 2.5 atm [1 atm = 101.29 kPa] of pressure before and after, respectively, "feeding" with H_2-CO_2). These pressure changes result from the metabolism of substrates into methane, as described by equations 1, 2, and 3:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (1)$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad (2)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (3)$$

In equation 1, 5 mol of gas is converted to 1 mol of gas, and the pressure drops with time. In the other reactions, a liquid-phase substrate is converted into a gas-phase product, and the pressure rises. With a pressure-lock syringe, a known gas sample volume (e.g., 0.30 ml) from a container of a known volume (e.g., 20.5-ml gas phase in a serum tube of a 26.5-ml total volume, with 6.0 ml of medium) is injected into a gas chromatograph, and the number of nanomoles in the sample (calculated from a standard curve) is multiplied by the ratio of volumes (68.33 in our example above) to obtain the total number of nanomoles per tube; this method is independent of pressure changes. Recently, an autoinjector sampling method has been described (15); this eliminates analytical errors arising from syringe use and injection variations between individuals but uses the same concept of a fixed volume ratio. Measurements not accounting for pressure variations cannot be acceptably accurate unless the sampled systems do not significantly vary in pressure from atmospheric pressure (e.g., many cell-free methanogenesis assays) or by a constant amount that is used as a correction factor.

The system described above has been used for the past 25 years to study methanogens, but most often analysis details such as the locking syringes are not described in the articles (1, 3–5, 7, 11–13, 16, 18, 21, 22). Mesophilic species studied are typically grown at 37°C, but there is an increasing number of studies of thermophilic species, grown especially at 65°C but also at 85 to 95°C (5, 8, 14, 19, 22). It has been assumed in the literature that GC analyses for methane from bottles and tubes at different temperatures yield the same results and that standard curves created at room temperature are valid for such measurements. We demonstrate clearly in this article that if cultures at their thermophilic growth temperature are analyzed for methane by the normal syringe method, an error large enough to affect experimental interpretations can be observed. This temperature-dependent error becomes appreciable (>15%) at 55 to 85°C. We discuss explanations and solutions for this problem.

Serum bottles (540 ml, no. 223952; Wheaton Scientific, Millville, N.J.) sealed with cutoff no. 2 black rubber stoppers and aluminum seals (no. 224187; Wheaton) were used for all experiments. Bottles were made anaerobic with nitrogen or hydrogen gas as described by Balch and Wolfe (1) and modified by the method of Daniels et al. (7) and, finally, pressurized with 10 lb/in^2 (gauge) of gas. A separate bottle was evacuated by water vacuum using a gas train, flushed with 5 lb/in^2 (gauge) of methane for 30 min, and continuously supplied with 20 lb/in^2 (gauge) of methane by using a pressure regulator and hose connection to the methane cylinder (CP grade; Air Products Co., Tamaqua, Pa.). From this bottle, both standard curve and experimental methane bottles were prepared; methane was transferred to each bottle with a 5-ml Pressure-Lok D gas syringe (no. 040035; Alltech Associates, Deerfield, Ill.) already flushed twice with methane. The standard curve was composed of eight methane concentrations and is further described with the results below. After overnight incubation at 25°C, the methane concentration in each experimental bottle was checked by a gas chromatograph, and all bottles having similar concentrations were used for further tests; this allowed elimination of bottles with leaking stoppers.

A GC-9A gas chromatograph (Shimadzu Co., Kyoto, Japan) was used with the following conditions: 200°C column and 250°C injector temperatures; 50 ml/min helium carrier gas flow; Carbosieve S-II (no. 1-0190; Supelco, Bellefonte, Pa.) stainless steel column; flame ionization detection with 0.5-kg/cm^2 air and hydrogen pressure. Injection volume was 100 μl, with a 250-μl gastight syringe (no. 1725LT; Hamilton,

* Corresponding author.

Reno, Nev.) with a Mininert syringe valve (no. 654051; Alltech) and a 25-gauge needle. The syringe was covered with silicon tubing 2 mm thick to prevent heat transfer from hand to syringe during sampling.

Bottle temperature was controlled with gyratory water baths. Samples were taken without removal of the bottles from the water. One water bath (type Grant SS-40-A3C(L); Science Electronic Inc., Miamisburg, Ohio) was shaken at 120 strokes per min to maintain temperature at or above 25°C. A modified water bath (type 3545; Lab-Line Instruments, Melrose Park, Ill.) was shaken at 160 rpm to maintain temperature at 4 or 15°C; to attain these cooler temperatures, a coil of copper tubing (ca. 3 m long, one-fourth of an inch outside diameter) was placed in the bottom of the bath and connected to an external refrigerated water bath circulator (type Lauda RMS20; Brinkmann Instruments, Königshofen, Germany).

Experimental bottles prepared as described above, containing neither liquid nor cells, were divided into two groups, each with three bottles. One group was kept at 25°C, and the other was kept at the temperature to be examined. Analysis was conducted three times for both groups at 12-h intervals after a 1-day preincubation. Sampling was standardized to reduce variation in the amount of gas removed; the plunger was quickly moved back and forth (half its length) three times and adjusted to the 100-μl level, and after a 10-s wait, the valve was closed and the sample was injected into the gas chromatograph.

Methane production by resuspended cells was carried out by methods modified from those described by Daniels and Zeikus (9). *Methanobacterium thermoautotrophicum* Marburg cells, grown in a 16-liter fermentor (6) and stored as a suspension at 4°C, were used to initiate methane production in 540-ml bottles. The three bottles held 50 ml of KMM-1 buffer solution containing (in millimolar concentration) KH_2PO_4 (96), K_2HPO_4 (40), $MgCl_2$ (0.1), NH_4Cl (5.0), NaCl (10), Na_2S (4.0), and resazurin (0.0022), at a final pH of 6.5. Bottles were made anaerobic by evacuation and flushing with pure hydrogen gas (1, 7), autoclaved at 121°C for 20 min, and then pressurized to 20 lb/in^2 (gauge) of H_2. After 3 ml of concentrated resuspended cells was inoculated into 50 ml of buffer and shaken at 63°C for 3 h, the bottles were flushed with pure hydrogen at 20 lb/in^2 (gauge) for 3 min to remove the methane present, and 0.4 ml of a Na_2S stock solution (0.5 M) was added to replace lost H_2S. Methane production was reinitiated by the addition of 1.5 ml of a 0.50 M stock solution of $NaHCO_3$, accounting for the addition of 750 μmol of CO_2 equivalent. The bottles were then incubated with shaking at 63°C, and gas samples were taken at intervals for GC analysis without removing bottles from the water bath. After each sampling at 63°C, the bottles were cooled rapidly to 25°C with ca. 12°C tap water and then incubated for 10 min in the 25°C water bath, and then another gas sample was taken for analysis.

In experiments aimed at developing a more convenient method for providing cells to other laboratories for producing ^{14}C-CH_4 (9), we provided bottles of resuspended cells of *M. thermoautotrophicum* strain Marburg with 250 μmol of unlabeled sodium bicarbonate in the presence of excess H_2 and no additional CO_2. When the cells were incubated and samples were taken at 63°C, it was observed that 270 to 300 μmol were measured by GC, representing 108 to 120% of the expected maximum methane. After prolonged incubation at 63°C and gas measurement from bottles cooled to near room temperature, close to 95% of the expected value was observed.

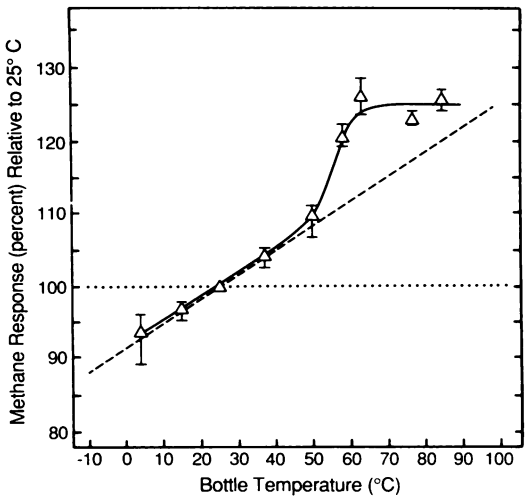

FIG. 1. Response in GC analysis for methane, relative to response with 25°C methane bottles, when sampled gas was in bottles at various temperatures. The dashed line indicates the calculated response if the gas law were to explain the observed deviation from 100%, assuming that the difference in temperatures of the syringe and the sampled bottle causes the deviation. The triangles indicate the averages of three replicate bottles in three separate measurements; the bottles contained about 193 μmol of methane. The bars above and below the triangles represent the extremes of methane averages from the experiments. The dotted line indicates the 100% value for methane, which is normally assumed in the literature.

To understand this phenomenon, a review of our analysis technique was undertaken. A new standard curve was established, including data collected on five separate days. The standard curve was linear in the range of 10 to 178 nmol of methane per injection, and the correlation coefficient was 0.996. Maximum variations between the average curve and individual points on one day were 9%. Variations as a function of the time of day (which might arise from small voltage fluctuations) were less than 5%. With the new standard curve for analysis of the resuspended-cell experiments, the unexpected extra methane was still observed.

A series of experiments were designed to examine the effects of gas temperature on GC response. Bottles (540 ml) without medium or cells were prepared dry, injected with known amounts of methane, and then analyzed while being kept at various temperatures. Figure 1 describes the results of these experiments. As the temperature rose from 25 to 85°C, there was a steady increase in response until 50°C, where a 109% response was seen. Above 50°C, the response was nonlinear and more extreme. The data suggest that cells incubated at 60 to 85°C should show an erroneous response of about 120 to 125% of that expected, which is consistent with our original unplanned experiments with resuspended cells. We thus examined this prediction more formally with methane-producing cells.

Resuspended cells of *M. thermoautotrophicum* were injected into 540-ml bottles containing KMM-1 buffer. After preincubation with pure hydrogen to remove traces of CO_2, the bottles were flushed thoroughly with hydrogen, and 750 μmol of $NaHCO_3$ was injected. Bottles were incubated with shaking at 63°C, cells were analyzed directly at 63°C, and then bottles were quickly cooled to 25°C and the cells were sampled a second time and analyzed again. As shown in Fig. 2, methane responses were always higher in the 63°C sam-

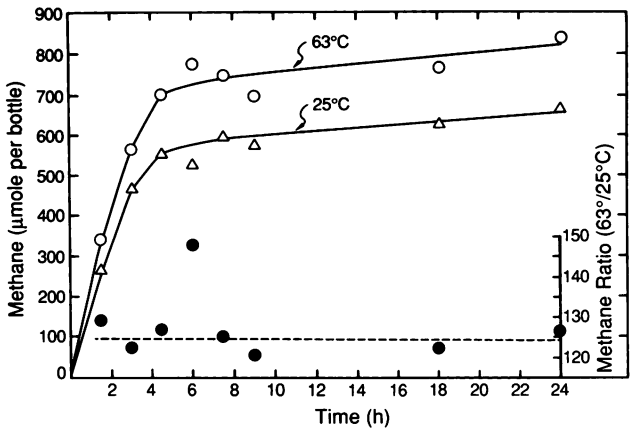

FIG. 2. Methane production by resuspended cells of *M. thermoautotrophicum*. The culture bottles were incubated at 63°C, but samples were taken both when the bottles were at 63°C and when they were cooled to 25°C for 10 min. ○, 63°C sampling; △, 25°C sampling; ●, ratio of methane measurement at 63°C compared with measurement at 25°C. Dashed line indicates the average excess methane production observed (124.6%) at the thermophilic sampling temperature.

pling, and the curves plotted with the two different sampling temperatures showed appreciable differences. The average excess methane response, as plotted with the dashed line in Fig. 2, was ca. 125% of the 25°C value. The near-final yield (25°C analysis) of 671 μmol was 90% of the bicarbonate added, consistent with an expected 4 to 8% assimilated into cell carbon and a few percent of the bicarbonate still unconsumed after 24 h. Several factors were considered in an effort to explain these unexpected data showing a repeatable excess methane response.

First, to explain our resuspended-cell results, we addressed the assumption that temperature-caused changes in methane solubility result in negligible changes in the total methane contained in the gas phase. This assumption is based upon informally discussed calculations of the amount of methane that can be dissolved in the medium compared with the amount in the gas phase. By using gas solubility data (17, 20) and the fact that 1 mol of gas occupies a 24-liter volume at 20°C at 1 atm of pressure (total), it can be calculated that at 20°C and 1 atm of methane (0 atm of gauge pressure), the dissolved methane is 1.4 mM; if a tube (26.5 ml) contains 6.0 ml of culture, then the gas phase (20.5 ml) would contain 0.854 mmol of methane, and the medium would contain 0.0084 mmol of dissolved methane; this represents only 1.0% of the tube's methane, i.e., 99.0% is in the gas phase. With temperature increases to 35, 70, and 100°C, the methane in solution decreases with a shift from 99.0% in the gas phase to 99.4, 99.5, and 99.6%, respectively. Thus, release of methane from solution during heating has a minimal effect on the amount of methane in the gas phase, and the practice to ignore dissolved methane is justified.

Second, we realized that the syringe was at room temperature, while the culture bottle was at the incubation temperature. Since sampling was done promptly (ca. 15 s), gas entering the syringe would be cooled by the glass; our room temperature is 23 to 25°C, independent of the day or season. Thus, we considered the ideal gas properties as described by the gas law (17):

$$PV = nRT \quad (4)$$

When gas is in a cooler environment, and pressure is the same, then the mole/volume ratio will be higher, i.e., the gas will be more concentrated. A similar phenomenon is expected if the vessel from which the sample is taken is colder than the syringe, i.e., the warmer syringe will result in the gas therein being less concentrated than the gas in the vessel. Our bottle volume did not significantly change over the temperatures examined: at room temperature and at 85°C, a bottle had an outside diameter of 57 mm. The pressures in the bottles and the syringe were the same, since they were connected via the needle. Thus, equation 4 can be used to derive equation 5, from which the molar quantities in syringe samples from bottles at different temperatures can be compared: constant = $PV/R = n_1T_1 = n_2T_2$, and $n_1/n_2 = T_2/T_1$, where T_1 is 273.2 + 25°C = 298.2 K, T_2 is 273.2 + x°C, n_1 is moles of methane in a constant volume at 25°C, n_2 is moles of methane in a constant volume at x°C, and y is methane response relative to 25°C, in percent.

$$y = \frac{(273.2 + x)}{298.2} \times 100 \quad (5)$$

These calculated data are plotted in Fig. 1 as a dashed line and fit our observed data from 4 to 50°C very well. Above this temperature, there was a transient jump, which leveled off at 70 to 85°C. Thus, our theory of temperature difference between the syringe and the sampled bottle explains part of the data but not the transient jump. We think this transient change is due to slight glass syringe barrel volume change due to heat transfer from the hot gas.

We examined two correction equations intended to account for nonideal gas behavior at high temperature and pressure: the Virial equation and the Benedict-Webb-Rubin equation (10, 17). Both show that there is less than a 0.5% change in methane behavior compared with that of an ideal gas from 25 to 100°C. Possible unusual phenomena that might explain the results were also investigated. Black rubber stoppers, cut into smaller pieces and placed in the bottles, neither released nor absorbed methane during heating. The presence of water in the bottles did not affect the measured methane. When H_2 instead of N_2 was used to replace air in the bottles, similar results were obtained. Analysis with syringes heated to the bottle temperature showed variable results, possibly arising from syringe temperature reduction during use, with corresponding syringe volume changes.

We conclude that in experiments with thermophilic methanogens, if samples are taken from culture containers maintained at the thermophilic temperatures, measurement errors can result when a standard curve prepared at room temperature is used. In reality, many researchers remove several culture containers from the water baths and take samples from the gas phases at various times afterwards, which can also result in variable results; we speculate that this phenomenon has not previously been reported because of this practice, in which some containers are measured at close to room temperature, and measurements of replicate vessels at different temperatures are averaged. For many purposes, the easiest solution would be to cool the containers to room temperature rapidly (e.g., under tap water) before analysis; although this may have unknown microbiological effects, several methanogens have responded well to being cooled and reheated, without lag phases (2; Fig. 2). Alternatively, a correction factor could be used, or a standard curve could be prepared at the thermophilic temperature, but the latter

option would be complicated if different incubation temperatures were used in the same laboratory for different organisms. Although we cannot fully explain the observed results, a significant portion of the temperature effect is caused by the differences in temperature between the culture container and the syringe, and this calculated effect accurately fits the data between 4 and 50°C and closely estimates the variation at 85°C.

This work was supported by contract N00014-88-K-0195 to L.D. from the Office of Naval Research.

ADDENDUM IN PROOF

After our article was accepted for publication, we learned of work published by Koch and Zinder (M. Koch and S. Zinder, Arch. Microbiol. **138**:263–272, 1984) that noted an overestimation of methane produced at 60°C. These investigators attributed the effect to the difference in temperature between bottle and syringe, as we did, but suggested that the cause of the problem was condensation of water vapor; they developed an empirical correction factor based on their culture work at 60°C. It may be noted that in our article we demonstrate that the phenomenon persists even with dry methane, as predicted by the gas law.

REFERENCES

1. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32**:781–791.
2. **Belay, N.** Unpublished data.
3. **Belay, N., and L. Daniels.** 1987. Production of ethane, ethylene, and acetylene from halogenated hydrocarbons by methanogenic bacteria. Appl. Environ. Microbiol. **53**:1604–1610.
4. **Belay, N., R. Sparling, and L. Daniels.** 1986. Relationship of formate to growth and methanogenesis by *Methanococcus thermolithotrophicus*. Appl. Environ. Microbiol. **52**:1080–1085.
5. **Brandis, A., R. K. Thauer, and K. O. Stetter.** 1981. Relatedness of strains ΔH and Marburg of *Methanobacterium thermoautotrophicum*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Orig. C **2**:311–317.
6. **Daniels, L., N. Belay, and B. Mukhopadhyay.** 1984. Considerations for the use and large-scale growth of methanogenic bacteria. Biotechnol. Bioeng. Symp. **14**:199–213.
7. **Daniels, L., N. Belay, and B. S. Rajagopal.** 1986. Assimilatory reduction of sulfate and sulfite by methanogenic bacteria. Appl. Environ. Microbiol. **51**:703–709.
8. **Daniels, L., R. Sparling, and G. D. Sprott.** 1984. The bioenergetics of methanogenesis. Biochim. Biophys. Acta **768**:113–163.
9. **Daniels, L., and J. G. Zeikus.** 1982. Convenient biological preparation of pure high specific activity ^{14}C-labelled methane. J. Labelled Compd. Radiopharm. **XX**:17–24.
10. **Dymond, J. H., and E. B. Smith.** 1980. The second virial coefficients of pure gases and mixtures: a critical compilation. Clarendon Press, Oxford.
11. **Huber, H., M. Thomm, H. Konig, G. Thies, and K. O. Stetter.** 1982. *Methanococcus thermolithotrophicus*, a novel thermophilic lithotrophic methanogen. Arch. Microbiol. **132**:47–50.
12. **Huber, R., M. Kurr, H. W. Jannasch, and K. O. Stetter.** 1989. A novel group of abyssal methanogenic archaebacteria (*Methanopyrus*) growing at 110°C. Nature (London) **342**:833–834.
13. **Jones, W. J., J. A. Leigh, F. Mayer, C. R. Woese, and R. S. Wolfe.** 1983. *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch. Microbiol. **136**:254–261.
14. **Jones, W. J., D. P. Nagle, Jr., and W. B. Whitman.** 1987. Methanogens and the diversity of archaebacteria. Microbiol. Rev. **51**:135–177.
15. **Liu, Y., D. R. Boone, and C. Choy.** 1990. *Methanohalophilus oregonense* sp. nov., a methylotrophic methanogen from an alkaline, saline aquifer. Int. J. Syst. Bacteriol. **40**:111–116.
16. **Nelson, D. R., and J. G. Zeikus.** 1974. Rapid method for the radioisotopic analysis of gaseous end products of anaerobic metabolism. Appl. Environ. Microbiol. **28**:258–261.
17. **Perry, R. H., D. W. Green, and J. O. Maloney (ed.).** 1984. Perry's chemical engineer's handbook, 6th ed. McGraw-Hill Book Co., New York.
18. **Rajagopal, B. S., and L. Daniels.** 1986. Investigation of mercaptans, organic sulfides, and inorganic sulfur compounds as sulfur sources for the growth of methanogenic bacteria. Curr. Microbiol. **14**:137–144.
19. **Thauer, R. K.** 1990. Energy metabolism of methanogenic bacteria. Biochim. Biophys. Acta **1018**:256–259.
20. **Weast, R. C., M. J. Astle, and W. H. Berger (ed.).** 1988. CRC handbook of chemistry and physics, 69th ed. CRC Press, Boca Raton, Fla.
21. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238**:2822–2826.
22. **Zeikus, J. G., and R. S. Wolfe.** 1972. *Methanobacterium thermoautotrophicus*, sp. n., an anaerobic, autotrophic, extreme thermophile. J. Bacteriol. **109**:707–713.