CONVENIENT BIOLOGICAL PREPARATION OF PURE HIGH SPECIFIC ACTIVITY ^{14}C-LABELLED METHANE

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SUMMARY

Radioactive methane of high specific activity was easily prepared using growing cultures or resuspended frozen cells of Methanobacterium thermoautotrophicum. This method gave a greater than 90% conversion of the substrate "$^{14}CO_2$"* into $^{14}CH_4$ using H_2 as a reductant, and is also applicable to the production of $^{13}CH_4$.

Key words: ^{14}C-methane, ^{13}C-methane, ^{3}H-methane, ^{2}H-methane, biological labelled methane

INTRODUCTION

Recent interest in the physiology, biochemistry and ecology of methane oxidizing bacteria and yeasts has produced a need for labelled methane to study metabolic pathways and environmental carbon flow (1, 2, 3 and references within). Some of these investigations have led to the discovery of novel pathways for one-carbon assimiliation (1, 3). Present work on these methylotrophs is particularly relevant to areas of biotechnology since single-cell protein production by cell growth

* No distinctions are made between CO_2, HCO_3^- and $CO_3^=$ when the symbol "CO_2" is used.

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on methane (or other one-carbon substrates) is an ongoing industrial venture; further, the alkane oxidation reactions of these organisms may be of industrial importance in the modification of some chemicals (3, 4). However, the limited availability and very high cost of labelled CH_4 can make some experiments unrealistic. Presently $^{14}CH_4$ is prepared commercially by inorganic catalytic reactions. A good general method of isotopic methane production using ruthenium-on-alumina catalysis has been described in this journal (5).

All known species of methane production bacteria use the following energy generating reaction:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

This reaction is highly favored thermodynamically, with $\Delta G^{o\prime} = -31.3$ kcal/rxn and thus has a Keq = 10^{20}. The general method of biological ^{14}C-methane production was proposed by the authors to other workers (6) who have reported their use of the methane in their experiments. Described here are the rationale and details of two simple and inexpensive methods taking advantage of the biological reaction using either growing or resuspended frozen cells of Methanobacterium thermoautotrophicum.

MATERIALS AND METHODS

Methanobacterium thermautotrophicum strain ΔH (ATCC # 29096) was grown in pressurized culture tubes (Bellco Glass, Vineland, NJ) as previously described (7) with a 3 atm overpressure of H_2/CO_2 (80:20 v/v). Anaerobic procedures with 23 gauge needles on 1 ml syringes were used in all cultural manipulations. Large cultures were grown in a 14 l New Brunswick fermentor continuously gassed with H_2/CO_2, using a previously described medium (8); cells were harvested aerobically with a Sharples continuous flow centrifuge and frozen immediately as pellets in liquid nitrogen and stored at -70°C. Care

should be taken when working with the pressurized systems and flammable gases but the tubes described and thick rubber hoses adequately clamped in place for use in gas transfer have presented no hazards in our labs. Quantitation of labelled and unlabelled gases was performed with a gas chromatograph equipped with a thermal conductivity detector and connected to a gas proportional counter (9). A thermal conductivity detector was used alone in the analysis of the experiment described in Table 2. $Na_2\ ^{14}CO_3$ (58 - 60 m Ci/m mole, Amersham/Searle) was used in the preparation of $^{14}CH_4$.

RESULTS AND DISCUSSION

We have developed two methods for the production of labeled methane.

Growing cell method: The following procedure was used to prepare $^{14}CH_4$ with the same specific activity as that of the $Na_2\ ^{14}CO_3$ used. Culture tubes that contained 10 ml of medium were inoculated with 1 ml of a log phase culture of M. thermoautotrophicum, pressurized to 3 atm overpressure with H_2/CO_2 and incubated with shaking at 65°C. The culture tube was repressurized with H_2/CO_2 after the absorbance at 660 nm reached 0.15-0.2. When the absorbance increased above 0.3, residual CO_2 was removed by gassing the tube vigorously with 100% H_2 for 5 min, then the tube was pressurized at 2 atm with 100% H_2. After incubating the culture as described above for one hour, the tube was gassed again with 100% H_2 to remove the CH_4 formed, and pressurized to 1 atm overpressure with H_2. An anaerobic solution of $Na_2\ ^{14}CO_3$ (prepared by repeated evacuation and flushing of the solution with N_2) was injected by syringe into the culture tube and the cells were incubated for 3-4 hours, at which time it was assured that all $^{14}CO_2$ was metabolized.

The specific activity of the $^{14}CH_4$ formed was shown in an experiment with low specific activity $Na_2\ ^{14}CO_3$ to be nearly identical with

that of the $Na_2\ ^{14}CO_3$ added (Table I). Labeled methane produced by this method was not contaminated by traces of $^{14}CO_2$, as shown in Figure 1. Although some "$^{14}CO_2$" was incorporated by the cells, the biological production of $^{14}CH_4$ has an efficiency of > 90%.

TABLE I. PRODUCTION OF $^{14}CH_4$ OF THE SAME SPECIFIC ACTIVITY AS ADDED $Na_2\ ^{14}CO_3$[1]

	Intial DPM present	Final DPM present	% of original DPM added	Specific activity (DPM/nmole)
CO_2	44.4×10^5	0	100	222.6
CH_4	0	41.7×10^5	94	214.8
Cells	0	0.46×10^5	1	Not determined

1 Experimental conditions were as described in the growing cell method.

The labeled methane formed can then be transferred to a separate container either by Pressure-Lok syringe (Precision Sampling Co., Baton Rouge, LA) (6; Mary Lindstrom, personal communication), or by transfer under vacuum with liquid N_2 as the freezing agent (10). Although the excess of H_2 and the resulting thermodynamics suggest the reaction should go to completion, possible remaining traces of "$^{14}CO_2$" may be trapped in the original solution by the addition of base. This technique has been used in the preparation of mCi amounts of $^{14}CH_4$.

Cell resuspension method: To demonstrate that the resuspension of cells would work in the production of methane, the experiment described in Table II was conducted. Experimental phase growing cells or cells from a resuspension of frozen pellets stored at -70°C were placed anaerobically in 5 ml of anaerobic 100 mM $K\text{-}PO_4$ buffer, 1 mM cysteine-HCl, 1 mM Na_2 S, and gased with H_2 via inlet and outlet needles (23 gauge). (Media with low buffer capacity should not be used since the raising of medium pH due to CO_2 removal may occur.) The frozen cell resuspension (0.2 g wet weight in 2 ml medium) was prepared by rapidly placing the frozen pellet into a dry tube, stoppering the tube and evacuating and flushing prior to cell thawing. Buffer (2 ml) was then added and the

tube was gassed thoroughly with H_2/CO_2, pressurized to 3 atm total gas and incubated at 62° for 1 hr prior to twnety-fold dilution in culture medium. The experimental tubes, as described in Table II, were gased with H_2 and examined at intervals for CH_4 production. All tubes exhibited adequate methanogenesis, and repeated gasing demonstrated that little methane was produced in the absence of added Na_2CO_3. Methanogenesis increased on addition of Na_2CO_3, and after an overnight incubation, 93-100% of the theoretical yield was achieved. The resuspension method was successfully used in the preparation of 5 mCi of $^{14}CH_4$ (data not shown).

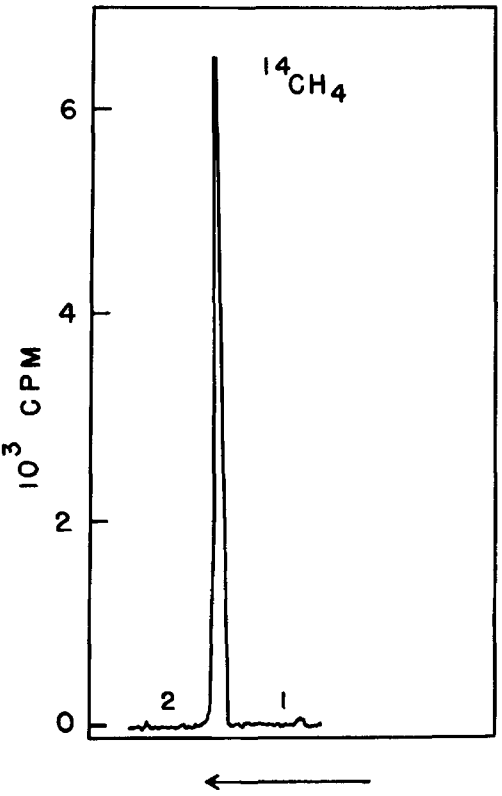

Fig. 1. Gas proportional counter tracing showing radioactive purity of $^{14}CH_4$. Radioactivity refers to that in a 0.2 ml sample of a 3100X diluted sample from a $^{14}CH_4$ preparation. 1 indicates the position of sample injection. 2 indicates the position at which $^{14}CO_2$ should appear. The arrow indicates the direction of the pen tracing.

Cells frozen for about one year at -70°C worked well in this system, and it is expected that cells will remain functional after storage for several years.

TABLE II. PRODUCTION OF METHANE BY SUSPENSIONS OF GROWING CELLS AND FROZEN PELLETS OF M. THERMOAUTOTROPHICUM

Sample[1]	Wet weight mg/tube	CH_4 Production (μmoles CH_4 per tube)				
		CO_2 removal incubation[2]		CH_4 generation incubation[3]		
		1 h	2 h	4 h'	16 h'	Theoretical yield
Growing cells	2.0	24	3	126	150	150
Frozen cells	10.4	32	1	114	140	150
Frozen cells	5.3	13	1	100	147	150

1 Samples were from a growing culture or a 20-fold diluted sample of resuspended frozen cells. Samples were placed in 5 ml of 100 mM KPO_4 buffer, pH 6.8, containing 1 mM cys-HCl, and 1 mM Na_2S.
2 Tubes were gassed with H_2 between the first and second hours of incubation (CO_2 removal).
3 Tubes were gassed with H_2 after the second hour of scrubbing incubation and were inoculated with 1 ml of 150 mM Na_2CO_3 at T_0; hr' represent times elapsed after this addition (CH_4 generation).

DISCUSSION

Both of the techniques work well for the production of radiochemically pure $^{14}CH_4$. The inorganic methods available to the biochemist or microbiologist for laboratory scale preparations (e.g. ref. 5) require somewhat unfamiliar techniques and extreme conditions. The $^{14}CH_4$ must also be free of O_2 if anaerobic CH_4 oxidation is to be studied. Since $Na_2{}^{14}CO_3$ is considerably less expensive than $^{14}CH_4$ an appreciable savings to the experimentor is possible. Also, the specific activity obtainable is virtually identical to that of the starting material, and is higher than that available in some commercial preparations. The growing cell method might be preferred by one who is properly equipped and trained for the growth of methanogens. However, a much simpler system is needed

for the use of resuspended frozen cells, and might be preferred by most experimentors. From approximately one gram of frozen cells (e.g. obtained from the first author's lab) many preparations of $^{14}CH_4$ can be made over a period of several years with minimal experimental apparatus (i.e. tightly stoppered sturdy tubes or vials, a water vacuum set up in line with a H_2 tank and a 3-way valve or stopcock, and some needles and syringes). Also, with thick suspensions and the original frozen pellet, anaerobicity is not so critical as with more dilute cultures. If a determination of the specific activity of the product methane is desired, the more commonly found scintillation counter can be used as an alternative to the proportional counter (9) using the method described by Zehnder et al. (11).

M. thermoautotrophicum was chosen for this preparation because of its ease and speed of growth, good yields, durability and minimal oxygen sensitivity; however any of a variety of other methanogens may also prove useful.

The synthesis of ^{13}C-methane with the same enrichment as the starting material would undoubtedly be possible using these methods. Also, as demonstrated previously, the preparation of C^3H_4 or C^2H_4 is possible using water as the source of isotope; H_2 gas does not contribute directly to the "hydrogen" in methane (12, 13).

Although the processes described above were performed in 26 ml tubes, culture bottles used in our laboratories (250 ml, Wheaton Scientific) can be used to produce mmole quantities. The rates shown in Table II imply that a dozen or less grams wet weight of cells should yield mole quantities of methane in about one day.

An addendum describing details relevant to those who are interested in this technique but who are unacquainted with anaerobic techniques is available from the first author.

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