

## MICROBIOLOGICAL PREPARATION OF $^3\text{H}$ -LABELLED METHANE

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### SUMMARY

Small quantities of tritiated methane were prepared by fermenting [methyl- $^3\text{H}$ ] methylamine hydrochloride using exponential phase, transiently starved, co-cultures of methanogenic bacteria. This method gave the expected 75% yield of  $\text{C}^3\text{H}_4$  from the substrate and produced  $\text{H}_2$ -free, low  $^3\text{H}_2\text{O}$  background  $\text{C}^3\text{H}_4$  for tracer studies.

**Key Words:**  $^3\text{H}$ -methane,  $^{14}\text{C}$ -methane, biologically labelled methane, methane oxidation.

### INTRODUCTION

An increase in atmospheric methane concentration has been reported recently (1); methane is a radiatively and chemically active gas and has the potential to modify climate through greenhouse warming as well as by modifying concentrations of other radiatively active gases through reactions with the OH radical. A search for biological sinks capable of modulating methane emissions has led to increased interest in the microbial ecology, physiology and biochemistry of aerobic methane oxidation as well as continued interest in the controversial and poorly understood phenomenon of anaerobic methane oxidation (2, 3). This has produced a need for labelled methane to study methane turnover in a variety of environments.

It is important that the pool size of reactants under study not be affected appreciably by added tracer in measurements of *in situ* rates. Radiocarbon labelled methane ( $^{14}\text{CH}_4$ ) can be synthesized with specific activities up to  $7.99 \times 10^{12}$  Bq/mmol (60 mCi/mmol) (4) and used to obtain reliable rates of methane oxidation in environments where methane concentration is in the mM range (2). However, in environments such as water columns where the methane is present in  $\mu\text{M}$  to nM concentrations, the use of  $^{14}\text{CH}_4$  could alter the methane pool size and require additional concentration/rate information. Tritiated

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methane ( $\text{C}^3\text{H}_4$ ) can be obtained at much higher specific activities (up to  $1.15 \times 10^{16}$  Bq/mmol, 86 Ci/mmol) and is more suitable for experiments in environments with low methane concentration. Geochemical studies on stable C and H isotopes in naturally occurring methane suggest that H isotope exchange between methane and water does not occur (5). Unfortunately,  $\text{C}^3\text{H}_4$  is expensive and is of limited commercial availability.

A commonly used procedure for the synthesis of  $^{14}\text{CH}_4$  has been previously presented by Daniels and Zeikus (4). They suggested that tritiated methane could be produced with this procedure by using tritiated water in the medium. Previous work showed that the hydrogen in methane originates from the aqueous medium, and not from headspace  $\text{H}_2$  (6, 7). This method has been attempted in this laboratory and was successful (unpublished results). Difficulties inherent in this method include: 1) The tritiated methane produced by this method would be contaminated by high levels of  $^3\text{H}_2\text{O}$  vapour and 2) large amounts of radioactivity would have to be added to the aqueous medium to obtain  $\text{C}^3\text{H}_4$  of high specific activity.

Another method for producing  $\text{C}^3\text{H}_4$  similar to the method reported here would employ methanogens that have the ability to produce methane from tritiated acetate. However, this method could present problems because methanogens that use acetate grow very slowly (Lacey Daniels, personal communication).

A few species of methanogenic bacteria use methanol and methylated amines for growth and methanogenesis (8). We describe the preparation of small quantities of high specific activity tritiated methane from [methyl- $^3\text{H}$ ] methylamine hydrochloride using co-cultures of strains of methanogenic bacteria similar to *Methanococcoides methylutens* and *Methanosarcina barkeri*.

#### MATERIALS AND METHODS

The two species of methanogenic bacteria used to synthesize tritiated methane have been previously isolated in pure culture (9). Using variations of the Hungate technique (10, 11), the same bacteria were obtained in co-culture for this work by serial dilution using anoxic marine sediment as a source of inoculum. Pure mono-cultures were not needed as both strains utilized methylamine to produce methane. Enrichment, isolation in co-culture and maintenance of the methanogenic bacteria was carried out in a slightly modified version of medium number 3 of Balch *et al.* (12). The medium used for this work

omitted the trace vitamins and contained only 0.25 g/L yeast extract and, in lieu of trypticase, contained 0.125 g/L of Bacto-Peptone (Difco). It also contained 0.78 g/L HEPES as a buffering agent. The medium was pre-reduced by boiling under a stream of  $\text{O}_2$ -free nitrogen and 16 ml was dispensed anaerobically (13) into "Balch" type test tubes (18 x 150 mm) which were sealed with butyl rubber stoppers secured by aluminum crimp seals (Bellco Glass, Vineland, NJ). After autoclaving, tubes were amended from sterile, anoxic stock solutions to reach the following final concentrations: ampicillin-sodium salt, 200  $\mu\text{g/ml}$ ; vancomycin, 100  $\mu\text{g/ml}$ ; streptomycin-sulfate, 100  $\mu\text{g/ml}$ ; sodium bicarbonate, 10 mM; methylamine hydrochloride 0.2% v/v; and sodium sulphide, 0.03% v/v. All reagents used were analytical grade (Sigma Chemical Co.). The methanogens used to produce  $\text{C}^3\text{H}_4$  were grown on a rotary shaker at room temperature.

Growth of the co-culture was monitored by gas chromatographic analysis of methane in subsamples of the culture headspace over time. Successive subculturing revealed that the late exponential growth phase was reached after three days, so co-cultures used later for the production of tritiated methane were harvested after three days of growth and transiently starved. The transient starvation was accomplished by centrifuging the cells and resuspending the pellet in fresh medium containing no methylamine. This medium replacement was done by first flushing the headspace of tubes with a sterile flow of oxygen-free nitrogen using 21 ga gassing and venting needles. The venting needle was positioned near the inside edge of the tube and stopper so that when the tube was inverted the used medium was expelled. Careful handling of the tube ensured that the pellet remained intact. A tube of sterile, fresh, substrate-free medium was then gassed and vented (as above) and connected in series to the tube containing the pellet. By inverting the tube containing the fresh medium, new medium was added to the pellet containing tube.

Two separate co-cultures were starved by the method outlined above and monitored for further methane production. When the rate of methane production approached zero (Fig. 1), an aliquot of tritiated methylamine hydrochloride solution was added by syringe. The [methyl- $^3$ ]methylamine hydrochloride,  $1.33 \times 10^{12}$  Bq, 10 mCi, ( $6.39 \times 10^{15}$  Bq/mmol; 48 Ci/mmol) was obtained commercially (Amersham Corporation, Arlington Heights, IL) and two separate syntheses were performed. The labelled substrate was transferred to a serum vial and made anoxic before addition by repeatedly gassing the headspace with

oxygen-free nitrogen. After the labelled methylamine hydrochloride was added to initiate the second synthesis, the vial that had contained the labelled substrate was rinsed with sterile anoxic distilled water; this rinse was added to the starved culture. The final liquid volume of both cultures was about 19 ml; the headspace volume was 8 ml. The cultures were incubated for one day and were checked for the production of  $C^3H_4$ . A sample of the headspace of both cultures was diluted 100-fold and was assayed by the method of Zehnder *et al.* (14). Biological activity was halted by the addition of 0.2 ml of 10 N  $H_2SO_4$ .

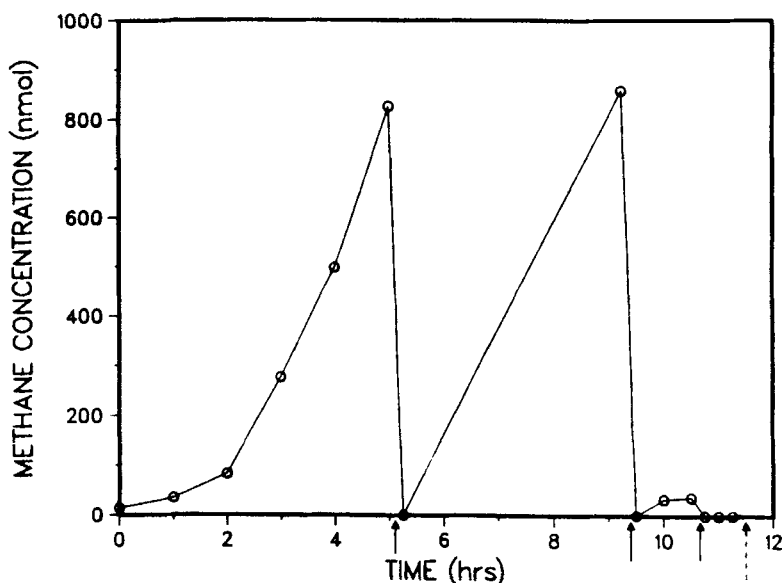


Fig 1. Total headspace methane production by the second transiently starved co-culture later used to produce tritiated methane. The solid arrows indicate time points when the headspace of the co-culture was flushed with  $O_2$ -free nitrogen for five minutes. The dashed arrow indicates time of label addition.

The headspace of the culture tubes was displaced by addition of anoxic distilled water into a 10-ml Glaspak syringe (Becton-Dickinson) fitted with a Mini-nert valve (Supelco). The syringe contents were then transferred to evacuated 8-ml serum vials fitted with black butyl rubber and aluminum crimp seals. These two vials each contained about 0.5 g of granular  $P_2O_5$  (J. T. Baker Chemical Co.) to absorb residual water vapour. Labelled methane was removed from these vials for field experiments; equal volumes of mercury were added following removal to maintain constant pressure.

## RESULTS AND DISCUSSION

Production of methane from methylamine with a methanogen co-culture should proceed according to the following overall fermentation equation:



so that complete reaction should convert 75% of the labelled methylamine into  $\text{C}^3\text{H}_4$ . The remaining label should be present as  $^3\text{H}_2\text{O}$  resulting from oxidation during the fermentation. Later assays of killed controls used in the estimation of both aerobic and anaerobic methane oxidation in the water column of the Black Sea revealed some contamination of the tritiated methane with tritiated water (about  $1.22 \times 10^6$  Bq/100  $\mu\text{l}$ , 9.18 nCi/100  $\mu\text{l}$ ). Table I summarizes the results of the syntheses.

Table I. Tritiated methane yield.

Final $\text{C}^3\text{H}_4$ Activity	% Yield <sup>1</sup>	Specific Activity
<u>Culture #1</u>		
$5.58 \times 10^{11}$ Bq (4.23 mCi)	84.6%	$3.74 \times 10^{14}$ Bq/mmol (2.81 Ci/mmol)
<u>Culture #2</u>		
$4.41 \times 10^{11}$ Bq (3.34 mCi)	66.8%	$4.59 \times 10^{14}$ Bq/mmol (3.44 Ci/mmol)
<u>Overall</u>		
$9.99 \times 10^{11}$ Bq (7.57 mCi)	75.7%	NA <sup>2</sup>

<sup>1</sup> These yields assume a starting activity of  $6.6 \times 10^{11}$  Bq (5 mCi) for each culture; in fact, the two cultures received different starting activities.

<sup>2</sup> NA, not applicable as the tritiated methane from the two syntheses was not pooled.

The results in Table I show that the synthesis went to completion since the overall expected 75% yield, as demonstrated by the activity of the  $\text{C}^3\text{H}_4$  product, was obtained. The  $\text{C}^3\text{H}_4$  specific activity, determined after a series of oxidation rate experiments, was lower than the  $4.75 \times 10^{15}$  Bq/mmol (36 Ci/mmol) expected. Production of unlabelled methane during the synthesis is the only process that can simultaneously account for the correct overall yield and lower specific activity, indicating that methane production from unlabelled substrates remaining after transient starvation must have occurred. Possibly, this can be minimized by longer periods of transient starvation. Also, there are indications

(unpublished observations) that elimination of the yeast extract from the medium used for transient starvation will further minimize unlabelled methane production.

Losses of  $C^3H_4$  due to dissolution in the distilled water used to transfer the methane from the culture vessel to the syringe also occurred; these can be minimized by using a saturated salt solution as the displacing fluid. For most experimental applications of  $C^3H_4$ , it is important to minimize the activity of  $^3H_2O$ , as this is the product of oxidation. The transfer should be conducted with agitation, allowing time for the  $^3H_2O$  to equilibrate and be diluted by the medium, reducing its gas phase activity.

Future attempts to produce tritiated methane might also make use of starved, resuspended, frozen cells similar to a procedure previously employed to produce  $^{14}CH_4$  (4).

Finally, it should be noted that any methanogens capable of producing methane from methyl groups could also be used to produce  $^{14}CH_4$ . C-14 methane produced by this method would be free of excess hydrogen (4) and would be valuable in experimental situations where added hydrogen is of concern. Cultures used to produce C-14 methane by this procedure would have to be rendered sufficiently alkaline to absorb the  $^{14}CO_2$  produced by this process from the headspace gas.

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