This is to certify that the

thesis entitled

KINETIC PARAMETERS OF THE CONVERSION OF METHANE PRECURSORS TO METHANE IN A HYPEREUTROPHIC LAKE SEDIMENT

presented by

R. F. Strayer

has been accepted towards fulfillment
of the requirements for

Ph.D degree in Microbiology

Joseph Tiedje
Major professor

Date August 10, 1977

MEU

METHANE PRODUCTION BY THE MICROFLORA OF THE ANAEROBIC PELAGIAL SEDIMENTS OF A HYPEREUTROPHIC LAKE

By

Richard Floyd Strayer

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1977

ABSTRACT

METHANE PRODUCTION BY THE MICROFLORA OF THE ANAEROBIC PELAGIAL SEDIMENTS OF A HYPEREUTROPHIC LAKE

By

Richard Floyd Strayer

Fluorescent antibody (FA) was prepared for a hydrogen-consuming methanogenic bacterium isolated from Wintergreen Lake pelagic sediment. The isolate resembles Methanobacterium formicicum. The FA did not cross react with nine other methanogens, including M. formicicum strains, or 24 heterotrophs, 18 of which had been isolated from Wintergreen Lake sediment. In a qualitative survey of different anaerobic habitats, FA reacting methanogens were detected in heat fixed smears of several different lake sediments and anaerobic sewage sludge but not in bovine rumen fluid. FA direct counts of the specific methanogen in Wintergreen Lake sediments were made on four different sampling dates and compared with five tube most probable number (MPN) estimates of the total methanogenic population that was present in the same samples. The FA counts ranged from 3.1×10^6 to $1.4 \times 10^7 \cdot g^{-1}$ dry sediment. The highest MPN estimates were at least an order of magnitude lower. These results indicate that a specific hydrogen-consuming methanogenic bacterium is among the predominant methane formers in this habitat.

Michaelis-Menten kinetic analysis was used to determine the kinetic parameters for the conversion of methane precursors to methane by the sediment microflora. For these experiments I designed an incubation

system that was not easily contaminated by air, that gave a large gas atmosphere reservoir so that added hydrogen was not significantly depleted during incubation, and that maximized the phase transfer of gaseous substrates and products.

Two independent methods were used to estimate the kinetic parameters for the conversion of hydrogen to methane. Direct linear plots were used to estimate parameters from initial velocity versus initial substrate concentration (v vs. S) experiments and an integrated solution to the Michaelis-Menten equation was used to estimate parameters from hydrogen and methane progress curves. Michaelis-Menten constants (K_m) ranged from 0.13 to 0.47% hydrogen for v vs. S experiments and 0.21 to 0.48% hydrogen for progress curve experiments. The mean K_m estimates for hydrogen consumption were 13 nmol $H_2 \cdot g^{-1}$ dry sediment for sediment collected in January and February and 29 nmol $H_2 \cdot g^{-1}$ for sediment collected in March. For the conversion of hydrogen to methane the average K_m was 23 nmol $H_2 \cdot g^{-1}$. For both v vs. S and progress curve experiments the maximum velocity (V_{max}) ranged from 1.1 to 5.9 μmol $H_2 \cdot g^{-1} \cdot h^{-1}$ for winter sediments. The V_{max} increased slightly from December to March.

A new method was devised to quantitate the very low concentrations of dissolved hydrogen in the sediment. The method involves the transferral of dissolved sediment gases to a carbon dioxide gas atmosphere. These sediment gases are then concentrated by absorption of the carbon dioxide by a high ionic-strength alkaline solution. For winter sediments the <u>in situ</u> dissolved hydrogen concentration ranged from 6 to 10 nmol$\cdot g^{-1}$. The natural rates of hydrogen consumption and hydrogen conversion to methane were estimated from these dissolved H_2 concentrations and the K_m and V_{max} estimates. For hydrogen consumption, the estimated

natural rate ranged from 430 to 1500 nmol $H_2 \cdot g^{-1} \cdot h^{-1}$. For the conversion of hydrogen to methane, the natural rate ranged from 72 to 290 nmol CH_4 produced$\cdot g^{-1} \cdot h^{-1}$. These results indicate that the methanogenic bacteria are capable of maintaining the low *in situ* hydrogen concentrations that are a necessary prerequisite for interspecies hydrogen transfer. The rate of hydrogen consumption by sediment microflora was high enough to lower moderately high hydrogen levels to normal concentrations within several hours.

Several potential hydrogen donors--formate, lactate, propionate, valine, and leucine--were tested for the ability to stimulate sediment methanogenesis. Only formate significantly altered the rate. Rates of formate conversion to methane were so rapid that accurate kinetic estimates could not be made. For example, at an incubation time of only 10 min, at least 47% of the added formate had been converted to methane. Shorter incubation times were tried but kinetic parameters could not be estimated even though stimulation of methanogenesis occurred.

The addition of unlabeled acetate had no effect on the rate of sediment methanogenesis but in other experiments [2-^{14}C] acetate was converted to labeled methane. Kinetic experiments with labeled acetate were used to resolve these apparently conflicting results and to estimate the kinetic parameters $K_m + S_n$, V_{max}, and turnover time, T_t. The results indicated that the conversion of acetate to methane was occurring at rates very near the maximum. The minimum natural rate of acetate conversion to methane was estimated from the minimum *in situ* dissolved acetate concentration (measured by Molongoski and Klug (unpublished data) for Wintergreen Lake pelagic sediment in the summer of 1976) and

the average turnover time estimated from the kinetic experiments. The minimum rate was estimated to be 0.108 $\mu mol \cdot g^{-1} \cdot h^{-1}$, which was 67% of the V_{max}. Using the average sediment dissolved acetate concentration for the entire summer of 1976, the estimated natural rate was 88% of the V_{max}. These results indicate that increases in heterotrophic acetate production in the sediment would lead to an increase in the acetate pool size since the acetate-using methanogenic bacteria are already converting this substrate at or very near the maximum rate.

A 15 min preincubation of sediment with 0.5% hydrogen in the gas phase had a pronounced effect on the kinetic parameters for the conversion of acetate to methane. When compared with parameters estimated from experiments with no hydrogen preincubation, the acetate pool size, as $K_m + S_n$, decreased by 37% and the turnover time for the acetate pool decreased by 43%. The V_{max} remained relatively constant. These results indicate that interspecies hydrogen transfer may be occurring in the sediment since less oxidized substrate, such as acetate, is produced in an uncoupled system. Further evidence that interspecies hydrogen transfer occurs was that a preincubation with hydrogen caused a 37% decrease in the amount of radioactive CO_2 produced from the heterotrophic metabolism of labeled valine by sediment microflora.

To my wife, Diane, for her encouragement and patience. To my parents for their moral support.

ACKNOWLEDGEMENTS

I am deeply grateful to Dr. J. M. Tiedje for his expert guidance and encouragement.

Special thanks are due to Dr. M. J. Klug for his helpful disucssions and for serving on my Guidance Committee.

I am also thankful to Drs. C. A. Reddy, H. L. Sadoff, L. L. Bieber, and J. A. Breznak for serving on my Guidance Committee.

My appreciation is extended to Drs. G. H. Lauff and M. J. Klug for making the facilities of the Kellogg Biological Station available.

I would like to thank the following people for supplying cultures: Drs. M. P. Bryant, J. G. Zeikus, M. J. B. Paynter, J. G. Ferry, M. J. Klug, and C. A. Reddy, and J. J. Molongoski.

I am also grateful for financial assistance from the Department of Microbiology and Public Health at Michigan State University, the National Institute of Health, and the National Science Foundation.

I am grateful to Dr. M. P. Bryant for taking me into his laboratory to teach me the Hungate anaerobic technique.

A special thanks is due to Sue Knoll for the task of typing the many revisions of this manuscript.

I owe too a special thanks to my friends, past and present, in the quite informal "Institute of Microbial Ecology", better known as Dr. Tiedje's research group. Their discussions on a variety of topics made my educational experience a much more rewarding one.

TABLE OF CONTENTS

		Page
LIST OF TABLES		vi
LIST OF FIGURES		viii
CHAPTER I.	REVIEW OF PERTINENT LITERATURE AND RATIONALE FOR EXPERIMENTAL APPROACH	1
	INTRODUCTION	1
	SUBSTRATES FOR METHANOGENIC BACTERIA IN PURE CULTURE	4
	METHANE PRODUCTION IN ANAEROBIC HABITATS	4
	Hydrogen as a substrate	4
	Acetate as a substrate	7
	INTERSPECIES H_2 TRANSFER	14
	RESEARCH REPORTED IN THIS DISSERTATION	15
	LITERATURE CITED	21
CHAPTER II.	APPLICATION OF THE FLUORESCENT ANTIBODY TECHNIQUE TO STUDY A METHANOGENIC BACTERIUM IN LAKE SEDIMENTS	24
	INTRODUCTION	24
	MATERIALS AND METHODS	25
	Media	25
	Anaerobic methodology	26
	Antigen preparation	26
	Antibody preparation	27
	FA stain of cultures and natural samples	27
	RESULTS	29
	Description of methanogenic organism purified from the sediment	29
	Tests on antibody prepared with the methanogen	30
	Qualitative survey of various habitats for FA reacting bacteria	34
	Direct count FA and MPN estimates of the methanogenic bacteria in sediments	34
	DISCUSSION	39
	LITERATURE CITED	43

Page

CHAPTER III. KINETIC PARAMETERS OF THE CONVERSION OF METHANE PRECURSORS TO METHANE IN A HYPEREUTROPHIC LAKE SEDIMENT 46

INTRODUCTION 46
MATERIALS AND METHODS. 49
Sampling 49
Incubation system. 49
Estimation of dissolved hydrogen 52
Gas analysis 53
Radioactive isotopes and analysis of radioactive gases. 53
Theory 54
RESULTS. 56
Kinetics of hydrogen conversion to methane 56
Other hydrogen donors and the stimulation of methanogenesis. 64
Kinetics of acetate conversion to methane. 64
DISCUSSION 83
LITERATURE CITED 90

LIST OF TABLES

Table		Page
	CHAPTER I	
1	Summary of reported examples of pure culture studies in which interspecies hydrogen transfer has been shown, and the effect of this metabolic coupling on the end products. The hypothesized interaction for natural sediments is included for comparative purposes	18
	CHAPTER II	
1	Control tests needed for the application of FA (from Schmidt (20)).	33
2	Microorganisms tested for cross-reactivity with FA preparation: no cross-reaction observed.	35
3	Number of methane producing bacteria in Wintergreen Lake sediment on different sampling dates as estimated by FA direct counts and by a five tube MPN technique using three different media. MPN tubes were scored as positive if methane was produced within 4 weeks.	38
	CHAPTER III	
1	Summary of kinetic experiments of the consumption of hydrogen and the conversion of hydrogen to methane by Wintergreen Lake sediments.	59
2	Ratio of hydrogen consumed to methane produced with decreasing hydrogen concentration calculated from the progress curves of Fig. 4.	67
3	Effect of potential hydrogen donors on the rate of sediment methanogenesis. Incubation temperature was 12 C	68
4	Effect of dissolved formate and dissolved acetate concentrations on the rate of sediment methanogenesis. Incubation time was 10 min at 23 C. Sediment was collected in the late winter	72

Table | Page

5 Kinetic parameters estimated from the regression of t/f versus A, as in Fig. 8. Experiment 1.-run within 4 days of collecting sediment in early July. Experiment 2.-run within 2 weeks of collecting sediment in late June 80

LIST OF FIGURES

Figure		Page
	CHAPTER I	
1	Generalized scheme of the anaerobic decomposition of organic matter	3
2	Effect of the fraction of the total volume occupied by the aqueous phase on the transfer of dissolved hydrogen to the vapor phase for incubation temperatures of 10 and 35 C. Arrows denote the percent aqueous phase in the incubation systems of Smith and Mah (27) and Cappenberg and Prins (7). Curves calculated from the equation of Flett et al. (12)	13
3	Comparison of the products formed and energy generated in the fermentation of glucose by Ruminococcus albus grown in mono-culture and in mixed culture with a methanogen. The figure is derived from Thauer et al. (29) but with the hydrogen utilizing Vibrio succinogenes replaced by a hydrogen-using methanogen.	17
	CHAPTER II	
1	Phase contrast photomicrograph of a short filament and single cells of a methanogenic bacterium isolated from Wintergreen Lake sediment and used to prepare the FA. Bar represents 10 um.	32
2	Photomicrograph of an FA-stained smear of the methanogenic isolate. Bar represents 10 um	32
3	Photomicrographs of immunofluorescing cells in FA stained smears of various habitats. (a) Wintergreen Lake pelagic sediment. (b) Sludge from an anaerobic sewage digestor. (c) Bovine rumen fluid. Bars represent 10 um	37

Figure | Page

CHAPTER III

1 Incubation system used to study the kinetics of methane production in lake sediments. The tube was specially made from a 20 x 200 mm culture tube with the top modified to accept a flange type butyl rubber stopper and Hungate-type screw cap. Tubes with 5 to 10 ml sediment were incubated in a near horizontal position on a rotary mixer as shown. 51

2 Effect of increasing hydrogen concentration , [S], on the rate of sediment methane production, v. The rates have been corrected for the control (no hydrogen added). Incubation time was 1.25 h at 10 C. Sediment was collected in the early winter. 61

3 Direct linear plot of the substrate, velocity data pairs plotted in Figure 2. 63

4 Progress curves of the headspace hydrogen and methane concentrations with time. Methane concentrations have been corrected for the control which had no hydrogen added. Incubation temperature was 12 C. Headspace (vapor) volume was about 65 ml. Sediment was collected in late winter 66

5 Effect of dissolved formate concentration of the rate of sediment methanogenesis. Methane production rates have been corrected for the rate of the control, which received an identical volume of oxygen free water but no formate. Incubation time was 1 h at 12 C. Sediment was collected in mid winter 70

6 Amount of methane produced by sediment with time at different concentrations of added dissolved acetate. Incubation temperature was 12 C. Sediment was collected in mid winter. 74

7 Production of labeled methane and carbon dioxide from [2-^{14}C] acetate (1 uCi). O - labeled methane produced in the absence of hydrogen ● - labeled carbon dioxide produced in the absence of hydrogen. Δ - labeled methane produced in the presence of 0.5% hydrogen. Hydrogen was added 15 min prior to the addition of labeled acetate. Incubation temperature was 12 C. Sediment was collected in June 77

Figure | Page

8 Effect of acetate concentration and hydrogen on the conversion of [2-^{14}C] acetate to methane by sediment. ● - labeled methane produced within 15 min in the absence of 0.5% hydrogen. O - labeled methane produced within 10 min in the presence of 0.5% hydrogen. The hydrogen was added 15 min prior to the acetate addition. Incubation temperature was 12 C. Sediment was collected in June 79

9 Effect of acetate concentration on the production of $^{14}CH_4$ from 2-^{14}C acetate by lake sediments. Each acetate concentration had the same specific activity of labeled acetate. Incubation time was 15 min at 12 C. Sediment was collected in June 82

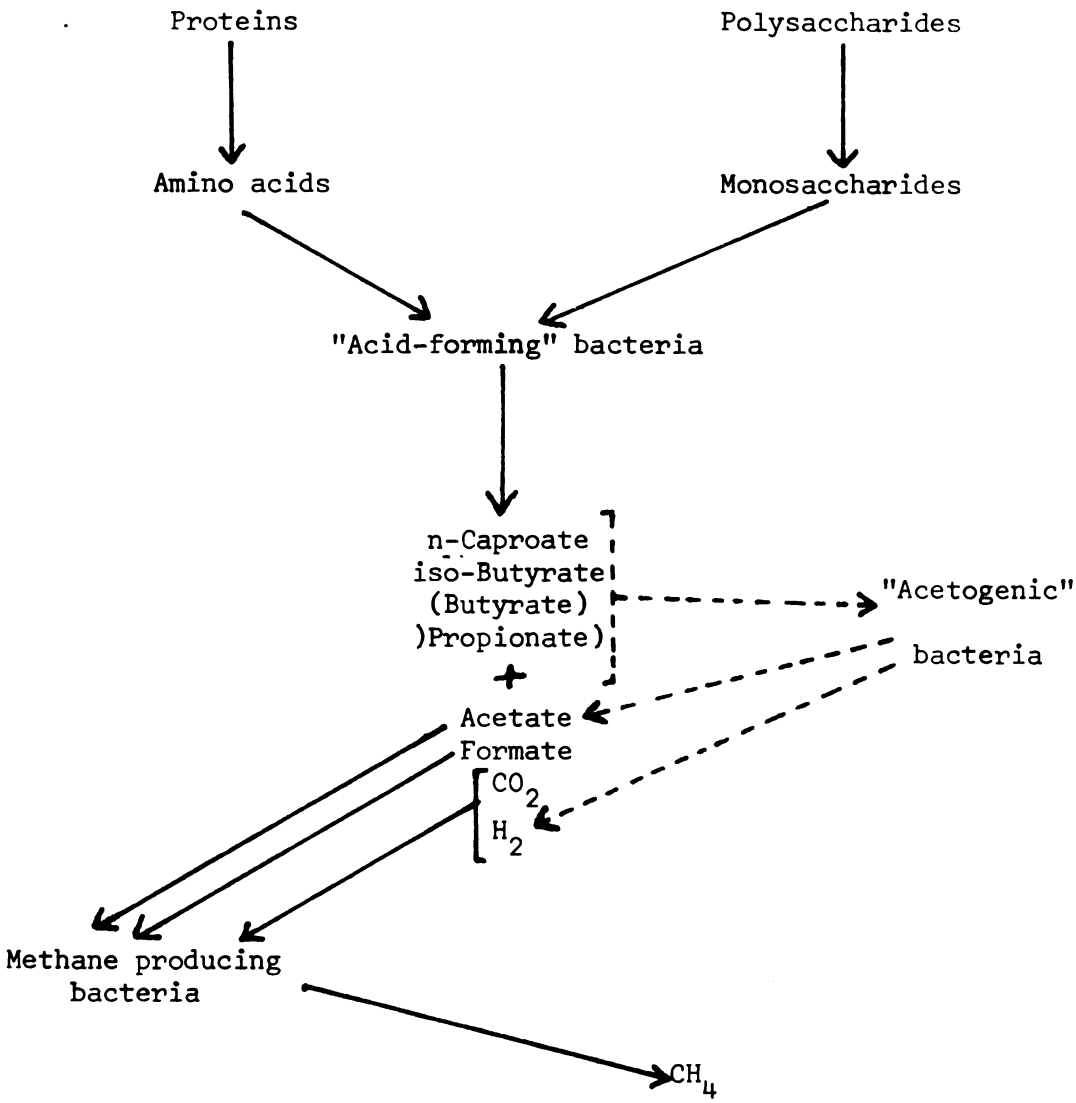
CHAPTER I

REVIEW OF PERTINENT LITERATURE AND RATIONALE FOR EXPERIMENTAL APPROACH

INTRODUCTION

The major end products of the fermentation of organic compounds by pure cultures of anaerobic heterotrophic bacteria are CO_2, H_2, short chain fatty acids, and other reduced molecules. In the anaerobic decomposition of organic matter in habitats such as the bovine rumen (14), sewage sludge digestors (18), and sediments of hypereutrophic lakes (33,5), some of these heterotrophic products are converted to methane. A generalized scheme of this decomposition chain is shown in Fig. 1. Typically, anaerobic decomposition is viewed as a two-stage process, with acid formation the initial phase and methane production the terminal phase. Bryant (2), however, has hypothesized a third phase which consists of "acetogenic" bacteria that convert short chain organic acids (and alcohols) to acetate and H_2. Although artifically separated into phases which are carried out by physiologically distinct microbial populations, anaerobic decomposition is better viewed as a continuum with metabolic interactions among and between the separate physiological groups. The most extensively studied metabolic interaction between anaerobic populations is interspecies hydrogen transfer (4,9,16,22,31, 35). This process has been studied with pure and mixed cultures isolated from a variety of anaerobic habitats, but no work has been done with

Figure 1. Generalized scheme of the anaerobic decomposition of organic matter.


Proteins
Polysaccharides
Amino acids
Monosaccharides
"Acid-forming" bacteria
n-Caproate
iso-Butyrate
(Butyrate)
)Propionate)
"Acetogenic"
bacteria
Acetate
Formate
CO_2
H_2
Methane producing
bacteria
CH_4

samples from natural habitats. The purpose of this chapter is to selectively review the pertinent literature related to the subjects of sediment methanogenesis and interspecies hydrogen transfer. I then discuss my hypothesis and the research approach and experiments designed to test this hypothesis.

Substrates for methanogenic bacteria in pure culture

Until 1967, methanogenic bacteria were thought to metabolize a variety of compounds to methane (1,28). These substrates included straight chain volatile fatty acids from formic to caproic, iso-and n-alcohols from methanol to pentanol, and the gases H_2, CO, and CO_2 (1,28). Then, Bryant et. al. (3) discovered that the supposedly pure culture of Methanobacterium omelianskii was actually a mixture of two distinct species; an ethanol-degrading bacterium (S organism) that formed acetate and hydrogen, and a methanogenic organism (Methanobacterium strain MoH) that could only use H_2 and CO_2 to produce methane. Elucidation of this mixed culture interaction has led to the observation by Wolfe (36), ..."(that methanogenic) organisms which use propionate, butyrate or higher fatty acids and alcohols are (likewise) not represented in pure culture at the present time." All known pure cultures of methanogenic bacteria can use H_2 and CO_2 to form methane (36,37). In addition, some species can use formate or CO and Methanosarcina barkeri can also utilize methanol and acetate (36).

Methane production in anaerobic habitats

Hydrogen as a substrate. Hydrogen and carbon dioxide are the principle substrates for methanogenesis in the bovine rumen (15). Formate, which is converted to H_2 and CO_2, accounts for 18% of the

hydrogen formed (15). The rest of the H_2 comes from the split of pyruvate into acetyl CoA, H_2 and CO_2 and from NADH via hydrogenase (14). Kinetic studies of the conversion of hydrogen to methane indicated that the K_s (substrate half-saturation constant) was about 1 μmole dissolved $H_2 \cdot l^{-1}$ and that the dissolved H_2 pool size was 1 $\mu mole \cdot l^{-1}$(15). Thus, H_2 was converted to methane at half the maximal rate. In a much earlier study (8), the maximal rate was determined to be 1.42 μmole $H_2 \cdot g^{-1} \cdot min^{-1}$. Hungate (14) has used this maximum rate to calculate that the turnover rate for H_2 conversion to methane in the rumen was 710 nmol $H_2 \cdot g^{-1} \cdot min^{-1}$ (equivalent to 178 nmol CH_4 produced $\cdot g^{-1} \cdot min^{-1}$) and that the pool turnover rate constant was 710 min^{-1}. However, the studies determining K_s and pool size were published 14 years after the study which determined the maximum rate, so these estimates of turnover rate and turnover rate constant may not be accurate. I have estimated the V_{max} from the 1/v vs. 1/s plots of Hungate's 1970 study (15) and obtained an average value of 2.9 μmoles CH_4 produced $\cdot g^{-1} \cdot h^{-1}$, which would be equivalent to 196 nmoles H_2 consumed $\cdot g^{-1} \cdot min^{-1}$. At an <u>in situ</u> H_2 concentration of 1μM, the turnover rate would be 98 nmoles $H_2 \cdot g^{-1} \cdot min^{-1}$ and the turnover rate constant would be 98 min^{-1}. Thus Hungate's published rate estimate is 7-fold higher than my corrected value for his data. For comparative purposes this rumen value is 42 times higher than the sediment H_2 turnover rate which I measured (Chapter III), when both are compared on a per g wet wt. basis.

Very little work has been done on the conversion of H_2 to CH_4 in anaerobic sewage sludge digestors because of the conviction that acetate is the predominent substrate for methanogenesis. However, one study does deserve critical discussion. Shea et al. (26) isolated a hydrogen

utilizing methanogen from sludge and studied the kinetics of its growth in continuous culture. Hydrogen and liquid flow rates were varied but the two rates were kept proportional. The lowest hydrogen concentration was about 1.9% (P_{H_2} = 14.3 mm Hg) and the highest was greater than 25% (extrapolated from their Fig. 4). Their results show a K_s of 569 mm Hg [based on hydrogen COD (chemical oxygen demand) removal basis] and 724 mm Hg (based on total COD removal). The maximum velocities were 24.8 mg H_2-COD removal · mg^{-1} volatile suspended solids (VSS) · day^{-1} and 31.7 mg total COD removed · mg^{-1}VSS·day^{-1}. Their K_m valves are equivalent to 75 and 95% H_2, respectively. The authors then note that hydrogen was generally less than 3% of the product gases in normal sludge digestion and consequently that the substrate removal activity of the hydrogen assimilating bacteria was less than 3% of the maximum. Their K_m estimates are 560 times greater than those of Hungate et al. (15), and are at values which would make many of the heterotrophic fermentations a thermodynamic impossibility. Clearly, something is amiss. A minimum concentration of 1.9% H_2 should place the system near maximum velocity. Perhaps this work should be disregarded because of obvious errors, however, it is the only published work on H_2 with sewage sludge organisms and it was felt that its probable inaccuracies should be brought to light.

Conversion of H_2 to methane by the microflora in lake sediments has also received little attention, but due more to a lack of investigations on this ecosystem than to interest in the fate of other methanogenic precursors. The majority of the current research has been done in the laboratory of J.G. Zeikus at Wisconsin. Winfrey et al. (33) have shown that the addition of hydrogen caused a significant stimulation in

sediment methanogenesis and that the amount of methane formed was proportional to the concentration of hydrogen added. The hydrogen concentrations that they added were rather high, 4.0% to 71%, and the shortest incubation time at which H_2 or CH_4 were measured was 24 h. However these investigators were not attempting a kinetic analysis but rather simply demonstrating the effects of H_2. Hydrogen was also shown to stimulate the conversion of ^{14}C labeled bicarbonate, formate, methanol and 1 and 2 labeled ^{14}C-acetate to methane (32). In a study of the inhibitory effects of sulfate on methane production in lake sediments, Winfrey and Zeikus (34) showed that the sulfate inhibition could be overcome by the addition of either H_2 or acetate. The authors proposed that competition for these substrates was the mechanism by which sulfate inhibited methanogenesis.

In a series of articles on sulfate-reducing and methane-producing bacteria in Lake Vechten sediment, Cappenberg (5,6,7) has neglected to report on hydrogen conversion to methane, but has determined that the majority of formate and hydrogen - CO_2 utilizing methanogens are located at a sediment depth of 2 to 3 cm, as opposed to acetate utilizing methanogens which are at 4 to 6 cm.

Acetate as a substrate. Methanosarcina barkeri is the only methanogenic bacterium in pure culture that can use acetate as its sole carbon and energy source (36). The morphological shape of the predominant bacterium in acetate enrichments that produce methane is often not a sarcina (11,18,21,30,39), but all attempts to isolate this bacterium(ia) have failed. The following reaction for acetate fermentation to methane (29)

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$$

$$\Delta G^{o'} = -7.4 \text{ kcal mol}^{-1}$$

shows that not enough energy is available for bacterial growth from this reaction (39). In a re-examination of acetate conversion to methane by Methanosarcina barkeri, Zeikus et al. (39) found that methane formation from acetate required the presence of hydrogen in the gas atmosphere and that methane was not formed when the bacteria were grown in a 100% N_2 or CO_2 atmosphere. Similar results were obtained with cultures of Methanobacterium thermoautotrophicum, which had not been previously shown to use acetate. The authors imply that both acetate and CO_2 are used by these bacteria to accept electrons and both substrates are reduced to methane in the presence of hydrogen. They did not determine, however, if the acetate was directly reduced to methane, or if it was first oxidized to CO_2 and then reduced. Either mechanism could explain their results. Zeikus et al. (39) have proposed the following reaction to explain their results

$$CH_3COO^- + 4H_2 + H^+ \rightarrow 2\ CH_4 + 2H_2O$$

$$\Delta G^{o'} = -39 \text{ kcal mol.}^{-1}$$

Other methanogens converting acetate to methane in the presence of H_2 include M. arbophilicum (37) and Methanospirillum hungatii strain GP1 (19).

The anaerobic habitat that has received the most attention with regard to acetate conversion to methane is the sewage sludge digestor. Jeris and McCarty (17) studied the fermentation of fatty acids, carbohydrates and proteins in order to determine the importance of acetic acid as an intermediate in sludge digestion. They concluded that, for all substrates tested, about 70% of the methane produced came from the

degradation of acetate. However, each experimental digestor was acclimated to the test substrate for a period of at least three months prior to use in a test run. Mah et al. (unpublished data cited in Chynoweth and Mah (10)), have criticized this practice since they found that euryoxic organisms which comprised less than 2.4% of the original sludge population became the most numerous types only 10 to 12 h after the addition of carbohydrate. Certainly a three month acclimation period would also lead to microbial populations quite different from those in the original sludge. Since single substrates were used by Jeris and McCarty (17) for each test run, the species diversity after three months would also be less than in the original sludge. These criticisms lead one to doubt the validity of their conclusion.

Smith and Mah (27) have also estimated that about 70% of the methane formed during sludge digestion comes from acetate. These investigators determined the acetate turnover rate constant by an isotope dilution technique. This turnover rate constant was multiplied by the acetate pool size to get the acetate turnover rate. They then determined the rate of total methane production for a separate sludge sample and compared this with the acetate turnover rate to obtain their conclusion that 70% of the methane came from acetate. Their assumption that all of the acetate is converted to methane is probably not valid. Any other fates of acetate such as oxidation by sulfate and sulfur reducers (20,32) were not considered. The presence of these bacteria in sludge has not been investigated but a strain of the sulfate-reducing, acetate oxidizing bacterium, Desulfotomaculum acetoxidans, has been isolated from piggery waste (32). A second alternative fate of acetate could be the reduction of this molecule and a second molecule of either

degradation of acetate. However, each experimental digestor was acclimated to the test substrate for a period of at least three months prior to use in a test run. Mah et al. (unpublished data cited in Chynoweth and Mah (10)), have criticized this practice since they found that euryoxic organisms which comprised less than 2.4% of the original sludge population became the most numerous types only 10 to 12 h after the addition of carbohydrate. Certainly a three month acclimation period would also lead to microbial populations quite different from those in the original sludge. Since single substrates were used by Jeris and McCarty (17) for each test run, the species diversity after three months would also be less than in the original sludge. These criticisms lead one to doubt the validity of their conclusion.

Smith and Mah (27) have also estimated that about 70% of the methane formed during sludge digestion comes from acetate. These investigators determined the acetate turnover rate constant by an isotope dilution technique. This turnover rate constant was multiplied by the acetate pool size to get the acetate turnover rate. They then determined the rate of total methane production for a separate sludge sample and compared this with the acetate turnover rate to obtain their conclusion that 70% of the methane came from acetate. Their assumption that all of the acetate is converted to methane is probably not valid. Any other fates of acetate such as oxidation by sulfate and sulfur reducers (20,32) were not considered. The presence of these bacteria in sludge has not been investigated but a strain of the sulfate-reducing, acetate oxidizing bacterium, Desulfotomaculum acetoxidans, has been isolated from piggery waste (32). A second alternative fate of acetate could be the reduction of this molecule and a second molecule of either

acetate, propionate, or butyrate to the respective products butyrate, valerate, or caproate (29). The change in the Gibb's free energy for each of these three reactions is -11.5 kcal/reaction (29). Evidence for the condensation and reduction of two acetate molecules was presented by Chynoweth and Mah (10) who found that radioactively labeled acetate was converted to butyrate in sludge.

Conversion of acetate to methane in lake sediments has been studied by Winfrey et al. (33) and Cappenberg and Prins (7). Winfrey et al. (33) found that [2 ^{14}C]-acetate added to sediment was quickly converted to methane and carbon dioxide. They did not mention their label recovery in these products, so I have extrapolated these data from their Fig. 4D and calculated that after 12 h of incubation 15% of the label was converted to methane and 9.4% was converted to carbon dioxide. The amount of label left in acetate or converted to other products should have been determined.

Cappenberg and Prins (7) used the same approach as Smith and Mah (27) to determine the turnover rate for acetate and the total rate at which methane was produced in the sediment. A difference was that Cappenberg and Prins (7) made corrections for the conversion of $^{14}CO_2$ to methane. The rate at which acetate was converted to methane by the sediment microflora was estimated to be 70% of the total methane production rate.

I believe the estimate that approximately 70% of the methane produced in sediment and sludge comes from acetate (7,27) is not valid and is probably too high. Both Smith and Mah (27) and Cappenberg and Prins (7) employed a manometric method to determine the total rate of methane production. Smith and Mah (27) used a 100 ml Warburg vessel

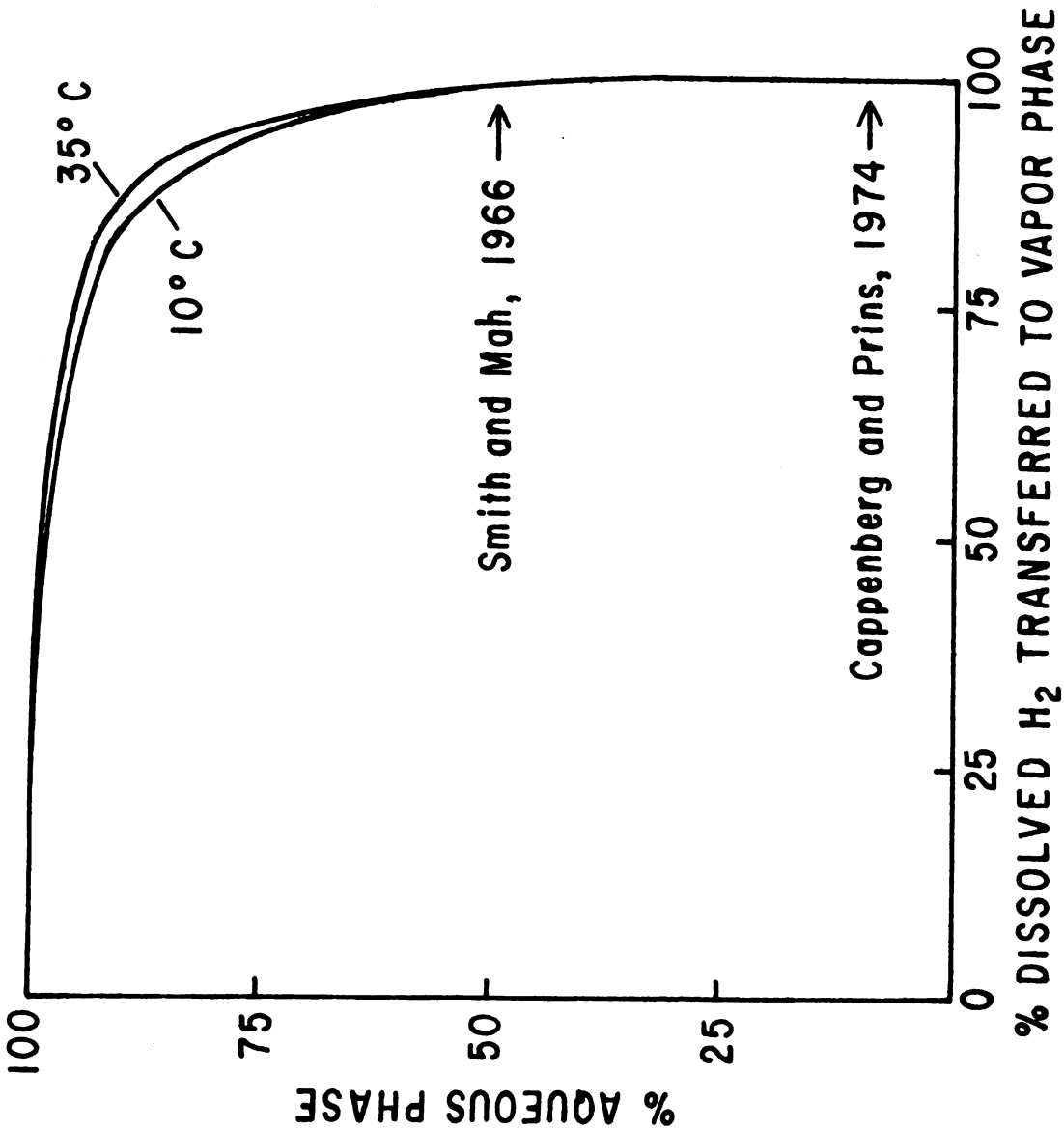
with 50 ml of sludge and an incubation temperature of 35 C. Cappenberg and Prins (7) used a 50 ml Warburg vessel and 3-4 g wet mud (3-4 ml?) at 10 C. The percent of the initial dissolved hydrogen transferred from the aqueous phase to the gaseous phase in these experimental vessels can be calculated from the equation of Flett et al. (12)

$$\frac{X}{M} = \frac{1}{(1+\alpha A/B)} \times 100 \qquad [1]$$

where X = volume of gas in the vapor phase, M = volume of gas in the aqueous phase, α = the Bunsen absorption coefficient at the incubation temperature, A = volume of the aqueous phase and B = volume of the vapor phase. I have used this equation to generate the 10 C and 35 C curves plotted in Fig. 2, and have designated the points where the values for the two experimental systems would be. Greater than 98% of the initial dissolved hydrogen in both systems should have been transferred to the headspace. This calculation means that the total methane production determined by Smith and Mah (27) and Cappenberg and Prins (7) does not include H_2 conversion to methane since less than 2% of the hydrogen concentration (original plus any H_2 produced during the incubation period) remains in the sediment.

The revelation that acetate may not contribute 70% of the methane carbon is extremely important for understanding anaerobic fermentations. With the new interest in replacement of fossil fuels with renewable energy sources (24), such an understanding is important to the design of systems for the optimum conversion of solid wastes (paper) and plant materials to methane. I suggest that a re-evaluation of the role of acetate and hydrogen in the production of methane in natural and man-made habitats is necessary.

Figure 2. Effect of the fraction of the total volume occupied by the aqueous phase on the transfer of dissolved hydrogen to the vapor phase for incubation temperatures of 10 and 35 C. Arrows denote the percent aqueous phase in the incubation systems of Smith and Mah (27) and Cappenberg and Prins (7). Curves calculated from the equation of Flett et al. (12).


35° C
10° C
Smith and Mah, 1966
Cappenberg and Prins, 1974
100
75
50
25
0
25
50
75
100
% AQUEOUS PHASE
% DISSOLVED H_2 TRANSFERRED TO VAPOR PHASE

From my review of the literature, the information on lake sediment methanogenesis was largely introductory and descriptive. Kinetic parameters are central to understanding flow and mechanisms in mixed culture systems and no such information for both hydrogen and acetate existed. Previous studies were typified by lengthy incubations at substrate concentrations far greater than natural levels, and sometimes at temperatures far above ambient, thus confounding the interpretation due to de novo synthesis or to other effects of an altered assay environment. Thus, an overall goal of my thesis research has been to introduce a greater level of sophistication into studies of sediment methanogenesis in order to understand the critical parameters controlling the process in nature.

Interspecies H_2 Transfer

The energy-yielding oxidation reactions of fermentative bacteria generate electrons in the form of reduced nicotinamide adenine dinucleotide (NADH). Since further fermentation can continue only if oxidation of NADH occurs, disposition of these electrons is necessary. In pure cultures, fermentation intermediates, such as pyruvate or electron acceptors derived from pyruvate, are usually used as electron sinks for this disposal, but an alternative for many bacteria is the formation of molecular hydrogen (13). But, the formation of H_2 from NADH is not thermodynamically favorable, with an apparent equilibrum constant of 6.7×10^{-4} at standard conditions of 1 atmosphere pressure for H_2 (35). This reaction does become favorable, however, when the partial pressure of hydrogen is below 10^{-3} to 10^{-4} atmospheres. The concept of interspecies hydrogen transfer is based on the maintenance of low partial

pressures of hydrogen by H_2 consuming microorganisms, such as the methanogenic bacteria (35). An hypothetical example of the effects of interspecies hydrogen transfer is presented in Fig. 3. This figure is adapted from Thauer, et al. (29), who constructed it using the data of Ianotti et al. (16) with Vibrio succinogenes as the H_2-consuming organism. I have replaced this organism with a H_2-consuming methanogenic bacterium.

In mono-culture Ruminococcus albus produces appreciable amounts of ethanol in order to oxidize NADH (Fig. 3). This deprives the microorganism of ATP regeneration via acetyl CoA. In co-culture with a methanogenic bacterium all of the NADH is used to produce H_2, which the methanogen keeps at a low partial pressure by reducing carbon dioxide to methane. When compared to the uncoupled fermentation, the coupled fermentation is characterized by:

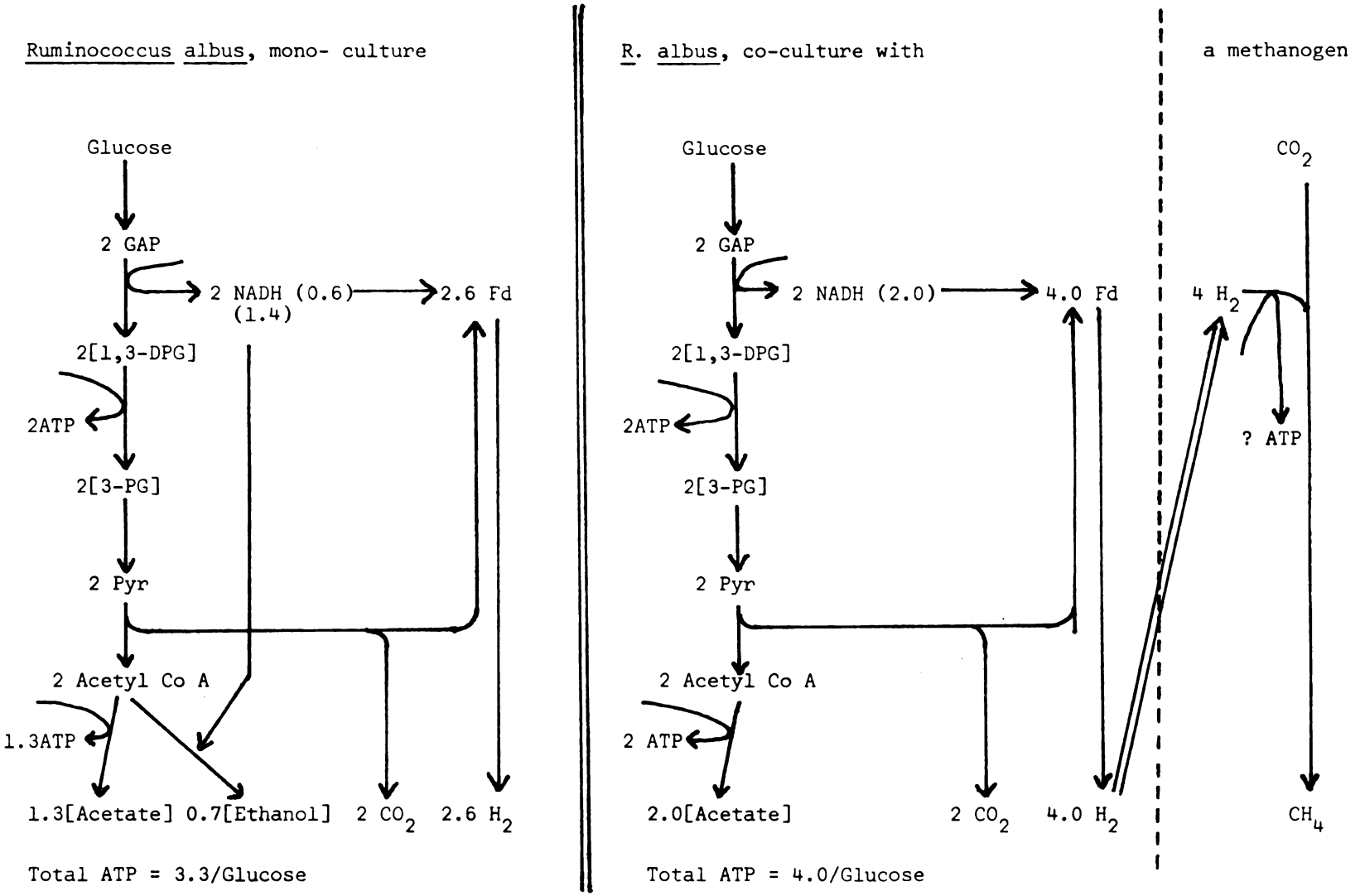
1. an increase in substrate utilization (not evident in Fig. 3, but see Reddy et al. (22))
2. a decrease in reduced fermentation products (ethanol)
3. an increase in oxidized fermentation products (acetate)
4. an increase in hydrogen produced as methane
5. an increase in energy production (R. albus 3.3 to 4.0 ATP, methanogen ? ATP) and thus enhanced growth of the interacting bacteria. (35)

A summary of reported examples of pure and mixed culture studies in which interspecies H_2 transfer has been shown is presented in Table 1.

Research reported in this dissertation

In the preceeding review, I have stressed the importance of the production of hydrogen and its subsequent utilization for methane

Figure 3. Comparison of the products formed and energy generated in the fermentation of glucose by Ruminococcus albus grown in mono-culture and in mixed culture with a methanogen. The figure is derived from Thauer et al. (29) but with the hydrogen utilizing Vibrio succinogenes replaced by a hydrogen-using methanogen.



Abbreviations; GAP, Glyceraldehyde phosphate; [1,3-DPG], 1,3-diphosphoglycerate; [3-PG], 3-phosphoglycerate: Pyr, pyruvate: Fd, Ferredoxin.

Table 2. Summary of reported examples of pure culture studies in which interspecies hydrogen transfer has been shown, and the effect of this metabolic coupling on the end products. Our hypothesized interaction for natural sediments is included for comparative purposes.

Original habitat of isolate	H_2 Producers		H_2 Utilizers		Major Products		Reference
	Organism	Substrate	Organism	Substrate	H_2-producer alone	H_2-producer + H_2-utilizer	
Rumen	Selenomonas ruminantium	carbohydrates	Methanobacterium M.O.H. or Methanobacterium ruminantium	CO_2, H_2	Not determined except that increased H_2 as CH_4 was observed in the mixed culture		(23)
Rumen	Ruminococcus flavefaciens	cellulose	Methanobacterium ruminantium	CO_2, H_2 formate	formate succinate acetate H_2 CO_2	acetate some succinate CH_4 CO_2	(34)
Rumen	Ruminococcus albus	carbohydrates (as glucose)	Vibrio succinogenes	fumarate, H_2	ethanol acetate H_2 CO_2	acetate H_2 as succinate CO_2	(16)
Sediment and Rumen	S organism	pyruvate	Methanobacterium ruminantium	CO_2, H_2	ethanol acetate CO_2 Some H_2	acetate CO_2 CH_4	(22)
Sediment	Desulfovibrio desulfuricans or D. vulgaris	ethanol or lactate	Methanobacterium M.O.H.	CO_2, H_2	None unless SO_4^+ or fumarate are present	CH_4 presumably acetate	(4)
Anaerobic sludge digestor	Clostridium thermocellum	cellulose	Methanobacterium thermoautotrophicum	CO_2, H_2	ethanol lactate acetate some CO_2, H_2	acetate CH_4	(30)
Rumen	Clostridium cellobioparum	glucose	Methanobacterium ruminantium	CO_2, H_2	lactate ethanol butyrate	acetate CH_4	(9)
Our hypothesized interaction in sediments	Saccharolytic and proteolytic clostridia	particulate organic matter, polymers, amino acids, and/or carbohydrates	Methane bacteria	H_2, CO_2 or H_2, acetate	reduced products such as volatile fatty acids some oxidized products CO_2 some H_2	CH_4 oxidized products such as acetate	

production in the anaerobic decomposition of organic matter. My hypothesis has been that interspecies hydrogen transfer is operative in lake sediments and of importance to the methanogenic and heterotrophic hydrogen-producing bacteria, and thus to the carbon and electron cycle of the lake.

My first objective was to determine if hydrogen consuming methanogenic bacteria were among the predominant methane formers. I used the autecological tool, the fluorescent antibody technique (25), to investigate this objective (Chapter II).

My second objective was to establish not only that hydrogen was a rate limiting substrate for methanogenesis, but that the methanogenic population was capable of maintaining the low partial pressures of hydrogen that are a necessary prerequisite for interspecies hydrogen transfer. A Michaelis-Menten kinetic analysis was selected (Chapter III), since the Michaelis-Menten constant, or Km, is an indication of the affinity of an enzyme for its substrate. Also needed was information on the natural rates of substrate utilization, substrate pool sizes and turnover times. An important part of this objective was to design an incubation system that was not easily contaminated by air, that gave a large substrate reservoir so that substrate concentration would not greatly change with time, and that maximized phase transfer of gaseous substrates and products.

My third objective was to determine the effects of hydrogen on various heterotrophic metabolic processes. Previous investigators have made the errors noted above because of their failure to perform experimentation with both of the prominent substrates involved in methane production. My approach was to use ^{14}C-labeled compounds in order to

study both the products and substrates involved (Chapter III). Acetate was selected not only because of its implied importance to methanogenesis, but also because its production is expected to increase if interspecies hydrogen transfer occurs. Valine was selected because of the possibility that the Stickland reaction, or the coupled oxidation of amino acids like valine with the reduction of amino acids like glycine and proline could be coupled with methane production instead.

LITERATURE CITED

1. Barker, H.A. 1956. Bacterial fermentations. Wiley. New York.

2. Bryant, M.P. 1976. The microbiology of anaerobic degradation and methanogenesis with special reference to sewage, p. 107-117. In H.L. Schlegel and J. Barnea (eds.) Microbiol energy conversion. Goltze, Gottingen.

3. Bryant, M.P., E.A. Wolin, M.J. Wolin, and R.S. Wolfe. 1967. Methanobacillus omelianskii, a symbiotic association of two bacteria. Arch. Mikrobiol. 59:20-31.

4. Bryant, M.P., L.L. Campbell, C.A. Reddy, and M.R. Crabill. 1977. Growth of desulfovibrio in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl. Environ. Microbiol. 33:1162-1169.

5. Cappenberg, T.E. 1974. Interrelationships between sulfate-reducing and methane-producing bacteria in bottom deposits of a freshwater lake. I. Field observations. Antonie van Leeuwenhoek J. Microbiol. Serology 40:285-295.

6. Cappenberg, T.E. 1974. Interrelationships between sulfate-reducing and methane-producing bacteria in bottom deposits of a freshwate lake. II. Inhibition experiments. Antonie van Leeuwenhoek J. Microbiol. Serol. 40:297-306.

7. Cappenberg, T.E. and R.A. Prins. 1974. Interrelationships between sulfate-reducing and methane-producing bacteria in bottom deposits of a freshwater lake. III. Experiments with ^{14}C labeled substrates. Antonie van Leeuwenhoek J. Microbiol. Serol. 40:457-469.

8. Carroll, E.J., and R.E. Hungate. 1955. Formate dissimilation and methane production in bovine rumen contents. Arch. Biochem. Biophys. 56:525-536.

9. Chung, K.-T. 1976. Inhibitory effects of H_2 on growth of Clostridium cellobioparum. Appl. Environ. Microbiol. 31:342-348.

10. Chynoweth, D.P. and R.A. Mah. 1971. Volatile acid formation in sludge digestion. In F.G. Pohland (ed.) Anaerobic biological treatment processes, Advances in Chemistry Series 105. American Chemical Society, Washington, D.C.

11. Ferry, J.G., and R.S. Wolfe. 1976. Anaerobic degradation of benzoate to methane by a microbial consortium. Arch. Microbiol. 107:33-40.

12. Flett, R.J., R.D. Hamilton, and N.E.R. Campbell. 1976. Aquatic acetylene-reduction techniques: Solutions to several problems. Can. J. Microbiol. 22:43-51.

13. Gray, C.T. and H. Gest. 1965. Biological formation of molecular hydrogen. Science 148:186-192.

14. Hungate, R.E. 1975. The rumen microbial ecosystem. Ann. Rev. Ecol. Systematics. 6:39-66.

15. Hungate, R.E., W. Smith, T. Bauchop, I. Yu, and J.C. Rabinowitz. 1970. Formate as an intermediate in the bovine rumen fermentation. J. Bacteriol. 102:389-397.

16. Ianotti, E.L., D. Kafkewitz, M.J. Wolin and M.P. Bryant. Glucose fermentation products of Ruminococcus albus grown in continuous culture with Vibrio succinogenes: Changes caused by interspecies transfer at H_2. J. Bacteriol. 114:1231-1240.

17. Jeris, J.S., and P.L. McCarty. 1965. The biochemistry of methane using ^{14}C tracers. Water Poll. Control Fed. J. 37:178-192.

18. Mylroie, R.L., and R.E. Hungate. 1954. Experiments on the methane bacteria in sludge. Can. J. Microbiol. 1:55-64.

19. Patel, G.B., L.A. Roth, L. vanden Berg, and D.S. Clark. 1976. Characterization of a strain of Methanobacterium hungatii. Can. J. Microbiol. 22:1404-1410.

20. Pfenning, N., and H. Biebl. 1976. Desulfuromonas acetoxidans gen. nov. and sp. nov., a new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Arch. Microbiol. 110:3-12.

21. Pretorius, W.A. 1972. The effect of formate on the growth of acetate utilizing bacteria. Water Res. 6:1213-1217.

22. Reddy, C.A., M.P. Bryant, and M.J. Wolin. 1972. Characteristics of S organism isolated from Methanobacillus omelianskii. J. Bacteriol. 109:539-545.

23. Scheifinger, C.C., B. Linehan, and M.J. Wolin. 1975. H_2 production by Selenomonas ruminantium in the absence and presence of methanogenic bacteria. Appl. Microbiol. 29:480-483.

24. Schlegel, H.L., and J. Barnea (ed). 1976. Microbiol energy conversion. Goltze, Gottingen.

25. Schmidt, E.L., R.O. Bankole, and B.B. Bohlool. 1968. Fluorescent antibody approach to the study of rhizobia in soil. J. Bacteriol. 95:1987-1992.

26. Shea, T.G., W.A. Pretorius, R.D. Cole, and E.A. Pearson. 1968. Kinetics of hydrogen assimilation in the methane fermentation. Water Research. 2:833-848.

27. Smith, P.H., and R.A. Mah. 1966. Kinetics of acetate metabolism during sludge digestion. Appl. Microbiol. 14:368-371.

28. Stadtman, T.C. 1967. Methane fermentation. Ann. Rev. Microbiol. 21:121-142.

29. Thauer, R.K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic bacteria. Bacteriol. Rev. 41:100-180.

30. van den Berg, L., G.B. Patel, D.S. Clark, and C.P. Lentz. 1976. Factors affecting methane formation from acetic acid by enriched methanogenic cultures. Can. J. Microbiol. 22:1312-1319.

31. Weimer, P.J. and J.G. Zeikus. 1977. Fermentation of cellulose and cellobiose by Clostridium thermocellum in the absence and presence of Methanobacterium thermoautotrophicum. Appl. Environ. Microbiol. 33:289-297.

32. Widdel, F., and N. Pfennig. 1977. A new anaerobic, sporing, acetate-oxidizing, sulfate-reducing bacterium, Desulfotomaculum (emend.) acetoxidans. Arch. Microbiol. 112:119-122.

33. Winfrey, M.R., D.R. Nelson, S.C. Klevickis, and J.G. Zeikus. 1977. Association of hydrogen metabolism with methanogenesis in Lake Mendota sediments. Appl. Environ. Microbiol. 33:312-318.

34. Winfrey, M.R. and J.G. Zeikus. 1977. Effect of sulfate on carbon and electron flow during microbiol methanogenesis in freshwater sediments. 33:275-281.

35. Wolin, M.J. 1974. Metabolic interactions among intestinal microorganisms. Amer. J. Clin. Nutr. 27:1320-1328.

36. Wolfe, R.S. 1971. Microbial formation of methane. Adv. Microbial Physiol. 6:107-145.

37. Zeikus, J.G., and D.L. Henning. 1975. Methanobacterium arbophilicum sp. nov. An obligate anaerobe isolated from wetwood of living trees. Antonie van Leeuwenhoek. 41:543-552.

38. Zeikus, J.G., and M.R. Winfrey. 1976. Temperature limitation of methanogenesis in aquatic sediments. Appl. Environ. Microbiol. 31:99-107.

39. Zeikus, J.G., P.J. Weimer, D.R. Nelson, and L. Daniels. 1975. Bacterial methanogenesis: Acetate as a methane precursor in pure culture. Arch. Microbiol. 104:129-134.

CHAPTER II

APPLICATION OF THE FLUORESCENT ANTIBODY TECHNIQUE TO STUDY A METHANOGENIC BACTERIUM IN LAKE SEDIMENTS

INTRODUCTION

Methods for enumerating methane producing bacteria in anaerobic habitats require anaerobic culture techniques, prolonged incubation times, detection of methane (19, 26) and, in the case of roll tube methods, some way of determining which colonies are responsible for methane production (8, 19). While these methods represent the state-of-the-art for estimating the total methanogenic population, autecological studies of specific species of methanogens are either extremely tedious, or cannot be done at all.

The fluorescent antibody (FA) technique, which is one of the most useful methods available for studying microbial autecology (11, 12, 21), has not been investigated with methane bacteria and has not been used in the anaerobic lake sediment habitat. One purpose of this paper is to report my evaluation of these applications of the FA technique. The second purpose is to compare two methods of enumeration - a fluorescent antibody direct count of a specific hydrogen-consuming methanogen and a most probable number (MPN) estimate of total methanogens in lake sediment. This comparison was made in order to determine if hydrogen-consuming methanogenic bacteria were among the predominant methane formers in this habitat.

MATERIALS AND METHODS

Media. The enrichment-purification (EP) medium for the methanogen contained the following per liter of distilled water: K_2HPO_4, 0.225 g; KH_2PO_4, 0.225 g; NH_4Cl, 0.185 g; NaCl, 0.45 g; $MgCl_2$-$6H_2O$, 0.076 g; $CaCl_2$-$2H_2O$, 0.060 g; trace elements solution (24), 10 ml; vitamin solution (24), 10 ml; 0.01% (w/v) resazurin solution, 1.0 ml; CO_2 equilibrated 8% (w/v) Na_2CO_3, 25 ml; cysteine-HCl, 0.5 g; Na_2S-$9H_2O$, 0.5 g. The gas atmosphere of all anaerobically prepared culture tubes was oxygen-free H_2-CO_2 (50:50, v/v). An incubation temperature of 28°C was used for enrichment and isolation.

Counts of FA reacting methanogens in the sediemnt were compared with estimates of the total methanogenic population made by the five tube MPN technique (1). Three different MPN media were employed. All had a mineral composition that was identical to that of the EP medium. In addition the following organic substrates were added per liter of medium: trypticase, 2.0 g; yeast extract, 2.0 g; Na formate, 2.0 g; Na acetate, 2.0 g. The three media differed in the reducing agent and/or growth factors that were added, per liter of medium; (i) cysteine-HCl, 1.0 g; (ii) a 24 h culture of Escherichia coli grown aerobically at 37°C in trypic soy broth (Difco), 40 ml; or (iii) Na_2S-$9H_2O$, 0.1 g; Ti(III) citrate (25), 0.6 mM Ti(III); 2-methyl butyric acid, isovaleric acid, and isobutyric acid, 0.1 mM of each. These reductants were chosen to avoid possible sulfide toxicity which has been noted by Cappenberg (8).

All reducing agents were added 24 h prior to inoculation with sediment. Preliminary experiments showed that the added E. coli would reduce methyl viologen within this time. The method of preparing

Ti(III) citrate (25) was modified such that all solutions were boiled under O_2-free nitrogen prior to mixing and all manipulations were done anaerobically.

Anaerobic methodology. The anaerobic culture and isolation techniques used were those described by Hungate (15) with the modifications of either Bryant and Robinson (7) or Macy, et al. (17). For some purification attempts I used an anaerobic glove chamber (2) (Coy Manufacturing Co., Ann Arbor, MI) with a gas atmosphere of H_2:N_2 (10:90, v/v). Petri plate agar (EP medium plus 1.5% agar w/v) streaks were incubated in anaerobic containers within the glove chamber. These containers were similar to those described by Edwards and McBride (10) and had a gas atmosphere of H_2:CO_2 (50:50, v/v). Exposure of the plates to the glove box atmosphere occurred only during inspection for growth and culture transfer to fresh medium.

Antigen preparation. The methanogenic culture that I used as the antigen in the preparation of fluorescent antibody (FA) was isolated from a dilution series of EP medium that had been inoculated with lake sediment. The sediment was collected by a gravity corer from the 6 m depth of Wintergreen Lake (Kalamazoo County, Michigan, R 9W, T 2N, Sec 8) in July. The medium was inoculated from a ten-fold dilution series of a homogeneous mixture of the upper 5 cm of the sediment core.

All atempts to obtain an axenic methanogenic culture were unsuccessful. However, contaminant levels could be kept at a minimum (less than 1% of the presumptive methanogen) by keeping the headspace H_2 concentrations high and by transferring the culture to fresh medium (10-fold dilution) every 2 days. Cells to be used as antigen were grown in 200 ml of EP medium, harvested by centrifugation and killed by suspending in 4% (v/v) formalin in 0.85% (w/v) sterile saline for 72 h.

Antibody preparation. The antigen suspension was injected into young adult male rabbits according to the schedule described by Schmidt (22). Blood was collected every two weeks from a puncture of the marginal vein of the ear. At each bleeding 30 ml of blood was collected in an equal volume of anticoagulent solution. Purification of the serum antibody and conjugation with fluorescein isothiocyanate (FITC) followed the precedure of Schmidt (22). The FITC to protein ratio was 1/50 (w/w) and protein was determined by the method of Lowry (16) with bovine serum albumin (BSA) as the standard. Excess FITC was removed from conjugated FA by passage through a Sephadex G-25 column that had been equilibrated with 0.12 M phosphate buffered saline, pH 7.2. The FA was collected in 5 ml portions, preserved with 0.01% thimerosal (w/v, Sigma Chemical Co.) and frozen until needed.

FA stain of cultures and natural samples. The microscope slide staining procedure of Schmidt (22) was used to stain heat fixed cultures or natural samples. Non-specific absorption of the FA was controlled by the prior addition of either rhodamine-conjugated gelatin (5) or 2% BSA. The FA stained smears were observed under a Leitz Ortholux microscope with incident ultraviolet (uv) light illumination. A 150 W Xenon lamp was used as the uv light source. The excitation filters were two KP490. The dichroic beam splitting mirror was a TK510 and the barrier filters were a K515 and a K510. Two thicknesses of Kodak Wratten gelatin filter No. 12 were added to the barrier filter assembly when photomicrographs were taken.

A modification of the soil procedure of Bohlool and Schmidt (6) was used to obtain direct counts of FA reacting bacteria in lake sediments. Ten ml of sediment, 90 ml of filter sterilized distilled water and 0.1 ml Tween 80 were mixed for 5 min in a blender. A flocculent (0.5 g

$Ca(OH)_2$, 1.25 g $MgCO_3$, dried overnight at 90 C) was added to this dispersed sediment which was then vigorously shaken by hand for 2 additional minutes. Two drops of Antifoam B emulsion (Sigma Chemical Co.) were added during the last 10 sec of shaking. The floc was allowed to settle, undisturbed, for 15 min. The supernatant liquid was then carefully removed by aspiration and a sample or, when necessary, dilution of the supernatant fluid was immediatley filtered through a black Sartorius 25 mm diameter membrane filter, 0.45 µm pore diameter. The procedure of Bohlool and Schmidt (6) as modified by Schmidt (2) was used to stain the bacteria on the filters with FA, except that 2% BSA was used to control non-specific absorption.

Stained bacteria were observed under the Leitz microscope with incident uv illumination as described above. Since the number of FA reacting bacteria per microscope field followed a Poisson distribution, these data were normalized with a square root transformation so that 95% confidence limits could be calculated. The bacteria on a minimum of three membrane filters were stained with FA on each sampling date and 100 microscope fields per filter were counted. The number of FA reacting organisms per gram dry weight of sediment was calculated using the equation of Bohlool and Schmidt (6).

Since microorganisms are removed by flocculation (21) I attempted to correct for this by determining the percentage of total sediment bacteria lost during this step. Total bacteria present before and after flocculation were determined by the acridine orange-epifluorescence, direct counting method of Daley and Hobbie (9). Only green fluorescing bacteria were counted.

For comparison with the FA direct counts, five tube MPN estimates of the sediment methanogenic bacterial population were made using the three MPN media described above. A 10-fold serial dilution of the sediment was made in EP medium with 1 min of vortex mixing between each dilution. MPN tubes were inoculated from this dilution series. The tubes were incubated for 4 weeks at 37°C. At the end of this time, each tube was analyzed for methane production as determined by flame ionization gas chromatography.

RESULTS

Description of methanogenic organism purified from the sediment. Fig. 1 is a phase contrast photomicrograph of single cells and a filament of the methanogen. The organism is gram positive and produces methane from hydrogen and carbon dioxide, and from formate. It does not produce methane from acetate or methanol in the absence of hydrogen. The optimum growth temperature is 37°C. Chains and filaments are common, often exceeding 60 µm in length. Cellular dimensions are 0.5 µm in diameter by 3 to 6 µm. Surface colonies are pale yellow to tan and flat. These colonies fluoresce blue-green when exposed to long wave uv light. This fluorescence is characteristic of methanogens and is due to oxidized factor 420, a compound which has only been found in methanogens (10). Individual cells of an actively growing culture exhibited a faint autofluorescence when observed microscopically under incident uv illumination. This allowed us to be certain that the morphotype reacting with the FA was a methanogen.

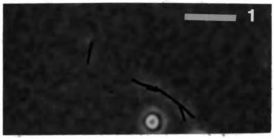
Tests on antibody prepared with the methanogen. Agglutination titers of serum collected from injected rabbits never exceeded 800. Although this value is low, Fig. 2 shows that FA prepared from this serum adequately stained the antigen. An FA stain of a culture in which the contaminants were deliberately allowed to proliferate showed that the contaminants did not react with the FA.

Table 1 lists the control tests which Schmidt (20) states are necessary for the application of FA. My results from these tests are listed in the right hand column. Several tests were run to satisfy the requirement that the FA should stain antigen cells grown under various conditions. The FA reactions of the methanogenic culture at different stages of the growth cycle and after different periods of starvation were all +4. An FA reaction of +4 was arbitrarily defined as the FA reaction of the original antigen preparation. A freshly stained smear of this antigen was used as a reference for each stain. The FA reaction of the methanogen culture grown at different temperatures ranged from +2 for cells exposed to 4°C to +4 for cells grown at 28 and 37°C. The morphology of the methanogenic bacteria changed at the different growth temperatures with single cells the predominant morphotype at 4°C and filaments the predominant morphotype at the higher temperatures.

Tests on the antibody (Table 1) included staining related and unrelated organisms to test the specificity of the FA preparation. The organisms tested are listed in Table 2. None were stained by the FA. Of particular importance to this study was the finding that no other organism isolated from Wintergreen Lake sediment reacted with the FA.

Figure 1. Phase contrast photomicrograph of a short filament and single cells of a methanogenic bacterium isolated from Wintergreen Lake sediment and used to prepare the FA. Bar represents 10 um.

Figure 2. Photomicrograph of an FA-stained smear of the methanogenic isolate. Bar represents 10 um.


1


2

Table 1. Control tests needed for the application of FA (from Schmidt (20)).

Component checked			Expected result	Actual result
Antigen	1.	Stain with "normal" serum FA	-	-
	2.	Stain with unconjugated homologous serum followed by homologous FA	-	-
	3.	Stain with homologous FA that has been absorbed with Ag cells	-	-
	4.	Stain of Ag cells grown under various conditions	?	+
Antibody	1.	FA stain of related organisms (see Table 2)	-	-
	2.	FA stain of unrelated organisms (see Table 2)	-	-

Qualitative survey of various habitats for FA reacting bacteria. Heat fixed smears of samples from several anaerobic methanogenic habitats were stained with FA to determine the distribution of this methanogenic strain. Samples stained included Burke Lake pelagic sediment, Wintergreen Lake pelagic and littoral sediments, anaerobic sewage sludge, and bovine rumen fluid. Photomicrographs of several of these FA stained samples are presented in Fig. 3. In all sediment and the anaerobic sewage sludge samples, the morphological shape shown in Fig. 3 a,b was the only one that was stained with FA. The only FA reacting organism in rumen fluid is shown in Fig. 3c.

Qualitative estimates of the FA staining reaction of cells in these habitats ranged from +1 in Burke Lake sediment to +3 in all Wintergreen Lake sediments, +3 to +4 in anaerobic sewage sludge and +2 in rumen fluid. Non-specific absorption of FA by all samples was minimal when they were pretreated with either rhodamine conjugated gelatin or BSA. Autofluorescent particles were not a problem in anaerobic sewage sludge or rumen samples, but were present in most of the sediment samples. These particles appeared to be partially degraded zooplankton and could easily be distinguished from FA reacting bacteria.

Direct count FA and MPN estimates of the methanogenic bacteria in lake sediment. Estimates of the methanogenic population in the upper 5 cm of sediment cores collected from Wintergreen Lake are presented in Table 3. Cores were collected at times of active methanogenesis. The October and March samples were taken just before lake turnover and after long peroids of anoxia. Following dispersion-flocculation, up to 10 ml of the supernatant could be passed through the membrane filters before they became clogged. For all FA membrane filter counts the number of

Table 2. Microorganisms tested for cross-reactivity with FA preparation; no cross-reaction observed.

Organism	Source of isolate	Contributor
Related organisms		
Methanobacterium strain M.o.H.	San Francisco Bay mud	M.P. Bryant
M. ruminantium strain PS	Anaerobic sewage sludge	M.P. Bryant
M. ruminantium strain M1	Rumen	M.P. Bryant
M. formicicum strain JF	Anaerobic sewage sludge, benzoate enrichment	J.G. Ferry
M. arbophilicum	Wetwood of trees	J.G. Zeikus
Methanobacterium sp.	Salt marsh sediment	M.J.B. Paynter
Methanosarcina MS	Aanerobic sewage sludge	M.P. Bryant
Methanosarcina U.B.S.	Lake Mendota sediment	J.G. Zeikus
Methanospirillum hungatii strain JF	Anaerobic sewage sludge, benzoate enrichment	M.P. Bryant
Unrelated organisms		
4 clostridial strains	Wintergreen Lake sediment	M.J. Klug
1 other obligate anaerobe	Wintergreen Lake sediment	M.J. Klug
6 facultative anaerobes	Wintergreen Lake sediment	M.J. Klug
4 dentrifying strains	Wintergreen Lake sediment	authors' lab
3 anaerobes isolated from a methanogenic enrichment	Wintergreen Lake sediment	authors' lab
bacterial smear of a sulfate-reducing enrichment	Wintergreen Lake sediment	authors' lab
6 *Bacteriodes* species	Various intestinal habitats	C.A. Reddy

Figure 3. Photomicrographs of immunofluorescing cells in FA stained smears of various habitats. (a) Wintergreen Lake pelagic sediment. (b) Sludge from an anaerobic sewage digestor. (c) Bovine rumen fluid. Bars represent 10 µm.

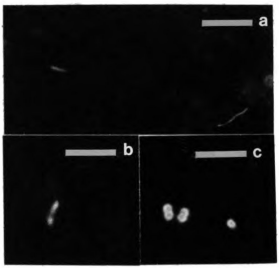

a
b
c

Table 3. Number of methane producing bacteria in Wintergreen Lake sediment on different sampling dates as estimated by FA direct counts and by a five tube MPN technique using three different media. MPN tubes were scored as positive if methane was produced within 4 weeks.

Sampling date	No. of methanogens x 10^{-4} per g dry sediment as determined by							
	FA direct count	(95% C.I.[a])	MPN with cysteine reductant	(95% C.I.)	MPN with *E. coli* reductant	(95% C.I.)	MPN with Ti(III) reductant	(95% C.I.)
25 Jun 1976	1400	(1300-1500)	10	(3.5-27)	ND[b]		ND	
3 Sep 1976	320	(300-330)	24	(6.4-59)	ND		ND	
16 Oct 1976	630	(590-680)	6.6	(2.3-17)	66	(23-170)	ND	
19 Mar 1977	310	(280-340)	2.6	(0.8-6.4)	11	(3.7-31)	16	(5.7-44)

[a] C.I. = Confidence interval

[b] ND = Not determined

bacteria per field followed a Poisson distribution (G-test for goodness of fit (23)). Additional MPN media were used after the June sampling date in an attempt to improve the MPN estimates over that obtained with the cysteine reduced medium.

Direct counts of acridine orange stained bacteria before and after the flocculation of dispersed sediment indicated that 15% of the bacteria were lost during flocculation. This loss was estimated using the sediment collected in March 1977. The FA counts presented in Table 3 have been corrected for this flocculation loss.

In order to determine if FA reacting bacteria were present in MPN tubes, the contents of high dilution, methane positive tubes inoculated with the October 16, 1976 sediment sample were stained with FA. FA reacting bacteria were present in all tubes tested.

DISCUSSION

As expected, a fluorescent antibody could be made that would react with the methane bacteria isolated (Fig. 2). The specificity of the FA was judged adequate since no other methanogen nor a variety of non-methanogens showed any cross-reaction with it (Table 2). FA reacting bacteria in sediment and anaerobic sewage sludge had the same morphology as the methanogen (Fig. 1, 2, 3a, 3b). The FA reacting organism in rumen fluid (Fig. 3c) did not; this coccoid cell was never observed in any stained preparations of our culture, lake sediments, or anaerobic sludge samples. When Hobson, et al. (14) stained sheep rumen contents with FA prepared for Selenomonas ruminantium they observed that, in addition to stained selenomonads, small cocci and occasionally large cocci also reacted. Their FA preparations cross-reacted with Veillo-

nella gazogenes and when rumen contents were stained with FA that had been absorbed with V. gazogenes no reaction was observed other than with selenomonad-like organisms. In a similar manner, it is likely that antibodies to microorganisms related to Veillonella gazogenes were responsible for the FA staining of the rumen organisms I observed. It is possible that these cross-reacting cocci are normal inhabitants of the rabbit intestinal tract.

The value of the FA technique for certain autecological studies may be limited by a high degree of strain specificity of the FA if there is a high degree of antigen diversity among the organisms responsible for the activity under study. Strain specific FA is not uncommon and has been used by Schmidt in studying Rhizobium (22) or noted by other investigators attempting to use FA in autecological studies of Thermoplasma (3), Sulfolobus (4), Bacillus sp. (13), and Butyrivibrio (18). There apparently is some strain or species specificity shown by the FA used in this study since it does not cross react with Methanobacterium strain M.o.H. and M. formicicum, which are morphologically similar to my isolate. However, my finding that FA stained cells of the correct morphology were present in all sediments and activated sludge that I examined suggests that the FA I prepared is not too strain specific and thus of use in the study of natural samples. Furthermore, the population of FA stained cells was at least as great, if not greater, than the population estimates obtained by the best MPN media (Table 3). Though there could be more numerous methanogens than either of these approaches detect, the present methodology suggests that the specificity of the FA I prepared is adequate for detecting isolatable sediment methanogenic microflora.

The intensity of staining that I observed varied. The weak staining of the methanogenic culture grown at 4°C and the observation of weakly FA stained bacteria in winter samples of Burke Lake pelagic sediment indicates that my FA may react poorly with cold adapted or extensively aged cells. My isolate apparently does not exhibit much antigenic variability since the FA readily stained sediment methanogens 1 1/2 to 2 1/2 years after their original isolation and it stained cells of laboratory cultures prepared under a variety of growth and starvation conditions.

No major problems were encountered with applying FA staining techniques to anaerobic lake sediments. Autofluorescent particles were initially a problem, but with experience it became easy to distinguish FA reacting bacteria. In March, after anoxic conditions had caused a winter kill, autofluorescent particles were absent.

Comparison of the two methods that I used for enumerating sediment methanogens (Table 3) shows that the membrane filter FA direct counts were generally an order of magnitude greater than MPN estimates. Several explanations could account for this difference. The most likely is that all viable methanogenic cells do not grow in MPN media. The differences in estimates among media indicate the important influence of media on results. Other explanations are that the FA may be detecting a substantial number of dead cells and that a slightly more vigorous method of release and dispersion of cells was employed with the FA method. An error which could increase the FA/MPN recovery ratio is an underestimation of flocculation losses. The value of 15%, which was determined for the predominant short rods in this sediment, may be too low for the more filamentous methanogenic cells.

My enumeration results, both by FA and MPN (Table 3), compare favorably with the results reported by others for different lakes. Zeikus and Winfrey (26) have estimated the number of methanogens in Lake Mendota sediments and found as few as $10^2 \cdot g^{-1}$ in shallow, winter sediments and as many as $10^6 \cdot g^{-1}$ in sediments underlying deeper waters in the summer. Although Cappenberg (8) determined the number of methanogenic bacteria with sediment depth in Lake Vechten, comparisons with my results are difficult to make, since his estimates are reported on the basis of the number of organisms per liter wet mud. Nevertheless, at a 5 cm sediment depth, the number of methane producing bacteria per ml of wet Lake Vechten mud ranged from 2×10^3 to 8×10^5.

Although the time required to isolate the microorganism of interest and to prepare and test the resulting FA is long, I believe that this autecological technique has proved useful for detection and enumeration of the methanogen. Recently, Ward and Frea (Abstr. Ann. Meet. Amer. Soc. Microbiol. 1977:169) have used FA techniques to detect other methanogenic species in Lake Erie sediments. Thus the FA techniques may be generally useful for autecological studies of methanogens.

LITERATURE CITED

1. American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1971. Standard methods for the examination of water and wastewater. American Public Health Association, Washington. 13th Ed.

2. Aranki, A., S. A. Seyd, E. B. Kenney, and R. Freter. 1969. Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box proceudre. Appl. Microbiol. 17:568-576.

3. Bohlool, B. B., and T. D. Brock. 1974. Immunofluorescence approach to the study of the ecology of Thermoplasma acidophilum in coal refuse material. Appl. Microbiol. 28:11-16.

4. Bohlool, B. B., and T. D. Brock. 1974. Population ecology of Sulfolubus acidocaldarius. II. Immunoecological studies. Arch. Microbiol. 97:181-194.

5. Bohlool, B. B., and E. L. Schmidt. 1968. Nonspecific staining: Its control in immunofluorescence examinations of soil. Science 162:1012-1014.

6. Bohlool, B. B., and E. L. Schmidt. 1973. A fluorescent antibody technique for determination of growth rates of bacteria in soil. In: T. Rosswall (ed.), Modern methods in the study of microbial ecology. Bull. Ecol. Res. Comm. (Stockholm) 17:336-338.

7. Bryant, M. P., and I. M. Robinson. 1961. An improved nonselective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. J. Dairy Sci. 44:1446-1456.

8. Cappenberg, T. E. 1974. Interrelations between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh water lake. I. Field observations. Antonie van Leeuwenhoek J. Microbiol. Serol. 40:285-295.

9. Daley, R. J., and J. E. Hobbie. 1975. Direct counts of aquatic bacteria by a modified epifluorescent technique. Limnol. Oceanogr. 20:875-882.

10. Edwards, T., and B. C. McBride. 1975. New method for the isolation and identification of methanogenic bacteria. Appl. Microbiol. 29:540-545.

11. Fliermans, C. B., B. B. Bohlool, and E. L. Schmidt. 1974. Autecological study of the chemoautotroph Nitrobacter by immunofluorescence: Appl. Microbiol. 27:124-129.

12. Fliermans, C. B., and E. L. Schmidt. 1975. Autoradiography and immunofluorescence combined for autecological study of single cell activity with Nitrobacter as a model system. Appl. Microbiol. 30:676-684.

13. Hill, I. R., and T.R.G. Gray. 1967. Application of the fluorescent-antibody technique to an ecological study of bacteria in soil. J. Bacteriol. 93:1888-1896.

14. Hobson, P. N., S. O. Mann, and W. Smith. 1962. Serological tests of a relationship between rumen selenomonads in vitro and in vivo. J. Gen. Microbiol. 29:265-270.

15. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. 14:1-49.

16. Lowry, O. H., N. Y. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

17. Macy, J. M., J. E. Snellen, and R. E. Hungate. 1972. Use of syringe methods for anaerobiosis. Amer. J. Clin. Nut. 25:1318-1323.

18. Margherita, S. S., R. E. Hungate, and H. Storz. 1964. Variation in rumen Butyrivibrio strains. J. Bacteriol. 87:1304-1308.

19. Paynter, M.J.B., and R. E. Hungate. 1968. Characterization of Methanobacterium mobilis, sp. n., isolated from the bovine rumen. J. Bacteriol. 95:1943-1951.

20. Schmidt, E. L. 1973. Fluorescent antibody technique for the study of microbial ecology. In: T. Rosswald (ed.) Modern methods in the study of microbial ecology. Bull. Ecol. Res. Comm. (Stockholm) 17:67-76.

21. Schmidt, E. L. 1974. Quantitative antecological study of microorganisms in soil by immunofluorescence. Soil Sci. 118:141-149.

22. Schmidt, E. L., R. O. Bankole, and B. B. Bohlool. 1968. Fluorescent-antibody approach to study of rhizobia in soil. J. Bacteriol. 95:1987-1992.

23. Sokal, R. R., and F. J. Rohlf. 1969. Biometry. W. H. Freeman and Co., San Francisco, CA.

24. Wolin, E. A., M. J. Wolin, and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. 238:2882-2886.

25. Zehnder, A.J.B., and K. Wuhrmann. 1976. Titanium (III) citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. Science 194:1165-1166.

26. Zeikus, J. G., and M. R. Winfrey. 1976. Temperature limitation of methanogenesis in aquatic sediments. Appl. Environ. Microbiol. 31:99-107.

CHAPTER III

KINETIC PARAMETERS OF THE CONVERSION OF METHANE PRECURSORS TO METHANE IN A HYPEREUTROPHIC LAKE SEDIMENT

INTRODUCTION

An integral part of the carbon cycle of hypereutrophic lakes is the anaerobic decomposition of organic matter in the sediment. The mechanisms controlling sediment methane production, which is the terminal process of this decomposition, have received recent attention by two laboratories (4,5,6,29,30,34). The addition of hydrogen, which is an immediate precursor of methane and a substrate for all known pure cultures of methanogenic bacteria (31), has been shown to cause an immediate stimulation of sediment methane formation (29). Thus hydrogen may be a limiting factor for this terminal metabolic process. However, not much else is known about hydrogen and its role in sediment metabolic processes. Yet to be determined are (i) the natural rates of hydrogen production and consumption, including conversion to methane, (ii) the *in situ* concentration of dissolved hydrogen, (iii) the fates of this dissolved hydrogen, (iv) the effects of various environmental parameters on sediment hydrogen metabolism, (v) and the effect of increases in the natural dissolved hydrogen concentration on other metabolic processes in the anaerobic decomposition of organic matter.

Some insight into the importance of hydrogen has been supplied by experiments with pure and mixed cultures (7,18,23,24,27,32). These experiments have shown that when hydrogen-producing heterotrophs are grown in the presence of hydrogen-consuming bacteria such as methanogens, a metabolic coupling exists between the two physiological groups

(32). This coupling, termed interspecies hydrogen transfer, results in an altered metabolism characterized by (i) an increase in substrate utilization, (ii) a decrease in reduced fermentation end products (iii) an increase in hydrogen produced (as methane) and, (iv) an increase in oxidized fermentation end products such as acetate (32).

Central to the concept of interspecies hydrogen transfer is the maintenance of low partial pressures of H_2 because the fermentative bacteria are assumed to produce molecular hydrogen from reduced pyridine nucleotide (NADH). This reaction is thermodynamically unfavorable at hydrogen partial pressures above 10^{-3} to 10^{-4} atmospheres (32). The dissolved hydrogen levels of anaerobic sediments are extremely low and often undetectable (29) thus, theoretically, interspecies hydrogen transfer can occur in this habitat. My hypothesis was that the methanogenic bacteria were capable of maintaining these low hydrogen concentrations. I predicted that the Michaelis-Menten constant, or K_m, for the conversion of hydrogen to methane must be low thus implying that the affinity of the methanogens for hydrogen was high. Hungate (16) has determined that the K_m for the conversion of H_2 to methane by the bovine rumen ecosystem and for Methanobacterium ruminantium in culture was very near the natural dissolved hydrogen concentration.

Acetate is thought by some to be another important precursor of the methane produced in lake sediments (4,5,6,29,30). Cappenberg and Prins (6) have calculated that about 70% of the methane produced by Lake Vechten sediments comes from acetate and Winfrey et al.(29) have shown that radioactively labeled [2-^{14}C]- acetate is rapidly converted to methane by the microflora in Lake Mendota sediments. It is to be noted, however, that the production and utilization of the oxidized fermentation

product, acetate, would also occur in lake sediments if interspecies hydrogen transfer were operative. Kinetic studies of the conversion of acetate to methane (6,26) have only estimated the turnover rate of the natural acetate pool by following the temporal disappearance of ^{14}C-labeled acetate added to this pool. These studies assumed that all of the acetate was being converted to methane, but this assumption was not verified. This could have been done by showing that the appearance of ^{14}C-labeled methane was equal to the disappearance of ^{14}C-labeled acetate. Also unknown are the ability of the acetate-utilizing methanogens to maintain low in situ concentrations of acetate, the effects of other environmental parameters on acetate conversion to methane, and the effects of acetate concentration on other metabolic processes in the sediment.

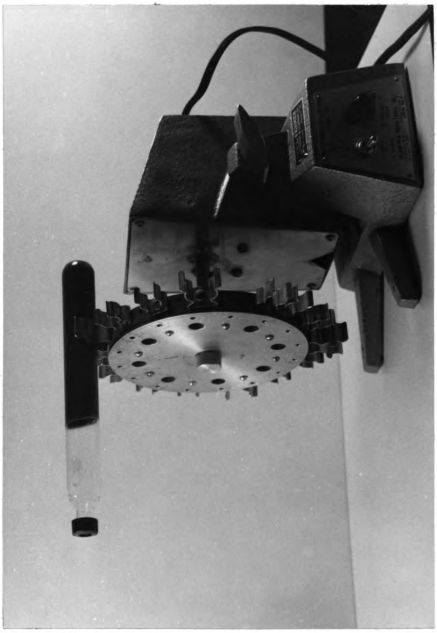
In this paper I report on the kinetic parameters, K_m, V_{max}, and turnover time, (T_t), for the conversion of hydrogen and acetate to methane by the microflora of an anaerobic pelagic lake sediment. Also reported are the effects of formate on sediment methanogenesis and the effects of hydrogen on the kinetics of acetate conversion to methane and on the heterotrophic decomposition of valine.

MATERIALS AND METHODS

Sampling. An Ekman dredge was used to collect samples from the pelagic sediment located within the 6 m depth contour of Wintergreen Lake. Mason jars were filled to overflowing with the collected sediment and the lid was tightly screwed in place. Sealed jars were transported to the laboratory in well insulated containers and stored at 4 C. Prior to use the sediment was transferred from the Mason jars to stoppered 1-liter reagent bottles. Transferral was done within an anaerobic glove chamber (Coy Manufacturing Co., Ann Arbor, MI.) that had a gas atmosphere of H_2/N_2 (10/90,v/v). The rubber stopper that sealed the reagent bottle was pierced by glass sampling and gassing tubes to which silicon rubber tubing was attached. The tubing was clamped to prevent the entry of air. After the reagent bottles were removed from the anaerobic chamber they were flushed with O_2-free argon (passed over hot reduced copper wire) to remove the H_2 in the headspace. Bottles were stored at 4 C. For experiments, the sediment in the bottles was sampled through the outlet tube with a 10 ml water-lubricated syringe that had been flushed with argon.

Incubation system. Because H_2 is an insoluble gas and because its diffusion from the gas phase into sediment is slow, I devised an incubation system (Fig. 1) that would minimize this limitation. A major feature is that the tubes are rotated at a near horizontal position so that a thin film (about 2 mm) of sediment accumulates on the sides, thus maximizing the contact between sediment and gas atmosphere. A second important feature is that there is a large headspace volume, which I found was necessary to ensure that the extremely low concentrations of added hydrogen were not significantly depleted during incubation.

Figure 1. Incubation system used to study the kinetics of methane production in lake sediments. The tube was specially made from a 20 x 200 mm culture tube with the top modified to accept a flange type butyl rubber stopper and Hungate-type screw cap. Tubes with 5 to 10 ml sediment were incubated in a near horizontal position on a rotary mixer as shown.



The tube was made from a 20 x 200 mm screw cap culture tube (Kimble Glass Co.). The top was modified to accept a flange type butyl rubber Hungate stopper (Bellco) held in place by a Hungate screw cap (Bellco). In operation tubes were flushed with oxygen-free argon and 5 to 10 ml of sediment were added anaerobically with a glass syringe. The tubes were sealed with the rubber stopper, vortexed for 1 min. while flushing with argon, and flushed for an additional 2 min. This step was necessary to remove the very high concentration of dissolved methane which concealed the rate at which methane was produced. Since the sediment pH increased from 6.8 before flushing to 7.4 after flushing, carbon dioxide (9.5% final volume, determined empirically and by calculations based on CO_2 solubilities) was added to return the pH to the original value.

All substrate additions were made anaerobically by syringe. The total volume of solutions, when added, never exceeded 6% of the sediment volume. All experiments were incubated at 10 to 14 C unless otherwise noted.

Estimation of dissolved hydrogen. The procedure that I used for extracting and concentrating the dissolved hydrogen in sediments is based, in principle, on the method developed by Hungate (15) for rumen fluid. The major difference is that instead of a water vapor gas phase, which is removed by condensation (15), I employed a CO_2 vapor phase, which was absorbed by a high ionic strength, alkaline solution.

Twenty ml of sediment was drawn into a 50 ml water-lubricated glass syringe. A syringe needle was attached and 30 ml of O_2-free CO_2 was slowly drawn into the syringe. The needle was then immediately inserted into a rubber stopper and the syringe shaken vigorously by hand to equilibrate the gas and aqueous phases. The gas phase was then injected

into an inverted 7.5 ml capped serum vial that had been filled with a solution of 20% NaCl (v/v) plus 1 M NaOH. The high ionic strength of this solution keeps most of the hydrogen in the vapor phase, and the alkali absorbs the carbon dioxide, thus concentrating the sediment gases in a volume of about 1.5 ml. The high ionic strength decreases the hydrogen solubility by about 1/2 (14) so it is important to keep the volume of the vial small so that re-solution of hydrogen would be minimal. My calculations (equations of Flett et al.(13)) have shown that, if in equilibrium with 7.5 ml of salt solution, 96% of the H_2 should be in the gas phase. An empty 10 ml syringe was also inserted through the serum cap to allow excess liquid to escape and to thus measure the volume of the gas phase at atmospheric pressure. The extraction and concentration steps were repeated twice with each sediment sample to ensure that all of the dissolved H_2 was removed.

Gas analysis. Methane was analyzed by a gas chromatograph (GC) equipped with a flame ionization detector. A 2 m Porapak Q column (Waters Associates) was operated at 60 C and helium was the carrier gas. Hydrogen was analyzed by a Carle Basic 8515 GC equipped with a microthermistor detector. Gas separation was made by a 1 m Porapak Q and 1 m Molecular Sieve 5A (Supelco) columns connected in series. Columns were operated at room temperature and argon was the carrier gas. For quantitation of low concentrations of H_2 the detector signal was further amplified by a 5/10 X operational amplifier which the laboratory had constructed.

Radioactive isotopes and analysis of radioactive gases. The following radioactive compounds were used: sodium [2-^{14}C] acetate, specific activity, 50 mCi mmol^{-1}(New England Nuclear); sodium [2-^{14}C] acetate,

specific activity 57.7 mCi $mmol^{-1}$ (Amersham/Searle); [U-^{14}C] valine, specific activity 280 mCi $mmol^{-1}$(Amersham/Searle). Radioactive methane and carbon dioxide in headspace gases were separated by injection of 1 ml samples into the Carle GC. Effluent gases from the GC were passed through a combustion furnace (Packard Tri-Carb) where methane was converted to CO_2 by passage over hot (750 C) oxidized copper wire. Carbon dioxide in the gas exiting the furnace was trapped in a solution that contained 9 ml methanol, 2 ml ethanolamine (Eastman, scintillation grade), and 6 ml scintillation solution (15 g 2,5-diphenyloxazole, (PPO), 1 g p-bis(0-methylstyryl)benezene (bis-MSB), 1 liter toluene). The radioactive CO_2 trapped in this solution was counted in a scintillation counter (Packard Tri-Carb) with an efficiency of 91%.

Theory. The assumption was made that the conversion of substrates to methane by the sediment microflora followed Michaelis-Menten kinetics according to the equation

$$v = \frac{V_{max} \cdot S}{K_m + S} \qquad [1]$$

The term v is the initial rate of methane production or substrate utilization, S is the initial concentration of substrate, V_{max} is the maximum initial velocity that can be attained, and K_m, the Michaelis constant, is the concentration of substrate that would give a rate equal to 1/2 the maximum velocity. To estimate the kinetic parameters, K_m and V_{max}, I employed the direct linear plot of Eisenthal and Cornish-Bowden (12). This non-parametric statistical method has the advantages that less assumptions are needed than with the methods which use least squares analysis, that it is simple and direct, and that the K_m, V_{max} estimates are less sensitive to outliers (12). Data pairs of substrate

(S_i) and initial velocity (v_i) are plotted as lines in K_m-V_{max} parameter space. Each intersection of these lines is considered an estimate of the K_m and V_{max} and the median value of these estimates is taken as the best estimate. The number of intersections is given by the equation

$$1/2\ n(n - 1) \qquad [2]$$

where n is the number of data pairs of S and v (12).

I have also employed progress curves (substrate or product vs. t) (25) as a second, independent method for determining the kinetic parameters for the conversion of hydrogen to methane. The following equation is an integrated solution to the Michaelis-Menten equation(25)

$$V_{max} \cdot t = K_m \cdot \ln\frac{S_o}{S_t} + (S_o - S_t) \qquad [3]$$

with S_o equal to the initial substrate concentration and S_t equal to the substrate concentration at time, t. Equation 3 can be converted to the form of a linear regression by dividing by t and rearranging (25) such that

$$\frac{\ln S_0/S_t}{t} = \frac{1}{K_m} \cdot \frac{S_o - S_t}{t} + \frac{V_{max}}{K_m} \qquad [4]$$

Equation 4 was used to analyze the data of both H_2 and CH_4 progress curves.

For kinetic experiments which involved the addition of labeled substrates and analysis of labeled gaseous products, the equation of Hobbie and Crawford (17) was employed

$$\frac{t}{f} = \frac{(K_m + S_n)}{V_{max}} + \frac{1}{V_{max}} \qquad [5]$$

with t equal to the incubation time, f equal to the fraction of added label that was converted to product, S_n equal to the <u>in situ</u> (natural)

substrate concentration, and A equal to the concentration of added (labeled and unlabeled) substrate. A regression of t/f versus A yields values for the $K_m + S_n$ at the x intercept, $(K_m + S_n)/V_{max}$ at the slope and the turnover time, T_t, at the y intercept (33). If S_n is known, the natural rate of substration utilization can be calculated from

$$v = S_n/T_t \qquad [6]$$

RESULTS

Kinetics of hydrogen conversion to methane. Concentrations of dissolved hydrogen in the lake sediment were determined for three samples collected at various times during the winter and for one sample collected in early June. No significant differences in concentration among samples were observed. The concentrations ranged from 6 to 10 nmol dissolved H_2 per gram dry sediment (0.7 to 1.2 umol dissolved $H_2 \cdot l^{-1}$ wet sediment).

A summary of the kinetic experiments that I performed with H_2 as substrate are presented in Table 1. Included are the approximate dates on which sediment was collected, the substrate or product that was analyzed, the range of substrate concentrations, the estimates of K_m, V_{max}, and T_t, and the method used to estimate these kinetic parameters.

Stimulation of the rate of sediment methanogenesis by increasing levels of hydrogen in the headspace gas usually resulted in a rectangular hyperbola (Fig. 2). Incubation times were kept short in order to ensure that initial hydrogen concentrations had not decreased by more than 10% at the end of the experiment. Incubation times were usually 1.25 h. The range of initial hydrogen concentrations was variable, but

never less than 0.13% nor greater than 2%. The kinetic parameters, K_m and V_{max}, were estimated from direct linear plots of the v,S data pairs (12) for each experiment. The data presented in Fig. 2 is shown as a direct linear plot in Fig. 3. Although K_m, V_{max} estimates can be made directly from the graph, I calculated the coordinates of each intersection from equations [5] and [6] of Cornish-Bowden and Eisenthal (10). Values for the K_m ranged from 0.13% (0.010 µmol dissolved $H_2 \cdot g^{-1}$) to 0.47% (0.033 µmol dissolved $H_2 \cdot g^{-1}$). The V_{max} ranged from 0.45 $\mu mol \cdot g^{-1} \cdot h^{-1}$ to 1.00 $\mu mol \cdot g^{-1} \cdot h^{-1}$ for methane production and 1.66 to 5.88 $\mu mol \cdot g^{-1} \cdot h^{-1}$ for H_2 consumption.

The alternative approach of using progress curves to obtain kinetic data was used because this method allows the determination to be done in a single experimental vessel and does not require concern over rapid substrate depletion at low substrate concentrations. An example of a progress curve for H_2 consumption and CH_4 production is presented in Fig. 4. The methane concentrations at each sampling have been corrected for the methane produced by the control which did not have hydrogen added. Concentrations of hydrogen and methane in the headspace was analyzed every 30-45 min depending on the number of replicates in each experiment. For the H_2 progress curve experiments (Table 1) the K_m estimates ranged from 0.21% H_2(0.010 µmol dissolved $H_2 \cdot g^{-1}$) to 0.48% H_2 (0.034 µmol dissolved $H_2 \cdot g^{-1}$) and the V_{max} ranged from 1.14 to 6.38 µmol H_2 consumed $\cdot g^{-1} \cdot h^{-1}$. For the H_2 progress curve shown in Fig. 4 the K_m was 0.29% and the V_{max} was 4.73 µmol $H_2 \cdot g^{-1} \cdot h^{-1}$. For the methane progress curve the K_m was 0.40% and the V_{max} was 1.13 µmol CH_4 produced $\cdot g^{-1} \cdot h^{-1}$. Although the ratio of H_2 V_{max} to CH_4 V_{max} is approximately 4 (the theoretical ratio for conversion of 4 moles of H_2 to 1 mole CH_4)

Table 1. Summary of kinetic experiments of the consumption of hydrogen and the conversion of hydrogen to methane by Wintergreen Lake sediments.

Date of Experiment	Date sediment was collected	Substrate or product analyzed	Initial H_2 concentration as % of headspace gas	Type of kinetic experiment	Incubation time (h)	K_m as % H_2	K_m as $\mu mol\ H_2 \cdot g^{-1}$	V_{max} $\mu mol\ H_2$ consumed or $\mu mol\ CH_4$ produced $\cdot g^{-1} \cdot h^{-1}$	T_t (h)	Method used to estimate K_m, V_{max}
13 Dec 76	Oct 76	CH_4	0.26 1.93	v vs. S	1.25	0.24	0.014	0.452	0.031	DL[a]
12 Jan 77	Jan 77	H_2	0.55	Progress curve	0-6	0.22	0.014	2.44	0.0057	IRE[b]
		H_2	0.55	Progress curve	0-6	0.24	0.015	3.00	0.0050	IRE[b]
5 Mar 77	Feb 77	H_2	0.22,0.10	Progress curve	0-3	0.21	0.010	1.18	0.0086	IRE[b]
		H_2	0.22,0.11	Progress curve	0-3	0.22	0.010	1.14	0.0085	IRE[b]
10 Mar 77	8 Mar 77	H_2	0.13-1.06	v vs. S	1.25	0.37	0.027	4.27	0.0064	DL
		CH_4				0.45	0.033	0.47	0.070	
11 Mar 77	8 Mar 77	H_2	0.14-0.70	v vs. S	1.25	0.38	0.028	5.51	0.0051	DL
		CH_4				0.13	0.010	0.49	0.020	
12 Mar 77	8 Mar 77	H_2	0.14-0.71	v vs. S	1.25	0.47	0.033	5.88	0.057	DL
		CH_4				0.38	0.027	0.69	0.039	
15 Mar 77	8 Mar 77	H_2	0.51	Progress curve	0-4.5	0.29	0.021	4.73	0.0044	IRE[b]
		CH_4				0.40	0.029	1.12	0.023	
25 Mar 77	8 Mar 77	H_2	0.66	Progress curve	0-5.25	0.48	0.034	6.38	0.0054	IRE[b]
		H_2	0.66	Progress curve	0-5.25	0.43	0.031	6.01	0.0051	IRE[b]

a direct linear plot
b Integrated rate equation of Segel (25)

Figure 2. Effect of increasing hydrogen concentration, [S], on the rate of sediment methane production, v. The rates have been corrected for the control (no hydrogen added). Incubation time was 1.25 h at 10 C. Sediment was collected in the early winter.

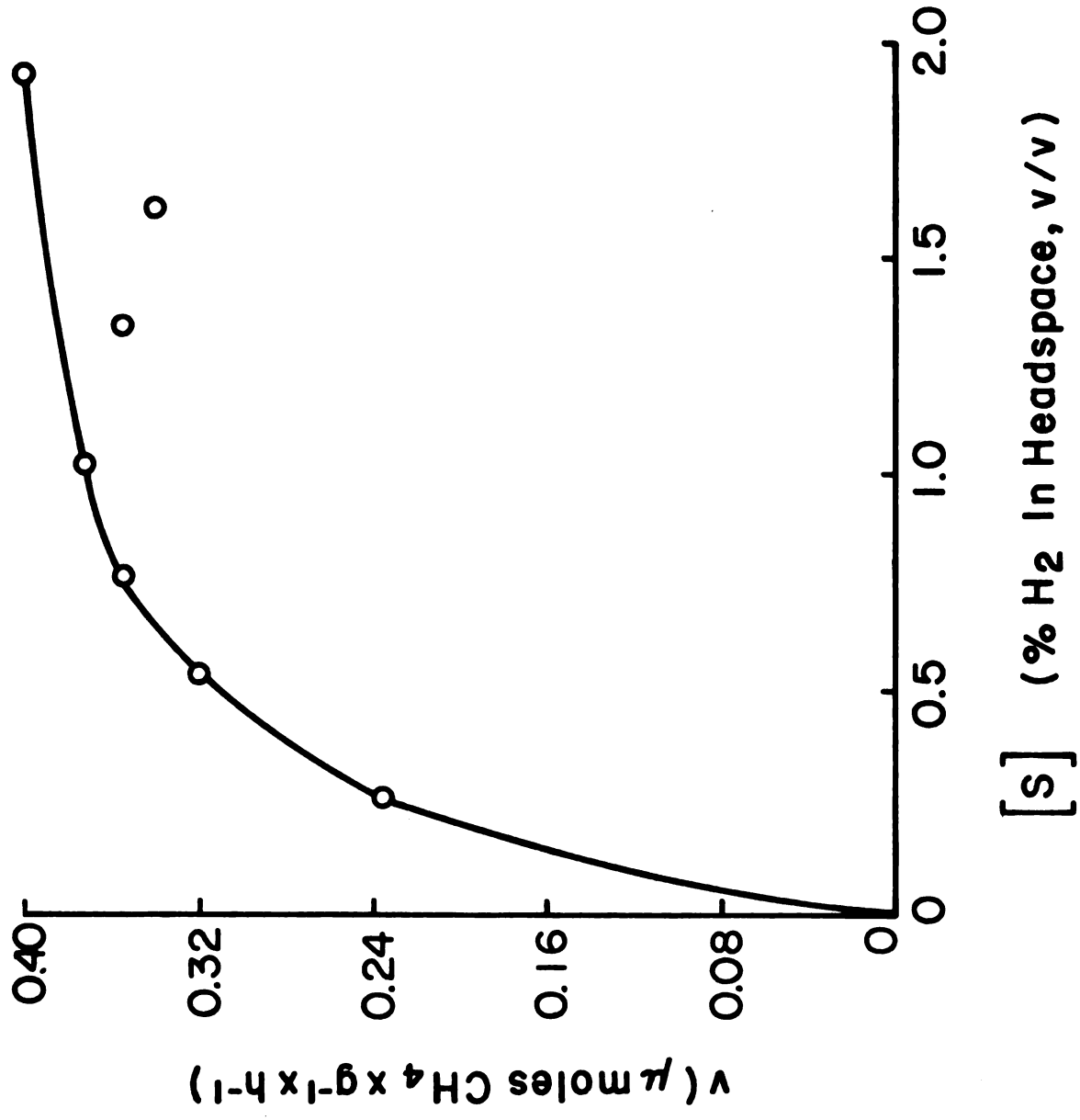
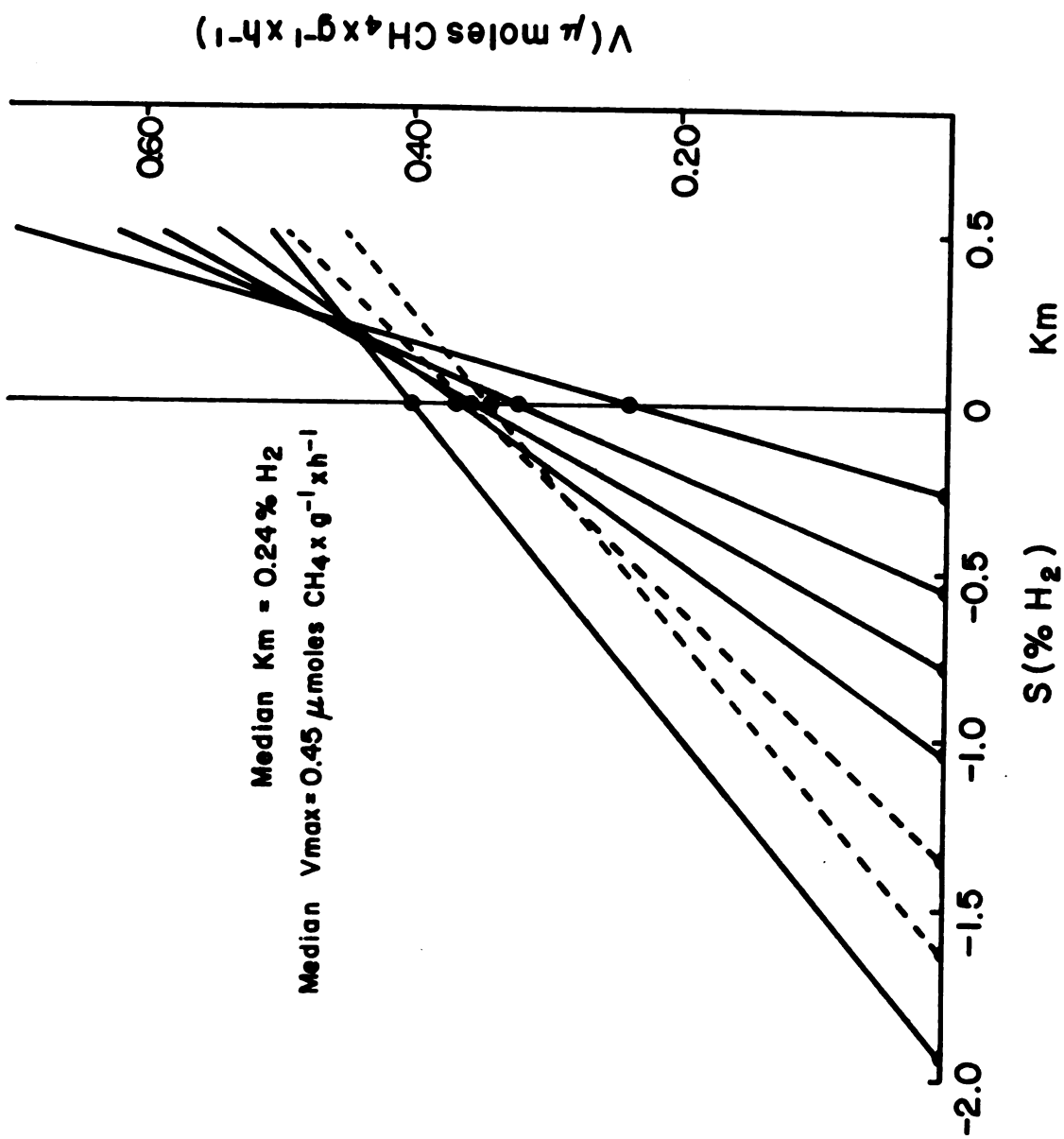

v (μ moles CH4 x g-1 x h-1)
0.40
0.32
0.24
0.16
0.08
0
0
0.5
1.0
1.5
2.0
[S] (% H2 In Headspace, v/v)

Figure 3. Direct linear plot of the substrate, velocity data pairs plotted in Figure 2.


V(μ moles CH4 x g-1 x h-1)
0.60
0.40
0.20
0.5
0
-0.5
-1.0
-1.5
-2.0
Km
S(% H2)
Median Km = 0.24 % H2
Median Vmax = 0.45 μmoles CH4 x g-1 x h-1

the ratio of hydrogen consumed to methane produced increased as the hydrogen concentration in the headspace decreased (Table 2).

Other hydrogen donors and the stimulation of methanogenesis. Table 3 shows a survey of the effect of various potential hydrogen donors on the rate of methanogenesis. Since only formate immediately stimulated methanogenesis, I attempted to determine the kinetic parameters for the conversion of this substrate to methane (Fig. 5). In this experiment, the response approaches a hyperbolic relationship. However, assuming that four moles of formate are needed to produce one mole of methane, at the lowest concentration of 1.16 μmol formate·g^{-1}, 87% of the formate was converted to methane within the 1 h incubation period. This percentage does not include other non-methane fates of formate. In an attempt to achieve a more valid temporal resolution, progress curves at several formate concentrations and with 2 min sampling intervals were employed. The experiment was, of necessity, performed at room temperature (23 C) next to the gas chromatograph. The rates of methane production, calculated for the 10 min sampling time (Table 4), still indicated that at least 47% of the formate had been consumed within this short time. At sampling times under 10 min the rates were variable but suggested that maximum velocities were reached somewhere between 260 and 400 μmol formate·g^{-1} sediment . K_m values could not be estimated.

Kinetics of acetate conversion to methane. The addition of between 1.23 to 12.1 μmol acetate·g^{-1} sediment did not stimulate the rate of sediment methanogenesis beyond the rate of the control with no addition (Fig. 6). This rate, calculated from the slope of the linear portion of the lines plotted in Fig. 6 averaged 0.307 μmol CH_4 produced ·g^{-1}·h^{-1}

Figure 4. Progress curves of the headspace hydrogen and methane concentrations with time. Methane concentrations have been corrected for the control which had no hydrogen added. Incubation temperature was 12 C. Headspace (vapor) volume was about 65 ml. Sediment was collected in late winter.

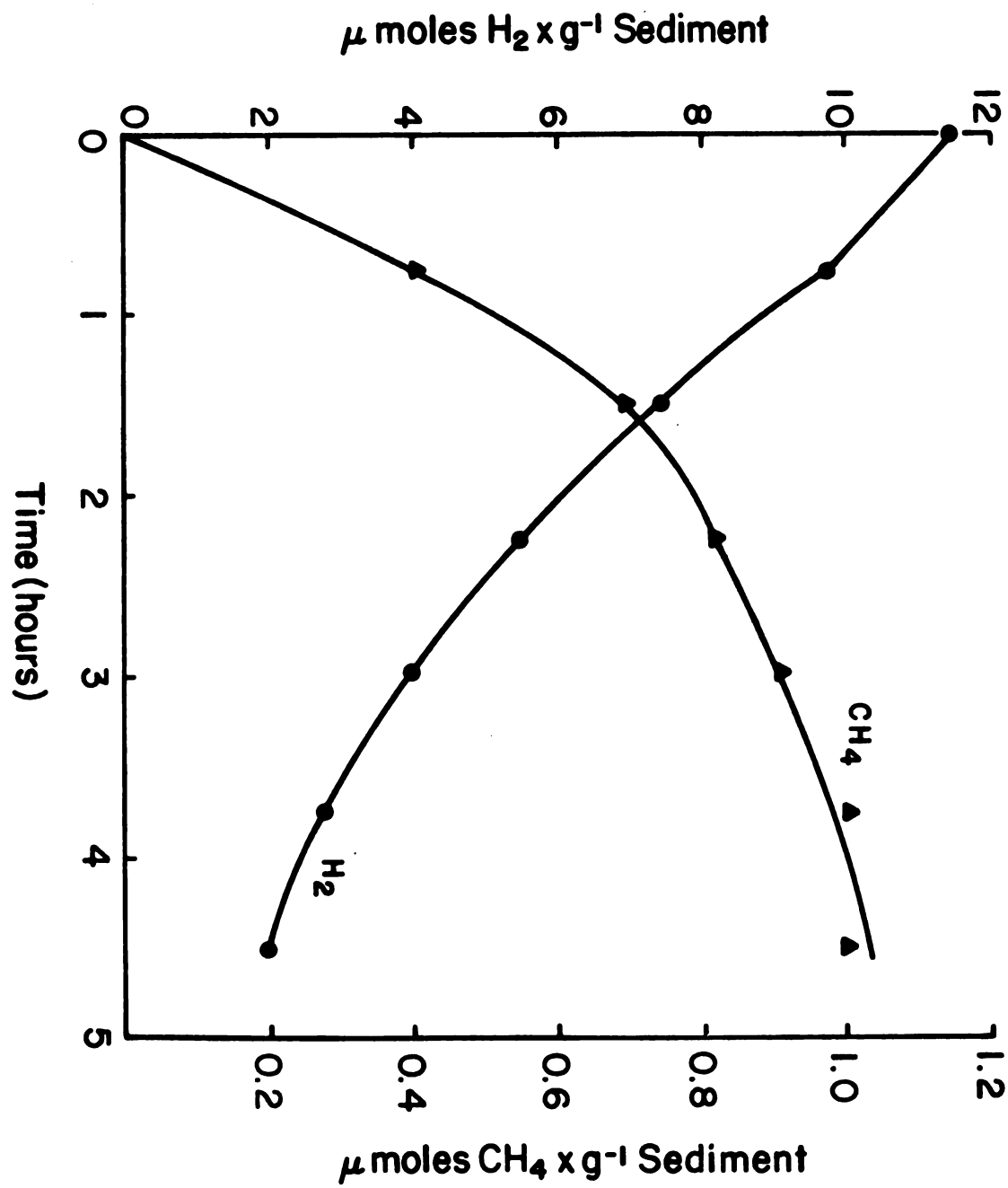

μ moles H_2 x g^{-1} Sediment
0
2
4
6
8
10
12
Time (hours)
0
1
2
3
4
5
H_2
CH_4
0.2
0.4
0.6
0.8
1.0
1.2
μ moles CH_4 x g^{-1} Sediment

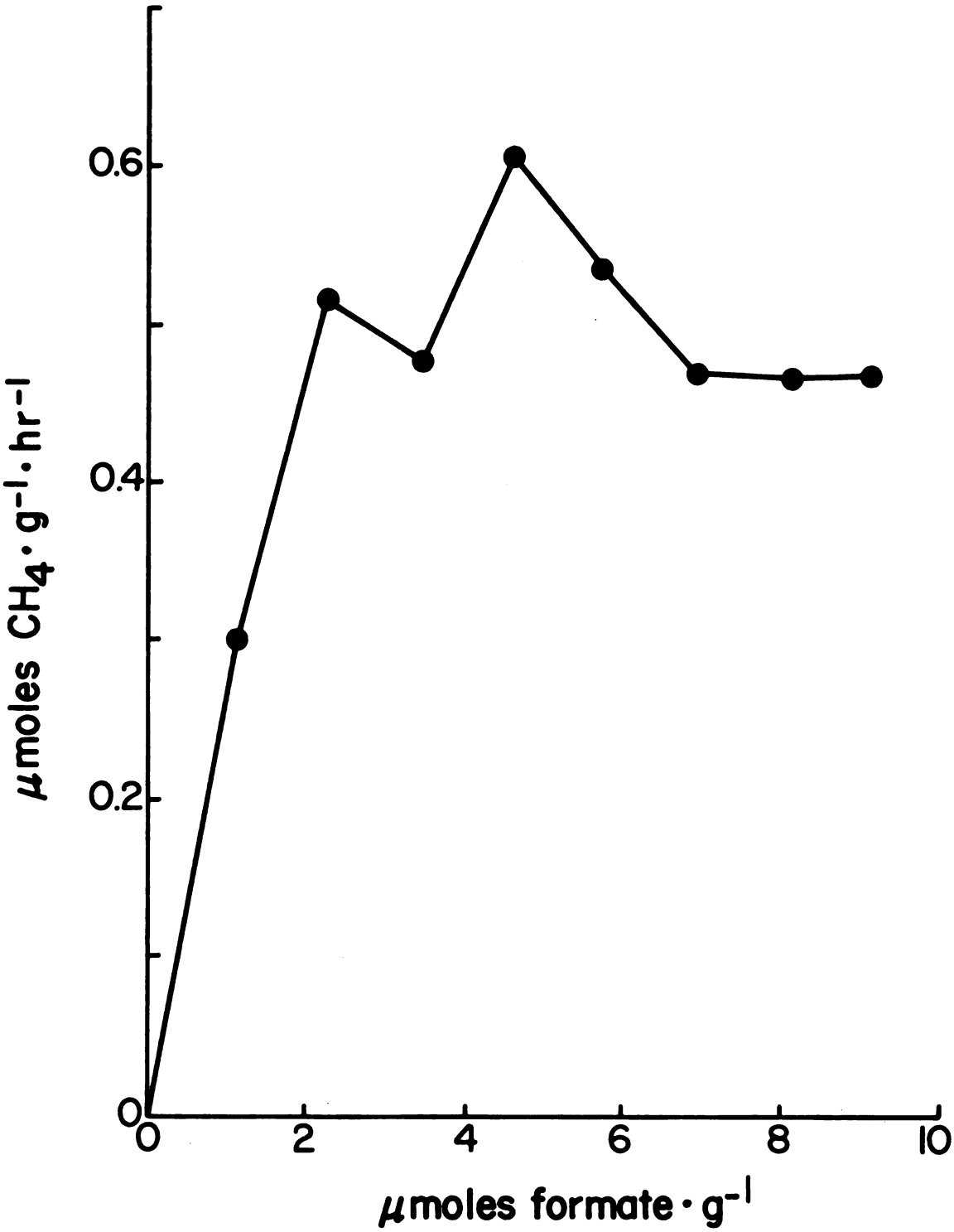
Table 2. Ratio of hydrogen consumed to methane produced with decreasing hydrogen concentration calculated from the progress curves of Fig. 4.

Time (h)	H_2 in headspace (%)	$\frac{H_2 \text{ consumed}}{CH_4 \text{ produced}}$
0.00	0.50	---
0.75	0.43	4.1
1.50	0.33	5.7
2.25	0.24	7.2
3.00	0.18	8.1
3.75	0.12	8.1
4.50	0.09	8.8

Table 3. Effect of potential hydrogen donors on the rate of sediment methanogenesis. Incubation temperature was]2 C.

Substrate	Concentration range ($\mu moles \cdot l^{-1}$)	Incubation time (h)	Stimulation (+ or -)
Formate	25-150	0.25	+++
	100-1000	1.0	+++
Lactate	40-280	6.0	-
		24.0	-
Propionate	40-280	6.0	-
		24.0	-
Valine	40-280	6.0	-
		24.0	-
Leucine	40-280	6.0	-
		24.0	-

Figure 5. Effect of dissolved formate concentration on the rate of sediment methanogenesis. Methane production rates have been corrected for the rate of the control, which received an identical volume of oxygen free water but no formate. Incubation time was 1 h at 12 C. Sediment was collected in mid winter.


0.6
0.4
0.2
0
0
2
4
6
8
10
μmoles $CH_4 \cdot g^{-1} \cdot hr^{-1}$
μmoles formate · g^{-1}

($\pm$ 4% s,n = 11). This lack of stimulation prompted me to attempt short term progress curves at lower concentrations and with methane measurements every 2 min. The results for the 10 min sampling time are presented in Table 4. Even under these conditions I could not detect significant acetate stimulation of methanogenesis.

In order to determine if the lack of acetate stimulation was due to the absence of acetate utilizing methanogens, I monitored the production of radioactively labeled methane and CO_2 from [2-^{14}C] acetate with time (Fig. 7). Also plotted is the production of labeled methane by sediment that had been preincubated with 0.5% H_2 for 15 min prior to the addition of labeled acetate. In both the presence and absence of added H_2, acetate was rapidly converted to methane, though the addition of H_2 caused an immediate conversion.

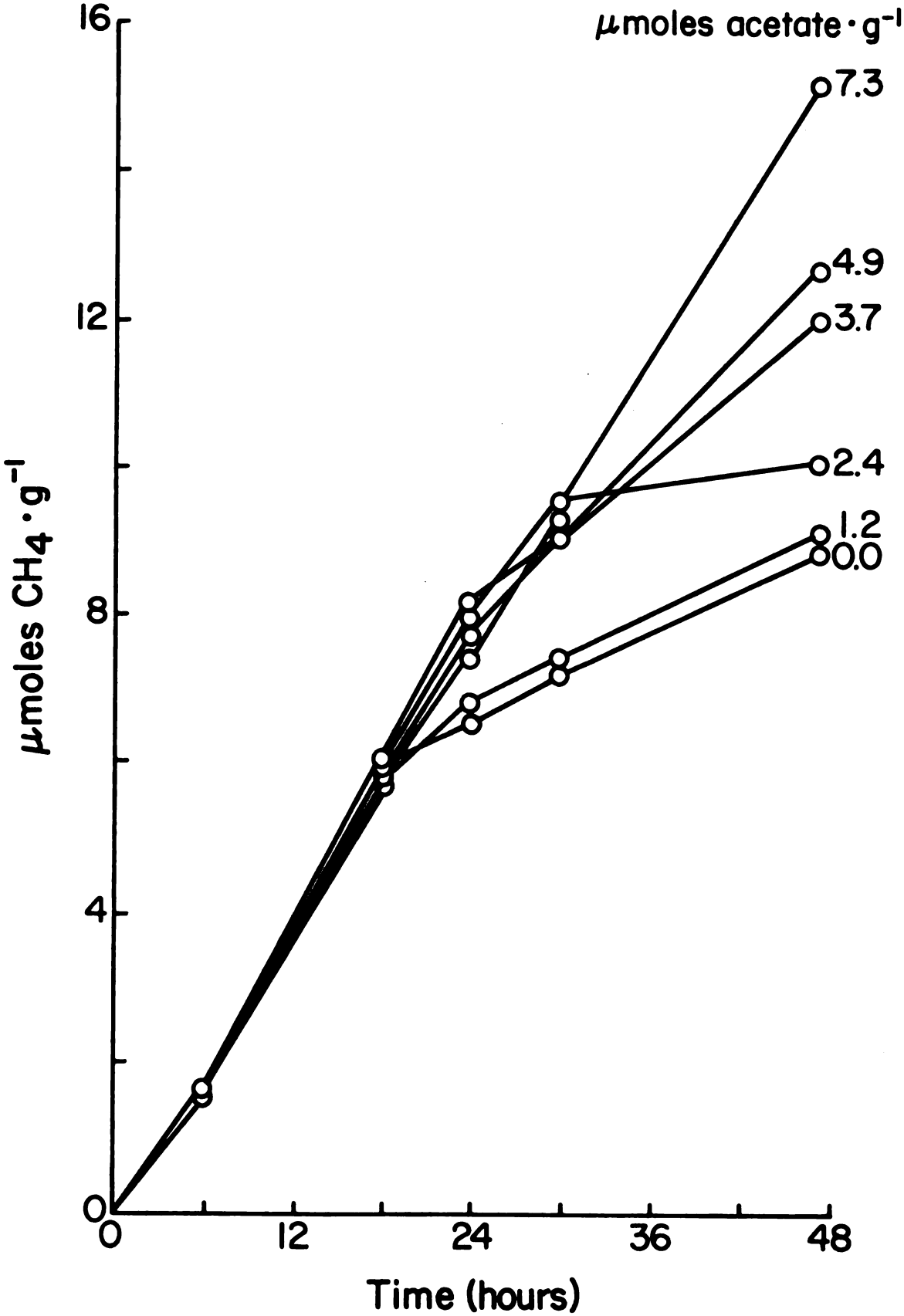
Because labeled methane appeared so rapidly after the addition of labeled acetate I ran kinetic experiments that were similar in approach to the uptake kinetic experiments described by Wright and Hobbie (33). A different amount of unlabeled acetate was added to each tube of sediment. Each tube then received the same amount (1 µCi) of [2-^{14}C] labeled acetate. After an incubation time of 10 to 15 min the amount of label in the headspace methane was determined. This radioactivity was used to calculate the term t/f of Hobbie and Crawford (17), which was plotted against the amount of labeled plus unlabeled acetate added to each tube. This plot is equivalent to a kinetic plot of s/v versus S. The results of this experiment, for the two cases of plus and minus preincubation with 0.5% H_2, are presented in Fig. 8. The kinetic parameters estimated from this plot and a similar experiment are presented in Table 5.

Table 4. Effect of dissolved formate and dissolved acetate concentrations on the rate of sediment methanogenesis. Incubation time was 10 min at 23 C. Sediment was collected in the late winter.

Substrate concentration (nmol · g^{-1})	Rate of methane formation	
	Formate (nmol $CH_4 \cdot g^{-1} \cdot h^{-1}$)	Acetate (nmol $CH_4 \cdot g^{-1} \cdot h^{-1}$)
0	220	250
130	340	170
270	520	330
400	776	280
530	850	300
660	ND*	310
800	950	330

*ND- not determined

Figure 6. Amount of methane produced by sediment with time at different concentrations of added dissolved acetate. Incubation temperature was 12 C. Sediment was collected in mid winter.


μmoles acetate · g⁻¹
7.3
4.9
3.7
2.4
1.2
0.0
μmoles CH4 · g⁻¹
16
12
8
4
0
0
12
24
36
48
Time (hours)

Since all results for acetate conversion to methane now indicated that the natural rate of conversion was at or very close to the V_{max}, a second type of labeled experiment was run, where each tube received the same specific activity of acetate but a different total acetate concentration. A plot of the amount of radioactive methane produced versus the acetate concentration should be in the form of a Michaelis-Menten rectangular hyperbola. The results of this experiment (Fig. 9) confirm that the rate of acetate conversion to methane is zero order with respect to acetate concentration.

Since the addition of hydrogen had a pronounced effect on the conversion of acetate to methane, the effect of H_2 on $^{14}CO_2$ production from [U-^{14}C] valine, a potential H_2 donor (Table 2) was examined. Four tubes, each containing 10 ml sediment, were incubated at 12 C for 15 min prior to the addition of 1 uCi labeled valine. Hydrogen (0.5% v/v) was added to two of the tubes at the start of the preincubation period. The amount of $^{14}CO_2$ present in the headspace gas was determined 15 min after the addition of the label. In the absence of hydrogen, 6.3×10^4 dpm $^{14}CO_2$ was detected in the headspace. The sediment exposed to 0.5% hydrogen produced only 4.0×10^4 dpm $^{14}CO_2$. This represents a 37% decrease in the production of labeled carbon dioxide from uniformly labeled valine.

Figure 7. Production of labeled methane and carbon dioxide from [2-^{14}C] acetate (1 μCi). O - labeled methane produced in the absence of hydrogen. ● - labeled carbon dioxide produced in the absence of hydrogen. Δ - labeled methane produced in the presence of 0.5% hydrogen. Hydrogen was added 15 min prior to the addition of labeled acetate. Incubation temperature was 12 C. Sediment was collected in June.

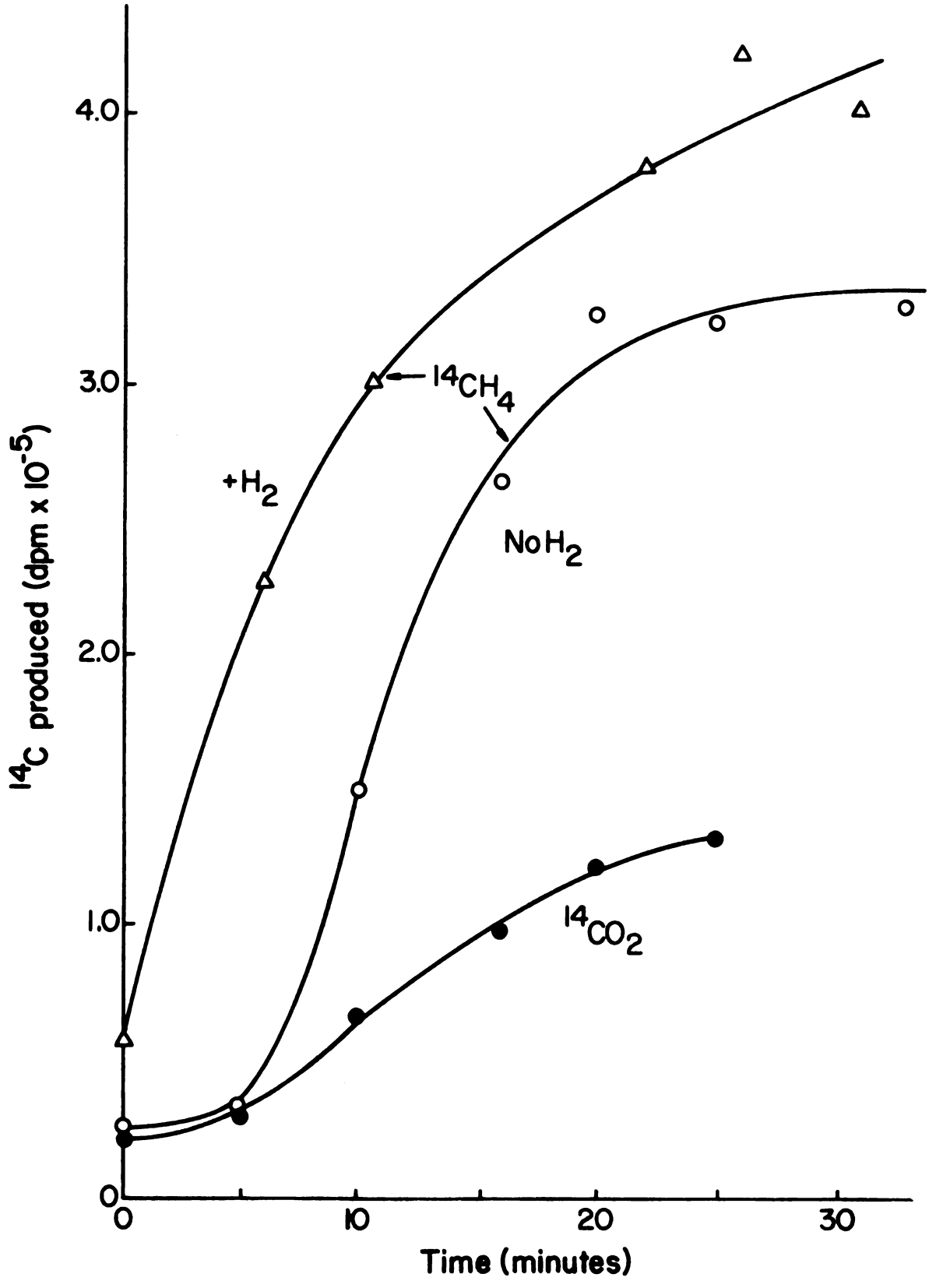

4.0
3.0
2.0
1.0
0
0
10
20
30
Time (minutes)
^{14}C produced (dpm x 10^{-5})
$^{14}CH_4$
+H_2
NoH_2
$^{14}CO_2$

Figure 8. Effect of acetate concentration and hydrogen on the conversion of [2-^{14}C] acetate to methane by sediment. ● - labeled methane produced within 15 min in the absence of 0.5% hydrogen. O - labeled methane produced within 10 min in the presence of 0.5% hydrogen. The hydrogen was added 15 min prior to the acetate addition. Incubation temperature was 12 C. Sediment was collected in June.

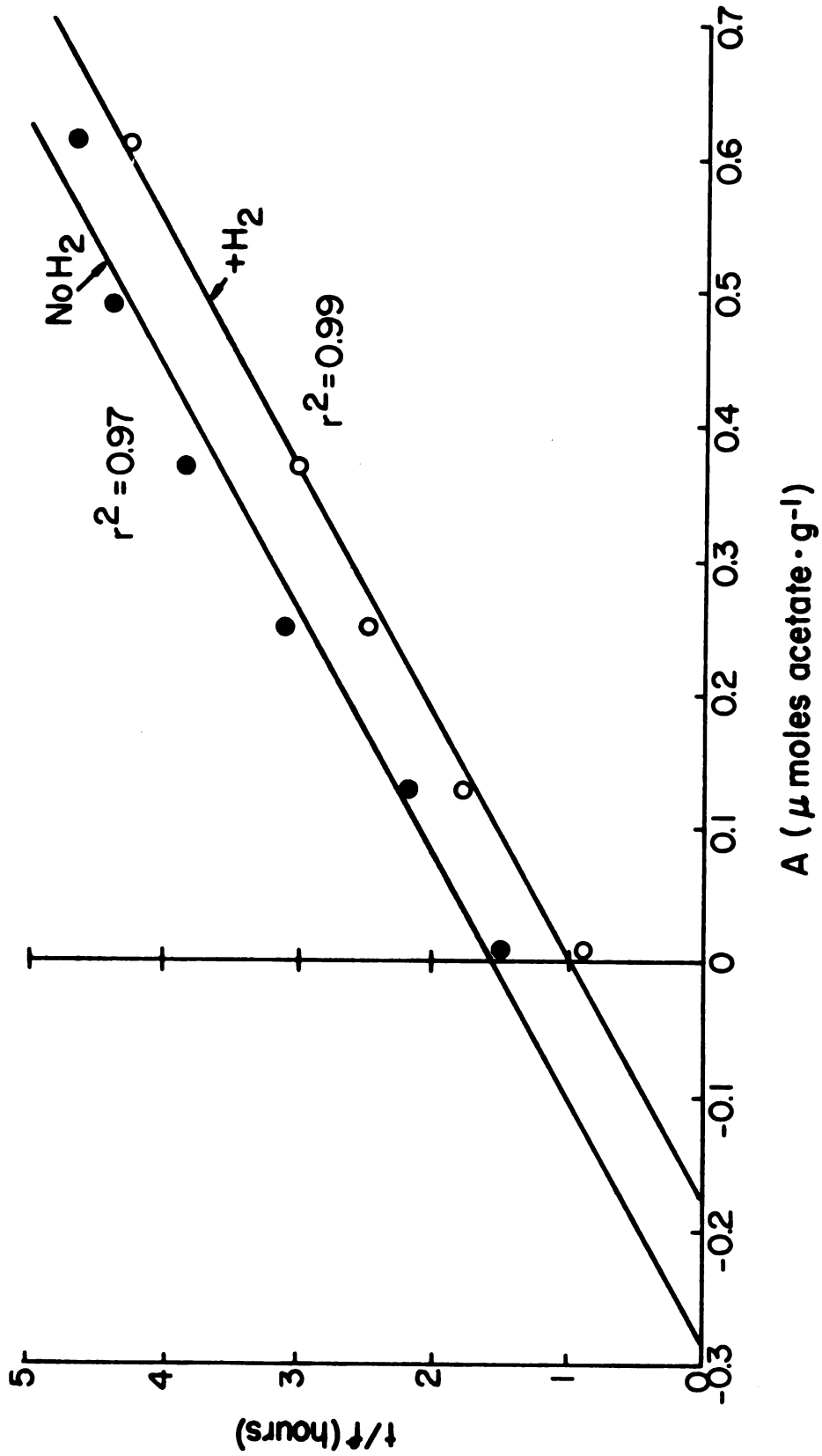
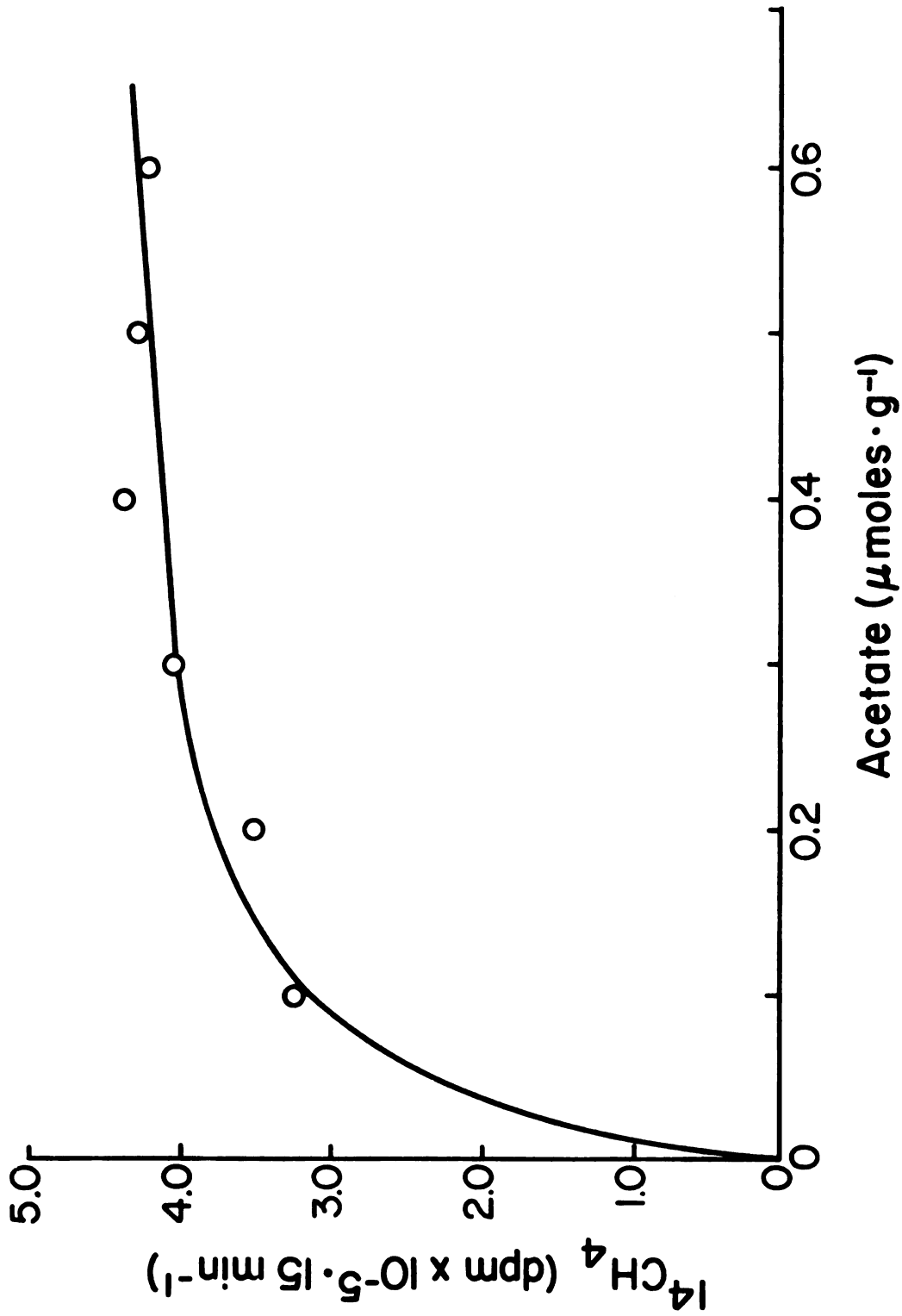

No H_2
+ H_2
r^2=0.97
r^2=0.99
t/f (hours)
A (μ moles acetate · g^{-1})
5
4
3
2
1
0
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7

Table 5. Kinetic parameters estimated from the regression of t/f versus A, as in Fig. 8. Experiment 1-run within 4 days of collecting sediment in early July. Experiment 2-run within 2 weeks of collecting sediment in late June.

	Preincubation with 0.5% H_2	$K_m + S_n$ (μmol acetate·g^{-1})	V_{max} (μmol CH_4·g^{-1}·h^{-1})	T_t (h)
Expt. 1	-	0.281	0.181	1.56
	+	0.177	0.181	0.98
Expt. 2	-	0.258	0.145	1.78
	+	0.165	0.189	0.91

Figure 9. Effect of acetate concentration on the production of $^{14}CH_4$ from [2-^{14}C] acetate by lake sediments. Each acetate concentration had the same specific activity of labeled acetate. Incubation time was 15 min at 12 C. Sediment was collected in June.


$^{14}CH_4$ (dpm x 10^{-5} · 15 min^{-1})
5.0
4.0
3.0
2.0
1.0
0
0
0.2
0.4
0.6
Acetate (μmoles · g^{-1})

DISCUSSION

The K_m estimates for the conversion of hydrogen to methane (Table 1) demonstrate that, if present in sufficient numbers, the methanogenic bacteria are capable of maintaining low concentrations of hydrogen in the sediment. Evidence that the methanogenic population is sufficient is indicated by the progress curve (Fig. 4) where moderately high initial hydrogen levels were decreased by 83% within 4.5 h. This rapid rate of consumption suggests that hydrogen limits methanogenesis and that hydrogen concentrations that may inhibit heterotrophic hydrogen production are quickly lowered to more favorable concentrations. In spite of the heterogeneity of sediments, I was able to obtain fairly reproducible K_m estimates among experiments employing different methods of analysis and with sediments collected on different dates (Table 1). These estimates must be considered the upper limit since transfer of hydrogen from the vapor phase to the aqueous sediment phase may still be rate limiting despite the design to optimize the transfer. The mean K_m for hydrogen consumption for the January and February sediment was 0.013 $\mu mol \cdot g^{-1}$ (or 1.6 $\mu mole \cdot l^{-1}$ wet sediment) and for the March sediment it was 0.029 $\mu mole \cdot g^{-1}$ (or 3.5 $\mu mole \cdot l^{-1}$ wet sediment). This compares favorably to the K_m value of 1 $\mu mole \cdot l^{-1}$ determined by Hungate et al. (16) for bovine rumen fluid and for Methanobacterium ruminantium. The mean K_m for methane production (Table 1) was 0.023 $\mu mol \cdot g^{-1}$ which does not vary significantly from the one for H_2 consumption.

The V_{max} for both the consumption of hydrogen and for the conversion of hydrogen to methane generally increased from December to March (Table 1). Since V_{max} is proportional to enzyme concentration this finding suggests that the population or activity of hydrogen consumers

and/or methane producers increased slightly throughout the Winter.

The *in situ* rate of hydrogen consumption can be calculated from the *in situ* hydrogen concentration, which was 6 to 10 nmol·g^{-1}, and the K_m and V_{max} (Table 1) according to equation [1]. The range in estimated natural velocities of H_2 consumption was 430 to 1530 nmol $H_2 \cdot g^{-1} \cdot h^{-1}$ and for H_2 conversion to methane it was 72 to 290 nmol $CH_4 \cdot g^{-1} \cdot h^{-1}$.

The non-methanogenic utilization of hydrogen by sediment microflora indicated in Table 2 has also been suggested by Winfrey et al. (29). A possible explanation is that the H_2 (and CO_2) are also being converted to acetate by an organism similar to the *Clostridium* isolated by Ohwaki and Hungate (20) from sewage sludge. A second possibility is that, with short term incubations, hydrogen consumption may be due to heterotrophic bacteria and the reaction $H_2 + NAD^+ \rightarrow NADH + H^+$. However, this reaction will be limited by the availability of organic electron acceptors, which are needed to recycle the catalytic amounts of NAD^+.

The potential hydrogen donors listed in Table 3 were selected for the following reasons. Formate was chosen because some methanogens can use it directly (31) and because it can be converted to hydrogen and carbon dioxide by a variety of heterotrophs (11). Lactate was selected because many heterotrophs can ferment it (11) and because Bryant et al. (3) have shown that with lactate, and in the absence of sulfate, interspecies hydrogen transfer occurs between *Desulfovibrio* and *Methanobacterium* strain MoH. Propionate was selected as a representative volatile fatty acid because Bryant (2) has hypothesized that there is an acetogenic population in anaerobic habitats that can convert these compounds and alcohols to acetate and hydrogen. The amino acids valine and leucine were selected because Molongoski and Klug (19) have shown that proteo-

lytic clostridia are the predominant isolatable heterotrophs in Wintergreen Lake sediments. The lack of significant stimulation of methane production by all but formate (Table 2) may indicate that either these substrates are unimportant hydrogen donors, or that they are already being turned over at maximum rates.

Although kinetic constants could not be estimated for the conversion of formate to methane, the almost immediate stimulation by low levels of formate (Table 4) suggests that this substrate could also be important in methane production. The rapid stimulation of sediment methanogenesis by formate has also been observed by Winfrey et al. (29). Formate concentrations in Wintergreen Lake sediments have been measured by Molongoski and Klug (unpublished data) and range from 13 to 63 $nmol \cdot g^{-1}$ during May to October. As with H_2, the methanogens may be responsible, in part, for maintaining these low concentrations. During short incubations and at added formate concentrations in excess of 130 $nmol \cdot g^{-1}$, I have detected only very low concentrations of hydrogen in the headspace gas. Since these hydrogen levels were not high enough to account for the observed stimulation of methanogenesis, I believe that formate may be directly used by the methanogenic bacteria present. This finding is consistent with the fluorescent antibody studies (Chapter II) which showed a formate and H_2 utilizing organism to predominate in these sediments.

The rate of conversion of acetate to methane appears to be quite close to the V_{max} for this reaction (Fig. 9, Table 4), which would explain the apparent lack of stimulation by additions of unlabeled acetate (Fig. 6). Natural concentrations of acetate in these sediments have been determined by Molongoski and Klug (unpublished data) to range

from 180-550 nmol·g^{-1} over the summer of 1976. Using the minimum measured acetate pool size and an average turnover time of 1.67 h (Table 5) then an estimate of the minimum natural velocity (equation 6) would be 108 nmol CH_4 produced ·g^{-1}·h^{-1}. This rate is 66% of the average V_{max} (Table 5) of 163 nmol CH_4 produced ·g^{-1}·h^{-1}, which further indicates that acetate is being converted to methane at rates very near the maximum. This discovery implies that the only way for an increase in the rate of acetate conversion to methane to occur would be by an increase in the population of acetate utilizing methanogens. But these bacteria have very slow growth rates (A. Zehnder, personal communication, and 22), so an increase in the rate of heterotrophic acetate production would lead to an increase in the acetate pool size.

My estimated minimum natural rate of acetate conversion to methane is approximately equivalent to 17 nmol · g^{-1} wet sediment·h^{-1}. Cappenberg (6) has estimated that acetate accounts for a rate of methane production of about 23 nmol·g^{-1} wet mud·h^{-1} in Lake Vechten sediments. This rate was about 70% of the apparent rate of total sediment methane production. Comparisons between the rates at which methane is formed from H_2 and from acetate by Wintergreen Lake sediments can not be made since the rate estimates were for samples collected at very different times of the year. The minimum rate at which hydrogen was converted to methane by winter sediment was about 67% of the rate at which acetate was converted to methane by sediments collected in June. The rate of H_2 conversion to methane by June sediments is unknown, but it probably greater than the minimum winter rate.

Cappenberg's (6) estimate that 70% of the methane comes from acetate is probably too high because the method he used to determine the

total rate of methane production underestimates the actual in situ rate. Cappenberg used a manometric method which involved placing 3-4 g wet sediment in a 50 ml Warburg vessel (6). I have used the equation of Flett et al. (13) and the Bunsen absorption coefficient for hydrogen at 10°C to calculate that, under Cappenberg's experimental conditions, 99.8% of the hydrogen initially dissolved in the sediment would have been transferred to the gas phase at equilibrium. Thus Cappenberg's estimate of the total methane production rate (0.034 $\mu mol \cdot g^{-1}$ wet $sediment \cdot h^{-1}$) (6) does not include conversion of dissolved hydrogen. Note that this same criticism applies to the results of Smith and Mah (26), who estimated that 70% of the methane produced in sludge came from acetate. For their experimental system I have calculated that 98.4% of the initial dissolved hydrogen was transferred to the gas phase.

Methane may not be the only fate of acetate in the sediment. Only 15% of the labeled acetate appeared as methane by the time labeled methane production ceased (Fig. 7). In other experiments (not shown) more of the label was converted to methane, 23% and 32%, but production also terminated at these values regardless of the length of incubation. Additional evidence for another fate of acetate comes from Winfrey et al. (29) in their study of Lake Mendota sediments. I have calculated that only 12% of the methyl labeled acetate that they added was converted to methane. Other possible acetate consuming organisms are the recently described sulfur-reducer, Desulfuromonas acetoxidans (21), and sulfate-reducer, Desulfotomaculum acetoxidans (28). However, only 6% of the labeled acetate I added was converted to carbon dioxide (Fig. 7), which still leaves nearly 80% to an unknown fate.

Hydrogen appears to stimulate the conversion of acetate to methane (Fig. 7), an observation also made by Winfrey et al. (29) for Lake Mendota sediments. However, the kinetic experiments (Fig. 8, Table 5) show that the preincubation period with 0.5% hydrogen caused a decrease in the acetate pool, as $K_m + S_n$, and a concomittant decrease in the acetate turnover time. The apparent stimulation in Fig. 7 is more likely due to a lower acetate pool and thus higher specific activity of the acetate than to an increase in the methanogenic conversion of acetate. This explanation fits with the hypothesis that interspecies hydrogen transfer occurs in the sediment since, in an uncoupled system, less oxidized substrate such as acetate is produced (32). Thus a decrease in the acetate pool would occur if the hydrogen that I added inhibited the heterotrophic production of acetate.

Further evidence that interspecies hydrogen transfer occurs in the lake sediment is that preincubation with hydrogen causes a 37% decrease in the amount of radioactive CO_2 produced from the heterotrophic metabolism of labeled valine. Under anaerobic conditions, valine is normally metabolized via the Stickland reaction to isobutyrate, with NAD^+ being reduced to $NADH^+$(1). The $NADH^+$ is oxidized back to NAD^+ by transferring the electrons to an electron accepting amino acid such as glycine or proline. The high hydrogen concentrations that I added to the sediment may have caused most of the NAD^+ to be in the reduced form and unable to accept electrons from valine, thus decreasing the production of $^{14}CO_2$. A second possibility is that the hydrogen had not effect on the Stickland reaction, but instead inhibited the further degradation of isobutyrate, the product of valine degradation via the Stickland reaction.

Cleland (9) has stated "... it is not practical to have the substrate which is an intermediate in a metabolic pathway present in levels much above its apparent Michaelis constant. Maintenance of a stable flow through a metabolic pathway is favored if each enzyme operates in the proportional region of its velocity versus concentration curve, so that a momentary rise in reactant concentration results in an increased reaction rate and tends to prevent further increase in concentration." (underline mine.) My experimental evidence shows that, for the anerobic sediment habitat, short term increases in hydrogen levels lead to increased reaction rates and thus prevention of further increase in dissolved hydrogen levels. However, for acetate, a short term increase in the pool size cannot cause a significant increase in the reaction rate since the rate is already near the maximum. Therefore, further increases in acetate concentration are not prevented.

LITERATURE CITED

1. Barker, H.A. 1961. Fermentations of nitrogenous organic compounds. In I.C. Gunsalus and R.Y. Stanior (eds.) The Bacteria. Vol. II: Metabolism. Academic Press, New York.

2. Bryant, M.P. 1976. The microbiology of anaerobic degradation and methanogenesis with special reference to sewage, p. 107-117. In H.L. Schlegel and J. Barnea (eds.) Microbial energy conversion. Goltze, Gottingen.

3. Bryant, M.P., L.L. Campbell, C.A. Reddy, and M.R. Crabill. 1977. Growth of desulforibrio in lactate or ethanol media low in sulfate and in association with H_2 - utilizing methanogenic bacteria. Appl. Environ. Microbiol. 33:1162-1169.

4. Cappenberg, T.E. 1974a. Interrelationships between sulfate-reducing and methane-producing bacteria in bottom deposits of a freshwater lake. I. Field observations. Antonie van Leeuwenhoek J. Microbiol. Serology 40:285-295.

5. Cappenberg, T.E. 1974b. Interrelationships between sulfate-reducing and methane-producing bacteria in bottom deposits of a freshwater lake. II. Inhibition experiments. Antonie van Leeuwenhoek J. Microbiol. Serol. 40:297-306.

6. Cappenberg, T.E. and R.A. Prins. 1974c. Interrelationships between sulfate-reducing and methane-producing bacteria in bottom deposits of a freshwater lake. III. Experiments with ^{14}C labeled substrates. Antonie van Leeuwenhoek J. Microbiol. Serol 40:457-469.

7. Chung, K.-T. 1976. Inhibitory effects of H_2 on growth of Clostridium cellobioparum. Appl. Environ. Microbiol. 31:342-348.

8. Chynoweth, D.P. and R.A. Mah. 1971. Volatile acid formation in sludge digestion. p. 41-54. In Anaerobic biological treatment processes. Adv. Chem. Ser. 105. F.G. Pohland (ed) American Chemical Society, Washington.

9. Cleland, W.W. 1967. Enzyme kinetics. Ann. Rev. Biochem. 36:77-112.

10. Cornish-Bowden, A., and R. Eisenthal. 1974. Statistical considerations in the estimation of enzyme kinetic parameters by direct linear plot and other methods. Biochem J. 139:721-730.

11. Doelle, H.W. 1969. Bacterial metabolism. Academic Press, New York.

12. Eisenthal, R., and A. Cornish-Bowden. 1974. The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. Biochem J. 139:715-720.

13. Flett, R.J., R.D. Hamilton, and N.E.R. Campbell. 1976. Aquatic acetylene-reduction techniques: Solutions to several problems. Can. J. Microbiol. 22:43-51.

14. Garrels, R.M., and C.L. Christ. 1965. Solutions, minerals and equilibria. Harper and Row. New York.

15. Hungate, R.E. 1967. Hydrogen as an intermediate in the rumen fermentation. Arch. Microbiol. 59:158-164.

16. Hungate, R.E., W. Smith, T. Bauchop, I.Yu, and J.C. Rabinowitz. 1970. Formate as an intermediate in the bovine rumen fermentation. J. Bacteriol. 102:389-397.

17. Hobbie, J.E., and C.C. Crawford. 1969. Respiration corrections for bacterial uptake of dissolved organic compounds in natural waters. Limnol. oceanogr. 14:528-532.

18. Ianotti, E.L., D. Kafkewitz, M.J. Wolin and M.P. Bryant. Glucose fermentation products of Ruminococcus albus grown in continuous culture with Vibrio succinogenes: Changes caused by interspecies transfer at H_2. J. Bacteriol. 114:1231-1240.

19. Molongoski, J.J. and M.J. Klug. 1976. Characterization of anaerobic heterotrophic bacteria isolated from freshwater lake sediments. Appl. Environ. Microbiol. 31:83-90.

20. Ohwaki, K. and R.E. Hungate. 1977. Hydrogen utilization by clostridia in sewage sludge. Appl. Environ. Microbiol. 33:1270-1274.

21. Pfenning, N., and H. Biebl. 1976. Desulfuromonas acetoxidans gen. nov. and sp. nov., a new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Arch. Microbiol. 110:3-12.

22. Pretorius, W.A. 1972. The effect of formate on the growth of acetate utilizing methanogenic bacteria. Water Research. 6:1213-1217.

23. Reddy, C.A., M.P. Bryant, and M.J. Wolin. 1972. Characteristics of S organism isolated from Methanobacillus omelianskii. J. Bacteriol. 109:539-545.

24. Scheifinger, C.C., B. Linehan, and M.J. Wolin. 1975. H_2 production by Selenomonas ruminantium in the absence and presence of methanogenic bacteria. Appl. Microbiol. 29:480-483.

25. Segel, I.H. 1968. Biochemical calculations. Wiley, New York.

26. Smith, P.H. and R.A. Mah. 1966. Kinetics of acetate metabolism during sludge digestion. Appl. Microbiol. 14:368-371.

27. Weimer, P.J. and J.G. Zeikus, 1977. Fermentation of cellulose and cellobiose by Clostridium thermocellum in the absence and presence of Methanobacterium thermoautotrophicum. Appl. Environ. Microbiol. 33:289-297.

28. Widdel, F., and N. Pfennig. 1977. A new anaerobic, sporing, acetate-oxidizing, sulfate-reducing bacterium, Desulfotomaculum (emend.) acetoxidans. Arch. Microbiol. 112:119122.

29. Winfrey, M.R., D.R. Nelson, S.C. Klevickis, and J.G. Zeikus. 1977. Association of hydrogen metabolism with methanogenesis in Lake Mendota sediments. Appl. Environ. Microbiol. 33:312-318.

30. Winfrey, M.R. and J.G. Zeikus. 1977. Effect of sulfate on carbon and electron flow during microbial methanogenesis in freshwater sediments. 33:275-281.

31. Wolfe, R.S. 1971. Microbial formation of methane. Adv. Microbial Physiol. 6:107-145.

32. Wolin, M.J. 1974. Metabolic interactions among intestinal microorganisms. Am. J. Clin. Nut. 27:1320-1328.

33. Wright, R.T. and J.E. Hobbie. 1966. Use of glucose and acetate by bacteria and algae on aquatic ecosystems. Ecology. 47:447-464.

34. Zeikus, J.G., and M.R. Winfrey. 1976. Temperature limitation of methanogenesis in aquatic sediments. Appl. Environ. Microbiol. 31:99-107.