

Kinetics of Butyrate, Acetate, and Hydrogen Metabolism in a Thermophilic, Anaerobic, Butyrate-Degrading Triculture

BIRGITTE K. AHRING†* AND PETER WESTERMANN

Department of General Microbiology, University of Copenhagen, Sølvgade 83 H, 1307 Copenhagen K, Denmark

Received 22 July 1986/Accepted 17 November 1986

Kinetics of butyrate, acetate, and hydrogen metabolism were determined with butyrate-limited, chemostat-grown tricultures of a thermophilic butyrate-utilizing bacterium together with *Methanobacterium thermoautotrophicum* and the TAM organism, a thermophilic acetate-utilizing methanogenic rod. Kinetic parameters were determined from progress curves fitted to the integrated form of the Michaelis-Menten equation. The apparent half-saturation constants, K_m , for butyrate, acetate, and dissolved hydrogen were 76 μM , 0.4 mM, and 8.5 μM , respectively. Butyrate and hydrogen were metabolized to a concentration of less than 1 μM , whereas acetate uptake usually ceased at a concentration of 25 to 75 μM , indicating a threshold level for acetate uptake. No significant differences in K_m values for butyrate degradation were found between chemostat- and batch-grown tricultures, although the maximum growth rate was somewhat higher in the batch cultures in which the medium was supplemented with yeast extract. Acetate utilization was found to be the rate-limiting reaction for complete degradation of butyrate to methane and carbon dioxide in continuous culture. Increasing the dilution rate resulted in a gradual accumulation of acetate. The results explain the low concentrations of butyrate and hydrogen normally found during anaerobic digestion and the observation that acetate is the first volatile fatty acid to accumulate upon a decrease in retention time or increase in organic loading of a digester.

Volatile fatty acids are important intermediates in the anaerobic conversion of organic matter to methane and carbon dioxide. Normally, propionate and butyrate account for 20% of the total methane produced in a digester (20). Failure of the anaerobic digestion process is usually indicated by a rapid increase in the concentration of volatile fatty acids with a concurrent decrease in methane production (1, 8, 20).

The degradation of propionate, butyrate, and longer-chained volatile fatty acids involves two groups of bacteria, the obligately hydrogen-producing acetogenic bacteria oxidizing the acids and the methane-producing bacteria utilizing the acetate and hydrogen produced (6, 9, 21-23). Owing to the unfavorable thermodynamics of fatty acid oxidation under standard conditions, the metabolism of the acetogenic bacteria demands a low partial pressure of hydrogen normally maintained by the hydrogen-utilizing methanogenic bacteria (8). The concentration of acetate and, hence, the activity of the aceticlastic methanogenic bacteria, have also been shown to influence the degradation of fatty acids, although relatively high concentrations of acetate (more than 80 mM) are needed to cause total inhibition (17).

Despite the importance of fatty acid degradation for methane fermentation, only a few studies have been carried out to elucidate the kinetics of these reactions. Lawrence and McCarty (19) obtained a K_m of 57 μM and a μ_{max} of 0.015 h^{-1} for butyrate and a K_m of 432 μM and a μ_{max} of 0.017 h^{-1} for propionate in experiments with mesophilic enrichment cultures metabolizing either butyrate or propionate. Heyes and Hall (13) examined the kinetics of propionate in mesophilic sludge digestors operated at different pHs and retention times and found that at least two subgroups, with distinctly

different growth kinetics, were present. The slower-growing subgroup had a K_m of 149 μM and a μ_{max} of 0.0054 h^{-1} , while the values for the faster-growing subgroup were 4.5 mM and 0.05 h^{-1} .

The present work was designed to allow us to study the kinetics of thermophilic butyrate metabolism. The choice of a triculture containing both a hydrogen-oxidizing and an aceticlastic methanogen together with the butyrate-degrading bacterium enabled us to examine the kinetics of all three catabolic reactions active during complete anaerobic degradation of butyrate. The determination of K_m for the conversion of hydrogen to methane in the triculture was of especial interest, since a high affinity for hydrogen, resulting in a low hydrogen partial pressure, is essential to the concept of interspecies hydrogen transfer. Schönheit et al. (26) determined the apparent K_m of hydrogen in the gas phase of a growing culture of *Methanobacterium thermoautotrophicum* to be approximately 20%. Assuming that the actual concentration of dissolved hydrogen was in equilibrium with the gas phase in these experiments, the K_m value of dissolved hydrogen (approximately 108 μM) is considerably higher than K_m values determined for pure cultures of mesophilic methanogens (1 to 10 μM) (18, 33). This could indicate that the concentration of dissolved hydrogen in cocultures containing *M. thermoautotrophicum* as the hydrogen-consuming bacterium could be too high to allow interspecies hydrogen transfer to occur.

MATERIALS AND METHODS

Source of organisms. The butyrate-degrading triculture was isolated from a stable butyrate enrichment culture initially inoculated with anaerobic sludge from a thermophilic (60°C) bench-scale digester as previously described (4).

Media and conditions for cultivation. Strictly anaerobic conditions were maintained throughout the handling of cell

* Corresponding author.

† Present address: Institute of Biotechnology, The Technical University of Denmark, 2800 Lyngby, Denmark.

cultures in all experiments. The anaerobic techniques used were essentially as described by Hungate (14) and modified by Bryant (7) and Balch and Wolfe (5).

The medium used for continuous cultivation of the triculture was the sulfate-free mineral medium used for enrichment and isolation of the thermophilic butyrate-degrading triculture containing butyrate as sole energy and carbon source (4). Unless stated otherwise, the butyrate concentration was 10 mM, added as sodium butyrate. Experiments with batch cultures were performed by using serum vials (50 ml, with 25 ml of medium) closed with butyl rubber stoppers and aluminum crimps, and the medium was supplemented with 0.1% yeast extract. The gas phase in the vials was 80% N₂-20% CO₂ pressurized to 1 atm (101.3 kPa) overpressure. All incubations were performed at 60°C, and experiments were run at least in triplicate.

Culture purity. Cultures were routinely checked for purity by microscopic examination and inoculation into thioglycolate medium (Difco Laboratories) and AC medium (Difco) inoculated under anaerobic and aerobic conditions which allowed no growth of the triculture.

Chemostat cultures. Continuous cultures were conducted in an all-glass growth vessel (Corning, U.K.) with a working volume of 1.0 liter. The fermentor was operated as a continuously stirred tank with no recycle, and the butyrate mineral medium was continuously fed corresponding to a dilution rate (*D*) of 0.01 h⁻¹. The outlet was through an overflow weir and an inverted siphon, and all tubing was butyl rubber. The pH was maintained at 7.2 by automatic titration with 1 M HCl. The total system was kept under a slight overpressure with a 80% N₂-20% CO₂ gas mixture.

The criteria for steady-state conditions in the chemostat were that all parameters must be held constant for at least five residence times and that no changes must be observed in the concentrations of either butyrate, acetate, or hydrogen.

Determination of kinetic constants. An integrated solution to the Michaelis-Menten equation (below) was used to determine kinetic constants from progress curves of butyrate-, acetate-, and hydrogenutilization (27), as follows:

$$\frac{\ln S_0/S_t}{t} = \left(\frac{-1}{K_m} \times \frac{S_0 - S_t}{t} \right) + \frac{V_{\max}}{K_m}$$

In this equation, *S*₀ is the initial substrate concentration, *S*_{*t*} is the substrate concentration at time *t*, *V*_{max} is the maximum initial velocity that can be attained, and *K*_{*m*} is the half-saturation constant.

In experiments with either butyrate or acetate added, the chemostat was allowed to reach steady state and then was given a slug of neutralized butyrate or acetate.

To avoid problems of phase transfer limitation between the gas phase and the liquid phase, hydrogen consumption was measured with continuously grown cultures transferred to sterile serum vials (10 ml of cultures of 50-ml serum vials). The vials were preincubated for 1 h under an atmosphere of 80% N₂-20% CO₂ and then given the appropriate hydrogen concentration in the gas phase by mixing 80% N₂-20% CO₂ with 5% H₂-75% N₂-20% CO₂, and the serum vials were pressurized to 1.5 atm (152 kPa) overpressure. Maximum contact between the culture and the gas phase was obtained by incubating the vials in a horizontal position in a reciprocating water bath.

The consumption rates of butyrate, acetate, and hydrogen with time were measured, and the experimental periods were kept within a few hours to eliminate interference from cell multiplication during the experiments.

A Lineweaver-Burke analysis was also used for determination of kinetic parameters. The metabolic rates of butyrate and acetate utilization were determined by daily measurements in vials with different initial concentrations of either butyrate or acetate inoculated with the triculture (1%, vol/vol). For determination of hydrogen kinetics, the chemostat cultures were incubated with different concentrations of hydrogen in the headspace gas as described above. The hydrogen concentrations in the vials were measured at approximately 10-min intervals, and the incubation time was kept low (less than 1 h) to ensure that the initial hydrogen concentration had not decreased significantly. The *V*_{max} values were calculated by using the growth yield calculated for growth of the triculture in chemostat culture.

Cell yields, enumeration of cells, and growth rates. Cell dry weight was determined by filtering 10- to 20-ml culture samples through preweighed membrane filters (pore size, 0.22 μm; Millipore Corp.) and drying them to a constant weight at 95°C. The increase in weight was taken as the dry weight.

Cell numbers of the three bacteria in the triculture during continuous cultivation were determined by using a counting chamber after appropriate dilution of the original sample. To estimate the biomass of the individual species, the numbers of *M. thermoautotrophicum* and the TAM organism found in the triculture were compared with a standard curve giving the correlation between the concentration of cells and the biomass obtained from pure-culture studies with the two bacteria. The biomass of the butyrate-degrading bacteria was then calculated as the difference between the cell dry weight of the triculture and the sum of the dry weight of *M. thermoautotrophicum* and the TAM organism.

Growth in batch cultures was monitored by measuring the specific butyrate consumption rate, which was calculated from the linear part of a semilogarithmic graph of butyrate concentration versus time.

Analytic procedures. Butyrate and acetate were detected by using a gas chromatograph with a flame ionization detector as previously described (1, 2). When the concentrations of butyrate and acetate in the cultures were less than 0.2 and 0.5 mM, respectively, the samples were concentrated prior to analysis. A 1.5- to 5-ml portion of the triculture was pressure-filtered through a 0.22-μm-pore-size membrane filter, and the pH was raised to 11 with KOH. The samples were then dried at 95°C and suspended in 0.1 to 0.5 ml of 2% H₃PO₄. Samples of 2 μl were injected into the gas chromatograph. The concentration of each compound was determined by comparing the obtained values with those of standards carried through the same concentration procedures.

Hydrogen was analyzed by using a gas chromatograph equipped with a thermal conductivity detector (4). Dissolved hydrogen in the triculture was determined after extraction and concentration by the method described by Robinson et al. (24). A correlation factor of 0.0133 between headspace and liquid phase was obtained by incubating 10 ml of sterile butyrate medium with different concentrations of hydrogen in the gas phase pressurized to 1.5 atm overpressure in a horizontal position in a reciprocating water bath as described for the experiments on hydrogen kinetics. The concentrations of dissolved hydrogen in the vials were determined after an incubation time of 1 h and were correlated to measurements of the concentrations in the gas phases.

All gas injections were carried out with Pressure-lok gastight syringes (Precision Sampling Corp.).

Gases and chemicals. High-purity gases were used throughout the experiments, and any traces of oxygen were

TABLE 1. Number, cell dry weight, and growth yield of the different metabolic groups of bacteria present in the thermophilic butyrate-degrading triculture

Metabolic group	No. of organisms/ml of triculture ^a	Cell dry wt/liter of triculture (g) ^b	Molar growth yield (g mol ⁻¹) ^c
<i>M. thermoautotrophicum</i>	9.4×10^{-7}	0.041	2.05
TAM organism	1.2×10^{-7}	0.0305	1.5
Butyrate degrader	2.1×10^{-7}	0.031	3.1
Coculture	1.2×10^{-8}	0.1025	10.25

^a Average of three enumerations.

^b Estimated from microscopic counts.

^c Expressed as the cell dry weight per mole of the respective substrate (hydrogen, acetate, and butyrate) utilized, assuming that 1 mol of butyrate yields 2 mol of acetate plus 2 mol of hydrogen.

removed by passing the gases through a heated copper column (300°C). All chemicals were reagent grade.

RESULTS

No attachment of the bacteria in the triculture to each other in discrete clumps or to the walls of the growth vessel was observed during continuous cultivation of the triculture for more than 1 year. The low dilution rate was necessary to obtain balanced growth among all three organisms, because an increase in dilution rate caused acetate and then butyrate to gradually accumulate when the dilution rate exceeded 0.02 h^{-1} .

Cell dry weights, doubling times, and enumeration. The numbers and dry weights of the three bacteria during continuous cultivation of the triculture are shown in Table 1. *M. thermoautotrophicum* was found to be the most numerous bacterium in the triculture, averaging 74% of the total bacteria. The population of butyrate-degrading bacteria was nearly twice as high as that of the TAM organism. Addition of hydrogen to the triculture resulted only in the growth of *M. thermoautotrophicum*, and enrichment cultures on hydrogen made from the triculture always contained *M. thermoautotrophicum* as the only bacterium present, indicating that this bacterium was the principal hydrogen-oxidizing bacterium in the triculture. The TAM organism utilizes hydrogen as well as acetate (2), but this is probably not important when the bacterium is cultivated with *M. thermoautotrophicum*.

Exact estimation of the cell mass of the three bacteria present during chemostat cultivation of the triculture was difficult because no separation of the bacteria could be obtained by differential centrifugation. The methods of biomass estimations with pure-culture studies of *M. thermoautotrophicum* and the TAM organism lead to problems of relating values found for different growth conditions. When the molar growth yields estimated for *M. thermoautotrophicum* and the TAM organism in the triculture (2.05 and 1.5 g/mol, respectively) (Table 1) were compared with values obtained with pure cultures of the two bacteria grown in batch cultures on 20 mM substrate (1.6 and 1.7 g/mol, respectively), the estimates seem reasonable.

A maximal growth rate of 0.032 h^{-1} , corresponding to a doubling time of 21.7 h, was found for growth in batch culture.

Measurements of dissolved hydrogen. Only low concentrations of hydrogen (less than 10^{-3} atm [101.3 Pa]) could be detected in the headspace during continuous and batch cultivation of the triculture. A concentration of $0.8 \mu\text{M}$ ($1.8 \times 10^{-5} \text{ atm}$ [1.82 Pa]) dissolved hydrogen was measured during continuous growth of the triculture.

TABLE 2. Kinetic parameters for the metabolism of butyrate, acetate, and hydrogen by the thermophilic butyrate-degrading triculture

Substrate	K_m (μM)	V_{\max} ($\text{mmol}^{-1} \text{ liter}^{-1} \text{ h}^{-1}$) ^a	Culture conditions (kinetics used)
Butyrate	76.4 ± 10.2	0.20 ± 0.02 (6.3)	BLC ^b (progress curve)
	65.2 ± 7.5	0.32 ± 0.02 (10.3)	Batch cultures (Lineweaver-Burke plot)
Acetate	399 ± 45.2	0.33 ± 0.05 (10.8)	BLC (progress curve)
	485 ± 20.2	0.22 ± 0.04 (7.1)	Batch cultures (Lineweaver-Burke plot)
Hydrogen	8.5 ± 0.6	5.4 ± 0.2 (132)	BLC (progress curve)
	6.8 ± 0.5	5.8 ± 0.2 (141)	BLC (Lineweaver-Burke plot)

^a Means of three independent determinations \pm standard deviations. Values in parentheses are expressed in millimoles per hour per gram (dry weight).

^b BLC, Long-term butyrate-limited chemostat culture.

Kinetics of butyrate, acetate, and hydrogen utilization. At the steady state, the triculture produced only methane and carbon dioxide, with hydrogen and acetate as intermediates.

Substrate kinetic parameters (K_m and V_{\max}) were determined with substrate-limited chemostat cultures at the steady state by measuring the consumption of butyrate, acetate, or hydrogen after a slug addition (approximately 400 μM butyrate, 1 mM acetate, and 15 μM dissolved hydrogen). Michaelis-Menten-type saturation kinetics were found for the consumption of all three substrates. A summary of the kinetic data obtained is presented in Table 2, together with the methods used to determine these parameters.

Figure 1 shows a representative progress curve of butyrate consumption by the triculture. The rate of consumption was concentration dependent below a concentration of approximately 100 μM . The calculated K_m was 85.2 μM butyrate, and the V_{\max} was 192.2 $\mu\text{M h}^{-1}$ ($6.2 \text{ mmol h}^{-1} \text{ g}$ [dry

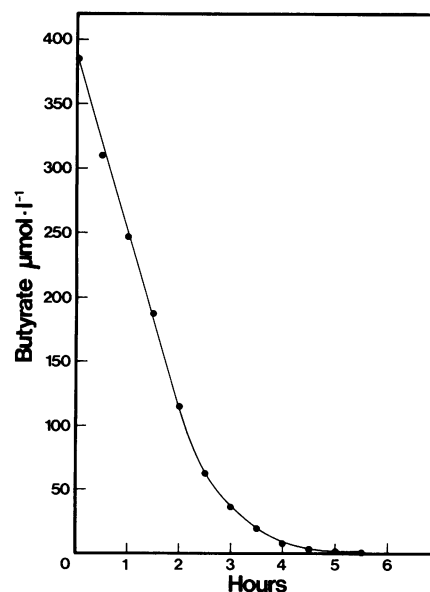


FIG. 1. Progress curve of butyrate degradation by the triculture with time.

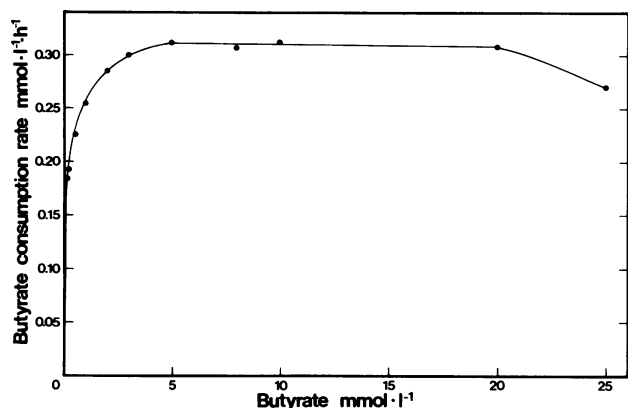


FIG. 2. Effects of increasing butyrate concentrations on the rate of butyrate consumption by the triculture. The results are means of triplicate determinations.

weight] $^{-1}$). Figure 2 shows the specific rates of butyrate degradation at different initial concentrations of butyrate from experiments with batch-grown tricultures. The specific degradation rate was maximal at approximately 3 mM butyrate and declined when the concentration was higher than 20 mM. No significant difference in K_m for butyrate degradation was found between batch- and chemostat-grown cells (Table 2). The maximum degradation rate, however, was somewhat higher for the batch-grown cells, primarily owing to the presence of yeast extract in the medium, which was found to enhance the specific rate of butyrate degradation (4).

A typical progress curve of acetate utilization is shown in Fig. 3; K_m and V_{max} were 0.39 mM and 0.34 mM h $^{-1}$ (10.81 mmol h $^{-1}$ g [dry weight] $^{-1}$), respectively. When the concentration of acetate was reduced to less than 0.5 to 0.7 mM, the degradation rate was slow when the concentration was less than 0.15 mM. Acetate consumption usually ceased when the concentration was approximately 10 to 20 times lower than

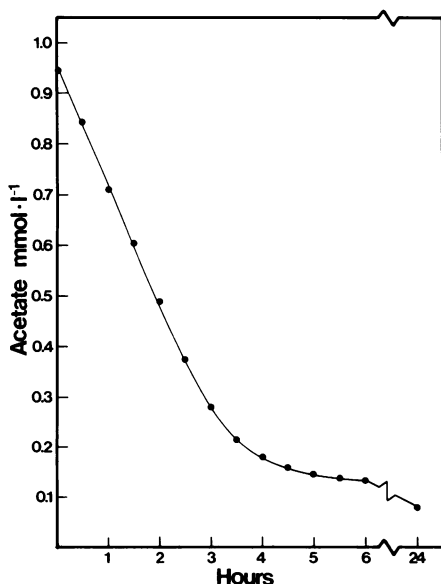


FIG. 3. Progress curve of acetate utilization by the triculture with time.

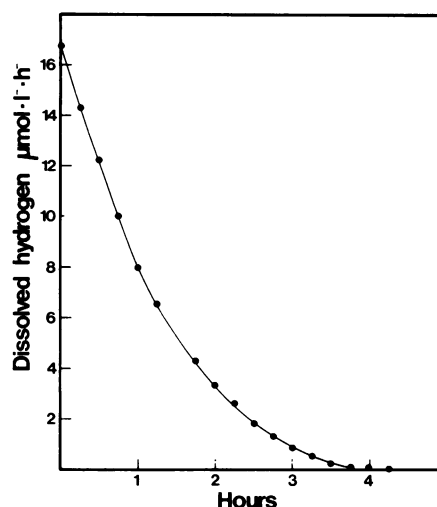


FIG. 4. Time course of aqueous-phase consumption of hydrogen by the triculture, calculated from gaseous-phase data.

the apparent K_m for acetate uptake (25 to 75 μ M acetate). The same threshold level for acetate uptake was found if the cultures were respiked with acetate. Energetically, acetate is a poor substrate (33); this might result in the energy required for acetate uptake at very low concentrations exceeding the energy gained from acetate metabolism, thereby limiting the acetate uptake at a certain threshold concentration. The K_m for acetate, obtained from batch-culture measurements of the specific acetate consumption rates of the triculture at different initial concentrations of acetate, showed no deviation from the values obtained from progress curves (Table 2) (data not shown). V_{max} , however, was somewhat lower than that found for pure cultures of the TAM organism.

A representative progress curve of hydrogen consumption is shown in Fig. 4, measured as the changes in the concentration of hydrogen in the headspaces of serum vials containing the coculture. The K_m was 1.52×10^{-2} atm (1.54 kPa) of hydrogen in the gas phase (9.0 μ M dissolved hydrogen), and the V_{max} was 5.4 mM hydrogen consumed h $^{-1}$ (0.13 mol h $^{-1}$ g [dry weight] $^{-1}$). The effect of increasing the concentration of hydrogen in the headspace gas on the rate of hydrogen consumption by the triculture is shown in Fig. 5; a K_m of 1.1×10^{-2} atm (1.11 kPa) of hydrogen (6.5 μ M dissolved hydrogen) and a V_{max} of 5.8 mM h $^{-1}$ (0.14 mol h $^{-1}$ g [dry weight] $^{-1}$) was found in this experiment.

DISCUSSION

Little is known about the bacteria involved in the utilization of fatty acids during the mineralization of organic matter to methane. The only thermophilic hydrogen-producing acetogenic bacteria previously described are butyrate-utilizing bacteria isolated with *M. thermoautotrophicum* as the principal hydrogen-utilizing bacterium (4, 12).

The present work is the first kinetic study of a defined culture degrading fatty acids. The half-saturation constant of butyrate utilization (76 μ M) is in good agreement with that obtained by Lawrence and McCarty (19) for mesophilic enrichment cultures on butyrate (57 μ M). The maximum specific growth, μ_{max} , of the thermophilic triculture (0.032 h $^{-1}$) was somewhat higher than a μ_{max} of 0.015 h $^{-1}$ reported for the mesophilic enrichment culture and much higher than the measured μ_{max} of 0.00825 h $^{-1}$ for the growth of

Syntrophomonas wolfei in coculture with *Methanospirillum hungatei*. It is, however, difficult to compare a triculture grown under conditions of both hydrogen and acetate elimination with a coculture in which acetate accumulates. Henson and Smith (12) reported that their thermophilic butyrate enrichments were more stable when the acetate was utilized, and results obtained from our studies on thermophilic butyrate degradation show that butyrate is utilized at a higher rate in the triculture containing the acetate-utilizing methanogen than is the coculture in which only *M. thermoautotrophicum* was present (4).

The K_m found for acetate utilization in the triculture (0.4 mM) was in the same order of magnitude as results obtained with pure cultures of the TAM organism (0.8 mM) (3) and similar to values obtained by Zinder et al. (34) for a thermophilic sludge digester with a thermophilic *Methanotherix* sp. as the dominant aceticlastic methanogen (0.3 mM). *Methanotherix soehngenii* was also reported to have a low K_m value (0.7 mM) (15), while both mesophilic and thermophilic cultures of *Methanosarcina* spp. have relatively high K_m values, ranging from 3 to 5 mM (33, 35). The degradation of acetate was found to be the rate-limiting process for complete degradation of butyrate by the triculture. The same conclusions were drawn by Kaspar and Wuhrmann (16) when they examined the kinetics of propionate, hydrogen, and acetate utilization in digesting sludge. A mean generation time of 34.7 h was calculated from the dilution rate of 0.02 h^{-1} causing acetate metabolism to cease. This value is much lower than the generation time of 60 h reported for pure cultures of the TAM organism and indicates that faster growth of this methanogen can be obtained by cultivating it with other bacteria.

The concentration of dissolved hydrogen measured in the coculture ($0.8 \mu\text{M}$) is in good agreement with a measured hydrogen concentration of about $1 \mu\text{M}$ in rumen and freshwater sediments (25, 31, 33) and less than 10^{-4} atm (10.13 Pa) hydrogen partial pressure in sewage sludge digester gas (16). This hydrogen concentration is sufficiently low to make fatty acid oxidation thermodynamically feasible (9, 21). As ex-

pected from the measured concentration of hydrogen in the triculture, the K_m values for hydrogen found in the present experiments (7 to $9 \mu\text{M}$) are of the same order of magnitude as the values found for pure cultures of mesophilic methanogens (1 to $6 \mu\text{M}$), freshwater sediments (3 to $8 \mu\text{M}$), rumen fluid (4 to $9 \mu\text{M}$), and digested sludge (4 to $7 \mu\text{M}$) (18, 25, 31, 33). On the other hand, the hydrogen concentration is considerably lower (more than 10 times) than the K_m value of 20% hydrogen in the gas phase determined for *M. thermoautotrophicum* by Schönheit et al. (26). The main reason for this great difference could be explained by phase transfer limitation under conditions of rapid utilization of hydrogen by the growing culture in the experiments with *M. thermoautotrophicum*, as also indicated by Kristjansson et al. (18). In our experiments, the hydrogen-utilizing methanogenic population was seriously restricted by the availability of hydrogen released during growth of the triculture in a chemostat with a long retention time and a low substrate concentration. This means that the rate of interface transfer between gas and liquid was rapid enough to supply the biological demand for hydrogen in the vials used for our kinetic studies, as reflected in a Michaelis-Menten saturation kinetics for hydrogen consumption. Another reason could be that *M. thermoautotrophicum* responds to substrate-limited growth in a chemostat by increasing its specific substrate uptake capacity (V_{\max}/K_m), as shown for other bacteria (11, 23). The maximum rate of hydrogen consumption found in the present experiments ($0.13 \text{ mol h}^{-1} \text{ g} [\text{dry weight}]^{-1}$) is in agreement with values reported for *Methanobrevibacter arboriphilus* but lower than the value of $0.43 \text{ mol h}^{-1} \text{ g} [\text{dry weight}]^{-1}$ estimated for *M. thermoautotrophicum* (33). This difference, however, could be caused by the suboptimal growth conditions for *M. thermoautotrophicum* found in our experiments.

Recently, Conrad et al. (10) hypothesized a juxtapositioning of the hydrogen-producing acetogenic bacteria and the hydrogen-consuming methanogens within flocs or consortia in sludge and sediments. In this manner, most of the hydrogen produced will not equilibrate with the common pool of hydrogen but will be directly consumed by the adjacent methanogens before it diffuses out of the floc matrix. Tomei et al. (32) described two mesophilic enrichment cultures of endosporeforming bacteria degrading butyrate in consortia with methanogens. One of the cocultures grew as an optically dense culture, while the other grew in clumps in an otherwise clear culture. During growth of our thermophilic butyrate-degrading triculture, no attachment of the bacteria to each other could be observed, and the bacteria were evenly dispersed in the culture medium when viewed under a microscope. The reason for the even distribution could be that none of the bacteria in our triculture were able to produce an extracellular polymer necessary for the forming of flocs or clumps or that the production of this extracellular polymer was suppressed because the hydrogen concentration of the triculture was low enough to allow butyrate degradation to proceed.

To ensure viability of the butyrate-degrading bacterium during batch cultivation, the triculture had to be transferred when the growth of this bacterium was within the exponential phase. Batch cultivation of the triculture by repeated transfers of inocula, therefore, rapidly resulted in problems in keeping the acetate-utilizing methanogen within the triculture as a consequence of the slower growth rate of the acetate-utilizing bacterium compared with that of the hydrogen- and the butyrate-oxidizing bacteria. The continuous-culture technique used in this study has proven to be a

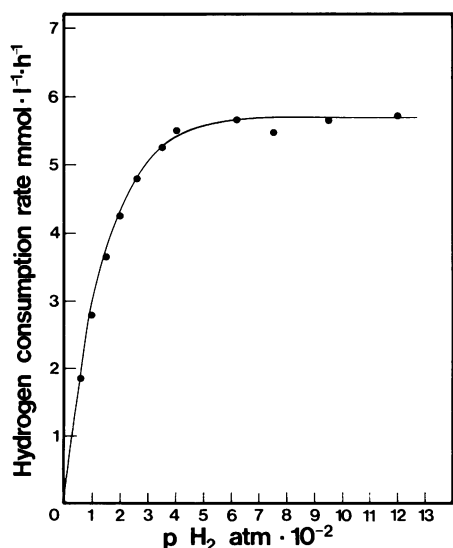


FIG. 5. Effect of increasing hydrogen concentrations on the rate of hydrogen consumption by the triculture. The results are means of triplicate determinations.

valuable device for kinetic studies during balanced growth of a defined mixed culture.

ACKNOWLEDGMENTS

We thank Karin Vestberg for excellent technical assistance.

This work was supported by grants no. 11-3888, 11-3982, and 11-5080 from the Danish Natural Science Research Council.

LITERATURE CITED

- Ahring, B. K., and P. Westermann. 1983. Toxicity of heavy metals to thermophilic anaerobic digestion. *Eur. J. Appl. Microbiol. Biotechnol.* **17**:365-370.
- Ahring, B. K., and P. Westermann. 1984. Isolation and characterization of a thermophilic, acetate-utilizing methanogenic bacterium. *FEMS Microbiol. Lett.* **25**:47-52.
- Ahring, B. K., and P. Westermann. 1985. Methanogenesis from acetate: physiology of a thermophilic, acetate-utilizing methanogenic bacterium. *FEMS Microbiol. Lett.* **28**:15-19.
- Ahring, B. K., and P. Westermann. 1987. Thermophilic anaerobic degradation of butyrate by a butyrate-utilizing bacterium in coculture with methanogens. *Appl. Environ. Microbiol.* **53**:429-433.
- Balch, W. E., and R. S. Wolfe. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethane-sulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* **32**:781-791.
- Boone, D. R., and M. P. Bryant. 1980. Propionate-degrading bacterium, *Syntrophobacter wolii* sp. nov. gen. nov., from methanogenic ecosystems. *Appl. Environ. Microbiol.* **40**:626-632.
- Bryant, M. P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. *Am. J. Clin. Nutr.* **25**:1324-1328.
- Bryant, M. P. 1979. Microbial methane production — theoretical aspects. *J. Anim. Sci.* **48**:193-201.
- Bryant, M. P., L. L. Campwell, C. A. Reddy, and M. R. Crabill. 1977. Growth of *Desulfovibrio* in lactate or ethanol media low in sulfate in association with H₂-utilizing methanogenic bacteria. *Appl. Environ. Microbiol.* **33**:1162-1169.
- Conrad, R., T. J. Phelps, and J. G. Zeikus. 1985. Gas metabolism evidence in support of the juxtaposition of hydrogen-producing and methanogenic bacteria in sewage sludge and lake sediments. *Appl. Environ. Microbiol.* **50**:595-601.
- Harder, W., and L. Dijkhuizen. 1982. Strategies of mixed substrate utilization in microorganisms. *Phil. Trans. R. Soc. Lond. Ser. B* **297**:459-480.
- Henson, J. M., and P. H. Smith. 1985. Isolation of a butyrate-utilizing bacterium in coculture with *Methanobacterium thermoautotrophicum* from a thermophilic digester. *Appl. Environ. Microbiol.* **49**:1461-1466.
- Heyes, R. H., and R. J. Hall. 1983. Kinetics of two subgroups of propionate-using organisms in anaerobic digestion. *Appl. Environ. Microbiol.* **46**:710-715.
- Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. *Bacteriol. Rev.* **14**:1-49.
- Huser, B. A., K. Wuhrmann, and A. J. B. Zehnder. 1982. *Methanotrix soehngenii* gen. nov. sp. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. *Arch. Microbiol.* **132**:1-9.
- Kaspar, H. F., and K. Wuhrmann. 1978. Kinetic parameters and relative turnovers of some important catabolic reactions in digesting sludge. *Appl. Environ. Microbiol.* **36**:1-7.
- Kaspar, H. F., and K. Wuhrmann. 1978. Product inhibition in sludge digestion. *Microb. Ecol.* **4**:241-248.
- Kristjansson, J. K., P. Schönheit, and R. K. Thauer. 1982. Different K_s values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: an explanation for the apparent inhibition of methanogenesis by sulfate. *Arch. Microbiol.* **131**:278-282.
- Lawrence, A. W., and P. L. McCarty. 1969. Kinetics of methane fermentation in anaerobic treatment. *J. Water Pollut. Control. Fed.* **41**:R1-R17.
- Mackie, R. I., and M. P. Bryant. 1981. Metabolic activity of fatty acid-oxidizing bacteria and the contribution of acetate, propionate, butyrate, and CO₂ to methanogenesis in cattle waste at 40 and 60°C. *Appl. Environ. Microbiol.* **41**:1363-1373.
- McInerney, M. J., and M. P. Bryant. 1981. Anaerobic degradation of lactate by syntrophic association of *Methanosarcina barkeri* and *Desulfovibrio* species and effect of H₂ on acetate degradation. *Appl. Environ. Microbiol.* **41**:346-354.
- McInerney, M. J., M. P. Bryant, R. B. Hespell, and J. W. Costerton. 1981. *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. *Appl. Environ. Microbiol.* **41**:1029-1039.
- McInerney, M. J., M. P. Bryant, and N. Pfennig. 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. *Arch. Microbiol.* **122**:129-135.
- Robinson, J. A., R. F. Strayer, and J. M. Tiedje. 1981. Method for measuring dissolved hydrogen in anaerobic ecosystems: application to the rumen. *Appl. Environ. Microbiol.* **41**:545-548.
- Robinson, J. A., and J. M. Tiedje. 1982. Kinetics of hydrogen consumption by rumen fluid, anaerobic digester sludge, and sediment. *Appl. Environ. Microbiol.* **44**:1374-1384.
- Schönheit, P., J. Moll, and R. K. Thauer. 1980. Growth parameters (K_s, μ_{max}, Y_s) of *Methanobacterium thermoautotrophicum*. *Arch. Microbiol.* **127**:59-65.
- Segel, I. H. 1968. *Biochemical calculations*. John Wiley & Sons, Inc., New York.
- Shelton, D. R., and J. M. Tiedje. 1984. Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. *Appl. Environ. Microbiol.* **48**:840-848.
- Smith, M. R., and R. A. Mah. 1978. Growth and methanogenesis by *Methanosarcina* strain 227 on acetate and methanol. *Appl. Environ. Microbiol.* **36**:870-879.
- Stieb, M., and B. Schink. 1985. Anaerobic oxidation of fatty acids by *Clostridium bryantii* sp. nov., a sporeforming obligately syntrophic bacterium. *Arch. Microbiol.* **140**:387-390.
- Strayer, R. F., and J. M. Tiedje. 1978. Kinetic parameters of the conversion of methane precursors to methane in a hyperthermophilic lake sediment. *Appl. Environ. Microbiol.* **36**:330-340.
- Tomei, F. A., J. S. Maki, and R. Mitchel. 1985. Interactions in syntrophic associations of endospore-forming butyrate-degrading bacteria and H₂-consuming bacteria. *Appl. Environ. Microbiol.* **50**:1244-1250.
- Zehnder, A. J. B., K. Ingvorsen, and T. Marti. 1982. Microbiology of methane bacteria, p. 45-68. *In* D. E. Hughes (ed.), *Anaerobic digestion 1981*. Elsevier/North Holland Biomedical Press, Amsterdam.
- Zinder, S. H., S. C. Cardwell, T. Anguish, M. Lee, and M. Koch. 1984. Methanogenesis in a thermophilic (58°C) anaerobic digester: *Methanotrix* sp. as an important acetoclastic methanogen. *Appl. Environ. Microbiol.* **47**:796-807.
- Zinder, S. H., and R. A. Mah. 1979. Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H₂-CO₂ for methanogenesis. *Appl. Environ. Microbiol.* **38**:996-1008.