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Metabolic Activity of Fatty Acid-Oxidizing Bacteria and the Contribution of Acetate, Propionate, Butyrate, and CO_2 to Methanogenesis in Cattle Waste at 40 and 60°C

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The quantitative contribution of fatty acids and CO_2 to methanogenesis was studied by using stirred, 3-liter bench-top digestors fed on a semicontinuous basis with cattle waste. The fermentations were carried out at 40 and 60°C under identical loading conditions (6 g of volatile solids per liter of reactor volume per day, 10-day retention time). In the thermophilic digestor, acetate turnover increased from a prefeeding level of 16 μM/min to a peak (49 μM/min) 1 h after feeding and then gradually decreased. Acetate turnover in the mesophilic digestor increased from 15 to 40 μM/min. Propionate turnover ranged from 2 to 5.2 and 1.5 to 4.5 μM/min in the thermophilic and mesophilic digestors, respectively. Butyrate turnover (0.7 to 1.2 μM/min) was similar in both digestors. The proportion of CH_4 produced via the methyl group of acetate varied with time after feeding and ranged from 72 to 75% in the mesophilic digestor and 75 to 86% in the thermophilic digestor. The contribution from CO_2 reduction was 24 to 29% and 19 to 27%, respectively. Propionate and butyrate turnover accounted for 20% of the total CH_4 produced. Acetate synthesis from CO_2 was greatest shortly after feeding and was higher in the thermophilic digestor (0.5 to 2.4 μM/min) than the mesophilic digestor (0.3 to 0.5 μM/min). Counts of fatty acid-degrading bacteria were related to their turnover activity.

In methane fermentation, anaerobically degradable organic matter is completely converted to CO_2 and methane. This biological process involves the complex metabolic interactions of three groups of bacteria. Fermentative bacteria hydrolyze the substrate polymers to simple soluble compounds which are fermented to volatile acids, CO_2, and H_2. The obligate H_2-producing acetogenic bacteria subsequently oxidize the propionate, butyrate, and longer-chain fatty acids to acetate, CO_2 and H_2. Finally, methanogens utilize the acetate and H_2 produced to form CH_4. The rate-limiting step in methane fermentation often involves the degradation of fatty acids since these begin to accumulate in digestors stressed by high organic matter loading rates or short retention times (or both). This, in turn, is closely related to the efficiency of H_2 utilization by methanogenic bacteria through interspecies hydrogen transfer. These principles have been discussed extensively in recent publications (9, 16, 19, 20; M. J. McInerney and M. P. Bryant, *in* S. S. Sofer and O. J. Zaborski (ed.), *Biomass Conversion Processes for Energy and Fuels*, in press).

Previous studies have shown that acetate is quantitatively the most important precursor during mesophilic anaerobic digestion of wastes and accounts for 60 to 80% of the CH_4 produced, depending on substrate composition (15, 21, 28, 32). The balance of the CH_4 produced is formed from CO_2 reduction (10, 15, 21, 31), although in some cases this has been estimated by difference (11, 28). The role of acetate, propionate, and CO_2 reduction in methanogenesis during sludge digestion has been assessed indirectly by a kinetic approach (16). However, the contribution of other volatile acids, propionate and butyrate in particular, has not been adequately investigated. Furthermore, these reactions have not been studied during thermophilic digestion. Recent work in this laboratory has focused on the documentation of the group of obligate H_2-producing acetogenic bacteria capable of oxidizing propionate, butyrate, and longer-chain fatty acids with the production of acetate, H_2, and, in the case of odd-numbered carbon sources, CO_2 (6, 19, 20). The present series of experiments was designed to determine the kinetics of acetate, propionate, and butyrate degradation and CO_2 reduction and their subsequent contribution to

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methanogenesis. The natural rates of these reactions were estimated by infusion of ^{14}C-labeled compounds into stirred bench-top fermentors fed on a semicontinuous basis with cattle waste. The fermentations were carried out at 40 and 60°C, temperatures which are considered near optimum for mesophilic and thermophilic digestion, respectively (24, 34).

MATERIALS AND METHODS

Digestors. Five-liter Microferm bench-top fermentors (New Brunswick Scientific Co., New Brunswick, N.J.) were used, each with a 3-liter working volume (Fig. 1). All tubing was glass, connected by thick-walled, narrow-bore, black butyl rubber, except the feed inlet and effluent outlet, which were wide-bore flexible rubber. One fermentor was maintained at 60°C for thermophilic studies, and the other was maintained at 40°C for mesophilic studies. The impellers were operated continuously at 260 rpm, except for a short time just before the once-daily feed, when the rate was increased to 600 rpm, and effluent of an equal volume to the fresh substrate to be added (300 ml to give a 10-day retention time) was removed. Digestor gas was collected in a sealed, calibrated, 10-liter bottle containing a 20% NaCl–0.5% citric acid solution to minimize CO_2 solubility, and the volume of gas was measured by fluid volume displacement. The gas volume was corrected to 760 mm Hg pressure at 0°C. The fermentor vessels were kept under slight positive pressure to minimize the chance of oxygen entry especially during substrate addition.

Substrate. A single batch of ca. 200 kg of dairy beef waste (feces and urine without bedding) was collected in December, 1978, from a 3-day accumulation on an open concrete surface. The steers were fed the same ration reported by Varel et al. (34). No antibiotics or other feed additives were included in the feed. The waste was ca. 24% total solids and 20% volatile solids (VS) when collected. After collection it was slurried and diluted to approximately 12% VS with tap water in a Waring blender. The blended slurry was then weighed into screw-top plastic bottles, each to provide the desired daily input of 18 g of VS per day per fermentor (6 g of VS per liter of reaction volume) and stored at −25°C until required. Just before daily feeding the substrate was thawed and brought to the required temperature. After removing the correct volume of effluent (300 ml), the fresh substrate was added, and rinsing with water used to obtain the same influent volume.

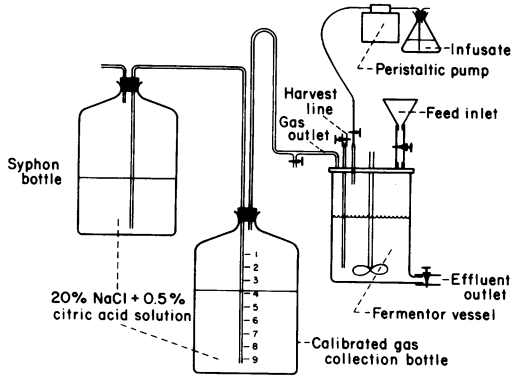

FIG. 1. *Schematic diagram of the Microferm fermentor with 3-liter working volume and calibrated bottle for measurement of gas volume.*

Infusion experiments with radioactive compounds. Sterile infusate in a 1-liter flask was infused at a constant measured rate (ca. 10 ml/h) into the fermentor vessel by means of a peristaltic pump (model 600–1200; Harvard Apparatus Co., Dover, Mass.). The infusate was prepared by using ca. 100 μCi of labeled compound, made up in 1 liter of 5 mM carrier, and sterilized by membrane filtration (0.2-μm pore size, 25-mm diameter; Amicon Corp., Lexington, Mass.). The infusion was started the evening before, to allow the radioactivity to plateau, and then continued for a 24-h period from one morning feed to the next. Infusion of labeled compounds did not significantly affect pool sizes.

Serial samples of digestor contents (30 ml) were removed at different times before and after feeding by means of a 35-ml syringe with a 6-mm nozzle attached to the harvest line of the fermentor with butyl rubber tubing. Care was taken to mix fluid remaining in the harvest line with that in the rest of the digestor before removing a sample and adding to glass bottles containing 0.25 ml of 10 M NaOH (final pH, ca. 10). The samples were centrifuged at 20,000 × *g* for 30 min, and the supernatant was stored in rubber-stoppered glass bottles at 4°C until required for analysis. Fermentor gas was sampled by using a 5-ml syringe with a 23-gauge needle (¾ in., ca. 18.5 mm) through the feed inlet tubing and injected into a glass tube sealed with a butyl rubber stopper and containing 20% NaCl–0.5% citric acid. This allowed repeated injections (0.5 ml) to be made on an adjacent gas chromatograph for concentration of CH_4 and CO_2 and for radioactivity analysis on a gas proportional counter. The infusion experiments were repeated twice with each radiolabeled chemical.

Chemicals. The following radioactive compounds were used: sodium [2-^{14}C]acetate (specific activity, 23.5 mCi/mmol), sodium [1-^{14}C]propionate (specific activity, 53 mCi/mmol), sodium [1-^{14}C]butyrate (specific activity, 12.7 mCi/mmol), and $NaH^{14}CO_3$ (specific activity, 57.5 mCi/mmol), obtained from Calatomic, Amersham, New England Nuclear Corp. (Boston, Mass.), and Amersham, respectively. All other chemicals were reagent grade and were obtained from commercial sources.

Analytical procedures. Total solids and VS were determined by a standard procedure (1). Total nitrogen was determined by the Kjeldahl method (1), and ammonia nitrogen was determined by the phenol-hypochlorite method (12). Cellulose, hemicellulose, and lignin were determined by the methods of Goering and van Soest (13). Lipids were determined by ether extraction (3). The concentrations of individual volatile fatty acids and lactate in the substrate were determined by the method of Salanitro and Muirhead (27).

The concentration of total volatile fatty acids was determined by steam distillation of 10 to 15 ml of

digestor fluid in a Markham still (7). Approximately 100 ml of distillate was collected and titrated with 0.1 M NaOH. The distillate was evaporated to dryness after addition of 1 to 2 drops of 10 M NaOH to bring the final pH to 10. The dry sodium salts were taken up in a known volume of water (5 ml). Radioactivity in total volatile acids was determined on two 1-ml portions, and the remaining 3 ml was used to determine activity and concentration of individual acids. Concentration of individual acids was determined by acidifying a sample of alkaline supernatant with 30% H_3PO_4, centrifuging, and filling septum-sealed, glass microvials for use with the automatic sampler (model 7671A) and a Hewlett Packard gas chromatograph (model 5830A). The volatile fatty acids were separated on a column of Chromosorb WAW, 10% SP 1000, and 1% H_3PO_4 (Supelco, Bellefonte, Pa.) with argon as the carrier gas. The separation of individual volatile fatty acids for activity analysis was carried out by partition chromatography with Sephadex LH 20 (Pharmacia, Uppsala, Sweden) and essentially the method of Van der Walt (33). Radioactivity was determined by adding an equal volume of Aquasol-2 (New England Nuclear Corp.) and counting in a Beckman Scintillation Counter (model LS 230) using external standard ratio for quench correction. Counting efficiencies were 85 to 95%.

Gas composition was analyzed with an Aerograph A100 gas chromatograph (Wilkens Instrument & Research, Berkeley, Calif.) with stainless steel column (5 ft by 0.25 in., ca. 1.5 m by 72.5 mm) packed with silica gel and connected to a thermal conductivity detector (bridge current, 200 mA) and recorder. Helium was used as carrier gas (flow rate, 60 ml/min). For analysis of specific activity in CH_4 and CO_2, the gases were separated on a Packard gas chromatograph (model 7800) equipped with a Porapak Q 100–120 mesh column and electron capture detector with helium as carrier gas (sensitivity, 10 nM CH_4 and 25 nM CO_2). Effluent from the gas chromatograph was fed directly into a Packard 894 gas proportional counter, and radioactivity was quantified by a multiplicative ion collection device. The following factors were optimal for the gas proportional counter: a time constant of 10 s and propane (chemically pure grade; Matheson Gas Products, Joliet, Illinois) used as quench gas at a flow rate of 6 ml/min. The outputs from the gas chromatograph detector and gas proportional counter were recorded on a dual pen recorder. On several occasions radioactivity in CH_4 and CO_2 was measured by liquid scintillation counting as described by Zehnder et al. (40).

Procedures for bacterial counts. Sterile anaerobic techniques for the preparation and use of media and details of the composition and preparation of stock solutions used have been described previously (8, 20). The roll tube medium for enumeration of total culturable and methanogenic bacteria was based on medium 174 (14). This medium contained (per 100 ml): 0.2 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 45 ml of clarified digestor fluid, 5 ml of Pfennig mineral solution, 2 ml of cysteine-sulfide-reducing solution, 0.1 ml of resazurin, 7 ml of $NaHCO_3$ solution, and 3.0 g of agar (Difco Laboratories, Detroit, Mich.) for thermophilic counts (2.0 g of agar for mesophilic counts). The medium was dispensed in 5-ml amounts into sterile tubes (18 by 150 mm) fitted with no. 1 black rubber stoppers and containing an 80% N_2–20% CO_2 gas phase. For the methanogenic bacteria the gas phase was replaced with 80% H_2–20% CO_2 at the time of inoculation. A liquid medium for most probable number determination of methanogens was prepared as above, but was incubated in a slanted position on a reciprocal shaker. The tubes for enumeration of methanogens were flushed with 80% H_2–20% CO_2 once weekly initially and twice weekly after 3 to 4 weeks of incubation.

The basal medium for most probable number determination of fatty acid degraders contained (per 100 ml); 5 ml of clarified fluid, 5 ml of Pfennig mineral solution, 0.1 ml of Pfennig metal solution, 2 ml of cysteine-sulfide-reducing solution, 0.1 ml of resazurin solution, and 7 ml of $NaHCO_3$ solution. The basal medium was supplemented with 0.3 g of sodium acetate, sodium propionate, or sodium butyrate for the three groups of bacteria. The tubes were incubated in a vertical position under an 80% N_2–20% CO_2 gas phase. Pure cultures of *Methanospirillum hungatei* (strain JF-1) and *Methanobacterium thermoautotrophicum* (strain ΔH) were kindly supplied by R. S. Tanner and R. S. Wolfe, Department of Microbiology, University of Illinois. They were maintained on slant cultures as described by McInerney et al. (20). The two pure strains were cultured in pressurized serum tubes using media and techniques described by Balch et al. (4).

Clarified digestor fluid was prepared as a single uniform batch by collecting effluent from the thermophilic and mesophilic digestors. The effluent was clarified by centrifugation (16,300 × *g*, 30 min, 4°C) and was stored at 4°C until required for medium preparation.

Effluent for enumeration of bacteria in the thermophilic and mesophilic digestors was collected just before the one daily feed and was blended for 1 min in a Waring blender which was vigorously gassed with 100% CO_2 (O_2 free). One milliliter was diluted by serial 10-fold dilutions in anaerobic diluent, and 1 ml of the appropriate dilutions was used to inoculate different media. Serial dilutions (10^{-4} to 10^{-9}) for inoculation of propionate and butyrate media also contained 0.5 ml of an actively growing culture of *M. hungatei* for the mesophilic counts and *M. thermoautotrophicum* for the thermophilic counts. All roll tubes and liquid media were incubated for 8 to 12 weeks at 40°C (mesophilic digestor) or 60°C (thermophilic digstor).

All colonies were counted after 4 and 8 weeks of incubation to determine the number of total culturable bacteria. There was very little increase in number of colonies, although they were more easily counted after 8 weeks of incubation. Methanogenic bacteria in roll tubes were examined and counted at 2-week intervals over the 8-week incubation period. All colonies which increased in size with 80% H_2–20% CO_2 flushing and were >2 mm in diameter were counted as methanogens. The size of colonies in the medium for total culturable bacteria with 80% N_2–20% CO_2 served as control. After 8 weeks of incubation CH_4 was determined in tubes of liquid medium 174 gassed with 80% H_2–20% CO_2 as compared with inoculated control

tubes gassed with 80% N_2–20% CO_2, and the methanogenic bacteria were quantified by most probable number (1). For the fatty acid degraders, optical density was measured at weekly intervals, and CH_4 was determined after 12 weeks of incubation. Positive tubes were identified as those with greater optical density and more CH_4 than inoculated control tubes which contained only the basal medium and no added substrate (acetate, propionate, or butyrate).

RESULTS

Substrate composition. The composition of the cattle waste used as substrate for the digestors is shown in Table 1. The waste contained 22.3% ash probably due to concrete scrapings obtained during collection. Cellulose, hemicellulose, and lignin accounted for 57% of the total solids and 73.4% of the VS. Ammonia formed was about 24% of the total nitrogen. Volatile acids (as acetic acid) comprised 3.6% of the VS, and the molar proportions of acetic, propionic, and butyric acids were 81:12:5. Lactate accounted for 1.5% of the VS.

Daily gas production in digestors. Both digestors were fed identical substrate at a loading rate of 6 g of VS per liter of reactor volume at a 10-day retention time, and cumulative gas production was measured over a 24-h cycle. Gas production was linear for the first 6 h after feeding and was significantly higher ($P > 0.95$) in the thermophilic digestor than in the mesophilic digestor during the last 20 h of the daily cycle. The eight experiments performed on each digestor covered a period of 8 to 12 months, and the gas production curves showed little variation and high stability, especially in the case of the thermophilic digestor. Total gas production per day was 16% higher in the thermophilic digestor than in the mesophilic digestor.

Methane and carbon dioxide production rates. The production rates for CH_4 and CO_2 were calculated for each experiment from the slope of a tangent drawn from the curve of total gas production at each of the sampling times to calculate the gas production rate. This value was multipled by the concentration of each gas in the headspace of the fermentor vessel to calculate the production rate of CH_4 and CO_2. The mean values ($n = 16$) for the two digestors are presented in Table 2. Methane production increased almost immediately after addition of daily substrate to the digestors and was 2 to 3 times greater 1 to 2 h after feeding than before feeding. Methane production rates were higher for the thermophilic digestor than for the mesophilic digestor at each of the sampling times, and the total over the 24 h cycle was 18% higher.

Fatty acid concentration and methane production. The concentration of acetate increased from a basal level of 1.93 and 3.51 mM to a peak of 6.15 and 8.24 mM 2 h after feeding in the thermophilic digestor and mesophilic digestor, respectively (Fig. 2). The levels showed a gradual decline to basal levels over the remainder of the 24-h cycle. Although the concentrations differed, the trends were similar for propionate and butyrate. The values ranged from 4.57 to 6.07 mM and 10.62 to 17.39 mM for propionate and from 0.29 to 0.57 mM and 0.46 to 0.84 mM for butyrate in the thermophilic and mesophilic digestors, respectively. The relatively high propionate levels in the mesophilic reactor were undoubtedly due to the relatively short retention time (10 days) for a mesophilic reactor with relatively high loading. Curves of cumulative methane production (Fig. 2) were linear for the first 6 h after addition of substrate to the thermophilic digestor. This linear portion was shorter (4 h) in the case of the mesophilic digestor.

Turnover of fatty acids. Table 3 presents

TABLE 1. *Composition of cattle waste used as substrate for digestors*[a]

Constituent	% in dry matter[b]
Organic matter (VS)	77.7
Cellulose	28.3
Hemicellulose	18.4
Lignin	10.3
Total nitrogen (crude protein)[c]	2.2 (13.5)
Ammonia nitrogen	0.52
Nonammonia crude protein[d]	10.0
Ether extract (crude lipid)	5.9
Volatile acid (as acetic acid)	2.8
Lactic acid	1.14

[a] See text for methods of analysis.
[b] Total solids; mean of 8 samples.
[c] Nitrogen × 6.25.
[d] (Total nitrogen − ammonia nitrogen) × 6.25.

TABLE 2. *Methane and carbon dioxide production rate in the mesophilic and thermophilic digestors*[a]

Time after feeding (h)	Production rate (μmol/min)			
	Mesophilic digestor		Thermophilic digestor	
	CH_4	CO_2	CH_4	CO_2
Before	21.04 ± 1.77[b]	13.21 ± 1.53	21.47 ± 1.71	13.77 ± 1.45
1–2	50.76 ± 4.46	39.98 ± 3.37	55.03 ± 2.41	46.01 ± 1.99
3.5–6	40.06 ± 2.89	27.88 ± 1.58	48.54 ± 1.17	39.29 ± 1.70
10–14	30.17 ± 1.35	19.57 ± 1.00	36.19 ± 1.23	25.93 ± 1.14
21–24	18.77 ± 1.27	11.55 ± 0.89	22.30 ± 1.22	13.95 ± 1.08

[a] Digestors were fed once daily with 6 g of VS per liter of digestor volume at a 10-day retention time. Data were calculated from the total gas production rate and gas composition.
[b] Each value in the table represents the mean ± standard deviation ($n = 16$).

results on acetate turnover obtained during continuous infusion of [2-^{14}C]acetate into the thermophilic digestor. Each experiment was performed in duplicate with each of the labeled compounds in the mesophilic and thermophilic digestors to determine turnover rates for acetate, propionate, and butyrate (Fig. 3). The most striking result was the large and very rapid increase in the turnover of acetate from a basal level of ca. 16 μM/min before feeding to a peak of ca. 49 μM/min 1 h after addition of fresh substrate in the thermophilic digestor, i.e., a threefold increase (Fig. 3B). The turnover rate gradually decreased over the remainder of the 24-h cycle. In the mesophilic digestor, the acetate turnover rate increased from a basal level of ca. 15 μM/min to a peak of ca. 40 μM/min 1 h after the daily feed (Fig. 3A). The turnover rate of propionate increased from a basal level of ca. 2 μM/min to a peak of 5.2 μM/min in the thermophilic digestor and from ca. 1.5 to 4.5 μM/min in the mesophilic digestor. The propionate turnover showed a broader peak than acetate, lasting for 3.5 to 6 h after the addition of fresh substrate. The butyrate turnover rates

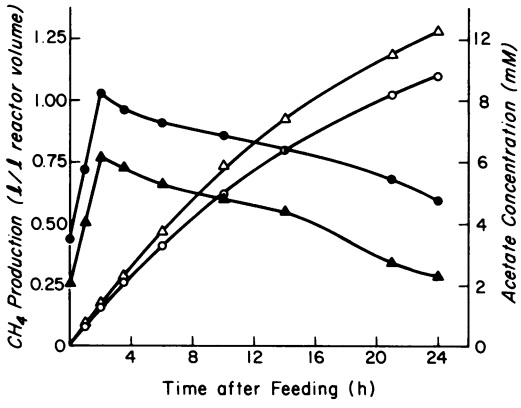

FIG. 2. *Methane production and acetate concentration during a 24-h cycle in digestors fed once daily with 6 g of VS per liter reactor volume at a 10-day retention time. Symbols: CH_4 production (○) and acetate concentration (●) in the mesophilic digestor; CH_4 production (△) and acetate concentration (▲) in the thermophilic digestor.*

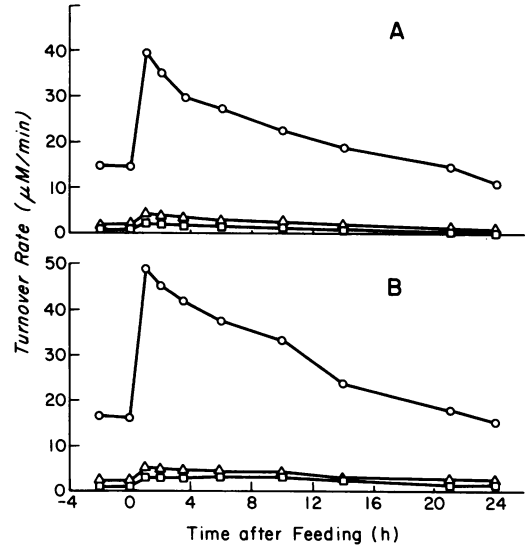

FIG. 3. *Turnover rates of fatty acids during a 24-h cycle in the mesophilic (A) and thermophilic (B) digestors fed once daily with 6 g of VS per liter of reactor volume at a 10-day retention time. Symbols: ○, acetate; △, propionate; □, butyrate. Each curve is the mean of two infusion experiments.*

TABLE 3. *Concentration, activity, specific activity, turnover rate of acetate, and percentage of methane formed via the methyl group of acetate over a 24-h cycle in the thermophilic digestor*[a]

Time (h)	Acetate concn (mM)	Sp act (dpm/μmol)		Turnover rate[b] of acetate (μmol/min)	% CH_4 formed via the methyl group of acetate	
		CH_4	Acetate		Sp act ratio[c]	CH_4 production[d]
−2	1.93	1,117	1,556	16.5	71.3	68.1
0	2.06	1,147	1,624	15.8	70.6	67.4
1	4.05	396	521	49.2	76.0	77.4
2	6.15	432	565	45.4	76.5	77.7
3.5	5.79	467	614	41.8	76.1	75.5
6	5.23	505	670	38.3	75.4	72.9
10	4.87	577	771	33.2	74.8	71.6
14	4.42	778	1,074	23.9	72.4	70.5
21	2.74	1,001	1,418	18.1	70.6	68.6
24	2.31	1,156	1,656	15.5	69.8	67.1

[a] Average of two infusion experiments with [2-^{14}C]acetate.
[b] Calculated as the ratio of infusion rate to specific activity, where infusion rate is 25,667 dpm/min.
[c] Calculated as (specific activity in CH_4/to specific activity in acetate) × 100.
[d] Calculated as (rate of acetate dissimilation/rate of CH_4 production) × 90.

were very similar in both digestors and ranged from a basal level of 0.7 to 0.9 μM/min to a peak of 1.5 to 1.7 μM/min. The peak in butyrate turnover was more delayed in the thermophilic digestor than the mesophilic digestor, occurring 6 h after the feed.

The percentage of CH_4 formed via the methyl group of acetate was calculated from the ratio of specific activities (Table 3). Results from the infusion experiments showed that approximately 90% (range, 88 to 94%) of the methyl group of acetate was actually converted to CH_4 and was not greatly affected by time after feeding. A small percentage of the label (3 to 5%) from [2-^{14}C]acetate was found in CO_2. Thus, a value of 90% was used to calculate the percentage of CH_4 produced via the methyl group of acetate from values for CH_4 production (Table 3). These values showed good agreement with those calculated from the specific activity ratio, but tended to be lower, except for peak fermentation 1 to 2 h after feeding, when they were higher. During infusion of [2-^{14}C]acetate a small percentage of the label (2 to 3%) was found in butyrate, and less than 1% was found in propionate.

Methane production from CO_2. From the ratio of specific activities of CH_4 and CO_2 calculated from data obtained from continuous infusion experiments with $NaH^{14}CO_3$ (Table 4), it can be seen that the percentage of CH_4 formed from CO_2 ranged from 23.2 to 29.4% in the mesophilic digestor and 18.5 to 27.4% in the thermophilic digestor. The trend was the same in both digestors, being lower after feeding. In the case of the mesophilic digestor the percentage decreased from a basal level of 28 to 29% to 23 to 24% between 1 and 3.5 h after feeding and then increased to the basal value by 6 h after feeding. The basal value was 26 to 27% in the thermophilic digestor, but decreased to 18.5 to 19.3% between 1 and 3.5 h after addition of fresh substrate. After 6 to 10 h of fermentation the values had increased to 21.1 to 22.7%, and after 14 h the basal value was reached.

Role of fatty acids and CO_2 as precursors of methane. The role of the methyl group of acetate and CO_2 as precursors of CH_4 are given in Table 5. The results show that approximately 10% more of the CH_4 produced during the different time periods after feeding was accounted for by acetate in the thermophilic digestor as compared with the mesophilic digestor. In both the mesophilic and thermophilic digestors the contribution of the methyl group of acetate to CH_4 formation decreased with time after feeding fresh substrate, whereas the contribution of CO_2 to CH_4 formation showed the reverse trend and increased with time after feeding.

The percentage of CH_4 accounted for by propionate and butyrate was calculated from turnover rates of each acid and CH_4 production rates, with the assumption that 1.75 mol of CH_4 was formed from 1 mol of propionate and that 2.5 mol of CH_4 was formed from 1 mol of butyrate (17). In the mesophilic digestor, 13% of the CH_4 was formed through propionate, and 8% of the CH_4 was found via butyrate. The values were 17% from propionate and 9% from butyrate in the thermophilic digestor. The contribution from propionate and butyrate generally showed a small increase with time after the once-daily feed. Since propionate and butyrate are intermediates which are oxidized by obligate H_2-producing acetogenic bacteria to form acetate plus H_2 plus CO_2, it was estimated that 50 to 63% of the CH_4 produced in the thermophilic and mesophilic digestors, respectively, was accounted for by the methyl group of acetate from sources other than propionate and butyrate.

Synthesis of acetate from CO_2. The contribution of CO_2 to the synthesis of acetate by the following reaction: $4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$, was calculated from the ratio

TABLE 4. *Concentration and specific activity of CO_2 and CH_4 and percentage of CH_4 formed from CO_2 in the mesophilic and thermophilic digestors*[a]

Digestor	Time after feeding (h)	CO_2		CH_4		% CH_4 formed from CO_2[b]
		Concn (μM)	Sp act (dpm/μmol)	Concn (μM)	Sp act (dpm/μmol)	
Mesophilic	1–5	8.91	4,099	11.37	984	24.0
	6–12	8.60	3,794	12.85	1,068	28.2
	13–24	8.34	3,562	13.21	1,030	28.9
Thermophilic	1–5	9.24	3.061	11.49	581	19.0
	6–12	9.49	3,115	12.31	682	21.9
	13–24	8.47	2,892	13.31	781	27.0

[a] Digestors were fed once daily with 6 g of VS per liter of digestor volume at a 10-day retention time. Data are the averages of two infusion experiments with $NaH^{14}CO_3$.

[b] Calculated as (specific activity in CH_4/specific activity in CO_2) × 100.

1/2 of specific activity in acetate/specific activity in CO_2 during the infusion experiments using $NaH^{14}CO_3$. The rate of acetate synthesized from CO_2 was then estimated from this ratio and the total acetate turnover. In the mesophilic digestor, the rate of acetate synthesis from CO_2 ranged from 0.2 to 0.5 μM/min and was highest after the once-daily feed. The thermophilic digestor showed the same trend, but with higher values ranging from 0.5 to 2.4 μM/min. The percentage of acetate formed from CO_2 was 1.3 to 2.0% and 3.0 to 5.3% in the mesophilic and thermophilic digestors, respectively (Table 6).

When net acetate turnover was calculated as total acetate turnover minus acetate synthesized from CO_2 and was used to determine the percentage of CH_4 formed from the methyl group of acetate; the values were 68.5 to 73.2% and 71.2 to 82.8% for the mesophilic and thermophilic digestors, respectively. These values were approximately 2 to 4% lower than values calculated from total acetate turnover by using the same assumption (see Table 3 for values in the thermophilic digestor).

Bacterial counts. The number of total culturable bacteria was 1.4 to 10^9 per ml of digestor content in the mesophilic digestor and was 10-fold higher (1.3×10^{10}) in the thermophilic digestor (Table 7). Counts of H_2-utilizing methanogens using the roll tube and most probable number techniques were in good agreement: 2.1 to 3.7×10^8 and 9.3×10^8 per ml of digestor contents in the mesophilic and thermophilic digestors, respectively. The counts of bacteria capable of degrading acetate with the production of CH_4 were 4.6×10^7 and 21.0×10^7 per ml of digestor contents in the mesophilic and thermophilic digestors, respectively (Table 7), approximately 25% of the count of the corresponding H_2-utilizing methanogens.

Counts of butyrate oxidizers were 5- to 10-fold higher than corresponding counts of propionate oxidizers.

TABLE 5. *Percentage of methane formed through different sources at various times after feeding in the mesophilic and thermophilic digestors*

Precursor	Time after feeding (h)	% of CH_4 accounted for by precursor	
		Mesophilic digestor	Thermophilic digestor
Acetate[a]	1–5	75	86
	6–12	72	80
	13–24	72	75
CO_2[b]	1–5	24	19
	6–12	28	22
	13–24	29	27
Propionate[c]	1–5	14	16
	6–12	13	16
	13–24	13	16
Butyrate[c]	1–5	8	7
	6–12	8	9
	13–24	9	9
Acetate from other sources[d]	1–5	53	63
	6–12	51	56
	13–24	50	50

[a] Calculated by assuming 1 mol of acetate formed 1 mol of CH_4.
[b] Results from Table 4.
[c] Calculated from turnover rates of the acids. CH_4 production rate, and assuming that 1 mol of propionate forms 1.75 mol of CH_4 and 1 mol of butyrate forms 2.5 mol of CH_4.
[d] Excluding contribution from propionate and butyrate.

TABLE 6. *Synthesis of acetate from CO_2 and percentage of methane formed from the methyl group of net acetate in the mesophilic and thermophilic digestors*[a]

Digestor	Time after feeding (h)	Acetate turnover (μM/min)	% Acetate synthesized from CO_2[b]	Acetate from CO_2 (μM/min)	Net acetate turnover (μM/min)	% CH_4 formed from methyl group of net acetate produced[c]
Mesophilic	1–5	35.1	1.5	0.5	34.6	64.9
	6–12	25.2	1.6	0.4	24.8	63.3
	13–24	15.2	1.8	0.3	14.9	62.1
Thermophilic	1–5	45.5	4.3	1.9	43.6	73.4
	6–12	35.8	3.4	1.2	34.6	69.6
	13–24	19.2	3.4	0.7	18.5	65.7

[a] Digestors were fed 6 g of VS per liter of digestor volume per day at a 10-day retention time.
[b] Average of two infusion experiments with $NaH^{14}CO_3$. Calculated as (1/2 specific activity in acetate/specific activity in CO_2) × 100.
[c] Calculated as (rate of net acetate dissimilation/rate of CH_4 production) × 90.

DISCUSSION

The kinetic studies on the conversion of fatty acids and CO_2 to methane measured the natural turnover rate of the fatty acid pools in the digestors by following the fate of infused ^{14}C-labeled acids and HCO_3^-. Acetate was the major precursor of the CH_4 produced. The contribution of the methyl group of acetate to methanogenesis decreased with time after feeding. In the mesophilic digestor, the value was 75% after feeding, but declined to the basal level of 72% between 13 and 24 h after feeding. Corresponding values in the thermophilic digestor were 5 to 10% higher, demonstrating that acetate was quantitatively more important as a CH_4 precursor at the higher temperature (Fig. 4). The regression lines show that, under the experimental conditions, with a CH_4 production rate of 40 μM/min the acetate turnover would be 28.7 and 32 μM/min in the mesophilic and thermophilic digestors, respectively. As predicted, more CH_4 was produced via acetate in the thermophilic digestor. The converse is also of interest since at the same acetate turnover (30 μM/min) the total CH_4 production would be higher in the mesophilic digestor (41.5 μM/min) as compared with the thermophilic digestor (37.7 μM/min). This reflects the greater contribution of CO_2 reduction to CH_4 production in the mesophilic digestor. The increasing slope of the thermophilic regression line is also consistent with the increased contribution of CO_2 to acetate synthesis at high acetate turnover.

TABLE 7. *Culture counts of different metabolic groups of bacteria present in the mesophilic and thermophilic digestors*[a]

Metabolic group	No./ml of digestor content[b]	
	Mesophilic digestor	Thermophilic digestor
Roll tubes		
Total culturable	1.4×10^9	1.3×10^{10}
Methanogens	3.7×10^8	10.4×10^8
Most probable number (3 tube)		
Methanogens	2.1×10^8	9.3×10^8
Acetate degraders	4.6×10^7	21.0×10^7
Propionate degraders[c]	0.9×10^6	1.5×10^6
Butyrate degraders[c]	0.5×10^7	1.5×10^7

[a] Digestors were fed 6 g of VS per liter of digestor volume per day at a 10-day retention time.

[b] Average of two experiments with three tubes per dilution in each experiment.

[c] Co-inoculated with *M. hungatei* (mesophilic) and *M. thermoautotrophicum* (thermophilic). See text for details of procedure.

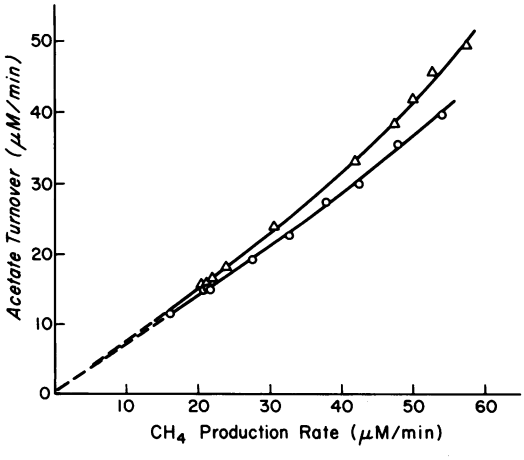

FIG. 4. *Relationship between CH_4 production rate (μM/min) and acetate turnover (μM/min) in the mesophilic* (○) *and thermophilic* (△) *digestor fed 6 g of VS per liter of reactor volume once daily at a 10-day retention time.*

Variations in the percentage of CH_4 formed from the methyl group of acetate can in part be accounted for by differences in methodology and assumptions used in calculations. It has been shown that, in anaerobic digestors (present studies) (21) and in pure cultures of *Methanosarcina barkeri* (35), approximately 90% of the methyl group of acetate appears as CH_4. Thus, studies which are carried out with the assumption that all acetate is being converted to CH_4 without verification (11, 29) are probably subject to error. If the value of 73% reported in several studies (15, 18, 28) were multiplied by 0.9 to account for the fraction of CH_4 arising from the methyl group of acetate, the result would be 66% and extremely close to the theoretical value for fats and carbohydrates. Similar errors can also be made when the contribution of CO_2 reduction to methanogenesis is determined as the difference between total CH_4 produced and CH_4 produced via acetate. In the present studies, the most reliable results are probably those derived directly from specific activity ratios.

A small amount (3 to 5%) of the methyl group of acetate was converted to CO_2. This has been reported previously in anaerobic digestors (21), lake sediments (10, 31, 37), and enrichment cultures of methane bacteria (29, 30). Oxidation of the methyl group of acetate to CO_2 is markedly increased in the presence of sulfate (37). *Desulfuromonas acetoxidans* and *Desulfomaculum acetoxidans*, sulfur- and sulfate-reducing bacteria capable of acetate oxidation to CO_2, have been isolated from several ecosystems including animal manure (26, 36). *Methanobacterium soehngenii*, commonly seen in digested sludge, is also able to oxidize the methyl group of acetate to CO_2 (41). Another possible source of labeled

CO_2 recovered during infusion of [2-^{14}C]acetate is anaerobic methane oxidation. Recent work has shown that all methanogenic bacteria are able to form and oxidize CH_4 simultaneously, and that anaerobic oxidation of CH_4 to CO_2 during methanogenesis in anerobic sediments and digested sewage sludge increased in the presence of iron and sulfate (38, 39). The carboxyl group of acetate is mainly converted to CO_2 during "acetate splitting," and this can in turn be reduced by H_2 to CH_4 (29, 30). Acetate is also a key intermediate in the metabolism of *M. barkeri* and can contribute up to 60% of the cell carbon formed during heterotrophic growth on acetate (36).

Lawrence (17) reported from kinetic studies performed in the laboratory of P. L. McCarty that 85% of the CH_4 formed from a complex waste was derived from acetic and propionic acids, and data from unpublished research in P. H. Smith's laboratory indicated that butyrate accounted for an additional 8%. However, there have been no published reports on the direct determination of turnover of these fatty acids and their contribution to methanogenesis during anaerobic degradation. Recent research has shown that propionate and butyrate are intermediates in the fermentation which are degraded to acetate, which is then converted to CH_4 (6, 16, 19, 20; McInerney et al., in press). These reactions are mediated by a group of organisms called obligate H_2-producing (proton-reducing) acetogenic bacteria (9, 19). The results in the present experiments show that propionate accounted for 13 and 17% of the CH_4 produced in the mesophilic and thermophilic digestors, respectively. Butyrate accounted for 7 to 9% of the CH_4 produced in both digestors. Together, these two acids would account for about 23% of the total methane produced. Radioactivity in CH_4 during infusion experiments with ^{14}C-labeled propionate and butyrate was not high enough for use in calculating specific activity ratios with confidence. However, when the value of 0.9 mol of CH_4 formed from 1 mol of acetate (produced as an intermediate) is used in the calculation the combined contribution of propionate and butyrate would be closer to 20%. Kaspar and Wuhrmann (16) estimated indirectly that approximately 15% of the CH_4 formed during mesophilic sludge digestion was from propionate via acetate and H_2. This value agrees closely with the direct estimate in the present study.

It was of interest to relate the numbers of fatty acid-oxidizing bacteria to their activity in the digestors measured during the infusion experiments (Table 8). The higher number of butyrate than propionate oxidizers is probably a reflection of the more favorable energetics of this reaction and, hence, possibly higher viability rather than an absolute difference. This accounts for the highest specific activity (3 μmol of propionate per min per 10^6 organisms) of the three fatty acids. The most probable number of butyrate degraders probably also included those organisms responsible for β-oxidizing longer-chain fatty acids present in the digestor substrate. The greater numbers of acetate, propionate, and butyrate degraders in the thermophilic digestor would be sufficient to account for the increased activity or turnover measured as compared with that in the mesophilic digestor, despite the lower specific activity of the thermophilic organisms. These results also document the presence of obligate H_2-producing bacteria which are active in the thermophilic temperature range (60°C). However, representatives of this group of organisms have so far only been isolated at mesophilic temperatures (6, 19, 20).

The production of CH_4 from CO_2 reduction showed the opposite trend to CH_4 production from acetate, being lower just after the addition of fresh substrate and increasing over the remainder of the cycle. Similar results were obtained by Mountfort and Asher (21), who explained their findings by the ratio of hydrogen to acetate produced during the degradation of complex organic matter determining the proportions of CH_4 produced from acetate and CO_2 in

TABLE 8. *Number, activity, and specific activity of fatty acid-oxidizing bacteria in the mesophilic and thermophilic digestors*[a]

Digestor	Fatty acid	No. of organisms per ml of digestor content	Activity (μmol/ml/min)	Sp act (μmol/min/10^6 organisms)
Mesophilic	Acetate	5×10^7	30	0.6
	Propionate	1×10^6	3	3
	Butyrate	5×10^6	1	0.2
Thermophilic	Acetate	20×10^7	40	0.2
	Propionate	1.5×10^6	4.5	3
	Butyrate	15×10^6	2	0.13

[a] Digestors were fed 6 g of VS per liter of digestor volume per day at a 10-day retention time.

the presence of HCO_3^--CO_2. The main compounds degraded in the early stages after feeding would be those giving a low ratio of hydrogen to acetate, whereas those degraded in the latter stages would give a higher ratio. Undoubtedly, the large initial burst of fermentation after addition of fresh substrate is responsible for the large increase in proportion of CH_4 formed from acetate which would not be found with continuous feeding. The addition of fresh substrate would provide a substantial amount of acetate, lactate, and bacterial protein which would influence the proportion of CH_4 formed from acetate. The production of hydrogen would also be highest at this time, although under the present experimental conditions of loading rate and retention time, would probably not be sufficient to influence CO_2 reduction. An important factor in the present experiments appears to be the activity of the bacteria synthesizing acetate from CO_2 and H_2, which activity was also higher after feeding. The synthesis of acetate from CO_2 after feeding was 0.5 and 1.6 to 2.4 μM/min in the mesophilic and thermophilic digestor, respectively, and accounted for 1.4 to 1.7 and 3.5 to 5.3% of the acetate synthesized in the two digestors. This would contribute to increased acetate turnover which was higher in the thermophilic digestor than the mesophilic digestor. Organisms which could be responsible for the synthesis of acetate from CO_2 in the thermophilic digestor are *Clostridium formicoaceticum*, *Clostridium thermoaceticum*, and organisms resembling *Clostridium aceticum* (2, 22, 23, 26). Recently, nonsporeforming acetogenic bacteria have been isolated from sediments and sludge and named *Acetobacterium woodii* (5). *Eubacterium limosum* isolated from the rumen and sewage sludge is able to synthesize acetate and butyrate from H_2 and CO_2 (B. R. S. Genthner and M. P. Bryant, Abstr. XV Rumen Function Conf. 1979, p. 10; B. R. S. Genthner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I64, p. 95; B. R. S.. Genthner, C. L. Davis, and M. P. Bryant, manuscript in preparation; R. S. Tanner, R. S. Wolfe, and C. R. Woese, manuscript in preparation).

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