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Changes in Proportions of Acetate and Carbon Dioxide Used as Methane Precursors During the Anaerobic Digestion of Bovine Waste

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In an anaerobic digestor which was fed daily with bovine waste, during the early stages after feeding (4 to 7 h) acetate (via the methyl group) accounted for almost 90% of the methane produced. As time after feeding increased, acetate declined as a precursor so that in the 12- to 14-h and 21- to 23-h periods after feeding the methyl group accounted for 80 and 73% of the methane produced, respectively. Measurements of methane production from CO_2 reduction showed that in the 2- to 12-h period after feeding, CO_2 accounted for 14% of the methane produced, whereas in the 12- to 24-h period it accounted for 27.5%. These results show that the percentages of methane accounted for by acetate and CO_2 vary with time after feeding the digestor.

Anaerobic digestion has been a common method of converting organic wastes to innocuous end products. For a review see Hobson et al. (6).

In New Zealand the production of methane from the digestion of animal wastes (mainly bovine waste) is becoming increasingly recognized as a supplementary energy supply on farms. Previous studies (7, 10, 16) have shown acetate to account for most (60 to 80%) of the methane produced in anaerobic digestors. Within this range the percentage of methane from acetate appears to be influenced by the composition of the starting material (7).

This paper examines in detail the ultimate precursors of methane during the digestion of bovine waste, and data are presented to show that during the period between daily feeding of an anaerobic digestor the proportion of methane accounted for by the different precursors varies.

MATERIALS AND METHODS

Substrate. Bovine feces (without urine and bedding) not more than 24 h old was collected from a dairy farm. Cows were grazed on pasture all year round. In the winter the feed was occasionally supplemented with a small amount of hay or silage. After collection the waste was diluted to approximately 5% volatile solids (VS) with biological oxygen demand water (1) and macerated in a Waring blender. Waste was stored in plastic bags at −25°C, and on the day before use it was thawed at room temperature. Just before feeding into an anaerobic digestor, waste was diluted to 2.5% VS and brought up to the same temperature as the digestor.

Operation of anaerobic digestor. The anaerobic digestor was used as a source of actively digesting waste for experiments. The digestor was of 7-liter capacity with gas, sample, and feed ports; contents were continuously agitated by a stirrer powered by a fermentor drive assembly unit (New Brunswick Scientific Co., New Brunswick, N.J.).

The digestor was initiated by the addition of 5 liters of cow waste (2.5% VS) and was maintained anaerobically at 37°C. Approximately 15 days after loading, maximum daily methane production was attained, and the digestor was thereafter operated on a semicontinuous basis by batch feeding samples of cow waste daily, using a retention time of 10 days. The loading rate was 2.5 g of VS/liter per day.

After 3 weeks of semicontinuous operation, daily gas production stabilized at 2.6 liters, 65 to 70% of which was methane with the remainder being CO_2. No hydrogen could be detected in the gas (detection limit, 0.1% [vol/vol]). Samples of actively digesting waste were used for experimental purposes after the digestor had been operated on a semicontinuous basis for 1 month.

Small-scale incubations. Radioactivity studies were carried out using small-scale incubations. At various times within 24 h of feeding the large digestor, samples (50 ml unless stated otherwise) of actively digesting waste were transferred to 130-ml Warburg-type vessels, using a large syringe (60 ml) with a wide-bore needle. The transfers were carried out under anaerobic conditions, and the vessels were gassed with a mixture of 70% N_2 and 30% CO_2 to exclude oxygen. After the transfers, vessels were sealed with glass stoppers lubricated with vaseline, and the contents were incubated at 37°C. In experiments that required vessels to have a center well for the absorption of CO_2, the stoppers were modified to include a passage to the center well for the addition of KOH. All flasks had a side arm plugged with a recessed butyl rubber stopper; gas samples were removed by penetrating this stopper with the needle of a glass piston syringe.

Analysis of cow waste. Total solids and VS were

determined by previously published procedures (1). Total nitrogen was determined by the Kjeldahl method (1), and ammonia nitrogen was determined by the phenol-hypochlorite method (4). For the latter method samples of waste were ground with glass beads, using a cell disintegrator (B. Braun, Melsungen, Germany), and then centrifuged at 7,000 × *g* for 10 min, and the supernatant was analyzed after being passed through a membrane filter (0.45-μm pore size; Millipore Corp.). Total volatile acids were determined after steam distillation by a previously published method (1). The procedure for preparing the sample for steam distillation was the same as that described below for the analysis of acetate. Cellulose, hemicellulose, and lignin were determined by the methods of Goering and van Soest (5). Lipids were determined by an ether extraction procedure (2).

Analyses of radioactive incubations. In experiments in which the rates of methane production and acetate dissimilation were determined, methane and the specific radioactivity of ^{14}C-labeled acetate were measured at various intervals after [2-^{14}C]acetate had been added to the 50-ml incubations. The amount of [2-^{14}C]acetate added was small (<0.03 μmol) so as not to affect the pool size of acetate already present (see Fig. 1 for acetate pool size).

Methane was determined by removing gas samples (1 ml) from flasks with a syringe and analyzing on a Fisher-Hamilton gas partitioner equipped with a thermal conductivity detector. Gas was fractionated at 25°C on dual columns; column 1 was packed with di-2-ethylhexylsebacate on Columnpak, and column 2 was packed with molecular sieve 13X. Helium was the carrier gas.

For the analysis of acetate, 4-ml samples were removed from digestors with a syringe and mixed with 0.5 ml of 50% H_2SO_4 at 0 to 2°C. Samples were centrifuged at 5,000 × *g* for 15 min at 0 to 2°C. For each sample the supernatant was removed, and the pellet was resuspended in 2 ml of water. The suspension was centrifuged again, and the supernatants from the two centrifugations were combined. The combined supernatant was steam distilled, and 100 ml of distillate was collected. (The recovery of acetate in the extraction and distillation steps was 98%.) The distillate was made alkaline with NaOH (pH 9 to 10) and evaporated to dryness on a rotary evaporator, and the residue was made up to 4 ml with 1 M formic acid. Acetic acid was determined by gas-liquid chromatography, using a method based on that of Mahadevan and Stenroos (8). The acid was separated at 180°C on a Poropak Q column coated with 2% H_3PO_4. Nitrogen was the carrier gas.

Radioactivity of acetate in 1 M formic acid was determined by liquid scintillation spectrometry. The composition of the scintillant was as described by Turner (15). Counts obtained were corrected for quenching and background. Counting efficiency was 90%. The radiochemical purity of acetate samples taken at various intervals from an incubation was greater than 95%. This was ascertained by celite column chromatography of the concentrated distillate according to the method described by Swim and Utter (14).

In experiments examining the fate of the label from the degradation of [2-^{14}C]acetate or the production of methane from $^{14}CO_2$, 2 ml of 3 M KOH was introduced at the end of incubation into the center well of vessels, and digestion was terminated by injection of 2 ml of 50% H_2SO_4 through the side-arm stopper. Vessels were held at 0 to 2°C for 2 h for complete absorption of CO_2; the resulting negative pressure was relieved by injecting CO_2-free nitrogen, using a glass piston syringe to give a slight positive pressure. The total volume of gas in the vessels was then determined by recording the excess gas forced into the syringe. For methane analysis a 4-ml volume of gas containing a known amount of methane was removed from a flask and injected into an evacuated tube stoppered with a butyl rubber bung. Excess unlabeled methane was also added in a known amount (0.2 mmol). Methane was combusted to CO_2 by flushing the gas from the tube with CO_2-free air and passing it over a heated cobalt oxide column at 800°C. CO_2 from the column was trapped in 3 M KOH and counted (counting efficiency, 86%). The scintillant was as previously described (15), except that Triton X-100 was replaced by methanol. To determine the recovery of CO_2 from the combustion process, 12% $BaCl_2$ was added to a portion of KOH containing absorbed CO_2; the resulting $BaCO_3$ precipitate was washed, collected on a tared filter disk and weighed after drying. Counts (as disintegrations per minute) were corrected for losses in recovery from the combustion process. Where the specific radioactivity of methane was required, the corrected disintegrations per minute was divided by the quantity of methane removed from the incubation flask.

CO_2 trapped in the center well of an incubation vessel was counted as above. Where a determination of the specific radioactivity was required, a portion of KOH containing CO_2 was treated with $BaCl_2$, and the resulting $BaCO_3$ was weighed as described above.

Chemicals. All chemicals were obtained from commercial sources and were of reagent grade. The radioisotopes $Na_2{}^{14}CO_3$ (specific activity, 60 mCi/mmol) and [2-^{14}C]acetate (specific activity, 56 mCi/mmol) were obtained from the Radiochemical Center, Amersham, England.

RESULTS

Composition of substrate. The chemical composition of cow waste is shown in Table 1.

TABLE 1. *Composition of dry matter of cow waste*

Component[a]	%
VS	82.0
Cellulose	16.0
Hemicellulose	10.5
Lignin	8.5
Total N	2.6
Ammonia-N	0.23
Non-ammonia crude protein[b]	14.8
Ether extract (crude lipid)	4.5
Volatile acid (as acetic)	1.8

[a] Methods for determining the components are given in the text.

[b] (Total N − ammonia-N) × 6.25.

The fiber constituents (cellulose, hemicellulose, and lignin) accounted for 43% of the VS. Non-ammonia crude protein accounted for 18% of the VS, and 5.5% of the VS was composed of lipid.

Methane production and acetate levels in large digestor. After an initial lag following feeding, methane production increased linearly and then declined slightly after 18 h (Fig. 1). Total methane production over the 24-h period was 1.7 liters. Acetate levels increased from 1.68 to 1.75 μmol/ml 0 to 2 h after feeding and then gradually decreased to 0.75 μmol/ml at 24 h. The initial increase in acetate levels coincided with a lag in methane production.

Acetate dissimilation and rate of methane production in small-scale incubations. Normally, actively digesting samples of cow waste were transferred to four Warburg-type flasks at selected intervals (3, 11, and 20 h) after feeding the large digestor. After incubation for 1 h, 0.028 μmol of [2-^{14}C]acetate (56 mCi/mmol) was injected into each flask. Samples of digestor contents were then removed at various intervals from one pair of flasks for acetate determinations, while gas samples were removed from the other pair of flasks for methane determinations. In several confirmatory experiments digestor contents were removed from only one flask instead of duplicate flasks. Normally, incubations were discontinued 2 h after injection of [2-^{14}C]-acetate.

For three experiments, representing the different times after feeding the digestor, plots of $\log_{10}$ of the specific radioactivity of acetate against incubation time gave straight lines (Fig. 2). Under conditions of constant pool size, the turnover rate constant (k) is equal to the slope (m) of the plots times 2.303. During the course of incubation in each of the above experiments,

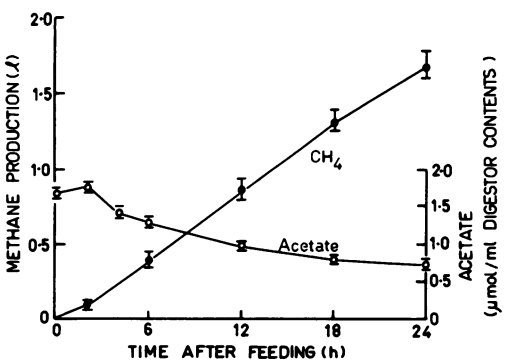

FIG. 1. *Methane production and pool size of acetate versus time after feeding large digestor. Symbols: (●) average of three determinations for methane production; (○) average of two determinations for acetate pool size. The bars represent the range.*

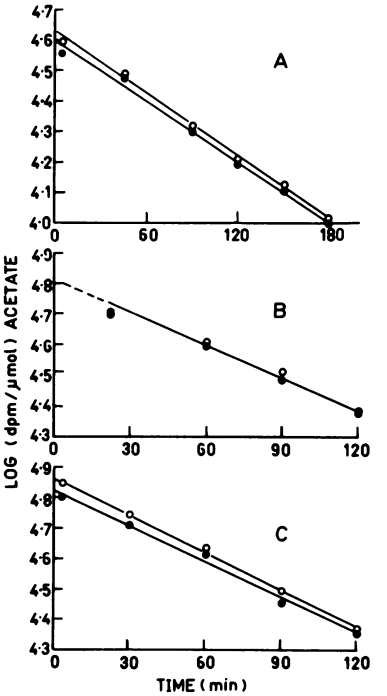

FIG. 2. *Decrease in specific radioactivity of acetate after addition of [2-^{14}C]acetate. (A) Plots for incubation representing a 4- to 7-h period after feeding. Average pool sizes were 1.31 (○) and 1.33 (●) μmol/ml. (B) Plots for 12 to 14 h after feeding. Average pool sizes were 1.03 (○) and 1.02 (●) μmol/ml. (C) Plots for 21 to 23 h after feeding. Average pool sizes were 0.72 (○) and 0.78 (●) μmol/ml.*

the pool size of acetate did not change appreciably and was similar to the pool size for the corresponding time intervals in the large digestor (see Fig. 1).

The turnover rate constants calculated from the plots described in Fig. 2 are presented in Table 2. Also presented are turnover rate constants calculated from similar plots (not shown) supporting the data obtained from Fig. 2. Examination of the data in Table 2 showed that although values for the turnover rate constant varied, they tended to increase with time after feeding. Conversely, the average values for the pool size of acetate decreased. For example, the average pool size of acetate for the 21- to 23-h period was almost half that determined for the 4- to 7-h period.

The rate of acetate dissimilation can be calculated by multiplying the turnover rate constant by the average pool size. Table 2 shows that the rate of acetate dissimilation declined as the time after feeding the digestor increased. For example, in the 21- to 23-h incubation period,

TABLE 2. *Comparison of rates of acetate dissimilation with rates of methane production at different times after feeding*

Expt	Period after feeding (h)	Turnover rate constant[a] (min^{-1})	Avg pool size of acetate[a] (μmol/ml)	Acetate dissimilation[a] (μmol/ml per min)	Methane production[b] (μmol/ml per min)
I	4–7	0.0075	1.32	0.0099	0.01
II[c]	4–7	0.0062	1.56	0.0097	0.0098
III	12–14	0.0088	1.025	0.009	0.01
IV[c]	12–14	0.0076	1.1	0.0084	0.0098
V	21–23	0.009	0.75	0.00675	0.0084

[a] Values for acetate in experiments I, III, and V are means and were determined from the plots in Fig. 2 for the corresponding times after feeding. The error in determining the means was 1% or less.

[b] Methane production rates in experiments I, III, and V were determined from the plots in Fig. 3 for the corresponding times after feeding.

[c] The volume of digestor contents added to incubation flasks was 80 ml.

the rate of acetate dissimilation was 0.69 of that calculated for the 4- to 7-h period.

Plots of methane production against incubation time for the experiments described in Fig. 2 are presented in Fig. 3. Rates of methane production determined from these plots and from other plots (not shown) obtained in confirmatory experiments are presented in Table 2. The results showed that the rate of methane production declined only in the latter stages after feeding.

Comparison of the rates of acetate dissimilation and methane production showed that with increased time after feeding the former declined whereas the latter was near constant. For example, in the 4- to 7-h incubation period the rate of acetate dissimilation was almost equal to the rate of methane production; however, in the 21- to 23-h period the rate of acetate dissimilation was only 0.8 that of methane production.

Incorporation of label into methane and CO_2 from [2-^{14}C]acetate. Values for the radioactivity in methane and CO_2 formed during the degradation of [2-^{14}C]acetate are presented in Table 3. Of the [2-^{14}C]acetate degraded during the 4- to 7-h period 3.8% of the label occurred in CO_2, whereas 9% occurred in methane. In the 21- to 23-h incubation period 88% occurred in methane, whereas 9.2% occurred in CO_2. The results showed that the fate of the methyl group of acetate did not appear to be significantly affected by time after feeding, and that approximately 90% of the methyl group was converted to methane.

Role of acetate as a precursor of methane, via the methyl group. Since in the degradation of acetate 0.9 mol of methane was formed per mol of acetate methyl group, the percentage of methane accounted for by acetate for any incubation period can be calculated from the following relationship: percent $CH_{4(Ac)}$ = [rate of acetate dissimilation (micromoles per milliliter per minute)/rate of methane production (micromoles per milliliter per minute)] × 0.9 × 100. Table 5 lists the percentage of methane accounted for by acetate (via the methyl group) in the various incubation periods. The results clearly showed that with increased time

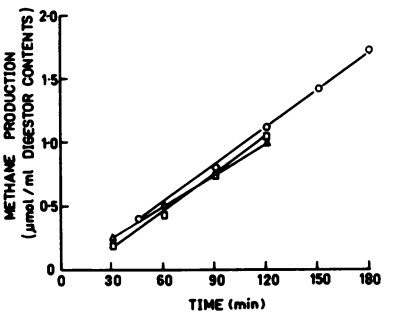

FIG. 3. *Methane production versus incubation time after addition of [2-^{14}C]acetate.* (○), (□), *and* (▲) *are each average values obtained for duplicate flasks for times after feeding of 4 to 7, 12 to 14, and 21 to 23 h, respectively.*

TABLE 3. *Incorporation of label into methane and carbon dioxide from [2-^{14}C]acetate*[a]

Period after feeding (h)	Incubation time (h)	[2-^{14}C]acetate[b] (dpm)	$^{14}CO_2$ (dpm)	$^{14}CH_4$ (dpm)
4–7	0	1.48×10^7		
	3	2.39×10^6	0.46×10^6	11.38×10^6
21–23	0	3.5×10^6		
	2	0.85×10^6	2.46×10^5	2.33×10^6

[a] Values represent the mean of two determinations.

[b] For the 4- to 7-h period 0.12 μmol of [2-^{14}C]acetate (56 mCi/mmol) was added to incubation flasks at zero time, whereas for the 21- to 23-h incubation 0.028 μmol of radioactive substrate of the same specific radioactivity was added. Values above are for total disintegrations per minute of acetate in flasks.

after feeding, acetate declined as a precursor of methane.

Methane production from CO_2. Actively digesting samples of cow waste (50 ml) were transferred to two flasks (130 ml) at each selected interval (1 and 11 h) after feeding the digestor. After the flasks were incubated for 1 h, $Na_2{}^{14}CO_3$ was injected into each. In one flask digestion was immediately terminated to obtain an initial estimation of the specific radioactivity of CO_2. In the other flask the contents were incubated for the appropriate time, and then the specific radioactivities of methane and CO_2 were determined. Over the selected incubation period (2 to 12 or 12 to 24 h) the specific radioactivity of CO_2 did not alter appreciably, and therefore an average value was taken when making a comparison with the specific radioactivity of methane (Table 4). For the 2- to 12-h period the ratio of the specific radioactivity of methane to that of CO_2 was 0.14, and in the 12- to 24-h period, 0.275. The percentage of methane accounted for by CO_2 in the former period was therefore 14%, and in the latter, 27.5% (Table 5). Thus, of the methane produced in the 2- to 12-h period 40.6 μmol was derived from CO_2, and in the 12- to 24-h period, 75.6 μmol. In the former the average rate of methane production from CO_2 was 4.06 μmol/h, and in the latter the average rate was 6.3 μmol/h. The results suggested that the activity of the H_2/CO_2-methanogenic bacteria was higher during the latter stages after feeding than in the early stages.

DISCUSSION

Under the conditions described here, there is little doubt that acetate accounts for most of the methane produced in the anaerobic digestion of bovine waste. Studies on the digestion of other types of organic waste have likewise shown acetate to be the major precursor of methane (7, 10, 16). In our digestor the percentage of methane accounted for by acetate (via the methyl group) decreased with time after feeding (Table 5). During the early stages after feeding (4 to 7 h) the methyl group of acetate accounted for almost 90% of the methane produced. However, as the time after feeding increased acetate declined as a precursor, so that in the 21- to 23-h period the methyl group accounted for only 73% of the methane produced.

The amount of methane formed from acetate is higher than would be predicted from the data in Table 1 if the theory of Jeris and McCarty (7) is followed. Although the composition of cow waste as listed in Table 1 is incomplete, from the combined protein, carbohydrate, and lipid fractions it could be predicted that during their degradation as much as 70% of the methane would be accounted for by acetate.

Values for the turnover rate constant of acetate (0.006 to 0.009 min^{-1}) are slightly higher than the value of 0.0052 min^{-1} reported by Smith and Mah (10) for the digestion of sewage sludge. Cappenberg and Prins (3) obtained a value of 0.0058 min^{-1} for acetate in mud from a freshwater lake. The rates of acetate dissimilation reported here are lower than the value of 0.024 μmol/ml per min previously reported for the digestion of sewage sludge (10).

During digestion the methyl group of acetate was mainly converted to methane, and the proportion of the methyl group converted did not alter appreciably with time after feeding. Previous studies with enrichment cultures of methanogenic bacteria have also shown that the methyl group of acetate is mainly converted to methane (11, 12). In the degradation of acetate reported here, a small amount (7%) of the methyl group was converted to CO_2. The pro-

TABLE 4. *Comparison of the specific radioactivity of $^{14}CH_4$ with $^{14}CO_2$ in the anaerobic digestion of cow waste*

Expt	Period after feeding (h)	Incubation time (h)	CO_2		CH_4		CH_4/CO_2[b]
			mmol[a]	dpm/mmol (× 10^{-6})	mmol	dpm/mmol (× 10^{-6})	
I[c]	2–12	0	1.69	3.07			
		10	1.88	3.03 (3.05)[d]	0.29	0.42	0.138
	12–24	0	1.99	2.63			
		12	1.91	2.50 (2.56)	0.29	0.78	0.304
II[c]	2–12	0	1.77	4.52			
		10	1.85	4.27 (4.4)	0.29	0.62	0.141
	12–24	0	1.96	4.38			
		12	1.90	3.74 (4.06)	0.26	1.00	0.246

[a] Measurement of the total amount of HCO_3^-/CO_2 in flask.

[b] Ratio of specific radioactivities.

[c] In experiment I 0.035 μmol of $Na_2{}^{14}CO_3$ (approximately 60 mCi/mmol) was injected into each digestor flask at zero time, whereas flasks in experiment II each received 0.05 μmol.

[d] Numbers in parentheses are averages.

TABLE 5. *Percentage of methane produced from acetate and CO_2 at various times after feeding*

Precursor	Period after feeding (h)	% Methane accounted for by precursor ± SD[a]
Acetate (via the methyl group)	4-7	89 ± 1.4[b]
	12-14	80.6 ± 1.6[b]
	21-23	72.8 ± 1.4[b]
CO_2	2-12	14 ± 0.7[c]
	12-24	27.5 ± 4[c]

[a] SD, Standard deviation.

[b] Means were determined from the data in Table 2 using the formula in text and are significantly different ($P < 0.01$).

[c] Means were determined from the data in Table 4 and are significantly different ($P < 0.05$).

duction of CO_2 from the methyl group of acetate has also been reported in enrichment cultures of methane bacteria (11, 12) and in lake sediments (3, 19). Winfrey and Zeikus (19) demonstrated that in the latter system there was a marked increase in the oxidation of the methyl group to CO_2 in the presence of sulfate. The small proportion of the methyl group converted to CO_2 reported here could be due to the activity of sulfate reducers. A sulfate-reducing bacterium, *Desulfotomaculum acetoxidans*, capable of oxidizing acetate to CO_2 has recently been isolated from bovine feces by Widdel (personal communication), and from mud by Widdel and Pfennig (18). The carboxyl group of acetate is mainly converted to CO_2 (3, 11, 12), and some of this evolved CO_2 can be further reduced to methane by addition of hydrogen.

Investigation of methane production from CO_2 showed that in the 2- to 12-h period 14% of the methane produced was accounted for by CO_2, whereas in the 12- to 24-h period the value was 27.5% (Table 5). These results are consistent with the decreased percentage of methane accounted for by acetate with increased time after feeding. The variation in the proportion of methane formed from CO_2 and acetate, related to time after feeding, has not been previously reported for other anaerobic digestors. One possible explanation relates to studies of Jeris and McCarty (7) and Weng and Jeris (17). These workers confirmed theoretical pathways by showing that more than 80% of methane can be derived from acetate during the degradation of certain amino acids (isoleucine and glutamic acid) whereas from the degradation of complex organic compounds the percentage of methane from acetate is lower. In the presence of HCO_3^-/CO_2 the proportions of methane from acetate and CO_2 appear to be determined by the ratio of hydrogen to acetate produced (7, 17). The higher the ratio the greater the proportion of methane formed from CO_2 reduction. Thus, in our digestor 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.

We have not investigated the degradation of other volatile acids such as butyrate and propionate because there is good evidence that in previously studied systems (for reviews see Pine [9] and Stafford [13]) these acids are degraded to acetate, which is in turn converted to methane. Furthermore, there have so far been no definitive reports of a methanogen capable of producing methane directly from such precursors.

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