Interrelations between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh-water lake. III. Experiments with ^{14}C-labeled substrates

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An ecological substrate relationship between sulfate-reducing and methane-producing bacteria in mud of Lake Vechten has been studied in experiments using ^{14}C-labeled acetate and lactate as substrates. Fluoroacetate strongly inhibited the formation of $^{14}CO_2$ from [U-^{14}C]-acetate and β-fluorolactate gave an inhibition of similar magnitude of the breakdown of [U-^{14}C]-L-lactate to $^{14}CO_2$ thus confirming earlier results on the specific action of these inhibitors.

The turnover-rate constant of L-lactate was 2.37 hr^{-1} and the average L-lactate pool size was 12.2 μg per gram of wet mud, giving a turnover rate of 28.9 μg of lactate/gram of mud per hr. The turnover-rate constant of acetate was 0.35 hr^{-1} and the average pool size was 5.7 μg per gram of wet mud, giving a rate of disappearance of 1.99 μg of acetate/gram of mud per hr. Estimations of the acetate turnover rate based upon the formation of $^{14}CO_2$ from [U-^{14}C]-acetate or [1–^{14}C]-acetate yielded figures of the same magnitude (range 0.45 to 1.74). These and other results suggest that only a portion of the lactate dissimilated is turned over through the acetate pool.

The ratio of $^{14}CO_2/^{14}CH_4$ produced from [U-^{14}C]-acetate by mud was 1.32; indicating that 0.862 moles of CH_4 and 1.138 moles of CO_2 are formed per mole of acetate. From the rate of disappearance of acetate (0.027 μmoles/gram wet mud per hr) and the rate of methane production (0.034 μmoles/gram wet mud per hr), it may be concluded that acetate is an important precursor of methanogenesis in mud (approximately 70%). A substrate relationship between the two groups of bacteria is likely since $^{14}CH_4$ was formed from [U-^{14}C]-L-lactate.

INTRODUCTION

Previous investigations indicated an ecological relationship between sulfate-reducing and methane-producing bacteria in mud of Lake Vechten (Cappenberg, 1974*a*, *b*). It was demonstrated that maximum numbers of the two bacterial groups occur at different depths and it was suggested that this was due to sensitivity of methane producers to hydrogen sulphide. The sulfate reducers were most abundant in the upper layers of the mud, the greater part of the methane producers were found deeper. Results of selective inhibition of methanogenic and sulfate-reducing processes in mud samples indicated that acetate is the main precursor of methanogenesis and lactate is the main source of energy for sulfate reduction. A substrate interrelationship between the two groups was postulated, since acetate is produced by sulfate reducers and is subsequently used by methane producers.

Further data on this substrate relationship were obtained with uniformly and non-uniformly labeled lactate and acetate as substrates in mud samples with and without the addition of specific inhibitors of sulfate-reducing and methanogenic processes. The end-products were assayed in short-term experiments in which the labeled substrates were not broken down fully and conclusions were drawn from the distribution of the radioactivity. Four requirements in the experiments must be met: (1) the amount of radioactive substrate added should not influence the pool size of this substrate, (2) the absence of induction phenomena, (3) complete mixing of the radioactive substrates, (4) linearity of response over time.

MATERIALS AND METHODS

Mud sampling. Undisturbed mud-cores were taken from Lake Vechten as described previously (Cappenberg, 1974*a*). The mud was used for the experiments either immediately or within 24 hr after storage at environmental temperatures in the dark. Previous experience indicated that no appreciable changes occurred in biochemical parameters of the mud samples during storage for 1 or 2 days at *in situ* temperatures.

Incubation technique. The incubations were conducted at the environmental temperatures (7–12 C) in 50-ml glass-stoppered Warburg vessels placed in a shaking waterbath (150 strokes/min). The center well of the vessels contained 0.2 ml of 10% KOH to absorb CO_2 and incubation was terminated by tipping 0.5 ml of 10 N H_2SO_4 from the sidearm into the main compartment. Shaking was continued for 5 min and the flasks were then placed in an icebath for 30 min to ensure complete uptake of CO_2 by the KOH.

To inoculate the vessels, mud samples (1–2 grams of wet mud) were collected

anaerobically from the mud cores as described earlier (Cappenberg, 1974*a*). The syringe was quickly weighed and then emptied into the main compartment of the vessels to start the incubation, and weighed again. To ensure a homogeneous distribution of mud and added labeled substrates, 1.0 ml of oxygen-free water had to be added to the vessels. This water had been boiled for several minutes and subsequently cooled under a stream of oxygen-free nitrogen. Anaerobiosis was maintained throughout the whole inoculation procedure by flushing with oxygen-free nitrogen. The nitrogen gas was freed from oxygen by passing it over a reduced copper column heated to 350 C. The glass stoppers used for closing the vessels were lubricated with vaseline. In some experiments when gas samples were taken for analysis, the glass stoppers were replaced by Suba-Seal caps.

Analyses. At the end of the incubations, the contents of the center well were transferred to counting vials, complete transfer being achieved by rinsing the well with 4 × 0.3 ml of water. The vials were assayed for radioactivity in a liquid scintillation counter (Tracerlab; Corumatic 25) after addition of 15 ml Unisolve 1 (Koch-Light Labs., Ltd., Colnbrook, England). All counts were corrected for background, for quenching and for the distillation of label during the incubations to the center wells.

In some experiments gas samples were taken from the gas phase of the vessels with a syringe inserted through the caps, after complete absorption of CO_2. These samples were slowly passed through a CuO column in quartz tubing and heated to 750 C to combust the methane to CO_2, which was then absorbed in an absorption train containing 2 N KOH and counted.

Mud samples that were analysed for radioactivity were inactivated with H_3PO_4 (final conc. ± 4 N) and from these acidified samples acetic acid and lactic acid were isolated by partition chromatography on cellulose powder columns (Whatman CF 11) eluted with mixtures of acetone in *n*-hexane (Hungate et al., 1970). The eluted fractions containing acetic acid or lactic acid were pooled separately, excess base was added, and the samples were dried in a rotating vacuum-evaporator. The acetate- or lactate-containing samples were redissolved and counted.

The concentrations of acetate and lactate in mud cores were assayed spectrophotometrically by specific enzymatic methods as described by Cappenberg (1974*b*).

Chemicals. All chemicals used were reagent-grade quality and obtained from commercial sources. The labeled substrates were purchased from the Radiochemical Centre, Amersham, England. The fluorolactate used was β-fluorolactate from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and the fluoroacetate from Calbiochem AG. (Lucerne, Switserland).

RESULTS

The results of pilot studies in which mud samples from different depths of the mud cores were incubated with labeled substrates in Warburg vessels, indicated that the formation of labeled CO_2 from these substrates was practically linear over a period of at least 2 hr. In all subsequent experiments therefore the incubation time was limited to 1–2 hr, except when stated otherwise.

Inhibition studies. In a previous report it was found that fluorolactate and fluoroacetate are potent inhibitors of lactate and acetate degradation in mud, respectively. In the presence of these inhibitors accumulation of lactate and acetate, respectively, in mud cores has been observed (Cappenberg, 1974*b*).

These findings have been tested again in a series of experiments using uniformly labeled L-lactate. The results (Table 1) show that fluorolactate strongly inhibited the formation of labeled CO_2 from L-lactate when incubated with mud samples from several depths of the mud core. Similar experiments were done with uniformly labeled acetate and fluoroacetate added to mud samples, which show similar results (Table 2).

Turnover of lactate. In mud, lactate is an intermediate, and previous studies have indicated that lactate is the main source of energy for sulfate reduction (Cappenberg, 1974*a*, *b*). From these studies it was concluded that the lactate pool size does not change appreciably and therefore, the rate of lactate dissimilation must equal its rate of formation. The magnitude of the lactate dissimilation was studied by the use of labeled L-lactate.

A large mud sample (46 grams) was removed from a mud core (upper 5 cm

Table 1. Effect of fluorolactate on the production of $^{14}CO_2$ from [U-^{14}C]-L (+) -lactate by mud from different depths[a]

Mud from layer at:	Substrate	Inhibitor	$^{14}CO_2$ produced/gram wet weight × hr cpm	
			Exp. 1	Exp. 2
−1 cm	L-lactate[b]	−	25,739	19,831
		+[c]	632	486
		Inhibition:	97.5%	97.5%
−5 cm	L-lactate	−	22,542	17,801
		+	1,345	1,263
		Inhibition:	94.0%	92.9%
−8 cm	L-lactate	−		5,311
		+		366
		Inhibition:		93.1%

[a] Incubation time: 1 hr at 7 C in exp. 1 and at 10 C in exp. 2.
[b] 0.1 μc of [U-^{14}C]-L(+)-lactate (spec. act. 45 m Ci/mmole) was added at zero time.
[c] Final concentration of fluorolactate: 0.015 mM.

Table 2. Effect of fluoroacetate on the production of $^{14}CO_2$ from [U-^{14}C]-acetate by mud from different depths[a]

Mud from layer at:	Substrate	Inhibitor	$^{14}CO_2$ produced/gram wet weight × hr cpm			
			Exp. 1	Exp. 2	Exp. 3	Exp. 4
−1 cm	acetate[b]	−	111,042	56,716	109,950	78,879
		+[c]	9,516	5,383	7,558	8,167
		inhibition:	91,6%	90.5%	93.1%	89.6%
−5 cm	acetate	−	82,095	124,557	77,368	120,431
		+	6,934	9,419	9,078	8,454
		inhibition:	91.6%	92.4%	88.3%	93.0%
−8 cm	acetate	−	74,047	86,276	64,574	55,914
		+	6,837	7,955	5,446	5,066
		inhibition:	90.8%	90.8%	91.6%	90.9%

[a] Incubation time: 5 hr 20 min at 13 C in exp. 1 and 2; 3 hr 30 min at 8 C in exp. 3 and 4.
[b] 0.2 μc of [U-^{14}C]-acetate (spec. act. 57 mCi/mmole) was added at zero time.
[c] Final concentration of fluoroacetate: 0.02 mM.

layer) and transferred to an erlenmeyer flask anaerobically. At zero time 0.0125 μmole of uniformly labeled Na-[^{14}C]-L-lactate was injected into the flask, the contents were rapidly mixed, and incubated at 10 C. Small subsamples (1–2 grams) were removed from the flask immediately after mixing and at 1-hr intervals. The subsamples were analyzed for radioactivity retained in the lactate after isolation with partition chromatography (see Materials and Methods section). The concentrations of L-lactate in the subsamples removed after 0, 1, 2, 3 and 4 hr were 15.0; 13.8; 9.0; 10.5 and 12.6 μg, respectively, per gram mud (wet weight), thus the small amount of labeled lactate added did not appreciably alter the lactate pool size.

When the ^{10}log of the cpm (counts per minute) in the lactate fraction (ordinate^y) was plotted against time in hourly intervals (abscissa^x), a straight line was obtained (correlation coefficient $r = -0.99$), which could be described by the formula: $y = -1.03x + 4.09$. The turnover rate (k) calculated from this plot was $k = 2.37\ hr^{-1}$. The dissimilation rate of an intermediate such as L-lactate is the product of the turnover rate times its pool size. The average L-lactate pool size during the experiment was 12.2 μg per gram of mud (wet weight), giving a rate of disappearance of L-lactate of 28.9 μg/gram of mud per hr.

Turnover of acetate. In mud, acetate is an intermediate and previous studies have indicated that acetate is the main precursor of methanogenesis (Cappenberg, 1974*a*, *b*). The turnover rate of acetate was also determined in the same experiment described above in which mud samples were incubated with uniformly labeled Na-[^{14}C]-L-lactate. Acetate formed from (labeled) lactate was isolated by partition chromatography from the same subsamples, the

acetate fractions were counted and the log values of the cpm in these fractions again plotted against time. The results are shown in Table 3 together with data obtained from a second experiment in which the turnover rate of acetate from the disappearance of [U-^{14}C]-acetate was estimated.

There is good agreement between the values obtained in these experiments using different substrates. In both experiments the acetate pool size remained constant. Since the turnover of lactate is a factor 10 $\times$ higher than the acetate turnover, this suggests that the larger part of the lactate turned over was converted to some other product than acetate.

The turnover of acetate was also measured in a number of experiments in which mud was incubated in Warburg vessels and in which the $^{14}CO_2$ produced from [1-^{14}C]-acetate or [U-^{14}C]-acetate was trapped. First, it was determined what the ratio of $^{14}CO_2/^{14}CH_4$ would be from both these radioactive substrates. From the results it is clear (Table 4) that labeled methane is formed from [1-^{14}C]-acetate. This result could not be explained if reduction of the methyl group of acetic acid were the sole mechanism of methanogenesis in mud. CO_2/H_2-fermenting methanogenic bacteria have been detected in our previous investigation (Cappenberg, 1974*a*). Therefore it seems likely that part of the carbon from the carboxyl group of acetate has been converted to methane by these bacteria. Probably CO_2 is an intermediate in this reaction. When the carboxyl carbon of acetate can only be converted into CO_2, we may conclude from the CO_2/CH_4 ratio that a fraction $1/7.43 = 0.134$ is reduced to CH_4.

There is also evidence from these results (Table 4), that oxidation of the methyl carbon of acetate to CO_2 occurs during mud digestion of acetate, since the ratio of $^{14}CO_2/^{14}CH_4$ found (1.32) is higher than the ratio (1.00) to be expected for acetate-splitting methanogenic organisms. If we assume that there is conversion of acetate by a split into CO_2 and CH_4 only and subsequent

Table 3. Kinetics of acetate metabolism in mud measured with two different tracer substrates

Tracer added	Plot formula	Corr. coeff.	Turnover rate hr^{-1}	Average acetate pool size µg/gram	Acetate dissimilation rate µg/gram per hr
[U-^{14}C]-L-lactate (spec. act. 45 mCi/mmole)	$y = -0.157x + 3.91$	−0.97	0.36	4.5	1.62
[U-^{14}C]-acetate (spec. art. 57 mCi/mmole)	$y = -0.146x + 3.50$	−0.99	0.34	6.9	2.35

Table 4. Ratio of $^{14}CO_2/^{14}CH_4$ in mud from a depth of −5 cm in the mud core calculated from [U-^{14}C]-acetate and [1-^{14}C]-acetate

Tracer used	$^{14}CO_2$	$^{14}CH_4$	$^{14}CO_2/^{14}CH_4$	Column No.
	cpm			
[1-^{14}C]-acetate (spec. act. 58 mCi/mmole)				
(0.1 μc)	68,083	13,904	4.89	1
(0.1 μc)	66,521	10,386	6.40	1
(0.2 μc)	112,559	14,574	7.72	2
(0.2 μc)	145,436	21,710	6.70	2
			Average 6.43	
[U-^{14}C]-acetate (spec. act. 57 mCi/mmole)				
(0.5 μc)	229,874	166,881	1.38	1
(0.5 μc)	222,408	173,119	1.29	2
(0.25 μc)	109,096	85,012	1.28	2
			Average 1.32	

reduction of CO_2 to CH_4 we would expect the formation of $1-0.134 = 0.866$ moles of CO_2 and $1 + 0.134 = 1.134$ moles of CH_4. Consequently the expected CO_2/CH_4 ratio would be $0.866/1.134 = 0.76$ which is much lower than the experimental value. This also indicates oxidation of methyl carbon to carbon dioxide. From these results we may conclude that the oxidation of methyl group or CH_4-oxidation to CO_2 is more important than the reduction of carboxyl group carbon to methane.

Evidence for the oxidation of acetate methyl carbon to CO_2 has been found also in a number of incubations with mud from the −5 cm layer (Table 5). When the radioactivity (cpm) in the $^{14}CO_2$ evolved from either [1-^{14}C]-acetate or [U-^{14}C]-acetate was expressed as a % of the radioactivity originally present in the carboxyl carbon of these substrates, higher values were always found with the [U-^{14}C]-acetate for the −5 cm layer in the mud, but not for either the −1 cm or of the −8 cm layers (Table 5).

The turnover of acetate has been measured in a number of experiments with [U-^{14}C]-acetate and in these experiments CO_2 was the only product analyzed for radioactivity. The ratio of $^{14}CO_2/^{14}CH_4$ produced from [U-^{14}C]-acetate by mud was 1.32 (Table 4) indicating that 0.862 moles of CH_4 and 1.138 moles of CO_2 are formed per mole of acetate. In order to calculate the rate of conversion of the labeled acetate from the $^{14}CO_2$ measurements a factor 1/1.138 was used. The results are shown in Table 6.

In view of the fact that only a portion of the lactate is turning over through the acetate pool, it can be concluded that estimation of the lactate turnover

Table 5. Production of $^{14}CO_2$ as percentage of the radioactivity from the carboxyl group carbon of [1-^{14}C]-acetate and [U-^{14}C]-acetate

Column No.	Depth of mud layer used	Substrate	cpm $^{14}CO_2$ as % original cpm C_1 evolved in 1 hr
1	−1	1-^{14}C-acetate	30.6
		U-^{14}C-acetate	23.8
2	−1	1-^{14}C-acetate	49.9
		U-^{14}C-acetate	49.1
1	−5	1-^{14}C-acetate	34.1
		U-^{14}C-acetate	42.5
2	−5	1-^{14}C-acetate	34.4
		U-^{14}C-acetate	42.3
3	−5	1-^{14}C-acetate	42.9
		U-^{14}C-acetate	60.8
4	−5	1-^{14}C-acetate	41.1
		U-^{14}C-acetate	58.3
1	−8	1-^{14}C-acetate	–
		U-^{14}C-acetate	58.2
2	−8	1-^{14}C-acetate	42.3
		U-^{14}C-acetate	44.9

Table 6. Turnover of acetate (μg/gram wet mud per hr) calculated from the production of $^{14}CO_2$ from [U-^{14}C]-acetate and the pool size of acetate (μg/gram wet mud) in mud from different depths. Turnover of acetate from the −1 cm layer not calculated as the pool size of acetate was not detectable (0–0.2 μg/gram wet mud).

Depth:		−5 cm		−8 cm	
		Pool size	Acetate turnover	Pool size	Acetate turnover
Exp. 1 column	1	–	–	11.8	0.64
13 C[a]	2	11.0	1.00	14.0	0.88
Exp. 2 column	1	8.7	0.75	–	–
8 C	2	6.0	0.83	12.0	0.75
Exp. 3. column	1	5.2	0.48	–	–
7 C	2	–	–	10.8	1.07
Exp. 3[b] column	1	5.2	0.50	–	–
7 C	2	–	–	10.8	1.34
Exp. 4 column	1	9.9	0.69	–	–
7 C	2	9.7	1.08	3.1	0.45
Exp. 9 column	1	5.4	1.65	–	–
10 C	2	6.1	1.69	–	–
Exp. 9[b] column	1	5.4	1.58	–	–
10 C	2	6.1	1.74	–	–

[a] Temperature of incubations.
[b] Based on 1-^{14}C-acetate data.

cannot be determined solely on the basis of formation of $^{14}CO_2$ from [U-^{14}C]-lactate. When the $^{14}CO_2$ and $^{14}CH_4$ were measured from [U-^{14}C]-lactate a ratio of 4.378 was found. The CO_2/CH_4 ratio of 1.32 found with [U-^{14}C]-acetate indicates that 0.862 moles of CH_4 and 1.138 moles of CO_2 are produced per mole of acetate. If we assume that lactate is completely converted into acetate and CO_2, we may expect 0.862 + 0.134 moles of CH_4 and 1.138 + 0.866 moles of CO_2. Consequently if this assumption was true a ratio of 2.012 might be expected (Table 7), showing that there is a discrepancy between theoretical and determined ratios which suggests that lactate is not completely converted through the acetate pool.

The turnover of lactate was measured in a series of experiments in which mud from different depths was incubated in Warburg vessels, trapping the $^{14}CO_2$ produced from [U-^{14}C]-lactate. A lactate turnover of 1.55 μg/gram wet mud per hr (mean of 7 exp.) at a depth of 1 cm in the mud core; of 0.61 μg/gram wet mud per hr (mean of 7 exp.) at −5 cm; and of 0.86 μg/gram wet mud per hr (mean of 4 exp.) at −8 cm were calculated. These calculations were based on the assumption that lactate is first converted quantitatively to acetate. The figures obtained in this way are much lower than the actual lactate turnover rate found (28.9 μg/gram wet mud per hr) by following the disappearance of lactate. These data show that measurement of the lactate turnover from determinations of $^{14}CO_2$ production from [U-^{14}C]-lactate cannot be relied upon as long as exact knowledge of the pathway of lactate breakdown in mud is lacking.

Table 7. Ratio of $^{14}CO_2/^{14}CH_4$ in mud from a depth of −1 cm in the mud core, calculated from [U-^{14}C]-lactate

Tracer used	$^{14}CO_2$	$^{14}CH_4$	$^{14}CO_2/^{14}CH_4$	Column No.
	cpm			
[U-^{14}C]-lactate	70,380	16,482	4.270	1
(0.5 μc)	74,494	16,768	4.443	1
	93,342	20,201	4.619	2
	67,216	16,089	4.179	2
			Average 4.378	

Theoretical ratio:

$$\begin{array}{lll} {}^{*}CH_3 & {}^{*}CH_3 \longrightarrow {}^{*}CH_4 & 1/2.32 \times 2 = 0.862 \\ | & | & \\ {}^{*}HCOH \longrightarrow & {}^{*}COOH \longrightarrow {}^{*}CO_2 & 1.32/2.32 \times 2 = 1.138 \\ | & & \\ {}^{*}COOH & {}^{*}CO_2 \searrow \begin{cases} {}^{*}CH_4 & 1/7.43 \times 1 = 0.134 \\ {}^{*}CO_2 & 6.43/7.43 \times 1 = 0.866 \end{cases} & \end{array}$$

$$\frac{{}^{*}CO_2}{{}^{*}CH_4} = \frac{(1.138 + 0.866)}{(0.862 + 0.134)} = \frac{2.004}{0.996} = 2.012$$

DISCUSSION

Previous investigations indicated an ecological relationship between sulfate-reducing and methane-producing bacteria in mud of Lake Vechten (Cappenberg, 1974*a*, *b*). When inhibitors of methanogenesis were added to mud samples, a decrease of the concentration of methane, an increase of the concentration of acetate and the presence of hydrogen gas was demonstrated. Similar experiments were performed with inhibitors of sulfate reduction, which showed an increase of the concentration of lactate and a decrease of the concentration of hydrogen sulphide. To obtain more experimental evidence, experiments were done with the same inhibitors added to mud samples incubated with uniformly labeled L-lactate or uniformly labeled acetate as substrates. The results (Table 1 and 2) showed that addition of fluorolactate or fluoroacetate strongly inhibited the degradation of lactate and acetate, respectively, from several depths of the mud core.

The turnover rate (k) of lactate, as given in the results, was 2.37 hr^{-1}. The average pool size in field studies of mud cores was 4.4 μg per gram of wet mud in the upper 5 cm (Cappenberg, 1974*b*), giving a rate of disappearance of L-lactate of 10.4 μg/gram wet mud per hr. The lactate pool size changed slightly in bottom deposits during the seasonal periodicity of Lake Vechten and therefore, the rate of lactate dissimilation must equal its rate of formation. As lactate is the main energy source of sulfate-reducing bacteria, a portion of the available lactate is converted into acetate and CO_2 which are subsequently used by methane-producing bacteria.

The turnover rate (k) of acetate (Table 3) was 0.35 hr^{-1}. The average acetate pool size in field studies was 5.2 μg per gram wet mud in the upper 5 cm (Cappenberg, 1974*b*), giving a rate of disappearance of acetate of 1.7 μg/gram wet mud per hr. The acetate pool size did not change appreciably in mud and also in the experiment with L-lactate there was no buildup of acetate. These observations suggest that the larger part of the lactate was converted to some product other than acetate, since the turnover rate of lactate is a factor 10 × higher than for acetate. It is suggested that this other product is a 3-carbon acid, e.g. propionic acid. Jeris and McCarty (1965) and Lawrence and McCarty (1969) using ^{14}C-tracers in methane fermentation experiments found that propionic acid was a major acid present in the degradation of carbohydrates and proteins in laboratory digesters.

Turnover rates of lactic or acetic acid have not previously been described in habitats similar to the sediments of Lake Vechten. Harrison, Wright and Morita (1971), Hall, Kleiber and Yesaki (1972), Sorokin and Kadota (1972), and Wood and Chua (1973) described methods for measuring the mineralization of

organic substrates in which labelled substrates were added to sediments. They obtained data of maximal velocity for mineralization (V_{max}), turnover time (T_t) and Michealis constant (K_m) for different substrates but not actual rates of turnover. Furthermore these experiments were not done under strictly anaerobic conditions. In another habitat (anaerobic sludge digestion) which is comparable to mud although richer in nutrients Smith and Mah (1966) calculated a turnover rate of 0.312 hr^{-1} of acetic acid and a rate of disappearance of 86.4 μg/ml wet sludge per hr. In the rumen, Gray, Jones and Pilgrim (1960) found a turnover rate of 0.45 hr^{-1} of acetic acid, calculated from the decline in specific activity of labeled acid; and Knox, Black and Kleiber (1967) found a turnover rate of 0.30 hr^{-1} using the isotope dilution technique. Rates of disappearance are not valid in this case, as most of the acetic acid will be absorbed in the rumen. Consequently the turnover rates of acetate in these habitats are of the same order as that now found for mud. However the rate of disappearance of acetate in these habitats is much higher.

Previous studies have shown that the rate of methane production in mud of Lake Vechten was 0.034 μmoles per gram wet mud per hr (Cappenberg, 1974*a*). The rate of disappearance of acetate is 0.027 μmoles per gram wet mud per hr. Assuming that 0.86 mole of methane is formed from 1 mole of acetic acid, under these conditions acetic acid would account for about 70% of the methane produced by the mud. Thus acetic acid is an important precursor of methanogenesis in mud. Jeris and McCarty (1965) concluded from their studies that about 70% of the methane produced in the anaerobic digestion process results from the degradation of acetic acid. Smith and Mah (1966), who studied the quantitative contribution of acetic acid to methane production in sludge using ^{14}C-labeled acetic acid, found that approximately 73% of the methane produced arose from this acid.

From the data in Table 4 it may be concluded that there is formation of methane not only from the reduction of the methyl group of acetic acid, but also from carbon from the carboxyl group of this acid, i.e. from CO_2. Previous studies showed that about 25% of the mixed population of the methanogenic bacteria in Lake Vechten are of the CO_2/H_2-fermenting types (Cappenberg, 1974*a*); therefore these bacteria may be assumed to contribute to the conversion of the carbon from the carboxyl group of acetate.

Also there is evidence (Table 4 and 5) that oxidation of the methyl carbon of acetate to CO_2 occurs during degradation of acetate in the mud, especially in the −5 cm layer of the bottom deposits. It is still unknown what kind of organisms play a role in this mechanism; possibly CH_4 is oxidized by sulfate-reducing bacteria as suggested by Cappenberg (1974*b*). It is also possible that radioactive CO_2 is produced from the methyl carbon of uniformly labeled

acetate that has been assimilated in the cell materials of the microorganisms. However, assimilation of the acetate carbon may be scant in comparison to dissimilation rates like it is for anaerobes in general.

The data of turnover of acetate in mud samples from different depths of the mud core (Table 6) indicated that the highest activity was obtained deeper in the mud. It had already been shown that maximum numbers of methanogenic bacteria are situated at depths of 3 to 6 cm in the mud (Cappenberg, 1974*a*), and that acetate-fermenting types are most abundant at a depth of 5 cm. As only part of the lactate is turning over through the acetate pool, the assessment of the lactate turnover cannot be based solely on the measurement of formation of labeled CO_2 from labeled lactate (Table 7). Although from these data no conclusions can be drawn regarding the turnover of lactate, they suggest highest activity in the upper layers of the mud. Observations indicated that sulfate-reducing bacteria are most abundant at depths of 0 to 2 cm in the mud (Cappenberg, 1974*a*).

The data in Table 7 suggest a substrate relationship between the two groups of bacteria since labeled methane was obtained from mud incubated with uniformly labeled lactate, although a higher ratio of labeled CO_2 and CH_4 was found than predicted by calculations. Definite answers cannot be given as long as exact knowledge of the pathway of lactate breakdown in mud is lacking.

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