

Kinetic Parameters and Relative Turnovers of Some Important Catabolic Reactions in Digesting Sludge

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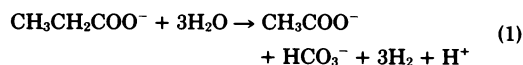
The kinetics of propionate degradation, acetate splitting, and hydrogen consumption in digesting sludge were investigated in a lab-scale digester. At natural steady-state conditions, the acetate-splitting systems in well-digested sludge were about half saturated. Propionate-degrading systems were saturated to only 10 to 15%, and hydrogen removal was less than 1% of the maximum possible rate. It was concluded that acetate splitting rather than "methanogenesis from fatty acids" is the rate-limiting reaction in the anaerobic degradation of dissolved organic matter and that a methanogenic anaerobic ecosystem is stabilized by its large unused capacity of hydrogen consumption which is "buffering" the partial pressure of dissolved hydrogen in the system at sufficiently low values to permit rapid fatty acid oxidation. A tentative scheme of the substrate flow in sludge digestion is presented. It suggests that acid formation coupled with hydrogen formation via pyridine dinucleotide oxidation yields the immediate substrates, namely acetate and hydrogen, for about 54% of the total methanogenesis.

Although extended experience in sludge digester operation has accumulated, the microbial ecology of this process is still poorly understood. Until recently, the anaerobic conversion of particulate, degradable organic compounds to methane and carbon dioxide was believed to comprise three steps: hydrolysis, acid formation, and methane production (1, 12). On this basis, methanogenesis from fatty acids was considered the rate-limiting step in the digestion of dissolved organics (7, 14), and the hydrolysis of insoluble polymers was regarded as rate limiting for the overall process of sludge digestion (6). However, new results (2, 18; H. F. Kaspar and K. Wuhrmann, *Microb. Ecol.*, in press) support the idea, first expressed by Bryant et al. (3) and later discussed extensively by Toerien et al. (20), of a further group of bacteria capable of degrading fatty acids to acetate, carbon dioxide, and hydrogen. Thus, the question as to the rate-limiting step in the digestion of organic matter was raised again.

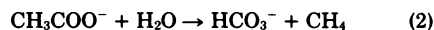
The present work examines the kinetics of the following three catabolic reactions of anaerobic decomposition.

(i) The degradation of fatty acids is examined; the oxidation of propionate is quantitatively (8, 18; H. F. Kaspar, Ph.D. thesis, Swiss Federal

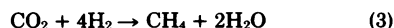
Institute of Technology, Zurich, 1977) and qualitatively (Kaspar, Ph.D. thesis; Kaspar and Wuhrmann, *Microb. Ecol.*, in press) of specific interest. Formally, it might be expressed as follows:



(ii) Methanogenesis by acetate splitting is examined; it is now well established that about 70% of the methane produced in sludge digestion is derived from the methyl group of acetate (2, 8, 13, 17) as follows:



(iii) Lithotrophic methane formation is examined (2, 22, 24) according to the following:



This reaction provides for the remaining 30% of the methane produced.

In this investigation, the kinetic parameters K_s and V_{max} of the three catabolic reactions were measured. The comparison of these values with observations on conversion rates in natural systems was thought to indicate the true rate-limiting reaction in the digestion of dissolved organic material. Product inhibition studies were carried out to quantitatively determine the substrate flow in sludge digestion, with special reference to hydrogen gas.

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MATERIALS AND METHODS

Sludge. Samples of well-digested sludge were taken from a municipal waste treatment plant (Kaspar and Wuhrmann, *Microb. Ecol.*, in press). In situ concentrations of acetate and propionate were around 0.5 mmol/liter and less than 0.05 mmol/liter, respectively. The hydrogen partial pressure in the digester gas was below 10^{-4} atm.

Chemicals. Sodium propionate (per analysis), sodium acetate (per analysis), and gases (H_2 , CO_2 , He) were obtained from commercial sources. Traces of oxygen in the gases were removed by passing them over heated copper (340°C) and through columns of Oxisorb (Messer Griesheim, Düsseldorf, West Germany [A. J. B. Zehnder, Ph.D. thesis, Swiss Institute of Technology, Zurich, 1976]). Final concentrations were ≤ 0.1 mg of O_2 per liter.

Apparatus. Batches of 7 liters of digested sludge were observed in a lab-scale digester (Bioengineering AG, Rüti, Switzerland). Before starting an experiment, the system was flushed with a mixture of 30% CO_2 and 70% He (for further technical details, see Kaspar, Ph.D. thesis). Sludge temperature was 33°C, and the pH was controlled at 7.4 by carbon dioxide dosage. Moistened mixtures of helium, hydrogen, and carbon dioxide were continuously equilibrated with the liquid phase of the sludge (two flat-blade stirrers [1,000 rpm] and four baffles in the fermentor vessel). Gas pressure was maintained at 1.03 to 1.06 atm. Sludge samples were drawn through a valve preventing the access of oxygen to the digester. Hydrogen sulfide and water vapor were removed from the digester gas by a silica gel filter. All tubes and connections were stainless steel (Hoke Inc., Cresskill, N.J.).

Sampling and transfer of the sludge from the municipal digester into the laboratory fermentor caused a slight disturbance of the steady-state conditions (a temporary rise of the hydrogen partial pressure to about 10^{-3} atm and an increase of the volatile fatty acid concentrations). Experiments were therefore started after an equilibration period of at least 24 h in the fermentor. The hydrogen partial pressure was then again less than 10^{-4} atm, and the concentrations of acetate and propionate had decreased to a steady-state level of about 0.2 mmol/liter, and less than 0.02 mmol/liter, respectively.

Analyses. The volume of gas leaving the digester was measured by a wet test gas meter (Elster, Mainz-Kastel, Germany). Gas analyses were performed at intervals of 1 h (two-column gas chromatograph, molecular sieve 5A, Porapak Q, thermoconductivity detector, Gow Mac Instruments Co. Ltd., Shannon Eire). Volatile fatty acids were analyzed with a Pye Unicam series 104 two-column chromatograph (Porapak QS, flame ionization detectors).

RESULTS

Kinetics of acetate degradation. Figure 1 exemplifies the type of experiments used for determining the kinetics of acetate degradation in digesting sludge. The initial steady-state acetate concentration $[(Ac)_0]$ and methane production rate (V_{0,CH_4}) yielded the steady-state acetate degradation rate ($V_{0,Ac}$), assuming $V_{0,Ac} \approx 0.7$

V_{0,CH_4} (8, 13, 17). The acetate concentration was then raised to 1 to 2 mmol/liter by adding sodium acetate. During the subsequent hours, the acetate degradation occurred at conditions of saturation ($V_{1,Ac}$), and its rate could be calculated as $V_{1,Ac} = V_{0,Ac} + \Delta(Ac)/\Delta t$. When the acetate concentration had dropped to values of less than 0.5 to 0.7 mmol/liter, its degradation became concentration dependent. This phase permitted the determination of the kinetic parameters $V_{max,Ac}$ and $K_{s,Ac}$ assuming a one-enzyme type of reaction. Lineweaver-Burk plots of measurements at intervals of 1 h gave very satisfactory linear regressions. Table 1 lists the results of five experiments. The initial average steady-state concentration of acetate was 0.26 mmol/liter, and the average steady-state degradation rate was 0.27 mmol/liter·h. Thus, the mean turnover rate of acetate was about 1/h. Saturation of the respective systems enhanced the acetate degradation rate by 56% to 0.41 mmol/liter·h. The calculated maximum rate was 0.63 mmol/liter·h, and the corresponding $K_{s,Ac}$ amounted to 0.32 mmol/liter. The difference between calculated and measured maximum rates might be explained by factors other than substrate concentration limiting the conversion in the phase of the linear decrease of acetate concentration.

Kinetics of hydrogen removal. A further series of experiments was performed to determine the kinetics of hydrogen turnover in digesting sludge (Fig. 2). Three hours before starting the experiment, the acetate concentration was increased to 2 to 3 mmol/liter by the addition of sodium acetate. In accordance with the results of the preceding experiments, a constant acetate degradation rate occurred which amounted to 80% of V_{1,CH_4} observed in the initial phase. Assuming that the total of CH_4 generated is the sum of CH_4 produced from acetate (equation 2) and from H_2 (equation 3), the rate of methane production from hydrogen was 0.2 V_{1,CH_4} , and the hydrogen removal rate on the basis of equation 3 (V_{0,H_2}) was 0.8 V_{1,CH_4} . The partial pressure of hydrogen was then increased by a continuous addition of this gas. After about 3 h, a new equilibrium of the hydrogen removal rate (V_{1,H_2}) corresponding to the new partial pressure (P_{1,H_2}) was established. Table 2 summarizes the results of five such experiments. Calculation of the kinetic parameters (Michaelis-Menten equation, Lineweaver-Burk regression) gave a K_{s,H_2} of 0.105 atm, and a V_{max,H_2} of 103 mmol/liter·h. At hydrogen pressures up to 0.7 atm and stirrer speeds of 2,000 rpm, the highest measured H_2 removal rate observed was 20.3 mmol/liter·h, which corresponds to a 24-fold enhancement compared with the natural steady-state turnover. This is still far from the

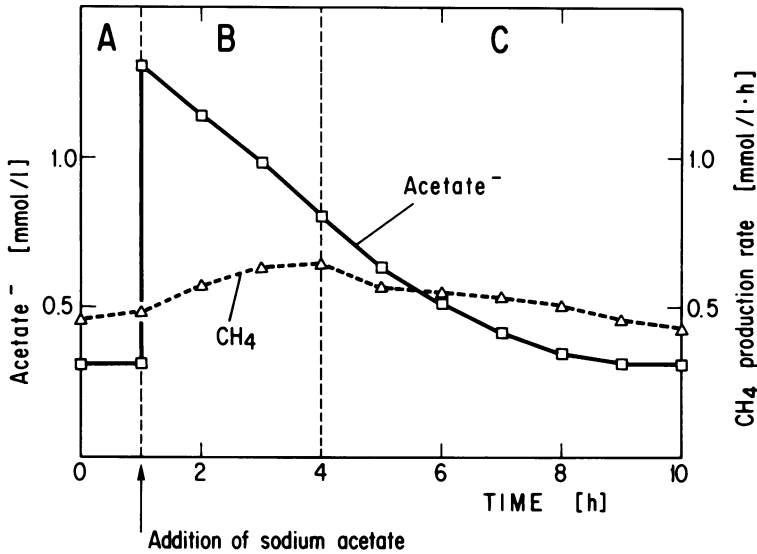


FIG. 1. Acetate degradation in digesting sludge. (A) Acetate concentration and methane production at natural steady-state conditions. (B) Acetate degradation at saturation of the system. (C) Acetate degradation at concentration-dependent conditions.

TABLE 1. Kinetics of acetate degradation in digesting sludge^a

Expt no.	(Ac) ₀ (mmol/liter)	V _{0,Ac} (mmol/liter·h)	V _{1,Ac} (mmol/liter·h)	V _{max,Ac} (mmol/liter·h)	K _{s,Ac} (mmol/liter)	r
1	0.31	0.32	0.48	0.71	0.21	0.987
2	0.42	0.41	0.52	0.86	0.39	0.941
3	0.23	0.27	0.38	0.58	0.28	0.973
4	0.20	0.20	0.38	0.61	0.45	0.981
5	0.16	0.17	0.30	0.41	0.25	0.977
Average	0.26	0.27	0.41	0.63	0.32	

^a (Ac)₂ and V_{0,Ac}, Concentration and degradation rates, respectively, of acetate at natural steady-state conditions; V_{1,Ac}, acetate degradation rate at system saturation; V_{max,Ac} and K_{s,Ac}, calculated maximum acetate degradation rate and half-saturation concentration, respectively; and r, correlation coefficient of Lineweaver-Burk regression.

calculated maximum rate of 103 mmol/liter·h. The difference is most probably due to the difficulty of achieving sufficiently high dissolution rates of H₂ in aqueous media.

Relative turnovers of propionate, acetate, and hydrogen. Experiments, as shown in Fig. 2, allowed the splitting of the total steady-state acetate production into three parts: acetate production which cannot be inhibited by increased H₂ pressures, acetate production from propionate, and acetate production by other reactions which can be inhibited by increased H₂ pressures (23; Kaspar and Wuhrmann, *Microb. Ecol.*, in press). An increase of the hydrogen partial pressure from <10⁻⁴ to ≥0.015 atm caused a linear accumulation of propionate and an accelerated decrease of the acetate concentration. Because no enhancement of propionate production due to the increase of P_{H₂} could be detected in earlier experiments (Kaspar and

Wuhrmann, *Microb. Ecol.*, in press), the rate of the propionate accumulation was assumed to be equal to the propionate turnover rate in the preceding steady state, and the seemingly enhanced acetate degradation could be interpreted as a partial inhibition of the acetate production. Table 3 shows the results of five experiments. Hydrogen pressures between 0.015 and 0.725 atm gave rise to a 50% average inhibition of the acetate production. The average propionate turnover rate (as determined by hydrogen inhibition) was 12% of the steady-state acetate turnover rate. With the aid of equations 1 and 3, 15% of the total steady-state methane production was computed to derive from propionate, which is confirmed by data published elsewhere (5, 11, 18). Similar calculations suggested that 21% of the total steady-state hydrogen evolution originates from propionate degradation.

Kinetics of propionate oxidation. The ki-

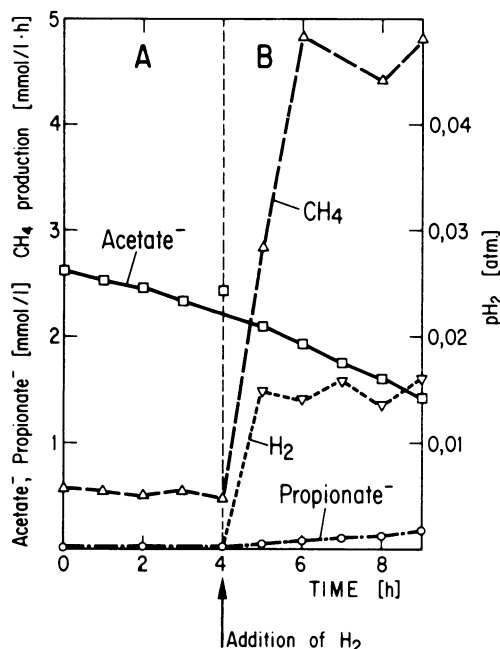


FIG. 2. Influence of increased hydrogen pressure on turnover of propionate, acetate, and hydrogen. (A) Acetate degradation and methane production at saturation of the acetate-degrading system. (B) Propionate accumulation, enhanced decrease of acetate concentration, and hydrogen consumption (i.e., methane production) at increased hydrogen pressure.

netics of the propionate turnover in digesting sludge were determined in analogy to the experiments with acetate (Fig. 3). As shown before, the propionate oxidation rate in the natural steady state of digesting sludge is about 12% of the acetate-splitting rate. Therefore, the steady-state propionate degradation rate could be expressed as $V_{0,Prop} = 0.08 V_{0,CH_4}$ (according to equations 1 and 3; measurements of V_{0,CH_4} in the first phase of the experiment). The average

value, as determined, was 0.03 mmol/liter · h. As already mentioned, the corresponding propionate concentration was less than 0.02 mmol/liter to the effect that the turnover rate was more than 1.5/h. When the propionate concentration was raised to 1 to 2 mmol/liter by the addition of sodium propionate, the degradation rate of the acid at saturation conditions increased by a factor of 6 up to an average of 0.19 mmol/liter · h. When the propionate removal rate finally became concentration dependent, kinetic parameters were determined in a manner similar to the experiments with acetate. The results were: maximum rate ($V_{max,Prop}$) between 0.16 and 0.30 mmol/liter · h, with an average of 0.23 mmol/liter · h; $K_{s,Prop}$ between 0.04 and 0.19 mmol/liter, with an average of 0.09 mmol/liter (average values of five experiments [Table 4]).

DISCUSSION

In the well-digested sewage sludge under investigation, the average steady-state concentration of acetate and its degradation rate were 0.26 mmol/liter and 0.27 mmol/liter · h, respectively. Assuming a one-substrate, one-enzyme reaction, the kinetic parameters found ($K_{s,Ac} = 0.32$

TABLE 2. Kinetics of hydrogen consumption in digesting sludge^a

Expt no.	P_{0,H_2} (atm)	V_{0,H_2} (mmol/liter · h)	P_{1,H_2} (atm)	V_{1,H_2} (mmol/liter · h)
1	$<10^{-4}$	0.88	2.12×10^{-2}	14.72
2	$<10^{-4}$	0.80	1.68×10^{-2}	14.04
3	$<10^{-4}$	0.76	1.04×10^{-2}	10.56
4	$<10^{-4}$	0.92	0.76×10^{-2}	7.08
5	$<10^{-4}$	0.88	0.38×10^{-2}	3.56

^a P_{0,H_2} and V_{0,H_2} , partial pressure and removal rate, respectively, of hydrogen at natural steady-state conditions; and V_{1,H_2} , hydrogen-removal rate at increased partial pressures P_{1,H_2} . From Lineweaver-Burk regression: $K_{s,H_2} = 0.105$ atm; $V_{max,H_2} = 103$ mmol/liter · h; $r = 0.995$.

TABLE 3. Influence of hydrogen on turnover of propionate and acetate^a

Expt no.	P_{0,H_2} (atm)	$V_{0,Ac}$ (mmol/liter · h)	$V_{1,Ac}$ (mmol/liter · h)	P_{1,H_2} (atm)	$V_{2,Ac}$ (mmol/liter · h)	$V_{0,Prop}$ (mmol/liter · h)	$V_{0,Prop}/V_{0,Ac}$ (%)	$(V_{2,Ac} - V_{1,Ac})/V_{0,Ac}$ (%)
1	$<10^{-4}$	0.21	0.29	0.015	0.39	0.03	14	48
2	$<10^{-4}$	0.21	0.43	0.339	0.54	0.03	14	52
3	$<10^{-4}$	0.39	0.64	0.420	0.82	0.04	10	46
4	$<10^{-4}$	0.22	0.41	0.512	0.51	0.03	14	46
5	$<10^{-4}$	0.12	0.23	0.725	0.29	0.01	8	58
Average							12	50

^a $V_{2,Ac}$, Apparent acetate degradation rate at increased hydrogen pressure; $V_{0,Prop}$, steady-state propionate degradation rate as determined by hydrogen inhibition; $V_{0,Prop}/V_{0,Ac}$, percentage of acetate produced from propionate; $(V_{2,Ac} - V_{1,Ac})/V_{0,Ac}$, percentage of acetate production being inhibited by increased hydrogen pressure.

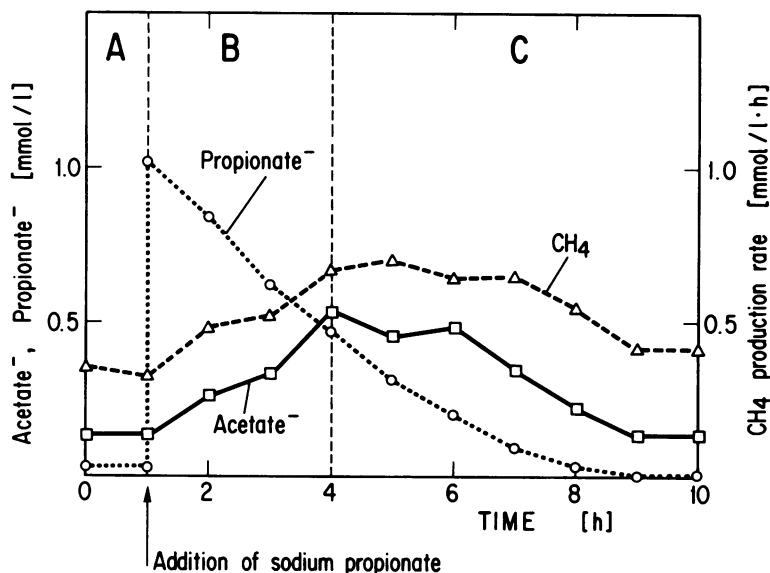


FIG. 3. Propionate degradation in digesting sludge. (A) Methane production and propionate and acetate concentrations at natural steady-state conditions. (B) Propionate degradation after saturation of the system. (C) Propionate degradation in concentration-dependent phase.

TABLE 4. Kinetics of propionate turnover in digesting sludge^a

Expt no.	$V_{0,Prop}$ (mmol/liter·h)	$V_{1,Prop}$ (mmol/liter·h)	$V_{max,Prop}$ (mmol/liter·h)	$K_{s,Prop}$ (mmol/liter)	r
1	0.04	0.22	0.303	0.191	0.983
2	0.03	0.21	0.232	0.048	0.993
3	0.03	0.21	0.269	0.121	0.991
4	0.03	0.18	0.204	0.070	0.998
5	0.02	0.13	0.161	0.044	0.994
Average	0.03	0.19	0.233	0.094	

^a $V_{0,Prop}$, Propionate removal rate at steady-state conditions; $V_{1,Prop}$, propionate removal rate at conditions of enzyme saturation; $V_{max,Prop}$ and $K_{s,Prop}$, calculated maximum propionate degradation rate and half-saturation concentration, respectively; and r , correlation coefficient of Lineweaver-Burk regression.

mmol/liter, $V_{max,Ac} = 0.63$ mmol/liter·h) indicate that the acetate-degrading system was slightly less than half-saturated under natural conditions (Table 1). Similar kinetic data were published for other digesting sludges (9, 15, 17) and enrichment cultures from various inocula (4, 21). In the same system, the natural steady-state concentration of propionate was less than 0.02 mmol/liter, and degradation occurred at a rate of about 0.03 mmol/liter·h. Average values of 0.09 mmol/liter and of 0.23 mmol/liter·h were found for $K_{s,Prop}$ and the maximum degradation rate ($V_{max,Prop}$), respectively. Hence, the propionate-oxidizing systems were only saturated to the extent of 10 to 15% (Table 4). Comparable values were published for enrichment cultures (10) and for digesting sludge (18). At natural hydrogen pressures in the original sludge of less

than 10^{-4} atm, its consumption rate was about 0.85 mmol/liter·h. The respective turnover rate, therefore, was $>1.1 \times 10^4$ /h. Values of $K_{s,H_2} = 0.105$ atm and of $V_{max,H_2} = 103$ mmol/liter·h were detected experimentally. These figures indicate that the actual hydrogen concentration and removal rates were more than 2 orders of magnitude smaller than the optimum values found for the hydrogen consumers in the system. The data are consistent with results published by Shea et al. (16) who concluded that in sludge digestion, the hydrogen removal rate is less than 3% of the maximum possible rate.

The experimental results indicate that the actual concentration of the main substrate for lithotrophic methanogenesis in well-digesting sludge, i.e., H_2 , is limiting the rate of its utilization to about 1% of the potential rate inherent

to the sludge biocenosis. Consequently, methane production from hydrogen is by no means rate limiting. Nevertheless, this reaction is of fundamental ecological significance because it "buffers" the hydrogen pressure in the system at values low enough to permit the exergonic oxidation of fatty acids (Kaspar and Wuhrmann, *Microb. Ecol.*, in press).

All essential reactions leading from dissolved organic matter to methane, namely, fermentation, fatty acid oxidation, acetate splitting, and lithotrophic methanogenesis, are catalyzed by individual enzyme systems of distinct groups of bacteria. In the sludge under study, the rate of propionate degradation could be accelerated by saturation of the propionate oxidation system by $480 \pm 108\%$ (average of seven assays). The respective relative acceleration for the acetate-splitting system was only $76 \pm 19\%$ (average of 10 assays). Hence, the degradation of acetate rather than "methanogenesis from fatty acids" is suggested to be the rate-limiting step in the reaction sequence of digestion of dissolved organic matter. We recognize, however, that in natural systems in which the hydrolysis of a variety of solid biopolymers is at the beginning of the degradation chain, the true rate-limiting reaction of the overall process is difficult to define.

A quantitative interpretation of the substrate flow in the digestion of dissolved organic material requires a common yardstick for measuring the various educts and products. A "theoretical chemical oxygen demand flow" may be established by introducing their oxidation equivalents, i.e., chemical oxygen demand. Based on our kinetic studies, the tentative scheme of Fig. 4 is proposed, indicating the substrate flow in digesting sewage sludge (of average composition, because it is found in conventional activated sludge treatment plants for predominantly domestic wastes). Because our knowledge on the biochemistry of H_2 formation during anaerobic degradation is still incomplete (19), the following two assumptions were made on the basis of thermodynamics (23; Kaspar and Wuhrmann, *Microb. Ecol.*, in press). (i) The reactions leading to hydrogen and acetate which can be inhibited by hydrogen pressures around 0.5 atm are embraced in a step tentatively called "oxidation, H_2 formation via pyridine nucleotide/ferredoxin systems" (although other pathways of H_2 formation may perhaps exist). (ii) Hydrogen and acetate formations which cannot be inhibited by H_2 pressures as high as 0.5 atm are assumed to start from pyruvate as an intermediate substrate (pyruvate oxidation by ferredoxin: e.g., acetylphosphate formation). With these simplifica-

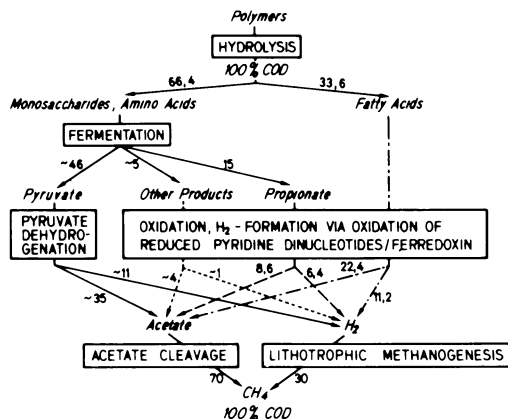


FIG. 4. Tentative scheme of substrate flow in steady-state anaerobic digestion of organic matter, with special reference to hydrogen production. Unit: percentage of total flow of theoretical chemical oxygen demand (COD), assuming a raw sludge from a conventional activated sludge plant treating domestic sewage of average composition.

tions in mind, it may be deduced from the relative turnovers and the stoichiometry according to equations 1 to 3 that hydrolysis products from fats account for about one-third of the total chemical oxygen demand flow, whereas the degradation of proteins and carbohydrates yields about two-thirds of the intermediate chemical oxygen demand. Various acidic fermentation products (especially propionate) plus fatty acids from fats would account for about 54% of the original chemical oxygen demand. These represent compounds which are further oxidized to acetate and hydrogen by electron transfer via pyridine dinucleotides (19). At least 63% of the total hydrogen evolution in the overall process would occur via this oxidation. Neither the organisms responsible for these oxidations nor any details of the mechanisms involved are known up to now (see, however, M. J. McInerney, M. P. Bryant, and N. Pfennig, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1978, I82, p. 94). Thus, hydrogen production from pyruvate dehydrogenation is quantitatively much less important than hydrogen production in final reactions requiring reduced nicotinamide adenine dinucleotide oxidation in pyridine dinucleotide/ferredoxin redox systems. It is obvious, therefore, that the bacteria capable of the anaerobic fatty acid oxidation coupled with hydrogen evolution are irreplaceable organisms in anaerobic methane-forming ecosystems. Their further investigation is urgently needed for a better understanding of sludge digestion and anaerobic mineralization processes in general.

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