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Pathways of Propionate Degradation by Enriched Methanogenic Cultures

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A mixed methanogenic culture was highly enriched in a growth medium containing propionate as the sole organic carbon and energy source. With this culture, the pathways of propionate degradation were studied by use of ^{14}C-radiotracers. Propionate was first metabolized to acetate, carbon dioxide, and hydrogen by nonmethanogenic organisms. Formate was not excreted. The carbon dioxide originated exclusively from the carboxyl group of propionate, whereas both [2-^{14}C]- and [3-^{14}C]propionate lead to the production of radioactive acetate. The methyl and carboxyl groups of the acetate produced were equally labeled, regardless of whether [2-^{14}C]- or [3-^{14}C]propionate was used. These observations suggest that in the culture, propionate was degraded through a randomizing pathway.

In methanogenic systems, propionate is degraded by a special group of microorganisms, the so-called obligate proton-reducing acetogens (2, 10, 12). These bacteria can only function if the partial pressure of hydrogen is kept low by hydrogen-consuming organisms. Boone and Bryant (1) isolated an organism which is able to degrade propionate in syntrophic culture with a hydrogen-consuming *Desulfovibrio* species. This organism, called *Syntrophobacter wolinii*, oxidizes propionate to acetate and presumably carbon dioxide and hydrogen (or formate). In a culture (called *Methanobacterium propionicum*) enriched by Stadtman and Barker (9), the ^{14}C-labeled carboxyl group of propionate appears exclusively in the carbon dioxide, whereas the accumulated acetate is practically free of labeled carbon. Buswell et al. (3), using an enriched propionate-degrading methanogenic culture and specifically labeled [^{14}C]propionate, demonstrated that after complete degradation of the fatty acid, all three carbons appear in both carbon dioxide and methane. This suggests that the entire degradation of propionate by a syntrophic microbial culture is not simply a sequence of two decarboxylation steps (first, propionate to acetate; second, decarboxylation of acetate by acetophilic methanogens) with a parallel removal of the hydrogen.

In this paper, we describe the exact labeling pattern of the products (acetate, carbon dioxide, and methane) formed by a highly enriched methanogenic culture during growth on propionate with different ^{14}C labels. In addition, we report that formate was not an obligate extracellular product of propionate catabolism.

Samples of digested sludge from the sewage treatment plant in Opfikon, Switzerland, were used to enrich for a propionate-degrading methanogenic culture. The sterile medium used for cultivation of the mixed culture (prepared from stock solutions) contained the following (grams per liter, except as noted): KH_2PO_4, 0.6; Na_2HPO_4, 1.33; NH_4Cl, 0.3; $CaCl_2 \cdot H_2O$, 0.11; $MgCl_2 \cdot 6H_2O$, 0.1; $NaHCO_3$, 4; cysteine hydrochloride, 0.05; $Na_2S \cdot 9H_2O$, 0.025; trace metal solution (13), 1 ml; vitamin solution (11), 1 ml; and sodium propionate, 3.9. The headspace was 80% N_2–20% CO_2 (73.5-kPa overpressure). The enrichment culture was routinely cultivated at 33°C in the dark on a shaker (80 rpm; amplitude, 5 cm) in serum vials (118 ml), each containing 30 ml of medium. Concentrations of methane and short-chain fatty acids were estimated with a gas chromatograph as described by Zehnder et al. (15). Preparative separation of propionate, acetate, and formate was performed with a high-pressure liquid chromatograph equipped with a spectrophotometer detector set at 210 nm and a reversed-phase column (4 by 250 mm) packed with Licrosorb RP-18, 5 μm (E. Merck AG, Darmstadt, Germany). Perchloric acid (0.01 N) or hydrochloric acid (0.01 N) was the carrier (flow rate, 2 ml/min; 20,000 kPa). Samples were filtered (0.2-μm membrane) and acidified (pH 2.2) with perchloric or hydrochloric acid,

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sparged with air for 2 min, and subsequently injected with a loop injector (Rheodyne 70-10 [Kontron, Zurich]; 1,060 μl). Gaseous [^{14}C]carbon dioxide and [^{14}C]methane were measured by the method of Zehnder et al. (14, 15). To determine the positions of labeled acetate formed during degradation of [2-^{14}C]- and [3-^{14}C]propionate, the two acids were separated from the medium with a high-pressure liquid chromatograph and HCl (0.01 N) as the carrier. The acetate fraction was collected, brought to pH 9 with NaOH, and evaporated to dryness (50°C). The residue was dissolved in 1 ml of distilled water, and the pH was adjusted to 7 with HCl. This sample was added to a culture of *Methanothrix soehngenii* (4), and the labeling pattern was determined from the $^{14}CO_2$ and $^{14}CH_4$ produced (13).

About a year was needed to establish an enrichment culture with a stable bacterial population. The initial enrichment culture was started by adding 5 ml of digested sludge to 45 ml of mineral salt medium. Every 3 weeks, 25 ml of the inoculated culture were transferred to 25 ml of fresh medium. After nine transfers, samples of the last inoculum were diluted according to the most-probable-number technique. The highest dilution (10^{-6}) showing growth was used for the study. The final population consisted of four distinctly different organisms. Three of them, which made up about 90% of the total cell number, were methanogens. One was autofluorescent when viewed by epifluorescence microscopy (coenzyme F_{420}), motile, and morphologically identical to *Methanospirillum hungatii*. The second, an autofluorescent nonmotile rod, resembled *Methanobrevibacter arboriphilus*. The third, a nonfluorescent large rod, was identified as the acetate-utilizing organism *Methanothrix soehngenii*. The fourth (about 10% of the whole population) was presumably the propionate-degrading acetogen. It was nonfluorescent, nonmotile, about 0.8 μm wide, and 2 to 3 μm long. It had rounded ends and did not form pairs, chains, or filaments. On the basis of chemostat experiments, the generation time of the propionate degrader was estimated to be 4.6 to 5.8 days (manuscript in preparation).

The labeling pattern of the degradation products derived from [1-^{14}C]-, [2^{14}C]-, and [3-^{14}C]propionate gave some information about the pathway by which propionate was metabolized in the mixed culture. We added 3.15-μCi amounts of the labeled propionate preparations to 118-ml serum vials containing 30-ml portions of mineral salt medium, 3.2 mM sodium propionate, and the washed mixed culture (biomass, 4.5 mg [dry weight]). The distribution of the radioactivity after 23 h of incubation at 33°C is shown in Table 1. To prevent considerable mineralization of the produced radioactive acetate, the vials were spiked at the start with 37.3 mM nonradioactive acetate. A high acetate concentration does not inhibit propionate oxidation (6; this study). The relatively large pool was very effective in preserving the labeled acetate (Table 1), which was further analyzed for label position with *Methanothrix soehngenii* (Table 2). Interestingly, the distribution of ^{14}C was the same, regardless of whether the acetate originated from [2-^{14}C]- or [3-^{14}C]propionate. To exclude the possibility of a mix-up of the two distinctly labeled propionates, the experiments were repeated with two different batches from the same supplier, and the same results were obtained in each case.

On the sole basis of the results shown in Table 1, we could not determine whether formate was an intermediate in anaerobic oxidation of propionate. Suppose propionate is metabolized through pyruvate; then acetyl coenzyme A (CoA), formate, or carbon dioxide plus hydrogen could be formed. Our culture, however, contained a methanogen able to convert formate to methane (*Methanospirillum hungatii*) and another able to split formate into hydrogen and carbon dioxide (*Methanothrix soehngenii*) (4). Thus, formate as a possible extracellular intermediate could not be detected in the experimental system yielding the results shown in Table 1. To minimize the influence of the methanogens on any formate produced, cultures (biomass, 1 mg [dry weight]) were incubated with 5 mM [1-^{14}C]propionate (1.1 μCi per vial), 37 mM ace-

TABLE 1. Distribution of radioactivity in products of propionate degradation by a mixed methanogenic population after 23 h of incubation[a]

Position of ^{14}C in propionate	Propionate (dpm × 10^6)[b]	Acetate (dpm × 10^6)[b]	Methane (dpm × 10^6)[c]	Carbon dioxide (dpm × 10^6)[c]	Recovery (%)
1	1.976	0.092	0.027	4.808	98.6
2	2.319	4.181	0.215	0.342	100.8
3	2.286	4.393	0.217	0.306	102.9

[a] Initial activity in the propionate, 7.0 × 10^6 dpm. Specific activity in each vial, 33 μCi/mmol. Only minor amounts (<0.1%) of propionate were assimilated, so assimilation values are not shown.

[b] Means of duplicate determinations are shown.

[c] Means of quadruplicate determinations are shown. The standard deviations in all cases were <5%.

TABLE 2. Label position in mixed-culture acetate (Table 1) incubated with *Methanothrix soehngenii*[a]

Original position of ^{14}C in propionate	Acetate (dpm × 10^3)		Carbon dioxide (dpm × 10^3)	Methane (dpm × 10^3)	^{14}C incorporated into biomass (dpm × 10^3)	Recovery (%)
	At start	After 5 days				
2	446.5	0	209.3[b]	205.1	4.1	93.7
3	347.6	0	166.7	161.2	3.7	95.4

[a] Incubation time, 5 days at 33°C.
[b] Mean values of triplicate determinations. Standard deviations in all cases were <6%.

tate, and 40 mM (unlabeled) sodium formate. Within the incubation period of 23 h, 0.45 mM propionate, 8.25 mM acetate, and 11 mM formate were consumed. No radioactivity could be detected in the formate fraction.

Two propionate degradation pathways which would lead to the labeling pattern observed in our experiments are known: the methylmalonyl-CoA pathway and the α-OH-glutarate pathway. In both, succinate is the only symmetrical intermediate. In the latter pathway, glyoxylate is condensed with propionyl-CoA to form α-OH-glutarate. Kay (5) showed that in an *Escherichia coli* mutant, propionate degradation via α-OH-glutarate is markedly stimulated by the addition of glyoxylate. In our enrichments, glyoxylate in different concentrations had no stimulatory effect on propionate catabolism (Table 3). In addition, no $^{14}CO_2$ was formed from [1-^{14}C]glyoxylate, and no radioactive glyoxylate was taken up and incorporated into the biomass. Higher concentrations of glyoxylate resulted in a virtually complete inhibition of propionate degradation. However, the hydrogenophilic and acetophilic methanogens were also strongly inhibited by glyoxylate.

The methylmalonyl-CoA pathway includes a CO_2 fixation reaction catalyzed by propionyl-CoA carboxylase. This CO_2 is lost again on the way to pyruvate. Thus, this pathway cannot be tested with whole cells and $^{14}CO_2$. On the basis of our results for the culture studies, we are unable to determine with certainty which pathway can be excluded from the scheme. [1-^{14}C]propionate did not lead to the formation of significant quantities of labeled acetate. [2-^{14}C]- and [3-^{14}C]propionate, however, were quantitatively converted to [^{14}C]acetate. The labeling pattern of this acetate suggests that propionate is degraded through either a randomizing pathway—i.e., one molecule in the pathway must be symmetrical—or different pathways operative at the same time and absolute velocity (e.g., half of the propionate passes through either acrylyl-CoA → lactate → acetate or acrylyl-CoA → β-OH-propionyl-CoA → malonic semialdehyde → acetate [for a detailed review of studies on propionate metabolism, see reference 7]). In all cases and under very different incubation conditions, the label was evenly distributed in the acetate, which exclusively represents the 2 and 3 carbons of the propionate (Table 1). This makes the possibility of two synchronous pathways rather unlikely.

Four electrons are released between succinate and pyruvate, presumably in the form of one reduced flavin and one reduced pyridine nucleotide each. For oxidation of pyruvate to acetyl-CoA, two different pathways in anaerobic microorganisms are known: the pyruvate-oxidoreductase system and the pyruvate-formate-lyase system. In the first, acetyl-CoA, CO_2, and reduced ferredoxin, flavin nucleotides, or possibly flavoproteins are formed (8). the products of the second are acetyl-CoA and formate. In the presence of a formate-hydrogen-lyase, CO_2 and

TABLE 3. Influence of sodium glyoxylate on propionate degradation and methane production by a mixed methanogenic culture

Propionate (mM)	Acetate (mM)	Headspace (80:20)	Glyoxylate (mM)	CH_4 produced (μmol/vial)[a]	Inhibition (%)
30	0	N_2-CO_2	0	66.75	0
30	0	N_2-CO_2	2.5[b]	31.3	53
30	0	N_2-CO_2	5	13.54	80
30	0	N_2-CO_2	10[b]	9.1	86
0	30	N_2-CO_2	0	49.6	0
0	30	N_2-CO_2	10	4.5	91
0	20	H_2-CO_2	0	1187	0
0	20	H_2-CO_2	10	70.9	94

[a] Means of duplicate determinations are shown. CH_4 production was measured after 75 h.
[b] When 3.3 μCi of [1-^{14}C]glyoxylate was added, no $^{14}CO_2$ was produced and no ^{14}C was incorporated.

H_2 are produced. Our experiments clearly excluded the possibility that formate is a free intermediate during oxidation of propionate.

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