Contribution of ^{13}C-NMR spectroscopy to the elucidation of pathways of propionate formation and degradation in methanogenic environments

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Abstract

Propionate is an important intermediate in the anaerobic degradation of complex organic matter to methane and carbon dioxide. The metabolism of propionate-forming and propionate-degrading bacteria is reviewed here. Propionate is formed during fermentation of polysaccharides, proteins and fats. The study of the fate of ^{13}C-labelled compounds by nuclear magnetic resonance (NMR) spectroscopy has contributed together with other techniques to the present knowledge of the metabolic routes which lead to propionate formation from these substrates. Since propionate oxidation under methanogenic conditions is thermodynamically difficult, propionate often accumulates when the rates of its formation and degradation are unbalanced. Bacteria which are able to degrade propionate to the methanogenic substrates acetate and hydrogen can only perform this reaction when the methanogens consume acetate and hydrogen efficiently. As a consequence, propionate can only be degraded by obligatory syntrophic consortia of microorganisms. NMR techniques were used to study the degradation of propionate by defined and less defined cultures of these syntrophic consortia. Different types of side-reactions were reported, like the reductive carboxylation to butyrate and the reductive acetylation to higher fatty acids.

Introduction

In methanogenic environments organic matter is degraded by consortia of physiologically different types of microorganisms (Stams 1994, Schink 1997). Fermenting bacteria degrade complex organic matter to a number of low-molecular weight compounds which are the substrates for methanogenic consortia. Propionate is a very important intermediate in organic matter mineralization. It has been estimated that propionate oxidation may account for 35% of the total methanogenesis in bioreactors (Koch et al. 1983; Gujer & Zehnder 1982). Generally, propionate is formed as one of the end products in the fermentation of a variety of organic compounds. These include sugars or compounds which are formed during sugar fermentation like lactate, ethanol, succinate and acetate. Propionate can also be formed by fermentation of certain amino acids. Glycerol and odd-chain higher fatty acids, which are formed by hydrolysis of fats, can be degraded to propionate as well.

For thermodynamical reasons, propionate cannot be degraded by single species in methanogenic environments. Its degradation to methane and carbon dioxide is performed by syntrophic consortia of acetogenic bacteria and methanogens. That is, the oxidation of propionate to acetate, CO_2 and H_2 by acetogens is only possible if the H_2 is directly used by methanogens to reduce CO_2 to CH_4. However, some conversion processes of propionate to higher fatty acids have been observed as well.

In methanogenic environments propionate formation and propionate oxidation occur simultaneously. ^{13}C-NMR can be applied to elucidate pathways in defined cultures of propionate-forming and propionate-degrading bacteria and in anaerobic environments

where propionate is formed and degraded simultaneously by mixed microbial communities. Propionate formation and degradation in anaerobic microorganisms is reviewed here, and where possible the contribution of ^{13}C-NMR is addressed.

Propionate formation

Propionate formation from sugars

During fermentative growth bacteria use metabolic intermediates as sinks for reducing equivalents. In the fermentation of a number of organic compounds propionate is a reduced end product. In the classical propionic acid fermentation propionate is formed as a result of the use of metabolic intermediates as electron acceptor in the conversion of sugars by, e.g., *Propionibacterium*, *Megasphaera*, *Veillonella* and *Pectinatus* species (Wood 1961; Thauer et al. 1977; Gottschalk 1986). It should be mentioned that the same pattern of fermentation products can also be obtained by sequential fermentation of sugars, involving different types of fermenting bacteria. Examples are the fermentation of sugars to lactate or to ethanol and CO_2 in the lactic acid or alcoholic fermentation, respectively, followed by lactate and ethanol fermentation to propionate and acetate (Table 1). The sequential lactic acid and propionic acid fermentation occurs in, e.g., the production of swiss cheese. Alcoholic fermentation of sugars is performed by many yeasts, fungi and bacteria. The conversion of ethanol in a propionic acid fermentation was first demonstrated for *Desulfobulbus propionicus*, a sulfate reducer which is able to grow by a propionic acid fermentation in the absence of sulfate (Laanbroek et al. 1982). Later on, it was also found in a number of other bacteria (Schink 1984; Schink et al. 1987; Tholozan et al. 1992).

Some sugar-fermenting bacteria form succinate as a minor or main product during sugar fermentation (Macy et al. 1978; Gottschalk 1986). Succinate may be converted further to propionate. Schink & Pfennig (1982) described *Propionigenium modestum*, a bacterium which grows by decarboxylation of succinate to propionate. The decarboxylation leads to the extrusion of sodium ions. In this way a sodium gradient across the cytoplasmic membrane is built up, and this is the driving force for ATP synthesis (Hilpert et al. 1984). Also other mesophilic bacteria and the moderately thermophilic *Selenomonas acidaminovorans* grow by decarboxylation of succinate (Denger & Schink, 1990; Janssen et al. 1996; Cheng et al. 1992).

Theoretically, glucose may also be fermented to acetate, CO_2 and hydrogen, which are subsequently further converted to propionate (Table 1). Such a reaction should be possible if a homoacetogenic bacterium is grown together with *D. propionicus* in the absence of sulfate. *Desulfobulbus* is able to convert acetate, CO_2 and H_2 to propionate (Laanbroek et al. 1982). It is known that homoacetogens have a poor affinity for hydrogen (Cord-Ruwisch et al. 1988). This would enable the sulfate reducer to consume the hydrogen formed from glucose by the acetogen.

Among the bacteria which perform a propionic acid fermentation from sugars or fermentation products derived from sugars, two different pathways of propionate formation are known (Ng & Hamilton 1971; Schweiger & Buckel 1984; Leaver et al. 1955; Wood 1961; Tholozan et al. 1994). These are the so called methylmalonyl-CoA pathway and the acryloyl-CoA pathway (Figure 1). Starting from lactate, bacteria like *Megasphaera elsdenii* and *Clostridium propionicum* oxidize part of the lactate to acetate, while the reducing equivalents are disposed of by reduction of lactate via HS-CoA derivatives in a linear route to propionate (Figure 1A). Fermentation of lactate labelled in the C3 atom yields only C3-labelled propionate, while C2-labelled propionate will be formed from C2-labelled lactate. Many other bacteria including *Propionibacterium* and *Veillonella* species, *D. propionicus* and *Pelobacter propionicus* form propionate in a pathway, in which carboxylation and decarboxylation reactions are involved (Figure 1B). In this methylmalonyl-CoA pathway the symmetrical molecules fumarate and succinate are intermediates. As a consequence, label originally present in the C3 atom of lactate will end up in equal amounts in the C2 and the C3 atom of propionate, and the same will happen if C2-labelled lactate is converted. This difference in the two pathways has been used in many studies to elucidate the pathway of propionate formation in anaerobes. A peculiarity is that the carboxylation and decarboxylation can be catalyzed either by one enzyme, a transcarboxylase, or by two separate carboxylases. A transcarboxylase is e.g. present in *Propionibacterium*, while *Veillonella* possesses sodium-dependent carboxylases (Gottschalk 1986; Houwen et al. 1991). Because label position in molecules can easily be determined by ^{13}C-NMR, the incorporation of ^{13}C bicarbonate into organic molecules can be used to determine the type of carboxylation and decarboxylation mechanism (Houwen et al. 1991). NMR spectroscopy was used to quantify the contribution of bacteria with different

Table 1. Direct and sequential fermentation of glucose to propionate and acetate. Gibbs free energy changes ($\Delta G^{0\prime}$) obtained from Thauer et al. (1977) are expressed in kJ per reaction

	$\Delta G^{0\prime}$
3 glucose $\rightarrow$ 4 propionate$^-$ + 2 acetate$^-$ + 2HCO$_3^-$ + 8H$^+$	−924
3 glucose $\rightarrow$ 6 lactate$^-$ + 6H$^+$	−594
6 lactate$^-$ $\rightarrow$ 4 propionate$^-$ + 2 acetate$^-$ + 2HCO$_3^-$ + 2H$^+$	−330
3 glucose + 6 H$_2$O $\rightarrow$ 6 ethanol + 6HCO$_3^-$ + 6 H$^+$	−677
6 ethanol + 4 HCO$_3^-$ $\rightarrow$ 4 propionate$^-$ + 2 acetate$^-$ + 2H$^+$ + 6H$_2$O	−247
3 glucose + 2 HCO$_3^-$ $\rightarrow$ 4 succinate^{2-} + 2 acetate$^-$ + 8H$^+$ + 4H$_2$O	−840
4 succinate^{2-} + 4H$_2$O $\rightarrow$ 4 propionate$^-$ + 4HCO$_3^-$	−84
3 glucose + 12H$_2$O $\rightarrow$ 6 acetate$^-$ + 6HCO$_3^-$ + 12H$^+$ + 12H$_2$	−619
4 acetate$^-$ + 4HCO$_3^-$ + 4H$^+$ + 12H$_2$ $\rightarrow$ 4 propionate$^-$ + 12H$_2$O	−305

propionate-forming pathways to propionate formation in complex environments. A very nice example is the work of Counotte et al. (1981) who were able to quantify the role of *Megasphaera* in propionate formation from lactate in the rumen. On the average *Megasphaera* contributed for about 75% to the fermentation of lactate, but this contribution largely depended on the diet.

The fermentation of ethanol (+CO$_2$) to acetate and propionate by *Desulfobulbus propionicus* and in *Pelobacter propionicus* were studied (Stams et al. 1984; Schink et al. 1987). In both bacteria propionate is formed from ethanol via the methylmalonyl-CoA pathway. It is assumed that ethanol is first oxidized to acetaldehyde. Then, acetaldehyde is partly oxidized to acetate and partly carboxylated to pyruvate, which thereafter is converted to propionate. The acryloyl-CoA pathway may also be involved in ethanol fermentation. This was demonstrated for *Clostridium neopropionicum* (Tholozan et al. 1992). In all these studies NMR was used as one of the principle techniques. The former two bacteria form equal amounts of C2- and C3-labelled propionate from C1-labelled ethanol, while in *C. neopropionicum* only C2-labelled propionate is formed from [1-^{13}C]-ethanol. Further evidence for the two pathways was obtained by measuring enzyme levels in cell free extracts.

Propionate formation from fats

In methanogenic environments fats are hydrolyzed to long chain fatty acids and glycerol. Long chain fatty acids are degraded by β-oxidation (McInerney 1988). In sequences of reactions acetyl-CoA groups are cleaved of from HS-CoA derivatives of long chain fatty acids. Consequently, propionyl-CoA groups are formed when odd-numbered fatty acids are degraded, resulting in the formation of propionate. Glycerol is an easily fermentable compound, which can be fermented in different ways. One type of fermentation is a propionic acid fermentation. For example, *Propionibacterium* is able to ferment glycerol to propionate.

Propionate formation from amino acids

Propionate can be formed in the fermentation of a variety of different amino acids (McInerney 1988). Certain amino acids can be fermented in a similar fermentation as described above for sugars or fermentation products formed from sugars. For example, after oxidative deamination of alanine and aspartate, pyruvate and oxaloacetate are formed, respectively. These intermediates can be partially oxidized to acetate and partially reduced to propionate leading to stoichiometries as given in Table 2 (Eqs. 1 and 2). Also glutamate may be converted reductively to propionate (Table 2, Eq. 3). This was described for *Selenomonas acidaminophila* (Nanninga et al. 1987). Reducing equivalents formed in the oxidation of glutamate to acetate are disposed of by formation of propionate. Interestingly, oxidative formation of propionate from amino acids has been observed as well. *Clostridium propionicum* and *Peptostreptococcus prevotii* ferment threonine oxidatively to propionate according to Eq. 4 of Table 2 (Tokushigo & Hayaishi 1972; Gottschalk 1986). *Acidaminobacter hydrogenoformans* and *Selenomonas acidaminovorans* form propionate oxidatively in the fermentation of glutamate and some other amino acids (e.g. histidine, arginine, citrulline) which are degraded through glutamate (Stams & Hansen 1984, Cheng et al. 1992). The reactions involved in glutamate fermentation in these two organisms are
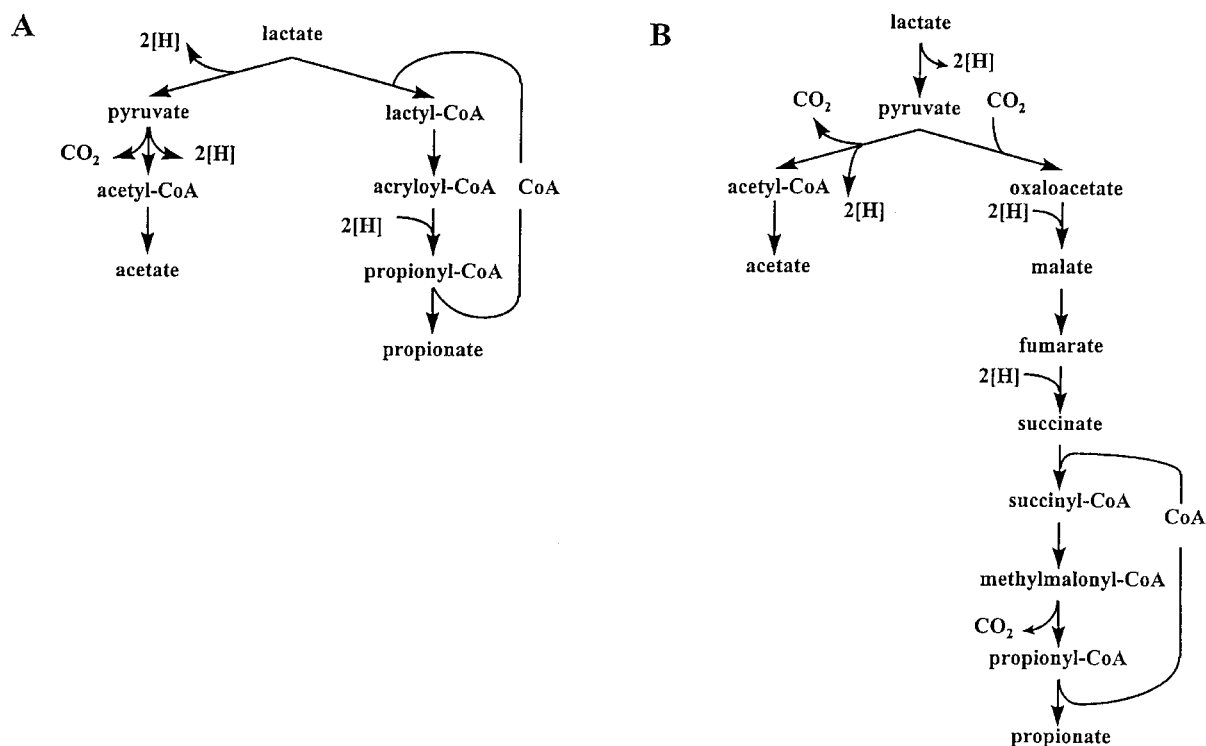
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Figure 1. Lactate fermentation to propionate and acetate via the acryloyl-CoA (A) and the methylmalonyl-CoA (B) pathway.

presented in Table 2 (Eqs. 5 and 6). In addition, we have enriched a propionate-degrading culture which is able, when growing syntrophically, to ferment glutamate oxidatively to propionate, which is then oxidized further to acetate as given by Eq. 7 of Table 2 (Plugge unpublished results).

Different pathways may be involved in the anaerobic degradation of glutamate. These are depicted in Figure 2. The β-methylaspartate pathway and the hydroxyglutarate pathway (Figure 2A and 2B) are the best known glutamate-degrading pathways (Buckel & Barker 1974; Barker 1981; Buckel & Miller 1987). In the first pathway, β-methylaspartate is formed, which is further metabolized to citramalate, and then cleaved into acetate and pyruvate. The pyruvate can be fermented further, e.g., in a propionic acid fermentation as is done by *S. acidaminophilum* (Nanninga et al. 1987) or in a butyric acid fermentation as occurs in, e.g., *Clostridium tetanomorphum* and some other Clostridia (Barker 1981). The hydroxyglutarate pathway only has been described for bacteria which ferment glutamate to acetate and butyrate, e.g., *Clostridium sporosphaeroides* and *Clostridium symbiosum* (Buckel 1980). Glutamate is oxidatively

deaminated to 2-oxoglutarate, which in a series of reactions is oxidatively decarboxylated to a crotonyl-CoA. Thereafter, this compound is partly oxidized to two acetate and partly reduced to butyrate. Other pathways for glutamate fermentation are possible as well (Figure 2C, 2D and 2E). After oxidative deamination to 2-oxoglutarate, a "reversed citric acid cycle" may be followed (Figure 2C). In this route 2-oxoglutarate is converted to isocitrate and citrate. Then, citrate is cleaved by citrate lyase to oxaloacetate and acetate. The oxaloacetate may be converted further, e.g., to propionate and acetate. 2-Oxoglutarate may also be oxidized further via the "citric acid cycle" to succinyl-CoA. The succinyl-CoA formed may be oxidized to acetate (Figure 2D, Table 2, Eq. 7) or may be decarboxylated to propionate (Figure 2E, Table 2, Eq. 6). In *A. hydrogenoformans* and *S. acidaminovorans*, acetate and propionate are formed according to Eqs. 5 and 6 of Table 2. According to the measured stoichiometry of conversion, propionate may be formed according to pathway depicted in Figure 2E. However, acetate may be formed according to the pathways as given in Figure 2A, 2B or 2C.

Table 2. Propionate formation during fermentation of amino acids. Gibbs free energy changes ($\Delta G^{0\prime}$) obtained from Thauer et al. (1977) are expressed in kJ per reaction

		$\Delta G^{0\prime}$
1.	$3 \text{ alanine} + 3\,H_2O \rightarrow 2 \text{ propionate}^- + \text{acetate}^- + HCO_3^- + 3H^+ + 3NH_4^+$	-130
2.	$3 \text{ aspartate}^- + 6H_2O \rightarrow 2 \text{ propionate}^- + \text{acetate}^- + 4HCO_3^- + 4H^+ + 3NH_4^+$	-192
3.	$3 \text{ glutamate}^- + 6H_2O \rightarrow \text{propionate}^- + 5 \text{ acetate}^- + 2HCO_3^- + 4H^+ + 3NH_4^+$	-178
4.	$\text{threonine} + 2\,H_2O \rightarrow \text{propionate}^- + HCO_3^- + H_2 + H^+ + NH_4^+$	-78
5.	$\text{glutamate}^- + 3H_2O \rightarrow 2 \text{ acetate}^- + HCO_3^- + H_2 + H^+ + NH_4^+$	-34
6.	$\text{glutamate}^- + 4H_2O \rightarrow \text{propionate}^- + 2\,HCO_3^- + 2H_2 + H^+ + NH_4^+$	-6
7.	$\text{glutamate}^- + 7H_2O \rightarrow \text{acetate}^- + 3\,HCO_3^- + 5H_2 + H^+ + NH_4^+$	$+70$

Based on measurements of enzyme activities *A. hydrogenoformans* seems to be able to form acetate in two different ways from glutamate, by the β-methylaspartate pathway and by the "reversed citric acid cycle", Figure 2A and 2C, respectively. The metabolism of this organism is strongly affected by the hydrogen partial pressure, as it uses protons for disposal of reducing equivalents. At a high hydrogen partial pressure glutamate is mainly fermented to acetate as shown in Table 2 (Eq. (5)). However, when this bacterium is grown in the presence of the hydrogen-utilizing *Methanobrevibacter arboriphilus*, a major part of the glutamate is degraded to propionate (Table 2, Eq. 6). The ratio acetate to propionate depends on the hydrogen partial pressure (Stams & Hansen 1984). The energetic barrier in the conversion to propionate is the oxidative deamination of glutamate to 2-oxoglutarate ($\Delta G^{\circ\prime} = +59.9$ kJ/mol). The conversion of 2-oxoglutarate to propionate is exergonic ($\Delta G^{\circ\prime} = -65.7$ kJ/mol). The energetic barrier can be taken away by cultivating the organism on 2-oxoglutarate. In this case propionate is formed in pure culture. Table 3 shows enzyme activities in cell free extracts of *A. hydrogenoformans* grown under different conditions (Stams et al. 1994). In glutamate-grown cells of pure cultures of *A. hydrogenoformans* two key enzymes of the β-methylaspartate pathway, β-methylaspartase and citramalate lyase, were present. Hydroxyglutarate dehydrogenase, a key enzyme of the hydroxyglutarate pathway of glutamate fermentation was not present. Isocitrate dehydrogenase, aconitase and citrate lyase which may be involved in glutamate fermentation to acetate via the "reversed citric acid cycle", were not detected or detected in very low activities in these glutamate-grown cells. In contrast, cells grown on 2-oxoglutarate or cells grown on glutamate in the presence of *M. arboriphilus* did not contain β-methylaspartase, whereas activities of enzymes of the "reversed citric acid cycle" had increased considerably compared with glutamate-grown cells. Enzymes involved in the oxidative formation of propionate via 2-oxoglutarate, succinyl-CoA, methylmalonyl-CoA and propionyl-CoA were present, which is in accordance with the pathway depicted in Figure 2E. Fumarate reductase, an enzyme which would be essential for a reductive formation of propionate could not be detected.

Interestingly, the fate of the different carbon atoms in glutamate is different when glutamate is metabolized to acetate and propionate through different pathways. Labelling studies were done to investigate glutamate fermentation of butyrate-forming bacteria (Buckel 1980), but to our knowledge propionate formation from labelled glutamate has not yet been studied. Following the glutamate-degrading pathways, the fate of carbon from glutamate is different. This is depicted in Figure 2 and Table 4. In the determination of the fate of carbon atoms two aspects were considered, namely that both the methylmalonyl-CoA and the acryloyl-CoA pathway may be involved in the conversion of intermediates like oxaloacetate, pyruvate and acetyl-CoA, and that citrate is a symmetrical molecule, which can be cleaved to acetate and oxaloacetate in two different ways. This cleavage may be different in different microorganisms. Thus, by using glutamate labelled in different C-atoms, the occurrence and importance of the different pathways in pure cultures of bacteria, defined mixed cultures and in complex microbial associations can be elucidated by NMR.
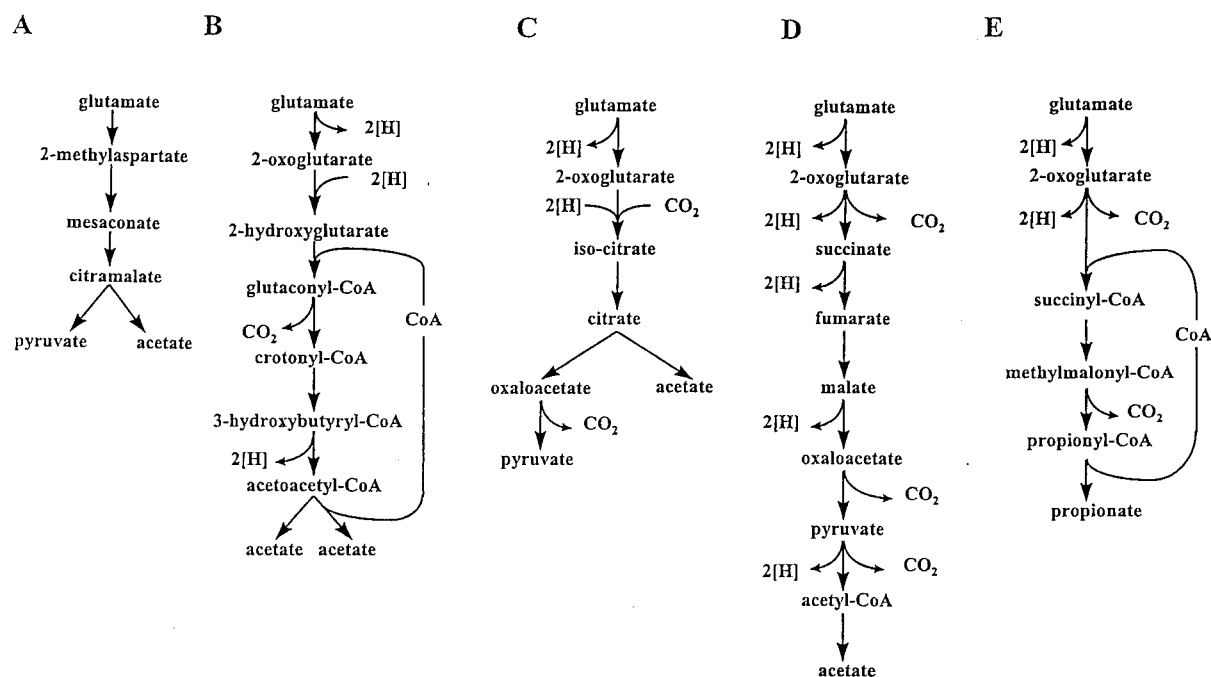
A   B   C   D   E

Figure 2. Fermentation of glutamate to acetate and propionate via β-methylaspartate pathway (A), hydroxyglutarate pathway (B) and reversed citric acid cycle (C) and to only acetate (D) or only propionate (E) by the citric acid cycle.

Table 3. Specific enzyme activities [μmol min^{-1} mg^{-1} protein] of *Acidaminobacter hydrogenoformans* cells grown under different conditions. Data obtained from Stams et al. (1994)

Enzyme	Glu[1]	2-OG	Glu[2] + M. arb
β-methylaspartase	0.24	0.00	0.00
Citramalate lyase	0.04	0.01	0.08
Glutamate dehydrogenase (NAD)	86.30	34.30	93.40
Isocitrate dehydrogenase (NADP)	0.12	1.24	1.12
Aconitase	0.00	0.16	0.26
Citrate lyase	0.20	0.17	0.48
2-oxoglutarate dehydrogenase (MV$^+$)	0.05	0.10	0.08
Propionyl-CoA carboxylase	0.13	0.08	0.39
Pyruvate dehydrogenase (MV$^+$)	3.43	4.06	1.86
Phosphotransacetylase	1.25	1.80	4.44
Acetate kinase	0.56	1.76	2.45
Malate dehydrogenase	0.78	3.38	2.26
Hydrogenase (MV$^+$)	1.14	5.27	4.19
Formate dehydrogenase (MV$^+$)	0.59	0.03	0.05

Hydroxyglutarate dehydrogenase (NAD-dependent), fumarase and fumarate reductase were not detected (<0.010 μmol min^{-1} mg^{-1} protein).
[1] Glu: glutamate; 2-OG: 2-oxoglutarate; M. arb: *Methanobrevibacter arboriphilus*.
[2] The specific activities are corrected for the contribution of protein and enzyme activities of the methanogen.

Table 4. Label position in propionate, acetate and carbon dioxide during glutamate fermentation via different pathways. Incorporation of label from CO_2 is indicated by *

$$^-OOC\underset{1}{-}\underset{2}{CHNH_3^+}\underset{3}{-CH_2}\underset{4}{-CH_2}\underset{5}{-COO^-}$$

	Propionate			Acetate	CO_2
	Acryloyl-CoA route	Methylmalonyl-CoA route	Direct oxidation		
Methylaspartate pathway	3 4 5 $CH_3-CH_2-COO^-$	4/3 3/4 */5 $CH_3-CH_2-COO^-$		2/3 1/4 CH_3-COO^-	5 CO_2
Hydroxyglutarate pathway			2/4 1/3 CH_3-COO^-	5 CO_2	
"Citric acid cycle"			3 4 2 $CH_3-CH_2-COO^-$	4/3 3/4 CH_3-COO^-	1,2,5 CO_2
"Reversed citric acid cycle"					
re-specific	4 3 * $CH_3-CH_2-COO^-$	3/4 4/3 5/* $CH_3-CH_2-COO^-$		2/4 1/3 CH_3-COO^-	5 CO_2
si-specific	2 3 * $CH_3-CH_2-COO^-$	3/2 2/3 1/* $CH_3-CH_2-COO^-$		4/2 5/3 CH_3-COO^-	1 CO_2

Propionate conversion

Syntrophic propionate oxidation

In methanogenic environments propionate is oxidized by syntrophic consortia of proton-reducing acetogenic bacteria and methanogens. *Syntrophobacter wolini* was the first described bacterium which grows in syntrophy with methanogens on propionate (Boone & Bryant 1980). The acetogens oxidize propionate to acetate and hydrogen, according to:

$$propionate^- + 3H_2O \rightarrow acetate^- + HCO_3^- + H^+ + 3H_2 \ (\Delta G^{0\prime} = +76.1 \text{ kJ})$$
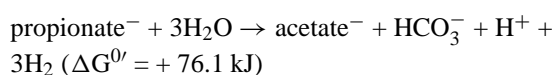
The Gibbs free energy change of this reaction is highly positive. Methanogens use hydrogen efficiently for the reduction of CO_2 to CH_4. In this way, they create a low hydrogen partial pressure, which enables the acetogen to grow. All propionate-oxidizing cultures which have been described thus far degrade propionate via the methylmalonyl-CoA pathway (Koch et al. 1983; Mah et al. 1990; Robbins 1988; Houwen et al. 1987; 1990; 1991; Wallrabenstein et al. 1994; 1995; Stams et al. 1993). The elucidation of this pathway has been done by using [14]C- and ^{13}C-labelled propionate, and by measuring enzyme levels in cell free extracts. The outcome of an *in vivo* NMR experiment with a syntrophic propionate-oxidizing culture as obtained by Houwen et al. (1987) is shown in Figure 3. Label in the C3 of propionate is incorporated in the C1 and the C2 of acetate, which indicates the presence of the methylmalonyl-CoA pathway. During propionate conversion succinate accumulates as an intermediate. Label in the C3 of propionate is randomized over the C2 and C3 of propionate as a result of the back reaction of succinate to propionate. The involvement of the methylmalonyl-CoA pathway was confirmed by enzyme measurements (Houwen et al. 1990). The pathway resembles the pathway of propionate oxidation in *Desulfobulbus propionicus* (Kremer & Hansen 1988; Stams et al. 1984). However, this bacterium is not able to grow syntrophically on propionate in the absence of sulfate (Widdel & Pfennig 1992). Interestingly, syntrophic propionate-oxidizing bacteria were found to be phylogenetically related to sulfate-reducing bacteria, and they were also able to grow on propionate by using sulfate as the electron acceptor (Harmsen et al. 1993; Wallrabenstein et al. 1994; 1995; Van Kuijk & Stams 1995). Surprisingly, *Desulforhabdus amnigenus*, a sulfate reducer which clusters with *Syntrophobacter* sp., is unable to grow by syntrophic propionate oxidation (Oude Elferink et al. 1995). The growth rate of syntrophic propionate oxidizer *Syntrophobacter wolinii* is rather low in comparison with that of *D. propionicus*, 0.1–0.2 versus 0.9–1.7 day^{-1}, respectively (Oude Elferink et al. 1994). Up to now, these physiological differences between *Syntrophobacter* sp. and *Desulfobulbus* sp. cannot be explained on a biochemical basis.

Energetically, succinate oxidation to fumarate coupled to proton reduction is the most difficult reaction step in syntrophic propionate oxidation. Therefore,
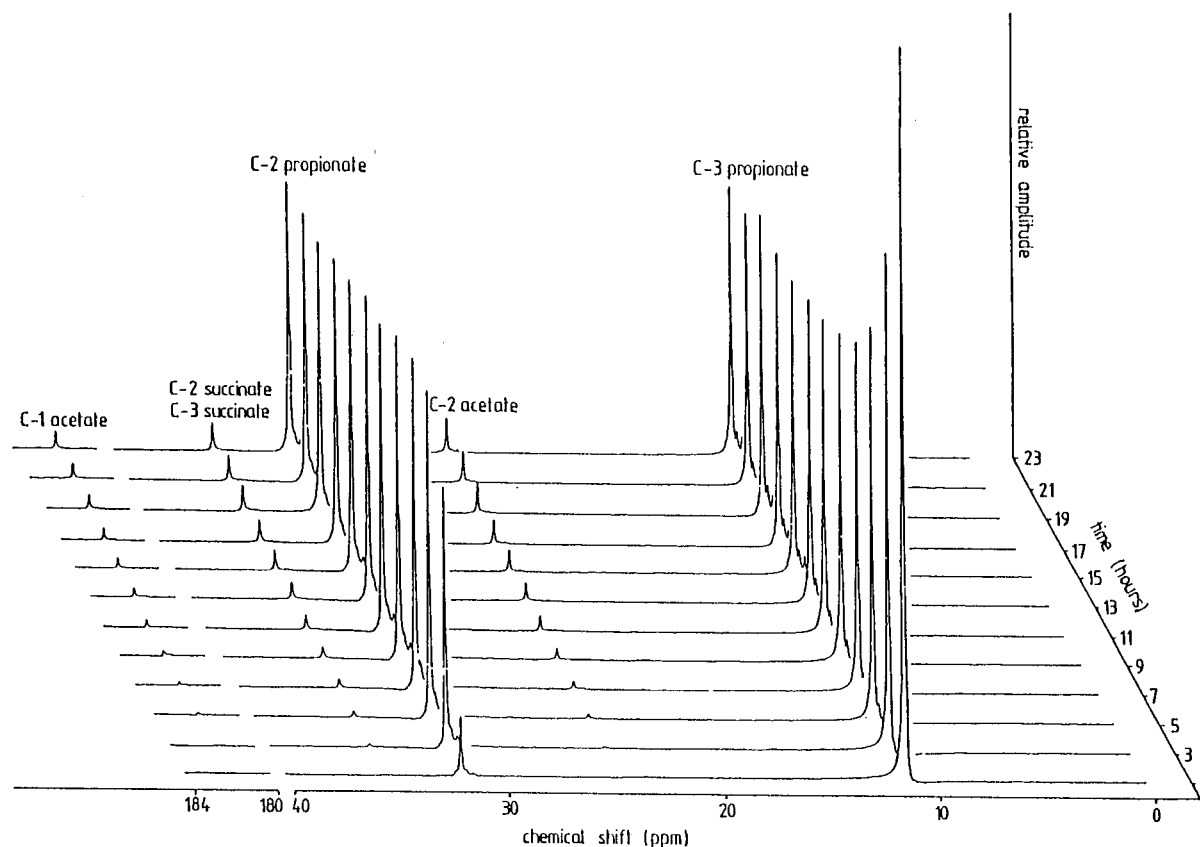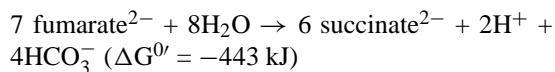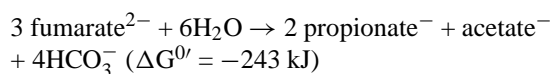
Figure 3. [2-^{13}C] propionate conversion by a methanogenic enrichment culture. Data obtained from Houwen et al. (1987).

propionate conversion may be enhanced by addition of fumarate. In this way, the bacteria may circumvent this energetically difficult step. The effect of fumarate on syntrophic propionate degradation was studied (Stams et al. 1993). As syntrophic oxidation is a rather slow process in comparison with fumarate fermentation, a clear effect of fumarate on propionate conversion could only be demonstrated with a highly purified culture. By using ^{13}C-labelled propionate and unlabelled fumarate it was shown that the propionate-oxidizing bacteria convert propionate to succinate and oxidize fumarate further to acetate. Remarkably, the propionate-oxiziding bacterium was also able to ferment fumarate. It appeared that fumarate was fermented according to:

7 fumarate^{2-} + 8H$_2$O → 6 succinate^{2-} + 2H$^+$ + 4HCO$_3^-$ (ΔG$^{0\prime}$ = −443 kJ)

and not according to:

3 fumarate^{2-} + 6H$_2$O → 2 propionate$^-$ + acetate$^-$ + 4HCO$_3^-$ (ΔG$^{0\prime}$ = −243 kJ)

which would be more likely for a bacterium which is able to oxidize propionate to acetate. By measuring enzyme activities it appeared that the bacterium activates propionate during propionate oxidation by means of a HS-Co-transferase and subsequently carboxylates propionyl-CoA by means of a transcarboxylase (Plugge et al. 1993). Consequently, fumarate fermentation to acetate and propionate according to the latter equation is biochemically not possible. By using ^{13}C-labelled acetate it was demonstrated by NMR that fumarate is oxidized to CO$_2$ via the acetyl-CoA cleavage pathway (Plugge et al. 1993) and not via the "citric acid cycle" as occurs in *Proteus rettgeri* and *Malonomonas rubra* (Kröger 1974; Dehning & Schink 1989). Presently, it is not known how widespread the observed propionate activation mechanism is among syntrophic propionate-oxidizing bacteria. *Syntrophobacter wolinii* seems to activate propionate by means of a kinase (Houwen et al. 1990).

Table 5. Labelling positions of propionate and butyrate during the degradation of ^{13}C-labelled propionate ($\pm$30 mM) by methanogenic sludge. Data were derived from Tholozan et al. (1988)

Intitial labelling of propionate	Time of sampling (h)	Propionate degraded (%)	Fractional distribution of ^{13}C atoms in:			
			Propionate		Butyrate	
			2-[13]C	3-[13]C	3-[13]C	4-[13]C
2-[13]C	8	43	0.90	0.10	1.00	0.00
	32	81	0.70	0.30	0.90	0.10
3-[13]C	8	35	0.05	0.95	–[a]	–
	32	76	0.10	0.90	0.00	1.00

[a] The butyrate concentration was too low to determine a fractional distribution.

Other propionate conversion reactions

Propionate oxidation is a relatively slow process and it is easily negatively affected by changes in the environmental conditions. For this reason, side reactions in its biodegradation route are possible as well. The formation of higher fatty acids like valerate and methylbutyrate by mixed microbial communities has been observed by using ^{13}C-labelled propionate (Lens et al. 1996; 1997). As higher fatty acids are degraded by β-oxidation, propionate will be formed back again from these fatty acids, when the conditions for their degradation are favourable. More interesting is the observation made by Tholozan et al. (1988) who found that in sludge from an industrial digester about 20% of the propionate was converted to butyrate. By using NMR techniques they were able to show that butyrate is formed by reductive carboxylation of propionate, as [2-^{13}C]- and [3-^{13}C]-propionate were converted to [3-^{13}C]- and [4-^{13}C]-butyrate, respectively (Table 5). A similar reductive carboxylation of propionate, involving 5–10% of the propionate pool, was also found for other sludges (Lens et al. 1996). Neither the exact biochemical mechanism nor the responsible microorganisms are known yet. The pathways of butyrate- and propionate-degradation as studied in pure and enriched cultures are clearly different. In addition, the syntrophic butyrate-degrading cultures which have been described thus far are unable to degrade propionate, and syntrophic propionate-degrading cultures do not degrade butyrate. Nevertheless, propionate and butyrate do affect each others degradation, as was shown by Lens et al. (1997).

Concluding remarks

In methanogenic environments propionate is an important intermediate. It is formed during fermentation of different organic compounds. Unlike propionate formation from sugars, the reductive and oxidative formation of propionate in the fermentation of amino acids has received little attention. Challenging for the future is to get more insight into amino acid fermentation in methanogenic environments, like bioreactors, freshwater sediments and the intestinal tract of animals and humans. The responsible microorganisms need to be isolated and characterized physiologically and biochemically. Also the pattern of products formed during amino acid fermentation by mixed microbial communities needs to be studied. In this respect, ^{13}C-NMR techniques are very useful, in particular because the fate of label can be followed by using specifically labelled amino acids. Many ^{13}C-labelled amino acids have become commercially available over the last decade. Currently unknown aspects of their degradation can be elucidated. Also the combined use of ^{13}C- and ^{15}N-NMR can be very attractive to get a complete picture of degradative pathways of N-containing organic compounds.

Our knowledge of the degradation of propionate in methanogenic environments is scarce. Although the major general principles of degradation are largely known (Schink 1997; Stams 1994), only very few propionate-degrading bacteria have been isolated and studied physiologically. However, thus far the work with the defined syntrophic cocultures does not reflect completely the observations made with complex microbial environments. Also here NMR can be used to get insight into the interactive degradation of propionate and other organic compounds. In this respect, it is worth to mention that the incorporation of labelled

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bicarbonate also may give very useful information concerning the different dissimilatory and assimilatory pathways.

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