# PHYSIOLOGY OF

# SYNTROPHIC PROPIONATE OXIDIZING BACTERIA

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# PHYSIOLOGY OF SYNTROPHIC PROPIONATE OXIDIZING BACTERIA

PROEFSCHRIFT

ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
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Ik wil iedereen bedanken die een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift.

I would like to thank everybody who contributed to the completion of this thesis.

## Stellingen

1. De toe- of afname van gelabeld waterstof en kooldioxide, kunnen niet zonder meer gebruikt worden als maat voor netto substraatkonsumptie.
   - Schink et al., (1983) Appl. Environ. Microbiol. 45, 1491-1500
   - Dit proefschrift

2. Het is niet juist specifieke enzymaktiviteiten, gemeten in celvrij extrakt, te extrapoleren naar in situ (fysiologische) kondities.

3. Met de titel: "A Proposed Pathway for Catabolism of Propionate in Methanogenic Cocultures", suggereert Robbins ten onrechte dat het hier om een nieuwe route gaat.
   - Robbins (1988) Appl. Environ. Microbiol. 54, 1300-1301

4. Het gebruik van een elektrode in kombinatie met (biologische) redox mediators, biedt zeer goede perspektieven voor fysiologisch onderzoek aan en het reinkweken van "obligaat syntrofe acetogene bakteriën".
   - Dutton (1978) Methods Enzymol. 54, 411-435
   - Srinivas et al. (1988) In: Erickson, L.E. and Fung, Y. (eds), Handbook on anaerobic fermentations. p 147-186
   - Emde en Schink (1990) Arch. Microbiol., in press

5. Azijnzuur en mierezuur zijn geen vetzuren.

6. Mensen die de term "interspecific hydrogen transfer" bezigen, zouden eens "The Origin of Species" van Charles Darwin moeten lezen.

7. Het aardige van wetenschap is dat je aan de ene kant een losse geest nodig hebt, die gekke dingen durft te bedenken, en het aan de andere kant noodzakelijk is om alles weer heel netjes te toetsen.
   - Prof. dr. C. de Jager, Elsevier 12, november 1988

8. Plato's wiskunde en de quantummechanica hebben gemeenschappelijk, dat beide de werkelijkheid slechts beperkt beschrijven.
   - Plato (± 400 v.C.) Timaios
   - Van Quantum tot Quark, Stichting Teleac (1989)

9. Schepping óf evolutie is een vals dilemma.
   - Evolutie en scheppingsgeloof, AMBO Boeken, Baarn (1978)

10. Echte humor is allesomvattend.
   - Herman Hesse (1927) Der Steppenwolf, Suhrkamp Taschenbuch 175, 17. Aflage. p 61-62 (1982)
   - Wim Sonneveld (1966) Interview met Mies Bouwman

11. Het oversteken van twee rivieren kan een hele overgang zijn.

12. Promoveren en karnaval vieren kunnen heel goed samengaan.

13. Die niet waagt, die niet wint.

Stellingen, behorend bij het proefschrift "Physiology of syntrophic propionate oxidizing bacteria"

Wageningen, 25 juni 1990 Frans Houwen

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Chapter 1

# GENERAL INTRODUCTION

## METHANOGENIC MINERALIZATION

Formation of organic matter by photosynthetic carbon dioxide reduction accounts for approximately 2.1 x $10^{14}$ gram organic carbon per day. This formation is counteracted by biological breakdown in animal and plant tissue, fungi and bacteria (Zehnder, 1982). The breakdown of organic matter is principally performed in two metabolic processes (Figure 1): 1) oxidation of the substrate leading to intermediary products (e.g. pyruvate, acetyl-CoA) and reduced cofactors (e.g. NAD(P)H, $FADH_2$, ferredoxines), and 2) reactions during which the reduced cofactors are reoxidized. The regeneration of the reduced primary electron acceptors is a prerequisite for substrate oxidation to proceed. In respiration processes, oxygen, metal ions, nitrogen, sulphur, carbon compounds, or protons are used as terminal electron acceptor. During fermentation, on the other hand, the electrons from the oxidation reactions are transferred to an organic intermediate which was formed in the same process. Whether oxidation or fermentation processes occur, is depending on the type of organism(s), the substrate(s) and the external electron acceptor(s) present (Zehnder and Svensson, 1986; Zehnder and Colberg, 1986). A natural population of bacteria will follow the concept of electron free energy: the energetical most favourable electron acceptor available for transfer of reducing equivalents (with the highest redox potential), will be used (Table 1) (Mah et al., 1977; Stumm, 1978; Zehnder, 1982; Cord-Ruwisch et al., 1988).

On molar basis, about 4.5% of the carbon fixed by photosyn-

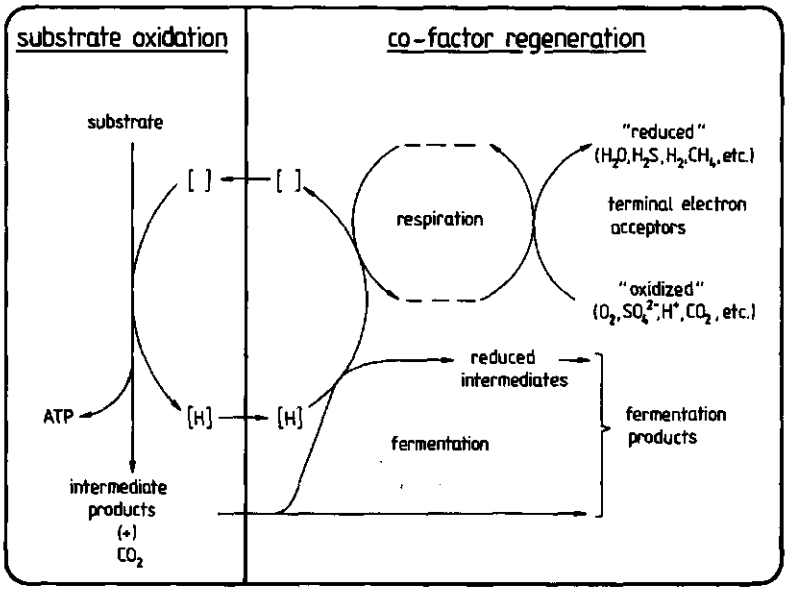


*Figure 1. The two basic processes involved in degradation of organic material. Reduced primary electron acceptors ([H] = NAD(P)H, $FADH_2$, etc), produced during oxidation of the substrate, are regenerated by either respiration or fermentation. Adapted after Zehnder and Svensson (1986).*

thesis is converted to methane (Vogels, 1979). Under methanogenic conditions, i.e. with protons and carbon dioxide as intermediary and ultimate electron acceptors, a great number of metabolically and phylogenetically distinct bacterial species cooperate in the degradation of complex organic matter to methane and carbon dioxide. The total process can be subdivided into three different steps (Figure 2) (Zehnder, 1978; Bryant, 1979; McInerney et al., 1980; Zehnder et al., 1982; Zeikus, 1982; Gujer and Zehnder, 1983). The first step (fermentative phase) is the hydrolysis of biopolymers into monomers and the degradation of these monomers to the main methanogenic substrates acetate, formate, carbon dioxide and hydrogen, and reduced organic products like fatty acids, alcohols and aromatic compounds. In the second step (acetogenic phase), syntrophic acetogenic bacteria metabolize these intermediairy organic products to acetate, formate, carbon dioxide and hydrogen, which are finally (methanogenic phase) converted by methane bacteria to methane (and carbon dioxide).

*Table 1. Various redox potentials of electron donors and electron acceptors involved in oxidation and fermentation of organic matter. E°', standard conditions, pH = 7, 25°C.*

| Redox compound | E°' (mV) |
|---|---|
| $SO_4^{2-}/HSO_3^-$ | -516 |
| acetyl-CoA/pyruvate$^-$ | -490 |
| $CO_2$/formate$^-$ | -432 |
| $H^+/H_2$ | -414 |
| ferredoxine ox/red | -398 |
| NAD(P)/NAD(P)H | -320 |
| $CO_2$/acetate$^-$ | -290 |
| S°/HS$^-$ | -270 |
| $CO_2/CH_4$ | -244 |
| $FAD/FADH_2$ | -220 |
| acetaldehyde/ethanol | -197 |
| pyruvate$^-$/lactate$^-$ | -190 |
| oxaloacetate$^{2-}$/malate$^{2-}$ | -172 |
| $HSO_3^-$/HS$^-$ | -116 |
| acrylyl-CoA/propionyl-CoA | -15 |
| fumarate$^{2-}$/succinate$^{2-}$ | +33 |
| $NO_2^-$/NO | +350 |
| $NO_3^-/NO_2^-$ | +433 |
| $Fe^{3+}/Fe^{2+}$ | +722 |
| $O_2/H_2O$ | +818 |

For many years, protons were thought to be the only electron carriers between the different types of organisms during methanogenesis. However, recently it was hypothesized that formate, as the product of carbon dioxide reduction, is a more important compound via which the electron flow takes place. The interspecies transfer of electrons is discussed in the next section.

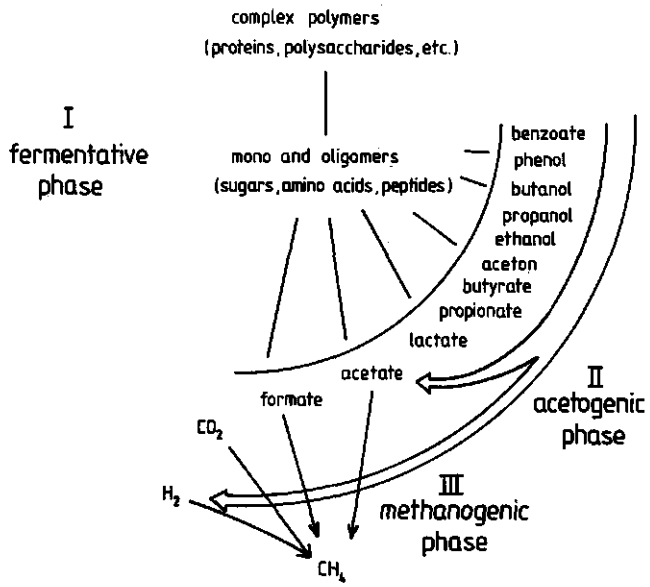


*Figure 2. Multistep scheme for the degradation of complex organic material under methanogenic conditions. Adapted after Vogels (1979).*

## INTERSPECIES ELECTRON TRANSFER

### Interspecies hydrogen transfer

In 1936, Barker described Methanobacillus (Methanobacterium) omelianskii as an organism that, in the presence of carbon dioxide, degraded ethanol to methane and acetate. Bryant et al. (1967) discovered that M. omelianskii in fact consisted of two organisms. The S-organism in this coculture oxidized ethanol to acetate and hydrogen, and the methanogen used this hydrogen for methane formation. The fact that the S-organism was not able to grow without the methane bacterium was explained in terms of change of free energy. Under thermodynamic standard conditions, the oxidation of ethanol to acetate and hydrogen is an endergonic reaction ($\Delta G^{\circ\prime} > 0$), which becomes exergonic ($\Delta G' < 0$) if the partial pressure of hydrogen is lowered (Bryant et al., 1967). In the syntrophic coculture of M. omelianskii the methane

bacterium consumes the hydrogen, produced by the S-organism, and thus in fact functions as an ultimate electron acceptor.
This first example of interspecies hydrogen transfer was one of the milestones in understanding the degradation of organic matter under methanogenic conditions as depicted in Figure 2. After this discovery it became generally accepted that alcohols other than methanol and fatty acids were not used by methanogens directly, but were anaerobically oxidized by obligate proton reducing acetogens. Today, however, some methanogenic species are known to oxidize alcohols in pure culture (Widdel, 1986; Zellner and Winter, 1987; Widdel et al., 1988).

Like ethanol oxidation, most of the reactions in the acetogenic phase of anaerobic decomposition of organic matter (Figure 2, step 2) are unfavourable under standard conditions (Table 2). However, as standard conditions do not describe the conditions prevailing in natural environments, the actual $\Delta G'$ should be considered. In case of a hypothetical reaction $aA + bB \longrightarrow cC + dD$, the $\Delta G'$ can be calculated according to the following equation:

$$\Delta G' = \Delta G^{\circ\prime} + RT \ln \frac{[C]^c [D]^d}{[A]^a [B]^b}$$

where the variables in brackets represent the molar concentration of a solute or the partial pressure of a gas. The concentration of the reactants and products can drastically change the free energy of the reactions. Of particular interest is hydrogen which often participates in acetogenic reactions in stoichiometrically large quantities (Table 2) and which can be present in a wide range of partial pressures. As hydrogen is a product in anaerobic oxidation of organic compounds, its production and utilization influences the course of reactions in anaerobic ecosystems (Wolin, 1974; 1976; McCarty, 1982; Zinder, 1984; Zehnder and Stumm, 1988; Dolfing, 1988). Figure 3 shows the dependence of the partial pressure of hydrogen on the free energy changes for oxidation of propionate, butyrate and

*Table 2. Oxidation reactions, with protons as the only electron acceptors, made possible by syntrophic interactions of acetogens and hydrogen consuming bacteria.*

| Reaction | Free energy[b] (kJ/mol) ΔG°' | ΔG' | $H_2$ producing organism | References[a] |
|---|---|---|---|---|
| $acetate^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$ | +104.6 | -9.4 | "Reversibacterium" | 19,34,41 |
| $propionate^- + 3H_2O \rightarrow acetate^- + HCO_3^- + 3H_2 + H^+$ | +76.1 | -9.4 | Syntrophobacter wolinii | 4,39 |
| | | | unnamed | 17 |
| $butyrate^- + 2H_2O \rightarrow 2\ acetate^- + 2H_2 + H^+$ | +48.1 | -8.9 | Syntrophomonas wolfei[d] | 20,21 |
| | | | Syntrophomonas sapovorans[e] | 27 |
| | | | Clostridium bryantii[f] | 32 |
| | | | Clostridium sp.[g] | 35 |
| | | | Strains SF-1[h] and NSF-2[i] | 11,28 |
| | | | unnamed | 1,14 |
| $isovalerate^- + HCO_3^- + H_2O \rightarrow 3\ acetate^- + H_2 + H^+$ | +20.2 | -8.3 | Strains Gralval and Gölval | 33 |
| $methanol + 2H_2O \rightarrow HCO_3^- + 3H_2 + H^+$ | +23.5 | -44.9 | Sporomusa acidovorans | 8 |
| $ethanol + H_2O \rightarrow acetate^- + 2H_2 + H^+$ | +9.6 | -47.4 | S-organism[j] | 6,26 |
| | | | Desulfovibrio sp. | 7 |
| | | | Pelobacter venetianus | 29 |
| | | | Pelobacter carbinolicus[k] | 30 |
| | | | Pelobacter carbinolicus[l] | 10 |
| | | | Thermoanaerobium brockii | 3 |
| | | | Strains GraETOH11[m] and GraProp11[m] | 12 |
| | | | unnamed | 25 |
| | | | "Reversibacterium" | 19,34,41 |
| $benzoate^- + 7H_2O \rightarrow 3\ acetate^- + HCO_3^- + 3H_2 + H^+$ | +53.0[c] | -15.4 | Syntrophus buswellii | 23,24 |
| | | | Strain BZ-1 and BZ-2 | 9,28 |
| | | | unnamed | 13,16 |
| | | | unnamed[n] | 36 |
| $phenol + 9H_2O \rightarrow 2\ acetate + 2HCO_3^- + 6H_2 + 4H^+$ | +111.1 | -60.1 | Strain P-2 | 2 |
| $alanine + 3H_2O \rightarrow acetate^- + HCO_3^- + 2H_2 + H^+ + NH_4^+$ | +7.5 | -49.5 | Acidaminobacter hydrogenoformans[o] | 31 |

| | | | | |
|---|---|---|---|---|
| | | | Eubacterium acidaminophilum[f] | 40 |
| | | | Clostridium sporogenes[q] | 37,38 |
| glutamate$^-$ + $4H_2O$ ⟶ propionate$^-$ + $2HCO_3^-$ + $2H_2$ + $H^+$ + $NH_4^+$ | -5.8 | -62.8 | Acidaminobacter hydrogenoformans[o] | 31 |
| glucose + $4H_2O$ ⟶ 2 acetate$^-$ + $2HCO_3^-$ + $4H_2$ + $4H^+$ | -206.3 | -320.3 | Strain PA-1[r] | 2,5 |
| fructose + $4H_2O$ ⟶ 2 acetate$^-$ + $2HCO_3^-$ + $4H_2$ + $4H^+$ | -206.3 | -320.3 | Syntrophococcus sucrumutans | 18 |
| $nC_6H_{12}O_6$ + $12nH_2O$ ⟶ $12nH_2$ + $6nHCO_3^-$ + $6nH^+$ (cellulose) | | | Acetivibrio cellulolyticus | 15 |
| lactate$^-$ + $2H_2O$ ⟶ acetate$^-$ + $HCO_3^-$ + $2H_2$ + $H^+$ | -4.2 | -61.2 | Desulfovibrio sp. | 7,22 |
| pyruvate$^-$ + $2H_2O$ ⟶ acetate$^-$ + $HCO_3^-$ + $H_2$ + $H^+$ | -47.3 | -75.8 | Strain PA-1[r] | 2 |
| malate$^-$ + $3H_2O$ ⟶ acetate$^-$ + $2HCO_3^-$ + $2H_2$ + $H^+$ | -26.4 | -83.4 | Acidaminobacter hydrogenoformans[o] | 31 |
| | | | Eubacterium acidaminophilum[p] | 40 |

[a] 1.Ahring and Westermann (1987[a,b]); 2. Barik et al. (1985); 3. Ben-Bassat et al. (1981); 4. Boone and Bryant (1980); 5. Brulla and Bryant (1989); 6. Bryant et al. (1967); 7. Bryant et al. (1977); 8. Cord-Ruwisch and Ollivier (1986); 9. Dolfing and Tiedje (1986); 10. Dubourguier et al. (1986); 11. Dwyer et al. (1988); 12. Eichler and Schink (1985); 13. Ferry and Wolfe (1976); 14. Henson and Smith (1985); 15. Khan (1980); 16. Knoll and Winter (1989); 17. Koch et al. (1983); 18. Krumholz and Bryant (1986); 19. Lee and Zinder (1988); 20. McInerney et al. (1979); 21. McInerney et al. (1981[a,b]); 22. McInerney and Bryant (1981); 23. Mountfort and Bryant (1982); 24. Mountfort et al. (1984); 25. Plugge et al. (1989); 26. Reddy et al. (1972[a]); 27. Roy et al. (1986); 28. Shelton and Tiedje (1984); 29. Schink and Stieb (1983); 30. Schink (1984); 31. Stams and Hansen (1984); 32. Stieb and Schink (1985); 33. Stieb and Schink (1986); 34. Thauer et al. (1989); 35. Tomei et al. (1985); 36. Tschech and Schink. (1986); 37. Wildenauer and Winter (1986); 38. Winter et al. (1987); 39. Xun and Bryant (1989); 40. Zindel et al. (1988); 41. Zinder and Koch (1984)

[b] $\Delta G°'$, standard conditions, pH = 7, 25 °C; $\Delta G'$, same as $\Delta G°'$ with the exception that $p_{H_2} = 10^{-5}$ atm.; after Thauer et al. (1977)

[c] After Thauer and Morris (1984)

[d] The organism degrades syntrophically saturated monocarboxylic fatty acids (4 - 8 carbon)

[e] The organism degrades syntrophically long chain fatty acids (4 - 18 carbon)

[f] The organism degrades syntrophically fatty acids (4 - 11 carbon) and 2-methylbutyrate
[g] Might be *Desulfotomaculum* species
[h] The organism degrades syntrophically fatty acids (4 - 6 carbon) and 2-methylbutyrate
[i] The organism degrades syntrophically fatty acids (4 - 6 carbon)
[j] The organism degrades syntrophically n-alcohols (2 - 5 carbon) and isobutanol
[k] The organism degrades syntrophically n-alcohols (2 - 4 carbon)
[l] The organism degrades syntrophically alcohols (2 - 5 carbon) and some diols
[m] The organism degrades syntrophically n-alcohols (2 - 5 carbon)
[n] Two different strains degrading syntrophically benzoate and hydroxybenzoate
[o] The organism degrades syntrophically some amino acids and malate
[p] The organism degrades syntrophically some amino acids, casamino acids, peptone, yeast extract and malate
[q] The organism degrades syntrophically alanine and isoleucine
[r] The organism degrades syntrophically glucose, pyruvate, fumarate and aspartate, and probably a range of lowly substituted aromatic compounds

ethanol, and on the methane formation from hydrogen plus bicarbonate, and sulphate reduction with hydrogen. Because three moles of hydrogen are produced per mole of propionate oxidized, the change of free energy of this reaction is stronger influenced by hydrogen than butyrate and ethanol oxidation. Decreasing the partial pressure of hydrogen leads to a decrease

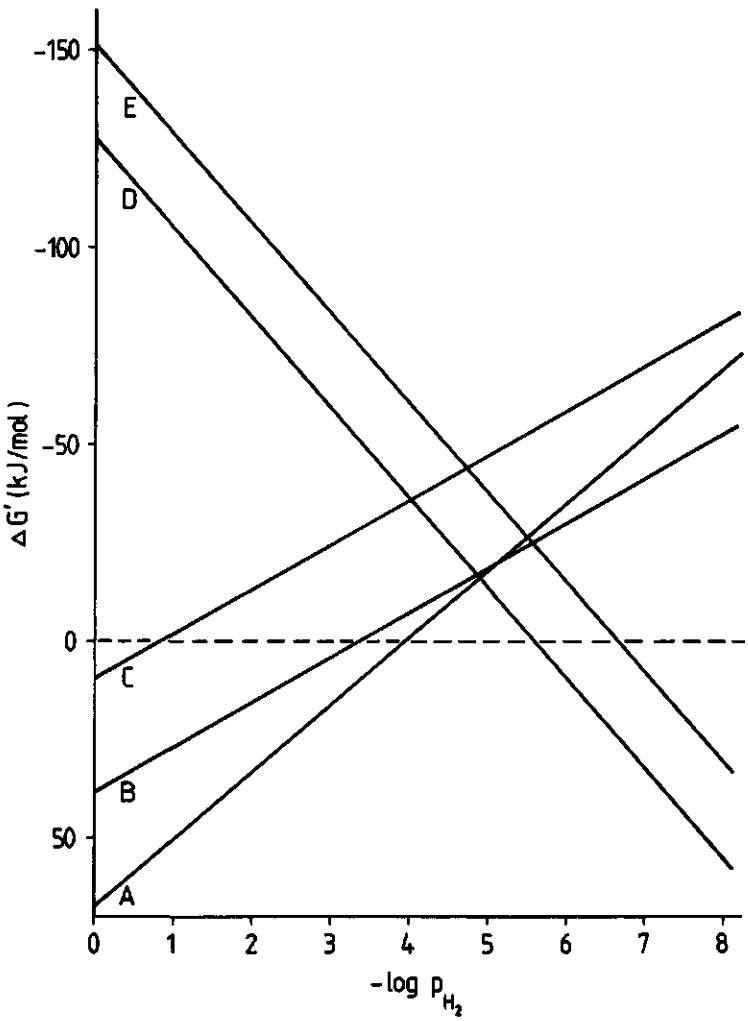


*Figure 3. Effect of the partial pressure of hydrogen ($p_{H_2}$) on the change of free energy (ΔG') for the reactions involving propionate (A), butyrate (B) and ethanol (C) oxidation, methane formation from hydrogen and bicarbonate (D), and sulphate reduction with hydrogen (E). Calculations were done assuming: [$HCO_3^-$] = 30 mM, all other concentrations were 20 mM; $p_{CH_4}$ = 0.7 atm.; T = 25 °C; pH = 7. The ΔG°-values to calculate this figure were from Thauer et al. (1977).*

of the $\Delta G'$ for the hydrogen producing organisms and an increase of the $\Delta G'$ for the hydrogenotrophic organisms (Figure 3). This implies that syntrophic associations can only exist within certain boundaries of the hydrogen partial pressure (McCarty, 1982; Zehnder and Stumm, 1988; Dolfing 1988). For syntrophic propionate oxidation under methanogenic conditions, these boundaries are $1.1 \times 10^{-4}$ atm. and $2.5 \times 10^{-6}$ atm. of hydrogen, whereas ethanol oxidation is possible over a wider range of hydrogen partial pressures. The narrow bounderies between which coexistence of hydrogenogenic acetogens and their hydrogenotrophic partners is possible, is reflected in the rather constant relative numbers of both types of organisms in cocultures (Bryant et al., 1967; Ferry and Wolfe, 1976; McInerney et al., 1979; Koch et al., 1983; Ahring and Westermann, 1987[a,b]; Dolfing and Tiedje, 1986, 1987; Houwen et al., 1990). Oxidation of hydrogen coupled to sulphate reduction is energetically more favourable than formation of methane from carbon dioxide reduction (Figure 3) (Dolfing, 1988). This may contribute to the higher growth rates which have been reported for obligate syntrophic acetogenic bacteria in coculture with sulphate reducers (McInerney et al., 1979; Boone and Bryant, 1980; Mountfort and Bryant, 1982; Houwen et al., 1990).

Besides allowing anaerobic oxidation of organic compounds, interspecies hydrogen transfer may also affect the metabolism of fermentative bacteria (Figure 2, first step). The classical example in this respect is Ruminococcus albus (Figure 4) (Iannotti et al., 1973). This organism ferments glucose to acetate, ethanol, carbon dioxide and hydrogen in pure culture. In coculture with Wolinella succinogenes, an organism that reduces fumarate to succinate, interspecies hydrogen transfer causes a shift in the intracellular electron flow and fermentation of glucose towards ethanol stops. The fermentation change caused by hydrogen utilization provides more energy for growth of the hydrogen producing species, because all of the shifts lead to an increased flow of pyruvate carbon to acetate carbon rather than to the electron sink fermentation product ethanol. Since one mole of ATP is associated with the production of one

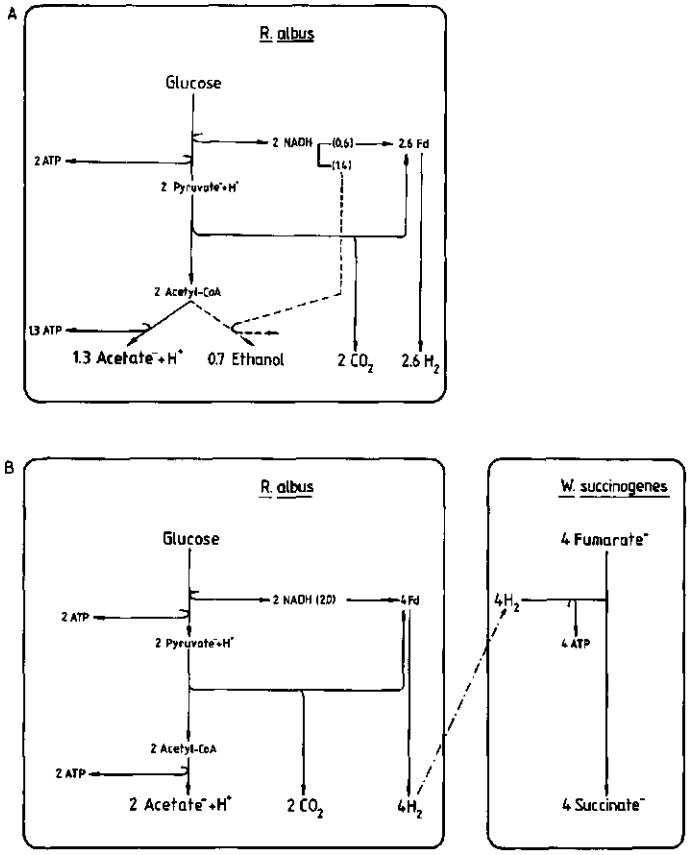


*Figure 4. Degradation of glucose by R. albus in pure culture (A) and in coculture with W. succinogenes (B). Interspecies hydrogen transfer causes a shift in electron and carbon flow, resulting in more ATP formation by the hydrogenogenic bacterium (Fd = ferredoxin) (Iannotti et al., 1973; Thauer et al., 1977).*

mole of acetate from pyruvate (Thauer et al., 1977), more ATP per mole of glucose is obtained in the coculture as compared to pure culture fermentation.

Many examples are known of fermentative organisms which change their metabolism depending on the partial pressure of hydrogen. In the presence of a hydrogenotrophic organism, causing low hydrogen partial pressures, these facultative proton reducing organisms are no longer obliged to dispose the electrons generated during oxidation of the substrate to carbon compounds (fermentation), but are free to produce hydrogen from NADH (respiration) and thereby generating more ATP per mole of

substrate oxidized (Wolin, 1974; 1976; Mah, 1982; Wolin and Miller, 1982).

Interspecies formate transfer

Thiele and Zeikus (1988) proposed that at least in some ecosystems the control of syntrophic electron flow during methanogenesis is achieved predominantly by interspecies formate transfer. A new model was put forward for electron flow during ethanol metabolism in digestor flocs with Desulfovibrio vulgaris and Methanobacterium formicicum as the interacting bacteria. In the localized mironiches high turnover constants for reducing equivalents (electrons) are developed. These turnovers are uncoupled from the hydrogen and/or formate pool outside the flocs. The main arguements for their hypothetical "formate-bicarbonate electron shuttle cycle" are: 1) turnovers of hydrogen pools were calculated to account for only a small part of the simultaneously occurring syntrophic electron transfer reaction rates; 2) the concentration of hydrogen in various ecosystems are below the threshold for carbon dioxide reducing methanogenic bacterial cultures; 3) the calculated thermodynamic free energies for several naturally occurring syntrophic methanogenic reactions are positive under in situ conditions; 4) thermodynamic calculations show that it could be energetically advantageous for syntrophic acetogens to preferentially excrete formate under in situ conditions; 5) most hydrogenotrophic methanogens are also able to use formate; at physiological hydrogen partial pressures, methane formation would be faster with formate than with hydrogen as methanogenic substrate; 6) some proton reducing bacteria are able to form formate.
The "bicarbonate-formate electron shuttle model" was further supported by production of $^{14}C$-formate from $^{14}CO_2$ in syntrophic ethanol and lactate degradation (Thiele and Zeikus, 1988). Moreover, dynamic reaction diffusion models of the flocs, used to analyze the consequences of aggregation for syntrophic

relations in methanogenic ecosystems, also were in favour of formate as the means of electron transfer in this ecosystem (Ozturk et al., 1989).
Boone et al. (1989) calculated that interspecies hydrogen transfer could not account for the observed degradation rates for butyrate and propionate oxidation (with concomitant methane production) in digestors and cocultures. Also in these systems, electron transfer was proposed to be achieved predominantly (98%) via formate, based on its higher diffusion coefficient in water compared to hydrogen.

Further physiological and biochemical studies are needed to give a better fundamental understanding of interspecies electron transfer. Whether hydrogen or formate is the most important electron carrier in complex anaerobic systems is difficult to determine. In complex associations of bacteria, many different organisms are present having either a formate hydrogen lyase or both a hydrogenase and formate dehydrogenase. Because of such enzyme systems, $H_2/CO_2$ and formate will easily be interconverted. The obligate syntrophic S-organism metabolizes formate to $H_2/CO_2$ in the presence of ethanol (Reddy et al., 1972b). Also Syntrophomonas wolfei and Methanothrix soehngenii are able to form $H_2$ from formate (Boone et al., 1989; Huser et al. 1982), whereas $H_2/CO_2$ and formate are interconverted by Desulfovibrio in the absence of sulphate (Stams and Zehnder, 1990) and by methanogens (Tzeng et al., 1975; Nishio et al., 1983; Eguchi et al., 1985; Sparling and Daniels., 1986; Tanner et al., 1989).
Diffusion calculations for hydrogen and formate are based on the assumption that interspecies electron transfer is determined by the thermodynamics of producing and consuming reactions (Ozturk et al., 1988; Boone et al., 1989). However, if the transfer of electrons is kinetically limited by enzymatic formation or consumption of hydrogen or formate at the cell surface, biological kinetic parameters have to be considered too. The determination of these parameters, e.g. the $K_m$ for hydrogenases, may be very complicated because of mass transfer limitation (Boone et al., 1989). Moreover, kinetic parameters might not be constant under different physiological conditions (Westermann et al.,

1989).
A clear argument against the necessity of formate transfer is that syntrophic ethanol and butyrate degrading cultures exist which grow in the presence of methanogens that are unable to oxidize formate (Bryant et al., 1967; McInerney et al., 1981[a]; Ahring and Westermann, 1987[a,b]).

Since formate is possibly important as interspecies electron carrier, the term "obligate proton reducing bacteria" should be used with caution. In the formate-bicarbonate electron shuttle cycle, not protons but carbon dioxide is reduced. Therefore, the term "obligate syntrophic acetogens" may be used for those organisms whose catabolic oxidations are only possible by the removal of electrons via either hydrogen or formate.

## APPROACHES TO STUDY THE PHYSIOLOGY AND BIOCHEMISTRY OF SYNTROPHIC BACTERIA; OUTLINE OF THIS THESIS

The work presented in this thesis deals with microbiological and biochemical aspects of syntrophic propionate oxidizing cocultures. Up to now, only a few obligate syntrophic acetogenic bacteria have been reported, which oxidize propionate to acetate, carbon dioxide and (presumably) hydrogen. Both Syntrophobacter wolinii (Boone and Bryant, 1980; Xun and Bryant, 1989) and the propionate oxidizing organism in the methanogenic coculture, originally enriched by Koch and coworkers (Koch et al., 1983; Houwen et al., 1987, 1990), are rod shaped, non-motile, and grow in coculture with sulphate reducing bacteria or methanogens. In contrast with S. wolinii, which is Gram-negative, the latter organism stains Gram-positive (Chapter 6).

Biochemical and physiological studies on obligate syntrophic acetogenic bacteria are difficult because of the presence of electron scavenging organisms. Three different approaches have been applied and tried to overcome this difficulty: 1) use of techniques which do not require pure cultures, e.g. the use of

specifically labelled compounds, 2) separation of cells by physical means, and 3) growth of the acetogen in pure culture by either using metabolic intermediates or artificial electron acceptors.

The use of labelled compounds is common to investigate biochemical pathways. $^{13}$C-NMR appeared to be a powerful tool to study propionate metabolism in anaerobic bacteria. Evidence was found for the involvement of the succinate pathway in propionate oxidation (Chapter 2 and 3), as has been shown by others (Buswell et al., 1951; Koch et al., 1983; Schink, 1985; Robbins, 1988). Moreover, with the inclusion of $H^{13}CO_3^-$ as second labelled compound, carboxylation reactions involved in propionate metabolism could be studied in a very easy way (Chapter 3). Even in complex biological systems the NMR technique appeared to be useful. It was possible to follow turnovers of different compounds in methanogenic granular sludge, after the addition of $^{13}$C-propionate and fumarate (Chapter 7).

To study enzyme activities in obligate syntrophic acetogens, very elegant ways were applied for the butyrate oxidizing Syntrophomonas wolfei. After syntrophic growth, cells were either separated from the hydrogenotrophic methanogens by gradient centrifugation (Beaty et al., 1987) or preferentially lyzed (Wofford et al., 1986). If a defined biculture is available, enzyme activities of the acetogen can also be determined by subtraction of the activities found for the hydrogen (or formate) consumer grown in pure culture. In this way several enzymes of the succinate pathway, including the key enzyme propionyl-CoA:oxaloacetate transcarboxylase, were demonstrated in S. wolinii (Chapter 6).

The nicest approach to study obligate syntrophic acetogens is to grow them in pure culture. S. wolfei, which oxidizes butyrate syntrophically, was grown axenic on crotonate, from which the CoA-ester is an intermediate in the degradation of butyrate (Beaty and McInerney, 1987). Attempts to grow the propionate oxidizing organism in a methanogenic coculture (Koch et al., 1983) on the metabolic intermediate pyruvate, failed because the organism does not grow on it, or growth on pyruvate is slower

compared with contaminating organisms present (Chapter 5).
Further, artificial systems have been described to remove hydrogen in biological systems: a) S. wolfei was shown to oxidize butyrate with simultaneous catalytic olefin reduction (Kaspar et al., 1987), b) in a slightly modified system Ahring and coworkers were able to grow S. wolfei in pure culture (Ahring, personal communication), c) Emde and Schink (1989) demonstrated the transfer of electrons to an electrode via a reoxidizable electron acceptor. The principle of a reoxidizable redox mediator was applied in a two-liquid-phase system, with a chemically synthesized electron acceptor (Chapter 4). Although the system was able to remove hydrogen from the gas phase and to remove electrons disposed during glutamate oxidation by Acidaminobacter hydrogenoformans, it was not possible to grow the propionate oxidizing organism.
If a pure culture of the acetogenic bacterium is available, interspecies electron transfer may be possible without contact of the two types of bacteria. The dialysis membrane method (Zindel et al., 1988) offers the possibility to perform physiological studies and to investigate enzyme activities after syntrophic growth without the interference of the hydrogen (or formate) consuming organism.

Chapter 2

# $^{13}$C-NMR STUDY OF PROPIONATE DEGRADATION BY A METHANOGENIC COCULTURE


## ABSTRACT

In vivo high-resolution $^{13}$C-NMR was used to study propionate oxidation in a methanogenic coculture. The addition of [3-$^{13}$C]-labelled propionate clearly showed succinate as an intermediate. The ultimate breakdown product acetate was labelled equally in the C-1 and C-2 positions. In addition, de novo synthesis of [2-$^{13}$C]-propionate was observed. The $^{13}$C-label randomized completely between the C-2 and C-3 of propionate. The results presented give further evidence for the involvement of the methylmalonyl-CoA pathway.



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## INTRODUCTION

During degradation of complex organic material under methanogenic conditions, about 15% of the total carbon is degraded via propionate as an intermediate (McCarty, 1964; Kaspar and Wuhrmann, 1978[a]; Gujer and Zehnder, 1983). Propionate is oxidized to acetate, carbon dioxide and hydrogen, according to the following equation:

$$CH_3CH_2COO^- + 3\ H_2O \longrightarrow CH_3COO^- + HCO_3^- + H^+ + 3\ H_2$$
$$\Delta G^{\circ\prime} = +80\ kJ/mol$$

Because of the extreme positive $\Delta G^{\circ\prime}$, the degradation of propionate is only feasible when the hydrogen is removed by other bacteria and its partial pressure is kept very low (Zehnder and Koch, 1983). Syntrophic cocultures of propionate oxidizing bacteria and hydrogen consuming anaerobes have been described by Boone and Bryant (1980).

Although there is some information about the kinetics of the breakdown of propionate (McCarty, 1964; Kaspar and Wuhrmann, 1978[a,b]; Zehnder and Koch, 1983; Heyes and Hall, 1983; Schink, 1985) very little is known about the biochemical pathway of the propionate oxidation. Based on results obtained with $^{14}$C-propionate labelled at different positions (Buswell et al., 1951; Koch et al., 1983; Schink, 1985) it is very likely that propionate is degraded via a pathway containing at least one symmetric intermediate, most likely succinate. However, direct evidence for this intermediate is lacking.

In this paper we present the results of an *in vivo* high-resolution $^{13}$C-NMR study with [3-$^{13}$C]-labelled propionate. The use of $^{13}$C-propionate offers the possibility to study the incorporation of $^{13}$C-label into intermediates and end products derived from propionate, simultaneously with the scrambling processes which are involved.

## MATERIALS AND METHODS

### Organisms and cultivation

The coculture used in this study, originally enriched by Koch et al. (1983), contained the propionate oxidizing organisms (approximately 10%), 2 populations of methanogenic bacteria and less than 1% of an unknown bacterium. In this coculture propionate is degraded to acetate, carbon dioxide and methane; acetate is not further metabolized.

The coculture was cultivated in a medium containing (in g/l unless otherwise stated): $KH_2PO_4$, 0.6; $Na_2HPO_4.2H_2O$, 1.68; $NH_4Cl$, 0.3; NaCl, 0.3; $CaCl_2.2H_2O$, 0.11; $MgCl_2.6H_2O$, 0.1; $NaHCO_3$, 4; $Na_2S.9H_2O$, 0.24; trace elements solution ($FeCl_2.4H_2O$, 2; $H_3BO_3$, 0.05; $ZnCl_2$, 0.05; $CuCl_2$, 0.03; $MnCl_2.4H_2O$, 0.05; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2.6H_2O$, 0.05; $NiCl_2$, 0.05; EDTA, 0.5; 1 ml concentrated HCl), 1 ml; vitamin solution (biotin, 2 mg; folic acid, 2 mg; pyridoxine HCl, 10 mg; riboflavin, 5 mg; thiamine HCl, 5 mg; niacin, 5 mg; cyanocobalamin, 5 mg; p-aminobenzoic acid, 5 mg; panthothenic acid, 5 mg), 1 ml and sodium propionate, 4.8. $SeO_2$ (0.1 mg) and $Na_2WO_4.H_2O$ (0.05 mg) were added as extra trace elements. The culture was grown in 10 l vessels containing 8 l medium under an atmosphere of $N_2/CO_2$ (80:20; 1.06 atm.) at 35 °C in the dark. The pH of the medium was 7.0-7.2.

### Preparation for the NMR

Cells were centrifuged and washed anaerobically with growth medium containing 5 mM sodium propionate to prevent starvation. Since FeS, included in the suspension, turned out to cause significant peak broadening in the NMR-spectra, removal of this substance was necessary. The 125 times concentrated cell suspension was therefore transferred to a test tube within an anaerobic glove box with a gasphase of $N_2/H_2$ (96:4). After

standing for four hours most of the FeS-precipitate had sedimented. Then, 2.0 ml of the supernatant was transferred to a NMR tube and 0.25 ml $^2H_2O$ was added to provide lock signal. This suspension contained approximately 8 x $10^{10}$ cells/ml. The NMR tube was closed with an over-seal stopper and the gas phase was changed to $N_2/CO_2$ (80:20; 1.2 atm.). At time zero 0.23 ml [3-$^{13}$C]-sodium propionate (90% enriched, Amersham, U.K.) was added with a syringe. The final propionate concentration was 45 mM.

High-resolution NMR

The $^{13}$C-NMR spectra were obtained at 75.46 MHz on a Bruker CXP-300 NMR spectrometer operating in the Fourier-transform mode, equipped with a 10 mm dedicated $^{13}$C-probe. Broadband proton-noise decoupling was applied and the measuring temperature was maintained at about 38 °C. Blocks of 7200 transients (1 h) were accumulated and sequentially stored on disc using 8000 data points, a 60° pulse (pulse time 12 µs) and a delay time of 0.5 s between the pulses. To obtain well-defined spectra zero-filling from 8 to 16 K data points was applied and a line broadening of 3 Hz was used. The chemical shift belonging to the resonance of the C-2 and C-3 of succinate (35.1 ppm) was used as internal standard. All resonances observed were assigned by adding in succession the corresponding compounds to lysed cells in medium. The areas of the resonances were obtained by integration. A balance of the $^{13}$C-label was calculated by relating the areas of all observed resonances to C-3 of propionate as described in the results section.

RESULTS

The time dependent incorporation of the $^{13}$C-label into

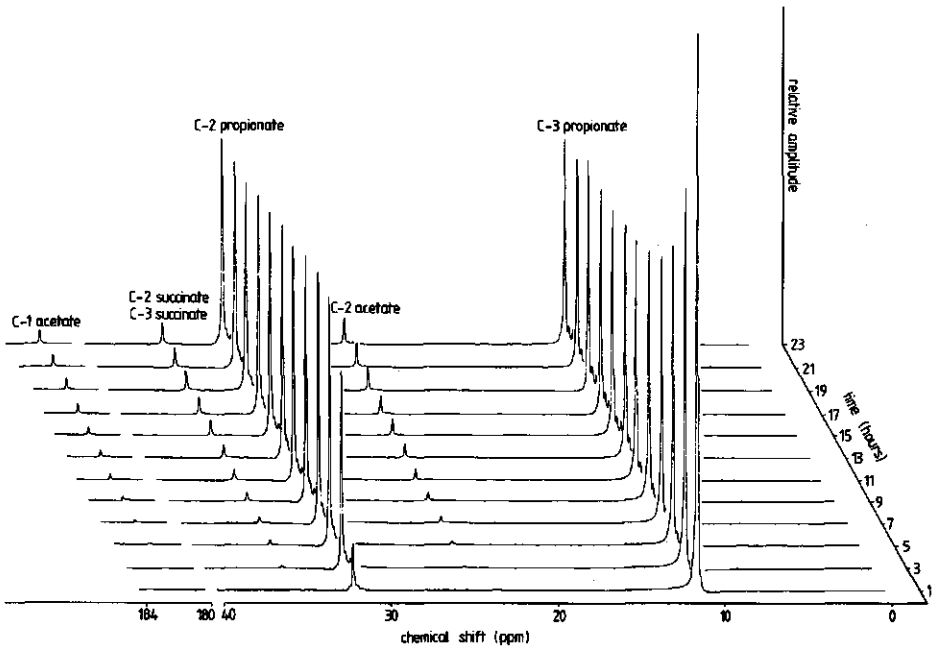


*Figure 1. Time course of the occurrence of $^{13}$C-label as measured in NMR-spectra during incubation of the propionate oxidizing coculture with [3-$^{13}$C]- propionate. Chemical shifts (ppm): 11.1, $^{13}CH_3CH_2COO^-$; 24.3, $^{13}CH_3COO^-$; 31.7,$CH_3{}^{13}CH_2COO^-$; 35.1, $^-OOC{}^{13}CH_2CH_2COO^-$; 182.2, $CH_3{}^{13}COO^-$.*

intermediates and end products after the addition of 45 mM [3-$^{13}$C]-labelled propionate to the coculture is shown in Figure 1. The main observations are: 1) The appearance of the $^{13}$C-label in the C-2 and C-3 carbon positions of succinate (35.1 ppm). Succinate became detectable after the first hour of incubation, accumulated for eighteen hours and remained constant thereafter (Figure 2). 2) Formation of low levels of acetate labelled at both C-atoms resonating at 24.3 (C-2) ppm and 182.2 (C-1), respectively. Label in acetate appeared after one hour and increased during the following twentythree hours of incubation. 3) Randomization of the $^{13}$C-label from [3-$^{13}$C]-propionate over the C-3 and C-2 carbon positions in propionate, resonating at 11.1 and 31.7 ppm, respectively. No resonance was observed at the C-1 resonance position of propionate at 185.6 ppm. The $^{13}$C-label appeared within a few minutes in the propionate C-2 position. Complete randomization was achieved within eight

hours. The concentration of the C-3 labelled propionate decreased simultaneously during these first eight hours, predominantly as a consequence of the randomization process. Propionate was used up slowly as a substrate during the experiment. 4) All resonances observed were composed exclusively of singlets, indicating that condensation reactions, producing $^{13}C$-$^{13}C$ moieties, can be excluded.

Figure 2 represents a compilation of the time courses of $^{13}C$-label depletion from the C-3 carbon position in propionate and its simultaneous incorporation into the C-2 position, the C-2 and C-1 positions of acetate and the C-3 and C-2 positions

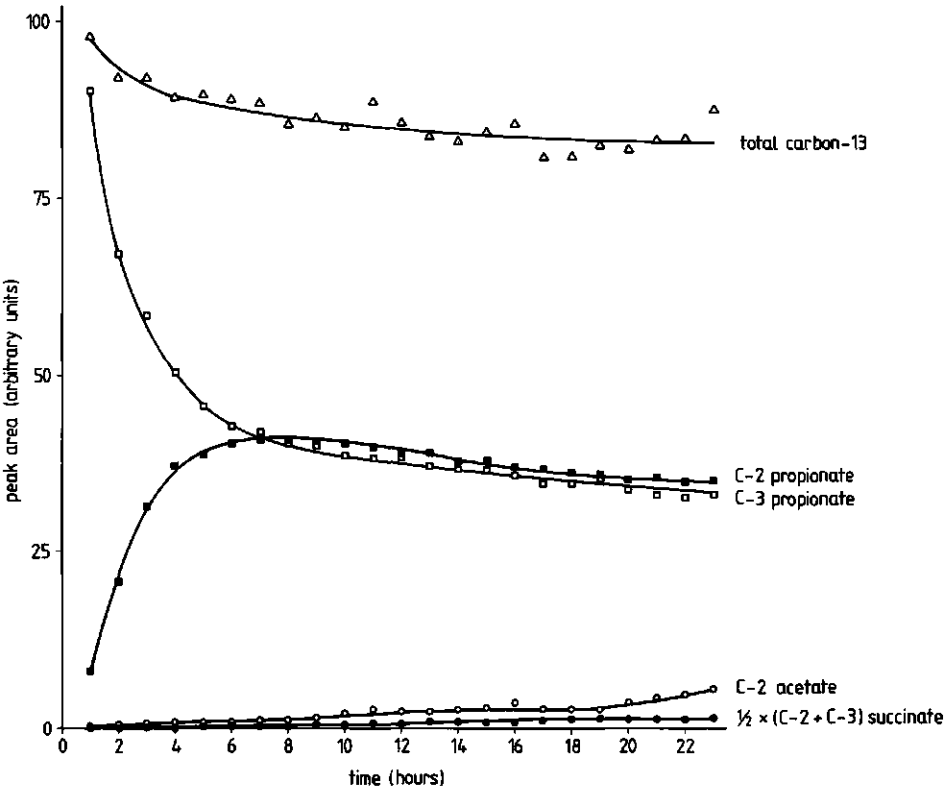


*Figure 2. Time course of the areas of the various $^{13}C$-resonances after adding 45 mM [3-$^{13}C$]-propionate to the coculture without correction for differences in relaxation behaviour. The upper trace represents the sum of the areas of all nuclei expressed in units of [3-$^{13}C$]-propionate (see text).*

*Table 1. Relative peak areas of the natural abundance $^{13}$C-NMR spectrum of an equimolar (40 mM) solution of propionate, acetate and succinate.*

| $^{13}$C-resonating nuclei | relative peak areas |
|---|---|
| C-3 propionate | 100.0 |
| C-2 propionate | 114.3 |
| C-1 propionate | 34.3 |
| C-2 acetate | 57.1 |
| C-1 acetate | 28.8 |
| 1/2 x (C-2 + C-3) succinate | 185.7 |
| 1/2 x (C-1 + C-4) succinate | 25.7 |

of succinate in an alternative fashion, by plotting the areas of the corresponding resonances. After completion of the scrambling over the C-3 and C-2 positions in propionate, the area measured for the C-2 resonance appeared systematically somewhat higher compared with that for the C-3 resonance. This finding can be accounted for by the small differences in relaxation between the two nuclei, which is apparent from the integrals of the natural abundance $^{13}$C-NMR spectrum of an equimolar (40 mM) solution of propionate, acetate and succinate in medium without cells, recorded under identical conditions as the cell spectra (Table 1). The area of the C-2 resonance of acetate is twice as high as that observed for the C-1 resonance of acetate (Table 1). This can also be deduced from the spectra shown in Figure 1 and thus confirms complete scrambling of $^{13}$C-label over both atoms in acetate.

The areas of the various resonances in Figure 2 have been corrected for the different relaxation behaviour of the corresponding nuclei on the basis of the differences in relaxation as derived from the natural abundance $^{13}$C-NMR spectrum (Table 1). From this the time dependent balance of $^{13}$C-label is obtained as the sum of the areas of all labelled nuclei expressed in units of [3-$^{13}$C]-propionate (Figure 2, upper trace). Not all the $^{13}$C-label was recovered during the course

of the experiment. This is most probably due to its incorporation into relatively immobile compounds (e.g. HSCoA-esters) which are beyond detection in high-resolution NMR.

At the end of the experiment, 0.3 ml of concentrated KOH (10 M) was added to the cell suspension, which was subsequently thoroughly shaken to adsorb all gaseous $^{13}CO_2$, to measure the amount of $^{13}C$-label incorporated in this compound. The contribution of gaseous $^{13}CO_2$ to the total $^{13}C$-balance appeared to be neglegible. This is in accordance with the observation that the coculture is not able to degrade acetate.

## DISCUSSION

The detection of succinate as an intermediate strongly suggests the involvement of the methylmalonyl-CoA pathway in the anaerobic propionate oxidation under methanogenic conditions, confirming the results obtained in earlier studies (Koch et al., 1983; Schink, 1985;). Because of the symmetry of succinate, randomization of the label in the product acetate and the substrate propionate occurs. The absence of typical $^{13}C$-$^{13}C$ couplings in the spectra shows that no condensation reactions of labelled products, e.g. acetate, are involved in the formation of succinate and propionate.

The rapid randomization of the label between the C-3 and C-2 atoms in propionate is intriguing. One explanation for this phenomenon might be that the reactions involved in the conversion of propionate to succinate are highly reversible. Since, in the interconversion of propionate and succinate both energy-requiring and energy-yielding reactions are involved, the propionate degrading organism must have a very efficient mechanism of energy conservation. However, the mechanism of this conservation is unknown. In this respect, it is interesting to note that the energy released from the decarboxylation of methylmalonyl-CoA in Veillonella alcalescens is conserved in

a $Na^+$-gradient across the cell membrane (Dimroth and Hilpert, 1984).

The fast randomization of propionate may also lead to a rapid exchange of the carboxyl group with $HCO_3^-$ from the environment. As a consequence, the formation of $^{14}CO_2$ from [1-$^{14}$C]-propionate cannot be taken as a measure for actual propionate breakdown. This observation explains the finding of Boone (1984) that there is difference in the apparent degradation rates of C-1 and C-2 labelled propionate.

The slow degradation of propionate during the twentythree hours of incubation may be caused by the high cell density used in the experiment. It has already been noted by Koch (personal communication) that above a certain cell density the degradation rate per cell declined.

Research is in progress to get further evidence for the methylmalonyl-CoA pathway by a) measuring intermediates with $^{13}$C- and $^{14}$C-labelled substrates and b) demonstration of the key enzymes.

ACKNOWLEDGEMENTS

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Chapter 3

# PROPIONATE METABOLISM IN ANAEROBIC BACTERIA: DETERMINATION OF CARBOXYLATION REACTIONS WITH $^{13}$C-NMR SPECTROSCOPY


## ABSTRACT

The role of carboxylation reactions in propionate metabolism was studied with in vivo high-resolution NMR in anaerobic propionate forming bacteria with well-established biochemical properties, and in two syntrophic propionate oxidizing cocultures. The inclusion of $^{13}$C-propionate and $H^{13}CO_3^-$ gave insight into the process of randomization at the level of propionate in relation to the type of the (de)carboxylating enzyme involved. Propionibacterium but not Veillonella and Desulfobulbus showed a propionate randomization in the absence of substrate. These differences are explained by the type of carboxylation mechanism and the energy state of the cells. Both syntrophic cocultures tested degrade propionate via the succinate pathway involving a transcarboxylase.



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In many anaerobic propionate forming and propionate degrading bacteria the succinate pathway is involved. In this route pyruvate or phosphoenolpyruvate, oxaloacetate, malate, fumarate, succinate, succinyl-CoA, methylmalonyl-CoA and propionyl-CoA are intermediates. Fermentation of sugars and lactate with propionate as major reduced product is carried out by several types of organisms, both Gram-positive and Gram-negative. In Propionibacterium spp. (Leaver et al., 1955; Allen et al., 1964), Veillonella alcalescens (Allen et al., 1963), Anaerovibrio lipolytica (Hobson, 1965), Selenomonas ruminantium (Paynter and Elsden, 1970), Arachnia propionica (Allen and Linehan, 1977), Bacteroides fragilis (Macy et al., 1978), Propionispira arboris (Schink et al., 1982; Thompson et al., 1984) and in Pelobacter propionicus (Schink et al., 1987) propionate is formed via the succinate pathway. In studies with $^{14}C$- and $^{13}C$-labelled compounds, evidence was also provided for the operation of the succinate pathway in syntrophic propionate oxidation (Buswell et al., 1951; Koch et al., 1983; Schink, 1985; Houwen et al., 1987). Desulfobulbus propionicus is able to do both, forming and oxidizing propionate via this pathway (Stams et al., 1984; Kremer and Hansen, 1988).

In the succinate pathway (de)carboxylation reactions play an important role. One of the main differences between the different bacteria is the presence or absence of a transcarboxylase. In Propionibacterium spp. (Swick and Wood, 1960; Wood, 1972), A. propionica (Allen and Linehan (1977), D. propionicus (Stams et al. 1984; Kremer and Hansen, 1988) and P. propionicus (Schink et al., 1987) the endergonic carboxylation of pyruvate to oxaloacetate is linked to the exergonic decarboxylation of methylmalonyl-CoA via a transcarboxylase. Contrary, in Veillonella alcalescens and Propionigenium modestum, the decarboxylation energy is conserved in a membrane-potential (Hilpert and Dimroth, 1982; Hilpert et al., 1984). The membrane-bound decarboxylases translocate $Na^+$-ions across the cell-membrane and

the formed $Na^+$-gradient drives ATP-synthesis via a sodium-ATPase (Dimroth, 1987).

By in vivo $^{13}C$-NMR it was shown for a methanogenic coculture, that propionate is equilibrated with succinate during propionate degradation (Houwen et al., 1987). Such a process, which was already recognized in propionate forming bacteria (Hettinga and Reinbold, 1972; Wood, 1972; 1981) leads to the formation of [2-$^{13}C$]-propionate from [3-$^{13}C$]-propionate, and vice versa. In Figure 1 the distribution of $^{13}C$-label from [3-$^{13}C$]-propionate

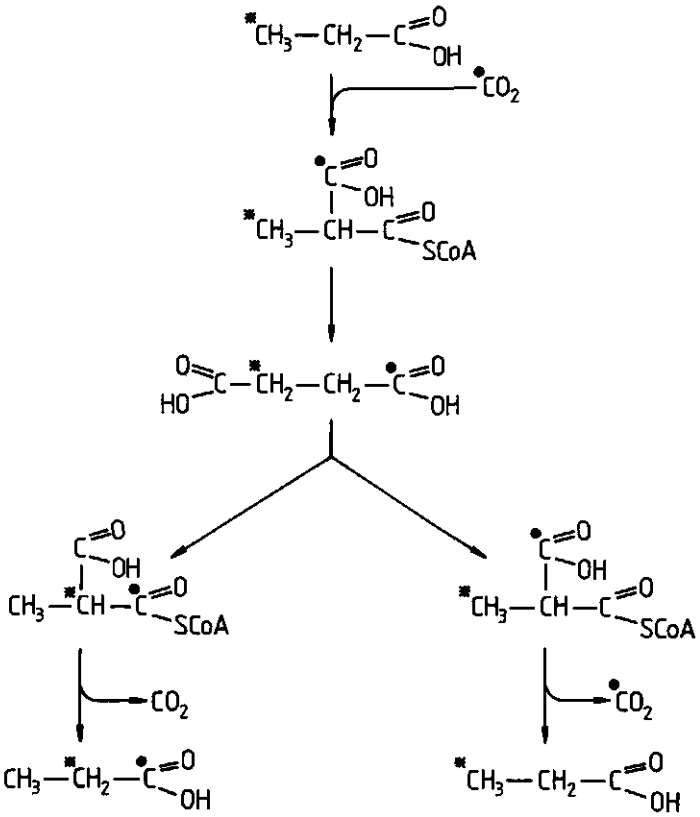

*Figure 1. Distribution of $^{13}C$-label from [3-$^{13}C$]-propionate and $H^{13}CO_3^-$ during interconversion of propionate and succinate with involvement of a carboxylase and a decarboxylase. Continued interconversion also yields the $C_1$-$C_3$ propionate isotopomer. However, propionate labelled at the C-2 and C-3 position ($C_2$-$C_3$ isotopomer) is not formed. In case of a transcarboxylase the incorporated $CO_2$ is expected not to be labelled. In high-resolution NMR the differently labelled molecules can be distinguished.*

and $H^{13}CO_3^-$ during the interconversion of propionate and succinate is shown (Barker, 1972). Because with in vivo $^{13}C$-NMR it is possibile to distinguish between the differently labelled molecules, the presence of labelled propionate and bicarbonate allowed to investigate the carboxylation reactions involved in the various propionate metabolisms.

## MATERIALS AND METHODS

### Organisms and cultivation

Two propionate oxidizing cocultures were used: a methanogenic coculture whose properties and cultivation were described before (Koch et al., 1983; Houwen et al., 1987) and a sulphidogenic biculture (DSM 2805) of Syntrophobacter wolinii and a Desulfovibrio species (Boone and Bryant, 1980). Cultivation was done in a medium containing (in g/l unless otherwise stated): sodium propionate 1.9; $Na_2SO_4$, 2.9; $Na_2HPO_4.2H_2O$, 0.53; $KH_2PO_4$, 0.41; $NH_4Cl$, 0.3; NaCl, 0.3; $CaCl_2.2H_2O$, 0.11; $MgCl_2.6H_2O$, 0.1; $NaHCO_3$, 4; $Na_2S.9H_2O$, 0.24; yeast extract, 0.2; 1 ml of a tenfold concentrated trace element solution described by Pfennig and Lippert (1966); 1 ml of the vitamin solution described by Stams et al. (1983); 1 ml of a mixture of $Na_2MoO_4$, $Na_2WoO_4$ and $SeO_2$ (each 0.1 mM) in 10 mM NaOH. Sodium lactate (1 mM) was added to stimulate the sulfate reducing bacterium. Desulfobulbus propionicus (DSM 2032) was a gift of D.R. Kremer, Department of Microbiology, University of Groningen, The Netherlands. The organism was cultivated in a medium containing (in g/l): sodium propionate 1.9; $Na_2SO_4$, 2.9; $Na_2HPO_4.2H_2O$, 0.53; $KH_2PO_4$, 0.41; $NH_4Cl$, 0.54; $CaCl_2.2H_2O$, 0.15; $MgCl_2.6H_2O$, 0.4; KCl, 0.3; $NaHCO_3$, 2.4; $Na_2S.9H_2O$, 0.48; yeast extract, 0.2. Trace elements and vitamins were the same as described above. Propionibacterium freudenreichii (DSM 20270) was cultivated in a medium containing (in g/l): sodium lactate, 5; caseine peptone, tryptic digest, 5;

yeast extract, 2,5; $NaHCO_3$, 1.7; sodium propionate, 1.9. The pH was adjusted to 7.0-7.2. The medium for the cultivation of Veillonella alcalescens (DSM 1399) contained (in g/l): sodium lactate, 5; yeast extract, 2.5; sodium thioglycollate, 0,5; $NaHCO_3$, 1.7; sodium propionate, 1.9. The pH was adjusted to 7.0. Megasphaerea elsdenii (DSM 20460) was kindly provided by M.C. Snoek, Department of Biochemistry, Agricultural University of Wageningen, The Netherlands. The organism was grown in a medium containing (in g/l): sodium lactate, 10; yeast extract, 6; sodium thioglycollate, 1; dissolved in tap water. The pH of the medium was adjusted to 7.4.
The two cocultures and V. alcalescens were grown at 37°C; the other organisms were cultivated at 30°C. Except for M. elsdenii, which was cultivated under $N_2$, the gas phase consisted of $N_2/CO_2$ (80:20).

Preparation for NMR

About 1.5 liter of the cultures was centrifuged anaerobically at 21,500 x g. All the following procedures were done in an anaerobic glove box with a gas phase of $N_2/H_2$ (96:4). The cell pellets of the methanogenic and sulphidogenic coculture and of D. propionicus were resuspended in their respective media without propionate but with $H^{13}CO_3^-$. Since FeS causes significant peak-broadening in the NMR-spectra, the suspensions were allowed to stand for 2 to 7 hours during which the FeS present in the medium could settle. After transfer to an NMR tube of the supernatant containing the cells, 10% (v/v) $^2H_2O$ was added to provide a lock signal. The NMR tube was closed with an overseal-stopper and the gas phase was changed to $N_2$ (1.2 atm.). At time zero, 50 mM [3-$^{13}$C]-propionate was added. The final densities of the cultures were 3.6 x $10^{10}$, 4.0 x $10^{10}$ and 2.3 x $10^{10}$ cells/ml, respectively.
Centrifuged cells of P. freudenreichii, V. alcalescens and M. elsdenii were resuspended in their respective media but without lactate, propionate and bicarbonate. The cell suspensions,

containing 3.7 x $10^{11}$, 2.5 x $10^{11}$ and 1.1 x $10^{10}$ cells/ml, respectively, were transfered to NMR tubes after addition of $^2H_2O$ (10% v/v), and the gas phase was changed to $N_2$ (1.2 atm.). A tenfold concentrated mixture of $H^{13}CO_3^-$ and sodium lactate was added to obtain concentrations of 20 mM and 2 mM, respectively. The formation of $^{13}C$-labelled compounds was followed for 45 (3 x 15) minutes by high-resolution NMR. [3-$^{13}C$]-propionate was added to a concentration of 20 mM and recording of NMR-spectra was continued for at least fourteen hours. Then, lactate was added to a concentration of 20 mM and the tubes were incubated further outside the NMR-apparatus. After prolonged incubation the cultures were analyzed again by NMR for one hour.

## High-resolution NMR

$^{13}C$-NMR spectra were obtained at 75.46 MHz on a Bruker CXP-300 NMR spectrometer operating in the Fourier-transform mode, using 10 mm o.d. NMR tubes. The Waltz pulse sequence was employed to decouple protons and the measuring temperature was maintained at about 30°C or 37°C, depending on the organism tested. NMR spectra of cells were collected by sequential storing of 1 hour periods (1800 transients) on disc in 8000 data points, using a 45° pulse angle (pulse duration 9 μs) and a pulse interval of 2 s. The chemical shift belonging to the C-3 of propionate (11.1 ppm) was used as internal standard.

## Chemicals

Sodium [3-$^{13}C$]-propionate (90% enriched) and $NaH^{13}CO_3$ (99% enriched) were purchased from Amersham, UK. Other chemicals were at least of analytical grade. Gases were obtained from Hoek Loos, Schiedam, The Netherlands.

## RESULTS

### Propionate oxidizing cultures

Incubation of the methanogenic coculture resulted in a complete degradation of the substrate within five hours (Figure 2). The acetate produced was labelled at both carbon atoms. Immediately after addition of $^{13}$C-propionate, randomization over the C-3 and C-2 carbons of propionate was observed. Succinate, which was previously shown to be an intermediate in propionate oxidation (Houwen et al., 1987), was not detected. Label from preadded bicarbonate was incorporated at the C-1 carbon position of propionate. The observed C-1 resonances were composed of a singlet and a doublet, corresponding to the single and double labelled propionate isotopomers $^{13}C_1$-$^{12}C_2$ and $^{13}C_1$-$^{13}C_2$,

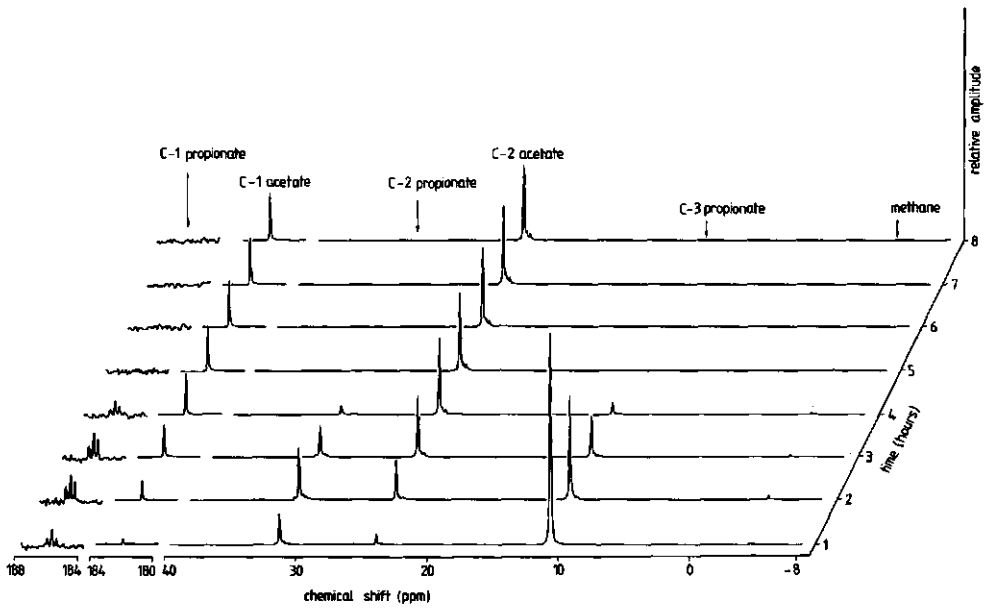


*Figure 2. Time course of the label incorporation as measured by $^{13}$C-NMR during the first 8 hours of incubation of the propionate oxidizing methanogenic coculture with [3-$^{13}$C]-propionate and $H^{13}CO_3^-$. The resonances belonging to the C-1 of propionate, composed of a singlet (central) and a doublet, are enlarged by a factor of 20 in vertical sense. $^{13}CO_2$ and $H^{13}CO_3^-$ are not shown.*

respectively. In the first hour, the contribution of the doublet was relatively small due to the initially higher concentration of C-3 labelled and unlabelled propionate (10% is not enriched) in contrast to C-2 labelled propionate. The presence of $H^{13}CO_3^-$ also resulted in the formation of $^{13}CH_4$ (-3.7 ppm), produced by the hydrogenotrophic methanogens. The decrease of this resonance with time can be accounted for by escape of the methane into the gas phase.

Figure 3A shows the time course of the various observed $^{13}C$-resonances by plotting their areas. Since they are not corrected for differences in relaxation behaviour and built up of Nuclear Overhauser Enhancement (NOE) between the various atomic nuclei, quantitative comparison between the labelled compounds by solely comparing integrals is inadequate. Serious errors can be made when quantitative relationships are derived from spectra recorded under rapid pulsing conditions, when these differences are not corrected for. To solve this problem and thus to enable quantitative comparison, the natural abundance $^{13}C$-NMR spectrum was recorded of an equimolar (40 mM) solution of propionate, acetate and succinate in medium as used for the methanogenic coculture. Spectra were recorded under identical conditions as described for the cell suspensions. Table 1 summarizes the integrated areas of the various nuclei relative to C-3 propionate, arbitrairily set to 100. Because $CO_2$ (and indirectly $HCO_3^-$) and $CH_4$ escape partially into the gas phase, their relative concentrations could quantitatively not be related to the other compounds.

In the methanogenic coculture propionate was completely randomized over the C-3 and C-2 atoms within the first three hours (Figure 3A, Table 1). The area of the C-2 carbon resonances of acetate is 2.1 times higher than of the C-1 carbon resonance, which shows that both carbon atoms are labelled to equal extend. Although the peak area of C-1 labelled propionate is very low, the amount of this compound after three hours is 17% of the amount of C-2 labelled propionate. In the fourth hour this percentage increased to 31%, probably due to the continued interconversion of propionate and succinate.

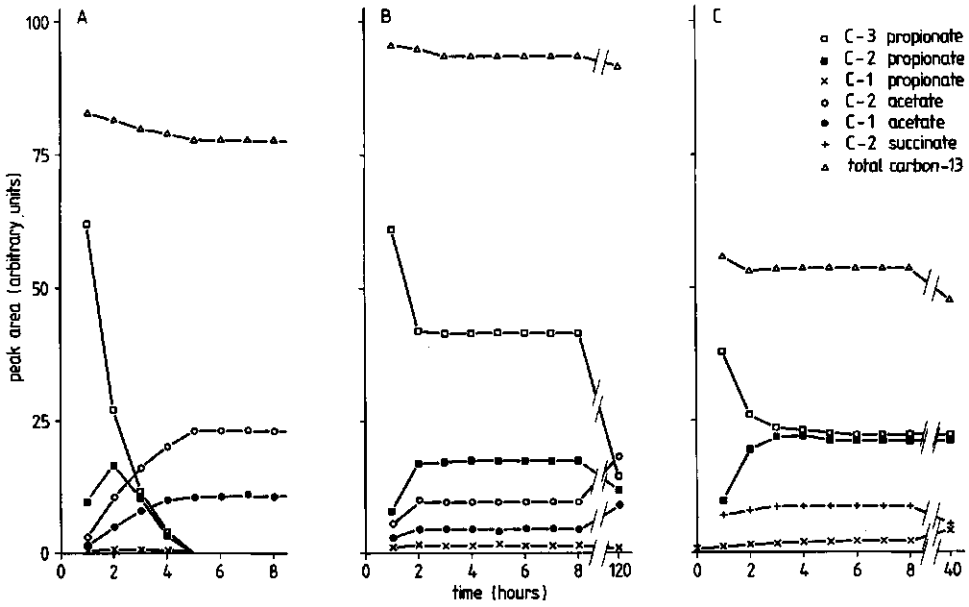


*Figure 3. Time course during the first 8 hours of the various $^{13}C$-resonances after addition of [3-$^{13}C$]-propionate and $H^{13}CO_3^-$ to: A) the methanogenic coculture; B) D. propionicus in the presence of 20 mM sulphate. After 15 hours extra sulphate (25 mM) was added and incubation was continued for another 5 days; C) P. freudenreichii in the absence of lactate. After 15 hours lactate (20 mM) was added and incubation was continued for another 25 hours.*
*The resonances are not corrected for differences in relaxation behaviour and NOE built up. The upper traces represent the sum of the areas of all nuclei expressed in units of [3-$^{13}C$]-propionate (see text). $^{13}CO_2$, $H^{13}CO_3^-$ and $^{13}CH_4$ are not shown and are not included in the total $^{13}C$-label.*

The sulphidogenic coculture degraded propionate very slowly; after nine days only 30% (15 mM) of the substrate had been oxidized to acetate. The $^{13}C$-label in the acetate was equally distributed over both C-atoms, indicating that also S. wolinii degrades propionate via the succinate pathway. Randomization of label over the C-3 and C-2 position of propionate had occurred to a degree of 80% in the same period. C-1 labelled propionate was present after one hour and its amount increased only twofold upon further incubation. After nine days label present at the C-1 carbon of propionate was 8.7% compared to the label at the C-2 carbon of propionate.

D. propionicus (Figure 3B) converted propionate stoichiome-

*Table 1. Relative peak areas of the natural abundance $^{13}$C-NMR spectrum of an equimolar (40 mM) solution of propionate, acetate and succinate. C-3 propionate was arbitrary set at 100. The third colum shows the chemical shifts of all nuclei discussed in the text.*

| $^{13}$C-resonating nuclei | relative peak area | chemical shift (ppm) |
|---|---|---|
| C-3 propionate | 100.0 | 11.1 |
| C-2 propionate | 89.5 | 31.7 |
| C-1 propionate | 38.7 | 185.6 |
| C-2 acetate | 57.9 | 24.3 |
| C-1 acetate | 27.6 | 182.2 |
| 1/2 x (C-2 + C-3) succinate | 144.7 | 35.1 |
| 1/2 x (C-1 + C-4) succinate | 49.7 | 183.2 |
| $H^{13}CO_3^-$ | | 161.2 |
| $^{13}CO_2$ | | 125.5 |
| $^{13}CH_4$ | | -3.7 |
| C-4 oxaloacetate | | 171.9 |

trically to acetate with concomitant reduction of sulphate. Net degradation of propionate and randomization of $^{13}$C-label over the C-3 and C-2 carbons started immediately after the addition of the substrate. Incorporation at the C-1 position of propionate (singlet + doublet) occurred as well. After three hours the randomization and the acetate formation stopped. It can be calculated from Figure 3B that at this time the sulphate must have been depleted. Addition of extra sulphate (25 mM) after fourteen hours, resulted in a further decrease of the total propionate pool, an increase of the acetate pool (labelled equally at both carbon atoms) and an almost complete randomization (96%) at the level of propionate. After five days of incubation not all the propionate had disappeared, most likely because of the high sulphide concentration. Label at the C-1 position was 19% compared to label at the C-2 carbon of propionate.

Propionate forming cultures

Incubation of P. freudenreichii in the presence of lactate (2 mM) and $H^{13}CO_3^-$ (without labelled propionate) resulted in incorporation of label at the C-1 resonance position of propionate, succinate and to some extent of acetate. All resonances observed were composed exclusively of singlets (data not shown).

Immediately after addition of [3-$^{13}$C]-propionate to the culture, label was incorporated into the C-2 carbon of propionate and also into the C-2/C-3 carbons of succinate (Figure 3C). The randomization of propionate had been completed within three hours of incubation. After this time, the degree of incorporation of $^{13}$C-label into the C-3 and C-2 carbon position in propionate remained constant. The amount of C-1 propionate (singlet + doublet) increased linearly during the whole experiment. This indicates that the interconversion of propionate and succinate continued even in the absence of the substrate lactate. After three hours C-1 labelled propionate was 18% of the amount of C-2 propionate. This percentage increased to 54% after prolonged incubation.

Addition of 20 mM lactate (after fifteen hours) and further incubation for another twenty-five hours led to a decrease of C-2 labelled succinate; most likely because the label was diluted by unlabelled succinate. Because the level of C-3 and C-2 propionate did not decrease (to the same extend) in this period, this indicates that both compounds were not in equilibrium anymore.

Unfortunately, spectral resolution was not good enough to quantify the integrals belonging to the resonances of the C-1 carbons of succinate and acetate. From the spectra it can be deduced that both compounds were present during the whole experiment and in amounts of less than 50% of the label incorporated in C-1 propionate.

In the experiment with P. freudenreichii, only 85% of the $^{13}$C-label (originating from [3-$^{13}$C]-propionate) was recovered after prolonged incubation. In all other experiments at least 93% was

recovered.

V. alcalescens incorporated $H^{13}CO_3^-$ in the presence of 2 mM lactate, leading to small amounts of C-1 propionate and C-4 oxaloacetate within 15 minutes. No increase of C-1 propionate was observed in the next 30 minutes, indicating that no lactate was present anymore. Even fifteen hours of incubation in the presence of [3-$^{13}$C]-labelled propionate led to a level of C-2 propionate which was merely detectable. From this it is clear that propionate and succinate are not equilibrated in V. alcalescens in the absence of substrate. After the addition of [3-$^{13}$C]-propionate, an increasing amount of C-4 oxaloacetate was formed (Figure 4), due to carboxylation of pyruvate. From the spectra it was deduced that the amount of this compound did not exceed 3% of the C-3 propionate pool. Incubation for twenty hours in the presence of 20 mM lactate resulted in a sevenfold increase of the amount of C-1 propionate. This corresponds to a

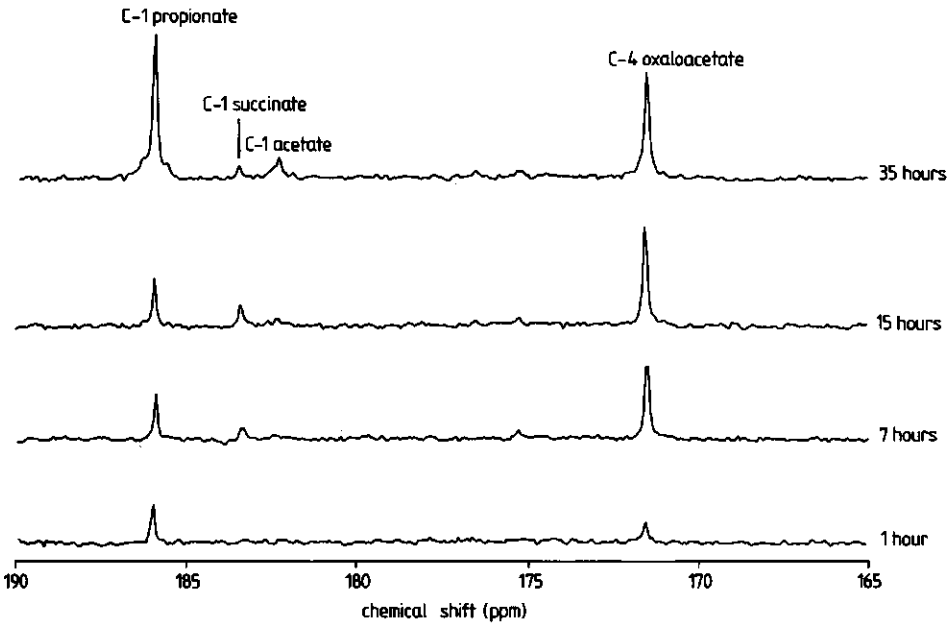


*Figure 4. Time course of the incorporation of $^{13}$C-label in carboxyl groups as mesured in NMR-spectra during incubation of V. alcalescens in the presence of $H^{13}CO_3^-$ and [3-$^{13}$C]-propionate. Between 15 and 35 hours of incubation lactate was present as substrate (see text).*

conversion of 14 mM lactate to propionate. Randomization of label in propionate was still only minor; 10% of the label was found at the C-2 carbon of propionate. Some label was found in the C-1 and C-2 carbon atoms of succinate and acetate. However, their amounts were always less than 50% of the label incorporated in C-1 propionate.

M. elsdenii did not incorporate any $^{13}C$-label at the C-2 position of propionate during four days of incubation. This is not unexpected because this organism uses the non-randomizing acrylate pathway for the conversion of lactate to propionate (Ladd and Walker, 1959).

## DISCUSSION

In this study the involvement of the (de)carboxylation reactions in propionate metabolism was investigated. The inclusion of $H^{13}CO_3^-$ in the in vivo NMR experiments gave insight into the process of randomization at the level of propionate in relation to the type of the (de)carboxylating enzyme involved.

In D. propionicus and P. freudenreichii the decarboxylation of methylmalonyl-CoA is coupled to the carboxylation of pyruvate via a transcarboxylase (Stams et al., 1984; Swick and Wood, 1960), and therefore incorporation of label at the C-1 position of propionate is not expected (Figure 1). However, during interconversion of propionate and succinate in these organisms, $^{13}C$-label was incorporated at the carboxyl group of propionate (and succinate). This may be explained in three different ways: 1) the transcarboxylases exchange to some extend $CO_2$ with the environment; 2) label from $H^{13}CO_3^-/^{13}CO_2$ is first incorporated into the C-4 carbon of oxaloacetate by anaplerotic enzymes and then transfered to propionyl-CoA. P. freudenreichii contains phosphoenolpyruvate carboxytransphosphorylase (Siu et al., 1961; Hettinga and Reinbold, 1972), whereas malic enzyme activity was found in D. propionicus (Stams et al., 1984); 3) the C-4

labelled oxaloacetate formed by anaplerotic enzymes is reduced to C-4(=C-1) labelled succinate. Which of these possibilities contribute(s) significantly to the incorporation of label into the C-1 of propionate, is not clear from our experiments.
At the time where complete randomization over the C-3 and C-2 atoms in propionate was achieved, the label at the C-1 position of propionate was 19% and 18% for D. propionicus and P. freudenrichii, respectively, compared to the other carbon atoms of propionate. This indicates that comparable reactions are involved in this process.
In V. alcalescens the amount of label at the C-1 position of propionate was during the whole experiment 1.5 - 2 times higher than at the C-2 position. Because in this organism $^{13}CO_2$ is incorporated into oxaloacetate by means of pyruvate carboxylase (De Vries et al., 1977) , all succinate formed will be labelled at the C-1 carbon. Half of the subsequent conversions to propionate yields C-1 labelled propionate (Figure 1). C-2 labelled propionate is formed during interconversion of [3-$^{13}$C]-propionate and succinate. However, because of the involvement of a decarboxylase and a (different) carboxylase during this interconversion (Galivan and Allen, 1968) equal amounts of C-1 labelled propionate are formed at the same time (Figure 1). Therefore, labelling at the C-1 carbon always will be higher than labelling at the C-2 carbon of propionate if the organism is incubated in the presence of [3-$^{13}$C]-propionate and $H^{13}CO_3^-$.

In the methanogenic coculture, after complete randomization, label at the C-1 position was 17% compared to the C-2 position of propionate. In the sulphidogenic coculture this percentage was 8.7% at the point where 80% randomization had occurred. Because of the similarity with D. propionicus and P. freudenreichii and the discrepancy with V. alcalescens, we conclude that both syntrophic propionate oxidizing organisms degrade propionate via the succinate pathway using a transcarboxylase. This conclusion is supported by enzyme activity measurements in cell free extracts (Chapter 6). Similar percentages as described above for incorporation or loss of label at or from the C-1 position of propionate were previously found for Propionibac-

terium sp. (Hettinga and Reinbold, 1972; Wood, 1972; 1981), in an anaerobic digestor and a continuous culture fermentor (Boone, 1984).

From the incorporation of label at the C-1 carbon of propionate a possible involvement of the randomizing α-OH-glutarate pathway, as discussed by Wegener et al. (1968), clearly can be excluded. Theoretically succinate might also be formed by a condensation of propionyl-CoA and glyoxylate with subsequent oxidation to succinic-semialdehyde-CoA (Rabin et al., 1965). Such a pathway could lead to label at the C-2 carbon of propionate, but not to label incorporation at the C-1 of propionate. Instead, glyoxylate labelled at the carboxyl group would be produced. In the five cultures with the succinate pathway, double (C-2 + C-1) labelled propionate was formed. This is a strong indication that methylmalonyl-CoA is an intermediate in all of them.

The interconversion of propionate and succinate gives to some extend information about the mechanism of energy conservation. In the propionate oxidizing organism in the methanogenic coculture, S. wolinii, D. propionicus and P. freudenreichii, a transcarboxylase is present. In these organisms incorporation at the C-2 position of propionate occurred to a great extend. D. propionicus did not degrade propionate when sulphate was depleted. Moreover, the interconversion of propionate and succinate stopped. Although no net energy is involved in transcarboxylation (Swick and Wood, 1960), activation of propionate and succinate requires catalytic amounts of ATP in this organism (Stams et al., 1984). Thus, because of other ATP-requiring reactions in the cell, interconversion will terminate if no energy is generated. In P. freudenreichii a succinyl-CoA:propionate transferase is present (Allen et al., 1964), therefore the activation of both compounds is not dependent on the ATP pool. This explains the continued incorporation of $^{13}C$-label at the C-1 carbon of propionate in the absence of substrate. V. alcalescens showed only minor formation of C-2 labelled propionate even in the presence of an energy substrate. This indicates that the $Na^+$-gradient across the cell membrane is

easily dissipated and is in this respect less efficient than a transcarboxylase. Because only a few organisms were tested, it remains unknown whether significant interconversion of propionate and succinate is restricted to propionate degrading and propionate forming bacteria having a transcarboxylase.

In P. freudenreichii only 85% of the label (originating from [3-$^{13}$C]-propionate) was recovered after incubation in the presence of substrate. This was exclusively due to a decrease of the amount of C-2 labelled succinate (Figure 3C). Formation of $^{13}CO_2$ from $^{13}$C-succinate via the citrate cycle in conjunction with the transcarboxylase cycle and/or a pathway involving the glyoxylate enzymes, as well as the citrate and transcarboxylase cycle enzymes, would explain the loss of visible label (Wood, 1981; Crow, 1987). Another posibility is the incorporation of label into relatively immobile compounds which are beyond detection in high-resolution NMR. The fact that the amount of labelled propionate (C-3 + C-2) did not decrease to the same extend, indicates that a labelled and an unlabelled propionate pool existed and that the former was not in equilibrium with succinate. Different pools of propionate and succinate were previously discussed for Propionibacterium arabinosum (Wood et al., 1956).

This study clearly shows the great value of the application of in vivo NMR in biological systems. Besides information about biochemical pathways involved in propionate metabolism, the use of a second labelled compound gave strong evidence for the presence of some important enzymes. Even in complex systems (mixed cultures) this technique offers the possibility to get detailed information about biochemical pathways which are operative.

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Chapter 4

# STUDY ON THE USE OF A TWO-LIQUID-PHASE ELECTRON REMOVAL SYSTEM FOR THE CULTIVATION OF PROTON REDUCING BACTERIA.


## ABSTRACT

A chemically modified hydrophylic viologen derivative (PVS) was tested as redox mediator in a two-liquid-phase system. Dibutylphtalate was used as organic phase including 2-anilino-1,4-naphtoquinone as terminal electron acceptor. Acidaminobacter hydrogenoformans fermented glutamate to acetate with electron transfer to 2-anilino-1,4-naphtoquinone. Results indicate that the obtained hydrogen partial pressure is not low enough to clearly influence the metabolism of the bacterium.



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## INTRODUCTION

Under methanogenic conditions obligate proton reducing bacteria play a key role in the degradation of organic matter. Intermediary products like propionate, butyrate, ethanol and lactate are converted by this group of organisms to the main methanogenic substrates acetate, carbon dioxide and hydrogen (Bryant et al., 1967; Kaspar and Wuhrmann, 1978a; Zehnder et al., 1982; Zeikus, 1982; Gujer and Zehnder, 1983). The oxidation of these intermediates, however, is thermodynamically unfavourable, and their breakdown is obligatorily linked to hydrogen consumption by methanogens (Zehnder, 1978; Bryant, 1979). Several cocultures have now been described in which this process of interspecies hydrogen transfer is essential (Chapter 1).

The presence of the hydrogenotrophic methanogens hampers physiological and biochemical studies on the obligate proton reducing bacteria. These studies would be greatly facilitated if the organisms could be grown in pure culture. For this purpose, non-biological hydrogen removal systems have to be developed. Syntrophomonas wolfei was shown to oxidize butyrate with simultaneous catalytic olefin reduction in the absence of interspecies hydrogen transfer (Kaspar et al., 1987). In a slightly modified system Ahring and coworkers were able to grow a thermophylic butyrate degrader in pure culture (Ahring, personal communication). Schink and coworkers (Emde et al., 1989; Emde and Schink, 1990) showed the transfer of electrons, formed in the fermentation of glycerol, lactate and propionate to an electrode via a reoxidizable electron acceptor.

Here we report the attempt to use an artificial electron acceptor for proton reducing bacteria. Propylviologen sulphonate (PVS), a water-soluble viologen derivative (Figure 1; Willner et al., 1981) was used as redox mediator in a two-liquid-phase system (Figure 2). 2-anilino-1,4-naphtaoquinone ($E^{\circ\prime} \approx -135$ mV) (Clark, 1960), dissolved in the organic phase dibutylphtalate, was used as terminal electron acceptor. First results are

presented for Acidaminobacter hydrogenoformans. This organism ferments glutamate to acetate and propionate. The ratio at which the two products are formed is dependent on the hydrogen partial pressure (Stams and Hansen, 1984). Therefore, the use of this organism is indicative for the hydrogen pressures which can be reached by the biphasic system.

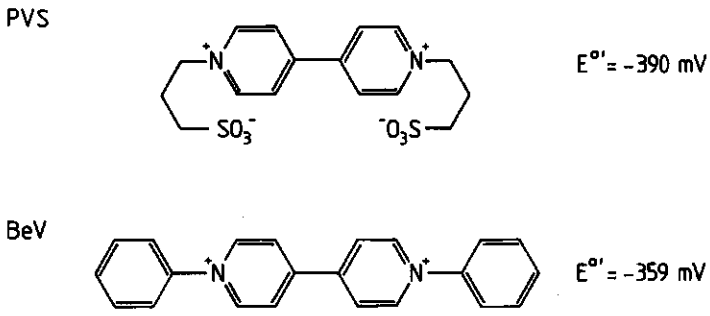



*Figure 1. Chemical structure of PVS and benzyl viologen. The hydrophylic viologen derivative can function as redox mediator with E°' = -390 mV. Because of the hydrophylic sulphonate group, PVS is supposed to be less toxic for microorganisms.*

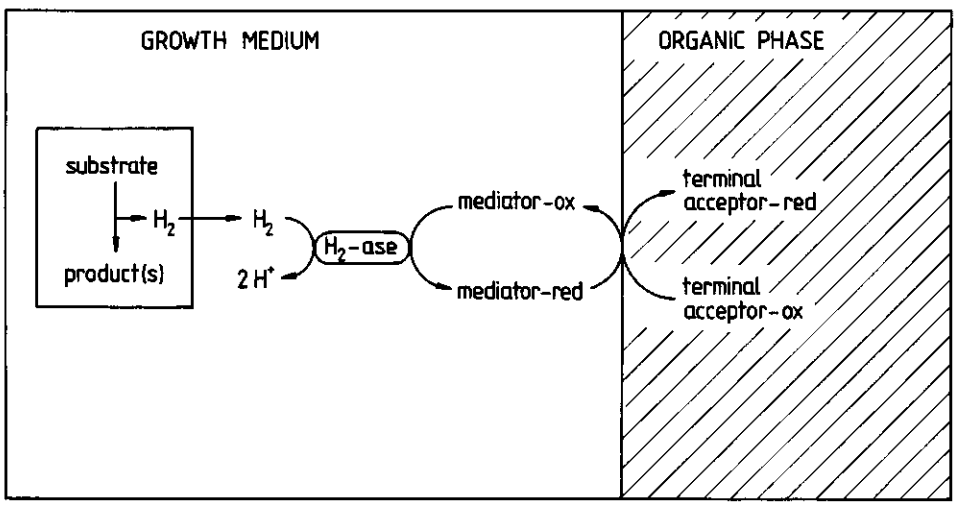



*Figure 2. Schematic representation of the two-liquid-phase system. Fermenting organisms are supposed to deliver hydrogen into the medium which is oxidized by means of an added hydrogenase with concomitant reduction of a mediator (PVS). Electron transfer into the organic phase is possible because of close contact between the two phases (shaking). 2-anilino-1,4-naphtoquinone was used as terminal acceptor.*

## MATERIALS AND METHODS

### Organisms and cultivation

Acidaminobacter hydrogenoformans (DSM 2784) was a gift from T.A. Hansen, Department of Microbiology, University of Groningen, The Netherlands. It was cultivated in the medium as described before (Houwen et al., 1987), except for the trace elements which were the same as described by Stams et al. (1983). The medium was supplemented with 0.2 g/l yeast extract. The organism was grown at 30 °C on α-ketoglutarate or glutamate (20 mM each).

Methanobrevibacter arboriphilus (DSM 744) was cultivated at 37 °C in the same medium as described before (Houwen et al., 1988), supplemented with 3 mM cysteine. A gas phase of $H_2/CO_2$ (80:20) was used as growth substrate.

### Materials

All chemicals used were of analytical grade. Gases were obtained from Hoek Loos, Schiedam, The Netherlands.

### Preparation of PVS

3.9 g 4,4-bipyridyl hydrate (25 mmole) was dissolved in 25 g 1,3-propane sultone (200 mmole) at 100 °C. After 1 hour 25 ml methanol was slowly added, hereby causing the product to crystalize. Cristals (5 g = 12 mmole) were collected, recrystalized from and washed with methanol. Part of the PVS used in this study was a gift from C. Laane, Department of Biochemistry, University of Wageningen, The Netherlands.

## Hydrogenase

The periplasmic hydrogenase of Desulfovibrio vulgaris (NCIB 8303), prepared essentially according to Van der Westen et al. (1980), was a gift from A.J. Pierik, Department of Biochemistry, Agricultural University of Wageningen, The Netherlands. The preparation contained 89 U hydrogenase and a total of 14.3 mg protein per ml. The enzyme preparation had been stored aerobically at -80 °C before use.

## Two-liquid-phase system

Medium (20 ml) was prepared anaerobically in vials (Aluglas Verenigde Bedrijven B.V., Amersfoort, The Netherlands) sealed with butyl rubber stoppers (Rubber B.V., Hilversum, The Netherlands) and a gas phase of $N_2/CO_2$ (80:20). The medium was supplemented with sodium glutamate (20 mM), PVS (1 mM) and hydrogenase (2 ml), and inoculated with 2 ml of cell suspension to give a final cell density of 2 x $10^8$ cells/ml and a volume of 25 ml.

The organic phase was prepared separately. 2-anilino-1,4-naphtoquinone (25 mM) was dissolved in dibutylphtalate, and 50 ml of this solution was transferred to 120 ml vials sealed with butyl rubber stoppers. The vials were flushed for at least 1 h with $N_2/CO_2$ (80:20) to remove oxygen.

Then, the media containing the organisms were transferred anaerobically to the organic phase and the pressure was brought to 1.7 atm. The vials were incubated horizontally while shaking at 130 rpm. Incubations were carried out at 37 °C for 30 days.

## Analytical methods

Propionate, acetate and formate were measured by HPLC. A Chrompack organic acids column (30 cm x 6.5 mm ID) was used. The mobile phase was 0.01 N $H_2SO_4$ with a flow rate of 0.8 ml/min.

The column temperature was 60 °C. 20 µl sample was injected using a Spectra Physics autosampler (SP 8775). Either UV-detection at 206 nm was done with a 2158 uvicord SD (LKB) or a differential refractometer (LKB 2142) was used. Propionate and acetate were also measured gaschromatographycally using a CP9000 (Chrompack, Middelburg) with a glass column (180 cm x 2 mm ID) filled with Chromosorb 101 (80 - 100 mesh). The carrier gas was nitrogen saturated with formic acid according to Ackman (1972). The temperatures of the injector, column and detector were 250 °C, 160 °C - 180 °C and 300 °C, respectively.

Glutamate was measured using a Biotronik LC 6000 E amino acid analyzer with a Darrum DC-GA resin column. A program for the separation of biological fluids was used (Spackman et al., 1958; Benson, 1973).

Hydrogen and methane were determined gaschromatographically as described before (Houwen et al., 1988).

## RESULTS AND DISCUSSION

The complete biphasic system (Figure 2) was first tested in the absence of cells. PVS was reduced if hydrogen was present in the gas phase (visible by blue colour formation). Mixing the medium with the organic phase resulted in an immediate oxidation of the PVS. Without shaking the PVS was reduced again. This cycle could be repeated until either hydrogen or the oxidized terminal acceptor were exhausted. This indicates that close contact between PVS and terminal acceptor is needed for electron transfer. Hydrogen was removed from the gas phase to a level below $2.3 \times 10^{-5}$ atmosphere; this value is the detection limit of our gaschromatograph.

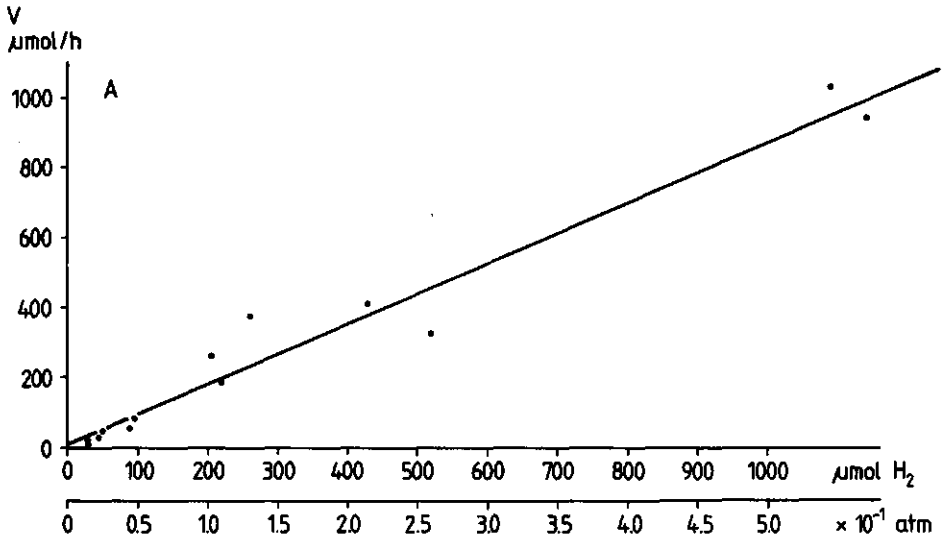
Acidaminobacter hydrogenoformans oxidized glutamate as shown in Table 1. In pure culture and in coculture with Methanobrevibacter arboriphilus, after eleven days glutamate had been converted almost completely. In pure culture glutamate was

*Table 1. Products formed in the oxidation of L-glutamate by A. hydrogenoformans in pure culture, in mixed culture with M. arboriphilus and in the two-liquid-phase system with 2-anilino-1,4-naphtoquinone as terminal electron acceptor. The mixed culture was started by inoculating A. hydrogenoformans in an already turbid culture of the methanogen. All incubations were at 37 °C. Samples were withdrawn after 11 and 30 days. In duplo experiments similar results were obtained as shown in the table. 148 μmol glutamate had been oxidized in the second two-liquid-phase experiment (30 days).*

| | Incubation-time (days) | Glutamate oxidized (μmol) | Products (μmol) Acetate | Propionate | Formate | $H_2$ | $CH_4$ |
|---|---|---|---|---|---|---|---|
| A. hydrogenoformans | 11 | 423 | 625 | 18 | 66 | 123 | 0 |
| | 30 | 521 | 740 | 20 | 60 | 163 | 0 |
| A. hydrogenoformans + M. arboriphilus | 11 | 471 | 564 | 87 | 0 | 0 | 143 |
| | 30 | 471 | 610 | 95 | 0 | 0 | 143 |
| A. hydrogenoformans in two-liquid-phase system | 11 | 71 | 98 | 0 | 0 | 0 | 0 |
| | 30 | 222 | 305 | 0 | 0 | 0 | 0 |

oxidized almost exclusively to acetate with formation of formate and hydrogen as electron sinks. In combination with M. arboriphilus, propionate was formed as second oxidized product and electrons were channelled to methane formation only. In the two-liquid-phase system with 2-anilino-1,4-naphtoquinone, acetate was recovered as the only fermentation product. No formate or hydrogen were detected. Although the carbon and redox balances of the conversions of glutamate are not 100% (Table 1) (Stams and Hansen, 1984), it still can be concluded that reducing equivalents were transferred from the organism into the organic phase. The formation of propionate by A. hydrogenoformans becomes thermodynamically more favourable than acetate formation at a hydrogen partial pressure of $10^{-5}$ atm. (Stams and Hansen, 1984). The fact that no propionate was measured as oxidized end product may be due to toxicity problems or to an inefficient transfer of electrons. At a partial pressure of hydrogen of $10^{-5}$ atm., the electrons have to be transferred to the electron acceptor at a redox potential (E') of at least -270 mV. As the redox midpoint potential (E°') of PVS is -390 mV (C. Laane, personal communication), the ratio $PVS_{ox}/PVS_{red}$ must be about 3 x $10^{4}$ in close vicinity of the organisms.

The two-liquid-phase-system was tested in more detail abiotically. Varying the initial amounts of hydrogen in the gas phase showed first order reaction kinetics for hydrogen removal with a reaction constant of 0.87 $h^{-1}$ (Figure 3A). First order kinetics can only be explained by diffusion limitation of hydrogen. Prolonged incubation of the abiotic system (shaking at 37 °C in the absence of hydrogen), drastically reduced the hydrogen removal rate. After eleven days, different amounts of hydrogen were removed according to Michaelis-Menten kinetics ($K_m$ ≈ 50 - 100 µmol; $V_{max}$ ≈ 2 µmol/h; Figure 3B). This observation may be explained by the decline of hydrogenase activity. Table 2 shows the calculated hydrogen removal rates of the system at the beginning and after eleven days of incubation at different hydrogen partial pressures. Without hydrogenase the biphasic system did not remove any hydrogen from the gas phase.
Between day eleven and day thirty, A. hydrogenoformans oxidized



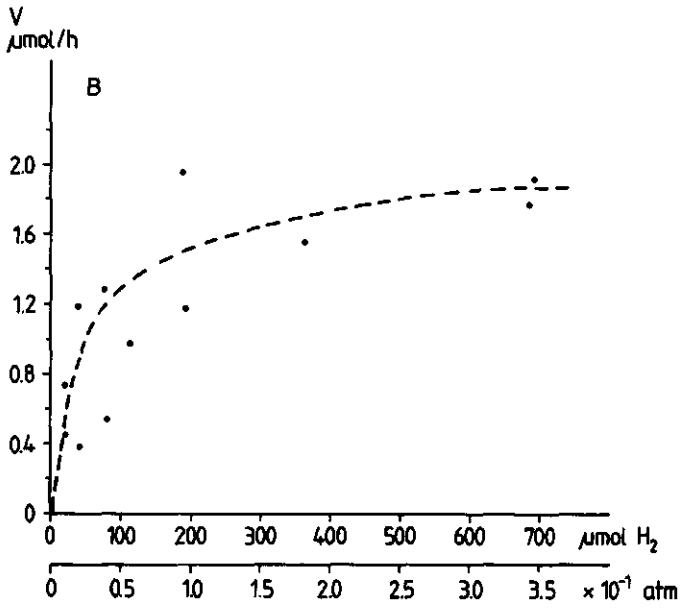




*Figure 3. Hydrogen removal rates versus initial concentrations measured for the abiotic system. At time zero (A) first order kinetics ($k = 0.87\ h^{-1}$) were observed. Prolonged incubation (B) drastically reduced the hydrogen removal rate by the system.*

*Table 2. Hydrogen removal rates of the biphasic system after 0 and 11 days of incubation (extrapolated from Figures 3A and 3B).*

| $p_{H_2}$ | V (µmol/h) | |
|---|---|---|
| | t = 0 | t = 11 |
| $10^{-4}$ | $1.7 \times 10^{-1}$ | $4 \times 10^{-3}$ |
| $2.3 \times 10^{-5}$ | $3.9 \times 10^{-2}$ | $9.2 \times 10^{-4}$ |
| $10^{-5}$ | $1.7 \times 10^{-2}$ | $4 \times 10^{-4}$ |

100 - 150 µmol glutamate, corresponding to an equal amount of hydrogen (or formate). As no hydrogen was found after eleven and after thirty days of incubation, this compound probably was not present during the period in between. Assuming a hydrogen removal rate at the detection limit of $9.2 \times 10^{-4}$ µmol/h (Table 2), less than 0.4% of the electrons could have been removed in the way as was tested in the abiotic system. Therefore, the bacterial hydrogenases probably were active in electron transfer to 2-anilino-1,4-naphtoquinone. Recently, for Propionibacterium freundereichii homoacetate and homopropionate fermentation was realized, using an electrode system with different redox mediators (Emde and Schink, 1990; personal communication). As this organism lacks a hydrogenase, these results show that transfer of electrons is possible bidirectionally via electron carriers that influence the intracellular redox potential, without the intervention of (a) hydrogenase(s). During glutamate oxidation of A. hydrogenoformans, direct interaction between PVS or 2-anilino-1,4-naphtoquinone (or both) with (an) electron carrier(s) from the bacterium also could explain the apparent discrepancy between the data from Table 1 and Table 2.

We were surprised to find in a control experiment, that PVS was not required for the abiotic systems to function. Hydrogen removal from the headspace occurred at the same rate independently of the presence of the redox mediator.

When A. hydrogenoformans was tested in a system with 2-anilino-

1,4-naphtoquinone as terminal electron acceptor but without PVS in the water phase, glutamate was oxidized completely to acetate within 5 days of incubation. Although part of the electrons had been transferred to the organic phase, 17 μmol ($5.8 \times 10^{-3}$ atm.) of hydrogen was measured in the gas phase at that time. In the parallel experiment with PVS, also all glutamate had been degraded but no hydrogen (or formate) was detected. Apparently the transfer of electrons from the bacteria to 2-anilino-1,4-naphtoquinone was less efficient in the absence of PVS.

If in the two-liquid-phase-system, 2-anilino-1,4-naphtoquinone was replaced by menadion (= vitamin $K_3$) ($E^{\circ\prime} \approx 0$ mV; Clark, 1960), no glutamate was oxidized by A. hydrogenoformans. With this terminal electron acceptor, the redox indicator resazurine ($E^{\circ\prime} \approx -51$ mV; Clark, 1960) present in the medium, turned pink. This was not the case with 2-anilino-1,4-naphtoquinone. Therefore, with menadion the redox potential of the medium may have been toxically high for the bacterium.

Although in principle the two-liquid-phase system has been shown to function as non-biological electron removal mechanism, more work is needed to improve the system.

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APPENDIX

PVS was also tested as redox mediator and as terminal electron acceptor to enable propionate oxidation in artificial

systems without hydrogenotrophic bacteria. Compared to benzyl viologen, PVS appeared to be much less toxic to a methanogenic propionate oxidizing coculture (Koch et al., 1983). No effect on propionate oxidation was observed from the addition of 0.01 mM PVS, whereas the same concentration of benzyl viologen was completely inhibitive. Because an unknown bacterium is present in this coculture (Houwen et al., 1987) it could not be investigated what the most sensitive organism was. When 2-Bromo-ethane-sulphonic acid was added to vials where considerable propionate degradation had occurred, methane formation stopped and some hydrogen accumulated in the headspace. The formation of hydrogen correlated with the concentration of PVS, indicating that PVS was reoxidized.

PVS was tested to function as terminal electron acceptor (in the medium) at concentrations of 0 - 40 mM. Although PVS was reduced by the organisms in all cases, propionate oxidation could not be measured.

Reduced PVS could easily be reoxidized by flushing the headspace with $N_2/CO_2$ (80:20). In a system to flush incubating cultures continuously, no significant propionate degradation was found in any of the vials after prolonged incubation. As redox mediator in the two-liquid-phase system, PVS also could not be applied successfully to oxidize propionate.

Chapter 5

# PYRUVATE AND FUMARATE CONVERSION BY A METHANOGENIC PROPIONATE OXIDIZING COCULTURE


## ABSTRACT

A stable, methanogenic propionate degrading coculture was tested for its ability to convert pyruvate and fumarate. Both substrates were degraded rapidly and without a lag phase. Acetate and propionate were formed from pyruvate, whereas fumarate was predominantly converted to malate and succinate. High-resolution $^{13}C$-NMR with [3-$^{13}C$]-pyruvate showed the involvement of a randomizing pathway in the formation of propionate. Two pyruvate fermenting organisms isolated from the coculture, did not degrade propionate in the presence of hydrogen consuming methanogens.



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# INTRODUCTION

During complete degradation of complex organic material under methanogenic conditions, about 15% of the total carbon is degraded via propionate as an intermediate (Kaspar and Wuhrmann, 1978[a]; Mackie and Bryant, 1981; Gujer and Zehnder, 1983). Propionate is oxidized to acetate, carbon dioxide and hydrogen, according to the following equation:

$$CH_3CH_2COO^- + 3\ H_2O \longrightarrow CH_3COO^- + HCO_3^- + H^+ + 3\ H_2$$

$$\Delta G^{\circ\prime} = +76.1\ kJ/mol\ (Thauer\ et\ al., 1977)$$

Due to the extreme positive $\Delta G^{\circ\prime}$, the degradation of propionate is only possible when hydrogen is removed by methanogenic bacteria (Zehnder, 1978; Bryant, 1979). Syntrophic cocultures of propionate oxidizing bacteria and hydrogen consuming anaerobes have been described (Boone and Bryant, 1980; Koch et al. 1983; Xun and Boone, 1989). Although some information is available about the kinetics of the breakdown (Kaspar and Wuhrmann, 1978[a,b]; Zehnder and Koch, 1983; Heyes and Hall, 1983; Schink, 1985), little is known about the biochemical pathway of propionate oxidation. Studies with $^{13}C$- and $^{14}C$-labelled propionate provided evidence for the operation of the succinate pathway (Buswell et al., 1951; Koch et al., 1983; Schink, 1985; Robbins, 1988; Houwen et al., 1987; 1990), a route which is also present in some other propionate forming and propionate degrading anaerobes (Chapter 3). In such a pathway fumarate and pyruvate are intermediates. The $\Delta G^{\circ\prime}$-values of the conversion of these intermediates to acetate, carbon dioxide and hydrogen are negative, and would allow growth of the propionate oxidizing organism in pure culture. Since it was shown that the obligate syntrophic butyrate oxidizer Syntrophomonas wolfei was able to grow on crotonate, from which the CoA-ester is an intermediate in the degradation pathway of butyrate (Beaty and McInerney, 1987), we decided to investigate the ability of a stable

propionate oxidizing coculture to convert pyruvate and fumarate.

## MATERIALS AND METHODS

### Organisms and cultivation

The propionate degrading coculture used in this study was originally enriched by Koch et al. (1983). It contained the propionate oxidizing bacterium (approx. 10%), two types of methanogenic bacteria (approx. 90%) and < 1% of unknown bacteria. This coculture degraded propionate to acetate, carbon dioxide and methane; acetate was not metabolized further.

Methanobrevibacter arboriphilus (DSM 744) and Methanospirillum hungatei (DSM 864) were purchased from the German Collection of Microorganisms (Braunschweig, FRG). Except for the growth substrate and two trace elements ($Na_2WO_4.H_2O$ and $SeO_2$ were not added), the medium had the same composition as described before (Houwen et al., 1987). For growth of M. hungatei, 2 grams bioTrypticase (bioMerieux) were added per liter medium. Pyruvate and fumarate were filter-sterilized as 2.5 M and 1 M solutions, respectively. Agar plates were prepared anaerobically in a glove box containing a gas phase of $N_2/H_2$ (96:4). Traces of oxygen were removed by passing the gas continuously over a BASF RO-20 palladium catalyst at room temperature. To the medium were added: agar (2%), sodium pyruvate (50 mM) and in some experiments 2-bromo-ethane-sulfonic acid (BrES) (50 mM). To allow stabilization of the pH (7.0- 7.2), the plates were stored for at least 12 hours in jars with a gas phase of 1.8 atm $N_2/CO_2$ (80:20) or 1.8 atm $H_2/CO_2$ (80:20).

### Incubation with pyruvate and fumarate

Propionate grown cells (720 ml) were transferred anaerobical-

ly and aseptically to 160 ml vials (Aluglas Verenigde Bedrijven B.V., Amersfoort, The Netherlands), closed with butyl rubber stoppers (Bellco, Vineland, USA). The vials were placed in a GSA-rotor (Sorvall) with 6 x 50 ml water. After centrifugation at 3000 rpm for two hours, the supernatant was removed aseptically and the cells were washed twice in a medium without carbon source. The final pellet was suspended in 20 ml of this medium and then 3 ml of the suspension was brought into 100 ml vials containing 30 ml medium with pyruvate or fumarate (40 - 50 mM) as substrates. The final cell density was 8 x $10^8$ cells/ml. Either 1.8 atm $N_2/CO_2$ (80:20) or $H_2/CO_2$ (80:20) was placed above the cultures. The vials were incubated at 35 °C. Samples were withdrawn weekly and analyzed as described below.

### $^{13}$C-NMR experiment

1.8 l of the propionate grown coculture was centrifuged and washed anaerobically with growth medium containing 5 mM sodium pyruvate. Since FeS in the suspension causes significant peak broadening in the NMR spectra, removal of this substance was necessary. The 100 times concentrated cell suspension was therefore transferred to a tube in the anaerobic glove box. After 15 hours almost all the visible FeS precipitate had sedimented. Then, 3.2 ml of the supernatant was transferred to an NMR tube and 0.4 ml $^2H_2O$ was added to provide a lock signal. This suspension contained approximately $10^{11}$ cells/ml. The NMR tube was closed with an overseal stopper, and the gas phase was changed to 1.2 atm $H_2/CO_2$ (80:20). At time zero 0.4 ml sodium [3-$^{13}$C]-pyruvate (99% enriched, MSD-Isotopes, FRG) was added with a syringe to give a concentration of 33 mM. High-resolution $^{13}$C-NMR was carried out as described before (Houwen et al., 1987).

Analytical methods

Propionate and acetate were measured gaschromatographically, using a Varian Aerograph 2400 with a chromosorb 101 column (200 cm x 2 mm) and a flame ionisation detector (FID). Samples were acidified by the addition of Amberlite IR 120($H^+$). 1 µl sample was injected together with 1 µl air. The carrier gas was nitrogen saturated with formic acid. The column temperature was 180 °C and the detector temperature 210 °C.

Hydrogen and methane were determined qualitatively with a Becker Packard 417 gaschromatograph which was operated with argon as the carrier gas. 0.2 ml from the gas phase were injected into the molecular sieve column 13X (1 m x 0.25 inch). The temperature of both the column and the detector (FID) were 100 °C.

Fumarate, malate and succinate were analyzed as methyl-esters with a Kipp GC system 8200 equiped with a pack sil 19B (10 m x 0.53 mm) column. 2 ml methanol was mixed with 1 ml sample and heated to 60 °C for 30 minutes. After cooling, 1 ml water and 0.5 ml chloroform were added and the mixture was vigorously shaken. 1.0 ul air and 0.5 ul from the chloroform-layer were injected into the gaschromatograph. Nitrogen was used as the carrier gas. The temperature of the column was 100 °C for 3 minutes and increased to 280 °C in 3 minutes. The injection temperature was 250 °C and the FID had a temperature of 325 °C. The inlet pressure was 10 kPa.

Pyruvate was measured according to Katsuki et al. (1971). The formation of 2,4-dinitrophenylhydrazone from pyruvate and 2,4-dinitrophenylhydrazine was measured spectrophotometrically at 416 nm.

## Incubation with pyruvate and fumarate

Cell suspensions of a stable propionate degrading methanogenic coculture were tested for the ability to convert pyruvate and fumarate. These experiments were performed in the presence of 2-Bromo-ethane-sulphonic acid (BrES) to prevent methanogenesis. With $N_2/CO_2$ in the gas phase (Figure 1A) pyruvate is converted within one week to acetate, propionate and a little hydrogen. In a separate experiment in which samples were withdrawn every two days, it was observed that pyruvate degradation started without a significant adaptation time. The degradation rate of pyruvate was in the same range as that of propionate; 35 mM of the latter compound was degraded in one week (results not shown). A slower conversion was observed in the presence of hydrogen (Figure 1B). In this case about equal amounts of acetate and propionate were formed.

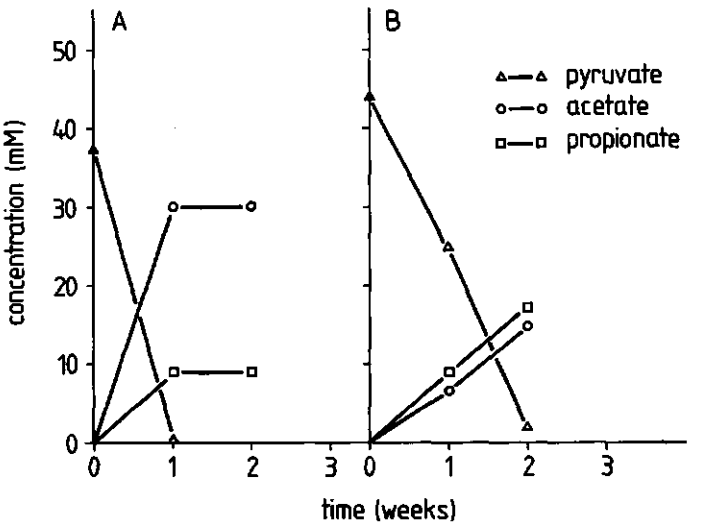


*Figure 1. Conversion of pyruvate by the coculture with $N_2/CO_2$ (A) or $H_2/CO_2$ (B) in the headspace.*

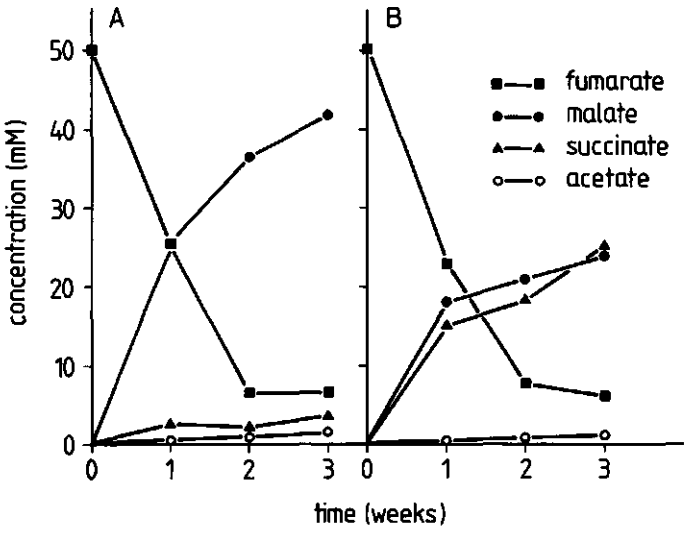


*Figure 2. Conversion of fumarate by the coculture with $N_2/CO_2$ (A) or $H_2/CO_2$ (B) in the headspace.*

Fumarate was degraded at the same rate in the presence or absence of hydrogen (Figure 2). Malate was the predominant product in the absence of hydrogen. Only small amounts of acetate and succinate were formed. In the presence of hydrogen, besides malate also succinate was a major product.

### $^{13}$C-NMR experiment

The time dependent incorporation of the $^{13}$C-label into intermediates and end products after the addition of 33 mM [3-$^{13}$C]-pyruvate to the propionate degrading coculture is shown in Figure 3. The amplitude of the resonances belonging to the $^{13}$C-labelled carbon at the C-3 position of pyruvate, resonating at 27.5 and 26.3 ppm (the second resonance is from hydrated pyruvate), decreased close to zero during the time course of the experiment.

$^{13}$C-label occurred at several positions in various compounds. These included the C-2 position of acetate (24.3 ppm), the C-3 and C-2 positions of propionate (11.2 and 31.7 ppm), the C-3/C-2

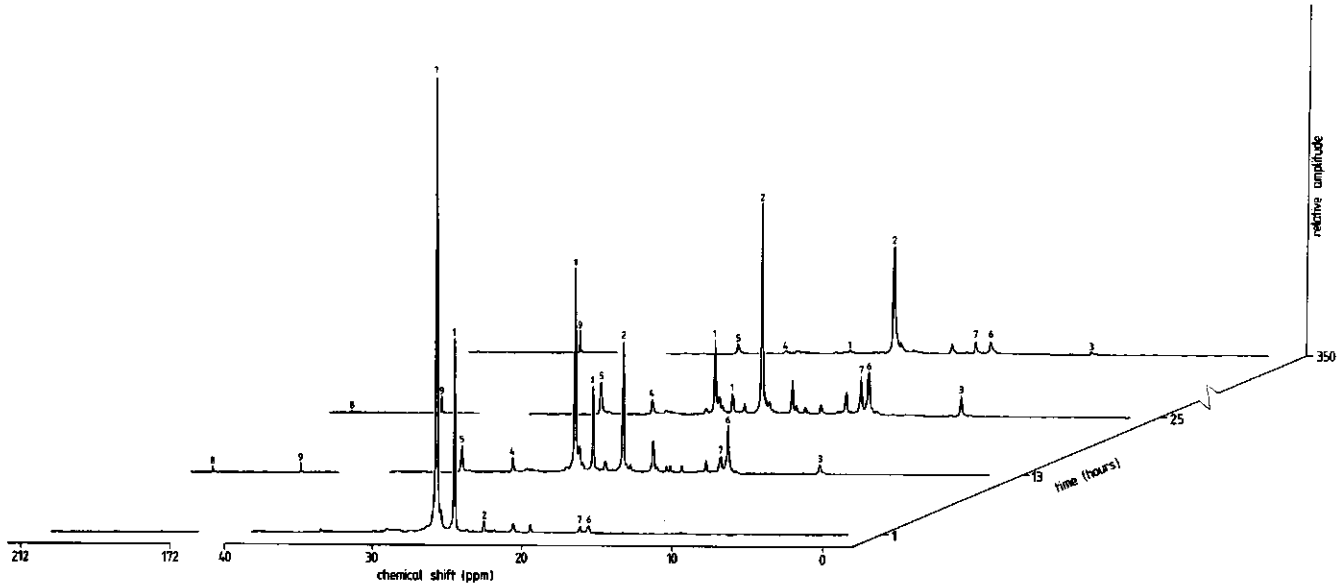


*Figure 3. Time course of the occurrence of $^{13}C$-label as measured in NMR-spectra during incubation of the coculture with [3-$^{13}C$]-pyruvate. Chemical shifts (ppm): 1) C-3 pyruvate (27.5 and 26.3), 2) C-2 acetate (24.3), 3) C-3 propionate (11.2), 4) C-2 propionate (31.7), 5) C-3/C-2 succinate (35.1), 6) C-3 alanine (17.3), 7) C-2 ethanol (17.8), 8) C-2 pyruvate (206.0) and 9) C-1 acetate (182.2).*

position of succinate (35.1 ppm), the C-3 position of alanine (17.3 ppm), the C-2 position of ethanol (17.8 ppm), the C-2 position of pyruvate (206.0 ppm) and the C-1 position of acetate (182.2 ppm). The high amplitude of the resonance of ethanol in the first hour of the experiment did not originate from $^{13}$C-pyruvate. It appeared to be a contamination of non-enriched ethanol as was clear from scanning for 15 minutes before adding the $^{13}$C-pyruvate. The ethanol must have originated from an open vial with ethanol inside the anaerobic glove box.

After 25 hours the coculture was further incubated outside the NMR apparatus to allow the methane bacteria to convert all hydrogen and thus allowing propionate to be degraded. After two weeks methane and a little hydrogen (and carbon dioxide) were present in the headspace. Pyruvate and the "intermediate" propionate had disappeared.

## Isolation of pyruvate fermenting organisms

The observation of a propionic acid fermentation involving a randomizing pathway, indicated that the propionate oxidizing organism in the coculture might be cultivated in pure culture on pyruvate. Therefore, different dilutions of the propionate degrading coculture were brought on agar plates containing pyruvate and BrES. Colonies appeared after 2 - 3 weeks of incubation. After three transfers on agar plates BrES was omitted because no methane was produced by the bacteria after transfer to liquid media with pyruvate and hydrogen. Colonies were tested for their ability to convert propionate in coculture with Methanospirillum hungatei and/or Methanobrevibacter arboriphilus. After eight weeks of incubation, no propionate degradation was observed.

In experiments performed to identify the nature of the pyruvate degrading bacterium, the isolate turned out to consist of two distinct types of rod-shaped bacteria; one curved rod was Gram-negative and the other Gram-positive. The former appeared to be a sulphate reducer, which could be obtained in pure culture via

dilution series in media with lactate and sulphate. Acetate was the only product with either lactate or pyruvate as the substrate. Fumarate, succinate and malate were not converted. The Gram-positive organism could be isolated in a similar fashion with glucose as carbon and energy source. Table 1 shows the product formation from some substrates which were tested as a first identification of the bacterium. Sulphate was not used as electron acceptor by this organism.

*Table 1. Product formation from some substrates by the isolated glucose fermenting bacterium. Substrates were added at 20 mM concentrations; yeast extract at 2 g/l. Numbers between brackets indicate concentrations in mM.*

| Substrate(s) | Product(s) |
|---|---|
| glucose | propionate (3.3) + lactate (3.0) acetate (1.2) + $H_2$ |
| glucose + $H_2$ | lactate (8.2) + propionate (6.4) + acetate (4.2) |
| glucose + fumarate[a] + $H_2$ | lactate (9.1) + propionate (6.1) + malate (4.1) |
| fumarate | malate (11.8) |
| fumarate + $H_2$ | malate (14.0) + succinate (<0.5) |
| pyruvate | propionate (4.3) + acetate (2.4) + $H_2$ |
| pyruvate + $H_2$ | propionate (7.0) + acetate (<0.5) |
| acetate + $H_2$ | - |
| yeast extract | propionate (1.5) + acetate (<0.5) + lactate (<0.5) |

[a]Acetate was not analyzed with fumarate as the (co)substrate

## DISCUSSION

The ability of a stable propionate degrading coculture to convert pyruvate and fumarate instantly was quite unexpected.

The coculture was isolated from agar-shake tubes and had been maintained for more than five years in media with propionate as sole energy and carbon source. The instant degradation of pyruvate and fumarate was an indication that the propionate degrader itself might be able to convert the two substrates. The idea was supported by the findings with $^{13}C$-NMR that a randomizing pathway is involved in both propionate degradation and propionate formation (Houwen et al., 1987; Chapter 3; this paper). Therefore, the pyruvate fermenting organism was isolated and tested on the ability to grow syntrophically on propionate. Unfortunately the isolate, which consisted of two different bacterial species, did not degrade measurable amounts of propionate in cocultures with methanogens. So far it remains unknown whether the syntrophic propionate oxidizer is able to grow on the metabolic intermediates pyruvate and fumarate. In any case, the organism cannot be easily isolated on these substrates because of its slower growth compared with the contaminating bacteria. Also the defined coculture described by Boone and Bryant (1980) could not be tested in this respect, because Syntrophobacter wolinii is maintained together with a Desulfovibrio strain. Desulfovibrio is known to metabolize pyruvate and fumarate.

The product formation by the coculture from pyruvate and fumarate in the presence and absence of hydrogen can partly be explained by the physiological properties of the isolated contaminating bacteria. The isolated sulphate reducer is able to ferment pyruvate to acetate, carbon dioxide and hydrogen, whereas the other isolate showed a typical propionic acid fermentation. In the presence of hydrogen, pyruvate conversion by the sulphate reducer is inhibited, whereas the metabolism of the propionic acid bacterium is shifted to more propionate formation. Desulfobulbus propionicus and Propionispira arboris, which under normal conditions form acetate and propionate in the expected stoichiometries, are able to form propionate as sole end product in the presence of hydrogen (Laanbroek et al., 1982; Stams et al., 1984; Thompson et al., 1984). The conversion of fumarate to succinate in the presence of hydrogen (Figure 2B;

Table 1), however, was not carried out by one of the two isolates at a comparable rate as was found for the coculture.

It remains unknown why the contaminants are maintained in the propionate oxidizing coculture after a huge number of transfers. The persistence of the sulphate reducer may be explained by the presence of small amounts of oxidized sulphur compounds in the medium. Sulphate reducers have a higher affinity for hydrogen than methanogens and they are even able to outcompete methanogens at low sulphate concentrations (Lovley and Klug, 1983). We were able to take advantage of the presence of the sulphate reducer and obtained a syntrophic sulphidogenic propionate degrading coculture, by repeated transfer of the coculture in propionate medium with sulphate (Houwen et al., 1990). The glucose degrader may endure in the coculture by growth on cell lysis products. The ability to grow on yeast extract is in accordance with this possibility (Table 1). Electronmicroscopic analysis of thin sections of propionate degrading cocultures always showed a high percentage of lyzed cells.

In the $^{13}C$-NMR experiment label occurred in the C-2 position of pyruvate and the C-1 position of acetate. This means that de novo synthesis of pyruvate from pyruvate had occurred via a symmetric intermediate, leading to randomization of the $^{13}C$-label between the C-3 and C-2 atoms. The label pattern in propionate shows that the same pathway is involved in propionate formation and in propionate oxidation by the coculture. Because these two processes probably are not carried out by the same organism, the analysis of enzyme activities in cell extracts of the coculture will not lead to conclusive results on enzyme involved in propionate oxidation. For this purpose, a defined biculture is absolutely required.

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Chapter 6

# ENZYMATIC EVIDENCE FOR THE INVOLVEMENT OF THE METHYLMALONYL-CoA PATHWAY IN PROPIONATE OXYDATION BY SYNTROPHOBACTER WOLINII


ABSTRACT

Enzyme measurements were carried out with crude cell-free extracts of the propionate oxidizing coculture of Syntrophobacter wolinii and Desulfovibrio G11. Using cell-free extracts of a pure culture of Desulfovibrio G11 as a blank, most enzymes involved in the methylmalonyl-CoA pathway for propionate oxidation, including a propionyl-CoA:oxaloacetate transcarboxylase, were demonstrated in S. wolinii.
S. wolinii had a lower cell yield than Desulfobulbus propionicus. This difference is explained in terms of energy conservation mechanisms. The sulphidogenic coculture with S. wolinii grew faster than a methanogenic coculture with a different propionate oxidizing organism. Substitution of the methanogens in the latter culture by hydrogenotrophic sulphate reducing bacteria, resulted in the same growth rate as measured for the Syntrophobacter-Desulfovibrio coculture.



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## INTRODUCTION

Propionate is an important intermediate in anaerobic breakdown of organic matter. Fermentation of carbohydrates or lactate with propionate as major reduced end product is carried by both Gram-positive and Gram-negative bacteria. Propionate can also be formed as a result of β-oxidation of odd numbered fatty acids, the fermentation of glycerol and of some amino acids, and the reduction of $C_1$ and $C_2$ compounds (Stams and Hansen, 1984; Schink, 1986)

In anaerobic environments in which sulphate is present, propionate is oxidized either to acetate and carbon dioxide by Desulfobulbus species (Laanbroek and Pfennig, 1981; Widdel and Pfennig, 1982; Samain et al., 1984) or completely to carbon dioxide by other types of sulphate reducing bacteria (Widdel, 1988). Desulfobulbus propionicus oxidizes propionate via the succinate pathway as shown in Figure 1 (Stams et al., 1984; Kremer and Hansen, 1988).

Under methanogenic conditions propionate is degraded by syntrophic consortia of bacteria. Propionate oxidation, coupled to proton reduction, is thermodynamically unfavourable and has to be linked to hydrogen removal by methanogens (hydrogen consuming anaerobes) (Zehnder, 1978; Bryant, 1979). Only a small number of syntrophic propionate oxidizing cocultures have been described (Boone and Bryant, 1980; Koch et al., 1983; Xun and Boone, 1989). Recently, a butyrate fermentation of propionate was reported as an alternative way of propionate degradation (Tholozan et al., 1988).

So far, studies on the biochemistry of syntrophic propionate oxidation were carried out using $^{14}C$- and $^{13}C$-labelled propionate. In all cases evidence was provided for the operation of a similar pathway as found for D. propionicus (Buswell et al., 1951; Koch et al., 1983; Schink, 1985; Robbins, 1988; Tholozan et al., 1988; Houwen et al., 1987, 1990). However, direct enzymatic evidence for this pathway is lacking. Syntrophobacter wolinii, which was used in this study, is an obligate proton

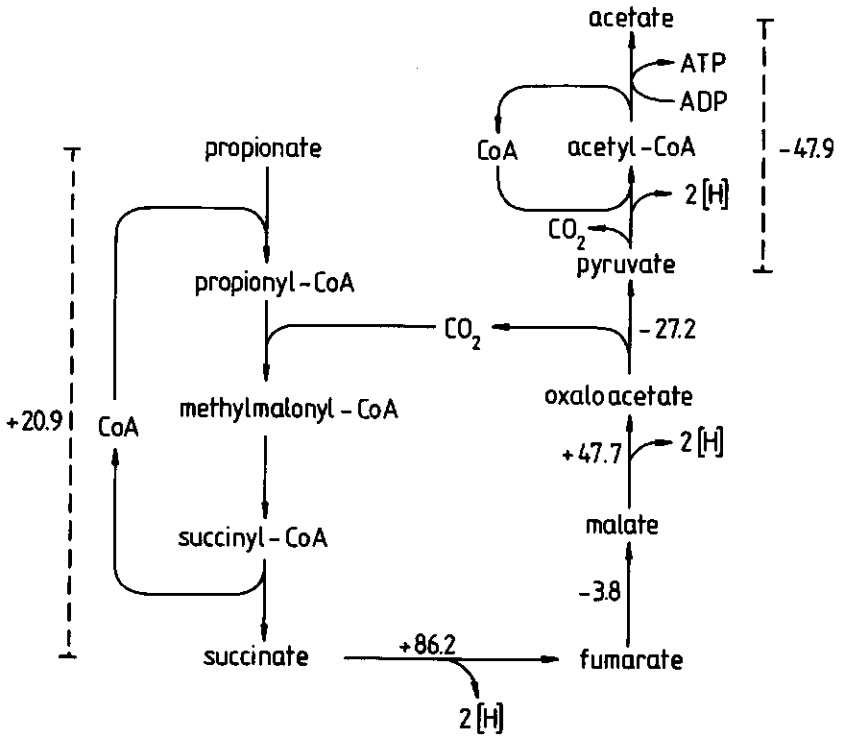


*Figure 1. The succinate pathway for propionate oxidation. $\Delta G^{o}$-values are from Thauer et al. (1977). A transcarboxylase and CoA-transferases are not necessarily present.*

reducing bacterium catabolizing propionate in a biculture with Desulfovibrio G11 (Boone and Bryant, 1980). In this paper we present the results of enzyme activity measurements in this coculture. A pure culture of the Desulfovibrio species was used as a blank to calculate the specific enzyme activities in the propionate oxidizing acetogen. To get insight into the energy metabolism of S. wolinii, its growth yield on propionate is compared with that of the propionate degrading D. propionicus. Further, the growth rate of the Syntrophobacter-Desulfovibrio coculture is compared with the growth rate of a second sulphidogenic coculture with a different propionate oxidizing organism, and with a methanogenic coculture also containing this second propionate oxidizer.

## MATERIALS AND METHODS

### Organisms and cultivation

In this study, three syntrophic propionate oxidizing cocultures were used. The defined sulphidogenic biculture of Syntrophobacter wolinii and Desulfovibrio G11 (Boone and Bryant, 1980). This culture (DSM 2805) was obtained from the German Collection of Microorganisms (Braunschweig, FRG) and was cultivated in a medium containing (in g/l unless otherwise stated): sodium propionate 1.9; $Na_2SO_4$, 2.9; $NaHPO_4.2H_2O$, 0.53; $KH_2PO_4$, 0.41; $NH_4Cl$, 0.3; NaCl, 0.3; $CaCl_2.2H_2O$, 0.11; $MgCl_2.6H_2O$, 0.1; $NaHCO_3$, 4; $Na_2S.9H_2O$, 0.24; yeast extract, 0.2; 1 ml of a tenfold concentrated trace element solution described by Pfennig and Lippert (1966); 1 ml of the vitamin solution described by Stams et al. (1983); 1 ml of a mixture of $Na_2MoO_4$, $Na_2WoO_4$ and $SeO_2$ (each 0.1 mM) in 10 mM NaOH. Sodium lactate (1 mM) was added to stimulate the sulphate reducing bacterium.
A methanogenic coculture, originally enriched by Koch et al. (1983) was cultivated as described before (Houwen et al., 1988). This culture is referred to as (culture) "Z". In contrast with S. wolinii, the propionate oxidizer in this culture stains Gram-positive. Because in this methanogenic culture a sulphate reducing bacterium appeared to be present (Houwen et al., 1988), the culture was also grown on propionate in the same medium in the presence of sulphate (2.9 g/l) and yeast extract (0.2 g/l). After repeated transfers, this culture was free of methanogens as evidenced by lack of methane production during incubation with hydrogen and absence of sulphate. This sulphidogenic coculture is referred to as (culture) "ZPS".
Desulfobulbus propionicus (DSM 2032) was a gift of D.R. Kremer, University of Groningen, The Netherlands. The organism was cultivated in a medium containing (in g/l): sodium propionate 1.9; $Na_2SO_4$, 2.9; $Na_2HPO_4.2H_2O$, 0.53; $KH_2PO_4$, 0.41; $NH_4Cl$, 0.54; $CaCl_2.2H_2O$, 0.15; $MgCl_2.6H_2O$, 0.4; KCl, 0.3; $NaHCO_3$, 2.4; $Na_2S.9H_2O$, 0.48; yeast extract, 0.2. Trace elements and vitamins

were the same as described for the Syntrophobacter-Desulfovibrio coculture.

## Growth rates and growth yields

The growth rates of the syntrophic propionate degrading cocultures were determined using the same media as described above, except that lactate was omitted from the Syntrophobacter-Desulfovibrio coculture and yeast extract (0.2 g/l) was also added to the methanogenic coculture. 15 ml of medium with 5% inoculum was incubated in 25-ml glass tubes. The optical density at 660 nm was measured directly with a Hitachi U-1100 spectrophotometer equiped with a test tube holder. Growth rates were based on five independent experiments.
Growth yields were determined for the Syntrophobacter-Desulfovibrio coculture and for D. propionicus. The presence of 1 mM lactate in the medium of the coculture was corrected for by subtraction of the yield on lactate (1 mM) only. For comparison D. propionicus was cultivated in the same medium as the coculture, without lactate. Growth yields were determined with a 5% inoculum and were averages of at least four experiments.

## Cell Counts

The relative numbers of the different organisms in the sulphidogenic cocultures were determined with a Leitz Diaplan D microscope equiped with a Philips LDK 12 camera and a video recorder (Sony VO 5630, U-matic). The relative number of methanogens in the methanogenic coculture was determined using a UV-microscope (Leitz, Dialux 20 EB).

## Preparation of cell-free extracts

Cells were harvested by centrifugation, washed once with 50

mM phosphate buffer with 2 mM $MgCl_2$, pH 7.1, and stored as wet cell paste anaerobically at -20 °C. Cells were thawed and brought into a French Press compartment in an anaerobic glove box with a gas phase of $N_2/H_2$ (96:4). The cells were broken at 1360 bar and the cell-debris was removed by centrifugation at 4000 rpm for 20 minutes. The supernatants were stored oxygen-free at 0 °C in glass tubes sealed with butyl rubber stoppers.

Enzyme assays

Enzyme assays were carried out anaerobically at 37 °C in a LKB 4053 kinetics spectrophotometer ultrospec K (Pharmacia Nederland b.v., Woerden). Cuvettes (1 ml) were closed with rubber stoppers and made anaerobic by flushing with $N_2$. Anaerobic buffer (unless otherwise stated, Tris-HCl at the same molarity and pH as the buffer mentioned in the cited references) and all other solutions were brought into the cuvette by syringe.
Thiokinase activity with propionate (E.C. 6.2.1.17), acetate (E.C. 6.2.1.1) and succinate (E.C. 6.2.1.5) were measured (at pH 8.5) as described by Oberlies et al. (1980). Kinase activities with these substrates (E.C. 2.7.2.1 for acetate kinase) were measured in the same way but with the omission of HSCoA. Propionyl-CoA:oxaloacetate transcarboxylase (E.C. 2.1.3.1), fumarase (E.C. 4.2.1.2), malate dehydrogenase (NADH-dependent, E.C. 1.1.1.37 and NADPH-dependent, E.C. 1.1.1.82) in the direction of malate formation, malic enzyme (E.C. 1.1.1.39) and succinate dehydrogenase (E.C. 1.3.99.1) with ferricyanide in a 100 mM Tris-HCl buffer, pH 7.4) were measured according to Stams et al. (1984). Fumarate reductase (E.C. 1.3.1.6) was assayed with reduced benzylviologen or NADH following the procedure of Boonstra et al. (1975). Hydrogenase (E.C. 1.12.1.2) was assayed as described for CO dehydrogenase by Daniels et al. (1977). Pyruvate dehydrogenase (E.C. 1.2.4.1) and formate dehydrogenase (E.C. 1.2.1.2) were measured according to Odom and Peck (1981). Pyruvate carboxylase (E.C. 6.4.4.1) was assayed according to

Scrutton et al. (1969), phosphoenolpyruvate carboxylase (E.C. 4.1.1.31) after Maeba and Sanwal (1969); isocitrate lyase (E.C. 4.1.3.1) after Dixon and Kornberg (1959); phosphotransacetylase (E.C. 2.3.1.8) after Oberlies et al. (1980) and NADH dehydrogenase (E.C. 1.6.99.3) was measured in a HEPES/KOH buffer, pH 7.5, with 0.05% Triton X-100 after Bergsma et al. (1982). Propionyl-CoA:succinate transferase was measured after Hilpert et al. (1984) in a 10 mM Na-arsenate buffer, pH 7.0. Propionyl-CoA carboxylase (2.1.3.1) was assayed, following NADH-oxidation at 340 nm, in a 100 mM Tris-HCl buffer pH 8.0 containing: $KHCO_3$ 50 mM, KCl 100 mM, $MgCl_2$ 4 mM, glutathione 2 mM, ATP 2 mM, phosphoenolpyruvate 1 mM, NADH 0.6 mM, lactate dehydrogenase 40 U, pyruvate kinase 10 U and propionyl-CoA 0.5 mM. The overall reaction from succinate to propionate was followed in a coupled assay as described by Stams et al. (1984). Both a 60 mM Tris-HCl buffer pH 7.4 and a 87.5 mM phosphate buffer pH 7.5 were used. Formate hydrogen lyase activity (E.C. 1.2.1.2) was measured in vials (25 ml) closed with butyl rubber stoppers. For this purpose 1 ml of cell-free extract was added to 2 ml 100 mM phosphate buffer pH 7.0 and hydrogen formation was measured after the addition of formate to give a concentration of 40 mM. Pyruvate formate lyase (E.C. 2.3.1.54) was measured in a buffer as described by Jungermann and Schön (1974), with 2 mM HSCoA and 30 mM pyruvate. 0.5 ml cell-free extract was added to 1 ml assay buffer in vials (10 ml) closed with butyl rubber stoppers.

## Analytical methods

Propionate and acetate were measured gaschromatographically or by HPLC; lactate was measured only by HPLC. A CP9000 gaschromatograph (Chrompack, Middelburg) was used with a glass column (180 cm x 2 mm ID) filled with Chromosorb 101 (80 - 100 mesh). The carrier gas was nitrogen saturated with formic acid according to Ackman (1972). The temperatures of the injector, column and detector were 250 °C, 160 °C - 180 °C and 300 °C, respectively. For HPLC (LKB 2150; Parmacia Nederland b.v.,

Woerden) a Chrompack organic acids column (30 cm x 6.5 mm ID) was used. The eluent was 0.01 N $H_2SO_4$ with a flow rate of 0.6 or 0.8 ml/min. The column temperature was 60 °C. 20 µl sample was injected using a SP 8775 autosampler (Spectra Physics, Eindhoven). Detection was done with an LKB 2158 uvicord SD (Pharmacia Nederland b.v., Woerden) at 206 nm, or an LKB 2142 differential refractometer (Pharmacia Nederland b.v., Woerden) was used.

Hydrogen and methane were measured gaschromatographically as described before (Houwen et al., 1988).

Protein in cell-free extracts was determined according to Bradford (1976). In the growth-yield experiment the microbiuret method (Kuenen and Veldkamp, 1972) was used.

Sulphide was measured according to Trüper and Schlegel (1964).

### Materials

Alle chemicals used were of analytical grade. Biochemicals and enzymes were purchased from Boehringer, Mannheim, FRG and Sigma Chemical Co., St. Louis, U.S.A. Gases were obtained from Hoek Loos, Schiedam, The Netherlands. Palladium catalist was a gift of BASF, Arnhem, The Netherlands.

## RESULTS AND DISCUSSION

### Enzyme activities

In view of the results obtained with $^{13}C$-NMR (Chapter 3) enzyme measurements were focussed on enzymes of the succinate pathway. The results for the Syntrophobacter-Desulfovibrio coculture are summarized in the first column of Table 1. The second column represents enzyme activities as measured in

*Table 1. Specific activities (μmol x min.$^{-1}$ x mg$^{-1}$ protein) of enzymes detected in the Syntrophobacter-Desulfovibrio coculture (column 1) and in a pure culture of the Desulfovibrio species (column 2). The third column represents the calculated values for S. wolinii (see text).*

| Enzymes | Coculture | Sulphate-reducer | S. wolinii (calculated) |
|---|---|---|---|
| propionate kinase | 0.120 | -[a] | 0.123 |
| propionyl-CoA carboxylase | < 0.02 | - | < 0.02 |
| propionyl-CoA:oxaloacetate transcarboxylase | 0.519 | - | 0.535 |
| succinate thiokinase | 0.315 | - | 0.325 |
| propionyl-CoA:succinate transferase | < 0.005 | - | < 0.005 |
| succinate dehydrogenase ($Fe(CN)_6^{3-}$) | 0.069 | - | 0.071 |
| fumarate reductase ($BV^+$)[d] | 0.013 | 0.011 | 0.013 |
| fumarase: | | | |
| fumarate disappearance | 1.04 | - | 1.07 |
| fumarate formation | 0.144 | 0.049 | 0.147 |
| malate dehydrogenase: | | | |
| NADH-dependent | 2.33 | 0.036 | 2.40 |
| NADPH-dependent | 0.191 | ND[b] | NC[c] |
| malic enzyme | 0.30 | 0.13 | 0.31 |
| pyruvate carboxylase | < 0.001 | - | < 0.001 |
| pyruvate dehydrogenase ($BV^{2+}$)[e] | 0.019 | 0.260 | 0.012 |
| phosphotransacetylase | 2.03 | 0.107 | 2.09 |
| acetate kinase | 0.116 | 0.331 | 0.109 |
| hydrogenase ($MV^{2+}$)[e] | 1.21 | 1.83 | 1.19 |
| formate dehydrogenase ($BV^{2+}$)[e] | 2.11 | 1.71 | 2.12 |
| NADH dehydrogenase (MTT)[g] | 0.020 | 0.008 | 0.02 |
| formate hydrogen lyase | 0.0043 | 0.0093 | 0.0041 |

[a] not detected

[b] not determined

[c]not calculated
[d]$BV^{+}$, reduced benzyl viologen
[e]$BV^{2+}$, oxidized benzyl viologen
[f]$MV^{2+}$, oxidized methyl viologen
[g]MTT, 3-(4'5'-dimethyl-thiazol-2-yl)-2,4-diphenyltetrazolium bromide
Enzymes not detected: Isocitrate lyase, pyruvate formate lyase, phosphoenolpyruvate carboxylase, propionate thiokinase, acetate thiokinase, succinate kinase.

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cell-free extracts of a pure culture of the hydrogenotrophic sulphate reducing bacterium. In the coculture the relative number of propionate oxidizers was very constant (Table 2). This made it possible to determine the contribution of S. wolinii to the total protein in the coculture. Based on mean protein content per cell in the pure culture of the sulphate reducer ($8.2 \times 10^{-11}$ mg), the total protein content in the coculture (62.4 mg/ml and 4.8 $10^{10}$ cells/ml) and the relative number (55%), it was calculated that about 97% of the protein in the coculture was from the propionate oxidizing organism. Column 3 (Table 1) gives the calculated specific enzyme activities of the syntrophic propionate degrader.

The results as shown in Table 1 are in agreement with the involvement of the succinate pathway for propionate oxidation by S. wolinii. Besides via methylmalonyl-CoA, propionate might be converted to succinate via α-OH-glutarate (Wegener et al., 1968). In this case, isocitrate lyase would play a key role (Rabin et al., 1965). Because of the high activity of propionyl-CoA:oxaloacate transcarboxylase and the absence of isocitrate lyase activity, we conclude that methylmalonyl-CoA is an intermediate in the conversion of propionate to succinate. This conclusion is supported by label patterns found during propionate conversion in the presence of both [3-$^{13}$C]-propionate and $H^{13}CO_3^-$ (Chapter 3).

The activity of the transcarboxylase as measured in S. wolinii is comparable to the activity in extracts of D.

propionicus (Stams et al., 1984). However, attempts to measure the overall conversion from succinate to propionate in a similar way (with 5 times more protein) as reported for D. propionicus, were not successful. Although S. wolinii is a propionate degrading organism, interconversion of propionate and succinate was observed (Chapter 3) and therefore propionate formation from succinate should be possible.

In the other syntrophic cultures (ZPS and Z), transcarboxylase activities of 0.006 and 0.036 μmol x $min^{-1}$ x $mg^{-1}$ were measured. The difference in activity beween these cultures, in which the same propionate oxidizing organism is present, may be due to differences in the protein contribution of the propionate degrader in the cell-free extracts.

In this study enzyme activities present in S. wolinii were calculated by subtracting specific activities of enzymes of different extracts. It remains unknown whether artefacts are introduced in this manner. Because Desulfovibrio G11 was grown on hydrogen and sulphate in the presence of propionate, the method is reliable if an enzyme is absent in the pure culture as is the case with e.g. the crusial enzymes transcarboxylase and succinate thiokinase. However, if an enzyme is present in the pure culture, results should be interpreted with caution. The high protein content of S. wolinii (97%) in the total protein of the cell-free extract was certainly favourable in this respect.

## Growth rates and growth yields

Figure 2 shows growth curves of the Syntrophobacter-Desulfovibrio coculture, the sulphidogenic coculture "ZPS" and the methanogenic coculture "Z". The growth rates determined from Figure 2, are given in Table 2. The Syntrophobacter-Desulfovibrio coculture had a higher growth rate than mentioned by Boone and Bryant (1980) (Table 2). This difference can be explained by differences in growth media and the slightly different temperature. Sulphide had a strong inhibiting effect on the growth of the Syntrophobacter-Desulfovibrio coculture and

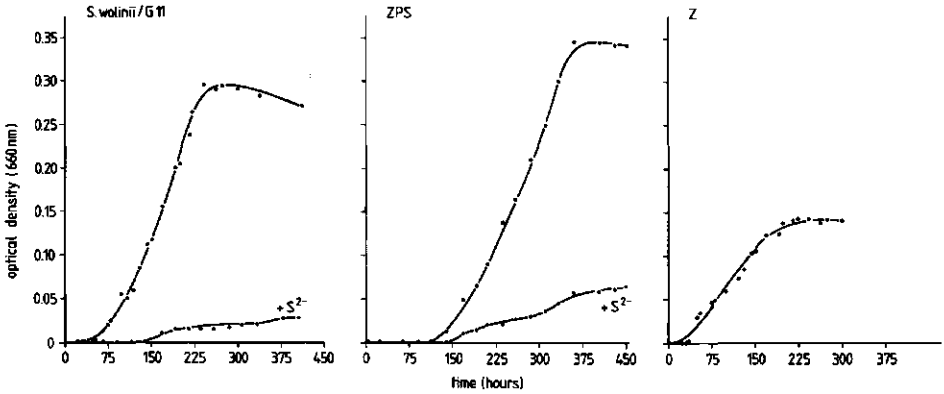


*Figure 2. Growth curves of the Syntrophobacter-Desulfovibrio coculture, ZPS and Z. For the sulphidogenic cocultures also growth curves were determined in the (initial) presence of 3 mM extra sulphide.*

on ZPS (Figure 2). Their specific growth rates decreased to 0.133 $day^{-1}$ and 0.187 $day^{-1}$, respectively. As both cocultures easily produce 10 mM sulphide during normal growth, the initial concentration of sulphide seems to be important. The propionate oxidizers grown together with hydrogenotrophic methanogens have a lower growth rate than with sulphate reducers as hydrogenotrophs (Table 2). The more negative Gibbs free energy change under sulphidogenic conditions compared to methanogenic conditions (equations (i) and (ii)) may have contributed to this difference (Thauer et al., 1977; Dolfing, 1988). Slower growth in coculture with methanogens was described earlier for S. wolinii (Boone and Bryant, 1980), the butyrate oxidizing Syntrophomonas wolfei (McInerney et al., 1979) and the benzoate degrading Syntrophus buswellii (Mountfort and Bryant, 1982).

(i) $4\ Pr^- + 3\ H_2O \longrightarrow 4\ Ac^- + 3\ CH_4 + HCO_3^- + H^+$

$\Delta G^{\circ\prime} = -102.4$ kJ/mol

(ii) $4\ Pr^- + 3\ SO_4^- \longrightarrow 4\ Ac^- + 3\ HS^- + HCO_3^- + H^+$

$\Delta G^{\circ\prime} = -151.3$ kJ/mol

The growth yield of D. propionicus was higher compared with that of the Syntrophobacter-Desulfovibrio coculture (Table 2).

*Table 2. Microbiological and biochemical characteristics of some propionate oxidizing cultures.*

| Organism(s) | % propionate oxidizer | Growth rate ($day^{-1}$) | Growth yield (g protein x $mol^{-1}$) |
|---|---|---|---|
| Desulfobulbus | 100 | | 1.69 |
| | | 0.89(1) | 1.95[a](1) |
| | | 1.66(2) | 2.17[a](2) |
| Syntrophobacter - Desulfovibrio G11 | 55 | 0.32 | 0.92 |
| | | 0.19(3) | |
| Methanospirillum | ND[b] | 0.10(3) | ND |
| Sulphidogenic coculture (ZPS) | 30 | 0.28 | ND |
| Methanogenic coculture (Z) | 10 | 0.23 | ND |

[a]With the assumption that 1 g cell carbon corresponds to 1 g protein
[b]Not determined
(1)After Stams et al. (1984)
(2)After Widdel and Pfennig (1982)
(3)After Boone and Bryant (1980)

Because the contribution of S. wolinii to the total protein in the cell-free extract was constant (97%), the yield of S. wolinii is about 0.89 g/mol. This is twice as low as the yield of D. propionicus.

During growth on propionate plus sulphate, D. propionicus conserves energy both at substrate level and by membrane-linked electron transport (Kremer and Hansen, 1988). The mechanism(s) by which energy conservation takes place in S. wolinii is

unknown. One ATP will be formed in the conversion of acetyl-CoA to acetate. However, reoxidation of reduced electron carriers is energetically difficult. Assuming that $H^+$ serves as electron acceptor, the partial pressure of hydrogen ($p_{H_2}$) is of great importance. At a $p_{H_2}$ of $10^{-5}$ atm. ($E'$ $2H^+/H_2$ = -272 mV), electrons derived from the conversion of pyruvate to acetyl-CoA ($E^{\circ\prime}$ = -490 mV) and of malate to oxaloacetate ($E^{\circ\prime}$ = -172 mV) can be disposed without loss of energy ($\Delta G'$ = -22.8 kJ/mol). At this $p_{H_2}$, however, the oxidation of succinate to fumarate ($E^{\circ\prime}$ = +33 mV) still costs 58 kJ/mol (corresponding to more than one ATP; Thauer et al., 1977). At an internal succinate/fumarate ratio of $10^5$ and a proton motive force of 150 mV to drive the presumably membrane-linked succinate dehydrogenase, this reaction would just be feasible. The amount of energy required to generate the proton motive force is not known; it can be speculated that it should be less than 1 ATP. Alternatively, the excretion of $HCO_3^-$ in symport with protons could contribute to the generation of the proton motive force. Various examples are known, involving membrane-linked enzymes, by which endergonic redox reactions are driven at the expense of energy (Thauer and Morris, 1984; Kröger et al., 1986; Häger and Bothe (1987).

ACKNOWLEDGEMENTS

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Chapter 7

# FATE OF PROPIONATE IN METHANOGENIC GRANULAR SLUDGE AT 55 °C IN THE PRESENCE AND ABSENCE OF FUMARATE.


## ABSTRACT

Mesophilic methanogenic granular sludge was investigated for its ability to degrade propionate at 55 °C. Propionate degradation started after an incubation period of fourteen days. Addition of fumarate as secondary substrate stimulated propionate metabolism. Propionate and succinate appeared to be direct precursors of each other. $^{13}C$-label randomized to some extent between C-3 and C-2 propionate and label appeared in succinate, indicating the involvement of the succinate pathway.



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# INTRODUCTION

Under methanogenic conditions, organic material is completely converted to methane and carbon dioxide in a sequence of reactions in which different physiological types of bacteria are involved (McInerney et al., 1980; Zeikus, 1982; Gujer and Zehnder, 1983). In a steady state situation, the concentration of intermediairy products and relative numbers of organisms is constant, and the most efficient route for breakdown of the substrate(s) is operative (McCarty et al., 1963; Grotenhuis et al., 1986; Smith and McCarty, 1989). To investigate the potential of the bacteria involved in the mineralization process, non-steady state conditions have to be created, e.g. by a shift in temperature or medium composition, a substrate pulse, or a pH shock. In this way, less efficient reactions are allowed to proceed resulting in transient accumulation of (new) products.

In anaerobic degradation of organic matter propionate is an important intermediate (McCarty et al., 1963; McCarty, 1964; Kaspar and Wuhrmann, 1978[a]; Mackie and Bryant, 1981; Balba and Nedwell, 1982). It is mainly formed as reduced end product during fermentation of carbohydrates and lactate. Most of the propionate is produced by decarboxylation of succinate (Wolin, 1974; Wood, 1981; Schink, 1985; 1986). Oxidation of propionate to acetate, carbon dioxide and hydrogen is endergonic under thermodynamic standard conditions ($\Delta G^{\circ\prime}$ = +76.1 kJ/mol; Thauer et al., 1977). The reaction only occurs when the partial pressure of hydrogen is kept extremely low by chemolitotrophic methanogens. Although propionate is a relative simple substrate, it is degraded slowly and therefore often causes problems in anaerobic wastewater treatment plants (Heyes and Hall, 1983; Gujer and Zehnder, 1983; Wiegant, 1986).

Tracer studies and enzymic analysis have shown that the succinate pathway, as depicted in Figure 1, is involved in syntrophic propionate oxidation (Buswell et al., 1951; Koch et al., 1983; Schink, 1985; Robbins, 1988; Houwen et al, 1987;

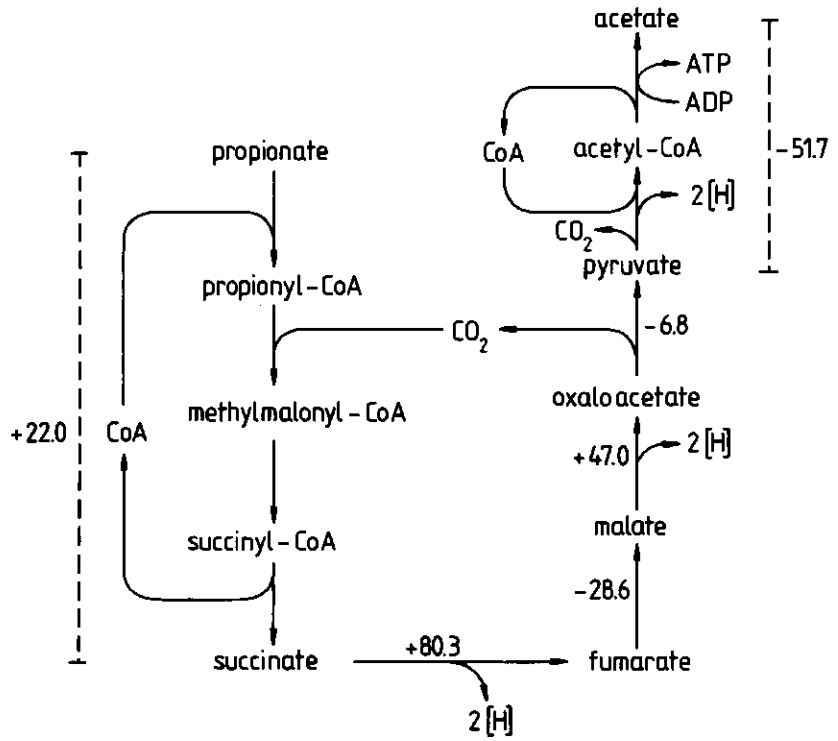


*Figure 1. The succinate pathway for propionate degradation. The presence of a transcarboxylase and CoA-transferases are hypothetical. $\Delta G^{o'}$-values for 55 °C were calculated using the van 't Hoff equation (Morel, 1983). Net ATP-production of the conversion may be less than one.*

1990). Figure 1 also shows that not all the reaction steps involved in propionate oxidation are thermodynamically unfavourable. It can be hypothesized that propionate oxidizers may grow at elevated hydrogen partial pressures on compounds like pyruvate, malate and fumarate. Moreover, in case a transcarboxylase is involved, the presence of fumarate may overcome the highly endergonic succinate oxidation step. Succinate would then be end product of propionate carboxylation, whereas fumarate would concomitantly be degraded to acetate.

Research was started to study propionate oxidation by mesophilic granular sludge under thermophilic conditions. As a consequence of the temperature shift the development of new populations of organisms is necessary. As these new organisms

have various growth rates, different transient phases are expected during substrate conversion. In order to assess the possible stimulatory effect of fumarate on propionate oxidation by granular sludge, experiments were performed in the presence and absence of fumarate. Preliminary experiments indicated an initial increase in the rate of propionate breakdown when fumarate was added as secundary substrate. High-resolution NMR with [3-$^{13}$C]-propionate was applied to make a distinction between propionate degradation and propionate formation.

## MATERIALS AND METHODS

### Methanogenic granular sludge

Methanogenic granular sludge was kindly provided by the CSM-sugar refinery at Breda, The Netherlands. It had been cultivated in a 30 $m^3$ UASB reactor at 32 °C on sugar-containing waste water (Pette and Versprille, 1982). Before use the sludge had been stored at 4 °C for several months.

### Medium

Incubations were carried out in a medium as described before (Houwen et al., 1987), except that yeast extract (0.2 g/l) was added and two trace elements ($SeO_2$ and $Na_2WO_4.2H_2O$) were not added. To keep the pH at 7.4 during incubations at 55 °C, the amount of $NaHCO_3$ was diminished to 2 g/l and $Na_2HPO_4$ was omitted. The gas phase consisted of $N_2/CO_2$ (80:20; 1.8 atm).

### Incubations

Granular sludge was disintegrated in a potter tube. In this

way small particles with a diameter of less than 0.5 mm were obtained. Serum vials (120 ml) were made anaerobic by flushing with $N_2/CO_2$ (80:20), and filled with 45 ml of anaerobic medium (without $Na_2S.9H_2O$). The medium had previously been made anaerobic by boiling, followed by cooling to room temperature under continuous flushing with $O_2$-free $N_2/CO_2$ (80:20). Disintegrated sludge (± 200 mg volatile suspended solids = dry weight minus ash) was added to the vials and then closed with butyl rubber stoppers and serum bottle caps. A gas phase of $N_2/CO_2$ (80:20) was placed at an overpressure of 0.8 atm. To remove traces of oxygen, $Na_2S.9H_2O$ was added by syringe to obtain a final concentration of 1 mM. Propionate, fumarate, or both were added to give final concentrations of 25 mM. Samples were withdrawn at different time intervals and analyzed as described below.

The incubations for NMR were carried out in 10 ml medium with 20 mM [3-$^{13}$C]-propionate (90% enriched, Amersham, U.K.) in the presence and absence of 40 mM fumarate. Samples (0.5 - 2 ml) were taken at various periods of time and centrifuged. Supernatants were diluted to 2 ml with water and 150 µl $^2H_2O$ was added to provide a lock signal. After the NMR measurements, the samples were analyzed for organic acids.

High-resolution NMR

$^{13}$C-NMR spectra of the supernatants were obtained on a Bruker CXP-300 pulsed Fourier-Transform spectrometer, equipped with a 10 mm $^{13}$C-probe. The Waltz pulse sequence was employed to decouple protons. Spectra were recorded during 20 min. at 25 °C using a 6 µs (30°) pulse and a delay time of 1 s.
To quantify the amount of label in the various carbon atoms, [2-$^{13}$C]-acetate (99,5% enriched, MSD-Isotopes, Canada) was added to a sample to a final concentration of 30 mM and its spectrum was recorded under indentical conditions as the supernatants. Corrections for differences in relaxation behaviour and Nuclear Overhauser Enhancement build up between the various atomic

nuclei, were made as described before (Houwen et al., 1987; Chapter 3).

Other analyctical methods

Propionate and acetate were measured both gaschromatographically (Houwen et al., 1988) and by HPLC. For HPLC a Chrompack organic acids column (30 cm x 6.5 mm ID) was used. The eluent was 0.01 N $H_2SO_4$ with a flow rate of 0.8 ml/min. The column temperature was 60 °C. 20 µl sample was injected using a Spectra Physics autosampler (SP 8775). UV-detection at 206 nm was done with a 2158 uvicord SD (LKB). HPLC was also used to quantify fumarate, malate and succinate.
Hydrogen and methane were determined by gaschromatography as described before (Houwen et al., 1988).

RESULTS

Incubation of disintegrated mesophilic granular sludge at 55 °C with 25 mM propionate led only to considerable degradation of propionate after more than twelve days of incubation (Figure 2A). As acetate was hardly detectable during the course of the experiment, cleavage of this compound into methane and carbon dioxide was not the rate limiting step in propionate oxidation. In a parallel experiment carried out at 37 °C, propionate oxidation started immediately, and complete degradation of the substrate was observed within thirty days (results not shown).
Fumarate was rapidly broken down within seven days at 55 °C yielding succinate and acetate as products (Figure 2B). Malate was intermediairy product during these first seven days of incubation. The acetate concentration hardly changed between day seven and fourteen. In this period, however, the succinate concentration decreased considerably with the simultaneous

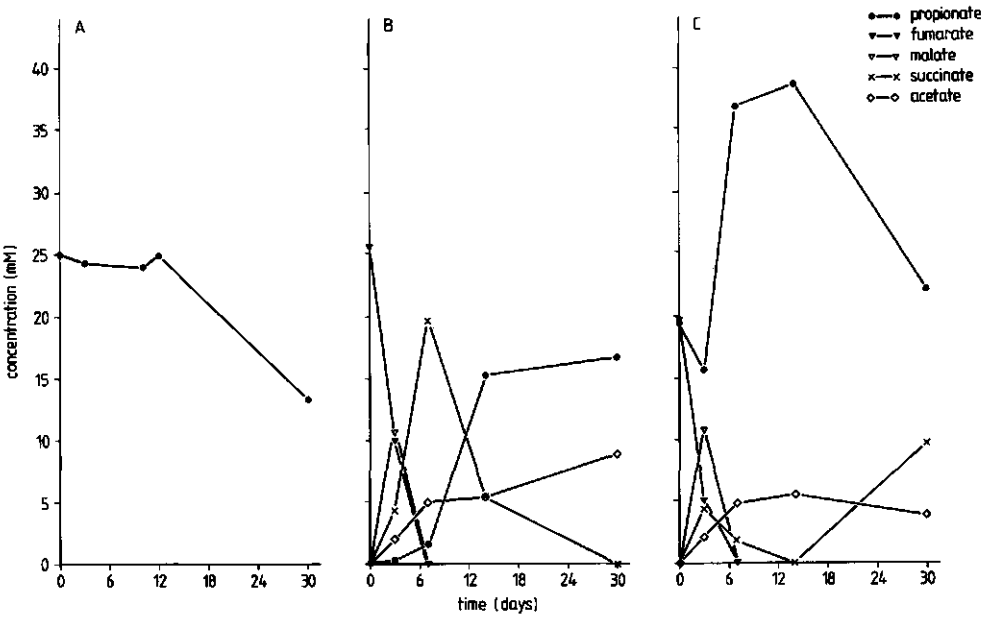


*Figure 2. Conversion of propionate (A), fumarate (B) and propionate plus fumarate (C) during incubation of disintegrated granular sludge at 55 °C, as measured by HPLC/GC methods.*

formation of propionate, indicating that propionate was formed directly from succinate.

When propionate and fumarate were both present as substrates (Figure 2C), the initial propionate oxidation rate was apparently higher than in the absence of fumarate. This phenomenon was found at all times this experiment was carried out. As observed in the incubation with fumarate alone, fumarate and malate are converted to succinate and presumably to acetate. The subsequent decarboxylation of succinate to propionate, however, now already occurred to a significant extent within seven days. After succinate became depleted within about fourteen days, the propionate concentration decreased again accompanied by an increase of the succinate concentration.

During the thermophilic incubations, both methane and hydrogen were measured in the gas phase. In all cases, considerable methane production was observed in the first three days of incubation, followed by a period of four days in which only

minor quantities of methane were formed. From seven days of incubation, continuous methane production was measured. Hydrogen was present at a high level in the first four days of incubation. After day four, this compound was hardly detectable. Under mesophilic conditions, no hydrogen was detected (results not shown).

With propionate and fumarate both present during incubation, propionate formation and propionate oxidation could not be distinguished, making it difficult to determine the influence of fumarate on the initial propionate oxidation rate. To distinguish between the two processes, [3-$^{13}$C]-labelled propionate was used as substrate in the presence and absence of fumarate. Figure 3A shows the $^{13}$C-label incorporation after incubation of disintegrated sludge with 18.5 mM [3-$^{13}$C]-propionate (90% enriched) and 40 mM fumarate (not enriched). A higher fumarate concentration was used to induce more initial acetogenic breakdown of this compound. In this way, the possible involve-

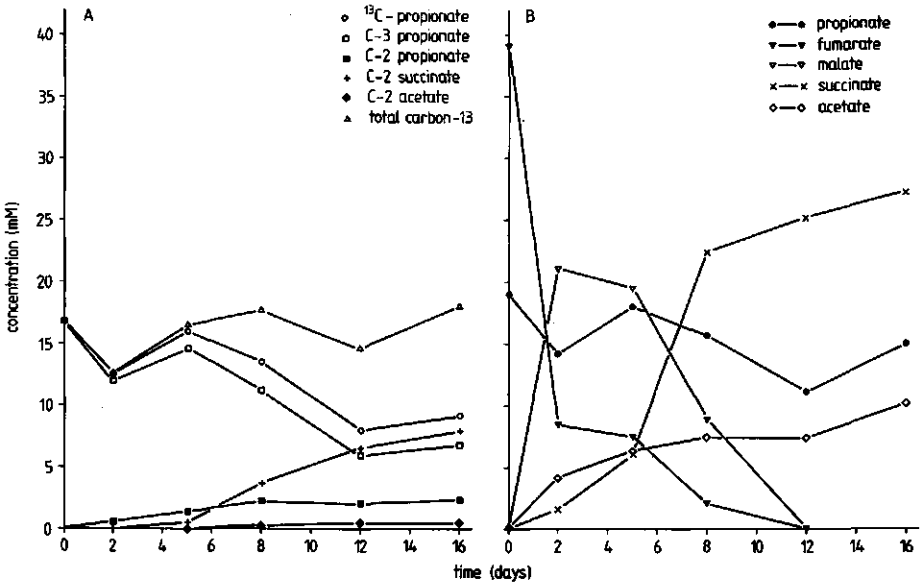


*Figure 3. $^{13}$C-label distribution during incubation of disintegrated granular sludge at 55 °C with [3-$^{13}$C]-propionate and fumarate, as measured by NMR (A). C-1 acetate (not shown) was present at the same concentration as C-2 acetate. The same samples were subsequently analyzed by HPLC/GC methods (B).*

ment of a transcarboxylase in propionate breakdown could be more effective. Between day zero and two, some C-3 propionate disappeared but was partly regenerated between day two and five, the $^{13}C$-label also being incorporated into the C-2 carbon positions of succinate and propionate and the C-2 and C-1 of acetate. About 0.5 mM of both C-2 and C-1 acetate were measured after sixteen days. This 1 mM $^{13}C$-labelled acetate corresponds to 10% of the total acetate as determined after sixteen days of incubation by HPLC/GC methods (Figure 3B). The succinate formation as the main product from fumarate (via malate) (Figure 3B), coincided with the onset of label incorporation at the C-2 carbon position of succinate and loss of label from C-3 propionate (Figure 3A). Apparently, propionate and succinate were interconverted considerably only after a certain threshold in the succinate concentration had been reached. The interconversion led to a dilution of the labelled propionate pool with unlabelled propionate and to incorporation of label into succinate. Because of the symmetric nature of succinate, decarboxylation of this compound also led to C-2 labelled propionate. During the whole experiment the net degradation of propionate as determined by HPLC/GC methods was about 4 mM.

In a parallel experiment with only $^{13}C$-propionate as substrate, the labelled propionate pool did not change during sixteen days (results not shown). Still, 4 mM of unlabelled acetate was measured which therefore must have been formed from endogeneous substrate. Although no labelled succinate was detected in this experiment, C-2 labelled propionate (1 mM) was found.

## DISCUSSION

Granular sludge, cultivated on a waste stream containing liquid sugar, was investigated for its ability to degrade propionate and fumarate under non-steady state conditions.

Disintegrated sludge degraded propionate at 55 °C only after a long incubation period (Figure 2A). As both hydrogen and methane were produced from the beginning of the incubations, they (initially) must have originated from endogeneous substrate.
The higher temperature limit for degradation by a mesophilic propionate degrading enrichment culture, of mesophilic sludge, and for chemolitotrophic methanogenesis was found to be 45 °C (Pfeffer, 1974; Zeikus and Winfrey, 1976; Mah et al., 1977; Boone and Xun, 1987). Above this temperature, not adaptation of the mesophilic organisms present, but growth of new (thermophilic) populations of bacteria is needed to allow substrate degradation. With fumarate as the sole substrate (Figure 2B), propionate was formed as reduced end product. Between fourteen and thirty days of incubation the propionate concentration remained constant. This indicates that at 55 °C, at least sixteen days were needed for the propionate oxidizers to grow considerably. This can also be concluded from Figures 2A and 2C. Contrary, fumarate degrading organisms (and methanogens) started to grow immmediately (Figure 2B and 2C).

Propionate degradation seemed to be initiated by the presence of fumarate (Figures 2C and 3). However, as part of the $^{13}C$-label was regenerated in propionate (Figure 3A), no net degradation had occurred. Labelled propionate therefore is initially most likely incorporated in large molecular weight, NMR-invisible, compounds and subsequently remobilized. This can occur by binding to enzymes or by incorporation into large molecular aggregates or biopolymers. Although similar patterns were found in the experiment with unlabelled propionate and fumarate compared to the NMR experiment (Figures 2C and 3B), all conversions appeared to be delayed in the latter experiment. The reason for this may be that the NMR experiment was carried out five months later, possibly resulting in a decrease of viable thermophilic bacterial populations.
In the experiment with $^{13}C$-propionate and fumarate as substrates, 10 mM acetate was recovered after sixteen days of incubation (Figure 3B). About 5 mM of acetate, originating from added substrate, had apparently been converted to methane and

carbon dioxide between day zero and sixteen. Assuming that another 4 mM acetate was produced from endogeneous substrate as was found in the parallel experiment with only $^{13}$C-propionate, a total of 19 mM (10 + 5 + 4) acetate would have been produced. Therefore, 45% - 50% of the acetate formed may have been degraded again by methanogenic bacteria. Only 1 mM of $^{13}$C-acetate was found (Figure 3A), which would correspond with a formation of 1.8 mM - 2.0 mM. In the same period (sixteen days) 4 mM of $^{13}$C-propionate had been degraded. This indicates that about half of the propionate is degraded directly to acetate, whereas the rest is converted to succinate with concomitant oxidation of fumarate to acetate. This is strengthened by the observation that no propionate was degraded in the parallel experiment. However, label in acetate may also have derived from reversible reactions in the fumarate converting bacteria. Because net degradation of propionate was minor, further work is needed to get evidence whether or not propionate carboxylation, leading to succinate, is linked to fumarate oxidation. So far, it cannot be said whether the propionate oxidation rate is enhanced by the presence of fumarate.

$^{13}$C-Label was incorporated at equal amounts into both carbon positions of acetate. Moreover, interconversion of propionate and succinate was demonstrated. This is in agreement with the involvement of the succinate pathway as shown in Figure 1. Similar results for propionate and succinate metabolism as described in this paper, were obtained in (tracer) experiments with other mixed populations of bacteria but also with pure cultures (Wood, 1972; Wolin, 1974; Boone, 1984; Schink, 1985; Schink et al., 1985; Robbins, 1988; Tholozan et al., 1988; Houwen et al., 1987; Chapter 3).

The formation of succinate from propionate was observed during incubation of sludge with both propionate and fumarate (Figure 2C). This is remarkable because at concentrations of 35 mM and 2.5 mM for propionate and succinate, respectively (and 20 mM for $HCO_3^-$), the $\Delta G'$ of this reaction is positive (+25.5 kJ/mol). A possible explanation is that intracellular and extracellular pools existed. Different pools for propionate and

succinate were also proposed for Propionibacterium species to explain label patterns (Wood, 1972) (Chapter 3). Alternatively, pathways involving propionate, acetate, carbon dioxide and hydrogen, which might be thermodynamically favourable, also could explain the succinate formation. As the conversion of malate to fumarate (Figures 2B, 2C and 3B) is highly endergonic under standard conditions at 55 °C ($\Delta G°'$ = +28.6 kJ/mol), also for these compounds different intra- and extracellular concentrations may have existed.

Because non-steady state conditions were prevailing in the experiments, intermediary products accumulated. During degradation of fumarate, succinate appeared to be a direct precursor of propionate (Figures 2B and 2C). A thermophilic bacterium has been isolated recently from these granules which can grow by the decarboxylation of succinate (Cheng Guangsheng et al., manuscript in preparation).

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## SUMMARY

Under methanogenic conditions, with protons and carbon dioxide as intermediate and ultimate electron acceptors, complex organic material is degraded in several steps to methane and carbon dioxide. About 15% of the total carbon compounds are degraded via propionate as an intermediate. Propionate is oxidized to acetate, carbon dioxide and hydrogen. For thermodynamical reasons, this reaction can only proceed if the partial pressure of hydrogen is kept very low. Hydrogenotrophic organisms, e.g. methane bacteria or sulphate reducing bacteria, are syntrophic partner in this process of interspecies hydrogen transfer. Recently, however, it was hypothesized that also formate could be an important compound via which the electrons are transferred to the partner organism.

The aim of this study was to obtain better fundamental understanding of biochemical and physiological aspects of obligate syntrophic propionate oxidizing bacteria. The presence of an obligate partner organism makes such studies very difficult. In this thesis different methods were used to overcome these difficulties: 1) techniques which do not require pure cultures, e.g. the use of specifically labelled compounds, 2) growth of the acetogen in pure culture by either using artificial electron acceptors or metabolic intermediates, and 3) determining the acetogen specific enzymes by subtracting the activities measured for the pure culture of the electron scavenging partner organism from the activities found in a defined biculture.

Using *in vivo* high-resolution $^{13}C$-NMR, evidence was found for the involvement of the succinate pathway in propionate oxidation by a methanogenic coculture (Chapter 1). The addition of [3-$^{13}C$]-labelled propionate clearly showed succinate as an intermediate, and the ultimate breakdown product acetate was labelled equally in the C-1 and C-2 positions. In addition, *de novo* synthesis of propionate from propionate was observed. The

$^{13}$C-label randomized completely between the C-3 and C-2 of propionate. Apparently propionate and succinate were interconverted at a high rate. These results were in accordance with the data published by others.

The interconversion of propionate and succinate offered the possibility to study the role of carboxylation reactions in propionate metabolism in some anaerobic bacteria (Chapter 3). This was done in a very easy way by the inclusion of [3-$^{13}$C]-propionate and $H^{13}CO_3^-$, which gave insight into the process of randomization and the types of (de)carboxylating enzymes involved. Both the propionate oxidizer in a methanogenic coculture and Syntrophobacter wolinii were shown to degrade propionate via the succinate pathway involving a transcarboxylase.

Chapter 4 deals with a two-liquid-phase electron removal system including the artificial, water soluble redox mediator propylviologen sulphonate (PVS). The organic phase dibutylphtalate was used as reservoir for the electron acceptor 2-anilino-1,4-naphtoquinone. In the abiotic two-liquid-phase system, electrons were transfered from the medium into the organic phase. The indicator organism Acidaminobacter hydrogenoformans oxidized glutamate to acetate without evolution of hydrogen (or formate). However, results indicated that the hydrogen partial pressure obtained by this method, was not low enough to clearly influence the metabolism of the bacterium. Besides possible toxicity problems, the relatively low midpoint redox potential of PVS (-390 mV) may have been the problem for efficient electron transfer at the required hydrogen partial pressure of $10^{-5}$ atm. to cause a shift in electron flow during glutamate oxidation. In a syntrophic propionate oxidizing coculture the electron scavenging methane bacteria could not be replaced by the artificial electron acceptor. PVS was tested both as redox mediator in the two-liquid-phase system, and as terminal electron acceptor.

The metabolic intermediates pyruvate and fumarate were tested for growth in pure culture of the propionate oxidizing organism in a methanogenic coculture (Chapter 5). A propionate fermenta-

tion was performed with pyruvate as the substrate. $^{13}$C-NMR showed the involvement of the succinate pathway in the formation of propionate. The isolated organism, however, did not oxidize propionate in coculture with hydrogenotrophic methanogens. Moreover, a sulphate reducer appeared to be present in the original coculture. A syntrophic sulphidogenic propionate oxidizing coculture was obtained by repeated transfer of the coculture in medium with propionate and sulphate. To test whether the (slow growing) obligate syntrophic acetogen can be grown on other substrates than propionate, could not be tested because of the contaminating organisms.

Chapter 6 is the first report on enzyme measurements in syntrophic propionate oxidation. As Syntrophobacter wolinii grows in a defined biculture with a Desulfovibrio species, it was possible to use cell-free extracts of a pure culture of the latter organism as a blanc. Most enzymes involved in the succinate pathway, including the key enzyme propionyl-CoA:oxaloacetate transcarboxylase, were demonstrated in S. wolinii. This confirms the results found by $^{13}$C-NMR (Chapter 3).
Further, S. wolinii appeared to have a lower growth yield than Desulfobulbus propionicus. This difference is explained in terms of energy conservation mechanisms. Comparison of growth rates of three syntrophic propionate oxidizing cocultures showed that hydrogenotrophic sulphate reducers are more efficient than methanogens during interspecies hydrogen transfer. The more negative Gibbs free energy change under sulphidogenic conditions compared to methanogenic conditions, is thought to contribute to this phenomenon.

The final chapter (7) deals with the use of $^{13}$C-NMR in a complex biological system. Propionate degradation was followed in mesophilic methanogenic granular sludge at 55°C. Because of the non-steady conditions, transient intermediary products accumulated in the medium. The addition of fumarate as secondary substrate stimulated propionate conversion. Propionate and succinate appeared to be direct precursors of each other during propionate metabolism. Selective labelling of one of the substrates offered the possibility to study turnovers of

different compounds. Moreover, interrelated biochemical processes could, in this way, be investigated in a relatively easy way.

## SAMENVATTING

Onder methanogene omstandigheden, met protonen en kooldioxide als intermediaire en uiteindelijke elektronenacceptoren, wordt organisch materiaal afgebroken in verschillende stappen tot methaan en kooldioxide. Ongeveer 15% van de totale koolstofverbindingen worden afgebroken via propionaat als intermediair. Propionaat wordt geoxideerd naar acetaat, kooldioxide en waterstof. Om thermodynamische redenen kan deze reaktie slechts verlopen als de partiële waterstofspanning zeer laag gehouden wordt. Hydrogenotrofe organismen, b.v. methaanbakteriën of sulfaat-reducerende bakteriën, zijn syntrofe partner in dit proces van "interspecies hydrogen transfer". Er is recentelijk echter gepostuleerd dat ook formiaat een belangrijke verbinding zou kunnen zijn waarmee de elektronen doorgegeven worden aan het partnerorganisme.

Het doel van deze studie was een beter fundamenteel inzicht te krijgen in de biochemische en fysiologische aspekten van obligaat syntrofe propionaat-oxiderende bakteriën. Deze studies worden bemoeilijkt door de aanwezigheid van het partnerorganisme. In dit proefschrift zijn verschillende methoden toegepast om deze moeilijkheden te omzeilen: 1) technieken die geen reinkulturen vereisen, b.v. het gebruik van specifiek gelabelde verbindingen, 2) groei van de acetogene bakterie in reinkweek door gebruik te maken van artificiële elektronenacceptoren of van metabole intermediairen, en 3) het verminderen van enzymaktiviteiten, gemeten in een gedefinieerde bikultuur, met de aktiviteiten die gevonden zijn voor een reinkultuur van het elektronen wegvangende partnerorganisme.

Met behulp van <u>in vivo</u> high-resolution $^{13}C$-NMR werd gevonden dat de succinaatroute betrokken is bij de propionaat-oxidatie door een methanogene cokultuur (Hoofdstuk 2). Toediening van [$^{13}$-C]-gelabeld propionaat liet zeer duidelijk succinaat zien als intermediair, en het uiteindelijke afbraakprodukt acetaat was gelijk gelabeld in de C-1 en de C-2 posities. Bovendien

werd de novo synthese van propionaat uit propionaat waargenomen. De $^{13}C$-label randomerizeerde volledig over de C-3 en C-2 van propionaat. Blijkbaar werden propionaat en succinaat zeer snel in elkaar omgezet. Deze resultaten waren in overeenstemming met data van anderen.

De snelle reversibele omzetting van propionaat en succinaat bood de mogelijkheid de rol van carboxyleringsreakties in het propionaat metabolisme van sommige anaërobe bakteriën te bestuderen (Hoofdstuk 3). Dit werd op een zeer eenvoudige manier gedaan door het toevoegen van [3-$^{13}C$]-propionaat en $H^{13}CO_3^-$, waardoor inzicht verkregen werd in het proces van randomerizatie en in de typen (de)carboxylerende enzymen die erbij betrokken zijn. Zowel de propionaat-oxideerder in een methanogene cokultuur als Syntrophobacter wolinii bleken propionaat te oxideren via de succinaatroute waarbij een transcarboxylase betrokken is.

Hoofdstuk 4 handelt over een elektronen wegvangend twee-fasen systeem met een artificiële, water oplosbare redox-mediator propylviologeen sulfonaat (PVS). De organische fase dibutylphtalaat werd gebruikt als reservoir voor de elektronenacceptor 2-anilino-1,4-naphtoquinone. In het abiotische twee-fasen systeem werden elektronen overgedragen vanuit het medium naar de organische fase. Het indikatororganisme Acidaminobacter hydrogenoformans oxideerde glutamaat naar acetaat zonder evolutie van waterstof (of formiaat). De resultaten wijzen er echter op dat de bereikte waterstofspanning niet laag genoeg was om het metabolisme van de bakterie duidelijk te beïnvloeden. Behalve mogelijke toxiciteitsproblemen, zou de relatief lage "midpoint redox potential" van PVS (-390 mV) een probleem kunnen zijn geweest voor een efficiënte elektronenoverdracht bij de vereiste waterstofspanning van $10^{-5}$ atm. om een verschuiving van de stroom van elektronen te bewerkstelligen tijdens glutamaat-oxidatie. In een syntrofe propionaat-oxiderende cokultuur konden de elektronen wegvangende methaanbakteriën niet vervangen worden door het gebruik van de artificiële elektronenacceptor. PVS werd getest als redox mediator in het twee-fasen systeem en als terminale elektronenacceptor.

De metabole intermediairen pyruvaat en fumaraat werden getest om het propionaat-oxiderende organisme in een methanogene cokultuur in reinkultuur te groeien (Hoofdstuk 5). Met pyruvaat als substraat werd een propionaat-fermentatie uitgevoerd. $^{13}$C-NMR liet zien dat bij de vorming van propionaat de succinaat-route betrokken was. Het geïsoleerde organisme oxideerde echter geen propionaat in cokultuur met hydrogenotrofe methaanbakteriën. Bovendien bleek een sulfaat-reduceerder aanwezig te zijn in de oorspronkelijke kultuur. Een syntrofe sulfidogene propionaat-oxiderende cokultuur werd verkregen door herhaalde overenting in medium met propionaat en sulfaat. Door de aanwezigheid van de contaminaties kon niet getest worden of de (langzaam groeiende) obligaat syntrofe acetogene bacterie kan groeien op andere substraten dan propionaat.

Hoofdstuk 6 is de eerste melding van enzymmetingen in syntrofe propionaat-oxidatie. Omdat Syntrophobacter wolinii in een gedefinieerde bikultuur groeit met een Desulfovibrio species, was het mogelijk celvrij extrakt van een reinkweek van het laatstgenoemde organisme te gebruiken als blanko. De meeste enzymen die betrokken zijn bij de succinaatroute, waaronder het sleutelenzym propinyl-CoA:oxaloacetaat transcarboxylase, werden aangetoond in S. wolinii. Dit bevestigt de resultaten die gevonden werden met $^{13}$C-NMR (Hoofdstuk 3).
Verder bleek S. wolinii een lagere groeiopbrengst te hebben dan Desulfobulbus propionicus. Dit verschil wordt verklaard in termen van mechanismen van energiekonservering. Door groeisnelheden van drie syntrofe propionaat-oxiderende cokulturen te vergelijken, bleek dat hydrogenotrofe sulfaat-reduceerders efficiënter zijn dan methanogenen tijdens interspecies waterstof overdracht. De meer negatieve verandering in de Gibbs vrij energie onder sulfidogene omstandigheden ten opzichte van methanogene omstandigheden, wordt verondersteld bij te dragen aan dit verschijnsel.

Het laatste hoofdstuk (7) gaat over het gebruik van $^{13}$C-NMR in een komplex biologisch systeem. Propionaat-afbraak werd gevolgd in mesofiel korrelslib bij 55 °C. Ten gevolge van de non-steady state kondities accumuleerden tijdelijk intermediaire

produkten in het medium. De toevoeging van fumaraat als tweede substraat, stimuleerde propionaat-omzetting. Propionaat en succinaat bleken direkte voorlopers te zijn van elkaar tijdens propionaat-metabolisme. Selektieve labelling van een van de substraten bood de mogelijkheid turnovers van verschillende verbindingen te bestuderen. Eveneens konden op deze manier met elkaar verbonden biochemische processen op een relatief eenvoudige wijze worden bestudeerd.

## SIMPEL GEZEGD

Dit laatste hoofdstuk is een aanhangsel bij mijn proefschrift.
Ik wil hierin voor niet-biologen op een hopelijk duidelijke manier vertellen waar ik me de laatste jaren mee heb bezig-gehouden.

Zolang de mens bestaat is hij bezig geweest zijn omgeving te verkennen. Uit deze nieuwsgierigheid zijn allerlei wetenschappen ontstaan, o.a. de natuurwetenschappen: natuurkunde, scheikunde en biologie.
Dit proefschrift houdt zich bezig met de mikrobiologie, een onderdeel van de biologie.
Het woord biologie stamt uit het Grieks: bios = leven, logos = kennis (de kennis van het leven).
Ieder onderdeel van de biologie houdt zich bezig met een bepaald onderwerp: dieren (mens), planten, schimmels, bakteriën.

Het bestuderen van bakteriën gebeurt door de mikrobiologie (micros = klein). Bakteriën komen overal voor zoals b.v. in voedsel (yoghurt, zuurkool, kaas, wijn, bier), het menselijk lichaam (tandplak, darmen), milieu (afvalwaterzuivering).
Wat is eigenlijk een bakterie?
Een bakterie is voor te stellen als een zak gevuld met water, waarbij de zak te vergelijken is met onze huid. Bakteriën komen in allerlei vormen voor (bollen, staven, kronkels, slierten) en vermenigvuldigen zich door deling 1 ⟶ 2, 2 ⟶ 4, 4 ⟶ 8, enz.
Als U bedenkt, dat in 1 $cm^3$ van de darmen in het menselijk lichaam 10.000.000 bakteriën zitten, is het wel duidelijk dat ze heel erg klein moeten zijn.

Wil ons lichaam kunnen funktioneren, moeten wij mensen eten en drinken. Het voedsel wordt door middel van verschillende processen in onze maag, darmen en uiteindelijk in de lichaams-

cellen, omgezet in koolzuurgas en water. Dit levert ons energie op en bovendien verschillende afvalstoffen.
Een bakterie voedt zich met molekulen; dit zijn de kleinste deeltjes waarin een stof kan voorkomen, b.v. $H_2O$ = water, $O_2$ = zuurstof, $CO_2$ = koolzuur, $CH_4$ = methaan = aardgas, $CH_3COOH$ = acetaat = azijn.
Het verwerken van molekulen door de bakterie gebeurt in verschillende stappen, vergelijkbaar met de spijsvertering bij de mens.
De bakterie waarover in dit proefschrift geschreven is, verwerkt propionaat ($CH_3CH_2COOH$) als voedsel.
De verschillende stappen waarin dit gebeurt, zijn weergegeven in de 1e figuur in hoofdstuk 7. Dit hele schema moet men zich in de bakterie voorstellen. We zien daar dat propionaat via 10 stappen verwerkt wordt tot acetaat. Dit levert energie (= ATP) op, waarvan de bakterie leeft, en tevens afvalstoffen. De afvalstoffen in dit geval zijn aangegeven met [H].
De bakterie moet moeite doen om deze afvalstoffen kwijt te raken. Gelukkig zijn er andere bakteriën (methaanbakteriën), die deze [H] als voedsel nodig hebben; ze maken hier methaan = aardgas van.
Beide bakteriën hebben elkaar dus nodig. Dit maakt het wel moeilijker om de bakterie die propionaat afbreekt, te onderzoeken. Een tweede moeilijkheid is dat deze bakterie niet tegen zuurstof kan, zodat altijd in een zuurstofvrije handschoenenkast en afgesloten flesjes gewerkt moet worden. Toch is het mogelijk om de verschillende stappen in het voedselverwerkingsproces in deze bakterie te bestuderen.
Hiervoor zijn 2 methoden mogelijk.
Bij de eerste methode is gebruik gemaakt van (radioaktief) gemerkt propionaat. Hiermee is het gelukt om succinaat als tussenprodukt en acetaat als eindprodukt aan te tonen (zie 1e figuur in hoofdstuk 7).
Bij de tweede methode kijkt men niet naar de tussenprodukten maar naar de tussenstappen. Als we de figuur bekijken, zien we dat de ene stof gemaakt wordt uit de vorige (propionaat ⟶ X ⟶ X ⟶ X ⟶ succinaat ⟶ fumaraat enz.) Voor iedere

stap (⟶) is een apart stuk gereedschap (= enzym) nodig. De bakterie heeft dus 10 verschillende enzymen nodig om propionaat in 10 stappen tot acetaat te kunnen verwerken. Lukt het nu om de aanwezigheid van alle enzymen in de bakterie aan te tonen, dan kan men aannemen dat de route klopt.

U zult zich afvragen waarom onderzoek gedaan is naar een bakterie die propionaat verwerkt. Propionaat is een belangrijke stof (molekuul) in de afvalwaterzuivering en vormt daar vaak een probleem omdat het (te) langzaam afgebroken wordt.
Dit onderzoek had tot doel een beter inzicht te krijgen in de manier waarop propionaat verwerkende bakteriën funktioneren. Het was niet de bedoeling direkt in de praktijk toepasbaar resultaat te krijgen. Het was dus geen toegepast onderzoek maar fundamenteel onderzoek.

Met veel plezier heb ik dit onderzoek gedaan.

## CURRICULUM VITAE

Frans Houwen werd op 27 februari 1958 geboren te Sevenum. Het diploma Atheneum B behaalde hij in 1977 aan het Peelland College te Deurne. In september van dat jaar begon hij met de studie biologie aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen werd in 1981 afgelegd. Zijn doctoraalfase bevatte als bijvakken Mikrobiologie (Prof. dr. ir. G.D. Vogels; onderzoek aan het methyltransferase systeem in Methanosarcina barkeri) en Biochemie (Prof. dr. H. Bloemendal en dr. A. Berns; kloneren van de preïntegratie-site van het murien leukemie virus) en het hoofdvak Genetika (Prof. dr. W. Hennig en dr. R.C. Brand; screenen op Y-chromosomale sequenties in een cDNA-kloonbank uit Drosophila hydei), en werd in 1985 afgesloten met het afleggen van het doctoraalexamen. In datzelfde jaar is hij begonnen als wetenschappelijk medewerker bij de vakgroep Mikrobiologie van de Landbouwuniversiteit Wageningen in het kader van een door de Stichting BION gefinancierd onderzoek onder leiding van Prof. dr. A.J.B. Zehnder en dr. ir. A.J.M. Stams. Dit proefschrift is hiervan het resultaat.

Tijdens zijn promotieperiode heeft hij deelgenomen aan "The Microbe 86, 14$^{th}$ International Congress of Microbiology" in Manchester (september 1986), "4$^{th}$ European Congress on Biotechnology" in Amsterdam (juni 1987), de workshop "Granular Anaerobic Sludge; Microbiology and Technology" in Lunteren (oktober 1987) en het "FEMS Symposium on Microbiology and Biochemistry of Strict Anaerobes Involved in Interspecific Hydrogen Transfer" in Marseille (september 1989).

Vanaf april 1990 is hij als wetenschappelijk medewerker (post-doc) verbonden aan de afdeling Mikrobiologie van de Landbouwuniversiteit in Uppsala, Zweden (Prof. dr. M.K. Lindberg en dr. B.H. Svensson) waar hij de invloed van ammonium op methaanbakteriën bestudeert.