First publ. in: FEMS Microbiology Letters 55 (1988), 2, pp. 119-124

Thermodynamics of hydrogen metabolism in methanogenic cocultures degrading ethanol or lactate

H.-J. Seitz [1], B. Schink [2] and R. Conrad [1]

[1] *Fakultät für Biologie, Universität Konstanz, Konstanz, F.R.G. and* [2] *Lehrstuhl Mikrobiologie I, Universität Tübingen, Tübingen, F.R.G.*

Key words: Hydrogen partial pressure; Proton reduction; Methanogenesis; Syntrophic coculture; Thermodynamics

1. SUMMARY

Pure cultures of *Desulfovibrio vulgaris* or *Pelobacter acetylenicus* do not grow with lactate or ethanol, respectively, under obligately proton- reducing conditions. However, a small part of these substrates was oxidized and molecular hydrogen was produced up to 4.2 and 3.2 kPa, respectively. During growth in syntrophic methanogenic cocultures with *Methanospirillum hungatei* as partner, maximum hydrogen partial pressures were significantly lower (0.7 to 2.5 kPa) than in the corresponding pure cultures. Calculation of Gibbs free energies for the prevailing culture conditions showed that H_2 partial pressures were kept in a range at which both, H_2-producing and H_2-consuming reactions, were thermodynamically permissive in pure as well as in syntrophic mixed cultures.

Correspondence to: H.-J. Seitz, Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, F.R.G.

2. INTRODUCTION

Anaerobic mineralization of organic matter to CO_2 and methane requires a complex food web of microorganisms [1,2]. Molecular hydrogen is a key intermediate in these degradation processes, and influences H_2-producing as well as hydrogen-consuming bacteria. It has been shown that a minimum H_2 partial pressure is necessary for net H_2 oxidation in pure cultures of hydrogen-utilizing bacteria [3,4]. On the other hand, it is well established by pure culture studies that increased H_2 partial pressures shift the electron flow in fermenting bacteria towards reduced organic compounds [5–7] or even completely inhibit oxidation of certain substrates which can only be oxidized via proton reduction [8–10].

H_2 concentrations permissive for anaerobic degradation of various substrates have been estimated from theoretical calculations [11–14] but have not yet been determined by measurements in defined cultures. We therefore measured H_2 metabolism of physiologically well-characterized fermenting bacteria under obligately proton-reducing conditions in pure culture as well as in coculture with a H_2-utilizing methanogenic bacterium. The data were used to calculate the

Gibbs free energies of the H_2 transformation at various growth phases. Our results show that H_2 partial pressures are being kept in a range at which both, H_2-producing and H_2-consuming reactions were thermodynamically permissive.

3. MATERIALS AND METHODS

3.1. Organisms and cultivation

Desulfovibrio vulgaris strain Marburg, DSM 2119, was kindly provided by Prof. Thauer, Marburg, F.R.G. *Pelobacter acetylenicus* strain WoAcyl, DSM 2348, and *Methanospirillum hungatei* strain M1h, were taken from the culture collection of our laboratory.

Cells were grown at an initial pH of 7.0–7.2 in the mineral medium described [15], except that Na_2S was increased to 1.5 mmol/l. Acetate (2 mmol/l) was added as additional carbon source if hydrogen was the sole electron donor. Prior to inoculation, media were reduced with a few crystals of sodium-dithionite (less than 0.1 mmol/l).

Precultures of hydrogen-consuming bacteria were grown in shaken 120 ml serum bottles under an atmosphere of H_2/CO_2 (80/20 v/v). *P. acetylenicus* was grown on acetoin (10 mmol/l) in 50 ml screw cap bottles without a gas phase. *D. vulgaris* was cultivated similarly on lactate (40 mmol/l) and limiting amounts of sulfate (5 mmol/l). Experiments with pure cultures and defined cocultures were carried out basically as recently described [4]. Purity controls were performed microscopically at the beginning and the end of each experiment.

3.2. Analytical procedures

Methane was measured with a Perkin Elmer gas chromatograph with flame ionization detector [16]. H_2 partial pressures above 100 Pa were analyzed in a gas chromatograph (Carlo Erba) with a thermal conductivity detector. H_2 partial pressures below 100 Pa were measured using a H_2 analyzer based on the HgO-Hg vapour conversion technique [17,18] with a lower detection limit of 0.2 mPa H_2. CO_2 was determined with an infrared analyzer (UNOR, Maihak, Hamburg, F.R.G.). Bicarbonate concentrations were calculated from CO_2 and pH using published equations [19]. Alcohols and volatile fatty acids were assayed by standard gas chromatography procedures with injector and detector temperatures being 130 or 170 °C, oven temperature being 100 or 130 °C, respectively. For analysis of volatile fatty acids, samples were acidified with formic acid to a final concentration of 0.5 mol formic acid/l [16]. Lactate was determined enzymatically using lactate dehydrogenase (Boehringer, Mannheim) according to [20]. Sulfide was determined by the methylene blue method [21]. The concentration of HS^- was calculated from total sulfide and the actual pH, assuming a pKa of 7.0. Sulfate was measured photometrically after precipitation with barium chloride [22, modified after 23].

The standard Gibbs free energies ($\Delta G^{o'}$) of H_2-producing and H_2-utilizing reactions were calculated from the tabulated Gibbs free energies of formation (ΔG_f) of the individual reactants and products [24]. H_2 and CH_4 were assumed as gaseous compounds; all other compounds as dissolved. The Gibbs free energy (ΔG) of a reaction under non-standard conditions was calculated from its standard Gibbs free energy ($\Delta G^{o'}$) and the actual partial pressures or concentrations of the reactants and products involved, the actual temperature and H^+-concentration [25].

4. RESULTS

4.1. H_2-metabolism in pure cultures

Pure cultures of *Desulfovibrio vulgaris* or *Pelobacter acetylenicus* were unable to grow by fermentation of lactate or ethanol, respectively, according to the following equations [24]:

(1) $\text{Ethanol} + H_2O \rightarrow \text{acetate}^- + H^+ + 2\ H_2$

$(\Delta G^{o'} = +9.6\ \text{kJ/mol ethanol})$

(2) $\text{Lactate}^- + 2\ H_2O \rightarrow$

$\text{acetate}^- + H^+ + HCO_3^- + 2\ H_2$

$(\Delta G^{o'} = -4.2\ \text{kJ/mol lactate})$

However, both strains were able to produce hydrogen which accumulated in the culture headspace to partial pressures of up to 3.2 kPa

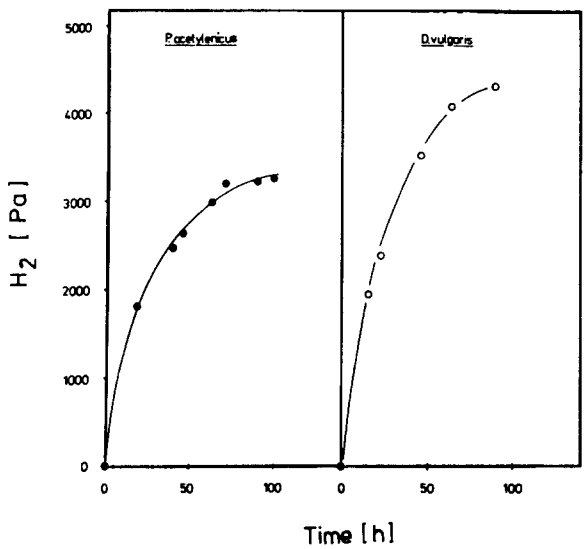

Fig. 1. Production of hydrogen from ethanol (●) or lactate (○) by pure cultures of *Pelobacter acetylenicus* and *Desulfovibrio vulgaris*. The data are mean values of duplicate experiments. The maximum theoretical H_2 partial pressure would be 24000 Pa, if the total amount of organic electron donor added (10 mmol/l) would have been fermented according to the equations given in RESULTS.

Table 1

Gibbs free energies of H_2-producing and H_2-consuming reactions in pure cultures of fermentative and methanogenic bacteria

Organism	Substrate	Maximum or minimum H_2 partial pressure (Pa)	ΔG (kJ/mol)
Desulfovibrio vulgaris	lactate (-sulfate)	4200	−34.6/lactate
Pelobacter acetylenicus	ethanol	3200	− 9.5/ethanol
Methanospirillum hungatei	hydrogen	2.5	−26.0/methane

with ethanol and up to 4.2 kPa with lactate as electron donor (Fig. 1). With both strains, hydrogen evolution ceased before the H_2 partial pressures reached values at which fermentation of lactate or of ethanol would become endergonic (Table 1).

In cell suspensions of *Methanospirillum hungatei* H_2 was oxidized according to the following equa-

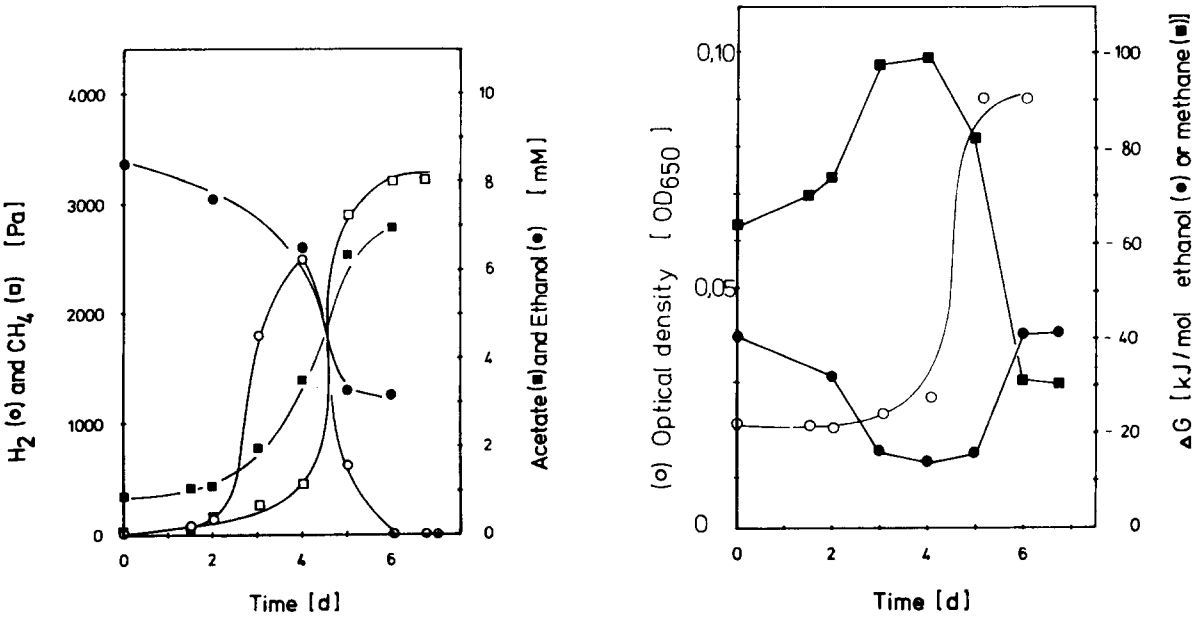

Fig. 2. Fermentation of ethanol by a homogeneously mixed coculture of *Pelobacter acetylenicus* and *Methanospirillum hungatei*: (a) time course of substrate degradation and product formation, (b) change of Gibbs free energies during syntrophic growth.

Table 2

Gibbs free energies of H_2-producing and H_2-consuming reactions in obligately syntrophic cocultures of fermentative and methanogenic bacteria

Coculture	Substrate	H_2 partial pressure (Pa)		ΔG (kJ/mol)
		maximum	minimum	
P. acetylenicus				−13.7/ethanol
+	ethanol	2500	2.8	
M. hungatei				−31.3/methane
D. vulgaris				−34.3/lactate
+	lactate	1800	2.2	
M. hungatei				−25.7/methane

tion [24]:

$$HCO_3^- + H^+ + 4\ H_2 \dashrightarrow CH_4 + 3\ H_2O$$

$$\times \qquad (\Delta G^{o'} = -135.6\ \text{kJ/mol methane})$$

H_2 oxidation stopped at H_2 partial pressures at which H_2-dependent production of methane was still exergonic (Table 1).

4.2. H_2-metabolism in syntrophic cocultures

In syntrophic coculture with the hydrogen-oxidizing *M. hungatei, D. vulgaris* and *P. acetylenicus* were able to grow with lactate or ethanol, respectively (Fig. 2). Hydrogen partial pressures during syntrophic substrate degradation were generally lower than the maximum value of the corresponding hydrogen producing strain. As a consequence, Gibbs free energies (ΔG) of lactate or ethanol fermentation usually were more exergonic in mixed than in pure cultures (Tables 1 and 2).

At the end of the exponential growth phase, hydrogen oxidation continued in the syntrophic culture until a minimum partial pressure was reached. Generally, this H_2 threshold partial pressure was identical to that observed in pure culture of the H_2-utilizing methanogen, and did neither depend on the H_2-producing strain nor on the electron donor (ethanol, lactate) used for H_2 production (Table 2).

5. DISCUSSION

In the absence of external electron acceptors, *Desulfovibrio vulgaris* and *Pelobacter acetylenicus* behaved like typical obligately syntrophic H^+-reducing bacteria. Thus, growth coupled to oxidation of organic substrates like lactate or ethanol was only possible with concomitant hydrogen removal. This reaction can be carried out by hydrogen-oxidizing bacteria in syntrophic coculture [10,26–30]. Recently, effective chemical mechanisms have also been demonstrated [31]. The necessity for H_2 removal can easily be explained by the fact that the H_2-producing reactions are endergonic or only poorly exergonic at standard conditions and pH 7.0, i.e., at a hydrogen partial pressure of approximately 100 kPa. During growth of these strains in syntrophic coculture with *M. hungatei,* H_2 partial pressures were about 40 to 150 times lower (0.7–2.5 kPa) and thus always kept in a range at which hydrogen producers as well as hydrogen consumers could obtain sufficient energy for growth (Fig. 2). This corresponds well with data based on H_2 measurements in various methanogenic environments [25].

While in homogeneously mixed cocultures H_2 partial pressures were always lower than the maximum partial pressures in pure cultures of the fermentative bacteria, this was different when H_2 producers were spatially separated from H_2 consumers [32]. Experiments with membrane-separated cultures recently demonstrated that H_2 partial pressures were dramatically higher in the compartment of the ethanol-fermenting *P. acetylenicus* than in the compartment of the hydrogen-utilizing *Acetobacterium woodii* [32]. Under these conditions H_2 partial pressures were reached which were almost identical to the maximum H_2 partial pressure in pure cultures of *P. acetylenicus,* presented in this study (Fig. 1).

Hydrogen partial pressures observed in syntrophic cocultures and consequently, Gibbs free energies (ΔG) available for H_2-producing and H_2-consuming reactions apparently were dependent on culture conditions and in addition, changed during incubation of the batch cultures. Therefore, it was not possible to correlate the observed Gibbs free energies (ΔG) with growth

yields of the fermenting or the methanogenic bacteria. We are presently establishing syntrophic chemostat cultures to study these relationships. However, our data demonstrate the thermodynamic boundary conditions of H_2-producing and H_2-consuming reactions.

Hydrogen-producing fermenting bacteria such as *P. acetylenicus* or *D. vulgaris* and H_2-consuming methanogenic bacteria such as *M. hungatei* did not metabolize H_2 beyond a particular partial pressure although actual Gibbs free energies (ΔG) would still have been exergonic. Thus, a certain minimum energy appears to be necessary for production or consumption of H_2 in these bacteria. In syntrophic cocultures, H_2 was kept in a range where fermentation as well as methanogenesis were exergonic by at least -13.7 to -34.3 kJ per reaction (Table 2). These Gibbs free energies (ΔG) represent about one to two third of the ΔG (-44 kJ mol^{-1}) necessary for the reversible synthesis of one ATP and thus, are consistent with the hypothesis of Thauer and Morris [33] that one third of ATP is the minimum biological energy quantum. In pure culture, *P. acetylenicus* produced H_2 from ethanol until the energy yield was as low as -9.5 kJ per mol of ethanol. However, ethanol fermentation had already stopped under these conditions.

The minimum Gibbs free energy (ΔG) of ethanol fermentation by *P. acetylenicus* was significantly lower than that of lactate fermentation by *D. vulgaris,* both in pure and in syntrophic mixed cultures. It is presently unknown whether the maximum possible H_2 partial pressure (or minimum ΔG) of fermenting bacteria is species-dependent or is determined by the biochemical degradation pathway of the substrate. Just recently it has been shown that energy is required to produce H_2 during the oxidation of lactate to pyruvate in *D. vulgaris* [34]. In case of H_2-consuming anaerobes, on the other hand, the minimum possible H_2 partial pressure (H_2 threshold) was shown to decrease with increasing redox potential of the electron acceptor used [4].

ACKNOWLEDGEMENTS

The authors are indebted to Prof. Dr. N. Pfennig for many helpful suggestions and for critically reading the manuscript. This study was supported by a grant of the Deutsche Forschungsgemeinschaft (Schwerpunkt 'Methanogene Bakterien').

REFERENCES

[1] Zehnder, A.J.B. (1978) In: Water Pollution Microbiology (R. Mitchell, ed.), pp. 349–376, John Wiley, New York.
[2] Zeikus, J.G. (1983) In: Microbes in their natural environments (J.H. Slater, and J.W. Whittenbury, eds.), pp. 423–462, Cambridge University Press, London.
[3] Lovley, D.R. (1985) Appl. Environ. Microbiol. 49, 1530–1531.
[4] Cord-Ruwisch, R., Seitz, H.J. and Conrad, R. (1988) Arch. Microbiol. 149, 350–357.
[5] Kim, B.H. and Zeikus, J.G. (1985) Dev. Ind. Microbiol. 26, 549–556.
[6] Zeikus, J.G. (1980) Ann. Rev. Microbiol. 34, 423–464.
[7] Ianotti, E.L., Kafkewitz, D., Wolin, M.J. and Bryant, M.P. (1973) J. Bacteriol. 114, 1231–1240.
[8] Johns, A.T. and Barker, H.A. (1960) J. Bacteriol. 80, 837–841.
[9] Ahring, B.K. and Westermann, P. (1987) Appl. Environ. Microbiol. 53, 429–433.
[10] Bryant, M.P., Wolin, E.A., Wolin, M.J. and Wolfe, R.S. (1967) Arch. Microbiol. 59, 20–31.
[11] Wolin, M.J. (1976) In: Symposium on microbial production and utilization of gases (H.G. Schlegel, G. Gottschalk, and N. Pfennig, eds.), pp. 141–150, E. Goltze, Göttingen.
[12] McInerney, M.J. and Bryant, M.P. (1980) In: Anaerobes and anaerobic infections (G. Gottschalk, ed.), pp. 117–126, Gustav Fischer Verlag, Stuttgart and New York.
[13] Speece, R.E. (1983) Environ. Sci. Technol. 17, 416A–427A.
[14] Harper, S.R. and Pohland, F.G. (1986) Biotechnol. Bioeng. 28, 585–602.
[15] Widdel, F. and Pfennig, N. (1981) Arch. Microbiol. 129, 395–400.
[16] Schink, B. and Pfennig, N. (1982) Arch. Microbiol. 133, 195–201.
[17] Seiler, W. (1978) In: Environmental Biogeochemistry and Geomicrobiology Vol. 3, (W.E. Krumbein, ed.), pp. 773–810. Ann Arbor Science Publishers, Ann Arbor, Michigan.
[18] Seiler, W., Giehl, H. and Roggendorf, P. (1980) Atmos. Technol. 12, 40–45.
[19] Stumm, W. and Morgan, J.J. (1981) Aquatic Chemistry, 2nd ed. John Wiley, New York.
[20] Bergmeyer, H.U. (Hsg) (1977) Grundlagen der enzymatischen Analyse, 1 Auflage. Verlag Chemie.
[21] Cline, E. (1969) Limnol. Oceanogr. 14, 454–458.
[22] Tabatabai, M.A. (1974) Sulphur Inst. J. 10, 11–13.
[23] Cypionka, H. and Pfennig, N. (1986) Arch. Microbiol. 143, 396–399.

[24] Thauer, R.K., Jungermann, K. and Decker, K. (1977) Bact. Rev. 41, 100–180.
[25] Conrad, R., Schink, B. and Phelps, T.J. (1987) FEMS Microb. Ecol. 38, 353–360.
[26] Bryant, M.P., Campbell, L.L., Reddy, C.A. and Crabill, M.R. (1977) Appl. Environ. Microbiol. 33, 1162–1169.
[27] McInerney, M.J. and Bryant, M.P. (1981) Appl. Environ. Microbiol. 41, 346–354.
[28] McInerney, M.J., Bryant, M.P., Hespell, R.B. and Costerton, J.W. (1981) Appl. Environ. Microbiol. 41, 1029–1039.
[29] Boone, D.R. and Bryant, M.P. (1980) Appl. Environ. Microbiol. 40, 626–632.
[30] Schink, B. (1985) Arch. Microbiol. 142, 295–301.
[31] Kaspar, H.F., Holland, A.J. and Mountfort, D.O. (1987) Arch. Microbiol. 147, 334–339.
[32] Stieb, M. and Schink, B. (1987) FEMS Microbiol. Ecol. 45, 71–76.
[33] Thauer, R.K. and Morris, J.G. (1984) Symp. Soc. Gen. Microbiol. 36, 123–168.
[34] Pankhania, I.P., Spormann, A.M., Hamilton, W.A. and Thauer, R.K. (1988) Arch. Microbiol. 150, 26–31.