The Influence of Extracellular Hydrogen on the Metabolism of *Bacteroides ruminicola*, *Anaerovibrio lipolytica* and *Selenomonas ruminantium*

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(Received 3 September 1979; revised 25 February 1980)

Strains of three anaerobic rumen bacteria, *Bacteroides ruminicola*, *Anaerovibrio lipolytica* and *Selenomonas ruminantium*, were able to use extracellular H₂ to reduce fumarate to succinate. Each bacterium possessed membrane-bound hydrogenase and fumarate reductase activity. Membrane-bound cytochrome *b* was reducible by H₂ and oxidizable by fumarate in each bacterium. The apparent *K_m* values for hydrogen of the hydrogenases were 4.5×10^{-6} M, 1.4×10^{-5} M and 4.4×10^{-5} M for *B. ruminicola*, *A. lipolytica* and *S. ruminantium*, respectively. The apparent *K_m* values for fumarate of the fumarate reductases were approximately 1.0×10^{-4} M for each bacterium.

INTRODUCTION

When inhibitors of rumen methanogenic bacteria are fed to ruminants the resulting depression of methane production is accompanied by an accumulation of H₂ and an increase in the proportion of propionic acid relative to acetic acid in the rumen (Demeyer & Van Nevel, 1975). It may be that the accumulating H₂ so decreases growth of those rumen bacteria which form acetate as their main product (Hungate, 1966) as to allow the propionate-producing bacteria to compete more successfully for fermentable substrate. Alternatively, the H₂ produced by the acetate-producing bacteria might be used directly by the propionate producers to increase the proportion of pyruvate which is reduced to propionate and thus increase the ratio of propionate to acetate produced.

In the present study several important propionate- and succinate-producing rumen bacteria were tested for their ability to utilize extracellular H₂ as a source of reducing power. Such activity has been shown to be the main source of energy for the non-fermentative rumen bacterium *Vibrio succinogenes* which requires an exogenous supply of H₂ (or formate) and fumarate (Wolin *et al.*, 1961). The reduction of fumarate to succinate by *V. succinogenes* is accompanied by electron transport-linked phosphorylation (Reddy & Peck, 1978).

METHODS

Anaerovibrio lipolytica 5S was the strain of Hobson (1965); it was grown on the medium described by Henderson (1971) with fructose as energy source. *Selenomonas ruminantium* strains 17 and WPL151/1 were isolated at the Rowett Research Institute as described by Hobson & Mann (1961); they were grown on medium H5 which was based on medium 1 of Kurihara *et al.* (1968) with rumen fluid replaced by distilled

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water and with 0.6% (w/v) glucose as the sole energy source. *Bacteroides ruminicola* 46/52 was the strain of Lysons *et al.* (1971) and *B. ruminicola* strains M3/12, M3/17 and 41/21 were isolated by Mr S. O. Mann at the Rowett Research Institute; these bacteria were grown on medium H5. *Megasphaera elsdenii* type 2 was the Large Coccus type 2 described by Hobson *et al.* (1958); it was grown on medium H5 or H5 with glucose replaced by 0.7% (w/v) sodium lactate.

Bacteria were grown anaerobically in batch cultures in vessels designed, as described by Jarvis *et al.* (1978), for direct turbidimetric measurements. The cultures were shaken in a New Brunswick G24 Environmental Incubator Shaker at 200 rev. min⁻¹ at 38 °C. Growth, under atmospheres of either O₂-free CO₂ or O₂-free H₂/CO₂ (80:20, v/v) was monitored until the end of exponential growth when samples were removed for measurement of bacterial dry weight, lactate and succinate concentrations and utilization of substrate (Henderson, 1975). Volatile fatty acids were assayed by gas-liquid chromatography (Fell *et al.*, 1968), concentrations being deduced from peak areas by comparison with standard curves.

Washed cell suspensions of bacteria were prepared under O₂-free N₂ atmospheres by centrifugation of large batch cultures (400 ml) and washing with 0.1 M-phosphate buffer pH 7.2 made anaerobic by flushing with O₂-free N₂. Broken cell suspensions were prepared anaerobically at 5 °C from washed bacteria by ultrasonic disruption using the Dawe Soniprobe (Dawe Instruments, London) set at maximum output (8 × 30 s bursts with 30 s cooling pauses). Unbroken bacteria were removed by centrifugation at 5000 *g* for 20 min. Membrane preparations were made from the broken cell suspensions by centrifugation at 100000 *g* for 120 min. The membrane pellet was resuspended in the original volume of 0.1 M-phosphate buffer pH 7.2. All preparations were stored under O₂-free N₂ at -60 °C.

Hydrogenase activity was assayed spectrophotometrically by measuring the increase in absorbance at 546 nm caused by the reduction of benzyl viologen ($\epsilon = 9750 \text{ l mol}^{-1} \text{ cm}^{-1}$). The reaction mixture, in a cuvette of 1 cm optical pathlength, consisted of 0.1 M-phosphate buffer pH 7.2, 1.66 mM-benzyl viologen and 0.05 ml enzyme preparation. The phosphate buffer used was made anaerobic by sparging with O₂-free N₂. Hydrogen was added as a saturated solution in 0.1 M-phosphate buffer pH 7.2; 2.0 ml of this solution were injected through a rubber stopper into the reaction mixture in the cuvette. Formate dehydrogenase was assayed by a similar method, with 1 mM-sodium formate in 0.1 M-phosphate buffer pH 7.2 replacing the H₂ solution. The final volumes in these assays were 3.0 ml. These methods were adapted from the formate dehydrogenase assay of Kröger & Innerhofer (1976). The fumarate reductase assay was adapted from that of Kröger & Innerhofer (1976). The reaction mixture (final volume 3.0 ml) consisted of 0.1 M-phosphate buffer pH 7.2, 0.166 mM-benzyl viologen, 0.166 mM-sodium dithionite and 0.05 ml enzyme preparation and the reaction was started by adding 6.66 mM-fumarate. Strict precautions were taken to ensure absence of O₂ and the gas phase was O₂-free N₂.

Cytochrome *b* was determined from reduced *minus* oxidized difference spectra (De Vries *et al.*, 1974), using the Cary 15 spectrophotometer (Cary Instruments, Calif., U.S.A.).

RESULTS AND DISCUSSION

Table 1 shows the effect of H₂/CO₂ (80:20, v/v) gas phase on the fermentation products of the bacteria grown in shaken batch cultures. In each strain of *B. ruminicola*, adding H₂ increased the proportion of succinate formed by fermentation of glucose and decreased the proportion of lactate. A small drop in the proportion of acetate formed was usually observed, but this was less consistent than the other changes. No significant changes in molar growth yield ($Y_{\text{substrate}}$) were observed.

With *A. lipolytica*, adding H₂ increased the proportions of propionate and succinate relative to acetate when fructose was fermented. The increase in molar growth yield in the presence of H₂ may have been due mainly to an observed increase in the growth rate of the bacteria in these cultures which would mean relatively less energy was expended on maintenance (Pirt, 1965).

The two strains of *S. ruminantium* had different fermentation patterns under CO₂ and reacted differently to the addition of H₂. *Selenomonas ruminantium* strain 17 produced no lactate, and under H₂/CO₂ atmospheres propionate production was stimulated at the expense of acetate production with only slight increases in succinate. *Selenomonas ruminantium* strain WPL151/1 produced lactate as a major product, and under H₂/CO₂ atmospheres propionate and succinate production increased at the expense of lactate. No significant effects on molar growth yields were recorded. The lack of

Table 1. Effect of extracellular H_2 on the fermentation products of rumen bacteria: *Bacteroides ruminicola*, *Anaerovibrio lipolytica*, *Selenomonas ruminantium* and *Megasphaera elsdenii*

Fermentation products are expressed as mol product (mol substrate fermented) ⁻¹ and molar growth yields ($Y_{\text{substrate}}$) as g bacterial dry wt (mol substrate fermented) ⁻¹ .										
Organism	Substrate*	Gas phase†	Acetate	Succinate	Lactate	$Y_{\text{substrate}}$				
<i>B. ruminicola</i> 46/52	Glc	CO ₂	0.09	0.16	1.34	33.9				
		H ₂ /CO ₂	0.05	0.46	0.94	32.2				
<i>B. ruminicola</i> M3/12	Glc	CO ₂	0.21	0.12	1.65	36.6				
		H ₂ /CO ₂	0.10	0.47	1.43	35.5				
<i>B. ruminicola</i> M3/17	Glc	CO ₂	0.11	0.19	1.70	46.0				
		H ₂ /CO ₂	0.15	0.45	1.41	40.5				
<i>B. ruminicola</i> 41/21	Glc	CO ₂	0.14	0.27	1.59	39.1				
		H ₂ /CO ₂	0.10	0.50	1.40	40.7				
<i>A. lipolytica</i> 5S	Fru	CO ₂	Acetate	Propionate	Succinate	Lactate	$Y_{\text{substrate}}$			
		H ₂ /CO ₂	0.66	0.63	0.13	0	51.6			
			0.49	1.20	0.20	0	58.9			
<i>S. ruminantium</i> 17	Glc	CO ₂	Acetate	Propionate	Succinate	Lactate	$Y_{\text{substrate}}$			
		H ₂ /CO ₂	0.53	0.79	0.22	0	48.5			
		CO ₂	0.31	1.07	0.25	0	46.4			
<i>S. ruminantium</i> WPL151/1	Glc	CO ₂	0.27	0.31	0.01	1.20	42.8			
		H ₂ /CO ₂	0.25	0.45	0.32	0.89	39.7			
<i>M. elsdenii</i>	Glc	CO ₂	Acetate	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate	Caproate	$Y_{\text{substrate}}$
		H ₂ /CO ₂	0.29	0.11	0.32	0.05	0.03	0.22	0.12	48.0
		CO ₂	0.35	0.19	0.36	0	0.02	0.11	0.16	50.77
<i>M. elsdenii</i>	Lac	CO ₂	0.42	0.33	0.12	0	0.13	0	0	12.0
		H ₂ /CO ₂	0.39	0.40	0.05	0	0.05	0	0	10.9

* Glucose (Glc), fructose (Fru) or Lactate (Lac), each at 6 g l⁻¹.

† In each experiment, H₂/CO₂ was 80:20 (v/v).

Table 2. *Hydrogenase activity and substrate affinity in whole cells and cell fractions of Bacteroides ruminicola, Anaerovibrio lipolytica and Selenomonas ruminantium*

Organism	Activity [μmol benzyl viologen reduced min^{-1} (g bacterial dry wt) $^{-1}$]		Activity after high-speed centrifugation [μmol benzyl viologen reduced min^{-1} (g protein) $^{-1}$]	
	Whole cells	Broken cell suspension	Pellet	Supernatant
<i>B. ruminicola</i>	54 (4.5×10^{-6})*	73 (6.0×10^{-6})	184	0
<i>A. lipolytica</i>	40 (1.4×10^{-5})	45 (ND)	67	0
<i>S. ruminantium</i>	105 (4.4×10^{-5})	130 (4.8×10^{-5})	227	0

ND, Not determined.

* Values in parentheses show apparent K_m for H_2 (M).

significant changes in growth yields with these changes in fermentation products was unexpected. Hobson & Summers (1972) suggested that propionate production would yield more ATP than acetate production and it was also assumed that propionate and succinate production would yield more ATP than lactate production.

Megasphaera elsdenii showed small increases in the proportion of propionate under H_2/CO_2 atmospheres, but these were not large enough to require the net uptake of extra-cellular H_2 and could have been achieved by use of H_2 formed in association with acetate production. Similar results were obtained by Van Nevel *et al.* (1974) with *M. elsdenii* grown under H_2 atmospheres.

The bacteria selected for further study were *B. ruminicola* strain 46/52, *A. lipolytica* strain 5S and *S. ruminantium* strain WPL151/1. Table 2 shows the hydrogenase activities associated with whole cells or cell fractions of these three bacteria. In each case there was some increase in the hydrogenase activity of cells following ultrasonic disruption. Also, in each case the activity was found entirely in the membrane pellet sedimented at 100000 g. In each bacterium the hydrogenase had a relatively broad pH optimum between pH 7.0 and 8.0; below pH 7.0 the activity decreased rapidly. To determine the apparent K_m for H_2 , a phosphate buffer solution was saturated with H_2 gas at 20 °C and atmospheric pressure and then portions of this solution were added to the assay mixture in anaerobic cuvettes. The saturated H_2 solution was calculated to contain 0.8 mM- H_2 (*Handbook of Chemistry and Physics*; CRC Press) which was very close to values determined by Erbes & Burris (1978). Table 2 shows that values obtained for the apparent K_m for H_2 did not vary significantly between whole bacteria and broken cell suspensions of the bacteria tested. In none of these bacteria could formate dehydrogenase activity be detected.

Hungate *et al.* (1970) reported a value of 1×10^{-6} M for the apparent K_m for H_2 of the hydrogenase of the rumen bacterium *Methanobacterium ruminantium*. In *B. ruminicola* the K_m was four to six times higher than that of *M. ruminantium*, while those of *A. lipolytica* and *S. ruminantium* were, respectively, 10 and 40 times higher. It follows that in competition for H_2 in the rumen, the methanogenic bacteria have an advantage over the propionate/succinate-producers studied here. If, however, methanogenesis were selectively inhibited by chemical additives, the hydrogenases of the propionate/succinate-producers should be capable of maintaining a very low H_2 tension, provided a sufficiency of electron acceptors were present.

As with the hydrogenase activities, the fumarate reductases of the three bacteria studied were associated with the membrane pellet (Table 3). Disruption of the cells resulted in two- to threefold increases in the activity of fumarate reductase. Addition of Triton X-100 (2 %, v/v) to the assays did not affect the fumarate reductase activity of the whole bacteria.

Table 3. *Fumarate reductase activity and substrate affinity in whole cells and cell fractions of Bacteroides ruminicola, Anaerovibrio lipolytica and Selenomonas ruminantium*

Organism	Activity [μmol benzyl viologen oxidized min^{-1} (g bacterial dry wt) $^{-1}$]		Activity after high-speed centrifugation [μmol benzyl viologen oxidized min^{-1} (g protein) $^{-1}$]	
	Whole cells	Broken cell suspension	Pellet	Supernatant
<i>B. ruminicola</i>	423 (1.5×10^{-4})*	800 (0.9×10^{-4})	1757	0
<i>A. lipolytica</i>	817 (1.7×10^{-4})	2600 (1.7×10^{-4})	2990	0
<i>S. ruminantium</i>	347 (1.8×10^{-4})	1100 (0.8×10^{-4})	2377	0

* Values in parentheses show apparent K_m for fumarate (M).

Fig. 1. Hydrogen-reduced minus air-oxidized difference spectra of ultrasonically disrupted *Bacteroides ruminicola*, *Anaerovibrio lipolytica* and *Selenomonas ruminantium*. Suspensions contained 10.5, 8.1 and 9.7 mg dry wt bacteria ml^{-1} , respectively.

The values obtained for the apparent K_m for fumarate (Table 3) were lower than values normally expected for the concentration of intracellular intermediates of the Krebs Cycle (Lowry *et al.*, 1971). It is possible, however, that the anaerobic bacteria used here, having an incomplete Krebs Cycle, could have difficulties in maintaining fumarate concentrations at this level.

There was very little difference between the K_m values for fumarate reductases measured using whole cells or broken cell suspensions. This contrasted with the fivefold decrease in K_m reported by Kröger (1977) for the fumarate reductase of *Vibrio succinogenes* following disruption of the bacteria. The increase in $V_{\max}$ for fumarate reductase in broken cell suspensions, however, supports the belief that in the bacteria studied here the transport of fumarate across intact membranes was limiting the reductase reaction.

Cytochrome *b* was present in each of the three bacteria and was shown to be associated with the membrane pellet. The amounts of cytochrome *b* were estimated to be 0.13, 0.24 and 0.10 μmol (g bacterial dry wt) $^{-1}$ in *B. ruminicola*, *A. lipolytica* and *S. ruminantium*, respectively.

When H_2 gas was bubbled into spectrophotometer cuvettes containing 2.5 ml of broken cell suspensions of these bacteria the cytochrome *b* was reduced to give the difference spectra shown in Fig. 1. The reduction obtained in each case was equal to that achieved by adding excess sodium dithionite. Reduction of cytochrome *b* was also obtained by adding 2.5 mM-NADH to broken cell suspensions but in each case the steady-state reduction

Table 4. Reduction of cytochrome *b* by H_2 and NADH and re-oxidation by fumarate, in *Bacteroides ruminicola*, *Anaerovibrio lipolytica* and *Selenomonas ruminantium*

The reduction of cytochrome *b* in broken cell suspensions was determined (i) after adding excess H_2 , (ii) after adding excess NADH (2.5 mM) and (iii) after adding excess fumarate (2.5 mM) to a preparation which had been fully reduced by H_2 . In each case, the percentage reduction of cytochrome *b* is expressed relative to the reduction caused by excess sodium dithionite.

Organism	Percentage reduction of cytochrome <i>b</i>		
	H_2	NADH	H_2 /fumarate
<i>B. ruminicola</i>	100	86	13
<i>A. lipolytica</i>	100	79	48
<i>S. ruminantium</i>	100	73	40

of cytochrome *b* was less than 100 % (Table 4). A substantial re-oxidation of the cytochrome *b* was obtained when excess sodium fumarate was added to the H_2 -reduced cell suspensions (Table 4).

The differences in the extent to which cytochrome *b* was reduced by H_2 and NADH suggest that there may be an extra cytochrome *b* component in the pathway for H_2 oxidation. Enoch & Lester (1975) found that the purified formate dehydrogenase of *Escherichia coli* contained a *b*-type cytochrome and Kröger & Innerhofer (1976) suggested that a similar association existed in *Vibrio succinogenes*. Preliminary studies on *B. ruminicola* and *S. ruminantium* using 2-heptyl-4-hydroxyquinoline *N*-oxide as an electron transport inhibitor also indicate the possibility of two cytochromes *b* in the chain linking H_2 oxidation with fumarate reduction, but further studies are required to clarify this. It is possible that in the ruminant animal where methanogenesis has been decreased by a specific inhibitor, the propionate/succinate-producing bacteria may be able to use H_2 diverted from methanogenesis and so prevent an excessive increase in H_2 tension. Should H_2 accumulate the acetate/ H_2 -producing bacteria (e.g. *Ruminococcus albus*) would ferment hexoses to acetate and ethanol with a loss of potential ATP production (Thauer *et al.*, 1977). The consequent loss of bacterial production may be responsible for the reported inhibitory effect of high H_2 concentrations on cellulolytic bacteria (Chung, 1976).

To ensure that the propionate/succinate-producing bacteria could utilize H_2 to their full potential the animals diet would have to contain a proportion of easily fermented carbohydrate to provide substrates for the bacteria. The formation of propionate rather than methane as the major reduced end-product of rumen fermentation would have a beneficial effect on the utilization of dietary energy by the animal.

I should like to thank Miss Shona Neil for skilled technical assistance during this study.

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