Influence of Hydrogen on the Fermentation in Rumen Protozoa, *Entodinium* Species

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Abstract The influence of H_2 on the fermentation of starch by rumen protozoa, mixture of *Entodinium* species, was studied in cultures *in vitro*. An experiment in which H_2 was continuously flown into cultures to provide the high partial pressure of H_2 , showed that the fermentation activity of entodinia is lowered by H_2 : Starch consumption and organic acid production were decreased. Simultaneously, a shift in the pattern of fermentation products was observed; acetate was decreased greatly, while butyrate was altered little, resulting in the decreased percentage of acetate and increased percentage of butyrate. In another experiment the co-existence of methanogenic bacteria, which were co-cultured to remove H_2 produced by entodinia, was found to enhance the fermentation activity of entodinia: Starch consumption, together with organic acid production, were increased. A great increase in acetate and little change in butyrate were also seen. Enhancement of H_2 production was shown, which could possibly be the reason for stimulated acetate production. A marked increase in the assumed ATP yield from fermentation was also noted. However, on the whole, the influence of the partial pressure of H_2 was not so great as expected. Implications of these results are discussed in detail.

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In order to understand the role played by protozoa in the metabolism that occurs in the rumen, especially in energy metabolism, *i.e.*, the energetic aspect of microbial cell synthesis and volatile fatty acid production, it may be of importance to have basic knowledge and adequate information on the fermentation process and growth rate of protozoa, and the factors affecting them.

In anaerobic organisms and facultative microbes growing anaerobically, it is generally believed that the rate of growth is primarily limited by the fermentation process, *i.e.*, the process of obtaining energy by means of oxidizing substrates¹⁾. In these organisms, fermentation activity is decisively affected by the readiness to dispose of electrons liberated from substrate oxidation. One of the most important modes of disposing of excess electrons has been known to be the formation of H_2 : Hydrogen is a common end product of carbohydrate fermentation in many bacterial^{2,3)} and several protozoal⁴⁻⁶⁾ species, including rumen microbes⁷⁾.

Some different mechanisms by which H_2 is formed have been known in different types of organisms, and the enzyme hydrogenase, which catalyzes H_2 formation, has been at least partially characterized in several species^{3,8-10)}. In several bacterial species, it has been shown that H_2 production is influenced by the partial pressure of H_2 , and stimulated by growth with H_2 -consuming organisms^{2,11)}. Furthermore, in

several species of H_2 -producing rumen bacteria, the shift in the pattern of fermentation by co-existence of a H_2 -consuming organism has been demonstrated¹²⁻¹⁵. Recently, a new example of intermicrobial H_2 transfer has been reported¹⁶: A rumen anaerobic fungus, which is an eukaryote, shifts the pattern of fermentation, when co-cultured with methanogens. The importance of the intermicrobial transfer of H_2 has been emphasized in relation to the fermentation in the rumen¹¹⁻¹⁶.

Rumen protozoa form H_2 as a major 'electron sink product', a product formed by accepting electrons⁷: Hydrogen formation is a substantial way of disposing of excess electrons. Whether this mechanism functions smoothly or not might greatly influence the fermentation activity, and consequently the growth rate.

Recently, VOGELS *et al.* have reported the close association of methanogenic bacteria with rumen entodiniomorphid protozoa, and have suggested that this enables an effective intermicrobial H_2 transfer by which both can grow faster¹⁷.

This paper reports the influence of H_2 on the fermentation in *Entodinium* species: In Experiment 1, the influence of H_2 flown into the cultures to provide the high partial pressure of H_2 was examined, being compared with the control in which N_2 was flown. In Experiment 2, the effect of methanogenic bacteria, which were co-cultured to remove H_2 produced by the protozoa, was investigated.

Materials and Methods

1. Collection and preparation of entodinia.

A Japanese native goat (*ca.* 25 kg) fitted with a permanent rumen cannula was used to collect protozoa. In order to obtain only *Entodinium* species, all other types of protozoa were removed from the rumen as follows: The goat was starved for 2 days, and then fed only commercial formula feed for cows for 5 days. During these 5 days 500 ml/day of 1% dioctyl sodium sulphosuccinate solution^{18,19} was administered into the rumen through a cannula. At the end of this purification period, only entodinia were detected in an extremely small number. Subsequently, diet was changed to a normal one consisting of alfalfa hay, green forage, and formula feed. Only entodinia, mainly *E. caudatum*, *E. simplex*, and *E. longinucleatum*, were seen to increase to a normal population density (*ca.* 10^6 /ml) in 2 weeks. These protozoa were collected and prepared as described previously²⁰.

2. Procedures and conditions of the culture of entodinia.

To prevent the contaminating bacteria from proliferating, washed protozoa were preincubated in buffered saline²¹ for 16 hr with a high concentration of antibiotics; 2,000 U/ml of penicillin G, 200 μ g/ml each of aminobenzylpenicillin, cephaloridin, streptomycin, and kanamycin.

In Experiment 1, these protozoa were cultured for 48 hr with 500 U/ml of penicillin G and 50 μ g/ml of cephaloridin, both of which can be considered to have no harmful effect on protozoa directly. Incubation was carried out under continuous bubbling with H_2 or N_2 using an apparatus illustrated in Fig. 1. In Experiment 2, protozoa treated as above were cultured for 48 hr with or without methanogenic

Fig. 1. Apparatus for the culture of entodinia used in Experiment 1. A: culture fluid (100 ml), B: 5% pyrogallol in 10% Na_2CO_3 , f: glass filter.

bacteria. Culturing was done shaking slowly in 300 ml round-bottom flasks containing 100 ml of culture fluid. Initial gas phase was O_2 -free N_2 . The method of collecting the gas produced, and other basic culture conditions have been described previously²². Entodinia inoculated in both experiments were approximately $2 \times 10^4/\text{ml}$ of culture fluid, and the initial number of methanogenic bacteria was nearly $10^8/\text{ml}$ of culture fluid.

3. Media used for the culture of entodinia.

The media used in Experiments 1 and 2 are shown in Table 1. Extracts of rumen bacteria were prepared as follows: One packed cell volume of mixed bacteria collected from the rumen was suspended in 4 volumes of buffered saline, and disrupted by ultra-sonication ($2\sim 3^\circ\text{C}$, 3 min). The homogenate was then centrifuged (20,000 g,

Table 1. Composition of the culture media for entodinia

	Medium 1	Medium 2
Buffered saline	85 ml	55 ml
Extracts of rumen bacteria	5 ml	5 ml
Suspension of heated <i>E. coli</i>	5 ml	5 ml
Trace mineral and vitamin solution	5 ml	5 ml
Methanogen cell suspension		30 ml
Starch particles with 0.1% β -sitosterol	0.5 g	0.5 g

15 min), and the supernatant was filtered through membrane filter (pore size, 0.45μ). The filtrate, which was stored at -80°C until needed, was used as the extracts. *Escherichia coli* cultured in nutrient broth²³⁾ and harvested at full growth was used as a protein source, after being heated at 80°C for 10 min. Beta-sitosterol which had been adsorbed to starch particles²⁴⁾ was added to the media. Trace mineral and vitamin solution has already been reported²⁵⁾. Methanogen cell suspensions were prepared as described below. Intact cells were used for co-cultures, and the heated (80°C , 10 min) ones were used for control, *i.e.*, mono-cultures.

4. Sources and culture of methanogenic bacteria.

Methanobrevibacterium ruminantium was kindly supplied by Dr. M. P. BRYANT, Department of Dairy Science, University of Illinois, and *Methanobacterium formicium* was generously given by the Laboratory of Microbiology and Fermentation, this department.

These bacteria were grown under O_2 -free H_2 - CO_2 (80:20) gas mixture in 30 ml of medium using butylrubber double-capped bottles (100 ml). The medium used for the culture of *M. ruminantium* was prepared according to SCHEIFINGER *et al.*¹³⁾ and LATHAM and WOLIN¹⁴⁾, slightly modified to consist of: Trypticase (BBL), 2 g; yeast extract (Difco), 2 g; Na-formate, 0.1 g; Na-acetate, 0.5 g; buffered saline, 200 ml; clarified rumen fluid, 400 ml; H_2O , 400 ml; cysteine-HCl, 0.5 g; and resazurin, 1 mg. Ten percent Na_2CO_3 solution was added to adjust the pH to 7.0. The medium for *M. formicium* was essentially the same as 'Medium 1' described by BALCH *et al.*²⁶⁾.

The culture mixture of each methanogen at its maximum growth was directly added to the culture medium for entodinia.

5. Analytical methods.

Formate and L-lactate were determined by the formate dehydrogenase²⁶⁾ and the lactate dehydrogenase²⁷⁾ methods, respectively. Acetate, propionate, and butyrate were determined by gas chromatography (GC) as described previously²²⁾. These organic acids were determined before and after incubation, and the difference between the two values of each acid was assumed to be the quantity produced.

Gas analysis was carried out by GC to measure H_2 and CH_4 produced, using C_2H_6 as an internal standard: At the end of incubation an aliquot of C_2H_6 was injected into the culture vessels, and after being thoroughly mixed²²⁾, an aliquot of the gas mixture was taken as a sample. GC was conducted at 30°C , using CO_2 as a carrier gas²²⁾.

Starch was determined as follows: Aliquots of the culture mixture, both before and after incubation, were taken and centrifuged (500 g, 5 min). The precipitate including starch particles and protozoal cells was hydrolyzed with 1 N HCl (90°C , 2 hr), and the liberated glucose was determined by the glucose oxidase method²⁸⁾.

The recovery of metabolic hydrogen, and the quantities of fermented hexose and ATP yield were calculated according to the procedures and assumptions described previously²²⁾.

Results

1) Influence of the high partial pressure of H_2 on the fermentation in entodinia.

Table 2. Fermentation of starch by entodinia under a flow of H₂ or N₂^{a)}

	N ₂	H ₂
	μ moles/vessel	μ moles/vessel
Starch consumed	376 $\pm$ 12	322 $\pm$ 8*
Organic acids produced	504 $\pm$ 16	444 $\pm$ 12*
Formate	11 $\pm$ 1 (2.2 $\pm$ 0.1)	9 $\pm$ 1 (2.0 $\pm$ 0.1)
Acetate	274 $\pm$ 12 (54.8 $\pm$ 0.9)	212 $\pm$ 8* (47.7 $\pm$ 0.4**)
Propionate	16 $\pm$ 1 (3.2 $\pm$ 0.1)	25 $\pm$ 2* (5.5 $\pm$ 0.1**)
Butyrate	182 $\pm$ 11 (36.1 $\pm$ 0.5)	172 $\pm$ 9 (38.7 $\pm$ 0.5*)
Lactate	19 $\pm$ 2 (3.8 $\pm$ 0.1)	26 $\pm$ 2 (5.9 $\pm$ 0.1**)
Hexose fermented	338 $\pm$ 11	304 $\pm$ 9
Assumed ATP yield	1,165 $\pm$ 27	1,041 $\pm$ 23*
ATP/hexose ^{b)}	3.45 $\pm$ 0.03	3.42 $\pm$ 0.02

a) The figures in the parentheses are percentages of the total amount. (means $\pm$ S.E., n=3) *: P < 0.05, **: P < 0.01. b) Assumed ATP yield/hexose fermented.

The data obtained in Experiment 1 are summarized in Table 2. When entodinia were constantly exposed to H₂, starch consumption was found to be slightly but significantly (P < 0.05) lower than when the protozoa were incubated under a flow of N₂. The total amount of organic acids produced was smaller, mainly due to the decrease in acetate production. Production of butyrate, another major product, was altered so little that the percentage of butyrate was shown to have increased.

Calculations based on the product values show that fermented hexose was slightly decreased by bubbling with H₂, but this is statistically insignificant (P < 0.05). Assumed ATP yield was significantly (P < 0.05) decreased by H₂. No significant difference was shown for ATP yield per fermented hexose.

2) Effect of the co-existence of methanogenic bacteria on the fermentation in entodinia.

Table 3 shows the results of Experiment 2 obtained using *M. ruminantium* as a H₂-consumer. Entodinia in the co-culture consumed significantly (P < 0.05) larger amount of starch. Simultaneously, in the co-culture an obvious increase in the total amount of organic acids was found: Acetate production remarkably increased, while butyrate production did not significantly do so. This caused a decrease in butyrate, expressed as a percentage. Formate was not detected in the co-culture, probably as a result of its consumption by the methanogen. Production of propionate and lactate was lower in the co-culture.

Calculation of the total H₂ produced by the protozoa, on the assumption that 1 mole of CH₄ is formed from 4 moles of H₂, showed that entodinia in the co-culture produced markedly larger amount of H₂. Significant increases in fermented hexose (P < 0.05) and assumed ATP yield (P < 0.01) were also shown.

Essentially the same results were obtained when *M. formicium* was used. (The data are omitted.)

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Table 3. Fermentation of starch by entodinia in mono-culture or co-culture with *Methanobrevibacterium ruminantium*^{a)}

	Mono-culture	Co-culture
	μ moles/vessel	μ moles/vessel
Starch consumed	896 $\pm$ 23	1,055 $\pm$ 28*
Organic acids produced	1,125 $\pm$ 42	1,321 $\pm$ 36*
Formate	25 $\pm$ 1 (2.2 $\pm$ 0.0)	0 $\pm$ 0 (0.0 $\pm$ 0.0)
Acetate	463 $\pm$ 29 (41.2 $\pm$ 1.1)	662 $\pm$ 34** (50.1 $\pm$ 1.4**)
Propionate	31 $\pm$ 2 (2.8 $\pm$ 0.1)	16 $\pm$ 2** (1.2 $\pm$ 0.0**)
Butyrate	577 $\pm$ 27 (51.3 $\pm$ 0.9)	626 $\pm$ 22 (47.4 $\pm$ 1.0*)
Lactate	29 $\pm$ 2 (2.6 $\pm$ 0.1)	17 $\pm$ 1* (1.3 $\pm$ 0.0**)
Total H ₂ produced ^{b)}	1,795 $\pm$ 46	2,470 $\pm$ 72**
H ₂	1,523 $\pm$ 28	38 $\pm$ 4
CH ₄	68 $\pm$ 4	608 $\pm$ 12
Metabolic hydrogen recovery (%)	93.0 $\pm$ 1.8	97.7 $\pm$ 2.3
Hexose fermented	839 $\pm$ 18	974 $\pm$ 27*
Hexose/starch ^{c)} (%)	93.6 $\pm$ 1.6	92.3 $\pm$ 2.3
Assumed ATP yield	2,779 $\pm$ 45	3,267 $\pm$ 59**
ATP/hexose	3.31 $\pm$ 0.01	3.35 $\pm$ 0.02

a) Expressions are the same as in Table 2. b) Total H₂=H₂+4CH₄. c) Hexose fermented/starch consumed.

The quantities of fermentation products in the co-culture presented in Table 3 are apparent values that were obtained by ignoring the quantities consumed by the methanogen as its carbon source. For this reason, a supplementary experiment was carried out to roughly estimate the consumption of organic acids by *M. ruminantium*, based on various postulations: Approximately the same number of this methanogen as used in Experiment 2 was incubated in the medium used for protozoal culture with added organic acids. Postulating that entodinia produce organic acids at a constant rate under the conditions employed, approximately half the values of the products recorded in Experiment 2 were added; acetate, 300 (μ moles/vessel); propionate, 10; butyrate, 300; and lactate, 10. Incubation was done under a gas phase of H₂-CO₂ (80:20). The amounts consumed during incubation for 48 hr were; acetate, 52 $\pm$ 4; propionate, 1 $\pm$ 1; butyrate, 16 $\pm$ 3; and lactate, 1 $\pm$ 0.

If the data in Table 3 are corrected by using these values, the following values can be obtained; acetate, 714 (μ moles/vessel); propionate, 17; butyrate, 642; and lactate, 18. Correction for formate is omitted, because this substance could be largely fermented by the methanogen²⁹⁾, and consequently recovered in CH₄. The results of the calculations using these corrected values are; total organic acids, 1,391 μ moles/vessel; metabolic hydrogen recovery, 94.4%; fermented hexose, 1,017 μ moles/vessel; assumed ATP yield, 3,423 μ moles/vessel and assumed ATP yield/fermented hexose, 3.37.

Thus, the difference in the values between the mono- and co-cultures is expanded by correcting the actually determined values. In this case, ATP yield per fermented hexose is significantly ($P < 0.05$) higher in the co-culture than the mono-culture,

which is due to the elevated value of acetate; acetate is a more oxidized substance than other fermentation products.

There may be little solid evidence for these postulations, but it can be certain that the corrected values are closer to the true values than the actually determined ones.

Discussion

It was shown in Experiment 1 that fermentation activity of entodinia is depressed by the high partial pressure of H_2 . More marked influence of H_2 was seen on the pattern of fermentation products: Acetate was significantly decreased, while the amount of butyrate was not altered. This probably indicates that formation of acetate is regulated in a different manner from that of butyrate, though both are considered to be formed *via* the same pathway, as far as acetyl-Co A. It has been reported that *Trichomonas foetus*³⁰⁾ and *Dasytricha ruminantium*³¹⁾ possess a unique organelle, 'hydrogenosome', in which acetate may be formed. If entodinia also have such an organelle, it may be possible that acetate is formed in this compartment, and butyrate in another one.

The amount of propionate and the percentage of lactate were higher in the culture receiving H_2 . However, it is not certain whether these trace products, together with formate, were formed by entodinia themselves; it may be possible that contaminating microbes were present in the protozoal culture, in spite of an effort to remove and suppress them. This point was not examined.

The influence of H_2 , on the whole, was not so great as expected. It may be likely that in the control culture bubbled with N_2 , H_2 concentration on the surface of protozoal cells was not reduced to the level at which H_2 has little influence on the fermentation in entodinia; above a certain level of H_2 , its inhibitory effects may not greatly differ.

As shown in Experiment 2, in the co-culture where a fairly low partial pressure of H_2 is considered to have been maintained, enhancement of acetate production and resultant fermentation activity was more marked. It is probable that this enhancement mainly resulted from the stimulation of H_2 production, though other explanations may be possible, *i.e.*, methanogenic bacteria exerted stimulatory effects on the metabolism of entodinia in other unknown manners.

However, again, the effect of the co-existence of methanogenic bacteria was not so great as expected. It has been reported that *Selenomonas ruminantium* produced approximately 100 times more H_2 , when co-cultured with a methanogen than in monoculture¹³⁾. In this¹⁵⁾ and other^{12,14)} H_2 -producing bacteria, the shift in the products caused by H_2 -consuming bacteria was qualitatively and/or quantitatively more drastic than that shown in this study. It is believed that these bacteria have a hydrogenase which catalyzes the formation of H_2 from reduced pyridine nucleotide:

This reaction is considered to be extremely sensitive to the partial pressure of H_2 ;

only at extremely low partial pressure of H_2 (below 10^{-3} atm), the formation of H_2 is favored^{7,32}).

No information is available at present on the mechanism of H_2 formation in entodinia, but the data obtained in the present study may possibly indicate that the manner of H_2 production in entodinia is different from that in the bacteria mentioned above. Recently, an investigation on the mechanism of H_2 production in *Dasytricha ruminantium* has been reported³¹), indicating that the H_2 -producing system in this organism is similar to that reported for *Trichomonas foetus*, which is rather akin to saccharolytic clostridia in respect to H_2 formation^{9,5,6,30}).

In conclusion, however, it is evident that the partial pressure of H_2 has an influence on the fermentation process of entodinia. Probably, the inhibitory effect of H_2 appears to be the end product inhibition that is seen in general. It was also proven that H_2 transfer from protozoa to methanogenic bacteria does take place, as suggested by VOGELS *et al.*¹⁷). As a consequence, entodinia co-cultured with a methanogen ferment carbohydrate at a higher rate, and trap more energy from the substrate. This may presumably suggest that in the presence of H_2 -consuming organisms, entodinia can grow faster than in their absence.

It seems to be important to take these facts into consideration, when the data obtained with a single or pure culture are generalized, or applied to the real rumen: Probably, such data do not accurately reflect the real state or nature occurring in the rumen that is an extremely complicated ecosystem.

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エントディニウム属の反芻胃纖毛虫の発酵に 及ぼす水素の影響

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酸素を終末電子受容体とすることができない嫌気性微生物においては、発酵の過程で遊離される電子の処理のされ易さが、発酵能に大きく影響を与え、その結果、生育速度が左右される。反芻胃纖毛虫は、電子処理の重要な方法として、 H_2 を產生するが、この H_2 生成反応は H_2 分圧の影響を受ける可能性があると考えられるので、この点を検討した。

ヤギの反芻胃から採取したエントディニウム属の混合纖毛虫を供試し、その発酵に対する H_2 の影響を、48時間の培養実験により調べた。 H_2 を連続的に通気することにより、 H_2 分圧を高くした場合には、 N_2 を通気した対照区よりも、でんぷん消費量および総有機酸生成量が有意に減少することが示された。また、発酵パターンにも変化が認められ、主要産物については、酪酸が殆ど変化しなかったのに対し、酢酸が顕著に減少し、その結果、生成割合では酪酸の減少、酪酸の増加という形とな

った。

次に、纖毛虫が產生した H_2 を速やかに除去する目的で、メタン菌の共存下で培養を行なったところ、メタン菌の共存区で発酵能が高くなることが示された。すなわち、でんぷん消費量、総有機酸生成量および H_2 生成量が増加した。この場合にも、酢酸の比率の激増と酪酸の比率の減少という発酵パターンの変化が認められた。また、ヘキソース発酵量と ATP 生成量の見積り値から、メタン菌の共存により、発酵能、すなわちエネルギー獲得能力が増大することが示唆され、したがって、生育速度も促進されるものと推測された。

しかし、全体的に、纖毛虫に対する H_2 の影響は著しく大きいものではなかった。これらの結果について、種々の点から考察を加えた。

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