Hydrogen production and transfer in the rumen

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Summary

Hydrogen is a central metabolite in rumen fermentation and its partial pressure is an important determinant of rumen methanogenesis. The balance of hydrogen ion (H^+) and dissolved hydrogen gas (H_2) concentrations directly determine the redox potential of the rumen and therefore the possible extent of oxidation of feedstuffs. Differential permeability of the bacterial membrane to H^+ and H_2 means the proportions of these metabolites in the extracellular fluid will differ from their proportions within the bacterium. Interpretation of extracellular H_2 concentration in the bulk fluid is also complicated by inter–species hydrogen transfer among H_2 producing and utilising rumen organisms which live in close physical (syntrophic) associations. The role of H_2 concentration in determining the array of fermentation products and the efficiency of energy capture by rumen organisms is discussed. Developing a greater understanding of rumen hydrogen metabolism may present new opportunities for the control of rumen methanogenesis.

Introduction

Because ruminant livestock contribute approximately 16% of global methane emissions (Moss 1993), rumen methanogenesis is increasingly a focus of nutritional research (Van Nevel and Demeyer, 1996; Mathison *et al.* 1998). Early studies demonstrated the rate of methane production was directly proportional to the rate of hydrogen uptake by rumen fluid (Czerkawski *et al.* 1972). The direct relationship between the partial pressure of hydrogen (p_{H2}) and methanogenesis suggests some research emphasis should rightly move from methanogenesis *per se* to factors affecting the availability of hydrogen within the rumen. Hydrogen for methane synthesis occurs in three key states in the rumen, these being hydrogen gas (H_2), reduced cofactors (such as NADH and NADPH), and as free protons. While hydrogen gas rarely accumulates to concentrations greater than 1% in headspace gas (Table 1), hydrogen in all its forms is a central regulator of rumen fermentation and for this reason it has been referred to as the 'currency' of rumen fermentation

Table 1 Reported composition of the rumen headspace gas.

Species	Nutrition	CO_2	CH_4	H_2	CO	O_2	N_2	H_2S
Cattle[2]	Unspecified	63.4	26.8	0.18	–	0.56	7	0.01
Sheep[1]	Hay –prefeed	47.1	36.2	0.033	nd	2.1	14.9	nd
	–feeding	24.5	12.0	0.046	nd	10.2	51.4	nd
	–2hr postfeed	47.5	33.0	0.062	nd	2.4	17.1	nd
Sheep[1]	Concentrate –prefeed	54.3	26.1	0.023	<0.001	4.5	16.8	nd
	–feeding	35.7	16.3	0.319	0.001	10.5	37.5	nd
	–2hr postfeed	68.4	24.8	0.135	0.001–0.01	1.5	5.3	nd
Cattle[3]	Non–bloated	56.4	0.07	23.82	0.15	1.92	17.41	nd
	Bloated	60.9	16.9	0.18	0.25	3.61	18.14	nd

Source: (1) Barry *et al.* 1977 (2) McArthur and Miltimore 1961 (3) Olson 1940; data for bloated cattle averaged for animals consuming sweet clover and lucerne; data for non–bloated animals averaged for cattle consuming sweet clover, lucerne, sudan grass and sorghum; nd, not determined.

(Czerkawski 1986). Additionally, the H^+/H_2 couple is used as the reference for microbial energetics and checking hydrogen balance has been the accepted way of validating results for *in vitro* incubations for over 20 years (Demeyer and Van Nevel 1975). Despite its importance, the kinetics of distribution and transfer of rumen hydrogen are poorly understood. This paper links basic chemical data and microbial biochemistry of hydrogen with its significance in the rumen and explores potential opportunities for improving animal production as a result of this understanding.

Distribution of hydrogen in the rumen

Examination of the distribution of hydrogen atoms in the rumen shows that over 90% of rumen hydrogen atoms reside in water (Table 2). The protons in water are not, however, free to exchange with other protons except when liberated or utilised in hydrogenation/hydration reactions. The same is true for other covalently bound protons such as those of the methyl group of acetate (Leng and Leonard 1965) which do not exchange with water in an aqueous solution. In rumen fluid however, substantial proton transfer occurs during microbial fermentation such that a substantial proportion of the hydrogen atoms in methane and hydrogen gas are derived from water (Figure 1). The difference in flux of substrate hydrogen and substrate carbon flux due to H^+ transfer means that tritiated and deuterated compounds are not useful as tracers of organic acid production in rumen fermentation studies (Leng and Leonard 1965; Hungate 1966).

Being a non–polar gas, H_2 is poorly soluble in water and its solubility is unaffected by pH since it does not ionise. Based on the solubility of hydrogen in water (IUPAC 1981) and on typical H_2 concentrations in the rumen headspace (Table 1) it can be estimated that at 39ºC, the concentration of dissolved hydrogen in bulk rumen fluid would vary between about 90 µM and 250 µM. The effects of headspace H_2 pressure on fermentation are equivocal. Czerkawski *et al.* (1972) changed the H_2 concentration in the headspace of rumen fluid incubations and showed H_2 to be utilised as fast as it was able to dissolve. However Nelson *et al.* (1960), reported no change in the CO_2: CH_4 ratio in rumen gas during sparging of the rumen with H_2, suggesting methane production did not respond to the concentration of H_2 in the rumen gas. The rate at which H_2 dissolves in solution may be an important difference in these studies. It is likely that cultures grown in closed atmospheres for long periods permit the dissolved and headspace gases to equilibrate which is not possible in the rumen due to H_2 being slow to dissolve. For this reason, fermentation patterns change in laboratory cultures in response to a higher H_2 pressure in the headspace (Van Nevel *et al.* 1974; Hillman *et al.* 1991) but not *in situ* (Nelson *et al.* 1960).

The mass transfer of dissolved hydrogen may be limiting to growth of methanogens if reliant on H_2 dissolved in the bulk medium, and so intimate (syntrophic) associations exist between many hydrogen– utilising microbes such as methanogens, and those which produce hydrogen (Conrad *et al.* 1985). Direct physical associations to facilitate syntrophy exist in the rumen such as those between rumen protozoa and endo– and ecto–symbiotic methanogens (Newbold *et al.* 1995; Ushida *et al.* 1997; Hegarty 1999). In sludge ecosystems, less than 10% of hydrogen transfer may

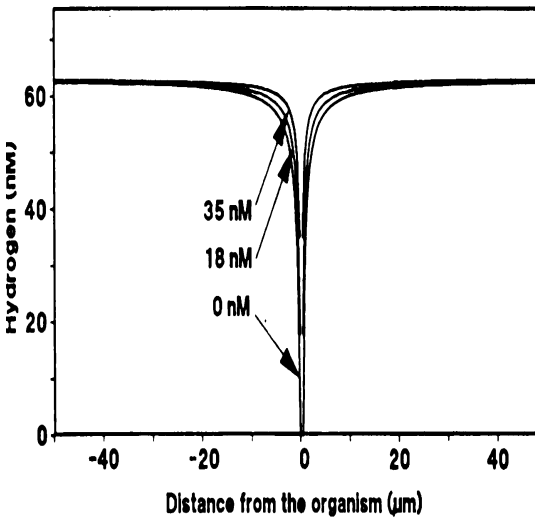


Figure 1 Changes in the specific activity (dpm per µg atom H) of H_2 (·), water (n) and methane (p) when rumen contents were incubated under unlabelled H_2 gas with ^3H_2O (Czerkawski 1975).

Table 2 Calculated distribution of hydrogen atoms in the rumen of a sheep assuming typical pool sizes of rumen digesta and metabolites.

Constituent	Pool size	H (g)	%
Water	4000 mL	444.0	91.6
Feed	450g DM	30.2	6.2
Microbes	163g DM	10.4	2.1
Methane	40mL	0.007	<0.1
Hydrogen gas	<1mL	<0.0001	<0.1

occur via H_2 dissolved in the bulk fluid (Conrad *et al.* 1985) and it should not be expected that hydrogen in the rumen gas space will equilibrate with, or be indicative of, the availability of dissolved hydrogen used by many rumen bacteria. The importance of close physical association in syntrophy was shown by Boone *et al.* (1989) using co–culture of a methanogen and a (H_2 liberating) butyrate degrader. By their calculations Boone and coworkers showed that unless microbes were within 10mm of each other, the presence of a H_2 producing microbe did not provide an advantage to the methanogen, other than by increasing the H_2 concentration in the bulk solution (Figure 2) The partial pressure of H_2 has substantial effects on the energetics of methanogens themselves and extremely low or high p_{H2} may cause the ATP yield to vary from 0.33 to 2 moles of ATP per mole of methane produced respectively (Keltjens and Vogels 1996).

Intracellular hydrogen ions

Since H_2 is non–polar it can be assumed to pass freely through microbial membranes, allowing intra– and extra–cellular H_2 concentrations to move towards, but probably not achieve, equilibrium. In contrast to H_2, the hydrogen ion concentration in the cytoplasm is a condition over which microbial cells exert tight control. The intracellular pH for all living cells is by necessity more alkaline than the surrounding environment. Extensive studies of *Streptococcus bovis* (Russell 1987) and *Streptococcus faecalis* (Harold *et al.* 1970) have found the internal pH for these organisms to be 0.4–1 unit higher than the medium. Intracellular pH is not a constant however, and acid–tolerant rumen bacteria allow their intracellular pH to decline in proportional to extracellular pH (Russell 1991).

Cytoplasmic pH is a function of trans–membrane proton migration (controlled by ATPase and symport systems) and production of acids within the cytoplasm (Booth 1985). Bacterial membranes have controlled permeability to protons, so passive inward diffusion of protons is minimal. The fact that bacteria import nutrients by either ion–driven or ATP driven transport (Russell and Strobel 1993) means maintenance of the pH differential across the membrane is critical for growth. The action of monensin in decreasing the trans–membrane Na^+/K^+ gradients can cause internal proton accumulation and decrease intracellular pH (Russell 1987). This change in proton gradient is associated with a reduced microbial growth efficiency which is most severe at low extracellular pH (Chow and Russell 1990).

Production of fermentation acids in the cytoplasm and their flux across the bacterial membrane play some part in intracellular pH regulation (Thauer *et al.* 1977). The inability of ionised acids to diffuse out of cells can cause accumulation and feedback inhibition of VFA production. Also, undissociated acids are lipophilic and can pass from the relatively acidic external environment into the cell carrying a proton and then, at the higher intracellular pH, dissociate releasing the proton into the cytosol. Because the ionised VFA cannot leave the cell, VFA entry can only serve as a means of introducing protons, reducing rather than improving the cells capacity to maintain a proton gradient across the membrane. Unless the cells do decrease their intracellular pH as external pH declines, extreme levels of intracellular VFA could accumulate (Russell and Strobel 1993). Build up of these acids intra–cellularly is thought to be one way by which the balance of end–products changes with external pH although the role of ADP/ATP and $NADH/NAD^+$ ratios in determining VFA balance must also be considered, as discussed later. Trans–membrane VFA entry may therefore serve more as a means of changing the pattern of VFA a cell produces via end–product inhibition, than as a means to control internal pH or uncoupling fermentation and growth.

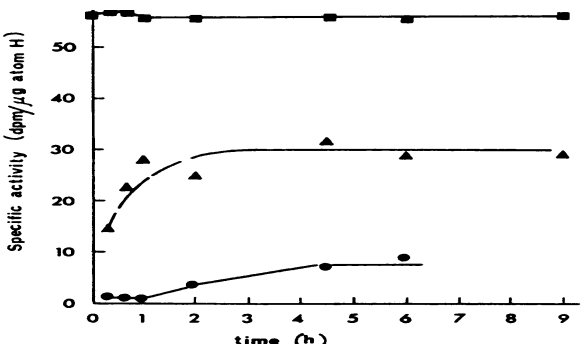


Figure 2 Diffusion of H_2 to a coccus 1 µm in diameter. Diffusion assumes a steady–state concentration profile of H_2 near a H_2 consuming cell with a cell surface concentration of 0, 17.5 or 35 nM and a bulk phase concentration of 63 nM (Boone *et al.* 1989).

Sources of rumen hydrogen

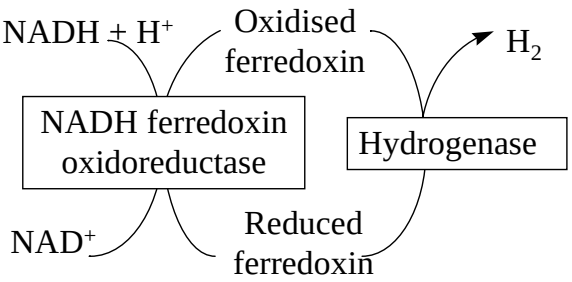
The atmosphere contains approximately 0.0005% H_2 and there is no H_2 carrier system in blood so inhaled or engulfed air will contribute little to H_2 in the rumen. A daily production of approximately 100 L of hydrogen gas may be anticipated in the rumen of a sheep on grounds that 4 moles of hydrogen are required per mole of methane produced and 25 L/d is a typical emission of methane by sheep (Pelchen and Peters 1998). Rumen H_2 is liberated by hydrogenase enzymes acting on reduced ferredoxin arising from either phosphoroclastic reactions (which do not involve NADH) or as part of a redox couple which allows reoxidation of reducing equivalents, such as NADH.

The bacterial hydrogenases which reduce protons to H_2 are single polypeptide chains (Van Dijk *et al.* 1979) or multi–subunit enzymes (Sawers 1994) which may contain iron/sulphur clusters and nickel. In rumen protozoa (Paul *et al.* 1990) and fungi (Yarlett *et al.* 1986) hydrogenase enzymes are principally located within the hydrogenosomes. The pyruvate synthase, hydrogenase

and lactate dehydrogenase enzymes (Yarlett *et al.* 1981) make the hydrogenosome the principal site of H_2 and CO_2 generation. The activity of protozoal and *Neocallimastix* hydrogenases are inhibited by carbon monoxide and a high pCO_2 drives fermentation towards H_2 consuming reactions such as butyrate and lactate production (Marvin–Sikkema *et al.* 1993; Ellis *et al.* 1991).

Oxidation of reducing equivalents

The principal means of regenerating NAD^+ from NADH is by the enzyme NADH ferredoxin oxidoreductase, coupled to a hydrogenase. The activity of NADH ferredoxin oxidoreductase is primarily controlled by concentration of dissolved H_2, being suppressed by a high p_{H2} (Gottschalk 1986).



Decarboxylation of pyruvate

Anaerobic decarboxylation of pyruvate in the rumen is achieved by two enzyme systems. Both the pyruvate ferredoxin oxidoreductase (pyruvate synthase) and pyruvate formate lyase enzymes liberate ATP without use of NADH as an intermediate electron carrier (Doelle 1975; Figure 3). The formate liberated by pyruvate formate lyase is however rapidly degraded in the rumen to CO_2 and H_2 and formate rarely accumulates (Hungate 1970). Formate may also be directly reduced by some rumen methanogens (Hungate *et al.* 1970). In ecosystems where these occur, formate may be a more important vehicle for interspecies hydrogen (electron) transfer for syntropic rumen organisms than is H_2 (Boon *et al.* 1989).

Additional to decarboxylation of pyruvate, hydrogen is also produced by oxidation of butyrate back to acetate (McInerny *et al.* 1981). This fermentation is conducted by a number of organisms (e.g. *Syntrophomonas wolfei*) and cycling of carbon between acetate and butyrate may provide a futile cycle to uncouple ATP production from cell synthesis. There is almost no secondary fermentation of propionate in the rumen (Rowe *et al.* 1985) and this is possibly due to the high energy cost of producing acetate from propionate ($\Delta G_o' = 71.7$ kJ/mole) compared to producing acetate from butyrate ($\Delta G_o' = 48.3$ kJ/mole; Boone *et al.* 1989)

Effects of pH and E_h on the availability of hydrogen gas to microbes

Considering the differences in intracellular and extracellular H^+ concentrations caused by the cell membrane, it is of some interest to understand what linkages, if any, exist between extracellular pH and intra–cellular H_2 availability. The microbial and biochemical mechanisms by which consumption of high levels of readily fermentable carbohydrates reduce rumen pH (increase extra–cellular proton concentration) are well understood (Owens *et al.* 1998).

The partial pressure of H_2 is directly related to both H^+ ion concentration (pH) as well as the redox potential of the rumen according to the Nernst Equation (equations 1, 2) which may be simplified for rumen conditions to equation 3 (Sauer and Teather 1987). Oxidation potential (E_o) is referenced against a standard hydrogen electrode and describes the capacity of a system to donate or accept electrons when the concentration of the oxidised and reduced forms are equal. Because these concentrations are unequal in biological systems, the redox potential (E_h) is calculated according to equation 1. Equation 3 demonstrates that redox potential will change in direct proportion to hydrogen ion concentration.

$$E_h = E_o - RT/F \ln([\text{oxidised form}]/[\text{reduced form}]) \quad (1)$$

$$E_h = E_o - RT/F \ln([H^+]/[H_2]) \quad (2)$$

$$E_h = 0.062 * \log [H^+]/p_{H2} \quad (3)$$

Equation 3 also shows the direct mathematical basis for why a rise in H_2 partial pressure is associated with a decrease in E_h when pH is held constant, as observed in continuous fermenters (Sauer and Teather 1987). In the ruminant animal, feeding causes a sharp rise in p_{H2} and this is associated with a rise in headspace H_2 concentration (μL/L) and a fall in E_h (Barry *et al.* 1977). The post–feeding rise in H_2 concentration occurs in spite of dilution through introduction of atmospheric N_2 and O_2 with feeding (these gases being up to 50% and 9% of rumen gas respectively). The strong positive association between hydrogen ion concentration in the rumen and p_{H2} observed by Barry *et al.* (1977) is unlikely to result from a direct chemical mechanism. Since production of H_2 is achieved through the action of intracellular hydrogenase enzymes, it is difficult to see how their activity could be altered by extracellular H^+ ion concentration. Instead it may reflect specific inhibition of methanogenesis as pH declines (Lana *et al.* 1998; Russell 1998) which then causes an accumulation of unused H_2 in the rumen.

The anti–methanogenic compounds which target the methanogen itself and cause H_2 accumulation reduce E_h, while those which inhibit methanogenesis

only by reducing H_2 availability (e.g. monensin) do not decrease E_h (Sauer and Teather 1987).

The impacts of p_{H2} on liberation of H_2 from the phosphoroclastic reactions and reduced cofactors are as follows. H_2 accumulation itself does not greatly inhibit hydrogenase enzymes and the phosphoroclastic production of acetate from pyruvate by pyruvate ferredoxin oxidoreductase is a fully reversible process. A high p_{H2} can therefore cause a direct feedback inhibition of the main acetate generating pathway in the rumen. A high p_{H2} also has a direct effect on the release of H_2 from NADH. The action of NADH oxidoreductase is subject to end–product inhibition by H_2. At low H_2 pressures ($p_{H2} < 1 \times 10^{-3}$ atm), H_2 liberation becomes thermodynamically feasible ($\Delta G_o' < 0$ mV) but at high p_{H2}, the reaction becomes endergonic and does not proceed (Gottschalk 1986). Because the action of NADH oxidoreductase is not reversible, NADH accumulates in times of high H_2 potential and so fermentation is stopped by lack of NAD^+.

Hydrogen availability as a control of rumen fermentation

Just as methanogenesis is directly proportional to p_{H2} in the rumen, so the balance of VFA produced may be expected to change with hydrogen availability both *in vitro* and *in vivo*. Within the cell, hydrogen concentration moderates the $NADH/NAD^+$ ratio which feeds back on fermentation pathways (Hino and Russell 1985). High substrate pressures which accelerate the glycolytic pathway induce a high $NADH/NAD^+$ ratio which stimulates propionate synthesis so oxidising NADH. If the ATP/ADP ratio is also high (as may occur due to a sudden influx of immediately fermentable substrate such as molasses), propionate synthesis is suppressed and NADH is instead utilised in butyrate production. Either butyrate or propionate synthesis will reduce the NADH ratio and allow accelerated acetate production to continue (Sutherland 1977).

Sutherland (1977) concluded that it was the free energy of inter–conversion which determined the flow of electrons and therefore the end–products produced by adaptable organisms. This is in keeping with the view that the relative rates of component processes are optimised for overall ATP gain or thermodynamic efficiency (Thauer *et al.* 1977), as well as to maintain internal homeostasis in the cell. Applying the Nernst equation, the energetically favourable product could be determined by the redox state of the cell as reflected in $NADH/NAD^+$ and ATP/ADP ratios as follows. The example of energetic optimisation of the acetate propionate balance (G_{ap}) is shown below.

$$G_{ap} = G'_{apo} + RT \ln ([\text{propionate}]/[\text{acetate}]) \\ *([ATP]/[ADP]*[PI])/([NADH]*[H^+][NAD^+])^4.[CO_2]^2$$

Figure 3 Reaction pathways which liberate H_2 from pyruvate in the rumen ecosystem.

Hydrogen effects on energy yield from fermentation

Ruminant nutritionists often lament that the rumen is an inefficient means of extracting energy for the animal from its diet, focusing on the fact that the anaerobic environment reduces the yield of ATP per mole of glucose from 38 to 2 ATP. When the complete animal/rumen unit is considered, however, there is little basis for such despondency. The efficiency with which released energy is captured in ATP from anaerobic fermentation (as far as it proceeds) is comparable to that of aerobes, being typically 25–50% compared to 44–59 % for aerobes (Thauer *et al.* 1977). A further similarity is that aerobes and anaerobes do not differ greatly in the efficiency of ATP use for cell growth and Y_{ATP} may be higher for some rumen bacteria than for aerobes (Russell and Wallace 1997). What does differ greatly is how far oxidation proceeds in the anaerobic compared to the aerobic environment. In the aerobic bacterium or mammalian cell, sugars are largely oxidised completely through to CO_2 while in the rumen, fermentation ceases at VFA. When the VFA are absorbed, the residual energy present in the fermentation products is made available to the host via aerobic respiration. Were rumen fermentation as extensive as in sewage fermenters, there would be no high energy compounds leaving the fermentation except methane, and no possibility of life for the ruminant.

An important metabolic difference between aerobes and anaerobes is the failure of the TCA cycle to fully operate in anaerobes. Possible explanations for why the TCA cycle does not operate in anaerobic environments are:

- The TCA cycle does not proceed because the oxidation of succinate to fumarate has an E_o' of 33 mV and only electron acceptors with a more positive redox potential can support the reaction (e.g. O_2, NO, NO_3, ubiquinone) and these are absent (Thauer *et al.* 1977)
- There is a deficiency of NAD^+ available, diminishing the NADH liberating steps in the TCA cycle
- The TCA cycle contains oxygen induced enzymes (Doelle 1975)

Of these possibilities, blocking of the cycle by accumulation of NADH and a concomitant lack of NAD^+ is considered to be the most important. Even if p_{H2} was reduced to minimal levels and regeneration of NAD^+ from NADH was rapid and complete, only methanogens, sulphate reducers and a few carbohydrate fermenting rumen bacteria have electron transport chains which would enable them to derive ATP from NADH generated by the TCA cycle (Russell and Wallace 1997). An active TCA cycle would then not itself increase ATP yield to most primary fermenters, although they would obtain one guanosine diphosphate from succinyl–CoA to succinate conversion and methanogens would capture some of the energy in hydrogen as ATP while reducing CO_2.

Conclusions

The partial pressure of hydrogen is a significant determinant of the rate of rumen methanogenesis and the array of VFA produced in the rumen. The presence of significant syntrophic relationships between a range of H_2 producing organisms and H_2 utilisers means the p_{H2} in the headspace is unlikely to be an accurate reflection of intracellular H_2 availability.

The balance of hydrogen ions (oxidised form of hydrogen) and H_2 (reduced form of hydrogen) determine the redox potential of the rumen and therefore the extent and products of fermentation. High H_2 concentrations induce a low redox potential allowing production of highly reduced end products (e.g. propionate, methane). When p_{H2} is low, the fermentation balance swings away from these to an acetate fermentation.

Strict control of the entry of hydrogen ions into bacteria at the membrane means extra–cellular pH does not have a direct chemical effect on H_2 generation within the cell. The increase in p_{H2} associated with low ruminal pH is considered to be, instead, a consequence of low pH being inhibitory to H_2 use by methanogens. Further understanding of factors which regulate H_2 availability in the rumen, particularly those reactions which utilise H_2, is likely to lead to recognition of new techniques to decrease methanogenesis in the rumen.

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