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Minimising the risk of nitrite toxicity in ruminants when dietary nitrate is used to mitigate methane emissions

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Executive summary

- Inorganic nitrate ingested by ruminants is reduced to nitrite then ammonia by rumen microbes. This two-step process deprives methanogens of hydrogen thus reducing methanogenesis while providing a nitrogen source for microbial growth.

- Absorption of the intermediate nitrite into the blood can be associated with hypoxia as a result of formation of methaemoglobin (MetHb) and other metabolically active intermediates such as nitric oxide. The syndrome is often termed *nitrite toxicity.*

- This review assesses how the risk of MetHb formation may be reduced in nitrate-fed ruminants to allow confidence in nitrate feeding as a commercial management practice.

- Potential control points were identified in regard to (1) reducing nitrite accumulation in the rumen contents and its entry into the blood and (2) reducing MetHb accumulation in the red blood cell.

- This report provides

    - Detailed appendices containing reviews of current knowledge and the underpinning literature

    - A summary of current knowledge and opportunities for manipulation about each control point

    - A list of critical gaps in understanding of nitrate and nitrite metabolism in ruminants and potential management interventions that require research to enable safer management of nitrate-fed ruminants

    - Description of the research questions needing answers to permit practical and safe management strategies for use of nitrate in diets for ruminants

- While there are only a few studies describing the metabolic capabilities of nitrate reducing microorganisms in the rumen, these studies together with studies of nitrate/nitrite metabolism in microbes in other environments, allowed the following conclusions to be drawn on how to minimize nitrite toxicity in ruminants.

    - High blood MetHb concentrations are a consequence of excretion of nitrite into the rumen fluid by microbes seeking to protect their internal metabolism from damage resulting from high intracellular nitrite concentrations.

    - The total supply of fermentable dietary N (including nitrate added to mitigate methane emissions) should not exceed the N requirement for net microbial synthesis in the rumen which is dependent on the fermentable energy availability (approximately 1.5 g N/MJ of ME). Higher N diets will increase the risk that increased urinary N output may promote release of greenhouse gases from the soil. In practice, this means that inclusion of supplementary nitrate in the diet of sheep and cattle usually becomes less appropriate when the crude protein content of the basal diet exceeds 10%.

- The presence of fermentable energy substrates for rumen microbes when nitrate is entering the rumen is pivotal to avoiding nitrite accumulation. A corollary is that the risk of toxicity upon ingestion of nitrate increases when fermentable energy in the diet (ME concentration in the dietary DM) is limited, *e.g.* in animals on low digestibility pastures.

- 'Detoxification' (removal) of nitrite in the rumen by conversion to ammonia will be accelerated if rapidly fermentable starch rather than fibrous substrate is provided to the rumen microbes.

- Managing the rate of nitrate supply to rumen microbes (*e.g.* by dispensing it using molasses blocks or with lipid or polymer 'slow release' coatings or by providing feed *ad libitum* rather than in pulses) appears effective in reducing methaemoglobinaemia and nitrite toxicity.

- There are no practical means of preventing uptake of nitrate by rumen organisms once nitrate is dissolved in the rumen fluid. However, pilot studies have demonstrated that supplying cultures of nitrite reducing strains of bacteria in supplements to concentrate-fed ruminants increases their capacity to remove the potentially toxic nitrite intermediate.

- Modelling multiple sets of published data provided consistent evidence that an unidentified nitrogenous intermediate is involved in the conversion of nitrate to nitrite. Identification of this intermediate may elicit new possibilities for intervention not yet documented.

- Removal from the rumen of nitrite in digesta flowing into the abomasum does occur. Losses via this route reduce the efficiency of methane mitigation potential; however, the amount removed is a small fraction of the total nitrate entry into rumen.

- Removal of nitrite by denitrification to N_2 in the rumen does not appear to occur. Small amounts of nitrous oxide (N_2O) are sometimes produced. However, further enhancement of nitrite conversion to N_2O would be unwise because N_2O has a higher global warming potential than methane, so it would reduce the net efficiency of methane mitigation.

- Some oxidation of nitrite to nitrate during its absorption through the gut wall probably occurs but has not been quantified; ways of accelerating this rate are not apparent.

- Little is known about the sites, mechanisms or inhibitors of nitrite absorption from the gastro-intestinal tract of ruminants, and no agents capable of blocking absorption into the blood stream have been identified for co-feeding with nitrate.

- There are no practical ways of reducing nitrite entry in the red blood cell. However, there is scope for preventing nitrite-promoted MetHb production by provision of intracellular anti-oxidants such as ascorbic acid, selenium. Vitamin E and curcumin that may reduce the redox pressure on MetHb formation in the red

cell. For these anti-oxidants to be used in practice, delivery mechanisms capable of producing sufficient absorbable active ingredient would need to be determined

- There is scope for accelerating the rate of regeneration of haemoglobin by provision of riboflavin to stimulate the NADPH-MetHb reductase pathway. This is the pathway stimulated by methylene blue, an effective therapeutic treatment that is, however, no longer allowable for treating livestock suffering from methaemoglobinaemia.
- Because MetHb reductase activity depends on phosphorylation reactions, it would be prudent to ensure that phosphorus supply is adequate before providing animals with dietary nitrate supplements as insufficient P may also reduce the rate of haemoglobin regeneration.
- There is between-animal variation in susceptibility to nitrite toxicity but whether this is due to differences in ruminal or mammalian metabolism is unknown. There may be potential for genetic selection directed to more nitrate-tolerant ruminants. The possibility that breeding for tolerance may be able to be ‘short-circuited’ by simple digesta transfer from low-susceptibility animals needs investigation.

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1 Recommendations on nitrate feeding strategies to minimise nitrite poisoning

This review was undertaken to identify key control points in the metabolism of nitrate and nitrite in ruminants and to consider options for research to find interventions at these control points that will make nitrate feeding safer and more effective. It was not within the scope of the review to consider on-farm management practices for feeding of nitrate. Nonetheless, the current 'best management practices for feeding nitrates to cattle' are summarised below with comments (*in italics*) on the presence of supporting/conflicting evidence within the review and of new aspects of management worthy of inclusion.

- 'If the basal feed (or water supply) contains or is likely to contain nitrate, do not provide supplementary nitrate. Feed with potentially high levels of nitrate include: Nitrogen-fertilized pasture; brassicas; hays made from cereal crops; weeds of stubble including Capeweed, Mintweed, Crown Beard, Pigweed, Red Root, Marshmallow and Fat Hen. Sometimes water can contain high levels of nitrate, particularly if it is drains from highly fertile soils, or has been contaminated with fertilizer or decaying organic matter.'
    - *The review provides a summary of natural sources of nitrate in Australian livestock systems (refer Appendix 4).*
- 'Introduce nitrate into the diet of your herd gradually. For the first two weeks, don't feed more than 25 grams of nitrate per adult animal per day (or 3.5 grams per kilo of dry matter intake per day). These amounts are based on an adult animal equivalent (450kg). Producers will need to account for the actual weight of their animals and feed accordingly.'
    - *The review identifies literature showing acclimation to nitrate may increase enzyme activities of nitrate and nitrite reductase and, more importantly, improve the ratio of nitrite to nitrate reductase activity. However, caution is advised in that high metHb levels are often observed in ruminants even though they have been ingesting nitrate for an extended period (Refer page 19).*
- 'After the adjustment period, never feed cattle more than the maximum safe level of nitrate. The maximum safe limit is 50 grams of nitrate per animal per day (or 7 grams per kilo of dry matter intake per day). Again, these amounts are based on an adult animal equivalent (450 kg). Producers will need to account for the actual weight of their animals and feed accordingly.'
    - *Inclusion levels are not directly addressed in this review, but levels should be determined in relation to the intake of ME and the presence of fermentable energy (and also P and S availability).*
    - 'Exercise caution when feeding nitrate to cattle that are on restricted intake, that have recently been fasted, or lack adequate levels of digestible dry matter.'

- *After feed restriction, nitrate is consumed faster and therefore the risk of nitrate poisoning is greater*. *There is a thorough review of literature referring to the need to provide fermentable carbohydrate in proportion to nitrate intake and in synchrony, and to ensure that ammonia is rapidly used for microbial growth thereby avoiding nitrite accumulation within rumen microbes and excretion of nitrite into the rumen fluid medium.*

- 'When feeding nitrates to grazing cattle, they should be given continuous access to the nitrate source (usually in the form of a lick-block). Avoid pulse-feeding nitrates to cattle.'

    - *There is good evidence that metHb formation and nitrite poisoning are related to the rate of nitrate ingestion (see page 14); high intakes over short periods can exacerbate nitrite build-up in rumen fluid; 'protection' of the nitrate source by coatings or other means (see p. 15) can be expected to prevent higher rates of nitrate conversion to nitrite by rumen microorganisms.*

- 'In a feedlot system where cattle are on total mixed rations, the nitrate should be dissolved in the liquid supplement, prior to mixing the ration, thus ensuing even distribution.'

    - *This is consistent with literature suggesting the benefits from synchronising time and level of nitrate supply with provision of fermentable carbohydrate to the rumen.*

- 'Ensure that cattle being fed nitrates also have adequate sulphur intake, in order to support rumen health. The ideal level is 2 g of sulphur per 100 g of nitrate.'

    - *Evidence for sulphur supply affecting nitrate metabolism within the normal levels of sulphur in ruminant feeds is lacking (refer page 15). Based on microbes in other ecosystems, an increase in nitrite reductase activity at higher sulphur levels might be expected, but it is not known if nitrogen-reducing, sulphur-oxidising microbes occur in the rumen*

- 'Stop feeding nitrates to cattle for 24 hours before any stressful or physically demanding activity, such as mustering.'

    - *This relates to regeneration of haemoglobin and the biology and management of this is reviewed (refer pages 15, 27-29). Higher concentrations of MetHb in the blood lead to hypoxia. Hypoxia will be more significant in animals subject to any form of exertion or stress. After of intake of nitrate in the diet ceases, MetHb levels should fall to low levels within 24 h.*

2 Recommendations for research to develop new opportunities for reducing methaemoglobin accumulation

A package of research is required to provide information to fill gaps in understanding of the fundamental biology of ruminal/animal nitrate metabolism, and to enable practical safe delivery of nitrate in the commercial world.

Based on the findings of this review, a research program is needed that enhances fundamental knowledge on ways to minimise ruminal nitrite absorption and MetHb accumulation in the blood. The project should:

1. Establish the sites, mechanisms and regulators of nitrate and nitrite absorption in the gastro-intestinal tract.
2. Demonstrate that synchronising fermentable substrate and nitrate entry into the rumen can prevent accumulation of ruminal nitrite in commercial animal production systems.
3. Evaluate the efficacy and practicality of 'slow-release' nitrate sources for ruminant feeding.
4. Quantify the levels of resilience to nitrate feeding between animals and identify the mechanism of that resilience. If it is rumen-based, can this resilience be transferred among animals?
5. Confirm that dietary riboflavin or other anti-oxidants accelerate haemoglobin regeneration and reduce the risk of nitrite toxicity.

Strategic research questions that may identify novel means of controlling nitrate and nitrate metabolism to reduce risk to animal health are listed below. It is recommended that as many as possible of these questions are addressed within the above research project:

1. What is the nature of the 'sequestration' of nitrate that precedes the appearance of nitrite after addition of a pulse dose of nitrate to rumen fluid *in vitro*?
2. Does dietary fibre bind rumen nitrite significantly in rumen fluid and reduce its rate of absorption?
3. Do VFA concentrations affect nitrate uptake by rumen bacteria? In like manner, do VFA also affect rate of nitrate or nitrite transfer through bacterial cells or gut wall?
4. Does the presence of Cl^- ion in molasses-salt blocks competitively inhibit nitrite absorption from the gut?
5. Are there factors (other than tungsten) that limit or enhance nitrate reduction rate in the rumen, *e.g.* enzyme inhibitors
6. Is the balance of reductase activities selectively modified by controlling the quantity of NADH available in the cell
7. Can cultures containing rumen microorganisms with enhanced nitrite reductase activity be dispensed in a practical way?
8. What is the natural occurrence of, and potential scope for oxidising nitrite to nitrate in the gut wall?
9. Does the most widely studied bacterial nitrite reductase system (Nir) occur in the rumen?

10. What factors reduce the quantity of nitrous oxide formed from nitrate supplements?
11. Clarify the relative metabolisable energy benefit/cost of urea supplementation (by urea hydrolysis) and iso-nitrogenous nitrate supplementation (by chemical reduction).
12. Investigate differences in the microbial species composition (nitrate reducing microbes versus other microbes; protozoal/fungal numbers/mL) between ruminants acclimated or not acclimated to dietary nitrate, or tolerant or intolerant of nitrate in the diet.
13. Determine whether protozoa/fungi play a major role in nitrate reduction in the rumen?
14. Investigate whether rumen archaea utilise nitrate directly.
15. Clarify whether the presence of NO_3 in rumen fluid inhibits uptake of acetate. Is the reverse also true?
16. Does NO_3/NO_2/NO reduce rumen turnover and contraction rate and contribute to reduced feed intake?
17. Investigate why about 25% of intra-peritoneally injected nitrite is converted to urea in mice, whereas virtually no urea is produced from a similar (i/p) injection of nitrate in mice.
18. Obtain proof-of-principle for safe delivery of nitrate via lick-blocks to cattle in extensive rangelands. (To support risk assessment, this will require investigation of the average intake and range of intakes of nitrate via blocks by individuals within the herd when offered a range of formulations during different seasonal conditions.)

3 Recommendations for research, in priority order

From the review, it appears that the greatest opportunity to reduce the risk of nitrite poisoning in ruminants (after all feeding management recommendations have been taken into account) are related to reducing the formation of methaemoglobin and increasing the regeneration of haemoglobin from methaemoglobin. Other strategies that may slow nitrate reduction or increase nitrite reduction in the rumen or slow the rate of nitrite absorption across the gut wall are also possible research targets. An understanding and exploitation of observed differences in the susceptibility to methaemoglobin formation between animals also provides research opportunities.

1. Demonstrate whether between animal differences in nitrite tolerance is due to the rumen microbes or to the animal

There is strong evidence that animals differ in their tolerance to nitrate supplementation and formation of methaemoglobin. However, it is not known whether the between-animal differences are due to different microbial populations in the rumen or to differences in animal physiology and metabolism. Understanding the nature of these between-animal differences would help either identify possible mitigation strategies or provide a basis for an animal breeding/selection program.

A possible research approach would be to identify extreme animals in methaemoglobin formation when given a nitrate supplement and exchange rumen contents between the animals to demonstrate whether differences in the rumen microbial population or animal

factors are responsible for the different tolerances to nitrate. If it is found that rumen factors contribute to tolerance, the possibility of developing cultures of rumen microbes with nitrite reducing capabilities (e.g. *Wolinella succinogenes*) should be considered. If mammalian metabolic factors are involved, there are possibilities for including various dietary additives in order to reduce the risk of nitrite poisoning.

2. Prevent formation of methaemoglobin from blood nitrite

Provision to the animal of antioxidants has potential to reduce the formation of methaemoglobin from nitrate. A range of antioxidants could be effective including vitamin E, ascorbic acid, selenite and curcumin.

3. Stimulate the regeneration of haemoglobin from methaemoglobin

Riboflavin, particularly in the presence of glucose, has been shown to stimulate the rate of the NADPH-dependent methaemoglobin reductase. Inorganic phosphorus is required in the biochemical pathways and must be provided in the diet. In addition, N-acetylcysteine is also known to stimulate the conversion of methaemoglobin to haemoglobin either via the same reactions or through the synthesis of glutathione.

Riboflavin is also an antioxidant. Thus the first research priority may be a multi-pronged supplementation of antioxidants and the other compounds listed that will both reduce the formation of methaemoglobin and stimulate the conversion of methaemoglobin to haemoglobin. Methods for delivering these compounds need to be devised so they are not metabolised by microbes in the rumen and are available to the animal.

4. Slow nitrate release and reduction rate in the rumen

Several strategies for coating or slowing the release of nitrate within the rumen have already been proposed and tested. These treatments include lipid and polymer coatings, sesame gum and other compounds. The extent of 'protection' will be important as 'over-protection' could result in a reduction in the potential for methane mitigation. The review also suggests that high concentrations of copper and/or cobalt in the rumen may reduce the rate of nitrate reduction to nitrite. These strategies could be evaluated in animals under Australian conditions with low energy availability from poor quality forages where nitrite poisoning is more likely. There is possibly a second advantage from feeding cobalt because it is known to stimulate red blood cell formation or erythropoiesis.

5. Speed nitrite reduction rate in the rumen

The review suggests there are some microorganisms such as *W. succinogenes* that have greater nitrite reducing capacity than others. Protozoa also appear to have considerable nitrate reducing activity. The exact identities of such organisms and their ability to persist in the rumen need to be clarified. The resulting information would provide an opportunity for intra-ruminal supplementation with microbial cultures to lower the concentration of nitrite in rumen fluid by speeding up its conversion to ammonia.

6. Slow nitrite absorption across the rumen wall

Evidence presented in the review suggests that nitrite is absorbed across the rumen wall by an active Cl^-/HCO_3^- pathway which is used for the transport of chlorine. Hence, sodium chloride containing supplements may help reduce the rate of nitrite absorption across the rumen wall.

4 Methane mitigation by dietary nitrate: focus of review

Dietary nitrate is being enthusiastically embraced in human nutrition, but for ruminant livestock, the future for nitrate supplementation is balanced between nitrate's desirable roles as a non-protein nitrogen (NPN) source and mitigator of enteric methane production (a potent greenhouse gas), and an undesirable health risk of nitrite (NO_2) poisoning. After considering possible feeding management strategies for mitigating methane emissions from ruminant livestock, the authors of a FAO review of the potential mitigation strategies (May *et al.* 2000a; Hristov *et al.* 2013) made the following observations with respect to the use of dietary NO_3.

Nitrates show promise as enteric CH_4 mitigation agents, particularly in low-protein diets that can benefit from nitrogen supplementation, but more studies are needed to fully understand their impact on whole-farm GHG emissions, animal productivity and animal health. Adaptation to these compounds is critical and toxicity may be an issue.

Inclusion of nitrate (NO_3) in the diet may provide positive benefits which, in an appropriate legislative context (outlined in Appendix 1) may bring economic benefits:

- The reduction of NO_3 in the rumen removes electrons from the electron carrier, NADH and returns it to its oxidised form, NAD^+. In rumen microbes, an adequate supply of NAD^+ is essential to enable them to digest feed by fermenting glucose and other sugars to pyruvate and short-chain fatty acids (acetate, propionate and butyrate) via the glycolytic pathway. In the absence of NO_3 which has a high affinity for electrons, this re-oxidation is maintained by the flow of electrons to CO_2 which is reduced to methane. Addition of NO_3 to the diet has been shown repeatedly to be an effective method of reducing methane emissions.

- The reduction of NO_3 to NO_2 and then to ammonia also provides the rumen microbes with a source of Non-Protein Nitrogen (NPN). NPN is used by microbes as a source of amino groups from which amino acids, proteins and other nitrogenous polymers are synthesised. In this respect, NO_3 can be used in diets as an alternative to urea - a widely used NPN supplement for grazing ruminants.

- It has been suggested that animals receiving iso-nitrogenous amounts of supplementary NO_3 in place of urea should retain more ME because fermentable energy is not emitted as methane. However, this benefit is probably small because some energy is used in reducing NO_3 to ammonia, two moles of which are already present in urea (see Appendix 3).

Importantly, a downside to the use of NO_3 in diets for ruminants, *viz*. the potential for NO_2 intoxication. Nitrate reduction in rumen microbes occurs via two major redox reactions, $NO_3 \rightarrow NO_2$ (Step 1) and $NO_2 \rightarrow NH_3$ (Step 2). Both steps are catalysed by intracellular enzymes, i.e. NO_3 reductase and NO_2 reductase, respectively. Importantly, Step 2 seems to be promoted by the presence of fermentable energy in the diet and by acclimation of the animal to dietary NO_3. If the rate of Step 1 exceeds the rate of Step 2, NO_2 will accumulate in the rumen contents and, because the NO_2 concentration in the blood is normally low, a NO_2 concentration gradient forms between the gut and bloodstream and NO_2 is absorbed. As the amount of NO_2 absorbed increases, MetHb concentrations in the bloodstream increase from normal values (1-2%) to values in excess of 5-10% which can have adverse clinical effects. In addition, there is an increase in the concentration of nitric oxide (NO) which has many clinical effects in the animal – some potentially beneficial and some detrimental.

Oxy-haemoglobin ($Hb\text{-}Fe^{2+}$; oxyHb) and MetHb in blood form a redox couple that acts catalytically as a NO_2 reductase, generating nitric oxide (NO) from NO_2. This enzyme system

responds to the oxygen level in the blood and catalyses the conversion of NO_2 to nitric oxide (NO) which in turn lowers vascular tone and, in addition, has many other physiological effects. The formation of MetHb in the blood is an important indicator of the likelihood of NO_2 poisoning. The MetHb is formed by the oxidation (loss of electrons) of ferrous (Fe^{2+}) haemoglobin (oxyHb) to ferric (Fe^{3+}) MetHb by reaction with NO_2. Each mole of oxyHb that is converted to MetHb causes the oxidation of 1.5 mol of NO_2 to NO_3 and consumes 1 mol of protons. MetHb reductase, a NADH-dependent enzyme, is responsible for converting MetHb back to oxyHb. As the ratio of MetHb to oxyHb increases, vascular tone decreases causing hypotension. Increased MetHb: oxyHb and lowered blood pressure both diminish the rate of oxygen transfer to body tissues despite some blood flow rate increases. In addition, the release of heme from MetHb can lead to inflammation.

The quantity of NO_2 in blood at any time depends not only on the rate of entry from the gut and endogenous sources, but also on its rate of removal. A single i/v dose of NO_2 is rapidly eliminated from the blood (Lewicki *et al.* 1994). Elimination of NO_2 occurs via several routes. In sheep, only 0.1% of the NO_2 administered intravenously was removed via the kidneys. The majority of the NO_2 was oxidised to NO_3 but some NO_2 may have been transferred to the digestive tract via saliva and other secretions (Lewicki *et al.* 1998). Isotope studies are required to identify if these excretory pathways occur in the same proportions with physiological levels of nitrite

In summary, inclusion of NO_3 in the diet does reduce methane emissions effectively (Nolan *et al.* 2010). However, there is a risk that, under certain circumstances, this mitigation strategy will induce NO_2 poisoning and hypoxia in animals. The consequence can be a loss of production or even death if the factors leading to this condition are not understood and managed effectively. This is especially important in animals subject to poor quality nutrition (Callaghan *et al.* 2014). The benefits in terms of methane mitigation from NO_3 inclusion in diets must be weighed against the risks of NO_2 accumulation in rumen contents and transfer to the bloodstream. The factors that could reduce these risks need to be understood and applied to management practices.

The focus in this review is to consolidate understanding of the metabolism of dietary NO_3 in the digestive tract and of MetHb in the blood, enabling identification of (i) potential control points to reduce the risk of NO_2 toxicity in NO_3-fed ruminants and (ii) potential strategies of intervention. The review is structured according to the hypothesis that the adverse consequences of NO_3 or NO_2 ingestion can be prevented by managing MetHb accumulation in blood by (1) reducing NO_2 entry into the blood and by (2) reducing MetHb concentration in the red blood cell. Critical control points regulating these two key processes are examined according to the framework displayed on the following page.

5 Review framework

"Strategies and critical control points by which the risk of methaemoglobinaemia may be controlled in NO_3-fed livestock"

Strategy 1: Reduce ruminal NO_2 accumulation and its entry into the bloodstream

(1) Reduce the rate of NO_2 production from NO_3

a. Reduce quantity of NO_3 ingested or its rate of release in the rumen

b. Decrease NO_3 uptake by rumen organisms (membrane transport)
c. Increase loss of NO_3 to lower digestive tract
d. Maximise NO_3-N use in microbial synthesis

(2) Accelerate rate of NO_2 conversion to ammonia by rumen microbes
a. Maximise the NO_2 reductase activity
b. Direct NO_2 to other end-products (e.g. N_2O)

(3) Prevent absorption of ruminal NO_2
a. Increase oxidation of NO_2 at the gut wall
b. Stimulate competitive inhibition of NO_2 absorption

<u>Strategy 2</u>: Reduce methaemoglobin concentration in the red blood cell (RBC)

(1) Slow the rate of methaemoglobin formation
c. Increase blood NO_2 loss via kidneys
d. Increase blood NO_2 transfer into the gut
e. Increase excretion of NO_3/NO_2 via saliva
f. Accelerate transformation of nitrite in the blood
g. Minimise NO_2 uptake by the red blood cells
h. Increase rate of oxidation of NO_2 to NO_3

(2) Accelerate the reduction of methaemoglobin back to haemoglobin
a. Accelerate the rate of regeneration of oxy-haemoglobin

These control strategies are considered in the following report and associated appendices. All items are considered, but may not be itemised individually subject to the quantity of information available. To aid application, key assessments are condensed into 'Take-home messages' at the conclusion of key sections.

<u>STRATEGY 1</u>: Reducing NO_2 entry into the blood

5.1 Reduce the rate of NO_2 production from NO_3

Most animal deaths associated with NO_2 toxicity have occurred in production environments where the quantity of feed ingested and its NO_3 content are not known. A review of the NO_3 intakes associated with (1) water, (2) feeds and forages and (3) feed supplements is provided in Appendix 1. Common NO_3 salts are readily soluble and are quickly ionised in the rumen, so it is necessary to balance the positive and negative possibilities - that is, to provide a quantity of NO_3 that is safe for the animal yet delivers significant methane mitigation?

As a general rule, the total NO_3 intake (entry into the rumen) should not exceed the <u>current</u> requirement for microbial synthesis of nitrogenous cell polymers by rumen microbes. This requirement depends on the current availability of energy substrates and other nutrients (including other sources of N) in the diet. The risk of poisoning is greater in animals subject to poor quality nutrition because of low substrate availability to support microbial growth.

A corollary to this rule is that the quantity of NO_3-N entering rumen contents should be synchronised to match the rate of microbial cell synthesis. Nitrate is generally rapidly released upon entry into the rumen whereas microbial growth increases for several hours. High rates of NO_3 intake and release in the rumen are therefore undesirable.

A reasonable maximum target value for NO_3 intake would be 2 g NO_3-N/MJ of ME intake (CSIRO 2007), equivalent to an inclusion rate of about 2% NO_3-N in a low-N diet with a digestibility of approximately 70%. This rate of NO_3 inclusion can be expected to reduce methane emissions by approximately 20 % but is higher than the currently approved methodology. Nitrate in excess of current microbial N requirement is wasted, leaving the rumen as ammonia (major pathway) or as NO_3 or NO_2 (which represent losses that, although relatively minor in quantity, can lead to NO_2 poisoning). Notably, nitrogen that is not incorporated into tissues or products is excreted in urine of grazing animals and can give rise to the generation of greenhouse gases from the soil that could negate the benefits of reduced methane emissions from the animal.

Recent observations at UNE provide further understanding on the value of matching nitrate and carbohydrate delivery as a basis for managing NO_3 supply. The studies summarised in Table 1 show that is it not just the quantity of NO_3 ingested but, critically, the manner in which it is offered that can help minimise potential increases in blood NO_2 and MetHb concentrations - our ultimate targets for preventing NO_2 poisoning (see also Appendix 6).

Table 1. Methaemoglobin concentrations in two studies of sheep given the same oaten chaff based diet. The diet was either offered* ad libitum *and was therefore accessible for the whole day (Study 1), or it was offered once per day and restricted to 1000 g (as fed)(Study 2)

Study	Sheep/group	Liveweight (kg)	Feed NO_3 conc. (% of DM)	DM intake (g/day)	MetHb conc (% at 2.5)
1	20	33.5±0.85	2.0	1182±69	1.60±0.1
2	6	31.8 ±3.7	2.0	882±90	14.0±1.6

Study 1: Godwin *et al.* (sheep fed *ad libitum,* paper in preparation)
Study 2: de Raphélis-Soissan *et al.* (sheep fed at 08:00 h, paper in preparation)

The contrast between the two studies indicates a diet containing 2% NO_3 can be safe and lead to minimal MetHb if continuously available *ad libitum* throughout the day (Study 1), or can lead to high MetHb if provided as a single meal (Study 2). This is a very subtle difference in feeding for a major difference in MetHb concentration, suggesting any period of low feed availability (even a few hours) may increase risk of MetHb formation. Presumably this is due to more rapid feed consumption once feed is available after a fasting period (Guerink *et al.* 1979). One strategy for achieving prolonged delivery to the rumen microbes is by mixing the NO_3 in the complete ration for *ad-libitum* fed livestock (de Raphélis-Soissan, paper in preparation), another is to provide the NO_3 in a protected form that is released more slowly to the rumen organisms.

Lipid- or polymer-coating of NO_3 salts offers a potential mechanism to enable once-a-day feeding of NO_3 to be undertaken more safely. Various techniques that have been used to produce slow-release fertilizer for use on pasture and crops might be adapted for ruminant feeding, e.g. urea-formaldehyde resin has been used as a component of fertilisers as a substantial portion of the N present in urea-formaldehyde resins is relatively insoluble in cold water. There have been attempts to adapt this process to develop slow-release urea

supplements for ruminants. A slow-release form of NO_3 is produced commercially by GRASP®, Curitiba-Paraná, Brazil. This product has been found to reduce the rate of NO_3 solubilisation in the rumen while still achieving expected levels of methane mitigation (C. Lee, pers. comm. – Agriculture and Agri-Food Canada, Lethbridge Research Centre, Lethbridge, Canada T1J4B1).

In the animal nutrition industry, encapsulation has only been in use for about a decade (Emanuelle 2006). Micro-encapsulation has been successfully applied to 'protect' natural compounds, like essential oils or vegetable extracts containing polyphenols with well-known antimicrobial properties. For example, Mamvura *et al.* (2014) used sesame gum to coat NO_3 and slow its rate of release in the rumen. Although such techniques have potential, adoption has been restricted because of the higher price of treated compared to untreated products (e.g. Addiscott (2005), p .60) and the low economic returns from methane mitigation.

Delivery of NO_3 in lick blocks is another practical means of restricting intake and prolonging the period of entry of NO_3 into the rumen. Challenges with lick blocks are that intake may decline with increasing NO_3 content and be variable between animals. Between-animal intakes can vary widely even under apparently similar conditions. For example, Cockwill *et al.* (2000) found that intake of molasses blocks (NO_3-free) by grazing beef cows averaged 445 g/d but ranged between 0 and 1650 g/d (SEM = 438 g/d). The existence of non-eaters and over-eaters has been a common finding in similar studies (Nolan *et al.* 1974). Intake of blocks containing NO_3 by cattle in a trial near Charleville was lower than of similar blocks containing urea (R.A. Leng, unpublished) suggesting NO_3-containing blocks are not as readily accepted by ruminants as blocks containing iso-nitrogenous amounts of urea. With sheep on a hay-based diet, intake was reduced as NO_3 concentrations increased, whereas intake of blocks containing urea increased over the same range of N inclusion (Li *et al.* 2012). Thus, the range of intakes may be smaller with blocks containing NO_3 and intake may also be extended over a longer period - both factors will reduce the likelihood of poisoning. This self-regulating impact of nitrate intake has also been shown in liquid supplements in cattle where NO_3 inclusion reduced molasses intake from licker drums (Goopy 2014).

Sokolowski *et al*. (1969) found that addition of inorganic sulfur (S) to a NO_3^-containing diet improved the utilisation of NO_3-N by lambs. In addition, the extra S facilitated NO_3 and NO_2 reduction to NH_3 in the rumen, thereby reducing the likelihood of methaemoglobinaemia (Leng 2010). van Zijderveld *et al*. (2010) showed that the addition of sulfate (SO_4)-S (0.85% total S in dry matter (DM)) to a diet containing NO_3 (2.6% NO_3 in DM) reduced methane production by ~21% compared with a diet containing NO_3 with no added sulfate. The study of Li *et al.* (2013) confirmed the effects of S on wool growth and methane production, but could not identify the expected interaction between NO_3 and S. There is, therefore, till a lack of clarity on whether higher S intakes can play a role in moderating nitrite toxicity.

<u>Take-home messages</u>:

1. ***Nitrate should be supplied in a total quantity not exceeding that required for microbial cell synthesis (2 g NO3/MJ ME intake) and provided in a diurnal profile matching the fermentable feed energy supply, with no periods in which feed is unavailable.***

2. ***Slowing the rate of nitrate solubilisation in the rumen may be achievable by chemical coating of the nitrate, but there is little data on this.***

3. ***While between-animal variation in supplement intake is recognised, and high nitrate blocks are not readily consumed, there is insufficient data on the level of NO3 intake and between-animal variation of in intake when ruminants are offered lick-blocks containing NO3. This is a key constraint to safe implementation of the current CFI by industry.***

5.2 Increase uptake of NO_3 by rumen organisms

A transport mechanism is required to carry NO_3 into the cell, *e.g.* NO_3/NO_2 porter (NNP) family TC#2.A.1.8. This could represent a site of intervention to reduce NO_3 uptake by rumen microbes and therefore its reduction to NO_2. Many bacterial species contain NarK-like transporters that act as either NO_3/proton symporters or NO_3/NO_2 antiporters. As yet, based on sequence homology, these transporters remain, in general, poorly characterised (Goddard *et al.* 2008). The NO_3 reductase systems are intracellular (discussed in more detail below) and NO_3 reduction is stimulated by high NO_3 concentrations in the microbial cytoplasm. However, some microbes have NO_3 or NO_2 reductase enzymes in their periplasm (the space between the cell membrane and the peptidoglycan layer).

There appear to be no industry-practical inhibitors of NO_3 loading systems, although inhibitors of these symport systems (Phenylglyoxal, Nigericin) are used for experimental purposes (*e.g.* Kucera 2005). Notably, these workers reported that substitution of butyrate or acetate for chloride in Tris-buffered assay media for anaerobic cultures of intact cells of *P. denitrificans* resulted in a marked inhibition at pH 7 of the proton motive force across the cytoplasmic membrane and an associated reduction in NO_3 uptake. The possibility of interactions between short-chain fatty acid concentrations in the rumen and NO_3 uptake by rumen microbes deserves further study. It is not known if VFA could similarly affect NO_3 uptake by mammalian cells.

***Take-home message*: It is unrealistic to prevent rumen microbes taking up nitrate but their rate of uptake may be affected by rumen VFA conditions in ways that are not yet well understood.**

5.3 Reducing activity of NO_3 reductase

Strickland (1931) found that, in washed suspensions of *E. coli,* NO_3 was reduced quantitatively to NO_2 by H_2. Woods (1938) found that two strains of *E. coli* and one strain of *Clostridium welchii* were able to reduce NO_3 quantitatively to ammonia. In this reaction, there was a transient appearance of the intermediate NO_2. There was initially a high rate of H_2 uptake during NO_2 formation followed by a slower uptake when NO_2 was reduced to ammonia.

In the rumen, NO_3 reductases are found in both bacteria and protozoa, with NO_3 metabolism of *Wolinella succinogenes* most intensively studied. The NO_3 reductases are of two types, being assimilatory (a process in which rate of NO_3 reduction is directly linked to microbial synthesis and is readily inhibited by high ammonia concentration) and dissimilatory (NO_3 reduction is uncoupled from microbial synthesis and is not inhibited by high ammonia concentration). There are three NO_3 reductase systems likely to be operating in rumen microbes. These are found in intracellular contents and in the periplasm. The biochemistry and enzymology of bacterial NO_3 reduction are described in more detail in Appendix 5. It should be noted that most of the information on reductases has been drawn from studies of non-rumen microbes and there is little published information on NO_3 reduction systems of predominant rumen organisms. Inhibitors of NO_3 reductases are known (*e.g*. Cu, Cr, Co, Pb cations, cyanide, thiocyanate, chloromercuribenzoate, p-hydroxybenzoate (http://www.brenda-enzymes.org/enzyme.php?ecno=1.7.1.1) but stopping NO_3 reduction completely would also prevent methane mitigation. For this reason, the desired outcome is to inhibit the rate of dissimilatory NO_3 reduction to NO_2 whilst maintaining or increasing the rate of reduction of NO_2 to ammonia (as outlined in the following sections).

***Take-home message*: Completely stopping ruminal nitrate reductase activity is neither practical nor desirable; however, we need strategies that reduce NO_3 reductase**

activity while increasing NO_2 reductase activity to avoid nitrite accumulation in rumen fluid.

5.4 Maximising NO_2 use in microbial synthesis

As part of the strategy aimed at avoiding MetHb formation, there is a requirement to minimise the ruminal absorption of NO_2 (the MetHb precursor) or its escape by outflow from the rumen. As already discussed, this can be done by synchronising the availability of NO_3 in the rumen with the availability of high energy substrates; the aim is to maximise assimilatory NO_3 and NO_2 reduction and minimise dissimilatory NO_2 reduction that is thought to raise rumen NO_2 concentration and, in turn, blood NO_2 concentration.

An overview of rumen fermentation and the pathways that generate NADH for these reductases is given in Appendix 3. The biochemistry of microbial cytoplasmic and periplasmic NO_2 reductases is described in greater detail in Appendix 5. From these combined understandings of N and energy metabolism, it is hypothesised that there may be opportunity to selectively modify the balance of reductase activities by controlling the quantity of NADH available in the cell.

Various studies have shown that the presence of rapidly fermentable carbohydrates can protect ruminants from NO_2 poisoning (Barnett and Bowman 1957; Holtenius 1957; Emerick *et al.* 1965; Bruning-Fann and Kaneene 1993) but the reasons for the 'protection' are not clear. The presence of the fermentable carbohydrate may stimulate changes in the microbial population that could favour NO_2 reduction (Yoshii *et al.* 2003); NO_3 and NO_2 reductions are also pH-dependent, having pH optima of 6.5 and 5.6, respectively (Tillman *et al.* 1965). The presence of more rapidly fermentable substrate in the diet should therefore be beneficial because it tends to lower rumen fluid pH which should, in turn, increase NO_2 reduction rate and reduce NO_3 reduction rate thereby lowering NO_3 concentration and absorption from the rumen. Another possibility is that, if NO_2 reduction is associated with N assimilation, it will be inhibited at higher ammonia concentrations which depend on the total fermentable N in the diet.

In many bacteria, both assimilatory and dissimilatory pathways exist. Nitrate and NO_2 reduction may occur by the NO_3 and NO_2 assimilatory enzyme (Nas; which reduces both), but the Nas enzyme, to our knowledge, has not yet been even looked for in rumen microbes. In contrast to dissimilatory enzymes, Nas is inhibited by high ammonia concentrations. Dissimilatory nitrate reductase (Nar, Nap) and nitrite reductase (Nir) enzymes are also present in rumen microbes and most are linked with an anaerobic electron transport chain (ETC). The nitrite reducing Nir enzyme, which has not yet been identified in rumen contents, is the only dissimilatory enzyme that does not depend on an ETC. The Nir reduction is an energy demanding reaction that is not inhibited by high NO_3 or NO_2 levels. Its major role is to prevent NO_2 poisoning in the cell. Nir utilizes NADH originating mainly from the glycolytic pathway as an electron donor. Higher rates of fermentation of carbohydrates via the glycolytic pathway which increase the NADH to NAD^+ ratio may, in turn, stimulate Nir activity (Iwamoto *et al.* 2001). In contrast, NO_3 reducing pathways, particularly Nar, are more energy efficient and less dependent on NADH. *Selenomonas ruminantium* utilises the Nar pathway (Asanuma *et al.* 2014) which is stimulated when there is a need to generate ATP via the ETC (Iwamoto *et al.* 2001). It is hypothesised that Nir may be enhanced relative to Nar when there is a source of readily available carbohydrates to generate a higher NADH:NAD^+ ratio in the microbial cell.

<u>Take-home message</u>: Lower pH should reduce risk of nitrite accumulation and this together with the higher NADH:NAD+ ratio in the rumen of sheep with highly fermentable energy intakes points to the risk of nitrite toxicity being least in grain-fed ruminants.

5.5 Acclimating the rumen microbial population to NO_3 in the diet

There is evidence that rumen microbial populations of cattle and sheep can adapt to the continuing presence of NO_3 in the diet if the amount of NO_3 ingested is gradually increased over days or weeks (Sinclair and Jones 1964; Farra and Satter 1971).

Lin *et al.* (2013) and Alaboudi and Jones (1985) found that there were changes in the composition of the rumen bacterial community of steers acclimated to dietary NO_3. Further, NO_3 disappeared more rapidly from incubations of rumen fluid from NO_3-adapted than from control steers.

Alaboudi and Jones (1985) compared the NO_3 and NO_2 reductase activities in rumen contents of sheep acclimated over a period of several weeks to dietary KNO_3 (2.5 g/kgW per d, equivalent to about 7% NO_3 in the diet as fed which, notably, is much higher than the concentration normally offered in practice). These workers found that the activities of both reductases were higher in rumen fluid from acclimated sheep but, significantly, the fraction of NO_3 reducing bacteria in rumen fluid was three-fold higher in the acclimated sheep, whereas the rate of NO_2 reduction was five-fold higher than in the control sheep. The higher NO_2 reduction activity in acclimated sheep means that their rumen NO_2 will be removed more quickly than in control sheep and peak NO_2 concentrations and rates of absorption into the bloodstream will be lower. After removal of NO_3 from the diet, the effects of acclimation declined and were no longer present after 3 weeks.

There are few other studies of the role of acclimation and the benefits of acclimation are still unclear. On present knowledge, it can be concluded that NO_2 concentration, absorption and accumulation in the bloodstream of acclimated ruminants, and the likelihood of NO_2 poisoning will probably be lower in acclimated ruminants than in non-acclimated ruminants. Nevertheless, this question requires clarification, as high MetHB levels have been regularly seen in sheep and cattle that have been on nitrate rich diets for weeks or months (*e.g.* Table 1) and MetHB climbs over time. Acclimation alone can't be relied on to ensure low MetHB.

Cockrum *et al.* (2010) studied the effects of dietary NO_3 (300 mg NO_3^-/kg BW daily or about 1.2% NO_3 in feed DM) on the performance of Suffolk ewes identified as being highly tolerant or less tolerant to the inclusion of NO_3^- in the diet. The tolerance ranking was based on individual performance and signs of NO_3^- poisoning. The inclusion of NO_3^- affected feed intake and intake was more variable in NO_3^- -treated ewes (CV = 59.3%) than in control ewes (CV = 13.6%) and intake was lower ($P < 0.001$) in NO_3-treated ewes than in control ewes. Intake of highly and lowly tolerant ewes was 82% and 23%, respectively, of the control (non-nitrate) ewes' intake.

The source of the NO_3/NO_2 tolerance may be related to differences in the microbial eco-system in the rumen or to factors associated with tissues, or both.

<u>Take-home message:</u> The likelihood of NO_2 poisoning will be lower in acclimated ruminants than in non-acclimated ruminants. Acclimation leads to significantly higher levels of both nitrate and nitrite reductase in the rumen; however, factors affecting the change in the balance of these two processes, ultimately affecting the extracellular nitrite concentration, are still not well understood. High MetHB levels of some sheep adapted to NO_3 indicate acclimation cannot be relied upon to ensure low MetHB. There is considerable between-animal variation in tolerance to dietary nitrate, but the heritability of this trait has not been studied.

5.6 Conversion of NO_2 to other end-products (e.g. N_2O)

Nitrous oxide (N_2O) is produced in small quantities in the human gut and it is also produced in the rumen of NO_3-fed ruminants (details in Appendix 8). The Nir enzyme converts NO_2 to nitric oxide (Moreno-Vivian *et al.* 1999). The greenhouse gas equivalents of N_2O and CH_4 are respectively 298 and 25 CO_2 equivalents (Forster *et al.* 2007). The much higher global warming potential of N_2O means that, if even a small percentage of dietary NO_3 is converted to N_2O rather than to ammonia, it could partly undo any methane mitigation effect arising from dietary NO_3 feeding. For example, for a sheep with a DM intake of 1 kg/d containing 2% NO_3, 20 g or 0.32 mol NO_3 is ingested. In theory, this NO_3 could lower CH_4 production by 0.32 mol or 129 g CO_2-equivalents. If, however, 1% of the dietary NO_3 (i.e. 0.2 g NO_2) were converted to N_2O, this would give rise to 0.071g N_2O, or 21.5 g CO_2-equivalents, lowering the effective methane mitigation effect by 17%.

In a study at UNE (de Raphélis-Soissan *et al.* 2014), sheep fed 1 kg/d of a diet containing 2% NO_3 produced 0.05 g N_2O (or 14.9 g CO_2-eq) per day. Thus, 0.14g NO_2 would have been converted to N_2O, meaning that 0.7% of their dietary NO_3 intake was excreted as N_2O. This would have reduced the effective methane mitigation by about 12%.

<u>Take-home message:</u> Nitrous oxide production in the rumen has been little studied; however, available studies suggest N_2O, in certain circumstances, may offset approximately 15% of mitigation arising from decreased methane production.

5.7 Nitrate absorption across the gut wall

Many reviewers appear to assume that NO_3 (and NO_2) are directly absorbed through the rumen wall of ruminants. However, Pfander *et al.* (1956) placed buffer solutions containing either NO_3 or NO_2 (at 670 mg/L) in the isolated rumen of anaesthetised sheep and found no evidence of either ion being removed from the rumen or appearing in the bloodstream.

Remarkably, there appear to be few more recent direct measurements of NO_3 and NO_2 uptake across the rumen wall. The argument that NO_3 is directly absorbed across the rumen wall seems to have been accepted on the basis that there is no obvious lag before the appearance of NO_3 in the blood when NO_3 is administered to the rumen. Similarly, it has been concluded that NO_2 is also absorbed across the rumen wall because NO_3 and MetHb concentrations increase almost immediately after NO_2 is placed in the rumen (Wang *et al.* 1961) (see Appendix 6). In the case of NO_2 uptake across the rumen wall, we (I.R. Godwin and J.V. Nolan, see Appendix 6) found that uptake of NO_2 did occur across the rumen wall of the isolated rumen in an anaesthesised sheep.

Wurmli *et al.* (1987) have suggested that NO_3 is probably absorbed across the rumen epithelium by a Cl^-/HCO_3^- exchange mechanism which is also the major route of Cl^- absorption. Absorption of Cl^- is almost completely inhibited in the presence of NO_3 but it is not known whether the reverse is also true. Whether the presence of NaCl in blocks may slow the rate of NO_2 uptake and reduce the risk of NO_2 poisoning deserves further study. Notably, NO_3 is also a potent inhibitor of acetate uptake (Aschenbach *et al.* 2009) and this could partly explain the increase in acetate:propionate ratio seen when NO_3 is used as a means of mitigating methane emissions in ruminants.

Nitrate and NO_2 absorption have been more extensively studied in non-ruminants. Sodium nitrite is rapidly absorbed from the stomach of mice, the rate constant being about 0.15/h and prevention of digesta flow to the duodenum with ligatures made no difference to overall absorption (Friedman *et al.* 1972). These findings fit with data from Grudzinski (1991) who found that as little as 10% of nitrite absorption occurred in perfused intestine of the rat. He also found that nitrite inhibited enterocyte Na-K-ATPase activity which may well influence the

sodium linked-active transport of many compounds. Hunault *et al.* (2009) found that, after oral administration in humans, between 95 and 98% of an aqueous solution of NO_2 was absorbed and that there was virtually no first pass effect of the liver.

In terms of NO_3 affecting the gut (in difference to the gut absorbing or reducing nitrate/nitrite), dietary nitrate and nitrite increase gastric mucosal protection in rats by increasing blood flow simply by increasing the non-enzymatic production of NO (Petersson *et al.* 2007). Also, NO causes relaxation of the oesophageal groove in calves (Barahona *et al.* 1998). Nitric oxide also reduces rumen turnover and contraction rate in sheep (Chen and Godwin 2013)

Take-home message:

1. ***Compounds secondary to NO3 metabolism, especially NO, may alter rumen dynamics and blood flow to the digestive tract, and in turn alter the expected behaviour of absorptive kinetics of the NO3/NO2.***

2. ***Research is needed to confirm the sites of NO3 and NO2 absorption and how absorption is regulated.***

STRATEGY 2: Reducing methaemoglobin accumulation

5.8 Slow the rate of methaemoglobin formation

5.8.1 Accelerate renal faecal and salivary excretion of nitrate/nitrite

In humans, NO_3 is absorbed from the upper GIT and 65-75% of an oral loading dose is excreted in the urine within 24 h while less than 1% is excreted in the faeces (Bartholomew and Hill 1984). Nitrate is readily filtered by the glomeruli and reabsorbed in the tubules. The concentration of NO_3 in human urine is 0.2-2.0 mmol/L while the concentration of NO_2 is virtually undetectable (Green *et al.* 1982). However, it should be noted that in humans urinary NO_3 excretion may exceed dietary NO_3 intake by a factor of 2 to 60 (Tannenbaum 1994). In a study using anaesthetised dogs with denervated kidneys, Godfrey and Majid (1998) showed that NO_3 excretion paralleled that of filtered load with no evidence of a tubular maxima, but as circulating NO_3 levels rose the percentage reabsorption showed a progressive decline. High levels of NO_3 entering the loop of Henle result in a reduced diluting capacity of the thick ascending limb due to a reduction in active chloride transport (Gutsche *et al.* 1984). Renal carbonic anhydrase is involved in the reabsorption of NO_2 because acetazolamide, an inhibitor of carbonic anhydrase, increases the excretion of NO_2, probably by blocking the production of acid-labile nitrito carbonate $ONOCOO^-$, the form in which NO_2 is actively transported in the kidney (Chobanyan-Jurgens *et al.* 2012). Use of the diuretic acetazolamide to increase NO_2 excretion is a possibility, but as ruminants grazing pasture generally have quite alkaline urine, the effect may be minimal.

Lewicki *et al.* (1994) gave an intravenous bolus dose of 400 µmol NO_2/kgW to 6 Polish wethers and monitored the changes in concentrations of NO_2 and NO_3 in plasma and urine for 30 h. The NO_3 and NO_2 were distributed in a volume of 12 L, equivalent to about 25% of the average liveweight of the sheep and therefore the distribution volume probably corresponded to extracellular fluid. The results highlighted the rapid turnover of the NO_2 pool

(t1/2 =0.49 h) and the slower turnover of the NO_3 pool. Urine excretion of NO_2 amounted to only 0.29% of the administered dose. Urine excretion of NO_3 was 13.8% of the NO_2 administered. The NO_3 was most likely formed by oxidation of the administered NO_2. These workers also measured urinary excretion of NO_3 following intravenous administration of 400 umol/kg W of $NaNO_3$. They found 23.6% of the NO_3 administered was excreted as NO_3 in urine and a further 14.8% of the NO_3-N was excreted as urea and 0.1% was excreted as ammonia (total 38.5%). These later results were consistent with the earlier study.

Schneider and Yeary (1975) gave a intravenous bolus dose of 20 mg NO_2/kgW (45 mmol) to 7 adult cross-bred sheep (weights ranging from 30-74 kg) and monitored the changes in concentrations of plasma NO_2 and NO_3 for up to 24 h.

Data of Lewicki *et al.* (1994) and Schneider and Yeary (1975) were fitted to kinetic models representing the elimination of plasma NO_2 by excretion and oxidation to NO_3 (represented by the appearance of NO_3-N in plasma) (See Appendix 6). It seems likely that the major route of elimination of NO_2 is by oxidation in the blood to NO_3 and the major route of elimination of NO3 from blood is by transfer into the gut.

About 25% of NO_2 injected intraperitoneally in mice appears as urea in the urine and about 50% as NO_3. On the other hand, virtually no urea is produced from intraperitoneally injected NO_3 (Yoshida *et al.* 1983). The mechanism for this conversion of NO_2 to urea is unclear and further work is required to properly characterise the pathways of NO_2 elimination from the bloodstream.

***Take-home message*: The pharmacokinetics of NO_3 and NO_2 not been well studied in any animal species. In ruminants, it seems likely that the major route of elimination of blood NO_2 is by oxidation to NO_3 and the major route of elimination of NO_3 from blood is by transfer into the gut. Some blood NO_3 is excreted in saliva but the quantitative significance of this transfer requires more detailed study in ruminants. Based on the information available, there appear to be no practical means of increasing the losses of NO_2 from blood by increasing the rate of excretion in urine or into the gut.**

5.8.2 Reduce nitrite entry into the red blood cell (RBC)

Nitrite is rapidly taken up by red cells (Hon *et al.* 2010). With plasma concentrations of less than 100 µmol/L there is virtually no accumulation of NO_2 in the red cell or MetHb formation (May *et al.* 2000a). At physiological pH, NO_2 is an anion (pK_a 3.2). Nitrite uptake by RBC is pH sensitive and is greater at pH 6.9 compared to pH 7.8, suggesting that the entry into cells is via a charge-sensitive mechanism (May *et al.* 2000a), with simple diffusion limited because of the negative charge. How does this anion traverse the reds cell membrane? Under normal conditions the concentration of NO_2 in RBC is about 300 nmol/L (Dejam *et al.* 2005) and as it is continually metabolised via the reactions detailed above, it must be continually imported from the plasma that has a normal NO_2 concentration of between 0 and 20 µmol/L, with strong evidence that it is normally below 500nmol/L (Dejam *et al.* 2005). Whole blood NO_2 levels range from 35 to 1193 nmol/L (Kehmeier *et al.* 2008). The RBC membrane proteins transport many ions and the (Band 3) anion exchanger represents nearly a quarter of these proteins. It has carboxyl and amine termini in the cytoplasm (Campanella *et al.* 2005). DeoxyHb has a higher affinity for the NH_2 terminus of Band 3 than does oxyHb, thus linking oxygen sensing with NO_2 metabolism.

However, May *et al.* (2000a) found that DIDS (4,4'-diisothiocyanato-2,2'-stilbenedisulphonate), a Band 3 transport inhibitor, did not affect NO_2 uptake by RBC. The glucose transport inhibitors, phloretin and cytochalasin were also without effect. DIDS does however, inhibit nitrite efflux from RBCs (Shingles *et al.* 1997), indicating that Band 3 may be involved in the transport of NO_2 out of RBC. Earlier studies suggest that the RBC membrane does not present a barrier to NO_2 entry as rates of MetHb formation in intact cells was equivalent to that of hemolysates of RBC (Zavodnik *et al.* 1999). The reaction with haemoglobin occurring at a slower rate than the rate of entry into the RBC (May *et al.* 2000a). There are normally present, in equilibrium, in solutions of NO_2 small amounts of gaseous nitrogen oxide species such as nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$), dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4; Fig. 1) (Zhao *et al.* 1994), all of which would readily diffuse across the cell membrane. Diffusion of HNO_2 would also be possible and its concentration would be increased with a decrease in pH, possibly accounting for the pH effect noted by Iwamoto *et al.* (2001) .

Removal of sodium and/or the inclusion of phosphate into incubation media reduces the uptake of NO_2 into RBC (May *et al.* 2000b), suggesting that the sodium-inorganic phosphate transporter system (Shoemaker *et al.* 1988) is involved in NO_2 uptake. May *et al.* (2000a) suggest that about 25% of NO_2 is taken up by this transporter and the remainder by diffusion of HNO_2 and the various gaseous species.

The rate of NO_2 uptake by RBC is also dependent on the oxygen status of the RBC. Uptake of NO_2 is enhanced in the deoxygenated RBC in humans (Vitturi *et al.* 2006) but not in those from rats (Feelisch *et al.* 2008), pigs (Jensen 2005), sheep (Blood and Power 2007) and fish (Jensen 2003). Nitrite influx into the red cell is increased with increased temperature as is the nitrite reductase activity of the haemoglobin (Fens *et al.* 2014).

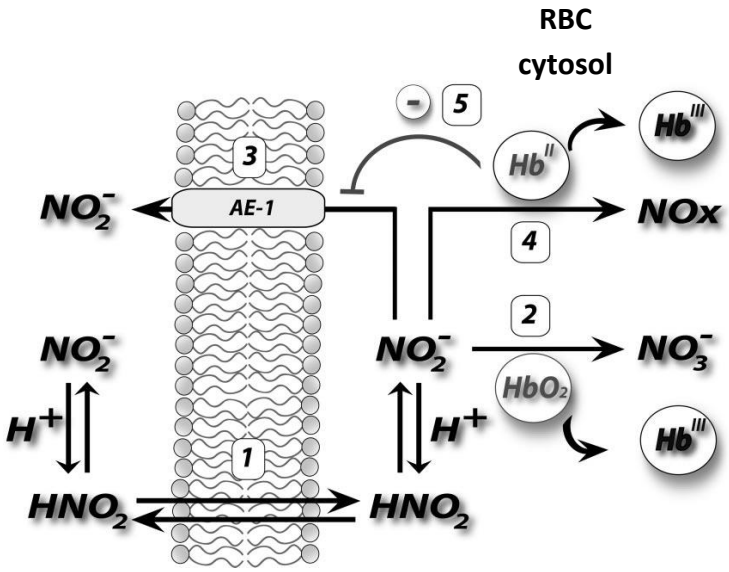


Fig. 1. S*cheme showing the proposed mechanism by which Hb deoxygenation regulates nitrite metabolism by RBCs.*
In step 1, nitrite moves into the RBC down an electrochemical/concentration gradient through either a channel and/or as nitrous acid. Under oxygenated conditions, the nitrite concentration gradient is regulated by intracellular reactions with oxyHb, resulting in nitrite oxidation (step 2) and export via anion exchanger (AE-1) (step 3). As RBCs desaturate, nitrite consumption is accelerated due to deoxyHb reactions, resulting in a species (NOx) that can ultimately produce NO after exiting the RBC (step 4) reaching maximal rates at the

Hb P50. Concomitantly, deoxyHb binds to and inhibits AE-1, thereby preventing export and maintaining intracellular nitrite levels (step 5)(Vitturi et al. 2009).

Interestingly, acetylcholine increases erythrocyte NO_2 levels and muscarinic antagonists decrease the levels via some interaction with the membrane enzyme acetylcholinesterase (Carvalho *et al.* 2004). The possibility of using muscarinic antagonists such as atropine or scopolamine to reduce NO_2 entry into red cells exists, but is unlikely to be practical due to the wide ranging side effects of muscarinic antagonists.

***Take-home message:* Entry of NO_2 into the RBC depends on pH, Na, P levels and temperature, but all are critical blood parameters not able to be substantially modified. Similarly, changing NO_2 entry by muscarinic antagonists like atropine or scopolamine would be associated with major metabolic changes in the animal so are not feasible.**

5.8.3 Antioxidant inhibition of methaemoglobin formation

When the capacity for oxyhemoglobin to consume NO_2 within cells is decreased, high levels of intracellular ascorbate can partially prevent NO_2-induced MetHb formation due to a direct reaction of ascorbate with NO_2. However, this reaction is relatively slow due to the ability of erythrocytes to scavenge NO_2 (May *et al.* 2000a).

Curcumin prevents NO_2-induced methaemoglobin formation in both haemolysates and intact red cells, but will not reverse methaemoglobin once formed (Unnikrishnan and Rao 1992), largely because it is an oxygen radical scavenger as well as a NO_2 scavenger (Unnikrishnan and Rao 1995). The efficacy of ascorbate or curcumin to prevent MetHb formation in ruminants is unknown, but neither is likely to escape the rumen fermentation process unscathed.

Take-home message: Antioxidants, such as ascorbate and curcumin, may reduce the natural oxidant pressure on haemoglobin, allowing greater 'buffering' from the onslaught induced by NO_2, but their practical application in ruminant animals may be difficult.

5.9 Accelerate transformation of nitrate/nitrite metabolism in blood and body tissues

Most recent research on NO_2/NO_3 metabolism is in the medical setting, due to its link to the production of NO and the many physiological effects that this mediator drives. Nitric oxide is normally considered to be generated from arginine via the activation of endothelial nitric oxide synthase (eNOS). The earlier literature suggests that NO_2 was a relatively inert by-product of NO metabolism (Kleinbongard *et al.* 2003); however the present view suggests that NO_2 is indeed a physiological mediator of NO signalling particularly during hypoxia (van Faassen *et al.* 2009). The reduction of NO_2 to NO can be stimulated by the addition of copper, with Cu^{2+} ions attached to albumin, being reduced by the body's own antioxidants to Cu^{1+} which then acts to reduce NO_2 non-enzymatically to NO (Oplander *et al.* 2013).

Nitrite is thought to be a storage form of NO in blood that can be transported to various tissues and delivered as NO during specific physiological or pathological conditions that require or manifest with vasodilation (Gladwin 2004). The NO is generated by the reduction of NO_2 by deoxyhaemoglobin in the red blood cell (Fig. 2).

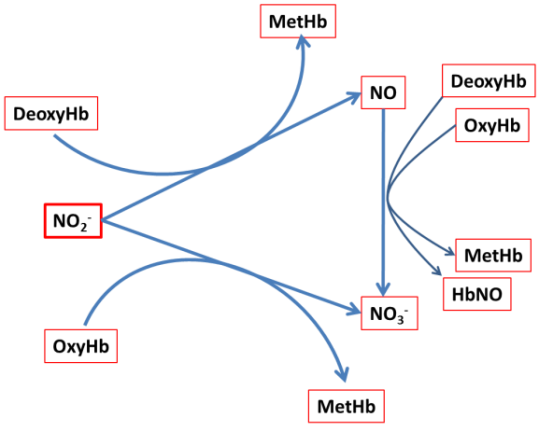


Fig. 2. ***Nitrite-haemoglobin interactions in the red blood cell (adapted from (Vega-Villa et al. 2013)***

Haemoglobin's primary role is as a reversible carrier of oxygen whose structure can be modulated between *relaxed* (R-state, high oxygen affinity) and *tense* (T-state, low-oxygen affinity) conformations. Oxyhaemoglobin and deoxyhaemoglobin scavenge NO with extreme rapidity. When well oxygenated, haemoglobin inhibits vessel dilation by NO_2, by scavenging NO. However when deoxygenated, haemoglobin does not inhibit nitrite-induced dilation. When the dilation is due to NO generation from a source independent of NO_2, both oxy- and deoxyhaemoglobin exhibit inhibition of vessel dilation (Dalsgaard *et al.* 2007; Isbell *et al.* 2007; Rodriguez *et al.* 2009; Vitturi *et al.* 2009). However there is evidence that NO_2 mediated vasodilation may be haemoglobin independent (Li *et al.* 2008).

The rate constants for NO scavenging by oxyHb (5×10^7 $M^{-1}s^{-1}$) and deoxyHb (3×10^7 $M^{-1}s^{-1}$) are very rapid and similar (Huang *et al.* 2007). The rate of NO_2 reduction by haemoglobin is three-fold faster with cell-free solutions of haemoglobin compared to intact red cell haemoglobin (Vitturi *et al.* 2009; Jensen and Rohde 2010). Stored blood also has a higher NO_2 reductase activity that results in a greater methaemoglobin concentration when exposed to nitrite (Almac *et al.* 2014). Early *in vitro* studies suggested that 1 mole of nitrite produces 2 moles of methaemoglobin (Greenberg *et al.* 1943).

The reaction of NO_2 with oxyHb is autocatalytic *in vitro*, but unlikely to be so under physiological conditions (Patel *et al.* 2011). The reaction progresses slowly at low NO_2 concentrations (k = 0.21–0.33 M−1 s−1) (Bellavia *et al.* 2013). At high NO_2 concentrations, the reaction becomes very efficient and autocatalytic (Rodkey 1976; Kosaka *et al.* 1979; Doyle *et al.* 1981).

The simplified equation:

$4NO_2^- + 4HbFe^{2+}\text{-}O_2 + 4H^+ \rightarrow 4\ HbFe^{3+} + 4NO_3^- + O_2 + 2H_2O$

has many intermediates of unknown importance and basically yields MetHb and NO_3.

The reaction of deoxyHb with NO_2 produces MetHb and iron nitrosyl haemoglobin (Hb-NO) in a two-step process where NO_2 is reduced by deoxyHb to yield MetHb and NO. The NO then goes on to bind to a vacant deoxyHb to yield Hb-NO.

$NO_2^- + HbFe^{2+} + H^+ \rightarrow NO + HbFe^{3+} + OH^-$

$NO + HbFe^{2+} \rightarrow HbFe^{2+}\text{-}NO$

When Hb is fully deoxygenated and in the T state it reacts slowly to reduce NO_2, but as each NO_2 molecule is reduced it generates both MetHb and NO, which in turn binds with another deoxyHb to form HbNO. When MetHb or HbNO are present as a tetramer then it is likely to promote a Hb conformational change to the R state. The more oxidation and nitrosylation of haems that occurs the more R state deoxyhaems will be present. The R state deoxyhaems show a faster reaction rate with NO_2 and hence the autocatalytic kinetics displayed (Huang *et al.* 2005b; Cantu-Medellin *et al.* 2011). This has the physiological implication of driving the production of NO more rapidly when oxygen tensions are low in tissue arterioles, resulting in vasodilation and greater blood flow (with oxygen delivery). Many intermediate compounds and reactions have been proposed, measured and largely dismissed as being of little consequence (Patel *et al.* 2011). It should be noted that gaseous NO when bubbled through fresh sheep blood led to substantial methaemoglobin formation largely from deoxyhaemoglobin (Iwamoto *et al.* 1994).

Take-home messages:

1. ***Nitrite is thought to be a storage form of NO in blood that can be transported to various tissues***

2. ***Preventing the interaction of blood NO_2 with haemoglobin is probably impossible and it should be noted that virtually all research on this topic is with 'physiological' concentrations of NO_2, not 'pharmacological' concentrations that arise with NO_3 feeding.***

5.10 Enhancing nitrogen oxide (NOx) efflux from the red cell

Much research has investigated the mechanism of NO escape from the RBC, because of its important role in vasodilation. Given the high concentration of haemoglobin in RBC (~20mmol/L) and its great propensity to scavenge NO, the lifespan of NO would only be about 1 µs and its diffusion distance would be about 0.1 µm (Cosby *et al.* 2003). As a consequence other mechanisms or species of NO efflux have been sought. Suggestions of export via the formation of nitrosothiols which are formed from N_2O_3 binding to thiols such as glutathione have been made (Nagababu *et al.* 2006). N_2O_3 is a strong nitrosating agent, which is in turn formed from the reaction of NO_2 with $HbFe^{3+}$-NO (Fernandez and Ford 2003) or alternatively $HbFe^{3+}$-NO_2 reacts with NO to form N_2O_3 (Basu *et al.* 2007). The N_2O_3 itself could also diffuse out of the red cell and dissociate into NO and NO_2 (Jeffers *et al.* 2005). Despite these suggestions mass spectrometry studies have shown no efflux of NO or N_2O_3 from red cells even when exposed to 16 mmol NO_2/L (Mikulski *et al.* 2010).

Nitrite exits the red cell via Band 3 protein as its movement is inhibited in red cell ghosts following the addition of DIDS (Shingles *et al.* 1997).

There appears to be no mechanism for enhancing NO_2 (and/or its metabolites) removal from the red cell. However, Fricker *et al.* (1997) suggest that some ruthenium (III) complexes are efficient at binding NO and would play a role in reducing the hypotension associated with NO_2 poisoning. Whether they would affect methaemoglobin formation is unknown. Use of agents such as cyanamide and disulfiram to reduce NO formation from tissue based haem-containing enzymes also reduces the effect of NO_2-induced hypotension (Whatman *et al.* 2013).

Take-home message: The red blood cell is normally an oxygen-rich environment so has a range of mechanisms to protect its functionality from damage by oxygen radicals, including NO. Antioxidants may be able to decrease MetHb concentration by preventing its formation and also by accelerating NO disposal.

5.11 Accelerating reduction of methaemoglobin back to haemoglobin in the red cell

Under normal circumstances, MetHb levels rarely exceed about 2% of total haemoglobin, simply because the small amounts produced by autoxidation or the action of oxidising chemicals are readily reduced by enzyme systems in the red cell. There are two enzyme systems capable of reducing MetHb.

The system normally most active is the NADH-dependent methaemoglobin reductase (NADH cytochrome b5 methaemoglobin reductase) (Agar and Harley 1972). With excessive NO_2 input as in NO_2 poisoning, this enzymatic reduction is simply overwhelmed and methaemoglobinaemia results. A second enzyme system that is normally inactive, NADPH-dependent methaemoglobin reductase, can be activated by the electron donor methylene blue. Methylene blue is reduced to leucomethylene blue and is oxidised back to methylene blue providing electrons for the non-enzymatic reduction of metHb back to haemoglobin (Janssen *et al.* 2004) (see Fig. 3).

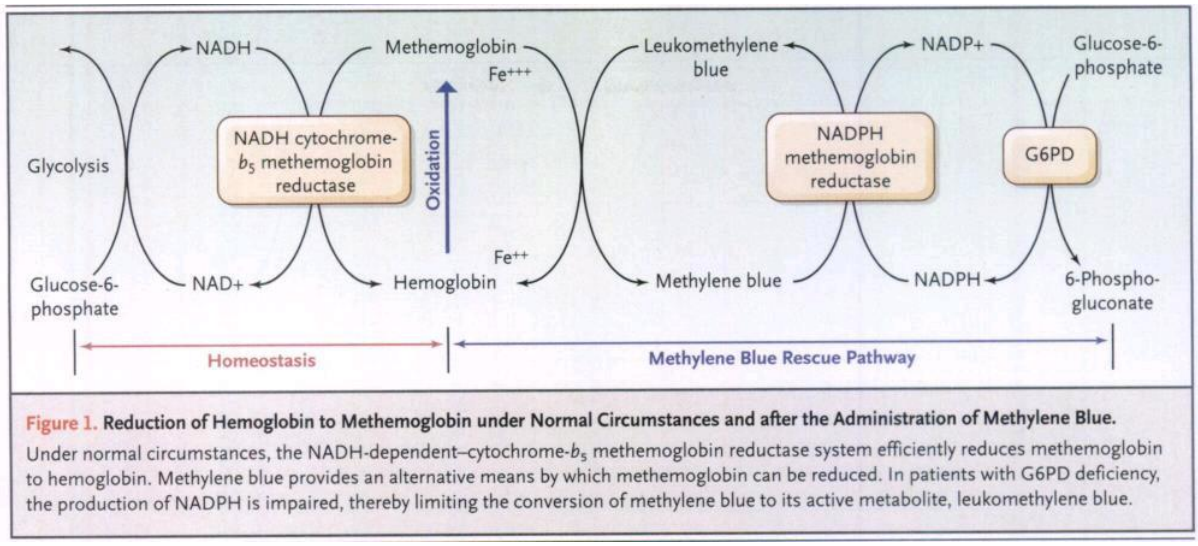


Figure 1. Reduction of Hemoglobin to Methemoglobin under Normal Circumstances and after the Administration of Methylene Blue.
Under normal circumstances, the NADH-dependent–cytochrome-b_5 methemoglobin reductase system efficiently reduces methemoglobin to hemoglobin. Methylene blue provides an alternative means by which methemoglobin can be reduced. In patients with G6PD deficiency, the production of NADPH is impaired, thereby limiting the conversion of methylene blue to its active metabolite, leukomethylene blue.

Fig. 3. ***Oxidation/reduction pathways for haemoglobin in red blood cells and partnership with methylene blue (Janssen et al. 2004)***

Methylene blue has been considered the standard treatment for acute methaemoglobinaemia (Janssen *et al.* 2004). Interestingly, using a modelling approach, Kinoshita *et al.* (2007) provided strong evidence that during physiological levels of methaemoglobin production, it is NADPH-methaemoglobin reductase activity that predominates and that NADH-methaemoglobin reductase only becomes active when methaemoglobin levels rise due to an overwhelming of NADPHMR as would happen in NO_2 poisoning. The inconsistency in modelled and measured data shows there is a lack of clarity on the relative importance of the NADH- and NADPH-linked reductase systems. Matsuki *et al.* (1978) suggest that this enzyme is a flavin reductase and showed that incubation of nitrite-treated red cells with added riboflavin reduced their MetHb levels much faster than controls. NADHMR is a soluble 245 amino acid protein found in the cytosol of the cell (Borgese *et al.* 1987) and is responsible for the majority of the reduction of MetHb in the red cell. Membrane bound varieties of the enzyme exist but play little role in the red cell (Borgese *et al.* 1987). There are three co-dominant alleles for NADH-methaemoglobin reductase in cattle, with all three present in *Bos indicus*, but only one present in *Bos taurus* (Fulton *et al.* 1978).

The second enzyme system found in the erythrocyte is NADPH dependent MetHb reductase is normally inactive but can be activated by electron donors such as methylene blue (Janssen *et al.* 2004). It normally contributes about 6% of the total methaemoglobin reduction. However, in the presence of methylene blue the activity of NADPH MetHb reductase is increased some 400-fold (Tomoda *et al.* 1980). This enzyme relies on the availability of NADPH generated by the pentose phosphate pathway, and clinically methylene blue is only used as an antidote to methaemoglobinaemia if the patient is tested and shown to have normal glucose-6-phosphate dehydrogenase (G6PD) activity. It is listed as the antidote for nitrite poisoning in standard veterinary textbooks (Brightling 2006). However, the drug is no longer recommended for use in animals destined for the food chain. It should also be noted that Dorset sheep have very low G6PD activity (Calabrese *et al.* 1983). G6PD deficiency in humans is commonly called *favism*, because consumption of fava (faba) beans generally results in a haemolytic episode due to the presence of a compound called divicine found in these beans. This compound has profound cytotoxic activity in G6PD erythrocytes, but paradoxically reduces methaemoglobin levels in normal erythrocytes, that have an active pentose phosphate pathway (Benatti *et al.* 1985).

5.11.1 Stimulation of the NADPH-methaemoglobin reductase pathway

This flavin-mediated reductase activity has been exploited in human medicine during the treatment of NADH MetHb reductase deficiency, where riboflavin is used as an alternative to methylene blue (Hultquist *et al.* 1993). The use of riboflavin as an alternative to methylene blue in ruminants is a realistic possibility. Riboflavin is synthesised in the rumen, but the effects of exogenous addition of riboflavin and its efficacy against NO_2 poisoning are unknown. Zinn *et al.* (1987) found that riboflavin supplementation of calves resulted in a slight increase in intestinal delivery and absorption of the vitamin.

NADH-MR relies on the production of NADH from the phosphorylation of glyceraldehyde-3-phosphate by glyceraldehyde-3-phosphate dehydrogenase in the glycolytic pathway to fuel the reaction with electrons. This step of the glycolytic pathway also requires the input of inorganic phosphate, whose addition enhances the glycolytic rate and the reduction of MetHb (Godwin *et al.* 2015). The glycolytic rate of erythrocytes is largely governed by the ratio of NAD/NADH. Hypophosphataemia associated with parturition is also known to cause methaemoglobinaemia in cattle (Ogawa *et al.* 1987).

Extracellular NADH can also aid in the reduction of methaemoglobin, if the levels of plasma lactate dehydrogenase are elevated by displacing the pseudo-equilibrium of the intracellular lactate dehydrogenase reaction so that intracellular NADH rises (Kennett *et al.* 2005). The glycolytic rate of erythrocytes increases by as much as 3-fold when the haemoglobin becomes deoxygenated (Giardina *et al.* 1995). The deoxygenated state producing a stimulation of glycolytic enzyme activity through the effect of an increased deoxyhaemoglobin N-terminus binding affinity with Band 3 membrane protein, which in turn releases membrane bound glycolytic enzymes (Low *et al.* 1993).

Treatment of acute methaemoglobinaemia in ruminants probably should involve intravenous administration of glucose to stimulate (or at least maintain) an adequate supply of NADH and/or NADPH from the glycolytic and pentose phosphate pathways. Blood glucose supply in ruminants is derived from gluconeogenic pathways rather than from direct gastrointestinal absorption and is generally lower than in non-ruminant species.

Take-home messages:

1. ***One of the great opportunities for reducing MetHb accumulation (by accelerating its reduction back to haemoglobin) may lie in accelerating the NADPH-MetHb reductase pathway as is currently achieved by using methylene blue. Riboflavin may offer an alternate means to accelerate this pathway.***

2. ***It should be noted that phosphorus deficiency in cattle is common in northern Australia, and it would be prudent to ensure that P supply is adequate before dietary nitrate supplementation.***

5.12 Genetic susceptibility of the red cell to nitrite

The activity of NADH–MetHb reductase is higher in foetal than adult sheep (Kempel *et al.* 1999) and declines after birth in most species (Vetrella *et al.* 1971; Agar *et al.* 1972). Fitting with this is the earlier observation by Emerick *et al.* (1965) who showed that pigs of different ages infused with NO_2 had the same initial peak of methaemoglobin, but the rate of decline was much faster in younger animals. The enzyme activity also increases over time in cattle exposed to dietary NO_3 (Godwin *et al.* 2015) and to rats exposed to NO_2 in drinking water (Csallany and Ayaz 1978). An early study with sheep showed that pre-treatment of animals with daily intraperitoneal injections of KNO_2 for 35 d delayed death, and increased the dosage of NO_2 required to cause a lethal increase in methaemoglobin (Diven *et al.* 1964). The potassium concentration of erythrocytes is a polymorphic trait in many ruminant species (Agar *et al.* 1972) and the rate of methaemoglobin formation is slower and its reduction faster in high potassium cells (Godwin 2014). Sheep also possess two normal adult haemoglobin types with different oxygen affinities that displays incomplete dominance in inheritance (Agar *et al.* 1972). Whether the propensity for these haemoglobin types to oxidise to methaemoglobin differs is unknown, but would likely be due to the differing oxygen affinities (Huang *et al.* 2005a). Sheep foetal haemoglobin has a greater NO_2 reductase activity probably to aid in vascular homeostasis via the production of NO (Blood and Power 2007). Newborn calves have a high blood and salivary NO_2: NO_3 concentration ratio, despite insignificant intakes of both compounds, and it has been suggested that this makes newborn calves more susceptible to NO_2 toxicity (Blum *et al.* 2001). Although bovine foetal haemoglobin is more susceptible to oxidation by NO_2 than is adult bovine haemoglobin (Betke *et al.* 1956), its higher NADH-methaemoglobin reductase activity (Lo and Agar 1986) compensates for that susceptibility. Erythrocytes from diabetic humans are less susceptible to NO_2-induced methaemoglobin than erythrocytes from healthy controls, probably as a result of differences in the levels of various antioxidant systems (Coleman *et al.* 1998).

Take-home message: Between-animal variation in susceptibility to NO_2 toxicity is known and some mechanisms by which animal genotypes may differ (e.g. haemoglobin, potassium and glutathione types) can explain these differences in part but not in whole. These specifically identified pathways currently do not offer a practical means of reducing MetHb risk, but there would be merit in understanding the mechanism of nitrate tolerance in sheep.

5.13 Other antioxidant enzyme systems in the red cell

Incubation of red cells with NO_2 increases lipid peroxidation, an indicator of oxidative reactivity (Batina *et al.* 1990). It has also been recently reported that another enzyme termed AOP2 or antioxidant protein, also exists within the haem pocket that also prevents the

oxidation of haemoglobin, both induced and spontaneous (Umbreit 2007). Its quantitative role is yet to be determined.

The erythrocyte also protects haemoglobin from oxidation by decomposing hydrogen peroxide produced within the cell by utilising the oxidation of reduced glutathione. Oxidising radicals are generally removed by the action of a suite of enzymes – superoxide dismutase, the red cell (May *et al.* 2000a) and does not lead to the precipitation of denatured haemoglobin as Heinz bodies (Sinha and Sleight 1968).

Sheep and cattle also possess genetically determined normal and deficient levels of glutathione (Tucker and Kilgour 1970); however there appears to be no difference in their red cell antioxidant enzyme activities (Suzuki and Agar 1983). Despite this, sheep red cells glutathione peroxidase and catalase. The disarming of oxygen radicals by these enzymes helps protect haemoglobin against oxidation from by-products of its own cargo – oxygen. Catalase predominates in the disposal of peroxide in the red cell (Gaetani *et al.* 1996) and acts as a general transported oxidant defence system mechanism (Agar *et al.* 1986). Russian research suggests that NO_2 at levels as low as 100 µmol/L inhibits catalase activity, but does not inhibit haemoglobin peroxidase activity. As a consequence, NO_2 leads to an increase in the rate of peroxide metabolised by haemoglobin, which further intensifies NO_2 oxidation and methaemoglobin formation (Titov and Petrenko 2003). Nitrite certainly leads to methaemoglobin formation, but otherwise does not particularly lead to strong oxidant stress within with high GSH levels have a more rapid rate of reduction of NO_2-induced methaemoglobin when incubated with glucose (O'Dea and Agar 1980).

5.13.1 Stimulation of erythrocyte antioxidant systems to prevent methaemoglobin formation

Sodium selenite greatly enhances methaemoglobin reduction by enhancing glutathione oxidation in rat erythrocytes (Iwata *et al.* 1977). More recent evidence suggests that NO_2 rapidly depletes cells of reduced glutathione, rendering them much more susceptible to MetHb formation (Michaelsen *et al.* 2009). The supplementation of animals with selenite as a methaemoglobin ameliorant is a possibility. Many substances outlined below may have an effect on methaemoglobin synthesis/reduction and whether the mostly minor effects are additive is unknown and research testing their combined efficacy could be fruitful.

Ascorbic acid reduces methaemoglobin formation in rats, humans and humans deficient in G6PD but not in sheep (Calabrese *et al.* 1983) and not consistently in cattle (Atyabi *et al.* 2012). Vitamin E was effective at reducing NO_2-induced methaemoglobin formation in cattle RBC (Atyabi *et al.* 2012). N-acetylcysteine is often used to treat methaemoglobinaemia in cats poisoned with paracetamol. It was assumed that N-acetylcysteine removed the paracetamol by way of a metabolic process involving glutathione (Gaunt *et al.* 1981). However, Wright *et al.* (1996) suggest that N-acetylcysteine either directly reduces methaemoglobin or stimulates the synthesis of glutathione within the red cell, which then reduces MetHb.

Nitrite activates K-Cl co-transporter in sheep LK erythrocytes and also decreases GSH levels - an effect that is characteristic of thiol-oxidising agents (Lauf *et al.* 1995). The activity of K-Cl co-transporter was positively correlated to the formation of MetHb (Adragna and Lauf 1998). Telmisartan, an angiotensin II receptor antagonist, produces a concentration dependent slowing of the rate of methaemoglobin formation in red cells incubated with NO_2 (Abbas *et al.* 2011).

In a preliminary report, Everse (1990) found that methaemoglobin in solution when incubated with methionine and riboflavin and irradiated with white light is reduced to haemoglobin very rapidly, but the presence of oxygen will lead to peroxide formation and reverse the process.

An early Russian study (Genkin and Volkov 1960) found that glutamate administered subcutaneously prior to subcutaneous administration of NO_2 markedly lowers the production of methaemoglobin in rats. This finding has recently been cited by an *in vitro* study investigating properties of haemoglobin-based oxygen carriers, which showed that both glutamate and tyrosine contribute to the stabilisation of distal histidine residues which

to Jainudeen *et al.* (1964) and Winter (1962)), long-term NO_3 feeding of cattle may result in compensatory erythropoiesis leading to a higher haemoglobin level and packed cell volume stabilise the haem pocket, preventing the formation of methaemoglobin (Wei *et al.* 2014). This is a surprising effect, however, because the levels of amino acids such as glutamine are unlikely to be limiting in ruminants. Accordingly, however, this was not observed by Godwin *et al.* (2015) who supplemented sheep with nitrate for 54 d, even though NADH-methaemoglobin reductase levels had increased.

Stimulation of erythropoiesis using substances such as $CoCl_2$ (Ebert and Jelkmann 2014) as occasionally used illegally in race horses, may be an alternative way of overcoming the lack of oxygen carriage caused by methaemoglobinaemia (although cobalt is known to cause a transient methaemoglobinaemia in rats (Horiguchi *et al.* 2004). Many substances stimulate methaemoglobin reduction *in vitro*, but are unlikely to be effective *in vivo*, largely because they are either not absorbed/available into the circulation and/or cannot traverse the red cell membrane. For example, inositol hexaphosphate greatly stimulates methaemoglobin reductase (Taketa and Chen 1977), but would not be absorbed from the gut, synthesised in the body or cross the red cell membrane.

***Take-home message*: As mentioned previously, anti-oxidants such as Vitamin E, selenite and N-acetylcysteine show promise as a means of reducing MetHb production while other means of protecting haemoglobin (e.g. by glutamate stabilisation) seem less practical. Stimulation of erythropoiesis using substances such as cobalt chloride also deserves further consideration.**

5.14 Nitrite intoxication

In addition to the reduced oxygen carrying capacity of the red blood cells due to the high concentration of MetHB, other effects of NO_2 intoxication include a marked drop in blood pressure (Asbury and Rhode 1964; Whatman *et al.* 2013) with an increase in the Frank-Starling stretch-induced intrinsic regulation of the cardiac output (Angelico *et al.* 2012).

Circulating NO_2 has a wide range of effects and readily increases cyclic GMP production, inhibits hepatic cytochrome P450 activity, and upregulates heat shock protein 70 and heme oxygenase-1 expression in a variety of tissues in a dose dependent manner (Bryan 2006). Rats intraperitoneally injected with NO_2 showed that nitrite rapidly entered the circulation and reached steady-state concentrations in most tissues in about 5 min. The increases in tissue NO_2 were also accompanied by increases in tissue NO_3 and nitrosation and nitrosylation products (Bryan 2006) but there is no indication of nitrosamine formation in meat from nitrate-fed animals in contrast to cured meats. Nitrite readily diffuses between blood and cerebrospinal fluid in lambs (Conahey *et al.* 2008).

Nitrite is also oxidised to NO_3 by ferrocytochrome c-cytochrome oxidase which effectively acts as a NO_2 oxidase (Paitian *et al.* 1985). The magnitude of this oxidation is unknown in mammals, but may be as high as 5.6 mmol/h per g of liver in trout (Doblander and Lackner 1996).

Following a modelling exercise using data collected in rats, Kohn *et al.* (2002) found that peak plasma NO_2 levels occurred 30 min after oral dosing and methaemoglobin levels peaked after 100 min.

Most data on NO_3 and NO_2 metabolism have been collected from naturally occurring levels of these compounds in non-ruminant animals. The pharmacodynamics and

pharmacokinetics of these compounds may be quite different when NO_3 is fed at methane mitigating levels (1-2% of DM) in ruminants.

<u>Take-home message</u>: Nitrite toxicity affects a wide range of species, yet the pharmacodynamics and pharmacokinetics of NO_2 are poorly understood in any species. Studies addressing this issue are urgently needed to both understand and treat NO_2 intoxication appropriately.

6 APPENDICES

Appendix 1 - NO_3 supplementation in Australian livestock – Legislative Framework

The Federal Government's ***Clean Energy Act 2011*** established a carbon pricing mechanism. This Act was repealed and the carbon pricing mechanism (carbon tax) abolished from 1 July 2014.

The Carbon Farming Initiative Amendment Bill 2014 established the Emissions Reduction Fund that commenced on 1 July 2014 to replace the carbon pricing mechanism and provide a transition for the Carbon Farming Initiative (CFI) by amending the: Carbon Credits (Carbon Farming Initiative) Act 2011. The 2014 Bill provided for:

"the Clean Energy Regulator to conduct auctions and enter into contracts to purchase emissions reductions; enable a broader range of emissions reduction projects to be approved; and amend the project eligibility criteria and processes for approving projects and crediting carbon credit units'. and also to establish 'transitional arrangements in relation to: the Register of Offsets Projects, which will be renamed the Emissions Reduction Fund Register (that) will include information about contracts to purchase emissions reductions; and existing Carbon Farming Initiative projects and methodologies and applications for new projects."

In effect, the Carbon Farming Initiative Amendment Bill 2014 has expanded the Carbon Credits (Carbon Farming Initiative) Act 2011 which defines the scope for the development of emissions avoidance activities that may be eligible for credits under the CFI scheme.

The CFI Bill lists three types of emissions avoidance projects that could be eligible under the CFI. Emissions avoidance projects fall under two primary types of methodology determinations: sequestration and emissions avoidance.

Introduced animal emissions avoidance projects are considered to be among such eligible projects. These are projects that promote the avoidance of emissions of methane from the digestive tract of an introduced animal or emissions of methane or nitrous oxide from the decomposition of introduced animal urine or dung.

'Reducing emissions from ruminant livestock by feeding NO_3 supplements' *is one such project that is included in the list of positive activities for emissions avoidance. When advising the Minister in May 2013, the Domestic Offsets Integrity Committee recommended that 'this proposed activity is suitable for inclusion on the Positive List' (of emissions avoidance measures), and has since been implemented in law (**http://www.comlaw.gov.au/Details/F2014L01129**). In giving this advice, the Committee noted: 'that there is potential for toxicity issues to arise in association with this activity in certain circumstances. This issue can and should be addressed through any methodology developed for this activity'.*

Implications of the legislation. In order to implement successful methane mitigation strategies based on feeding NO_3 in the diets of ruminant livestock, a more detailed understanding of changes in the metabolism of the rumen in response to dietary NO_3 and in the types of microorganisms and their capabilities and interactions is required. In addition, the potential for NO_2 poisoning of supplemented animals needs to be successfully addressed.

Appendix 2 - Fermentation by rumen microorganisms

The main microbes in the rumen include bacteria, protozoa and fungi and archaea. These microbes obtain the nutrients they need from forages, concentrates and supplements ingested by the animal. The major components in these feeds are complex carbohydrates, proteins and lipids that digested by the rumen microbes under anaerobic conditions (fermentation). The fermentation process generates ATP that is required by the rumen microorganisms for their maintenance and to synthesise the various polymers needed for the microbes to grow. However, because of the anaerobic conditions, surplus end-products, including volatile fatty acids, ammonia and the microbial polymers retain relatively large amounts of metabolisable energy that, after absorption from the gut, become available for further oxidation under aerobic conditions by the ruminant host. Gases such as CO_2, methane and H_2 produced in the rumen are directly eructated or inhaled and later exhaled from the lungs. The fermentative pathways found in mixed microbial fermentations of bacteria, protozoa, fungi and methanogens are described in Fig. 2.1. The fermentative properties of many of the bacterial species found in the rumen have been described (Table 2.1, from Russell and Hespell (1981).

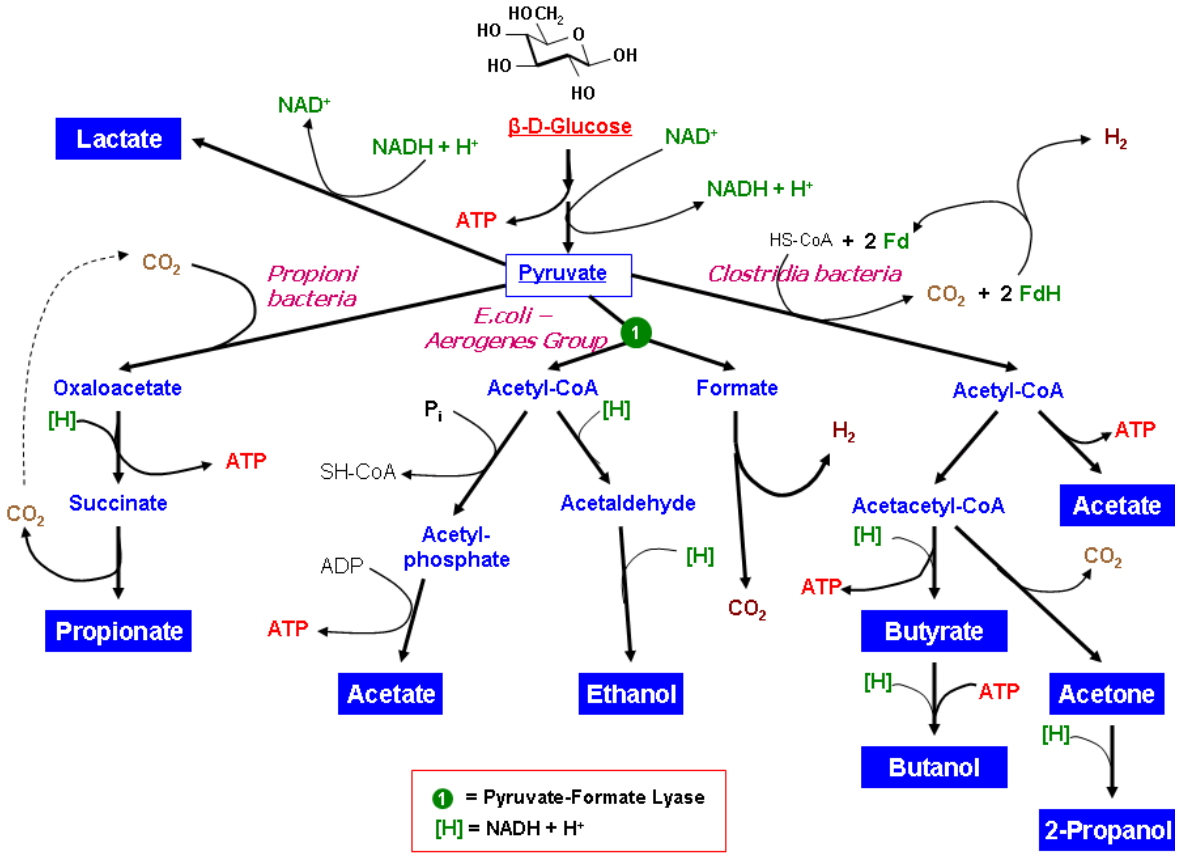


Fig. 2.1. The pathways of microbial mixed-acid fermentations (from **http://basic-microbiology.blogspot.com.au/2011/11/fermentations.html**

Table 2.1. Fermentative properties of different species of bacteria found in the rumen (after Russell and Hespell (1981)

Bacterial species	Function	Products
Fibrobacter (Bacteriodes) succinogenes	C,A	F,A,S
Ruminococcus albus	C,X	F,A,E,H,C
Ruminococcus flavefaciens	C,X	F,A,S,H
Butrivibrio fibrisolvens	C,X,P,R	F,A,L,B,E,H,C
Clostridium lochheadii	C,PR	F,A,B,E,H,C
Streptococcus bovis	A,S,SS,PR	L,A,F
Ruminobacter (Bacteriodes) amylophilus	A,P,P,R	F,A,S
Prevotella (Bacteriodes) ruminocola	A,X,P,PR	F,A,P,S
Succinomonas amylolytica	A,D	A,S
Selenomonas ruminantium	A,SS,GU,LU,PR	A,L,P,H,C
Lachnospira multiparus	P,PR,A	F,A,E,L,H,C
Succinivibrio dextranosolvens	P,D	F,A,L,S
Methanobrevibacter ruminantium	M,HU	M
Methanosarcina bakeri	M,HU	MC
Treponema bryantii	P,SS	F,A,L,S,E
Megasphera elsdenii	SS,LU	A,P,B,V,CP,H,C
Lactobacillus sp	SS	L
Anaerovibriolypolytica	L,GU	A,P,S
Eubacterium rumantium	SS	F,A,B,C
Oxalobacter formigens	O	F,C
Wolinella succinogenes	HU	S,C

Major substrates utilised: C –cellulose; X-xylan; A-amylose; D dextrin; P-pectinolytic; PR-protein; L-lipid; M-CO_2 (methanogen); GU-glucose utilising; LU-lactose utilising; SS- soluble sugars; HU-hydrogen utilising; O-oxalate

Products formed. F-formate; A-acetate; E-ethanol; P-propionate: L_lactate; B-butyrate; S-succinate; V- valerate; CP-caproate; H-hydrogen; C-CO_2 ; M-methane.

The fermentation process begins when complex carbohydrates (starch and cellulose) are first hydrolysed to di- and mono-saccharides by extracellular enzymes before being imported into cells. Hexoses and other soluble sugars are actively transported into cells. Glucose is imported, for example, either by a highly specific glucose transport system or by a phosphoenolpyruvate-glucose phosphotransferase system. A facilitated-diffusion mechanism is responsible for glucose transport in *Streptococcus bovis* when glucose concentrations are high (Russell 1990).

Glucose and other hexoses are fermented to pyruvate, mainly by the Embden-Meyerhoff-Parnas glycolytic pathway (Fig. 2.1) or via the Pentose Phosphate pathway. For each mole of hexose fermented, 2 mol NAD^+ are reduced to NADH + H^+ and 2 mol of ATP are generated. If fermentation is rapid and NADH builds up in the cell, pyruvate may be reduced to lactate with no net formation of NADH. However, most of the pyruvate formed is usually converted to formate or acetyl-CoA. Two cytoplasmic enzymes are responsible for the degradation of pyruvate, *viz.* lyase (PFL), both of which generate ATP without using NADH as an intermediate electron carrier (Doelle 1975) The acetyl-CoA is converted to short chain fatty acids including acetate, propionate and butyrate. Smaller amounts of higher and

branched chain fatty acids are also produced, (e.g. valerate, iso-valerate, isobutyrate). Acetyl-CoA cleavage is catalysed, by phosphotransacetylase (PTA) and acetate kinase (ACK), to acetate or to butyrate via acetoacetyl-CoA. Notably, some strains of rumen bacteria cannot transform acetyl-CoA into acetate and so must reduce it to ethanol (Allison *et al.* 1964). In this case, the acetyl-CoA is reduced to ethanol in two steps, by aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH). A build-up of ethanol occurs only under particular conditions, and generally not in mixed cultures. Ethanol production by, for example, *R. albus* is eliminated with a corresponding increase in acetate and H_2 formation (Iannotti *et al.* 1973). The lactate, ethanol and VFA produced in the cytoplasm are exported to the external medium.

Formate synthesised in the cytoplasm must be removed to avoid intracellular acidosis, so it is either catabolised to CO_2 and H_2 by cytoplasmic formate dehydrogenase, or it is exported via the FocA channel to the periplasmic space where it is degraded to CO_2 and H_2 by formate:hydrogen lyase (Lu *et al.* 2011).

During the oxidation of hexoses, the energy required for microbial maintenance and growth is released by substrate-level phosphorylation in two key reactions, *viz.* the NAD-linked dehydrogenation of glyceraldehyde-3-phosphate, and the cleavage of pyruvate by the pyruvate-formate lyase. Depending on the ATP requirements for maintenance of microbial cells, the amounts of microbial biomass formed can vary from about 11 to 20 g cell dry matter per mol ATP generated (Hespell and Bryant 1979).

Acetate, propionate and butyrate in rumen fluid are absorbed and are major sources of oxidisable substrate (energy) for ruminant tissues. The microbial cells formed in the rumen flow to the intestines and are digested and absorbed. These cells are approximately 50% crude protein and are a major source of essential amino acids for the host animal but also provide the animal with additional oxidisable substrates and other nutrients (Leng and Nolan 1984).

Hydrogen production and transfer in the rumen

The principal means of regenerating NAD^+ from NADH in rumen microorganisms is by NADH-ferredoxin oxido-reductase coupled to a hydrogenase (Fig. 2.2).

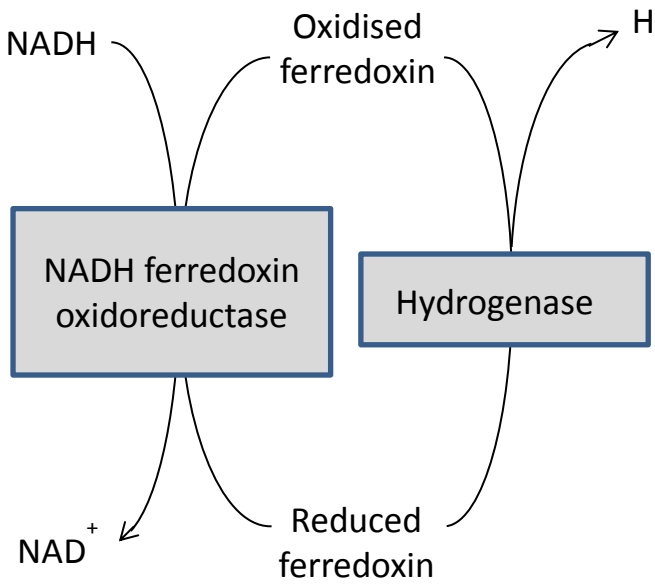


Fig. 2.2. NADH ferredoxin oxidoreductase coupled to hydrogenase is responsible for H_2 generation in rumen bacteria when concentration of H_2 in the cell is low

The reduction of protons by NADH ferredoxin oxidoreductase generates H_2 and can only occur when the concentration of H_2 is sufficiently low (Iannotti *et al.* 1973). Anaerobic conditions are essential; the reaction is thermodynamically unfavourable under standard conditions and the activity of this oxidoreductase is suppressed by high p_{H2} (Gottschalk 1986). H_2 is also produced in the periplasm by formate:hydrogen lyase.

Bacterial hydrogenases are single polypeptide chains (Wu and Mandrand 1993) or enzymes with multiple sub-units (Sawers 1994) and may contain iron/sulphur clusters and nickel. In rumen protozoa (Paul *et al.* 1990) and fungi (Yarlett *et al.* 1986), hydrogenases are mainly located in membrane-enclosed organelles known as hydrogenosomes. These are the principal sites of H_2, acetate, CO_2 and ATP generation by reactions catalyzed by pyruvate:ferredoxin oxido-reductase, hydrogenase and lactate dehydrogenase (Muller and Lindmark 1978).

In the context of this review, it should be noted that the reduction of NO_3 to ammonia also regenerates NAD^+. This should help promote a more efficient fermentation and digestion of feed materials, especially the more rapidly fermented carbohydrates.

Appendix 3 - Chemistry – oxidation states of nitrogen in the environment

Nitrogenous forms in the environment

A small fraction of the N_2 in the atmosphere is incorporated into nitrogenous polymers in living organisms and is inter-converted between more oxidised and more reduced forms. NO_3 (NO_3^-) is the most oxidised nitrogen compound in the biosphere, oxidation state +5. It serves as a nutrient and an electron acceptor for many bacteria, archaea and some eukaryotes. Nitrogen is found in reduced forms in proteins and nucleic acids in all living organisms where its oxidation state is the same as ammonium (NH_4^+), -3). The inter-conversions carried out by living cells involve both oxidative and reductive processes catalysed by various enzymes shown in Fig. 3.1.

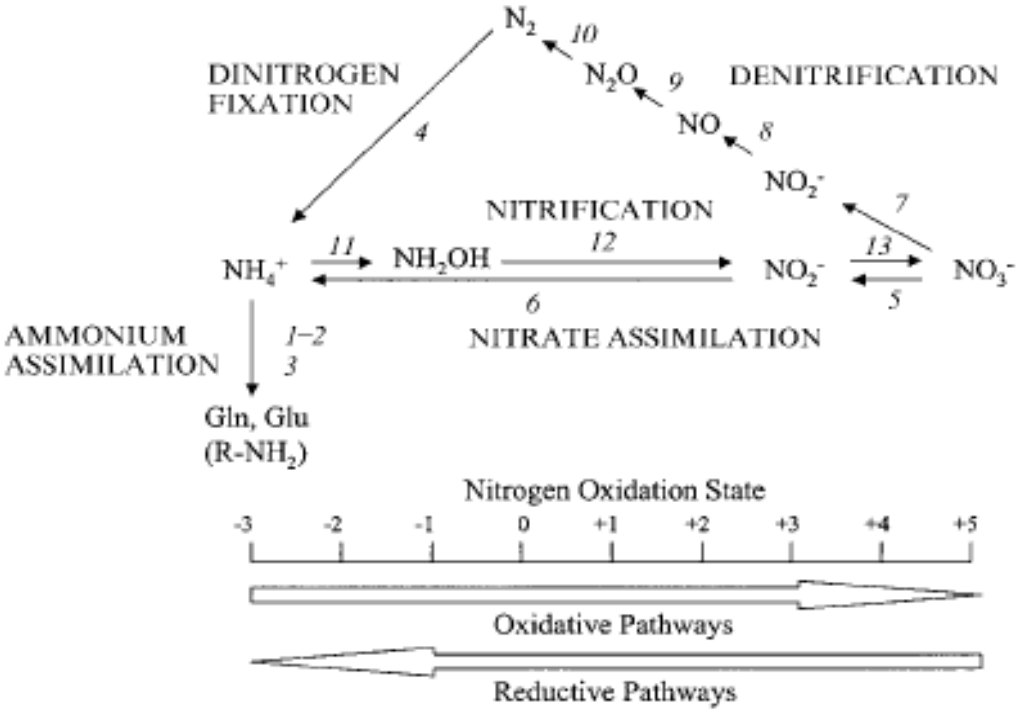


Fig. 3.1. The N cycle in nature showing the N compounds arranged according to their oxidation states with arrows showing the principal oxidative and reductive pathways

Enzyme key: 1–2, glutamine synthetase–glutamate synthase (GS–GOGAT); 3, glutamate dehydrogenase (GDH); 4, nitrogenase; 5, assimilatory NO3 reductase (Nas); 6, assimilatory NO2 reductase (sirohaem-Nir); 7, dissimilatory and respiratory NO3 reductases (Nap and Nar); 8, respiratory NO2 reductases (Cu-Nir and cd1-Nir); 9, nitric oxide reductase (Nor); 10, nitrous oxide reductase (Nos); 11, ammonia monooxygenase; 12, hydroxylamine oxidase; 13, NO2 oxidase.(from Cabello et al. (2004)

After its reduction to NH_4^+, NO_3^- can also be used as a source of N by archaea, bacteria, fungi, algae and higher plants (Moreno-Vivian and Ferguson 1998). Most living organisms can incorporate NH_4^+ into amino acids, proteins and other nitrogenous polymers.

Nitrate as an oxidising agent

The focus in this review is on the development of appropriate management practices for supplementing ruminant livestock with NO_3 or NO_2 to reduce the formation of methane - a potent greenhouse gas. Briefly, the reduction of NO_3 and NO_2 by rumen microorganisms can utilise electrons that might otherwise be used in the reduction of CO_2 to methane, thereby reducing methane emissions. The reduction of CO_2 to methane in the rumen requires 8 mol electrons, e.g. $4H_2$, *viz*.

$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$ $\Delta G0' = -175$ kJ

Reduction of NO_3 to NH_4^+ by rumen microbes also uses $4H_2$ and the NH_4^+ formed can also be used as a nitrogen source for growth. In addition, the reduction of NO_3 to NH_4^+ is energetically more favourable under standard conditions, *viz.*

$4H_2 + NO_3^- + 2H^+ \rightarrow NH_4^+ + 3H_2O$ $\Delta G0' = -598$ kJ

In Reaction 2, NO_3 is first reduced to NO_2 that is then reduced to NH_4^+. If NO_3 reduction is faster than NO_3 reduction, NO_2 will accumulate (Dawson and Allison 1988). Importantly, NO_2 inhibits growth of many rumen microorganisms, especially methanogens, cellulolytic bacteria and protozoa (Iwamoto *et al.* 2002). In ruminants, NO_3 may therefore potentially affect digestibility and the efficiency of microbial growth, although the evidence on this is conflicting. Animals can potentially become susceptible to NO_2 intoxication if NO_2 accumulates in rumen fluid and is absorbed into the bloodstream (Allison and Reddy 1984). This can lead to NO_2 poisoning in the animal with unwanted reductions in production and sometimes death.

Appendix 4 - Naturally occurring sources of NO_3 in non-supplemented diets

Drinking water for livestock in Australia can be run-off water in steams or collected in dams, or bore water. Information on N in Australian water sources is very limited. Information on contaminants in water can be found in a review commissioned by MLA entitled *Water quality effects on ruminant health and productivity* (Kurup *et al.* 2011). Monitoring of NO_3 in groundwater is inconsistent and patchy in Australia. Nevertheless, NO_3 contamination of ground water is apparently widespread in both rural and urban areas and is associated with, for example, grazing animals, dairying and fertilizer applications and effluent disposal. Non-agricultural sources of NO_3 include lawn fertilizers, septic systems, and domestic animals in residential areas. Additionally, airborne nitrogen compounds given off by industry and automobiles are deposited on the land in precipitation and dry particles. The median NO_3-N concentration is low, i.e. less than 2 mg NO_3-N/L (Lawrence, 1983), but NO_3-N concentrations from 0.001 to 29 mg/L have been recorded. Higher concentrations have usually been found in areas of intensive agriculture with a history of repeated annual applications of nitrogenous fertiliser (watercorporation.com.au). In Western Australia, elevated NO_3 concentrations are ascribed to naturally occurring plant decay underground and various municipal localities have been granted exemption from compliance with the NO_3 guidelines by the Department of Health. Nitrate from agricultural fertilizers and animal manure can pass through the soil and reach underlying aquifers so that concentrations in these aquifers often exceed 10 mg NO_3-N/L. Nitrate can persist in ground water for decades and can continue to accumulate and reach levels exceeding 100 mg NO_3-N/L as more N is applied to the land surface every year (LWRRDC 1999).

NO_3 concentrations in feeds and forages

Cereal grains and protein concentrates rarely contain NO_3 in sufficient concentrations to be of concern for livestock feeding and are low-risk feeds. However, various forages can accumulate NO_3 under particular conditions. In addition, certain weeds commonly found in pastures or crop fields, e.g. dock (*Rumex* spp) and Johnson grass (*Sorghum vulgare*) can also accumulate NO_3. Nitrate concentrations are typically higher in stems and lower in leaves (where there is more NO_3 reductase activity) and are extremely low in seeds (Pfister 1988). In grasses, the majority of the NO_3 is in the stem. In pearl millet, for example, the stems contained three times more NO_3 than leaves (Krejsa *et al.* 1987) (Krejsa *et al.* 1987)and in maize and rye grass stems, the highest concentrations have been detected nearer to the ground. As a result, feeding forage crops presents the greatest risk of poisoning.

Accumulation of NO_3 in plants is influenced by a number of factors that are listed below.

Plant species. More than 80 species including a number of common agricultural crops can accumulate NO_3 to high levels under extreme conditions. Cultivars of perennial rye grass and the sorghum family (sorghum, sudan, pearl millet and their crosses) are particularly prone to NO_3 accumulation.

Stage of maturity. Under normal growing conditions, NO_3 concentrations in plants tend to decrease as plants mature. Nitrate concentrations are higher in young plants (or in regrowth) and lower in mature plants (Pfister 1988).

Fertilizer application. Nitrate concentration in plants is directly related to levels of application of NO_3 fertilizer. An outbreak of nitrite poisoning that occurred in Qld after hungry sheep and cattle were held in yards where *Dactyloctenium radulans* (button grass) was growing was attributed to their intake of forage with a high NO_3 concentration (from 4.0 to 12.9%) resulting from plant uptake of N from faeces and urine (McKenzie *et al.* 2004).

Plant growing conditions. Nitrate levels increase in plants when conditions limit plant growth but still permit the uptake of NO_3. Thus, cold temperatures, frost damage, drought and treatment with growth retarding herbicides may elevate NO_3 concentrations in plant materials (Krejsa *et al.* 1987). Overcast conditions can increase plant NO_3 concentrations because NO_3 reductase activity is low during periods of poor illumination that limit the rate of photosynthesis (Pfister 1988).

Soil moisture content affects uptake and utilization of NO_3 by plants. During drier periods, when plant growth is limited, plants continue to take up NO_3 but have reduced NO_3 reductase activity (Pfister 1988). The effect of water stress on NO_3 content of pastures is pertinent to Australia. Pfister (1988) reported high levels of NO_3 in plants shortly after the end of a severe drought. Nitrate concentrations in stems of water-stressed pearl millet increased from approximately 5 g/kg to 9 g/kg within two days of the commencement of irrigation (Krejsa *et al.* 1987). Up to 7 to14 d may be required for NO_3 concentrations in pasture to return to low levels following drought-ending rains (Fjell *et al.* 1991). Other factors such as soil mineral content and herbicide treatment also affect NO_3 accumulation (Pfister 1988).

Nevertheless, under normal growth conditions, the concentrations of NO_3 in forages are low and are insignificant in relation to the amount of fermentable nitrogen required for the microbes to efficiently digest the biomass in the rumen. Only rarely can NO_3 concentrations be expected to become high enough to exceed the rumen requirements for fermentable nitrogen. The exception to this may be in temperate areas with high rainfall and heavy dressings of N fertilizers. In such cases, NO_3-N concentrations as high as 2.5% of forage DM (or 45% of the total N in the forage) have been recorded (O'Donovan and Conway 1968).

Addition of NO_3 to feed or supplements

Nitrate, like urea, can be used as a dietary non-protein N (NPN) supplement that is converted in the rumen to ammonia. Ammonia is a major source of N for amino acid synthesis, and protein and other polymers in growing rumen bacteria and fungi. Protozoa also metabolise NO_3 and produce ammonia, but they excrete the ammonia and use amino acids synthesised from other precursors. In comparison with urea, NO_3 has the advantage that it reduces methane emissions in ruminants. However, currently it is about twice as expensive as urea per unit N

During periods of grazing on green forages, ruminants tend to ingest forages quickly, swallowing boluses without excessive chewing and later comminute the feed whilst resting and ruminating. After entering the rumen, plant cells may survive intact for several hours and may undergo changes due to stress that result in rapid proteolysis and release of N into the rumen environment (Kingston-Smith and Theodorou 2000). When plants with high NO_3 concentrations are ingested, the effect of this stress on the rate of release of NO_3 into rumen fluid is unknown. Nevertheless, it is reasonable to expect that the duration of NO_3 release will be extended when the NO_3 enters the rumen in living cells. This situation can be contrasted with that arising when soluble inorganic NO_3 salts are ingested when the peak concentrations of NO_3 after ingestion of the same quantity of NO_3 are likely to be higher.

Supplementary NO_3 can be included in the diet of ruminants in various ways. Solutions containing NO_3 can be sprayed onto chopped hays or in to concentrates during mixing. Nitrate can be included in blocks or provided in drinking water. When these materials are ingested, NO_3 (being highly soluble) will be quickly released into the rumen fluid producing a sudden peak in NO_3 concentration. It is therefore likely the NO_3 will become available before the fermentation of the meal has had time to generate new ATP for microbial growth and before the need for assimilation of NO_3-N to meet cell synthesis has become significant.

Appendix 5 - NO_3 and NO_2 metabolism in ruminal microorganisms

NO_3 reduction by rumen microbes

To gain energy for maintenance and growth, microorganisms oxidise feed substrates using O_2 or other electron acceptors. Because the rumen is highly anaerobic, rumen microbes usually shed unwanted electrons by reducing CO_2 to form methane. However, NO_3 and NO_2 have a higher affinity for electrons than does CO_2 and so, when present, are potential alternative electron acceptors. Their inclusion in the diet of ruminants can be expected to reduce their methane emissions. However, when the rate of reduction of NO_3 to NO_2 exceeds the rate of NO_2 reduction to ammonia, NO_2 can build-up in the rumen fluid and NO_2 absorption into the bloodstream can lead to NO_2 intoxication in the animal.

Bacteria

Some of the bacteria capable of reducing NO_3 and NO_2 in the rumen include. *Selenomonas ruminantium*, *Veillonella parvula*, *Wolinella succinogenes* (Stewart and Bryant 1988) and *Veillonella Alcalescens (Inderlied and Delwiche 1973)*, *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* (Chaucheyras-Durand *et al.* 2010). Allison and Reddy (1984) identified 5 major groups of bacteria from sheep that had been adapted to dietary NO_3. Two groups that exhibited the ability to reduce NO_3 and metabolise formate were presumptively identified as selenomonads; a third group could metabolise formate and reduce NO_3 and produced mainly lactate. A fourth group identified as *Anaerovibrio* spp could not reduce NO_3 and produced mainly propionate from glucose.

Asanuma *et al.* (2002) has argued that many other NO_3/NO_2 reducing bacterial species must be present in the rumen. There may however be strain differences within species. Yoshii *et al.* (2003) identified four distinct types of *S. ruminantium* (Nos 7, 12, 15 and 22) based on their relative abilities to reduce NO_3 and NO_2. For all strains, NO_3 reductase activity was higher than NO_2 reductase activity and the specific activity of respiratory NO_3 reductase was higher than of the assimilatory reductases. NO_3 reduction rate was enhanced in *S. ruminantium* when cultured with an amylolytic sp. in a medium containing starch and, as a result, NO_2 accumulated. On the other hand, Asanuma *et al.* (2003) isolated a new NO_2-reducing bacterial strain, and demonstrated that NO_2 reduction in the rumen could be enhanced by introducing the new bacterial variant. This offers a possibility that cultures of NO_2 reducing strains of bacteria could be developed for feeding to livestock known to be at risk of NO_2 poisoning.

Protozoa and fungi

Iwamoto *et al.* (2001) found that protozoa have considerable ability to reduce NO_3 and NO_2 in rumen contents (although some of this activity could be due to intracellular bacteria). They found that, under certain circumstances, NO_2 removal by reduction to ammonia is increased in the presence of protozoa (perhaps because they remove starch and reduce populations of amylolytic bacteria). Yoshida *et al.* (1982) concluded that protozoa play a very important role in NO_2 reduction in the rumen. They also argued that protozoa may use lactate as a hydrogen donor for NO_3 and NO_2 reduction.

Lin *et al.* (2011) found rates of NO_3 removal by a protozoa-rich fraction were similar to those in the whole rumen contents, implying that protozoa were highly active in reducing NO_3. In contrast, NO_3 removal by a bacteria-rich fraction from the same rumen contents *in vitro* was slower for the first 12 h after which it was similar to whole rumen contents. A fungi-rich fraction appeared to metabolise NO_3, but only slowly.

Aerobic fungi express two pathways of dissimilatory nitrate reduction in response to low environmental oxygen tension. Takaya (2002) found the fungus *Fusarium oxysporum*

expressed the pathway of respiratory nitrate denitrification catalyzed by nitrate reductase and nitrite reductase. These enzymes are coupled with ATP generation through the respiratory chain and produce nitric oxide.

Archaea

Some members of the archaea have the ability to metabolise NO_3, but whether rumen methanogens have NO_3 reducing abilities is apparently unknown. If they do, it is conceivable that, when NO_3 is available, they might switch from reducing CO_2 to methane, to reducing NO_3 to ammonia. This would, in part, explain the methane mitigating effect of dietary NO_3 supplementation.

In the archaea, NO_3 reduction processes and especially their regulation are less well understood than in bacteria (Cabello *et al.* 2004). In Nature, dissimilatory NO_3 reduction appears to be more common than NO_3 assimilation (Martinez-Espinosa *et al.* 2001) and genes coding for putative NO_3 transporters, and NO_3 and NO_2 reductases have been found in their sequenced genomes. These sequences contain MGD cofactor encoding genes and the archaeal NO_3 reductases appear to be molybdo-enzymes (Cabello *et al.* 2004). Genome sequencing has revealed that ABC-type NO_3 transport systems are widespread in archaea but, being ATP-powered are energy–expensive, making their involvement in dissimilatory NO_3 reduction unlikely (Moir and Wood 2001). Nevertheless, NO_3 uptake may not be required for respiratory NO_3 reduction in archaea (*cf*. bacteria), but the putative ATP-dependent transporters could be involved in NO_3 assimilation (Cabello *et al.* 2004).

NO_3 and NO_2 reducing enzymes in rumen microorganisms

It is important to recognise that much of our knowledge of NO_3 reducing systems (especially genomic descriptions) in bacteria have been obtained with microorganisms that may not be predominant species in the rumen. The following information is subject to this caveat.

NO_3 reductases. Three distinct types of NO_3 reductases catalyze the two-electron reduction of NO_3 to NO_2 in bacteria, *viz.* a periplasmic dissimilatory reductase (Nap), a membrane-bound respiratory reductase (Nar) and a cytoplasmic assimilatory (Nas), reductase. All three types of reductases can be present in the same organism and contain a molybdenum cofactor at their active sites.

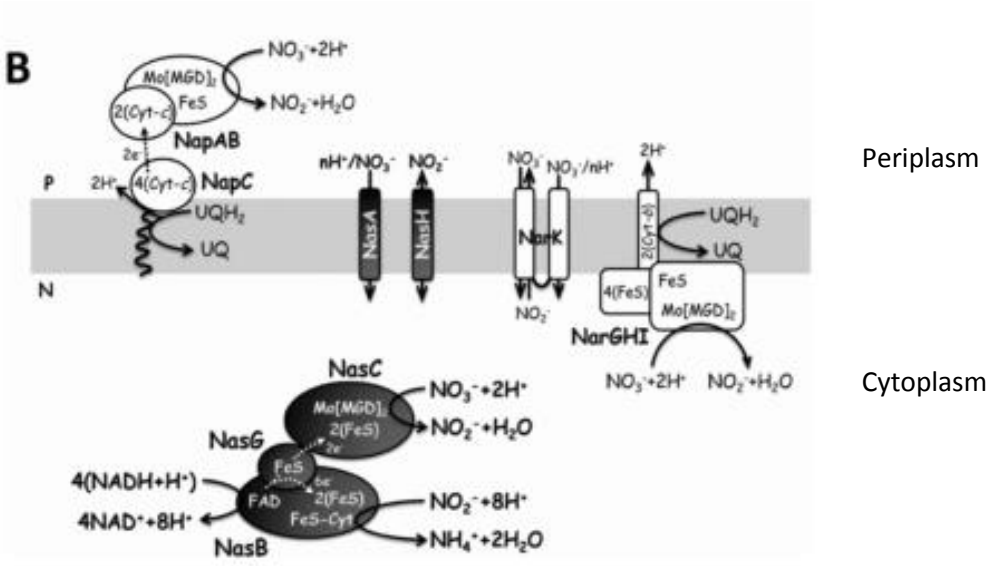


Fig. 5.1. Reduction of NO_3 to NO_2 during assimilatory and dissimilatory (cytoplasmic or periplasmic) pathways operated by Nap, Nar and Nas (Gates et al. 2011b)

It seems desirable to define an enzyme in its functional context rather than to categorise it because, depending on the metabolic fate of NO_2, any NO_3 reductase can have different functions under various conditions. Moreno-Vivian and Ferguson (1998) describe how distinctions can be made between assimilatory, dissimilatory and respiratory functions.

The periplasmic dissimilatory Nap is widespread in gram-negative bacteria. This reductase has a dissimilatory function and does not seem to have a significant role in NO_3 assimilation or anaerobic respiration. The Nap system probably facilitates the generation of a proton motive force (PMF) when the electrons from NADH are passed through NADH dehydrogenase (Richardson and Watmough 1999) and acts as a dissimilatory enzyme that helps bacteria maintain an appropriate redox potential for fermentation processes and optimal growth (Sears *et al.* 1997). Nap may also assist bacteria to transition from aerobic to anaerobic metabolism, and to make use of alternate reductants (Siddiqui et al 1993). In addition, Nap may enable the bacteria to use high NO_2 levels to inhibit the growth of competing bacteria (Siddiqui *et al.* 1993).

The respiratory membrane-bound Nar is also dissimilatory and generates a transmembrane PMF that supports ATP synthesis (Moreno-Vivian *et al.* 1999). The respiratory Nar and Nrf both require electron donors such as hydrogen or formate, whereas Nap and Nir require fermentative substrates such as glucose or lactate (Simon 2002).

Nitrate reduction in the cytoplasm depends on the Nas reductase (Fig. 3.1). *NasB* and *NasC* genes encode a cytoplasmic NAD(P)H dependent reductase in heterotrophic bacteria and fungi. The presence of NO_3 and NO_2 stimulates the activity of Nas; the enzyme is however inhibited by high ammonia concentrations (Moreno-Vivian *et al.* 1999). This enzyme is only active when ammonium is required for polymer synthesis in the cell but it may be important when ammonia concentration in the external medium is low. An important question in relation to rumen microbes: *Is ammonia formed by this process used without being first excreted*?

NO_2 reductases. Two types of respiratory NO_2 reductases are found in bacteria, *viz*. the Nrf encoded enzyme which reduces NO_2 to ammonia, and Nir which converts NO_2 to nitric oxide (Moreno-Vivian *et al.* 1999). Nitrite formed by NO_3 reduction can be reduced to ammonium or to nitric oxide (NO) by different types of NO_2 reductases (Brittain *et al.* 1992). There is no evidence that denitrification occurs in the rumen (Kaspar and Tiedje 1981) but some non-denitrifying organisms have soluble NO_3 reductases in the cytoplasm that reduce NO_3 to NO_2 or nitric oxide (NO) and, depending on the species, use ferredoxin, $FADH_2$ or NADH as the electron donors. A number of microbes produce N_2O during NO_3 reduction. To enable these systems to operate, transporters are needed to import the NO_3 and export the NO_2 or NO.

In *E. coli*, a cytoplasmic NADH-dependent enzyme encoded by the *nirB* gene (Harborne *et al.* 1992) catalyses the reduction of NO_2 to ammonium thereby preventing toxic accumulation of NO_2 and regenerating NAD^+.

Fig. 5.2 shows the cytoplasmic NO_2 reducing pathway of Nrf in the rumen microbe, *Wolinella succinogenes* which uses a non-fermentable substrate (formate or dihydrogen) to generate an electrochemical proton potential across the cytoplasmic membrane.

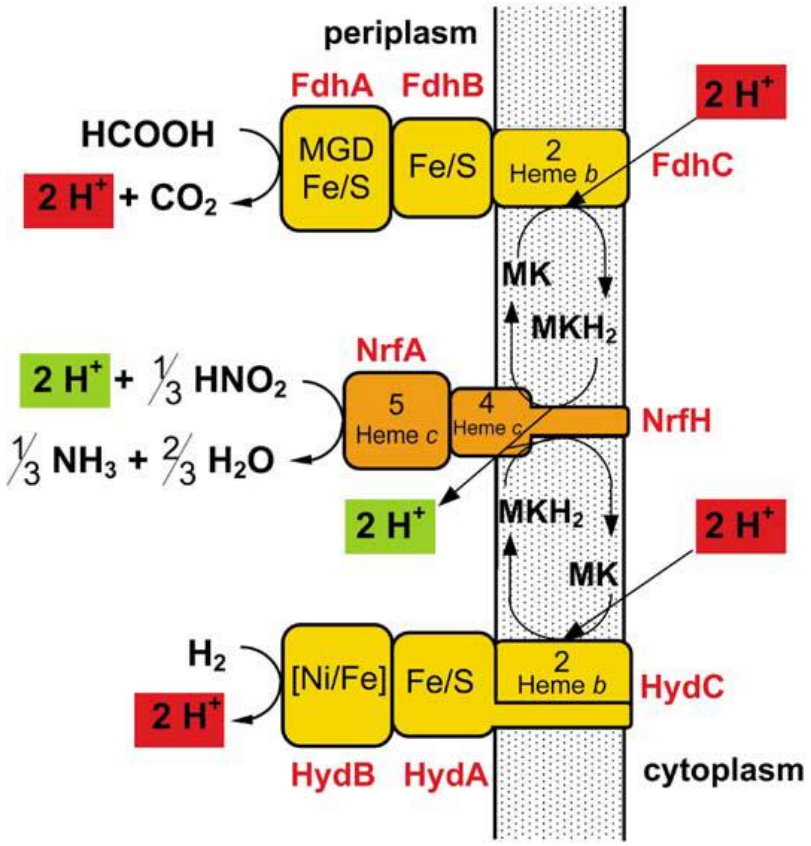


Fig. 5.2. Respiratory NO_2 reduction in* W. succinogenes*. Formate dehydrogenase (Fdh), respiratory NO_2 reductase (Nrf) and Hydrogenase (Hyd) are represented with their respective subunits (FdhA, FdhB; NrfA, NrfH; HydB, HydA, HydC) MK: menaquinone (Simon 2002)

Importantly, *W. succinogenes* is a genuine rumen bacterium and populations of this microbe increase in animals being acclimated to dietary NO_3.

A summary of the different NO_3 and NO_2 reductase enzymes found in anaerobic bacteria is given in Table 5.1.

Table 5.1 Types of enzymes involved in nitrate metabolism and their functions in anaerobic microbes

Characteristic	Assimilatory, NO_2^-	Dissimilatory		
		NO_2 respiration		NO_2 reduction
		Nir	Nrf	
NO_2 reductase	Assimilatory Nas	Respiratory Nir	Respiratory Nrf	Dissimilatory Nir
Location	Cytoplasm	Periplasm	Periplasm	Cytoplasm
Reaction catalyzed	NO_2^--->NH_4^+	NO_2^-→NO	NO_2^-→NH_4^+	NO_2^-→NH_4^+
Structural genes	*nasB*[a]/*nirA*[b]	*nirS/nirK*	*nrfA*	*nirBD*
Prosthetic groups	FAD[c], FeS[d], siroheme	cytcd_1[e]/Cu	cyt*c*	FAD, FeS, siroheme
NO_2 transport	Yes	No	No	Yes
Function	Biosynthesis of N compounds	PMF (denitrification)	PMF (ammonification)	2H ⇓[f] and NO2 detoxification
Regulation[g]				
O_2	No	Yes	Yes	Yes
NH_4^+	Yes	No	No	No
NO_3^-/NO_2^-	Yes	Yes	Yes	Yes

[a]Following the gene designation in *K. oxytoca* for the NADH-NO_2 reductase.
[b]Following the gene designation in cyanobacteria for the ferredoxin-NO_2 reductase.
[c]FAD is present in the NADH-NO_2 reductases, but it is absent from the cyanobacterial assimilatory ferredoxin-dependent NO_2 reductase.
[d]FeS, iron-sulfur centers.
[e]cytcd_1, cytochrome cd_1 complex.
[f]2H ⇓, dissipation of reducing power.
[g]Some differences in regulation in prokaryotic organisms have been reported.

Transport mechanisms

Biological membranes are highly impermeable to polar or charged molecules, so dedicated channels or transporters are needed to enable ions to move across the cytoplasmic membrane(Lu *et al.* 2013). Cation channels in particular must have a high degree of selectivity so that they can exclude the smallest cation, *i.e.* the proton, yet allow larger cations to pass, thereby enabling proton potentials across the membrane to be maintained

(Lu *et al.* 2013). The formate-NO_3 transporters in microbial cells move various small anions such as Cl^-, formate, NO_3 and hydrosulphide through the membrane. Notably, the FocA channel is co-transcribed with pyruvate-formate lyase which catalyses the cleavage of pyruvate to acetyl-CoA and formate (Lu *et al.* 2013).

Owing to the cytoplasmic location of the Nas enzyme, NO_3 transport across the cytoplasmic membrate is required before NO_3 reduction by this enzyme can occur (Moreno-Vivian *et al.* 1999). Two types of NO_3 transporters are involved in bacterial assimilatory NO_3 reduction: the ATP-dependent ABC transporters; and the monomeric NarK-type PMF-dependent transporters. In most bacterial Nas systems, NO_3 seems to be transported by an ABC-type transporter consisting of an integral membrane subunit, a cytoplasmic ATP-binding component and a periplasmic substrate-binding protein (Moir and Wood 2001). Nitrate and NO_2 transport systems (and genes coding for their components) have been thoroughly studied in cyanobacteria and *Klebsiella*. However, in the rumen microbe, *B. subtilis,* an electrogenic NO_3 uptake mediated by a different transporter, the *nasA* gene product is present (Ogawa *et al.* 1995).

Ammonium can move across membranes by diffusion (especially in alkaline media).The enzymes responsible for ammonium assimilation (GS-GOGAT and GDH) are present in all living organisms but ammonium transporters (Amt) are found in bacteria, archaea and eukarya. Amt proteins constitute an active NH_4^+ uniport dependent on a PMF that may also facilitate diffusion of ammonia in both directions across the plasma membrane (Soupene *et al.* 2002; Cabello *et al.* 2004). In this way, ammonium ions, particularly those generated by dissimilatory NO_3 reductases can be used by other microbes for synthetic purposes.

Assimilatory reduction of NO_3 to ammonia

Nitrate assimilation is a mechanism allowing N to be incorporated into bacterial polymers during their growth (Lundberg *et al.* 2004; Lundberg *et al.* 2009). The overall process requires reducing equivalents, i.e. 8 electrons, for the assimilatory reduction of NO_3 to ammonia

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$$

$$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$$

Nitrite reduction is performed by a cytoplasmic NasB sirohaem-dependent NO_2 reductase (Lin & Stewart 1998) or NirBD (Berks *et al.* 1995)or periplasmic enzymes encoded by the nrf operon – the latter also being involved in 'NO_2 dissimilation'. One of these enzymes is the multiheme cytochrome *c* NO_2 reductase of *E. coli*, encoded by the *nrf* operon. This enzyme catalyses the formate-dependent NO_2 reduction to ammonium (Einsle *et al.* 2002). Both the formate- and NADH-dependent pathways (Nrf and Nir, respectively) appear to be totally repressed during aerobic growth, but are partially induced during anaerobic growth in the absence of NO_2; and they are induced further and become more active when NO_2 is present (Page *et al.* 1990).

The assimilatory reductases from most heterotrophic bacteria are apparently dependent on the pool of cytoplasmic reduced pyridine nucleotides which enables them to be coupled to organic carbon catabolism (Moreno-Vivian and Flores 2007). Assimilatory NO_3 reduction would potentially dispose of some excess reductant present in a reduced organic carbon pool (Kirchman 2000). However, the biochemical mechanism by which assimilatory NO_3 reductases of heterotrophic bacteria access this pool of cellular reductants is not well understood, particularly because analysis of the primary structure of these proteins suggests that they do not have NADH or NADPH binding domains (Lin and Stewart 1998; Richardson *et al.* 2001; Gates *et al.* 2011a) identified a key role for a putative Rieske-type [2Fe-2S] ferredoxin in *P. denitrificans* that is widely conserved in other bacterial Nas systems and showed that this protein is essential for coupling of NADH oxidation to both NO_3 and NO_2

reduction. For assimilatory NO_3 reduction, electrons were probably derived from NADH within the cytoplasm. Electrons from FAD-containing NO_2 reductase (NasB) flow to the NO_3 reductase (NasC), possibly via the small ferredoxin (NasG). In the Nas system, there are two transporters; NasA and NasH, predicted to be involved in NO_3 and NO_2 transport, respectively. This hypothesis is depicted in Fig. 6.

Dissimilatory reduction of NO_3 to ammonia

Dissimilatory NO_3 reduction to ammonia (DNRA) occurs in the rumen because of its low redox potential, especially when there is a ready source of fermentable substrates. Microorganisms benefit from DNRA which is used (a) to detoxify the accumulated NO_2, (b) to serve as an electron sink allowing the re-oxidation of NADH, and (c) to release energy through electron transport phosphorylation (ETP). Under NO_3-limiting conditions, the need for an electron sink is more important and thus NO_2 is converted to ammonium.

The main energy-producing step of DNRA is the reduction of NO_3 to NO_2. The reduction of NO_2 to ammonium is catalysed by soluble periplasmic NO_2 reductase which is not NADH-dependent and which prevents conservation of energy. Therefore, when fermentable organic matter is limiting, NO_2 is not converted to ammonium and therefore accumulates (Tiedje 1988). Nevertheless, some soluble NO_2 reductases, e.g. sulfite reductase, are NADH-linked. In the presence of sulphide, NO_2 reduction to ammonium may be enhanced because sulphide is an alternative substrate for the nitrite reduction pathway (Brittain *et al.* 1992). The presence of sulfide in the environment also enhances DNRA systems (Brunet and Garcia-Gill 1996).

DNRA activity is higher in carbon rich, electron acceptor poor environments, and so anaerobic environments are best for DNRA populations. DNRA becomes the main NO_3 and NO_2 reduction pathway at chemical oxygen demand and nitrogen ratio (COD/N) values greater than 53 (Akunna *et al.* 1992). All of the microorganisms carrying out DNRA are able to couple H_2, or formate oxidation to ATP production via DNRA (Brunet and Garcia-Gill 1996). Various physiological electron donors for DNRA have been identified, and include compounds such as glucose, pyruvate, lactate, glycerol, and formate, as well as inorganic electron donors such as H_2

In the rumen of animals grazing on dry mature roughages, or in penned animals given basal diets of low digestibility straws and agricultural by-products, nitrogen for cell growth may often be limiting. Under these circumstances, when NO_3 is provided as the major source of nitrogen in the diet, it is likely that NO_3 assimilation pathways would predominate. However, if NO_3 is being supplemented with a view to methane mitigation, its rate of release in the rumen may determine whether DNRA processes occur. If supplementary NO_3 is ingested in a single meal, the rate of NO_3 release may well exceed the amount currently required for cell synthesis. In this case, DRNS may predominate when intake is highest - soon after the meal is offered, or when a grazing bout commences -and NO_2 may accumulate.

A summary of the enzymes and transporters known in the paradigm microbe, *E. Coli* is given in Fig. 5.3.

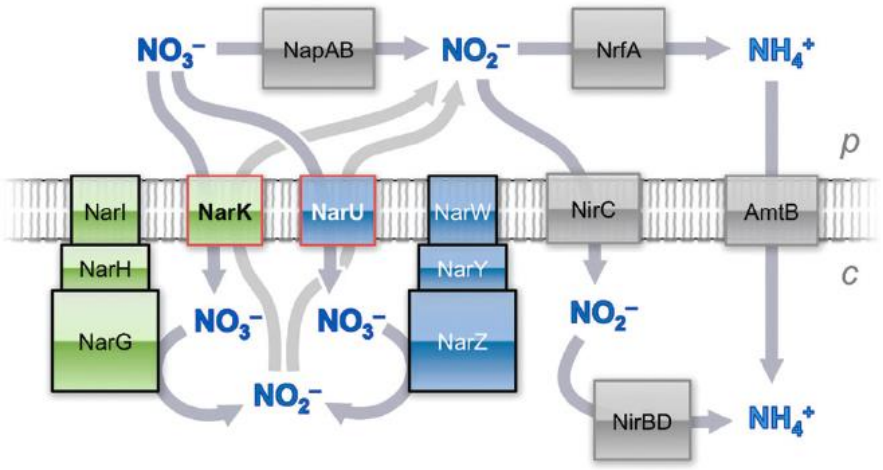


***Fig. 5.3. A diagram showing the known enzyme and transporter molecules of N metabolism in* E. Coli (after Andrade and Einsle (2013).** Nitrate reductases NarGHI and NarZYW face towards the cytoplasm. Nitrate is imported and the toxic product, NO_2, is exported into the periplasm by the exchanger NarK and NarU. The periplasmic NO_3 and NO_2 reductases NapAB and NrfA serve in detoxification, while the $NH4^+$ transporter AmtB and the cytoplasmic NO_2 reductase NirBD, with the corresponding NO_2 channel NirC, are required for assimilation N for bacterial polymer synthesis.

Appendix 6 - The fate of NO_3 salts after intraruminal dosing NO_3 and NO_2 kinetics

Pathways of nitrate metabolism in the body

After NO_3 enters the rumen and is dissolved in the rumen fluid, it is reduced by microbes to NO_2 and then to ammonia. These compounds are incorporated into microbial materials, absorbed through the rumen wall, and pass distally from the rumen in digesta. After materials enter the bloodstream they may be eliminated via the kidneys or by recycling to the gut. The pathways are represented in Fig. 5.1.

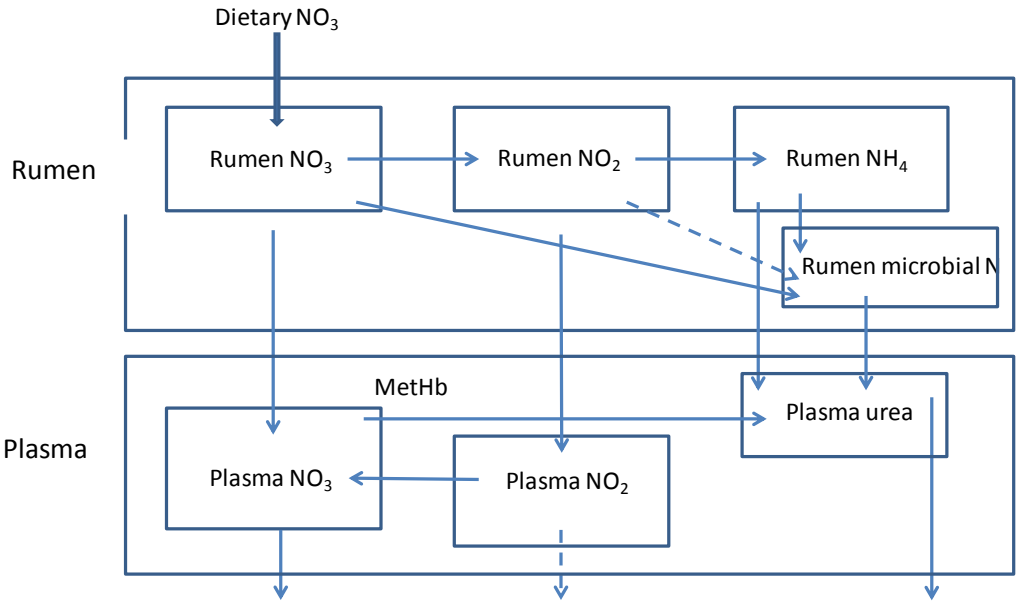


Fig. 6.1. Pathways by which dietary NO_3 may pass through the rumen contents and blood plasma in ruminants

University of New England (UNE) studies

At UNE in 2009, we studied the rate of disappearance of NO_3 dosed into the rumen of 8 sheep and found the decrease in concentration of NO_3 via all pathways was well described by a single exponential function in all animals. This suggests NO_3 removal is a concentration-dependent process. Other published studies also show that NO_3 removal from rumen fluid is concentration-dependent. However, there are published studies that suggest that NO_2 is not formed immediately by reduction of the disappearing NO_3 (Leng 2008). This result implies a possible transient (unknown) N intermediate exists in conversion of NO_3 to NO_2. Some of these studies are re-examined in more detail below.

Re-analysis of data from Jameson (1958)

Jameson (1958) gave a single intraruminal dose of 25 g KNO_3 to two grazing sheep (24 and 36 kg) and monitored the resulting appearance of NO_2 and ammonia in rumen contents. Unfortunately, there was too much variation in ammonia concentration to enable a three-compartment model to be fitted with confidence. A two-compartmental model was fitted to the data using WinSAAM, incorporating an assumption that rumen fluid was replaced once per day (k value = 0.042/h).The fitted data for a two-compartment model showing disappearance of NO_3 and appearance of NO_2 is given below.

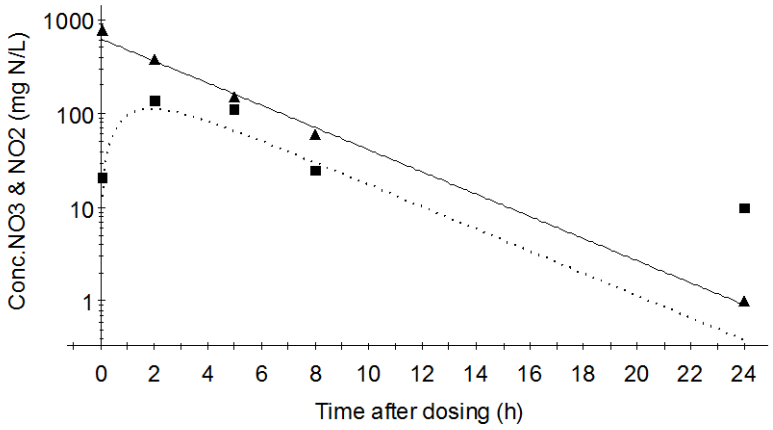


Fig. 6.2. Solution for a two-compartmental model describing the perturbations in concentrations of NO_3 and NO_2 in rumen fluid of sheep after a single intraruminal injection of 25 g KNO_3

The model suggests the NO_3 dose was diluted in a rumen fluid volume of 6.6 L (which is reasonable) and the NO_3 disappearance from the rumen fluid was well described by a single exponential function ($t_{1/2}$ = 2.5 h). The data suggest 85% of the NO_3 was converted to NO_2 (concentration peaked after about 2 h) and 15% of the NO_3 was either incorporated directly by rumen microbes or flowed to the lower gut. For the NO_2 pool, 5% remained in microbes or passed to the lower gut and about 95% was presumably reduced to ammonia. There were too few data to confidently determine whether or not there was any delay before NO_2 reduction and NO_3 appearance. The last point for the secondary NO_2 pool is poorly fitted and suggests that concentration-dependent kinetics may not apply to NO_2 turnover.

Re-analysis of data from Wang et al. (1961)

Wang *et al.* (1961) injected 70 g KNO_3 intraruminally into a cow and monitored the concentration of NO_3, NO_2 and ammonia in rumen contents.

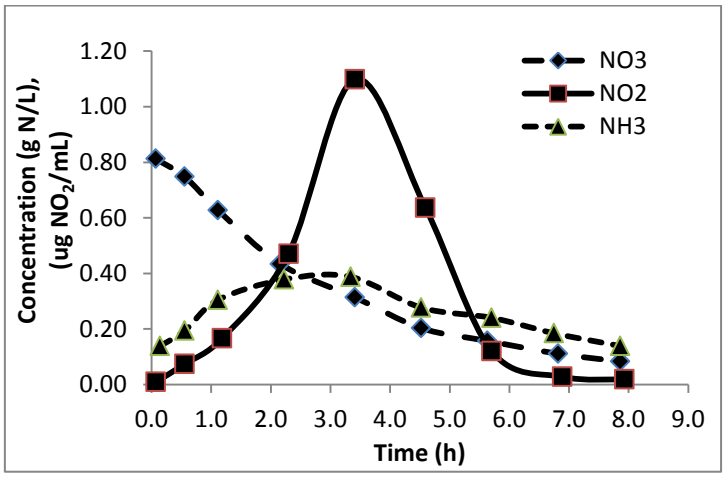


Fig. 6.3. Nitrate (mg N/L), NO_2 (ug N/L) and ammonia (mg N/L) concentrations in rumen fluid after an addition of 70 g KNO_3 (42 g NO_3 or 9.5 g N) to the rumen of a Hereford cow (Wang et al. 1961)

The NO_3 concentration declined rapidly ($t_{1/2}$ about 2.3 h) with appearance of NO_2 and ammonia which reached their peak values after about 3 h.

When results of Wang and co-workers were fitted to a two-compartment model (NO_3 and ammonia in rumen fluid) using WinSAAM (Fig. 6.4), the estimated rumen fluid volume (12 L) was much lower than expected for a cow weighing approx. 350 kg. The reason for this discrepancy is not known but no further analysis was undertaken with these data. It does appear, however, that most of the NO_3 dose was reduced to, and recovered in, rumen fluid ammonia.

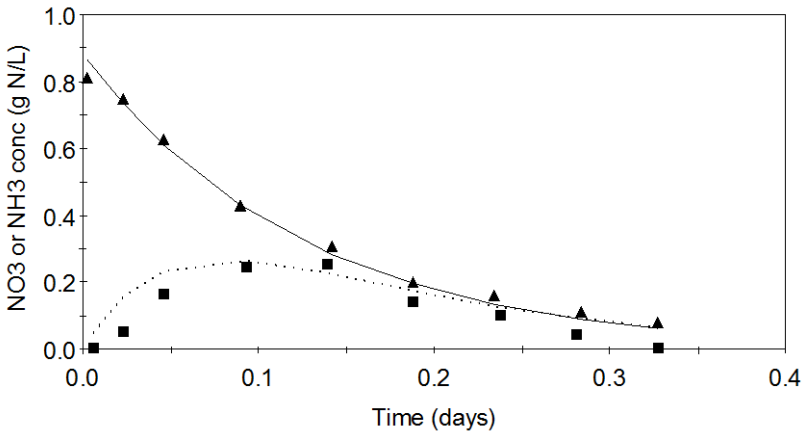


Fig. 6.4. Decline in concentration of NO_3 (▲) and increase and decrease in concentration of ammonia (■) in a cow after intraruminal dosing with 70 g KNO_3 (Wang et al. 1961)

Further data of Wang and co-workers in Fig. 6.5 shows the appearance of MetHb in the blood (peak %). Notably, the dilution of ^{15}N in the combined [NO_3+NO_2] pool was quite rapid, indicating that considerable quantities of unlabelled NO_3 and/or NO_2 were entering the rumen - a point that was not discussed by the authors but is difficult to explain unless it is from endogenous sources. The enrichment of [NO_3+NO_2]-N was not a simple exponential decay, reflecting the effect of analysing both NO_3-N and NO_2 N in the one sample and fitting the average enrichment.

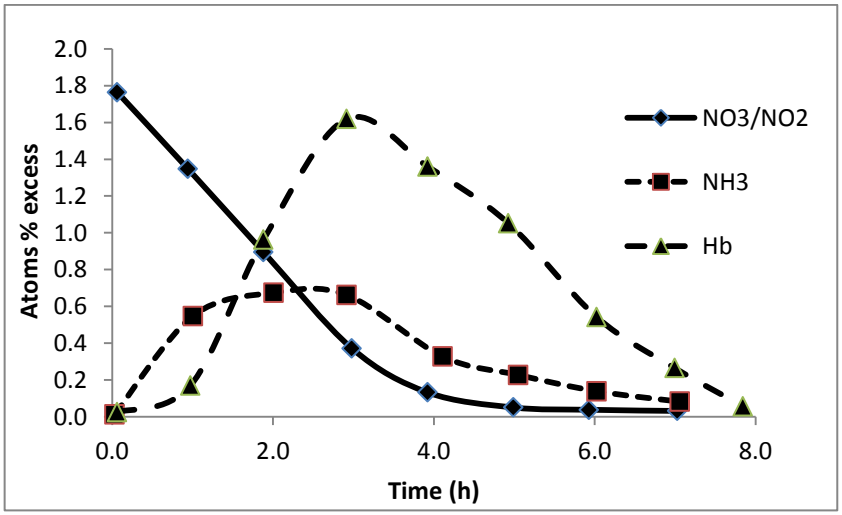


Fig. 6.5. The decay in enrichment of [NO_3 + NO_2]-N (♦) and the appearance of labelled ammonia-N (■) in rumen fluid after an intraruminal dose of 120 g $^{15}KNO_3$ (1.7 atoms % excess). The appearance of MetHb (peak approx. 16%) is also given (▲). (after Wang et al. (1961)

The results of this experiment suggest the peak for rumen NO_3 was about 2 h which was also approximately the time of the peak ammonia enrichment.

Re-analysis of data from Alaboudi and Jones (1985)

Alaboudi and Jones (1985) offered acclimated sheep (50-60 kg) two meals, each of 600 g of a hay/grain diet, each day at 1000 and 1600 h. The diet contained 1.5 kg KNO_3/kg W providing a total of 50 g NO_3 each day, i.e. diet contained 4.2% NO_3. The changes in NO_3 and NO_2 concentration in rumen fluid and in blood MetHb concentration after a meal are given in the figure below.

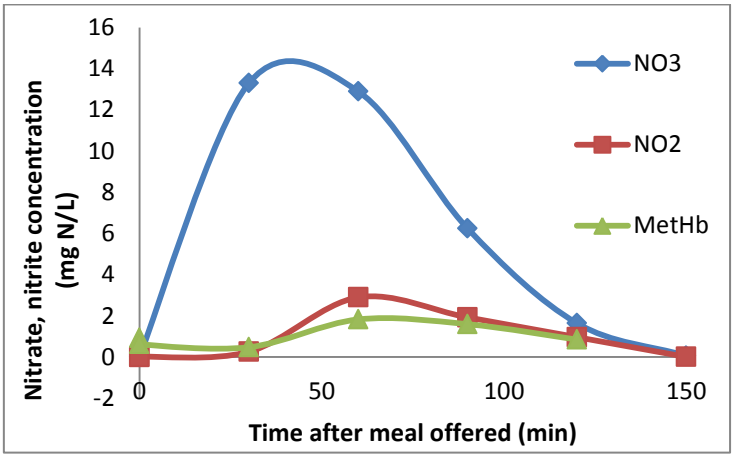


Fig. 6.6. The increase in NO_3 and NO_2 concentration in rumen fluid and in blood methaemoglobin concentration after acclimated sheep were offered 600 g of hay:grain containing 25 g NO_3 (Alaboudi and Jones 1985)

The rate of ingestion of the meal is not known, and so the pattern of build-up in NO_3 concentration that occurred in the rumen could not be modelled easily and may not be well represented by the fitted curve. However, it is clear the peak in NO_2 concentration occurred later than the NO_3 peak. The peak in blood MetHb concentration appears to be closer in time to the peak for rumen fluid NO_2, suggesting absorption of NO_2 occurs rapidly (and probably directly from the rumen). Despite the relatively high concentration of NO_3 in the diet, the formation of MetHb was quite low and of little clinical significance. Notably, the diet contained 50% grain (oats and barley).

Re-analysis of data from Barnett and Bowman (1957)

Barnett and Bowman (1957) used rumen fluid collected from an abattoir to set up artificial rumens to which they added different substrates and examined the kinetics of added NO_3. The results are given in Figs 1-4 below (Fig. 1, no supplement, Fig. 2, powdered cellulose, Fig. 3, glucose, Fig. 4, dried grass).

Leng (2008) noticed that the NO_3 removed apparently did not appear immediately in NO_2 or ammonia. Notably, the NO_3 that disappeared in the first 5 h, appeared later (from 5-30 h) as NO_2, so that NO_2 concentration (mM) at 35 h of incubation had increased to almost half the NO_3-N concentration (mM) at the start of the incubation period. Leng suggested there might be an initial sequestration of NO_3 or NO_2 within rumen microbial cells and that a considerable fraction of the NO_3-N dose might be released later as NO_2. It is also possible that initially the rate of NO_3 reduction to ammonia keeps pace with the rate of NO_2 appearance from NO_3, but that the rate of NO_2 reduction slows over time due to NH_3 accumulation or shortage of substrate, then NO_2 builds up, creating the impression that there is a delay in NO_2 appearance. There is also evidence that NO_2 binds to fibre (Shahidi and Hong 1993; Amarowicz *et al.* 1996) and so fibre in rumen contents may 'sequester' NO_2 until its binding capacity is saturated.

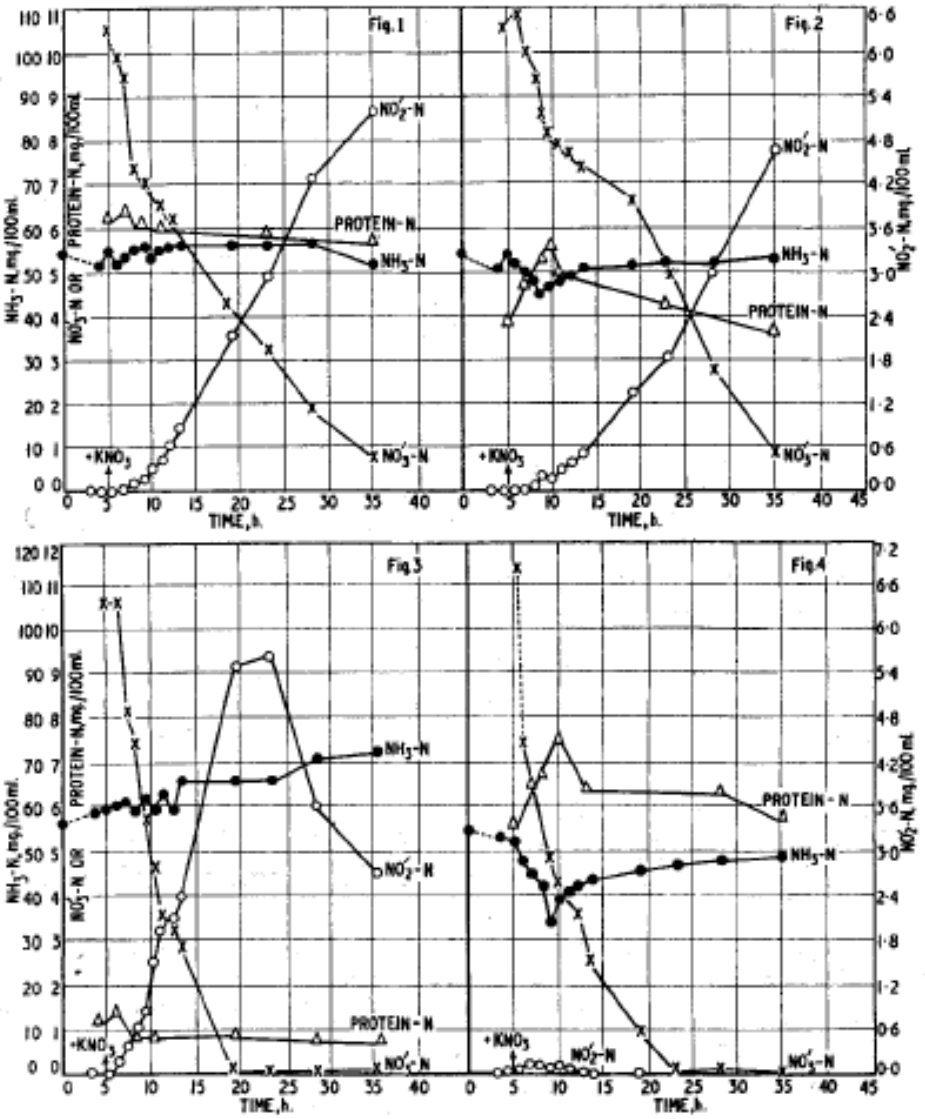


FIG. 1.—*Value for NO'_3-N, NO'_2-N, NH_3-N and protein-N in the artificial rumens 1 and 2. The results are averages of very similar values obtained with each rumen*

× mg. NO'_3-N/100 ml. rumen fluid
○ mg. NO'_2-N/100 ml. rumen fluid
● mg. NH_3-N/100 ml. rumen fluid
△ mg. protein-N/100 ml. rumen fluid

FIG. 2.—*Values as in Fig. 1 for rumen 3*
FIG. 3.—*Values as in Fig. 1 for rumen 4*
FIG. 4.—*Values as in Fig. 1 for rumen 5*

Fig. 6.7. Effect addition of different substrates to rumen contents *in vitro (Barnett and Bowman 1957)*

Re-analysis of data from Lewis (1951)

We re-evaluated a similar experiment (Lewis 1951) in which NO_3 (12 g $NaNO_3$, 8.75g NO_3) was dosed into the rumen of a wether (3 years old given 1.6 kg/d meadow hay) 16 h after feeding. We used WinSAAM to fit a two-compartment model describing the disappearance of NO_3 and the appearance of NO_2 after the NO_3 dose was administered into the rumen. The situation appeared similar to that found by Barnett and Bowman (1957) in that NO_2 did not appear immediately. To obtain a good fit to the data, it was necessary to program a 'lag' of

0.93 h between the NO_3 and NO_2 compartments. The fitted data are shown in the Fig. 6.8 below.

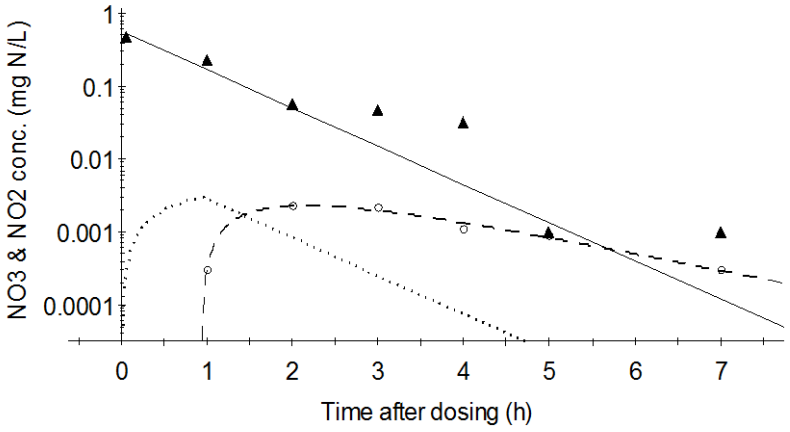


Fig. 6.8. The output from a model fitted in WinSAAM showing the decrease in NO_3 concentration in rumen fluid (▲) after administration of 8.75 g NO_3 into the rumen and the appearance, after a delay, of NO_2 in rumen fluid (○, data from Fig 1, (Lewis 1951). The dotted line with no experimental data represents the NO_3 in a putative pool thought to be responsible for the delay in appearance of NO_2

As in other studies, the disappearance of NO_3 appeared to be concentration-dependent but was more rapid than in the studies discussed above ($t_{1/2}$ = 0.5 h). However, the delay in the appearance of NO_2 is difficult to explain if only concentration-dependent kinetics are applied. It is possible that nitrite reductase activity would be better explained by applying Michaelis-Menten kinetics but, even with this assumption, it is difficult to reconcile the results.

Notably, the model indicates that the formation of rumen fluid NO_2 was not quantitative and there was a substantial loss of NO_3 before its conversion to NO_2 [L(0,1) versus L(2,1)]. (This would in part represent the flow of NO_3 to the lower digestive tract and its absorption across the rumen wall.)

Interestingly, Woods (1938) made the following observation: *Experiments on the effect of pH on the initial rate of H_2 uptake with NO_3 yielded a curious result. It was found that below pH 7.3 there is a marked lag period of about 5 min. before the rate becomes linear*. *This lag period is less marked or absent at pH 7.3 and above. This phenomenon has been observed with two other batches of organisms and no explanation can be offered for it.*

UNE studies by Nolan, Hegarty and Li *(paper in preparation)*

In the published work on reduction of NO_3 in the rumen re-evaluated above, the quantities of NO_3 administered have been delivered in relatively large meals. (Even with the ^{15}N studies of plasma NO_3 kinetics by Lewicki *et al.* (1998) relatively large amounts of NO_3 with a low ^{15}N enrichment were used.) It seems probable that the quantities of NO_3 fed, and in particular, the NO_2 produced, may have altered the conditions in the rumen fluid to the extent that microbial activities were altered or compromised.

At UNE, we studied the kinetics of a single intra-ruminal dose of $^{15}NO_3$ in 8 sheep given a diet of chaffed oaten hay (9.7 MJ ME, 60 g CP, 2.9 g NO_3^-/kg DM) containing iso-nitrogenous additions of either NO_3 (2.5% of DM) or urea-N (1.2% of DM). However, the

ration was given in small meals (31 g) at hourly intervals, so that the sheep probably ingested small amounts of NO_3 every hour. The $^{15}NO_3$ administered was a small fraction of the total NO_3 entering the rumen.

A 3-compartment model (NO_3, ammonia and bacterial N isolated from rumen fluid) was used to describe the transfers of N from NO_3^- to NH_3 and bacterial N in the rumen of the sheep. Enrichments of N in rumen NH_3 and bacterial compartments after a single intraruminal administration of $^{15}NO_3^-$ or $^{15}NH_3$ were visually and statistically well-fitted by this model. The model solutions enabled the completeness of reduction of ruminal NO_3^- to NH_3 to be estimated; in addition, NH_3 and bacterial compartment masses and N transfers between them were quantified.

A – ^{15}N-ammonia B – ^{15}N-NO_3

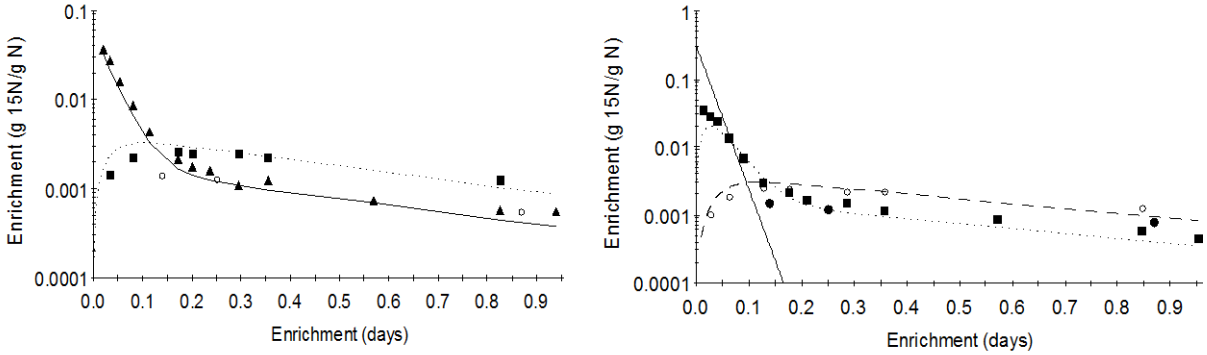


Fig. 6.9. Output from a 3-compartment rumen model for a sheep (shown as lines over-plotted on experimental data represented by symbols) for enrichment of NH_3-N (▲) and bacterial N (■) after intraruminal injection of $^{15}NH_4$-Cl (A), and NO_3-N (no data, simulated solid line), NH_3-N (▲) and bacterial N (■) after intraruminal injection of the same amount of ^{15}N as $K^{15}NO_3$ (B). The sheep was ingesting a diet of chaffed oaten hay containing 1.2% urea and 0.06% NO_3 in the dry matter. All 4 sets of enrichment data were fitted simultaneously using the one model.

In contrast to the results of the 'intraruminal NO_3 loading' experiments discussed above, these results suggest the small amount of NO_3 administered was rapidly reduced to NO_3 and then to ammonia in the rumen – this is evident from the apparently rapid decay in NO_3 enrichment and the almost immediate appearance of label in rumen fluid ammonia (Fig. 6.9).

The solutions for the model output for sheep given iso-nitrogenous amounts of urea and or NO_3 are given in the figures 6.10 ad 6.11 below.

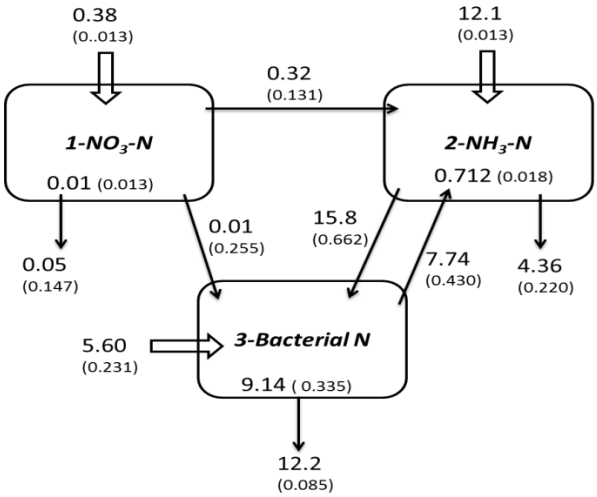


Fig. 6.10. <u>Urea-supplemented sheep</u>. A steady state model showing compartment sizes (M(i), g N) and inter-compartmental flow rates (R(i,j), g N/d) in the rumen fluid of sheep (mean ± SE). The model was fitted to experimental tracer data from compartments 2 and 3 for each of the sheep given a diet of chaffed oaten hay supplemented with 12 g urea or 5.52 g urea-N/d (see example of fit for one of the sheep in Fig. 2a).

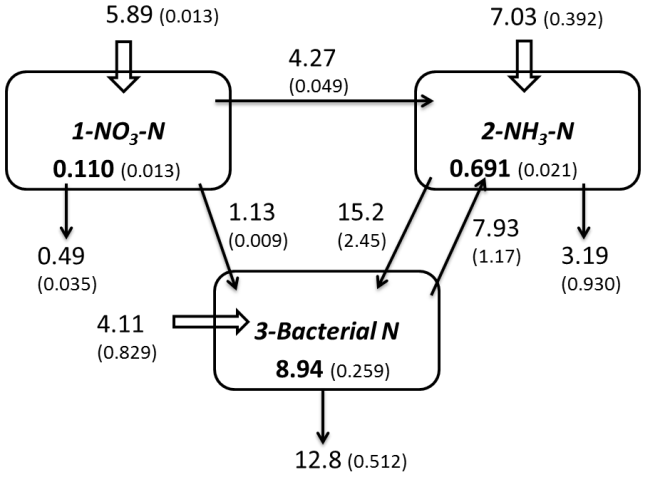


Fig. 6.11. <u>NO_3-supplemented sheep</u>. A steady state model showing compartment sizes (M(i), g N) and inter-compartmental flow rates (R(l,j), g N/d) in the rumen of sheep (mean ± SE). The model was fitted to experimental tracer data from compartments 2 and 3 for each of the sheep given a diet of chaffed oaten hay and a supplement of KNO_3 supplying 24.4 g NO_3 or 5.52 g N/d (see example of fit for one of the sheep in Fig. 2b).

Some NO_3^--N (about 27%) of that entering the NO_3 compartment) was not converted to NH_3 and therefore was unavailable to oxidize metabolic H_2 and thereby reduce methane emissions;. 73% of the NO_3^- entering the rumen was chemically reduced to NH_3 while the remaining 27% was directly assimilated by bacteria (19%) or absorbed across the gut wall or removed as NO_3 or NO_2 in digesta flowing out of the rumen (8%).

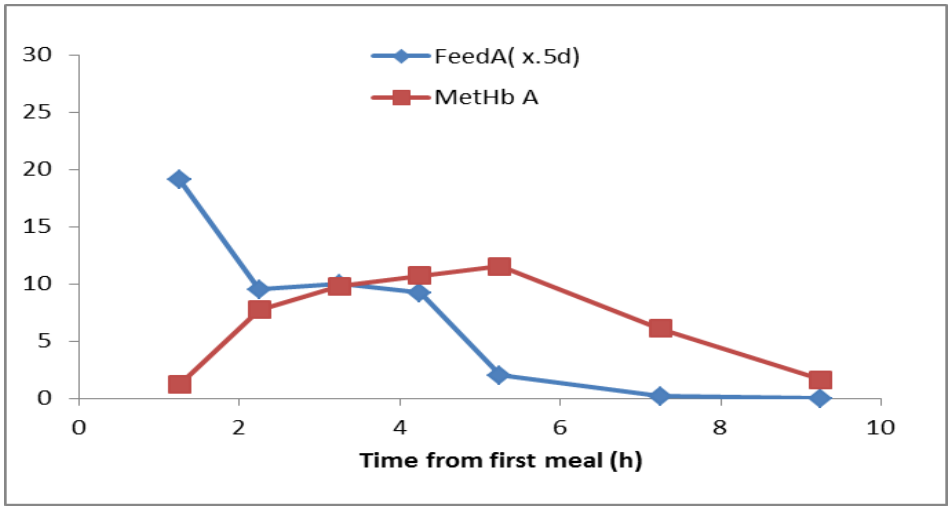
The results show that only a small fraction of the NO_3 reduced in the rumen was directly assimilated by rumen microbes (about 6% of the N incorporated by microbes in the sheep fed NO_3). If assimilatory processes occurred within individual microbes (i.e. NO_3 reduction to ammonia and ammonia incorporation into cell polymers), then ^{15}N in labelled NO_3 taken up by microbes would be incorporated into ^{15}N-labelled cell polymers without first labelling ^{15}N-ammonia in the rumen fluid medium. There is very little information on the relative importance of assimilatory and dissimilatory pathways of NO_3 reduction in mixed rumen populations of microorganisms (see Lin and Stewart (1998)). According to our study, most of the ^{15}N administered intraruminally as $^{15}NO_3$ is quickly reduced to ^{15}N-ammonia which then entered rumen fluid before being taken up by microbes for incorporation into cell polymers. This suggests the microbes performing the NO_3 reduction excreted NO_2 or ammonia into rumen fluid and other microbes assimilated the rumen ammonia for cell polymer synthesis.

Effect of rate of ingestion of dietary NO_3 on MetHb formation

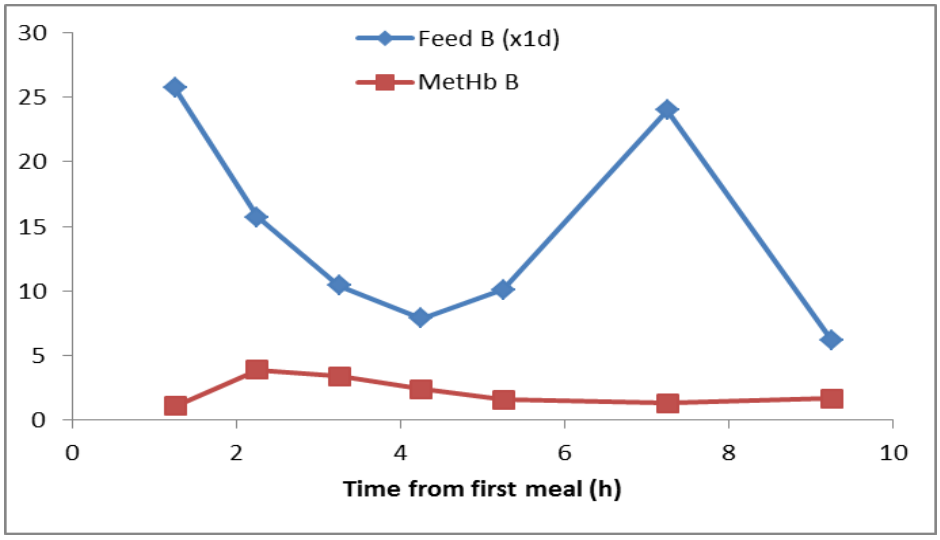
At UNE, de Raphélis-Soissan *et al*. (paper in preparation) offered sheep 1000 g oaten chaff containing NO_3 daily as either as a single meal offered at 08:00 h, as 2 meals of 500 g offered at 08:00 and 20:00 h, or as meals of 83.3 g at hourly intervals.

The concentration of MetHb was always below a clinically significant concentration (<12%) but was closely dependent on the rate of ingestion (and probably absorption) of feed/NO_3 (see Fig. 6.12 below). It can be concluded that nitrite build-up in rumen fluid and nitrite absorption is closely relate to the rate of NO_3 entry into the rumen fluid at any point during the day. Management procedures that reduce the size of the pulses of NO_3 entering the rumen and spread the entry of NO_3 over a longer period will be likely to reduce the peak concentrations of NO_2 in rumen fluid, and so reduce the risk of NO_3 poisoning. Coated forms of NO_3 may help achieve this goal.

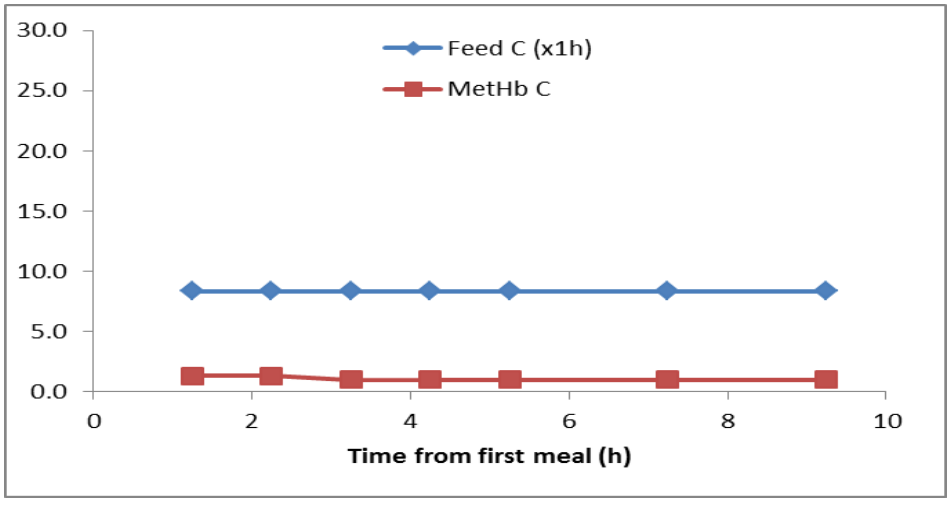
Feed intake recorded at intervals after feed containing NO_3 was first offered to sheep at 08:00 h and the response in MetHb concentrations in blood is shown in Fig. 6.12 with the same responses from similar sheep fed *ad libitum*.



(a)



(b)



(c)

Fig. 6.12. Feed intake (g, blue line) recorded at intervals after feed containing NO_3 was first offered to sheep at 08:00 h and the response in MetHb concentrations in blood monitored (red line). Figures show responses of sheep to (a) a single meal of 1000 g offered at 08:00 h, (b) as 2 meals of 500 g offered at 08:00 and 20:00 h, or (c) as meals of 83.3 g at hourly intervals

Intake rate after a single meal offered in the morning was initially quite high and this form of feeding produced a higher MetHb peak concentration than when the same quantity of feed was offered twice daily. When feed intake was relatively uniform throughout the day, MetHb remained at baseline levels. These findings are similar to those of (Guerink *et al.* 1979) who found that MetHb concentration increased with rate of forage intake.

In a study of fed-lot cattle, Velazco *et al.* (2014) found that NO_3-fed cattle ingested more meals over the day than cattle given meals containing the same quantity of N as urea (14.7 vs 7.39 meals/day; $P < 0.05$) and the meals were smaller (0.77 vs 1.82 kg/meal). The reason for the difference in intake patterns is not clear. It is possible that cattle become aware of the adverse metabolic consequences of ingesting NO_3 too quickly and modify their feeding behavior to reduce this effect (du Toit1 *et al.* 1991).

Conclusion. The results show that MetHb concentration in blood is closely associated with patterns of NO_3 ingestion over time. It is possible that cattle respond to adverse metabolic consequences when they ingest NO_3 too quickly and subsequently modify their feeding behavior, taking more frequent but smaller meals, to reduce this effect. However, there are differences between sheep in the magnitude of the MetHb response to similar intakes of NO_3 and feeding behaviour of animals given NO_3-supplemented diets may show corresponding between-animal differences.

Appendix 7 – Absorption of rumen NO_2 from the gut

UNE, Godwin/Nolan Expt – April 2014 (anaesthesised sheep)

One study (Pfander *et al.* 1956) showed NO_3 and NO_2 were not absorbed from the rumen and, surprisingly, there is little other direct evidence of NO_3 and NO_2 absorption from the rumen, reticulum and omasum. Nevertheless, there is a general consensus in more recent publications that this occurs.

At UNE, we (I.R. Godwin, J.V. Nolan, unpublished) studied the removal of NO_2 from the rumen contents and appearance of NO_3 and MetHb in blood of an anaesthesised sheep after 5 g KNO_2 was administered intraruminally. The rumen was tied off at the pylorus to prevent distal flow of rumen contents and to eliminate the potential for post-ruminal absorption of NO_2 or ammonia. The results are shown in Fig. 7.1 below.

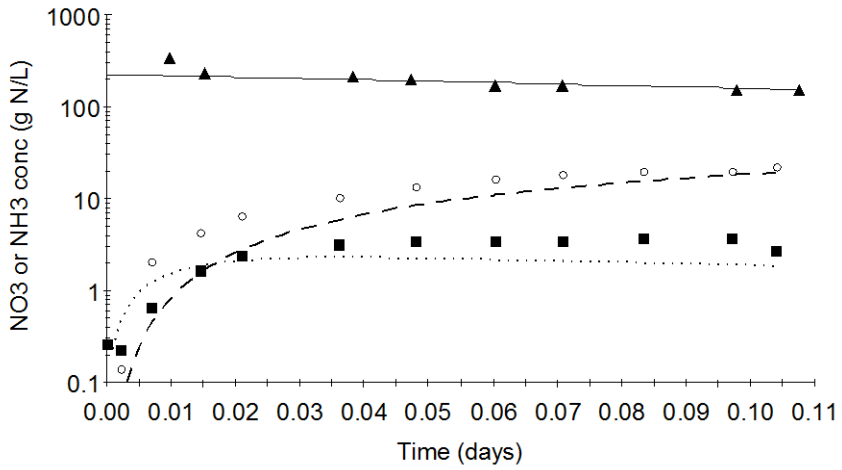


Fig. 7.1. Disappearance of ruminal NO_2 (▲) after an intra-ruminal dose of 1018 mg NO_2-N and appearance of NO_2 (■) and NO_3 (○) in blood

The over-plotted lines in Fig 7.1 are the solution to a model fitted to the experimental data using WinSAAM.

Table 7.1. Rate constants fitted by WinSAAM to a three-compartment model describing loss of NO_3 from the rumen compartment and appearance of NO_2 and NO_3 in the blood of an anaesthesised sheep.

Category	Form	Solution	Minimum	Maximum	FSD
L(0,1)	D	1.00E-05/d			
L(2,1)	A	3.51	0.1	100	0.330
L(3,2)	A	93.1	0.1	1000	0.343
L(0,3)	A	0.099	0.099	10	92.1
P(1)	A	4.5 Litres	4.5	8	0.082
P(2)	A	14.7	2.7	15	0.382
P(3)	D	14.7			

Conclusion. NO_2 was absorbed directly from the rumen, albeit slowly ($t_{1/2}$ = 4.7 h), contradicting earlier reports by Pfander *et al.* (1956). All NO_2-N removed was later detected in blood NO_2 and later blood NO_3, suggesting reduction of NO_3 to ruminal ammonia (L(0,1)) was negligible. This could indicate that rumen microbial activity was compromised by the added NO_2 which produced an unphysiologically high initial concentration of NO_2 in the rumen. On the other hand, most of the NO_2 entering the blood was quickly oxidised to NO_3.

Factors affecting NO_2 (and NO_3) concentration in the blood – re-analysis of published studies of pathways of removal of NO_2 (and NO_3) from the bloodstream

NO_2 enters the blood from endogenous sources and by uptake from the gut. The rate of removal of blood NO_2 has been the subject of a number of studies (e.g. (Lewis 1951; Barnett and Bowman 1957; Farra and Satter 1971; Schneider and Yeary 1975; Wagner *et al.* 1983). There is a general consensus that NO_2 is oxidised to NO_3, or excreted via the kidneys, or excreted into the gut as depicted in Fig. 7.2

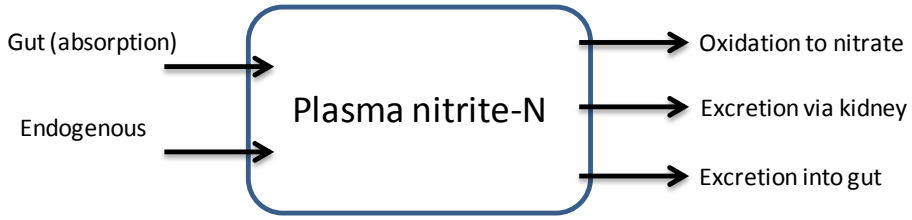


Fig. 7.2. Entry and excretion of plasma NO_2-N in ruminants

Reanalysis of data of Lewicki et al. (1994) and Schneider and Yeary (1975)

Data of Lewicki *et al.* (1994) and Schneider and Yeary (1975) were fitted to kinetic models representing the elimination of plasma NO_2 by excretion and oxidation to NO_3 (represented by the appearance of NO_3-N in plasma).

Lewicki *et al.* (1994) gave an intravenous bolus dose of 400 µmol NO_2/kgW to 6 Polish wethers and monitored the changes in concentrations of NO_2 and NO_3 in plasma and urine for 30 h. The results were first fitted to a simple two-compartment model using WinSAAM but the data for the NO_3 pool were poorly fitted and are not shown here. A better fit (Fig. 7.3) was obtained by including an intermediate pool, even though the actual identity of such a pool was unclear. The NO_3 and NO_2 were distributed in a volume of 12 L, equivalent to about 25% of the average liveweight of the sheep and therefore the distribution volume probably represented extracellular fluid. The results highlight the rapid turnover of the NO_2 pool and the slower turnover of the NO_3 pool.

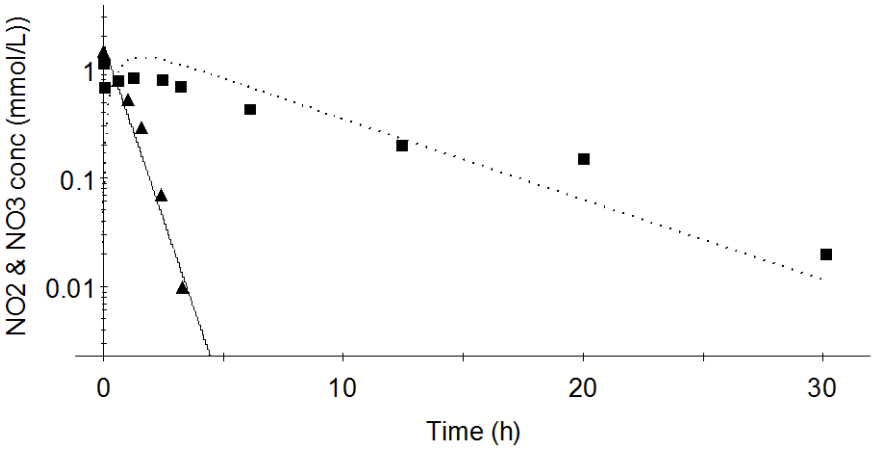


Fig. 7.3. Elimination of NO_2-N from plasma of sheep and the appearance of plasma NO_3 (data of Lewicki *et al.* (1994))

Schneider and Yeary (1975) gave a intravenous bolus dose of 20 mg NO_2/kgW (45 mmol) to 7 adult cross-bred sheep (weights ranging from 30-74 kg) and monitored the changes in concentrations of plasma NO_2 and NO_3 for up to 24 h. Their data were less well distributed throughout the sampling period than data of (Lewicki *et al.* 1994) but were fitted without the need for inclusion of an intermediate pool. The fitted curves are over-plotted on the experimental data in Fig. 7.4.

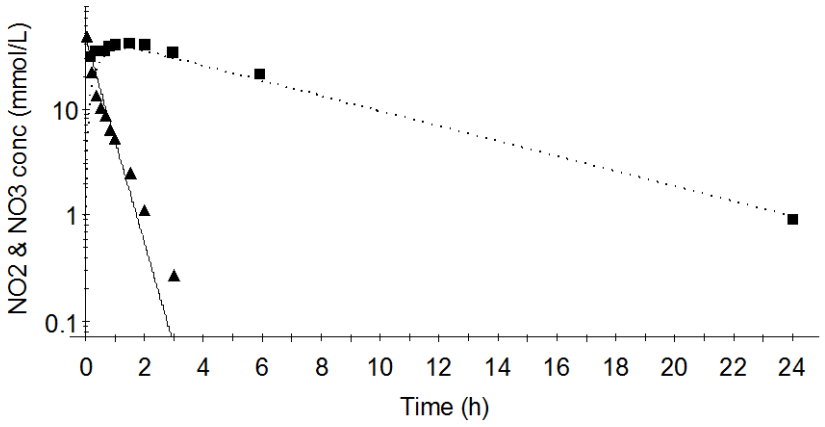


Fig. 7.4. Elimination of NO_2-N from the plasma of sheep and the appearance of some of this N as plasma NO_3 after oxidation (data of Schneider and Yeary (1975)

The $t_{1/2}$ for NO_2 in the plasma compartment was 0.47 h, but elimination of NO_3 was slower ($t_{1/2}$ of 4.2 h).

Conclusions. In general, the results of both plasma NO_2 challenge studies were similar. The bolus NO_2 doses, in the quantities used in these studies, were rapidly eliminated from the plasma ($t_{1/2}$ values, 0.48 h) and there was a concurrent increase in blood NO_3 concentration which, in comparison with the NO_2 pool, had a relatively slow turnover rate (also see analysis below of data of (Lewicki *et al.* 1998). Variability in the data prevented

strong conclusions to be made about the kinetics of elimination of plasma NO_2 by non-oxidative routes, e.g. by excretion via the kidneys.

Kinetics of plasma NO_3 elimination

Lewicki *et al.* (1998) administered an intravenous dose of ^{15}N-NO_3 (95.4% atom % excess; 0.4 mmol NO_3/kgW) to sheep (51 kg) and monitored plasma NO_3, urinary NO_3, ammonia and urea concentrations for 50 h. These findings fit a hypothesis that there is transfer of NO_3 into the gut followed by microbial reduction of the NO_3 to ammonia and then absorption of the ammonia leading to synthesis of urea in the liver. In accord with this hypothesis, blood urea was measurably enriched after 15 min and reached a maximum enrichment after 6 h. The results have been re-evaluated by fitting a kinetic model to the published data and the figure shows the transfer of plasma NO_3 to urea. The model allows for the remaining urea to be transferred out of the animal tissues (presumably mainly by urinary urea excretion, even though urea itself is also known to be transferred to the gut).

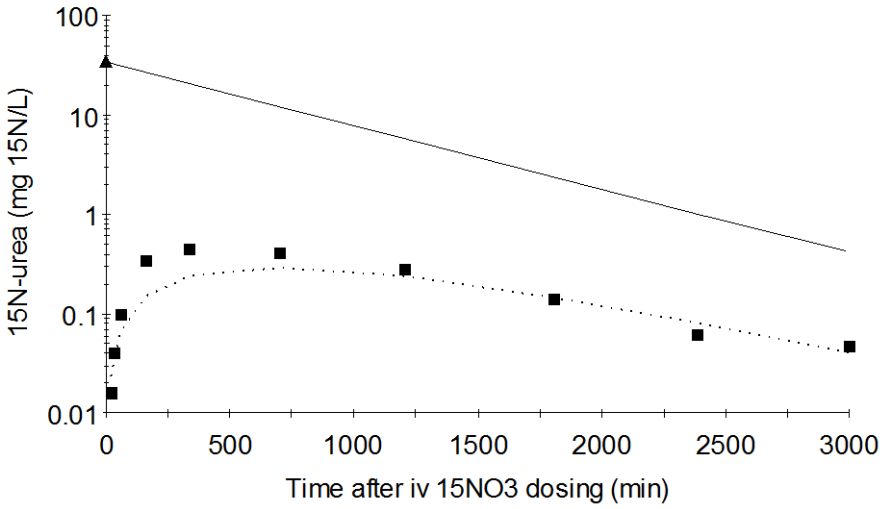


Fig. 7.5. Appearance of ^{15}N-urea in plasma (■) of a sheep after the sheep was given an intravenous dose of $^{15}NO_3$. The model was used to predict the rate of loss of ^{15}N from plasma $^{15}NO_3^-$ (◢).

In this study, NO_3 turnover ($t_{1/2}$ of approx. 8 h) was again much slower than the turnover of NO_2. The volume of distribution of urea and NO_3 was about 10 L which is consistent with published studies suggesting that NO_3 and NO2 are distributed in extracellular and interstitial fluid. The total excretion in urine of the NO_3-N dose in all forms of nitrogenous materials by the end of the 50-hour sampling period was only 24% of that administered.

According to this model, only about 2.5% of the N leaving the plasma NO_3 pool was directly transferred to plasma urea. However, by the end of the sampling period, 14 % of the NO_3 dose was excreted as urea in urine. Thus some labelled urea may also have been recycled to the rumen and lower digestive tract and converted to labelled ammonia which is then converted again to urea. The fraction excreted as ammonia after 50 h was 0.1%. The total recovery of ^{15}N was only 38% of that administered. Of the remaining ^{15}N in the body (62% of that administered as $^{15}NO_3$), some was presumably transferred to the gut as already suggested, but the ammonia was apparently ultimately incorporated into tissue proteins and other materials. The fate of the remaining ^{15}N is, however, uncertain. According to some researchers, it is possible NO_3-N is incorporated locally in tissues, but this suggestion requires further investigation.

Appendix 8 - NO_3 reduction in the human intestine: Production of nitrous oxide and nitric oxide

Dissimilatory NO_3 reduction to ammonium (DNRA) is considered to be the main NO_3 reducing process in human faecal microbiota (Allison and Macfarlane 1988; Vermeiren *et al.* 2009). Denitrification pathways have been demonstrated but in the study of Allison and Macfarlane (1988) accounted for only 3% of the reduced NO_3. On the other hand, when NO_2 was added to faecal slurries, 23% of the NO_2 was recovered as gaseous products (Allison and Macfarlane 1988). These workers concluded that large quantities of NO_2 may be preferentially denitrified in the colon. When ^{15}N-NO_3 was added, here was almost complete recovery of ^{15}N in ammonia in microbial environments (Vermeiren *et al.* 2009). Nevertheless, small amounts of NO and N_2O are produced during NO_3 reduction in the colon. It was first suggested that NO and N_2O may be side products of DNRA (Vermeiren *et al.* 2009), but a later study showed that two biological processes can generate NO (see

Fig. ***8.1***). The bacterial reduction of NO_3 to NO_2 and the production of H_2S by the fermentation of readily available sulphur sources (cysteine or methionine) are both required for the chemical process producing NO (Vermeiren *et al.* 2012a). Excluding starch from the diet increased NO production (Vermeiren *et al.* 2012a), possibly because fermentation of sulphur containing amino acids was increased. This in turn may have resulted in higher H_2S levels and therefore a higher NO production.

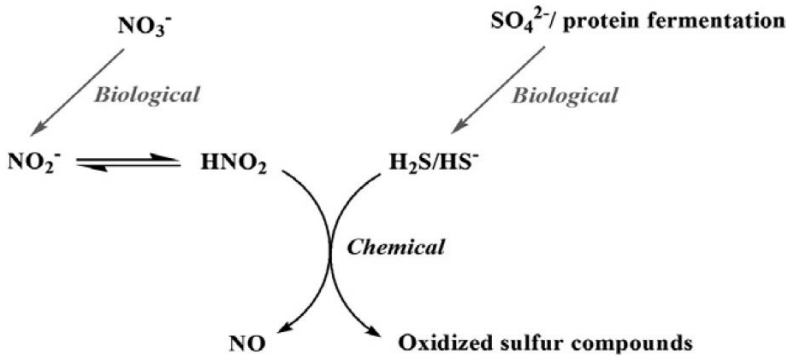


Fig. 8.1: Proposed biological and chemical processes leading to NO production in the human large intestine (Vermeiren et al. 2012a)

The addition of NO_3 to faecal slurries reduced both hydrogen and methane accumulation (Vermeiren *et al.* 2012b). In this study, NO_3 acted as an electron acceptor, with NO_3 reduction out-competing CO_2 reduction and methanogenesis. In addition, there was evidence of a direct inhibition of the methanogens by NO_3 and NO_2. The VFA profile was altered with NO_3 increasing the acetate to butyrate ratio. Allison and Macfarlane (1988) argued that changes in VFA profile may not be linked only to the inhibition of methanogenesis because the same observation was made in individuals that do not produce methane.

Nitrous oxide production in the rumen

The pathway of denitrification shown below apparently does not occur in rumen microbes and N_2 is not a final product

$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

However, as in the human colon, the production of N_2O from NO_3 and NO_2 does occur. Some rumen bacteria including *Wolinella succinogenes* appear to perform a dissimilatory reduction of NO_2 to ammonia which is then secreted into the medium (Averill 1996).

Appendix 9 - Methane production in the rumen

Methanogens belong to the phylum Euryarchaeota of the domain Archaea, and are divided into the following five orders, namely *Methanosarcinales*, *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales* and *Methanopyrales*. Unlike bacteria, methanogens lack peptidoglycan in the cell wall which is replaced in different orders by pseudomurein, heteropolysaccharide or protein (Balch *et al.* 1979). All methanogens have coenzyme F420, which is necessary for hydrogenase and formate dehydrogenase, Methanogens also require coenzyme M, which they either import or produce themselves (Rouviere and Wolfe 1988).

Methanogen populations in the rumen (and in model rumen systems operated over a 240-h period) were studied by means of the small subunit (SSU) rRNA phylogenetic framework for group-specific enumerations. Members of the family Methanobacteriaceae were the most abundant methanogens in the rumen, accounting for 89% (± 1.0%) of total population of archaea in the rumen fluid and 99% (± 1.8%) of archaea in a protozoal fraction from rumen fluid. The percentage of archaea in the model rumen systems after 48 h declined from 84% (± 8.5%) to 54% (± 7.8%) which could be explained by the loss of protozoa from these systems. It has been estimated that methanogens associated with protozoa are responsible for 9 to 37% of the methane production in the rumen (Finlay *et al.* 1994). Anaerobic fungi such as *Neocallimastix frontalis* have also been found to transfer H_2 to associated methanogens with the result that their enzymatic activity is increased and metabolism is shifted towards acetate production (Mountfort and Asher 1985).

Methanogens have symbiotic relationships with bacteria, protozoa and fungi in the rumen involving interspecies H_2 transfer. Protozoa have both intracellular and extracellular associations with methanogens (Sharp *et al.* 1998). Unlike H^+, H_2 generated by oxidation of NADH in the cytoplasm or by cleavage of formate in the periplasmic space can be transferred between different microbes in the rumen).

As mentioned earlier, H_2 is a non-polar gas so is sparingly soluble in water and, because it does not ionise, is unaffected by pH. The dissolved H_2 is a source of energy for methanogens which use it to reduce CO_2 to methane as follows:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

Dissolved H_2 in rumen fluid is highest after feeding when methane production is also highest (Clapperton and Czerkawski 1969) and a linear relationship has been found between dissolved H_2 concentrations and rates of methane formation (Hungate 1967).

Formate entering rumen fluid is generally converted quickly to H_2 and CO_2 (Hungate *et al.* 1970) and most methanogens in the rumen utilize H_2 and CO_2. However, under some conditions, formate can be converted to methane without prior oxidation to CO_2 (Fina *et al.* 1960) according to the equation.

$$4HCOOH \text{ -- } 3CO_2 + CH_4 + 2H_2O$$

Boone *et al.* (1989) calculated the potential for H_2 and formate diffusion between microbes and argued that at H_2 concentrations commonly found in nature, H_2 would not diffuse rapidly enough to dispersed methanogenic cells to account for the normal rate of methane synthesis. These workers argued that formate diffuses more rapidly than H_2 so may be a primary carrier for inter-species electron transfer between syntropic microbes. In ecosystems with no methanogens, formate is rapidly degraded to CO_2 and H_2 and rarely accumulates (Hungate *et al.* 1970).

Some methanogens use alternative substrates. For example, *Methanosarcina* grows only slowly on H_2 and CO_2 but uses methanol and methylamines for methanogenesis (Hungate *et al.* 1970). Methanol that is produced by fermentation of diets that contain pectins (Neumann *et al.* 1999). Volatile fatty acids (VFA) are potential substrates for methanogenesis (*e.g.* in biogas fermenters) but are not commonly used as substrates for methanogenesis in the rumen as the time taken for their conversion to H_2 and CO_2 is incompatible with a relatively fast digesta turnover.

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