THE POTENTIAL OF FEEDING NITRATE TO REDUCE ENTERIC METHANE PRODUCTION IN RUMINANTS

By

R A LENG AO, PhD, DRurSc .
Emeritus Professor
University of New England

A Report
The Department of Climate Change,
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Australia

Bionutric PTY LTD
ABN:79 559 285 529
PO Box 361
Coolum Beach
QLD 4673, Australia
Email rleng@ozemail.com.au
Web site www.penambulbooks.com

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Drs Roger Hegarty and Simon Bird have made important inputs and Professor John Nolan assisted in numerous ways, constructively criticizing aspects of the report which became an extremely complex mission once I recognized the importance of reviewing literature outside of the rumen ecology, where I am much less comfortable with the science. I wish to acknowledge the sections 2.3. and 2.4 were taken almost *verbatim* from the first class review of Lundberg ***et al*** (2004).

The metabolism of nitrate in ecological niches such as those pertaining to oil fields came to my attention through my interest in the study of Peak Oil and I believe it provides in sights into why nitrate toxicity occurs at times in grazing ruminants. The review has been an exciting challenge which I see as remaining in-complete and a continuing process to be reviewed as new information is generated.

All figures had to be drawn from graphs presented in the various publications. These were magnified so as to allow readings as accurately as possible but are therefore not necessarily 100% accurate.

An important contribution to the report was made by Helen McLennan who efficiently obtained much of the literature and finally formatted the manuscript.

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THE POTENTIAL OF FEEDING NITRATE T0 REDUCE ENTERIC METHANE PRODUCTION IN RUMINANTS

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OUTLINE OF CONCEPT

In the rumen and elsewhere, continuous microbial fermentation is contingent on the reoxidation of cofactors generated when organic matter is fermented to volatile fatty acids (VFA) with the synthesis of microbial cells. Reduced cofactors surplus to those needed in production of the fermentation end products (synthesis of cells and reduced end products such as propionate) must be re-oxidised to allow fermentation to continue. Under the anaerobic conditions and high carbon to nitrogen ratios in the rumen, the cofactors must be oxidised before re-use by electron transfer to acceptors other than oxygen. The major hydrogen (electron) sink in the rumen is methane produced by the reduction of carbon dioxide using reduced co-enzymes such as NADH as the electron source.

Nitrate can replace carbon dioxide as an electron acceptor with the generation of another reduced product -- in this case, ammonia, i.e. nitrate is reduced to nitrite and then to ammonia. The reactions by which electrons are transferred to produce methane or ammonia are given at the end of this section.

Bacteria that reduce nitrate to ammonia are more active in the rumen when substantial amounts of nitrate have been included in a diet for extended periods. Availability of rumen ammonia is often a primary deficiency in diets fed to ruminants. Ammonia is usually generated from degradation of dietary protein or by supplementation with urea. Nitrate could potentially replace urea in diets to provide a nitrogen source for microbial protein production and growth.

Nitrate in ruminant feeds (particularly lush pasture) can generate the condition, methaemoglobinaemia, which reduces feed intake and productivity and can be fatal. The toxic syndrome results from absorption of nitrite produced in the rumen from nitrate. The potential toxicity of nitrate has led to an emphasis in research on understanding the toxicity syndrome and this has clouded the issue as to whether nitrate might replace other fermentable nitrogen sources in ruminant diets. There appear to be no studies of the value of nitrate as a sole or major N resource in ruminants under practical feeding conditions.

The research evidence leaves little doubt that nitrate, when included at sufficient concentrations in a diet so as to maintain optimum fermentation rate, can largely prevent enteric methane production from ruminants. The barrier to its use in practice is the association of nitrate in a feed with nitrite poisoning. However, nitrite accumulation appears to only occur when relatively large quantities of nitrate are suddenly introduced directly into the rumen of animals not accustomed to nitrate in their feed

Nitrate is extremely rapidly cleared from rumen fluid when a load is injected into the rumen and the rapid removal appears to be the major factor associated with nitrite accumulation. Nitrite accumulation may also be greater where the animals are consuming diets high in crude protein which is associated with high rumen fluid ammonia and hydrogen sulphide concentrations. When nitrate is fed as a component of the diet, and the animal has been adapted to nitrate by introducing it gradually over time, nitrite accumulation does not occur in the rumen. These conditions favour an assimilatory nitrate reduction system in nitrate-metabolising bacteria where ammonia is produced at a rate equal to that of the assimilation rate into microbial growth. This process is suppressed and repressed by ammonia.

A review of the literature indicates that the key to success in using nitrate to reduce enteric methane production from ruminants is minimising nitrite accumulation in rumen fluid. There is strong evidence that nitrite accumulation from nitrate is minimal in the rumen when two requirements are met, viz. the rumen is adjusted or acclimated to nitrate in the diet and sulphur to nitrate ratios in the feed are balanced to maintain the activity of sulphur reducing bacteria (SRB) which also reduce nitrite to ammonia. It appears that this applies to feeds with crude protein levels that spread across the normal range.

There is research on non-rumen anaerobic systems that indicates that nitrite accumulation is strongly influenced by the population densities of specialised microbes that reduce nitrate to nitrite and oxidise sulphide to sulphate when further reducing nitrite to ammonia. These are termed nitrate reducing sulphide oxidising bacteria (NR-SOB). Reduction of sulphate to hydrogen sulphide is curtailed by a high nitrate load that appears to stimulate other nitrate reducing organisms which out compete sulphur reducing organisms for electrons. Such an interaction in the rumen of animals on high nitrate/protein diets may explain the nitrate toxicity syndrome in ruminants.

Reactions

$NO_3^- + 4H_2 + 2H^+ \rightarrow NH_4^+ + 3H_2O$ (8 electrons accepted per mol nitrate)

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

1 mol of nitrate would produce 1 mol of ammonia and reduce methane production by 1mol or 16g or 22.4L of methane.

Methane production by sheep (40kg) on a good quality diet has been reported in McAlister and Newbold (2008) to be 21.6L/kg feed consumed. To simplify any calculation, if this estimate is adjusted to 22.4 L/kg it represents a mol of methane per kg of feed. If nitrate were to be used to inhibit methane then theoretically the requirements would be 1 mol of nitrate or 101g K-nitrate/kg of feed providing 14gN or 87.5g of crude protein in a diet. The requirements for crude protein vary with digestibility of a diet and are usually in the range of 12-18%. The calculations merely show that nitrate can provide the amount of reducing power in the rumen to theoretically dispose of all metabolic hydrogen produced at levels that are lower than N requirements in the feed. Thus if addition of nitrate to a feed prioritizes the disposal of metabolic hydrogen then as a rule of thumb for each 1% inclusion of K-nitrate in a diet the corresponding decrease in methane production is 10%. In the scientific literature there are a number of reports in which addition of 3-5% nitrate to already adequately balanced diets is tolerated by sheep without any ill effects.

Is the project worth pursuing?

This author has no doubt that the project is worth pursuing. The key element, i.e. that nitrate can be the sole or major source of fermentable nitrogen in the rumen without any ill-health in the recipient animals, is already proven albeit with poor quality feeds which only supported low productivity in lambs and young goats. Research indicates that methane will be substantially inhibited where nitrate is a major N source in a diet. With a small research input to define the necessary management and husbandry requirements, nitrate could be recommended for use wherever ruminants are supported on low protein forages.

The potential to use nitrate to reduce enteric methane production in ruminants on good quality diets that are balanced for crude protein and therefore support levels of production approaching the animal's genetic capacity, also appears to be entirely feasible. Research in the early 1960s (see section on feeding trials below) showed that there were no ill-effects in lambs growing at up to 240g/day of adding 4% K-nitrate to the diet. If nitrate reduction was the dominant pathway of electron uptake in the rumen this could have reduced methane production by as much as 40% but this has to be tested. Similarly 4% K-nitrate in a high protein forage based diet fed to

sheep had no ill effects provided the nitrate was introduced over a period of 10-20d. Nitrate may be best used as mixtures of their salts

The potential to use nitrate to replace urea is also significant in the annual dry season supplements provided to cattle and sheep grazing dry mature pastures or crop stubbles throughout Australia. However, a major task will be the development of delivery strategies. Under present husbandry practices cattle and sheep, particularly breeding stock may be supplemented for 3-6 months of the year depending on location and particular weather patterns. The breeding herd makes up the single largest group of grazing ruminants and cattle and sheep produce 59 and 25% of Australian enteric methane emissions respectively for these species.

Not all ruminants are supplemented in the dry season, however, the potential for carbon credits to lift the use of supplements is high from two aspects, farmers and graziers have been irritated by the critics, who have used enteric methane production as a weapon against the industry and secondly the potential income from carbon credits will offset some of the costs of supplementation in the future.

As an example of the potential reduction in methane production by cattle the following is given. A mature cow may produce daily 250 liters of methane which is approximately 160g or say 60kg/year.If there was an annual saving of 25-50% of this methane it represents 15-30kg methane or assuming methane is 21 times more effective greenhouse gas than carbon dioxide, 315-630kg equivalents as carbon dioxide. Assuming $30/tonne carbon credits, the carbon value is $10-$20. The cost of supplementation with a molasses urea block at the present time is around 20 cents/d or $18-36 per head per season. The calculations are probably over optimistic but clearly there is a major incentive to apply such a strategy should the research outcomes be as positive as the present speculation. Similar calculations apply to sheep.

Such a supplementation strategy for livestock if successful could have enormous effects on methane inventories in the developing countries where livestock are multi-purpose providing draught power, meat, milk and fiber. These animals are accessible to the feeding strategy because they are mostly hand fed, poor quality forages that are deficient in fermentable nitrogen and they produce a high proportion of the atmospheric methane.

The need for such technology is increased world wide if Peak oil, resource depletion and global warming (see Leng 2008) lead to:

a. A downturn in pig and poultry production which is highly probable for a number of reasons including the high cost of feed and the incidence of disease communicable to humans. There should be a re-emphasize of the unique and important nutritional properties of red meats which will need to expand but this is really only feasible by basing the diets on biomass such as straw.

b. Production levels per animal are reduced as resources such as feed grain and protein meals become more expensive and therefore less used.

c. An increased use of animal traction occurs, requiring greater numbers of mature livestock. This is already happening in many developing countries.

It is already forecast that meat production will need to be doubled by 2030 to meet world population increase (FAO 2008).The competition for grains (or crop land) for food for humans, feed for animals or feedstock for biofuel production (see Leng 2008) indicates that grain will be too expensive to feed to animals and that much of this extra meat must be generated from biomass resources presently underutilized. These, largely cellulosic (fibrous) residues, can only be used by herbivores, in particular the ruminant, by virtue of their fermentative digestive system. Recent developments in the industrial processing of crop residues to improve their feeding value (Davis *et al* 2007) have pioneered the way for future expansion of ruminant production without impinging on land use for other food production purposes. These systems must be expanded to fill the coming gap in meat production relative to demand and they can also be used to finish ruminants in drought, however, the additional enteric methane production may be a major impediment to any such development. The ability to replace urea by nitrate as the fermentable nitrogen source would

reduce enteric methane production and would increase the acceptability of these production systems in the future.

Non-technical summary

Methane, produced and released into the atmosphere from many biological systems is a greenhouse gas which has about 21 times more capacity to retain heat than carbon dioxide, the major green house gas. As a greenhouse gas, methane is some 16 times more problematic than carbon dioxide when the turnover times of the two gases in the atmosphere are taken into account. Globally ruminants produce some 80 million tonnes of methane annually, accounting for some 28% of anthropogenic (man made) methane emissions. Clearly the adoptions of management practices for ruminants which achieve major reductions in methane release without economic penalty are issues of high priority.

In current feeding systems for ruminants, the production of methane is an inevitable consequence of fermentative digestion. The rumen microbial consortium has to dispose of hydrogen that is produced in fermentation because it would inhibit the digestion of feeds if it was allowed to accumulate. The rumen microbial ecosystem achieves this principally through the presence of methanogens that reduce carbon dioxide to methane.

Recognition that supplements of nitrate in ruminant diets compete successfully for the reductive processes in the rumen which normally generate methane, is an exceptionally promising development, given that in addition to inhibiting methane production, the end products of nitrate production is ammonia. Ammonia is an essential nutrient for the rumen microbial population as it is the major source of the nitrogen for protein synthesis and hence growth of microbial cells. Microbial protein in turn is the source of amino acids absorbed and used for animal growth, reproduction and milk production. Urea, which is quickly converted to ammonia in the rumen, is often included in ruminant diets as a nitrogen supplement. The replacement of urea with nitrates in ruminant diets is now entirely feasible, subject to supplement management. A further useful feature of this development is that small amounts of nitrogen oxides produced in nitrate metabolism in the rumen inhibit the activities of some methanogens, potentially increasing the level of inhibition of methane production where nitrate is only a proportion of the total fermentable N in a diet.

Earlier research on the possible use of nitrate in ruminant diets did much to stifle interest in this area by focussing attention on the risks of nitrite poisoning in ruminants that result from the production of nitrite from nitrate in the rumen. After reappraisal of the available information, it is clear that nitrite only accumulates in rumen fluid in amounts where it becomes toxic when animals are not acclimated to nitrate feeding by slowly increasing dietary nitrate over a week or so. Acclimation is similarly necessary when urea is introduced into ruminant diets. In short, nitrite poisoning is not a threat to well managed livestock fed nitrates if they are first acclimated and then continuously maintained on this N source.

The adoption of nitrate feeding in ruminants will be dependent on normal least cost feeding considerations taking into account future potential carbon credits that may be earned, but if competitive with urea, the obvious advantages of reduced methane production without economic loss are highly promising. In fact, complete inhibition of methane production could increase utilisation of digested feed energy by up to 15%, if dry matter digestibility is not compromised.

EXECUTIVE SUMMARY

1. Globally ruminants produce around 80 million tonnes of methane annually, which accounts for about 28% of anthropogenic methane emissions.
2. Enteric methane is produced by reduction of carbon dioxide during fermentative digestion in the rumen, a process dependent on oxidation of reduced cofactors generated in the chemical pathways that convert feed substrates to volatile fatty acids (VFA) and microbial biomass. The VFA are a major source of substrate for energy transactions in ruminant animals and the latter being roughly 60% protein is the major source of essential amino acids for their growth, reproduction and milk and fibre production.
3. Hydrogen (or more specifically high-potential electrons associated with hydrogen) is readily appropriated by methanogenic bacteria that use it to reduce carbon dioxide to methane which is quickly lost from the rumen fluid to the gas cap and removed by eructation. The generation of oxidised co-factors when methane is produced enables digestion of feed by microbes to be continuous and therefore feed intake is optimised.
4. It is well established that bacteria from the rumen are able to use nitrate and sulphate as electron acceptors instead of carbon dioxide and when they use alternative electron acceptors methane production will be reduced.
5. Nitrate would be the choice of alternative electron acceptor because it is reduced to ammonia, the key compound needed for microbial cell synthesis, whereas sulphate is reduced to hydrogen sulphide. Hydrogen sulphide does not accumulate appreciably in rumen fluid and is excreted in a similar way to methane.
6. Nitrate has been rejected as a potential N source for ruminants because nitrite poisoning has occurred when nitrate was administered to animals without prior acclimation. Nitrite is anti-nutritional because it is absorbed and causes mild to extreme methaemoglobinaemia. The symptoms varying from a loss of production to death of the animal
7. From the literature reviewed, it is concluded that nitrite accumulation in the rumen only results when the rumen is overloaded with nitrate or when nitrate intake is increased suddenly. When nitrate is increased in a diet stepwise on a daily basis, adaptation occurs in the rumen with increased capacity to metabolise nitrate and insignificant amounts of nitrite accumulate.
8. In the majority of research where nitrate has been administered to ruminants, the objectives have been to study the nitrate toxicity syndrome. In order to simulate the field conditions, nitrate has been administered as an intra-ruminal load in animals unaccustomed to consuming nitrate. This “toxicological approach” appears to have masked the high potential of nitrate as an alternative fermentable nitrogen source to urea.
9. Sheep can “tolerate” (without any signs of toxicity) up to 4% nitrate in high quality concentrate or forage based diets that support high levels of growth with or without the added nitrate. At this inclusion rate in a diet significant reduction in methane could result but the research objectives in the published research were not focussed on this aspect and methane production was not measured.
10. In sheep acclimated to nitrate the capacity of the rumen microbial communities to use nitrate and nitrite are increased by up tenfold
11. Nitrite accumulation in the rumen was insignificant and transitory when ruminants were adapted to nitrate that was included as a component of the mixed diet. Nitrite did not accumulate in the rumen of acclimated sheep even with dose rates equivalent to those required to supply fermentable nitrogen at rates that would support maximum

fermentative digestion of feed and at rates that could eliminate methane production totally.

12. Nitrate reduction by micro-organisms in natural and contrived environmental situations that have economic consequences (e.g. oil fields, sediments, sewage works, biodigestors etc) has led to an enormous literature on the microbiology, biochemistry and genetics of these organisms which are not only widespread but are extremely diverse in their metabolic strategies.
13. Researches of microbial physiology and biochemistry have described several microbial enzymes that effect the reduction of nitrate to ammonia in the rumen and other anaerobic ecosystems.
14. These enzymes are characterised as nitrate and nitrite reductases and often are present in the same organism.
15. Dissimilatory nitrate reductase functions as an electron acceptor re-oxidising reduced coenzymes and playing the same role as would be played by methanogenesis in some ecosystems.
16. Assimilatory nitrate reductase produces ammonia intracellular at rates commensurate with cellular growth.
17. Recently, an assimilatory nitrate reductase that is also coupled with oxidation of sulphide to sulphur or sulphate has been identified in bacteria from a number of anaerobic ecosystems. The most studied organism occurs in oil-contaminated water where the oil organics are being fermented. These organisms are known as nitrate reducing, sulphide oxidising bacteria (NR-SOB). They have been shown to produce ammonia and sulphate or poly- sulphur under nitrate-limited growth media conditions and when nitrate is in excess and sulphide is limiting they produce nitrite. Organisms with these capabilities appear to be present in the rumen. For example *W. succinogenese* has the capacity to oxidise hydrogen sulphide and reduce nitrate and nitrite. The organism uses the conversion of nitrite to ammonia to generate ATP for growth.
18. Sulphur reducing bacteria (SRB) and nitrate reducing bacteria (NRB) are diverse and are found together in many anaerobic ecosystems including the rumen. There are major interactions between nitrate and sulphate metabolism in the consortiums of micro-organisms that are present in these diverse anaerobic ecosystems. Of major importance is that many of the SRB appear to have dual roles, i.e. they reduce inorganic and organic sulphur and also most of the species also actively reduce nitrite to ammonia. However, in a high nitrate medium, these organisms or their sulphur and nitrite reducing abilities appear to be suppressed. In the oil-field studies, the nitrate suppression of sulphur reduction in the sulphur reducing organisms decreases hydrogen sulphide production and stimulates the NR-SOB to produce nitrite from nitrate. This inhibition is strengthened in substrate limited media (low amounts of fermentable carbohydrate providing limited electron donors). It is hypothesised that a similar interaction may explain the accumulation of nitrite when nitrate is suddenly introduced into the rumen.
19. It is concluded that when animals are acclimated slowly to dietary nitrate, nitrite accumulation in the rumen is avoided and toxicities are not experienced.
20. Use of nitrate as a supplement for to grazing animals will require the development of supplementary feeding strategies that either permits the animal frequent access to nitrate, or a slow-release nitrate formulation is provided in the supplements that are ingested less frequently, (Slow release nitrate preparations are already in use particularly for production of turf). The same problems beset managers wanting to use urea as nitrogen supplement and there are a number of strategies already developed that may be brought to bear on this area for research.

21. There are numerous basal feed resources throughout the world used for ruminant production that are deficient in crude protein and require supplementation with a source of fermentable nitrogen to balance the diets for production. These include traditional grain based feeding systems as adopted by the US and Australian beef feedlot industry. Urea has been the source of additional rumen ammonia. However, with careful slow adjustment (a requirement which also applies to urea feeding), there seems to be no impediment to replacing urea with nitrate and possibly with better results (more efficient microbial growth and less energy losses as methane).In addition in the grazing livestock industries nitrate has major potential to replace urea in block licks, loose mixes and liquid mixes which have proved so successful in promoting growth in animals fed crop residues, stubbles and dry pastures throughout the world.

22. Numerous possibilities exist for replacing urea with nitrate in unconventional high energy, low protein forage crops that are being used for ruminant production in various parts of the world, for example sugar cane and molasses or cassava-based diets.

23. Recent technical developments using straw treated with alkali to improve its digestibility may promote production systems for ruminants, where replacing urea with nitrate is entirely feasible. With these new treatment technologies, production levels approach those obtained on grain based feedlot diets. Some 35 million tonnes of straw is produced annually in Australia and with the adoption of the new technologies, the straw presently wasted, often by burning, could support 5- 10 million cattle. The present use of straw treated or other wise as a ruminant feed is enhanced by supplementing the diets with urea. Inhibition of methane by replacing urea with nitrate in these diets is an attractive proposition that could assist such industries to develop, particularly if credit is provided for reduction in greenhouse gas emissions. Sugar cane and cassava thrive on the coastal fringe of Queensland and could potentially be the feed of choice for fattening cattle for the overseas meat trade, or in drought when there is the need to de-stock pastures and provide other means of supporting, in particular, the breeding stock.

24. The application of nitrate to ruminants under ideal pasture grazing conditions is not likely to be practical for a number of reasons including the potential for toxicities, the probable lack of intake of high nitrate supplements and the increased load of nitrogen in animal excreta which may increase NO_x release into the atmosphere. However, research leading to the successful application of nitrate in low protein diets could provide the incentive for the development of pasture species designed to improve pasture biomass low in protein. This would require a paradigm shift in pasture plant breeding and management.

LITERATURE REVIEW

1.1 Introduction

Ruminants have evolved to utilise diets high in 'plant structural cell biomass' of which most of the energy is present in the structural carbohydrates and to a lesser extent simple sugars. The content of structural carbohydrates depends on the maturity of the plant and these complex carbohydrates are also only slowly degraded by microbial enzymes. The rumen digestive tract has evolved to capably ferment a wide range of feed materials with the production of microbial biomass and a number of fermentation end products including methane and the volatile organic acids. Microbial cells, synthesised in the rumen, provide the majority of the animal's requirements for essential amino acids when microbes are washed out of the rumen and are digested and absorbed from the intestines. The supply of microbial protein from the rumen often requires augmentation with dietary bypass protein to ensure optimal animal production from a particular feed. The volatile fatty acids and plant fats (which are relatively unchanged in the rumen) provide the majority of the energy substrates for maintenance and synthesis of new tissue (see Preston and Leng 1985).

On balanced diets, the rumen thus provides a sustainable microbial habitat, generally consisting of interactive colonies of bacteria, anaerobic fungi and protozoa. The efficiency of this ecosystem depends on maintaining a low redox potential essential for anaerobic fermentation. The reactions involved in the pathways of carbohydrate and protein degradation generate reduced cofactors, which, if not oxidized, feed back and inhibit fermentation. Various strategies have evolved in the rumen to oxidize the reduced cofactors which results in hydrogen release to the medium. Strictly anaerobic Archaea (methanogens) use this hydrogen to reduce carbon dioxide to methane with the generation of ATP which provides the organisms energy requirements for maintenance and cell growth. However numerous rumen organisms are able to utilize energetically more favorable electron acceptors such as nitrate and sulphate generating ammonia and hydrogen sulphide respectively.

In ruminants (sheep, cattle, goats) on a range of diets and therefore diverse rumen ecosystems, it has been clearly demonstrated that the major electron sinks in the rumen milieu include: 1) growth in the microbial biomass, 2) production of propionate, 3) hydrogenation of unsaturated fatty acids and 4) the generation of methane by the Archaea (see Hungate 1965). The latter requiring the release/transfer of hydrogen to methanogens by microbes stepwise fermenting the components and intermediate products produced from feed materials.

The question posed is whether it is possible to eliminate or significantly reduce methane production by feeding material, such as nitrate, that provides an alternative electron acceptor whilst still maintaining the efficiency of the fermentative digestion. The latter directly relates to nutritional value of a feed. There are a range of compounds that could provide electron acceptors which have not been studied in detail for this role. Nitrate/nitrite and sulphate have potential to be reduced in the rumen but researchers have been distracted by focusing on the toxic effects of nitrite produced from nitrate in the rumen under some circumstances leading to economic loss by lowered production and death of animals. The potential benefits from using nitrate as alternative electron acceptor in the diets of ruminants has received little attention, particularly where it may also supply a high proportion of the total N in a feed.

1.2 Sources of nitrates in the diets of ruminants

Nitrate salts and ammonium nitrate are commercial fertilizers available throughout Australia.

Nitrate is a common component of crude protein in a variety feeds consumed by ruminants. The most common source is from forage with cultivars from the Perennial Rye Grass and Sorghum family (Sorghum, sudan, pearl millet and their crosses) being particularly high

accumulators of nitrate. Under certain conditions, a wide variety of plants may accumulate quantities of nitrate. In addition to normal livestock feeds, certain weeds that commonly inhabit pastures or crop fields can also accumulate nitrates. Cereal grains and protein concentrates rarely contain appreciable nitrate concentrations.

Nitrate is not distributed evenly in plants. For example, nitrates are typically higher in stems, lower in leaves (more nitrate reductase activity) and extremely low in seeds (Fjell *et al*, 1991; Pflster, 1988). In pearl millet, the stems contained three times more nitrate than leaves (Krejsa *et al*, 1987) and in maize and rye grass stems there is a gradient in nitrate up the stem with the highest concentrations in the lowest parts. The majority of the nitrate is in the stem in all grasses.

Forage maturity plays an important role in that nitrates are higher in young growth (or re-growth) but lower in mature plant tissues (Pfister 1988; Fjell *et al* 1991). Sunlight is also important because nitrate reductase activity of plants is low during periods of poor illumination which also limits the rate of photosynthesis (Pfister 1988).

Soil moisture affects uptake and utilization of nitrates by plants. During periods of low rain fall and therefore reduced growth, plants continue to take up nitrate but have reduced nitrate reductase activity (Pfister 1988).Of particular practical importance to Australia is the effect of water stress on nitrate content of pastures. Krejsa *et al* (1987) and Pfister (1988) reported large accumulations of nitrate in plants shortly after a severe drought. Stem nitrate concentrations in water-stressed pearl millet increased from approximately 5g/kg to 9g/kg within two days of the commencement of irrigation (Krejsa *et al*, 1987). As many as 7 - 14 d may be required for nitrate levels 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 nitrate accumulation (Pfister, 1988).

Nitrates may be present in pastures used by ruminants on a continuous basis. Under normal growth conditions the concentrations of nitrate 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. It is only under particular conditions that nitrates become high enough to be close to the rumen requirements for fermentable nitrogen, but even then they are rarely a high percentage of the total crude protein in the feed (see Wright and Davison 1964). The exception to this maybe in temperate areas with high rainfall and heavy dressings of N fertilizers when nitrate N has been recorded as high as 2.5% of the dry matter of pasture and 45% of the total N in the forage (O'Donovan and Conway 1968). When nitrates are present in feed and become toxic they are usually associated with excessive levels of crude protein in the feed and high ammonia concentrations in the rumen of animals that consume the forage.

However, even at high protein levels in a diet, research clearly shows that nitrates consumed by sheep on diets high in crude protein, are converted to ammonia in the rumen providing fermentable nitrogen for microbial growth (Lewis 1951; Wang et al 1961).

1.3 Toxicity of nitrate and other sources of non protein nitrogen in ruminant diets

The majority of research on nitrate in ruminant diets has been focused on its toxic role in the animal. Since nitrate is the major precursor of nitrogenous compounds in plants including protein, it is safe to assume that under grazing conditions where pastures are growing that ruminants would ingest some nitrate on a continuous basis. This varies from very small amounts to the large concentrations that under certain conditions are known to become toxic (see values in Table 1). O'Donovan and Conway (1968) in Ireland showed that with high rates of application of nitrogen fertilizers that crude protein in the pasture sometimes exceeded 6% N (3.9-6.4%) with up to 45% (from 5-45%) as nitrate -N. Recent work by Lovett *et al* (2004) found that for each 1% increase in crude protein (from 13-23%CP) in perennial rye grass from N fertilizer application to the soil, nitrate in forage increased linearly by 0.035g/kg dry matter (from 0.04 to 3.6 g/kg DM) with an R^2 of 0.94. The point being made is that nitrate is not an uncommon substrate in rumen

fermentation and there are numerous rumen organisms that can utilize it for growth. These organisms are probably permanent components of the microbial ecosystem that is the rumen (Cheng et al 1985).

Table 1. Some values recorded for the nitrate concentrations in forages (%DM) consumed by ruminants (Faulkner and Hutjens 1989).

	No of Analysis	Average nitrate (%)	Low nitrate (%)	High nitrate (%)
Lucerne Hay				
Dehydrated	430	0.24	0.06	0.84
Hay	56	0.24	0.06	0.6
Silage	13	0.12	0	0.36
Maize plant				
Green-chop	11	0.78	0.12	1.72
Silage	66	0.48	0	2.64
Stalks	12	1.20	0.3	3.60
Oats				
Hay	11	0.78	0	2.4
Silage	3	0.54	0	3.6
Pasture				
Bluestem	619	0.06	0	0.12
Brome grass	3	0.48	0.06	1.32
Clover		0.30	0.18	0.48
Sudan grass				
Green-chop	16	1.56	0.12	2.88
Hay	12	0.3	0	1.98
Silage	2	0.18	0.12	0.24
Sorghum				
Stalks	11	0.24	0	1.62
Silage	40	0.30	0	0.90

Nitrate toxicity in ruminants occurs irregularly but seldom frequently in most grazing situations. It is usually associated in ruminants with sudden increases in nitrate intake from lush green feed that is also high in crude protein. Nitrate poisoning is associated with green “high quality” pasture plants that have impeded photosynthesis or in plant foliages that accumulate nitrates and which animals are suddenly forced to rely on for feed (see Wright and Davison 1964). In all studies reviewed, where nitrate poisoning has been a problem’ the crude protein content of the feed is generally high between 18 and 38%, where rumen ammonia levels are consistently high often up to 3 times the recommend levels for optimum microbial growth (Preston and Leng 1985).

In a large number of studies going back to the early part of the 20th century it has been demonstrated that nitrate is relatively innocuous and it is nitrite produced by microbial metabolism in the rumen that is the toxic agent and that on absorption from the rumen binds to haemoglobin of red blood cells limiting tissue supplies of oxygen (Bradley et al 1939).

1.4 Urea and nitrate toxicity: General considerations

Urea has been the preferred source of non protein N to supply fermentable nitrogen (ammonia) to ruminants on diets that are deficient in crude protein since McDonald (1948) first demonstrated the key role of ammonia as the N source for microbial protein assimilation. It is now fully accepted that most rumen microbes can use ammonia as a sole N source for cell growth (Allison 1969). Virtanen (1966) demonstrated that urea could be the sole source of nitrogen in a diet fed to dairy cows and therefore microbial cell growth in the rumen could supply all the essential amino acids for cows producing moderate amounts of milk. Just as with urea, nitrate is converted to ammonia by rumen organisms (see Lewis 1951) and is a potent source of ammonia

for bacterial growth, but at the levels reported in perennial rye grass, the calculated reduction in methane was about 1% which is close to that measured (Lovett *et al* 2004).

Urea toxicity results when the rate of ammonia production from urea exceeds its rate of microbial assimilation; rumen fluid concentrations of ammonia rise and it is absorbed in excess of the capacity of the liver to convert it to urea and excrete it in the urine. Under these circumstances peripheral blood ammonia levels increase and directly affect the central nervous system. Urea toxicity is more correctly termed ammonia toxicity and normally occurs where animals are either previously underfed or on low crude protein diets and not acclimated (adapted) to urea when they over consume urea or urea containing feeds.

Nitrate poisoning is also a misnomer as the toxic principle is nitrite produced from nitrate by rumen microorganisms under some circumstances. When the absorption rate of nitrite exceeds the capacity of the red blood cells to bind and oxidize nitrite to nitrate, the per cent methaemoglobin in red blood cells increases. This in turn reduces the oxygen carrying capacity of the blood and eventually results in hypoxia of the essential organs and death. In practice, nitrate toxicity is associated with high protein diets where nitrate in the feed boosts the crude protein content of the diet (nitrate accumulates in forages growing on fertile soils at times of normally high growth rates and high protein content) and therefore there is the probability that nitrate and protein intakes interact to produce the syndrome.

Ruminants adapt to high urea intakes and also self medicate the amounts of urea they consume to their requirements (Loosli and McDonald 1968). The rate of nitrate metabolized in the rumen is increased many fold by slowly adjusting nitrate in a sheep's diets (Alaboudi and Jones 1985). On most high quality feeds the ammonia requirements for microbial growth are met from protein hydrolysis in the rumen which can also be very rapidly produced particularly where the feed supply allows the development of hyper ammonia producing organisms (Russell et al 1988). Although rare, on high protein diets ammonia toxicity may occur, especially in animals fed intermittently high protein supplements such as lupin grain in large amounts (personal observations).

1.5 Toxic dose of urea and nitrate in ruminants

The toxic dose of any compound is assessed as the minimum dose that when abruptly injected is lethal to 50% of the experimental animals. There are numerous studies throughout the literature on the toxic dose of nitrate for ruminants. The results shown in Table 2 are a small representative sample and have been taken from Hibbeard et al (1994). There is little value in the reported LD50 estimates as the dose of nitrate that can be tolerated depends on a range of factors that will be discussed in the body of this report. Cattle appear to be more susceptible to nitrate poisoning compared with sheep. A number of authors have suggested that the upper limit of nitrate intake is about 1g/kg live weight in animals not accustomed to receiving nitrate in their diet. Since the capacity to metabolize nitrate is increased by slow acclimation by 3-10 fold, the upper limit for nitrate could be as high as 10g nitrate /kg live weight. Thus a 50kg un-acclimated sheep consuming 1500g forage per day would be at toxic limits at 50g nitrate or 11.3 g N. This would be the equivalent of consuming 20g urea. An acclimated sheep could consume say up to 10 times this representing the nitrate being equivalent of 120 g urea per day. The measured toxic dose of urea in un-acclimated ruminants is actually higher than that of nitrate around 450 mg/kg (22.5g /50kg sheep) (Booth and McDonald 1982). Stephenson et al (1992) recorded ammonia toxicities in sheep fed poor quality hay when 20g urea was administered into the rumen of 40kg sheep.

The requirements for fermentable N for a theoretically 100% efficient rumen fermentation (microbial growth is optimal) is approximately 30gN/kg organic matter digested or 15gN/kg dry matter intake at 50% digestibility. However in actually measured microbial growth efficiencies the efficiency is approximately 0.3-0.5 of theoretical efficiency. From these calculations it appears that nitrate could replace all the urea in a diet where sheep and cattle are acclimated to the nitrate without generating nitrite toxicity.

The literature on the toxicity of nitrate containing feeds for pregnant and lactating ruminants is large but contradictory. Some researchers have reported that abortions occurred during and sometimes following periods where nitrate was consumed in forage by breeding livestock. Others report no effects. In terms of milk yield, the literature is equally conflicting. A good discussion of the effects of nitrate in feeds consumed by lactating and breeding stock is presented by Crawford et al (1966). However, as is so often the case with the applied research on nitrate toxicity, the adverse effects of nitrate in a diet are associated with sudden intake of a large amount of nitrate (see later) and the accumulation of nitrite in the rumen.

Table 2. Some estimated toxic doses of nitrates for ruminants. In all cases these were measured in both laboratory trials and in field trials where outbreaks of nitrate poisoning had occurred and animals were on green pasture or fed hay with high crude protein content (in most cases the hay or pasture crude protein is not stated). Only a small proportion of the available information on lethal doses of nitrate is reported here (see Hibberd et al 1994).

Dose (mg nitrate/kg body weight)	Reference
198-998	O'Hara and Fraser, 1975
326	Bradley *et al* 1940
716	Crawford *et al* 1966
706-988	Wnght and Davison, 1964
308	Deeb and Sloan, 1975
499	Ruhr and Osweiler, 1986
345-260	Setchell and Williams 1968

A point of major significance is that the mineral composition of the forage is variable in grazing systems and the finding (Tillman *et al* 1965) that the level of molybdenum in a diet had a highly significant effect on nitrite accumulation in the rumen of sheep fed nitrate, suggests that this element may be a factor in the conflicting field data on the incidence and severity of nitrate toxicity. There were suggestions of a relationship between Mo and the high incidence of nitrate poisoning that occurred in areas of Victoria where wide scale application of the use of molybdenum occurred in the 1940s on the soils that were molybdenum deficient. It is interesting in this respect that molybdenum was applied to pastures to supply a sufficient pool in soil to last for 10 years before a second application was recommended. Over those 10 years, there could be a large variation in forage molybdenum. The effect of molybdenum on nitrate utilization in the rumen is discussed more fully later.

The conclusion is that the lethal dose of nitrate as reported in the literature is extremely variable. There are therefore numbers of extraneous factors that affect the utilization of nitrate by ruminants. A most pertinent point, however, is that in animals unaccustomed to the nitrogen source, nitrate may be no more toxic than urea under the same feeding conditions. Farmers around the world have learned how to manage urea supplementation of ruminant feeds and to minimize the risks. The risks of nitrate poisoning may be similarly managed without undue losses in productivity.

Any strategy developed to use nitrate to replace urea as a major N in ruminant diets, low in true protein would have world-wide application. These diets could include grains, sugar cane, mature forage and crop residues including straw. An ability to use nitrate and thereby eliminate enteric methane release to the atmosphere could mean that there would be a paradigm shift in the approach to breeding forages for grazing systems, i.e. towards species with high digestible biomass and low crude protein yields. Providing N as nitrate also opens up the potential for alkali treatment to increase the digestibility of straws and other agricultural by-products low in protein. The major point here is that the reduced methane production when such diets are fed must offset the cost (in greenhouse gas terms) of the treatment.

If the fermentable nitrogen in a low protein diet can be provided by nitrate, then the effect on enteric methane production could be dramatic, even totally eliminating enteric methane production from ruminant animals. However adding smaller amounts of nitrate to otherwise balanced diets should clearly reduce methanogenesis. In the following discussion, the potential and consequences of replacing fermentable nitrogen with nitrate are examined. The immediate research and the practical development requirements follow later in a second report.

2 BACKGROUND

2.1 Nitrate metabolism in anaerobic environments

Nitrate reduction in anaerobic systems occurs by three distinct pathways: dissimilatory nitrate reduction to nitrogen gas (denitrification), and dissimilatory and assimilatory nitrate reduction to ammonia. Assimilatory nitrate/nitrite reduction is also referred to as respiratory nitrate or nitrite ammonification. Assimilatory nitrate reduction is referred to in this presentation by any of the three terms according to its use by authors whose research is being discussed. Denitrification does not occur in the rumen, but when nitrate is present in rumen fluid, small amounts of nitrogen oxides are produced. The control mechanisms likely to determine the dominant reductase activities in the rumen are complex. However, considerable information is available on anaerobic metabolism in other natural environments that may provide in sights to the control of the, perhaps simpler, rumen systems for reduction of nitrate and nitrite to ammonia.

2.1.1 Denitrification

Denitrification proceeds in a stepwise manner, i.e. nitrate (NO_3^-) is reduced to nitrite ($N0_2^-$), nitric oxide (NO), nitrous oxide (NO_2), and nitrogen gas (N_2).

2.1.2 Dissimilatory nitrate reduction to ammonia (DNRA)

Dissimilatory nitrate reduction is the production of ammonia by reduction of nitrate and/or nitrite which occurs when redox potential values in the medium are low (Knowles, 1982), often in the presence of sulphide (Brunet and Garcia-Gill, 1996) or high organic matter concentrations (Hungate 1965: Akunna *et al* 1993) and is affected by the nature of the carbon source (Akunna *et al* 1993). Dissimilatory reduction is unaffected by ammonia in the culture medium and rapid conversion of nitrate to ammonia occurs even at high concentrations of ammonia. Its primary function appears to be to re-oxidize reduced pyridine nucleotides (e.g. NADH) which limit the rate of growth of microorganisms (Prakash and Sadana 1972).

2.1.3 Assimilatory nitrate reduction to ammonia (ANRA)

Assimilatory nitrate reductase involves enzymes that catalyze the reduction of nitrate to nitrite then to ammonia. The term assimilatory refers to the fact that the product of the enzymatic activity remains in the organism. Nitrate is reduced to nitrite by NADH reduction and nitrite is reduced to ammonia by assimilatory nitrite ammonification which is coupled to ATP formation (see for review, Simon 2002). In this case, high ammonia concentrations have an inhibitory effect on assimilatory nitrate reductase, thus ensuring that the microorganism produces just sufficient ammonia to meet its requirements for synthesizing organic-N compounds (Payne 1973) or stores the ammonia intracellular as amino N that can be readily mobilized as a source of ammonia.

Nitrate is an important source of ammonia for assimilation and many species of plants, fungi, and bacteria can incorporate nitrate through the combined action of dissimilatory nitrate reductase and assimilatory nitrite reductase. Typically, these enzymes are induced by nitrate or nitrite and are also subject to general regulation by ammonia, as in nitrogen metabolite repression in fungi (Cole and Brown 1980; Dunn-Coleman *et al* 1984). The enzymes are soluble, oxygen tension has little effect on the process, and ammonia is formed only at rates that can be assimilated into cell organic nitrogen. Their presence in the rumen has not been studied as such, however the nitrate reducing enzymes in bacteria in the rumen of sheep was reported to be membrane bound (Reddy and Allison (1981) quoted by Allison and Reddy (1984)). A microorganism isolated from the bovine rumen by Wolin *et al* (1961), *Wolinella succinogenese* (formally *Vibrio*), has been the most thoroughly studied organism that carries out assimilatory or respiratory nitrite ammonification (Simon 2002).

A recent demonstration that goats maintained a positive N balance on a diet of straw/molasses where nitrate provided 90% of the dietary N (see Figure 14) suggests that the

assimilatory NRB could potentially play a major role in rumen fermentative digestion when ammonia is limiting. In studies in which nitrate was the sole fermentable N source to sheep fed purified diets (Tillman *et al* 1965) nitrate was able to supply all the nitrogen needed for organic nitrogen assimilation in the rumen (this is discussed more fully later*).* Assimilatory nitrate reduction has important advantages for the efficiency of energy use in microorganisms compared with methane generation because the energy that is lost in methane is conserved in microbial biomass and the molar growth yield should be higher than for fermentations supported by other sources of ammonia, including protein and urea.

2.2 Conditions supporting the nitrate reduction pathway in microorganisms

Dissimilatory and assimilatory nitrite reduction are the predominant nitrate reduction pathways when organic matter content is a major fermentable energy source and fermentable nitrogen is not limiting and incubation time or the turnover time of the contents of ecosystem is short (for example in the rumen). In natural systems with long turnover times and where VFAs are the main electron donors, denitrification (nitrate is converted to nitrogen gas) is more dominant than dissimilatory nitrate reduction to ammonia (for example in waste water treatment systems, flooded rice paddy and biodigestors).

In the latter systems it has been reported that nitrate and/or reduced N-oxides, such as nitrite, nitric oxide, and nitrous oxide, suppress methane production (Akunna *et al* 1994; Klüber and Conrad 1998a; Klüber and Conrad 1998b; Clarens *et al* 1998; El-Mahrouki and Watson-Craik, 2004). The suppression is greatest with nitrous oxide (see Table 3). The apparent non-enzymatic production of nitrogen oxides when nitrate is metabolized in the rumen has implications for the control of methanogenesis (Kaspar and Tiedje 1981). This is discussed further below.

The predominant pathway of nitrate metabolism in the rumen is uncertain but has always been assumed or even asserted to be dissimilatory nitrate reduction to ammonia, the overall two-step reduction of nitrate shown below,

$$NO_3^- + 2H^+ \rightarrow H_2O + NO_2 \quad \text{Equation 1}$$

$$NO_2^- + 6H^+ \rightarrow H_2O + NH_3 \quad \text{Equation 2}$$

Organisms capable of nitrite ammonification usually have the ability to reduce nitrate to nitrite in dissimilatory metabolism (Simon 2002), nitrite being a suitable electron acceptor for anaerobic respiration. Formate and hydrogen are the common electron donors in assimilatory nitrite ammonification. These substrates are oxidized according to equations 3 and 4 below:

The sulphate reducing organisms (SRB) are an exception with the majority of the *Desulfovibrio* species using nitrite but not nitrate (Mitchell *et al* 1986). Recently sulphide has been shown to function as an electron donor for respiratory nitrite ammonification in nitrate reducing-sulphide oxidizing bacteria (NR-SOB) that oxidize hydrogen sulphide directly (Hubert and Voordouw 2007) (equation 5).

$$3HCO^-_2 + NO^-_2 + 5H^+ \rightarrow 3\,CO_2 + NH^+_4 + 2H_2O \quad \text{Equation 3}$$

$$3H_2 + NO^-_2 + 2\,H^+ \rightarrow NH^+_4 + 2H_2O \quad \text{Equation 4}$$

$$3HS^- + NO_2^- + 5H^+ \rightarrow 3S^0 + NH^+_4 + 2H_2O \quad \text{Equation 5}$$

The reaction in equation 5 suggests that nitrate or nitrite uptake could be stimulated by a source of sulphide produced locally. As most organisms that reduce sulphate are also capable of reducing nitrate or nitrite, the feeding of nitrate and sulphate is likely to have important interactions

and, in some environments, sulphate reducing organisms are inhibited by nitrite. For this reason sulphur metabolism in the rumen is outlined below.

2.3 Multiple enzymes catalyze parallel pathways of nitrate metabolism (after Lundberg *et al* 2004)

A feature of respiratory bacterial nitrate metabolism is that individual reaction can be catalyzed by multiple enzymes that differ depending on the organism and environmental conditions. This makes any discussion of nitrate reduction extremely complex. In addition, little is known of the cellular organizations of these enzymes in bacteria that are normally present in the rumen or that colonize the rumen when nitrate becomes a significant proportion of the dietary crude protein.

The detailed biochemistry of the nitrate/nitrite reductase is being investigated intensively using specialized technology. A complete review of current knowledge in this area is beyond the scope of this document, so the section below has been extracted from a major review (see Lundberg 2004) to provide references and additional information. This is an area that will require research inputs when and if a successful feeding regimen is developed for nitrate. At least three types of nitrate reductase catalyze the reduction of nitrate to nitrite, viz.

- A soluble, assimilatory nitrate reductase (NAS) that is present in the cytoplasm.
- An energy-conserving nitrate reductase (NAR, for example NarG, which is encoded by the first gene of the narGHJI operon) with catalytic sites located in the cytoplasm which are associated with the cytoplasmic membrane and from which they receive electrons for nitrate reduction.
- A soluble, periplasmic nitrate reductases (NAP) that is found in many Gram-negative bacteria.

All three types are molybdo-proteins and some bacteria, for example, *Paracoccus pantotrophus*, possess all three types. *Escherichia coli* and *Salmonella enterica* serovar *Typhimurium* also synthesize three different nitrate reductases. Although they lack an assimilatory nitrate reductase, the genes that encode the membrane-associated nitrate reductase are duplicated (the narG and narZ operons) and differentially regulated, and they also express a periplasmic nitrate reductase, Nap, which is encoded by the napFDAGHBC operon.

2.4 Bacterial nitrite reduction (after Lundberg 2004)

Nitrite reduction is the reaction that defines whether bacteria catalyze denitrification or nitrate reduction to ammonia, and in each case, two distinct classes of nitrite reductase are involved. All nitrite reductases are synthesized preferentially during anaerobic growth. The denitrification of nitrite to nitric oxide is catalysed by the copper-containing NirK or the cytochrome *cd* nitrite reductase NirS, both of which are located in the periplasm. Two biochemically distinct nitrite reductases catalyse the reduction of nitrite to ammonia. The NADH-dependent NirBD nitrite reductase reduces nitrite directly to ammonia in the cytoplasm of some bacteria (for example, Gram-negative enteric bacteria and Gram positive bacteria such as *Staphylococcus carnosus* and *Bacillus subtilis*). The role of NirBD is to detoxify nitrite that is generated by NarG (the membrane-associated nitrate reductase) during anaerobic growth in the presence of nitrate concentrations that are much greater than those usually found in animals. More widely distributed is the cytochrome *c* nitrite reductase Nrf, which catalyses the reduction of nitrite to ammonia in the periplasm of Gram-negative bacteria. This enzyme is the terminal component of an electron-transfer pathway in which electrons are transferred from physiological substrates, especially formate (hence the designation Nrf, for nitrite reduction by formate). Nap, the periplasmic nitrate reductase, and Nrf, the periplasmic nitrite reductase, are coordinately regulated to provide a pathway for the reduction of nitrate to ammonia in the periplasm. It is rare for any single species to be able to catalyse both denitrification and nitrate reduction to ammonia. Although there are also few reports of the occurrence of both NirK and NirS in the same species, some enteric bacteria,

such as *E. coli* and *S. typhimurium*, encode both the cytoplasmic nitrite reductase NirBD and the periplasmic nitrite reductase Nrf, and therefore have both a cytoplasmic pathway and a periplasmic pathway for the reduction of nitrate via nitrite to ammonia. High concentrations of nitrate induce expression of the cytoplasmic pathway which is encoded by the *narGHJI* and *nirBD* operons, but repress the periplasmic pathway which is encoded by the *Nap* and *Nrf* operons. At very low external concentrations of nitrate and nitrite, similar to the concentrations found in body fluids, it is the Nap-Nrf periplasmic pathway that is induced rather than the cytoplasmic pathway. However, it is likely that the cytoplasmic NarG enzyme is responsible for the accumulation of nitrite from nitrate by lingual microorganisms. Chemostat competition experiments have established that the ability to reduce nitrate and nitrite in the periplasm confers a selective advantage on strains of bacteria that are able to express only the cytoplasmic pathway. Consequently, nitrate reduction in the periplasm of enteric bacteria is believed to be the physiologically significant pathway. Even bacteria that lack denitrification pathways and are known to reduce nitrite directly to ammonia generate relatively high concentrations of nitric oxide (NO). Although unproven, it is possible that this NO is produced by one of the enzymes that reduce nitrite to ammonia (NrfA or NirBD).

2.5 Sulphur metabolism in the rumen

SRB in the rumen utilize anaerobic respiration pathways for bioenergetics processes. The SRB are grouped by the mechanism used to reduce sulphate, i.e. assimilatory processes or dissimilatory processes. In general, the dissimilatory reduction of sulphur compounds is used for the generation of ATP, while the assimilatory process reduces sulphur compounds for incorporation into other organic compounds necessary for cell survival (Odom and Singleton, 1993). In the rumen, SRB from both the assimilatory and dissimilatory groups exist, and the latter are responsible for the reduction of sulphur to hydrogen sulphite and hydrogen sulphide. Similarly NRB in the rumen appear to belong to both the dissimilatory and assimilatory groups. Although many bacteria can produce sulphide, organisms from the *Desulfovibrio* and *Desulfotomaculum* genus are most likely the predominant sulphate-reducing bacteria (Cumming *et al* 1995b).

In the rumen, the extent of dissimilatory sulphate reduction is proportional and limited by the amount of sulphur containing compounds. The predominant sulphide compounds formed in the dissimilatory process are S_2^-, S^o, HS^-, or HSO_3^- (Odom and Singleton, 1993). The pKa values for these compounds are around 7.0 (the pKa for H_2S is 7.2). Because the pH range of a 'normal' rumen is between 6 and 7, these reduced forms of sulphide are readily protonated and therefore most of the sulphide present in the rumen is in the gas phase as hydrogen sulphide (H_2S). Only small amounts remain in the liquid phase in a variety of sulphide containing compounds. Hungate (1965) suggested that, under normal feeding conditions, if the gas phase hydrogen sulphide is equilibrated with rumen fluid, its concentration would be about 0.1mM. However, in cattle fed high levels of sulphate that induced polioencephalomalacia, hydrogen sulphide in rumen fluid attained 0.3 mM (Gould *et al* 1997). Hydrogen sulphide is eructated from the gas space: eructation is followed by inhalation of the gases into the lungs where a proportion of the hydrogen sulphide is absorbed and converted into sulphate (Dougherty *et al* 1965).

Most of the sulphur in herbage is contained in methionine and cysteine in plant proteins. Most of the proteins in fresh plant materials are highly soluble and are rapidly fermented on entry into the rumen. In studies with dairy cows on perennial ryegrass pastures, Dewhurst *et al* (2007) demonstrated that cysteine-S was more readily converted to hydrogen sulphide at rates greater than that of methionine-S which, in turn, was faster than the conversion of sulphate-S to hydrogen sulphide when the same amount of sulphur in these forms was added to the rumen. The concentration of hydrogen sulphide in rumen gases increased with increasing dose rates of sulphur compounds administered into to a cow's rumen and peaked in rumen gases 30-40 min after administration of cysteine (Dewhurst *et al* 2007). Inorganic sulphur as sulphate is converted to hydrogen sulphide but there is a time lag between introduction of sulphate and peak production of sulphide in cattle of up to 10d (Gould *et al* 1997). The population of SRB and NR-SOB in the rumen will be controlled largely by the true protein content in the herbage fed ruminant and the

normally low level of sulphide because of the release to the gas space may maintain only minor populations of NR-SOB.

2.6 Potential interaction of SRB and NRB

Where nitrate concentrations are high in an anaerobic ecosystem, it is probable that the heterotrophic NRB out-compete SRB for degradable organic electron donors and thus inhibit SRB (see Nikolic *et al* (1984). SRB have also been shown to be inhibited by high molybdenum concentrations in an incubation medium (see Bryden and Bray 1972). The inhibition probably occurs because of the removal of S by Mo-S interactions.

When ruminants receive a nitrate load in the rumen either as an experimental simulation of the toxicity syndrome or for the study of nitrate metabolism, in the period after mixing, nitrate levels decline precipitously for up to 1h and then steadily decrease thereafter (see Figure 3). For example, 25-35g $NaNO_3$ administered as a drench to sheep will give nitrate concentrations of 60-80mM in the rumen fluid. This concentration decreases in 1h to about 20 mM and then falls to zero over the next 8 h (see Figures 2 and 3). During this early uptake of nitrate, it is apparent that in most studies that nitrite is produced and accumulates in the fluid medium. Nitrite does not accumulate when the same amount of nitrate is given in the feed which is probably ingested throughout the day and therefore is more slowly released in the rumen (Alaboudi and Jones 1985).

When cysteine was administered with nitrate into the rumen of sheep, nitrite did not accumulate in rumen fluid (Takahashi *et al* 1998). However, feeding nitrate in a well-balanced diet increased the excretion of sulphur to the extent that sheep went from positive to negative sulphur balance. A return to a positive balance was achieved by increasing sulphur levels in the diet (see Sokolowski *et al* 1969). These findings suggest a strong interaction of nitrate and sulphur utilization in the rumen. Similarly, in other ecosystems where the organisms are actively metabolizing nitrate, high levels of nitrate are accompanied by a transient build up of nitrite concentrations in the fluid medium. Studies with *Desulfovibrio desulphuricans*, which can reduce either nitrate or sulphate, showed that the addition of nitrate to the incubation medium totally inhibited sulphur reduction (Krekeler and Cypionka 1995) possibly by competition for electron donors.

In oil wells, sea water, which contains appreciable quantities of sulphate, is used to flush out more oil as the well depletes and decompresses. Addition of nitrate in such systems reduces or inhibits hydrogen sulphide release (Grigoryan and Voordouw 2008). The understanding of the interaction of NRB and SRB and nitrate reducing, sulphide oxidizing bacteria (NR-SOB) that has been revealed by research aimed at oil well management has thrown up some interesting aspects that may provide insights applicable to the rumen system. These ideas are developed in the next section.

2.7 The microbial interaction in oil fields, managed to minimize hydrogen sulphide release, as a potential model for the metabolism of nitrate in the rumen

Sulphate–nitrate interrelationships in anaerobic environments are complex. The microbiological interactions when nitrate is injected into ‘spent’ oil fields to prevent souring (production of hydrogen sulphide gas) appear to be similar to the changes that occur in the rumen when nitrate is suddenly introduced.

Nitrate-reducing bacteria (NRB) can be categorized according to whether they use organic (hNRB) or inorganic (NR-SOB) electron donors and whether nitrate reduction proceeds via denitrification or dissimilatory nitrate reduction.

Nitrate injection in depleted oil fields charged with water and containing organic carbon (oil organics) changes the microbial community in the anaerobic zone from mainly SRB to one enriched in NRB, which include a NR-SOB that oxidize hydrogen sulphide directly (Equation 5)

and the heterotrophic NRB (hNRB) (which reduce nitrate as shown in Equations 3 and 4, or by denitrification). The NRB appear to effectively compete with SRB for degradable organic electron donors (Figure 1) and thus potentially inhibit SRB metabolism. The fermentable organic products of oil degradation are represented by lactate which is a primary electron donor when it is oxidized to acetate. Both types of NRB also promote inhibition of SRB via production of nitrite by nitrate reductase. The majority of sulphate reducing *Desulfovibrio* species reduce nitrite but not nitrate (Mitchell *et al* 1986).

Co-culture experiments using *Sulphurospirillum* (a NR-SOB with very similar attributes to *W. succinogeneses*, this is discussed later) and *Desulfovibrio* species. (SRB, species of which can be isolated from the rumen) revealed that the former out competed the latter when these could not overcome nitrite inhibition, either because their cell density was too low (Haveman *et al* 2004) or because they lacked nitrite reductase (Greene *et al* 2003). Thus, nitrite production appears to be essential for the competitive success of hNRB, whereas nitrite removal is equally essential for the competitive success of SRB when these two groups are competing in the same culture for common electron donors. Nitrite is only an intermediate in the reduction of nitrate to ammonia by *Sulphurospirillum* species but it is a substrate for many *Desulfovibrio* species that use nitrite but not nitrate (Mitchell *et al* 1986). Nitrate-limiting conditions promote respiratory ammonia production with little nitrite accumulation in pure cultures of *Sulphurospirillum* spp. This is consistent with the absence of residual nitrite at lower nitrate concentrations in the incubation medium when nitrate reduction to ammonia was catalyzed by *Sulphurospirillum*-dominated communities.

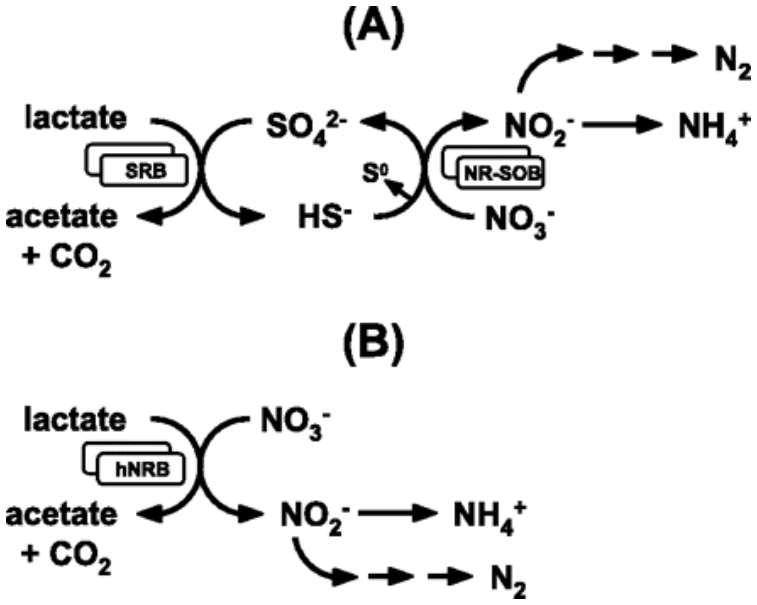
With the *Sulphurospirillum* spp used in these studies, it appears that it was the ratio of fermentable organic matter (lactate) to nitrate that determined whether nitrate reduction yielded nitrite or ammonia. When the ratio was high (nitrate limiting) more ammonia was produced, whereas when the ratio was low (surplus nitrate), nitrite production was favored. At the same time the requirements for hydrogen sulphide by *Sulphurospirillum* must be met and at high nitrate to sulphide ratios in the medium, the main products were nitrite and sulphate (Greene *et al* 2003).This results in decreasing concentrations of both nitrate and hydrogen sulphide in the incubation medium.

2.8 Nitrate-sulphur interactions in the rumen environment

The microbiological changes in the rumen when nitrate is introduced have received little attention. SRB, particularly *Desulfovibrio* spp, are usually present in significant numbers in the rumen and intestinal contents of animals including humans (Forsberg 1980; Gibson 1990), but their cell numbers will be limited by the sulphur or nitrate content of a diet. The low hydrogen sulphide content of rumen fluid (around 0.1 mM) (Hungate 1965) would tend to lower the possibility of a NR-SOB being a significant component of the rumen milieu. However, cysteine-S is rapidly reduced to hydrogen sulphide in the rumen and when cysteine was injected with a nitrate load, accumulation of nitrite in the rumen of sheep was inhibited (Takahashi *et al* 1998). The ability of SRB to reduce nitrite to ammonia is widespread, but only a few strains are capable of dissimilatory nitrate reduction. The nitrate using SRB often have an inducible periplasmic nitrate reductase (Nap, reducing nitrate to nitrite) and a constitutive periplasmic cytochrome *c* nitrite reductase (Nrf). Thus Nap is absent but Nrf is present in cells growing by dissimilatory sulphate reduction (Liu and Peck, 1981; Seitz and Cypionka, 1986). The ability to reduce sulphite to sulphide by nitrite reductase and, conversely, nitrite to ammonia by sulphite reductase is also common in these bacteria, possibly because both are six electron reductions of substrates of similar size and charge. The high affinity of dissimilatory sulphate reductase for nitrite and its low turnover number may allow nitrite to serve as a competitive inhibitor of sulphate reduction under physiological conditions. Greene *et al* (2003) produced data strongly supporting the concept that the function of periplasmic Nrf in SRB is to prevent inhibition of dissimilatory sulphate reduction, the key energy-generating process, by reducing nitrite to ammonia. SRB such as *Desulfovibrio* species and NR-SOB may interact as shown in Fig. 1.

The *Desulfovibrio* species use electron donors such as formate or lactate to reduce sulphate to sulphide, and the NR-SOB uses nitrite as the electron acceptor to reoxidize the excreted sulphide to sulphate. Because nitrate is reduced initially to nitrite (Fig. 1), nitrite may accumulate, inhibiting dissimilatory sulphate reduction. This inhibition can be prevented or overcome by the nitrite reductase activity of the SRB, which reduces nitrite to ammonia. In the rumen the electrons for this reduction are ultimately provided by reduced cofactors generated in the Embden-Meyerhof pathways of carbohydrate fermentation. Thus, together an SRB and NR-SOB co-culture could catalyze the oxidation of organic matter with nitrate through a sulphide intermediate.

Figure 1 Impact of nitrate on the oil field sulphur cycle.
(A) Sulphide produced by SRB activity can be recycled to sulphate or sulphur by NR-SOB reducing nitrate to nitrogen (denitrification) or ammonia (DNRA). (B) hNRB compete with SRB for organic electron donors, such as lactate, excluding sulphide production by SRB and possibly lowering their capacity to switch to nitrite reduction. Many SRB and hNRB oxidize lactate incompletely to acetate and CO2 as shown. The overall reactions in panels A and B are the same: the oxidation of lactate with nitrate (from Hubert and Voordouw 2007). SRB and NRB are present in the rumen and, if NR-SOB is also present, then similar end-products could be produced without denitrification because of the shorter turnover time of rumen contents than of the oil fields water medium.



2.9 Nitric and nitrous oxides and methanogenesis

Nitric oxide and nitrous oxide are produced *in vitro* in small quantities in rumen liquor from cattle given nitrate but this appears to be a by-product of dissimilatory nitrite reduction to ammonium rather than denitrification (Kaspar and Tiedje1981). Even bacteria that lack denitrification pathways and are known to reduce nitrite directly to ammonia, produce relatively high concentrations of nitric oxide (NO) (Ji and Hollocher 1989).

W. succinogenese, isolated from bovine rumen, is capable of transforming nitrate to ammonia harnessing all eight electrons but it also transforms nitrite to nitrous oxide and gaseous nitrogen (Yoshinari 1980), illustrating that there are organisms which can utilize the intermediates of the ammonification pathways in the rumen. It is now clear that a number of non-denitrifying organisms can produce nitrous oxide during nitrate reduction (Bleakly and Teidje 1982; Teidje 1988) and nitrous oxide may therefore accumulate in rumen fluid when nitrate is added in the feed. The complete inhibition of methanogenesis in some methanogens in culture by minute concentrations of nitrous oxide (see Table 3) has implications for the feeding of nitrate to ruminants and may be a mechanism that helps direct electrons to ammonification rather than

methanogenesis (Kluber and Conrad 1998a,b).The biochemical etiology is obscure; however it is worthy of some research to check whether there is a critical level of nitrate in a diet above which the reduction in methanogenesis is greater than would be theoretically possible if nitrate was merely acting as an electron acceptor.

Table 3 Inhibitory effects of N-oxides on *Methanosarcina barkeri* and *Methanobacterium bryantii* (a, Klüber and Conrad, 1998; b, Clarens *et al*, 1998)

N-oxide	Methanogenic Species	N-oxide Concentration	Residual Methanogenic Activity (%)
Nitrate	*Ms. barkeri* *Mb. bryantii* *M. mazei*	30 mM 30 mM 14.3 mM	24[a] 41[b] 54[a]
Nitrite	*Ms. barkeri* *Mb. bryantii*	0.1 mM 1 mM	Complete inhibition[a] 50[a]
Nitric oxide	*Ms. barkeri* *Mb. bryantii*	1.7 µM 0.8-1.7 µM	Complete inhibition[a] Complete inhibition[a]
Nitrous oxide	*Ms. barkeri* *Mb. bryantii*	0.95 mM > 95 µM	10[a] Complete inhibition[a]

The amounts of the nitrogen oxides produced are low and no specific side reactions or non-enzyme transformations appear to be involved in natural conditions (see Zumft 1993). However, there is no knowledge of the concentrations of nitrogen oxides in rumen fluid when nitrate is present in substantial amounts in a diet

Inhibition of some methanogens by N-oxides has been identified as the main mechanism involved in the suppression of methanogenesis in rice cultivation (Roy and Conrad 1999) but is unexplored in digestive systems that depend on microbial fermentation. In addition to competition, nitrite and the intermediates of dissimilatory nitrate reduction and denitrification are known to inhibit various bacterial species (Klüber and Conrad 1998). This is highly important for the rumen system as inhibition of ruminal organisms may lead to specific inhibition or decrease of the digestion rate of feed and a lowering of the production of fermentation end-products (largely VFA and microbial biomass) required by the animal for its sustenance. The inhibitory effects of N-oxides on cultures of *Methanosarcina barkeri* and *Methanobacterium bryantii* are shown in Table 3. The main point is that extremely low concentrations of N-oxides are required to inhibit these methanogens. It is feasible that some methanogens would be unable to establish in the rumen under such conditions and it is speculated that perhaps this inhibition could be beneficial to the survival of a flora containing acetogens which would also limit methane production (see Klieve and Ouwerkerk 2007).

2.10 Are there other sources of nitrogen oxides that could enter the rumen?

All animals, including humans, ingest some nitrates in their feed and the potential for generation of nitrogen oxides is important in many physiological and biochemical reactions. The role of nitrogen oxides in the inhibition of enteric methanogenesis is an area of interest.

Recent research has shown in humans that dietary nitrate (which is estimated to be about 2-3 mmol/d) is absorbed from the stomach and small intestine into plasma. It is actively concentrated 10 fold from the plasma into the saliva (Spiegelhalder *et al* 1976; Tannenbaum *et al* 1976; Duncan *et al* 1995) and then secreted in saliva into the mouth and upper intestinal tract. Approximately 25% of dietary nitrate is recycled via this entero-salivary circulation (Spiegelhalder *et al* 1976; Tannenbaum *et al* 1976). The dorsal surface of the tongue provides a habitat for symbiotic nitrate-reducing bacteria which reduce nitrate to nitrite (Sasaki & Matano 1979; Duncan

et al 1995). This population is increased by high nitrate content in food (see McKnight *et al* 1999). Thus, from the salivary and dietary nitrate, nitrite is generated in the mouth and the amount increases with nitrate availability (Lundberg *et al* 1994; McKnight *et al* 1997). Nitrate is not reduced in the mouth of the germ free rat (Duncan *et al* 1995). This research establishes the role of the bacteria resident on the tongue. Nitrite mixed in saliva is carried with food into the stomach where in the acid environment, it is converted to nitrous acid (HNO2) which in turn breaks down to various nitrogen oxides (see also Lundberg *et al* 2004).

Studies of nitrate/nitrite metabolism in ruminants appear to have been confined to the conversion of nitrate to nitrite in the rumen and further reduction to ammonia. It has been automatically assumed that nitrate and nitrite are absorbed directly from the rumen and nitrite binds to the haemoglobin in red blood cell diminishing their ability to carry oxygen to the tissues. Absorbed nitrate will be carried to various parts of the body and some may be excreted via urine but equally it may be concentrated in saliva. Intravenously injected ^{15}N labeled nitrate was only slowly lost from the body of sheep, with only a small proportion (40%) of label lost in the urine over 50h. However, label appeared in urine urea quite quickly, indicating that the injected nitrate had been converted to ammonia in the digestive tract (Lewicki *et al* 1998).These authors suggested that nitrate was stored in the body. In the ruminant, the anatomy of the animal dictates that dietary and salivary nitrate first enters the rumen where rapid uptake by ruminal organisms and there is potential for absorption across the rumen wall and movement with digesta to the omasum and abomasum occurs (this is discussed in the next section). The role of the salivary glands and lingual microbes in producing nitrite is unknown. Elevated plasma levels of nitrate in animals given a pulse load of nitrate into the rumen may have major effects not previously recognized (see metabolism of nitrate in the whole animal). Regurgitation of digesta (rumination) may also play a role by returning feed particles and rumen fluid containing nitrate to the mouth where nitrite could also be formed from nitrate by microbial action of lingual bacteria.

Under normal feeding conditions, little nitrate or nitrite appears in rumen fluid but measurable amounts occur when nitrate is added to a diet, particularly if the animal has not been accustomed to dietary nitrate (see section on metabolism of nitrate). Under these circumstances some of the nitrate /nitrite in rumen fluid will flow down the tract into the acidic abomasum There is a large absorption of water and solutes in the omasum prior to digesta entering the abomasum but the extent of further movement of nitrate/nitrite into the acid abomasum is unknown. The extent of any conversion of nitrite to nitric oxides in the abomasum is also unknown. Probably the major uncertainty is the extent to which nitrate is absorbed and recycled to the digestive tract. There is, however, the possibility that, when sheep are loaded with nitrate, absorbed nitrate circulates through saliva and may affect the nitrite entry into rumen fluid. Nitrate recycling through absorption and concentration into saliva could be a highly beneficial in extending the availability of nitrate nitrogen with time to meet the requirements of the rumen organisms.

A review of *in vivo* studies where nitrate has been fed or administered directly into the rumen of cattle or sheep in large quantities indicates that there is certainly an apparent absorption of both nitrate and nitrite, however the contribution of nitrate and nitrite in feed to nitrate and nitrite in pools in the animal body have not been quantified.

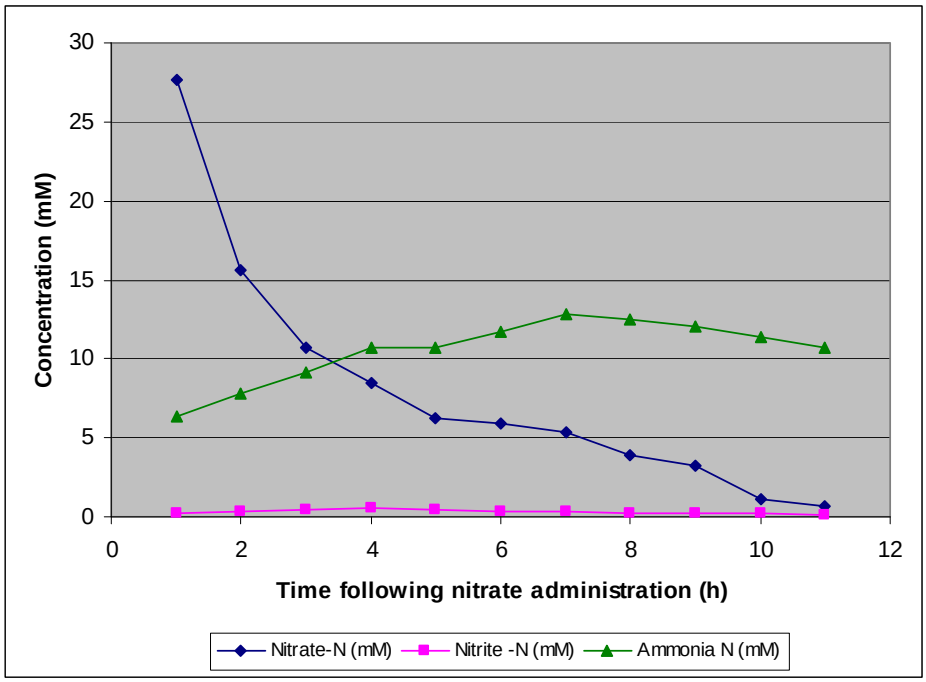
3 REVIEW OF NITRATE/NITRITE METABOLISM IN RUMINANTS.

3.1 Metabolism in the rumen of nitrate and nitrite

Lewis (1951) studied nitrate metabolism in the rumen of intact sheep by injecting a nitrate load into the rumen of sheep 16h after they had ingested their daily ration. The sheep had not been previously accustomed to nitrate in their feed. The effects of the nitrate load on rumen fluid concentrations of nitrate, nitrite and ammonia are shown in Figure 2. Notably, the increase in ammonia concentration in rumen fluid was quite slow and nitrite concentration was always low.

Since then a number of research groups have examined the clearance of intra-ruminal nitrate doses in fed animals (see Figure 3). In almost all similar experiments, the animals used were on feed but had not been adapted to nitrate (see for examples Takahashi and Young 1991; Sar *et al* 2004a,b,Sar *et a*l 2006). However in the studies of Tillman *et al* (1965) the sheep were given a diet for 10 d with nitrate as the sole source of nitrogen in a purified diet. The sheep were then fasted for 12 h and 500g feed was suspended in 2 L of water (the feed contained 32.5g of KNO_3 and 32.5 g of $NaNO_3$) and administered (force fed) through the rumen fistula. The nitrate load used was more than twice the amount given in the other experiments reported in Figure 3.

Figure 2. Changes in nitrate, nitrite and ammonia concentrations in rumen fluid of sheep fasted for 16 h and injected intra-ruminally with 25g sodium nitrate (Lewis 1951).



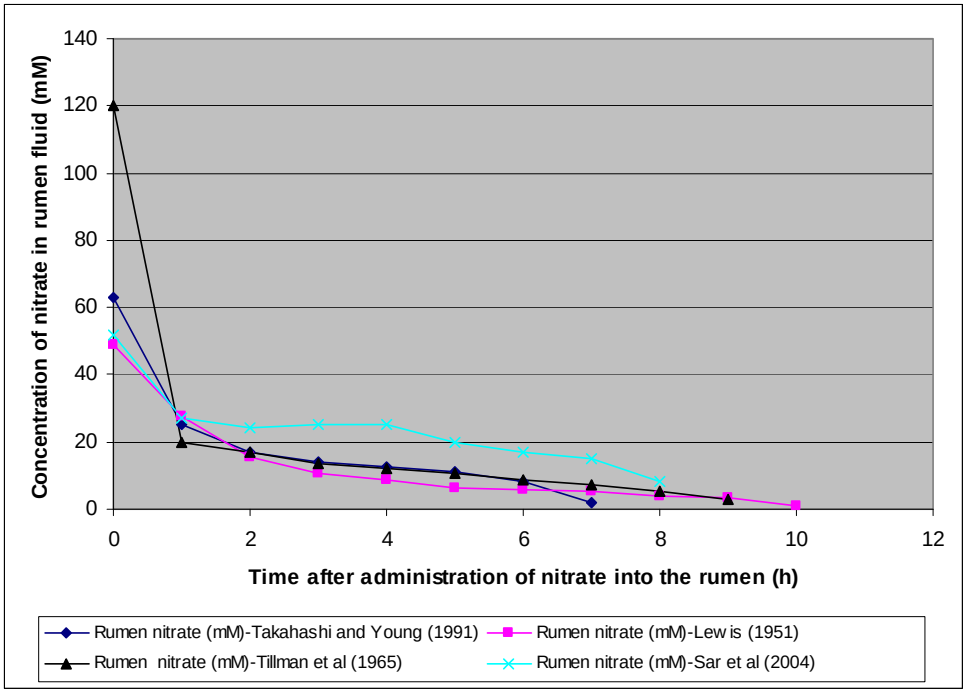
In Figure 3, an estimate of the zero time nitrate concentration in the sheep's rumen fluid is included. This is calculated from the dose given and an assumed rumen volume and assuming the nitrate had mixed instantaneously in the rumen contents. The calculated concentration of nitrate at zero time has a small error associated with the assumption of rumen volume (±10%). However, including the calculated nitrate concentration at the time of injection of nitrate changes considerably the perception of nitrate clearance. Initially there appears to be a high rate of disappearance of nitrate from the rumen, especially when the higher amount of nitrate was given in the study of Tillman *et al* (1965) where within 1 h of administration, up to 80% of the nitrate dose had disappeared from the rumen contents. These sheep were acclimated to nitrate feeding but, in all the studies the rates of decline in rumen nitrate after the first hour were relatively slow whether the animals had been accustomed to dietary nitrate or not. The production of ammonia seems unlikely to be a major cause of this initial rapid rate of disappearance, as ammonia levels

increased relatively slowly and continuously over a time interval of 8 h or more whereas nitrite accumulation peaked at 2-4 h (see Figure 20). Apparently, a number of processes were acting simultaneously to remove nitrate from the fluid medium in the rumen.

Urea is quantitatively and rapidly converted to ammonia in the rumen and the pattern of ammonia accumulation from a urea load in the rumen should be qualitatively similar to that occurring from a nitrate load if dissimilatory nitrate reduction to ammonia is the primary reason for the rapid clearance of nitrate.

The patterns of ammonia accumulation in the experiments shown in Figure 3 can be contrasted to patterns of ammonia accumulation in which urea has been injected directly into the rumen. Kaye *et al* (2001) administered a single intra-ruminal injection of urea (0.4g /kg live weight) to four breeds of sheep and one breed of goat that had been fasted for 24h and showed that rumen ammonia concentration increased 7 fold in the following 30 min. The increase in rumen fluid ammonia concentration should have been about 100 mmol/L if all the urea was hydrolyzed and the resulting ammonia had mixed instantaneously in the rumen fluid. The measured values averaged 80% (range 65-100%) of the expected theoretical value in the 15 animals used in the study. Stephenson *et al* (1992) also examined rumen fluid ammonia accumulation in Merino sheep fed low protein hay following urea administration to the rumen. Between 10 and 20g urea were administered in water via a rumen cannula and, assuming a typical rumen volume for such sheep of 4L, the peak ammonia level at 30 min post injection averaged 67% (range 57-79%) of the theoretical concentration if all the urea had been instantaneously converted to ammonia and mixed in the rumen contents.

Figure 3. Clearance of nitrate from rumen fluid of sheep following administration of a nitrate load in different studies

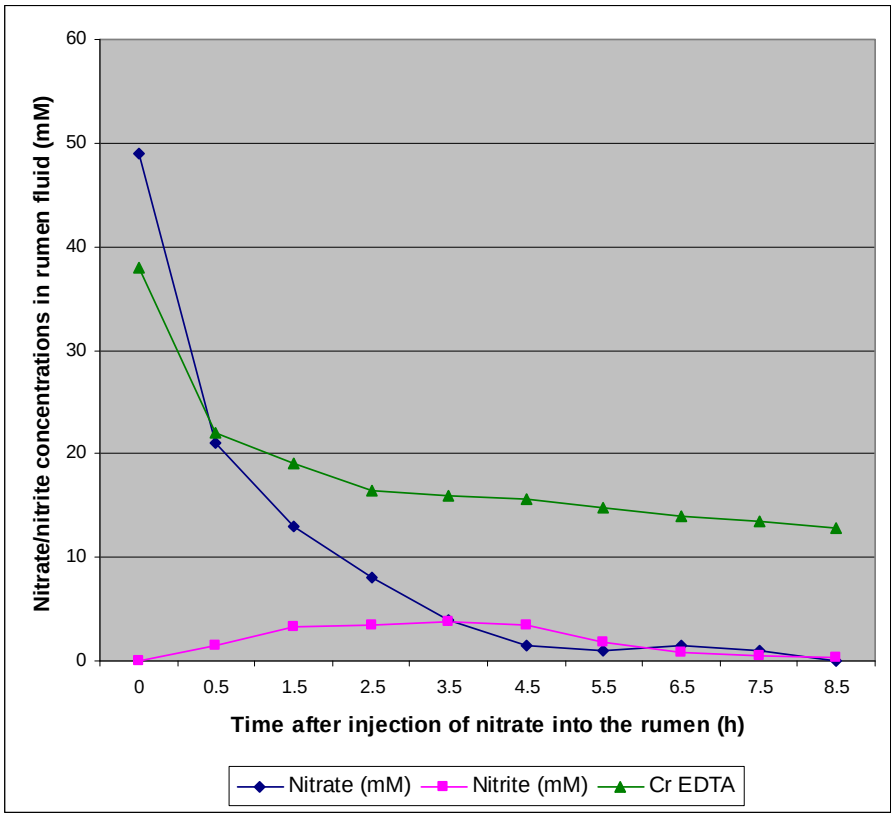


The rapid loss of nitrate from rumen fluid has gone unrecognized because researchers did not calculate the nitrate present at the time of nitrate administration. Calculations can be made on the basis that 1 mol nitrate will be reduced by rumen microorganisms to 1 mol of ammonia. Thus, in the experiment of Tillman *et al* (1965), if all the nitrate disappearing in the first hour had been converted to ammonia, the concentration in rumen fluid would have been elevated by about 100 mmol/L or by allowing an approximate 75% of the ammonia generated entering the rumen ammonia pool as was recorded for urea (Stephenson *et al* 1992;Kaye *et al* 2001) the theoretical elevation should have been 75 mmol/L but it actually had only increased over the first hour by 2 mmol/L and continued to increase over the next 6 h post administration of the nitrate by a further 6

mmol/L (see Figure 21). This is a pattern of increase rumen ammonia totally different to that when urea load is administered into the rumen.

The apparent slow accumulation of ammonia in rumen fluid when a nitrate load is applied to the rumen in comparison to the rapid accumulation of ammonia following administration of a urea load is difficult to explain. It appears to suggest that nitrate disappearing from rumen fluid was not quantitatively converted to ammonia which is then released to rumen fluid as may be expected if nitrate reduction was by the dissimilatory pathway.

Figure 4. The theoretical dilution of CrEDTA in the rumen in relation to the changes in nitrite and nitrate levels in the rumen of a sheep following administration of 25 g Na-nitrate intra-ruminally (after Sar *et al* 2004a).

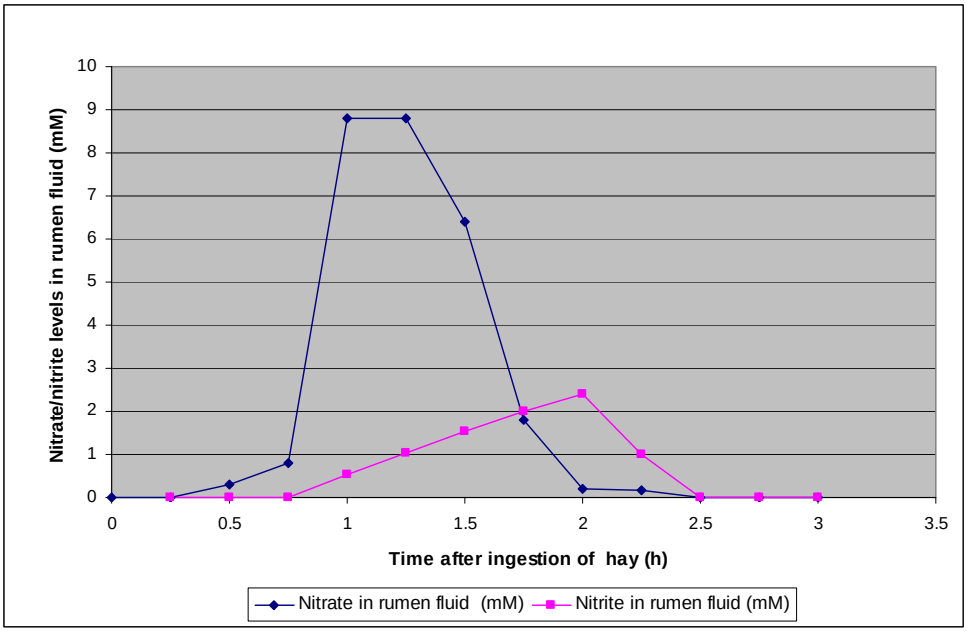


To emphasize that the apparent rapid clearance of nitrate is not associated with mixing of the dose in rumen fluid, more recent data from Sar *et al* (2005) is shown in Figure 4. Superimposition of a characteristic dilution curve for the inert non- absorbable marker, Cr-EDTA, injected into the rumen (taken from Downes and McDonald (1964) working with similar sheep) shows the appreciable differences between nitrate clearance and mixing. This is interpreted to show that, after administration of nitrate into the rumen, there is an initial rapid (net) rate of disappearance of nitrate and then a much slower rate of disappearance. The results are typical of those in the literature and show an accumulation and decline of nitrite in rumen fluid. Unfortunately Sar *et al* (2005) did not report the changes that occurred in rumen ammonia levels, but in previous publications where similar nitrate doses were applied to sheep on similar diets, rumen fluid ammonia concentrations rose over 8h from 13 mM to over 23 mM. Which appears to be not compatible with the concept that dissimilatory nitrate reduction is the primary pathway of removal of nitrate from rumen fluid when nitrate is administered in large doses? This is discussed further towards the end of this paper.

3.2 Rumen fluid nitrate and nitrite concentrations in cattle receiving nitrate in feed or as a drench

In studies with cows fed a mixture of hay and concentrates in two meals per day (Kemp *et al* 1977), the change in nitrite and nitrate concentration in rumen fluid are shown in Figure 5 after the animals had ingested a meal of high nitrate forage (over 45 min) in the morning. Prior to this experiment the animals had been on this feeding regime for 18 d. Although the cattle were accustomed to a daily dose of nitrate, the nitrate and nitrite concentrations appearing in rumen fluid were transient and both were cleared within 2-3 h (see Figure 5). A similar pattern of nitrate and nitrite change was observed where the animals were dosed with nitrate directly into the rumen. Thus between doses of nitrate there was no, or little nitrate in the rumen for 20h. Acclimation by rumen organisms to nitrate is discussed further below.

Figure 5. Changes in nitrate and nitrite in rumen fluid of a cow following ingestion of hay containing 82g of K-nitrate (Kemp et al 1977)

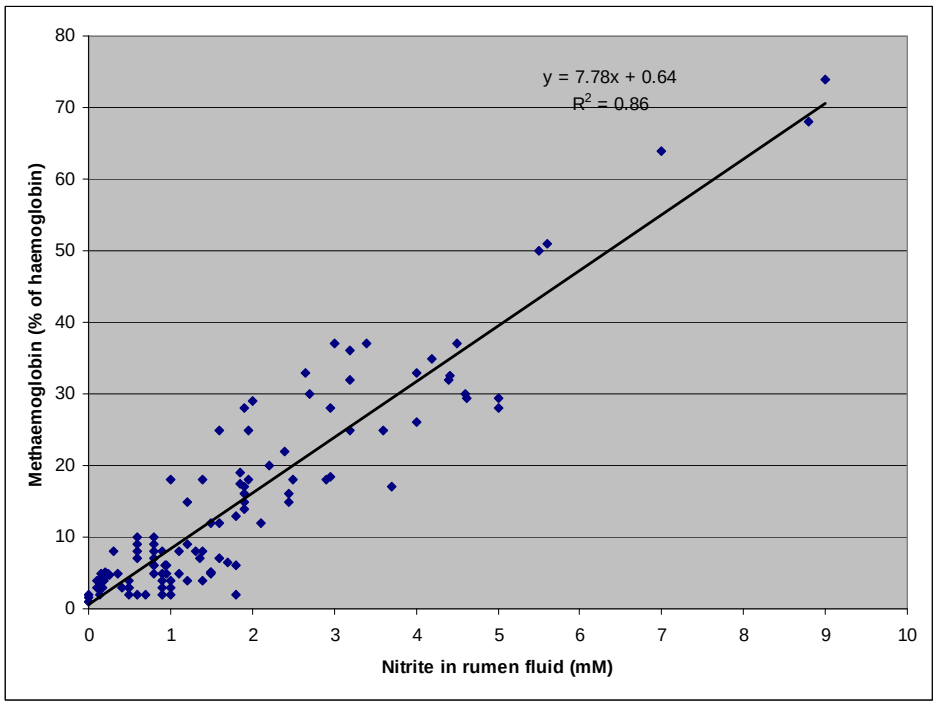


Kemp *et al* (1977) studied cattle given a variety of feeds in 32 feeding trials and summarized the relationship between the concentration of methaemoglobin in blood and peak nitrite in rumen fluid. The combined results indicate there is a strong relationship between peak rumen nitrite concentration and blood methaemoglobin as a percentage of total blood haemoglobin (Figure 6).

Crawford *et al* (1966) had previously collated data from several studies, relating nitrate intake and methaemoglobinaemia in cattle and although a sigmoid response curve was reported, the data were derived from animals that had lethal levels of methaemoglobin and required an injection of methylene blue in order to survive. In Figure 6, data are included only where nitrate ingestion resulted in sub-lethal methaemoglobin levels (arbitrarily less than 40% methaemoglobinaemia). The relationship in Figure 7 shows that a small increase in intake of nitrate can result in an exponential increase in blood methaemoglobin. Geurink *et al* (1989) have published an almost identical relationship.

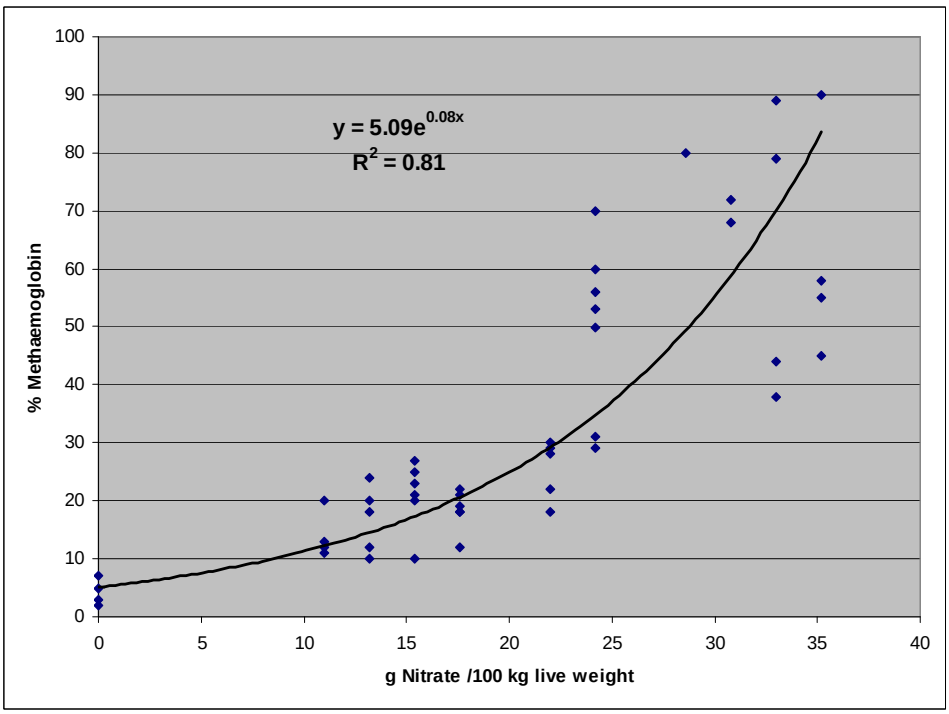
A comparison of feeding nitrate sprayed onto hay as against drenching a similar load of nitrate, indicated that the rate of intake of nitrate is an important issue as it could be expected that nitrite production in the rumen will depend on the concentration of nitrate in the rumen at any one time (see Figure 8). In feeding trials with dairy heifers, the lethal dose of nitrate in feed was found to be 3 times that injected directly into the rumen or about 1g/kg live weight (Crawford *et al* 1966).

Figure 6. Relationship between the peak concentration of nitrite in rumen fluid and methaemoglobin in the blood of cows fed various rations with added K-nitrate including hay, turnip, freshly mown grass or concentrate (Kemp et al 1977).



Kemp *et al* (1977) have criticized these results, pointing out that the cattle took some considerable time to consume the feed, and produced data showing the toxic dose of nitrate was much lower than the values suggested by Crawford *et al* (1966). As already discussed in their studies the nitrate was only available in the rumen for a short time each day and the nitrate or nitrite may not have been present for sufficient time to induce changes in the numbers or activities of rumen bacteria that utilize nitrate and nitrite as electron acceptors.

Figure 7. Changes in methaemoglobin in blood of cattle with increasing amounts of nitrate entering the rumen (after Crawford et al 1966).



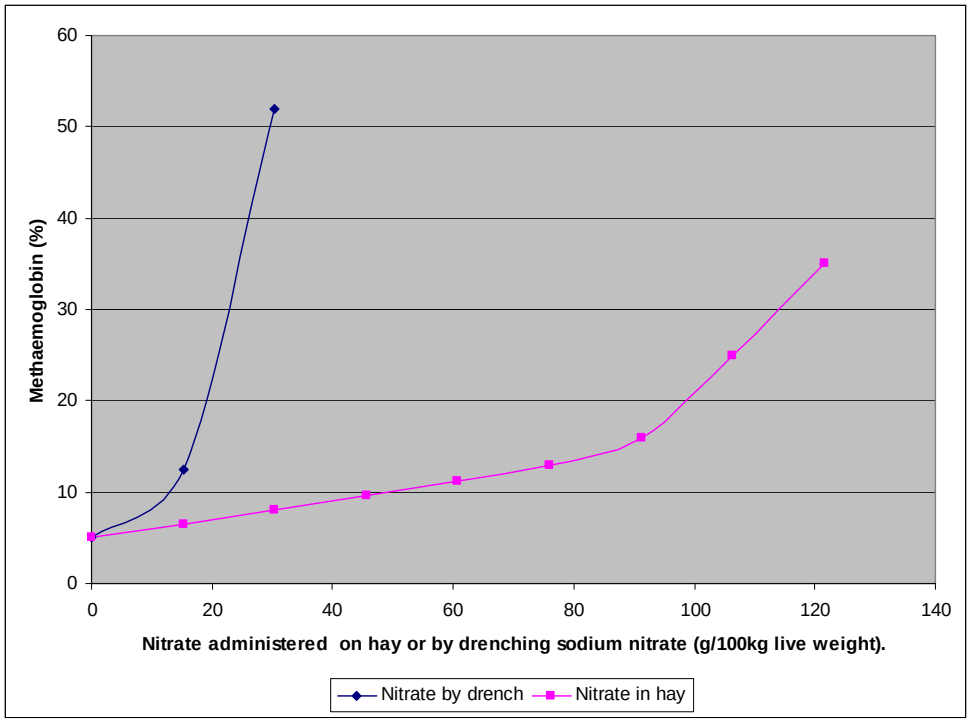
Geurink *et al* (1989) pointed out that the rate of diffusion of nitrate from the feed in the rumen was an important factor affecting how much methaemoglobin accumulated in blood. The rate of diffusion of nitrate from a fodder together with the rate of consumption of the forage interacts to affect the level of nitrite appearing in rumen fluid and therefore the amount of nitrite absorbed and hence the percentage methaemoglobin produced.

3.3 Evidence for nitrate recycling to the rumen after nitrate loading of the rumen

There have been few isotope dilution studies that have examined the dynamics of nitrate-N entering the rumen in the same way that urea and ammonia N have been studied (see Nolan and Leng 1972).

From several studies reported in Figures 2, 3, 4 and 5 above, a nitrate load added to the rumen of sheep and cattle is cleared within 2- 8 h, the rate apparently depending on the amount given, the level of adaptation of the animal to nitrate and perhaps also species. Wang *et al* (1961) used ^{15} N-labeled nitrate to provide information on nitrate metabolism (see Figure 9). The cows were given 100g KNO_3 daily via a rumen fistula 1 h before feed was given. This was done for several days and then on one day, 120 g KNO_3 labeled with ^{15}N was given before feeding. The surprising result was that the ^{15} N enrichment (atoms percent excess) in isolated nitrate-N plus nitrite-N from the rumen following administration of the salt declined quickly, indicating that unlabelled nitrate or nitrite was entering the nitrate pool in the rumen from sources other than the nitrate added that day in the labeled dose, or that nitrate/nitrite was in equilibrium with some other N source in the rumen.

Figure 8. Comparison of methaemoglobin produced in dairy heifers fed hay containing nitrate or drenched with an aqueous solution of nitrate (Crawford *et al* 1966).



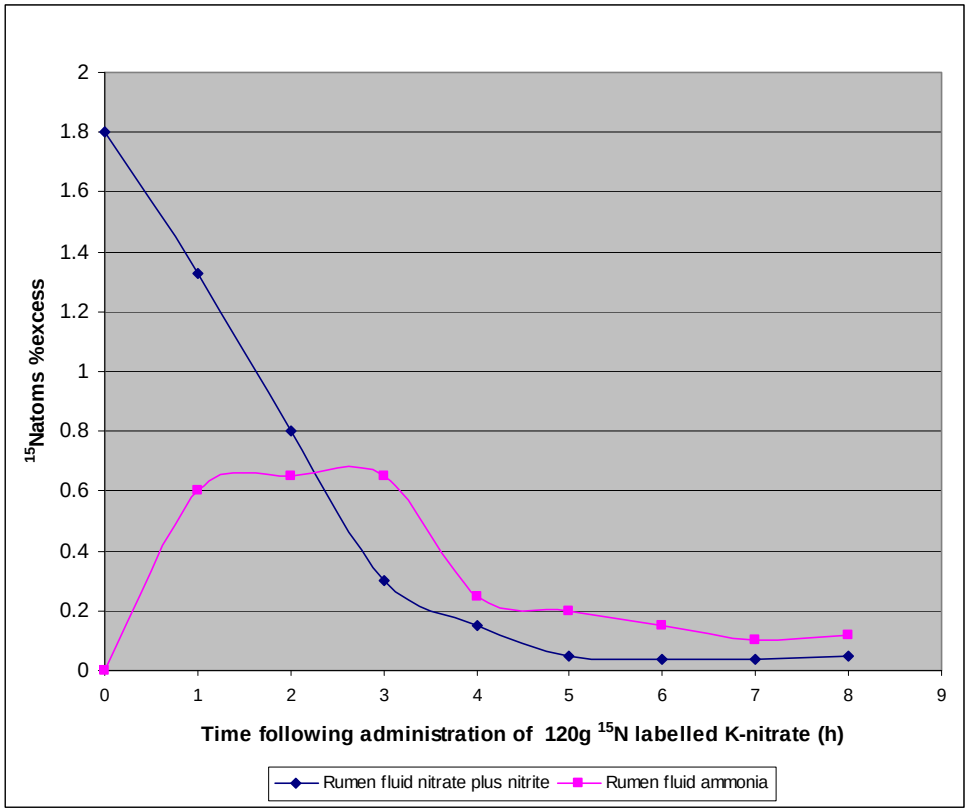
Before discussing the implications of this research it must be emphasized that the study reported has weaknesses in the experimental approach that impose constraints on the conclusions that can be drawn from this paper. A list of criticism would include:

1. The study reported only one animal that was given ^{15}N tracer in the nitrate load.

2. The nitrate load was given to a cow in the morning prior to the feed (hay, concentrate) and no information was provided on composition, on how much of either ingredient was consumed and over what time interval relative to the dose that was administered.
3. The animal was by no means in a steady state; normally a requirement of tracer studies, nor was it truly a tracer study and is more appropriately categorized as a clearance study.
4. There was no reported analysis for nitrate in the hay or concentrate or when the hay and concentrate were consumed relative to the dose being given. If nitrate was present in hay then the dilution curves produced may merely reflect the absorption from this source.

Despite the criticisms this appears to be the sole tracer study of ^{15}N-nitrate metabolism in the rumen *in vivo* and so an attempt is made to tease out any insights it may provide. It also emphasizes the lack of critical knowledge of the dynamic aspects of nitrate metabolism and the urgency with which this is needed to facilitate the use of nitrate in feeds.

Figure 9. Changes in 15N in ammonia and nitrate plus nitrite in rumen fluid of a cow injected intra-ruminally with 120g K-nitrate labelled with 15N (Wang et al 1961).



The dilution of ^{15}N-labelled nitrate shown in Figure 9 can be explained if quantities of unlabelled nitrate were being recycled to the rumen from the amounts absorbed on the previous day(s). The nitrate was administered through the rumen fistula in 500ml of water and was almost completely removed from the rumen within 6h, some of the nitrate administered 24 h previously may have been retained (stored) and recycled to the rumen. Nitrate/nitrite in rumen fluid was not detectable beyond about 8 h post administration and therefore it would be necessary for the 'recycled nitrate' to enter the rumen at rates equal to its uptake by microorganisms. In this respect the studies of Lewicki *et al* (1998) suggest that ^{15}N labeled nitrate, injected intravenously into sheep was only slowly cleared from blood with little being excreted in the urine even after 50h (40% of the injected dose). The destination of the remaining nitrate-N (60%) was not examined. The amount of nitrate injected was small relative to the amounts injected in rumen clearance

studies (see Figures 2 and 3). However, the results support the suggestion that nitrate absorbed from the rumen is recycled to the digestive tract since ^{15}N appeared in urinary urea-N within 15 min and continued to increase up to 6h post injection of the ^{15}N-nitrate. This could be produced in the rumen or lower digestive tract colonized by bacteria from absorbed ammonia, or ammonia generated from deamination of amino acids from digested NRB that metabolized nitrate by the assimilatory pathway. The most likely explanation is that circulating nitrate is absorbed into saliva and secreted into the rumen, where large populations of microbes are present. The small amount of ^{15}N-nitrate injected would be diluted more than 100 times if it mixed in the fermentable N pool in the rumen and was incorporated into microbial protein. However, the apparent continuing circulation of nitrate in blood for up to 50 h post injection could indicate that some nitrate is stored within the body of sheep supporting the conclusions drawn from the studies of Wang et al (1961).

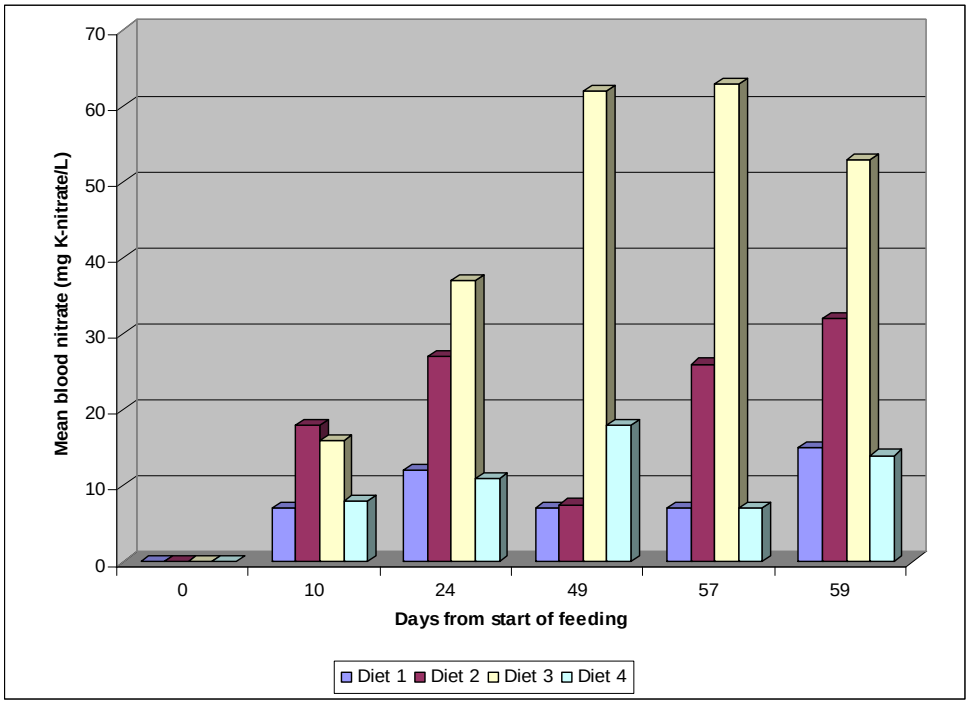
In a study of nitrate utilization by cattle consuming Sudan hay (containing 3.2% w/w KNO_3) and concentrates, Clarke *et al* (1970) showed that blood nitrate concentrations increased from the time the nitrate containing feed was introduced (Figure 10). The rate of accumulation of nitrate was higher with the inclusion of soybean meal (diets 2 and 3) than with urea (diets 1 and 4) (Figure 10). Simon *et al* (1959) reported blood nitrate levels in cattle fed hay supplemented with 140g/day K NO_3, of 55.6 and 187 mg/L in two cows. Takahashi and Young (1991) reported peak plasma nitrate levels of 11mgN/l in sheep administered intra-ruminally with 30g Na NO_3.

The point has been made earlier that there could have been a high level of nitrate in the diet used by Wang et al (1961) that was not recognized. This would have been highly unlucky as normally hay contains little nitrate (see Table 1). If nitrate was absent from the hay used in this study then a number of hypotheses can be developed for testing, that may explain the apparent dilution of ^{15}N-nitrate in the rumen of this cow, including the following:

1. Nitrate is absorbed readily from the rumen and enters pools in the body that release nitrate into the circulating blood to be returned to the rumen over a prolonged period (this appears to be the most plausible from Wang et al (1961) and is given some support by Lewicki et al 1998) and also the publications where nitrate accumulation in blood and excretion in urine have been reported (see Figure 10 and also the earlier section on nitrate toxicity).
2. That nitrate is temporally sequestered or stored (as against nitrite being bound to protein) in rumen organisms and this pool of nitrate is released into rumen fluid as the concentration of nitrate declines following the once a day administration. This and the concept of protein binding are supported to some extent by the apparent rapid disappearance of nitrate from the rumen of sheep and cattle after a load has been administered. This is discussed further at the end of this manuscript.
3. That nitrate or nitrite is reversibly bound to protein in the rumen when high levels of nitrate are administered and that nitrite - or more likely both nitrite and nitrate - could be released slowly as the dose is cleared from the rumen. Nitrite in particular is a highly reactive molecule and binds to a number of proteins. Incorporation of the stable isotope ^{15}N from nitrite has been shown to occur in both bovine serum albumin and the muscle protein myosin (see Woolford *et al* 1975). If nitrite binding to protein (either of dietary or microbiological origin) occurred in the rumen, then the level of nitrite in the rumen fluid would not be an indicator of nitrite production. The flux of nitrogen through the nitrate to nitrite reactions maybe many fold greater than previously believed and could account for the rapid decrease in nitrate concentrations and the dilution curves obtained following ^{15}N labelled nitrate injection by Wang *et al* (1961).
4. Nitrate injected into the fluid pool in the rumen is transferred to another nitrogen pool in the rumen. A remote possibility is associated with a recently discovered anaerobic ammonium oxidizing bacteria that exists at the oxic –anoxic interfaces between water bodies and sediments (see Kuenen and Jetten 2001) which might well be acting at the interfaces of feed and rumen contents especially when the rumen contents are regurgitated, re-chewed and re swallowed (rumination). The quantity of feed that enters the regurgitation cycle can be large. Ulyatt (1982) showed in sheep fed lucerne hay that the dry matter in feed passed on average about twice through the rumination

cycle. Each bolas is probably exposed to aerobic and anaerobic interfaces. A possibility is that following ingestion, feed nitrate is partially converted to ammonia in the rumen contents and mixes with ammonia from other sources. Ammonia then moves with rumen contents in rumination to the mouth where anaerobic conditions must be compromised, particularly at the interfaces in the cud or bolus where ammonia could be partially oxidized to nitrite by aerobic nitrifiers. At the same time nitrite and nitrate could be secreted in saliva. The nitrite could then re-enter the anaerobic rumen in the bolus and could be partially responsible for the build up of nitrite that is responsible for toxicities. A full ammonia oxidation in the mouth is unlikely because of the slow final rate of the anammox reaction (see Kuenen and Jetton 2001). The shapes of the dilution curve (atoms % excess) for ammonia and nitrate/nitrite are suggestive of a possible pooling of nitrate/nitrite in bacteria or some other pool prior to reduction to ammonia (Figure 9).

Figure 10. Mean blood nitrate concentration with time after introduction of cattle to diets containing nitrate (Clarke et al 1970). Diets 1 and 4 contained high urea. Diets 3and 4 were contained soybean meal as a major N component.



Even when nitrate intake is low, nitrite could be absorbed and then bind with the haemoglobin of red blood cells. These cells can oxidize the nitrite to nitrate which is then released to plasma as shown in the following reaction:

$$Hb(O_2)_4 + 4NO^{2-} + 4H^+ \rightarrow MetHb + 4NO_3^- + 2H_2O + O \quad \text{(Equation 6)}$$

If the ruminant concentrates plasma nitrate in saliva in the same way as humans do (see Duncan *et al* 1995) then the nitrate will be recycled to the rumen via this mechanism. Recycling of nitrogen is important in ruminants, enabling them to survive on diets with much less protein than monogastric animals, e.g. pigs, but despite many studies over the last half century, the ways that nitrogen is returned to the rumen are not fully understood. Nitrate-nitrite recycling from the digestive tract to the blood in ruminants is a worthy area for research and the size of the haemoglobin pool suggests that nitrite nitrogen that is absorbed, bound by haemoglobin, oxidized to nitrate and recycled to the rumen by these mechanisms could be potentially an advantage allowing a more continuous supply of fermentable-N, provided transportation of sufficient oxygen to the tissues was not compromised.

Nitrate that enters blood appears to be distributed in extracellular fluid (see Lewicki et al 1998). Therefore the amount of nitrate stored in the body could be considerable (100mg nitrate/L in an extracellular fluid volume that is 25% of body weight would represent 10g nitrate stored in a 400kg cow). Nitrate present at these levels in extra cellular fluid would be returned at sites along the GI tract and also excreted in urine but only 30-40% of an intravenous dose of ^{15}N labeled nitrate was accounted for by excretion in urine indicating substantial secretion of nitrate into the GI tract and capture by bacteria or some form of storage in the body. Although clearance rates of nitrate from blood plasma was slow, when a small dose rates was intravenously administered, high intakes of nitrate in dairy cows and sheep fed high energy and high crude protein diets resulted in substantial nitrate excretion in urine (see for example Sebaugh *et al* 1970; Setchell and Williams 1962).

3.4 Summary of whole animal studies

Slow adjustment of ruminants to nitrate (acclimation) in their feed has a major effect on nitrate disappearance and nitrite production in rumen fluid. The typical result for peak nitrate and nitrite in rumen fluid after a load of nitrate is given either as a single amount through the rumen fistula to sheep unaccustomed to nitrate in their diet is illustrated by the results of Takahashi *et al* (1998). The outcomes were very different when Aloubadi and Jones (1985) slowly adapted their sheep to nitrate and fed a similar amount of nitrate in two daily meals (Table 4). Acclimated sheep fed nitrate appear to have had much reduced peak rumen fluid levels of nitrate and nitrite compared to un-acclimated sheep. If the nitrate added to the rumen of these sheep as a dose or contained in the morning feed had mixed instantaneously in rumen fluid, the nitrate level should have been between 60 and 70 m-mol/L in both cases(Takahashi and Young 1991 compared to Alaboudi and Jones 1985). The clearance of nitrate from the rumen was rapid following injection into the rumen (over 50%) in the first hour. Where a similar amount of nitrate was fed in a meal (half the daily intake) in sheep accustomed to a nitrate containing diet, concentrations of nitrate in rumen fluid peaked 30 min after feeding at a mere 0.95 m-mol and declined to zero within 2 h. The injected amount of nitrate (in studies of Takahashi *et al* 1998) was similar to the amount fed in the meal which was half the daily ration (Alaboudi and Jones 1985). The apparently extremely rapid rate of nitrate removal in acclimated sheep was also apparently associated with minimal nitrite accumulation (0.2m-mol/L in fed acclimated sheep and 4 m-mol/L in un-acclimated sheep dosed with nitrate).

One result from Takahashi and colleagues is more similar to the results of Alaboudi and Jones (1985) where, in the later studies, the animal was administered a dose of nitrate approximately double the dose given in the other research shown in Table 4. Taken together, the results of both groups of workers indicate that the degree of acclimation and amount of nitrate administered interact to affect nitrite accumulation in the rumen of sheep. The acclimation of animals and their rumen microbes to nitrate in feed is further discussed below.

The results from Sar *et al* (2004b) are so different to previous results reported from that group that it could be that the units on the graphs reported in the paper may be in error. If the values for rumen nitrate and nitrite concentrations (μg/ml) were actually nitrate-N and nitrite-N, then the peak nitrate would be 9.3 mmol/L and peak nitrite 2.0 mmol/L and the results would then fit with their other studies in sheep not acclimated to nitrate.

Combining nitrate with a dose of cysteine decreased nitrate clearance from the rumen and inhibited nitrite build-up in rumen contents (Table 4-Takahashi *et al* 1998).

Table 4. A comparison of the outcomes of dosing sheep with nitrate without prior acclimation and in acclimated sheep.

Dose of nitrate (g/d)	Peak nitrate in rumen fluid (mM)	Peak nitrite in rumen fluid (mM)	Ruminal nitrate (mM) if mixing of salt was instantaneous	Author	Comments
25 ($NaNO_3$)	28	0.47	49	Lewis (1951)	Nitrate was introduced into the rumen directly 16h after being fed
24.4 ($NaNO_3$)	2.1	0.61	52	Sar *et al* (2004b)	Sheep were fed twice daily and nitrate introduced 30 min after the morning feed
30 ($NaNO_3$)	10	2.4	59	Takahashi and Young (1991)	Sheep were fed chopped grass hay morning and evening
30 ($NaNO_3$) + cysteine	36	0.1	59	Takahashi and Young (1991)	As above but sheep received a dose of cysteine along with the nitrate
32 ($NaNO_3$)	20	4	68	Takahashi *et al* (1998)	Nitrate was injected directly into the rumen of un-acclimated sheep 30 min after ration of hay/alfalfa was given
32 ($NaNO_3$) +cysteine	25	0.1	68	Takahashi *et al* (1998)	As above but sheep received a dose of cysteine with the nitrate
41 (KNO_3)	0.95	0.2	68	Alaboudi and Jones (1985)	Nitrate was fed as part of the diet. Feed was given twice daily at 1000h and 1600h. Total daily dose was 82g K-nitrate fed in hay/grain based diet

3.5 Conclusions from whole animal studies

When nitrate is introduced into the rumen, there is a rapid disappearance of nitrate from the rumen fluid that is related to the amount given and the time taken for the delivery. In the published research, these effects have been masked by the mixing of nitrate in the rumen fluid which should be relatively quick and the delay in sampling the rumen after a load of nitrate was administered. The indications in the studies reported in Table 4 are that nitrate disappears rapidly during this period in both cattle and sheep. The possibilities are that:

- Nitrate is rapidly taken up by bacteria and metabolized to ammonia via an induced assimilatory nitrate reductase in rumen microorganisms with rapid growth or that nitrate is stored, for example as amino N to support growth but over a more prolonged period of time
- Nitrate-N is temporally sequestered in microorganisms in the rumen
- Nitrite and possibly nitrate are bound by proteins in the rumen
- Large amounts of nitrate and nitrite are absorbed directly across the rumen wall
- Outflow of digesta to the omasum and abomasum is increased.

Of these possibilities, a combination of 1) rapid growth of NRB or rapid uptake by bacteria with intra cellular accumulation and assimilatory reduction to ammonia and 2) absorption into blood, appear to be the most likely reason for the high initial loss of nitrate from rumen fluid. It is difficult, however, to accept the rapid or explosive (exponential) growth of NRB because of the need for a concomitant rapid fermentation of organic substrates that would be necessary to meet the demand for organic electron donors to remove nitrate at the rates observed. This particularly applies to the research of Lewis (1951) where 16 h fasted sheep were used

The rates of accumulation of ammonia following nitrate administration into the rumen appear to be too slow for the dissimilatory pathway to be dominant. A number of reports indicate blood nitrate levels increase after a high dose of nitrate is placed in the rumen, but no reports appear to consider that nitrite (or nitrate formed by oxidation of nitrate) could recycle nitrate to the rumen either via the rumen epithelium or, as in humans, via the saliva. Nitrate absorption does occur but is unlikely to be the main mechanism of removal of nitrate. However, the rate of absorption is probably concentration-dependent and therefore absorption may be involved in the initial rapid disappearance of nitrate after a nitrate load is suddenly placed in the rumen.

Concentration or storage of nitrate in rumen microbes appear to be unlikely, however, it has been reported to occur in organisms in other ecosystems, for example growth inhibited cells of *Pseudomonas florescence* concentrated nitrate 6 fold (Betlach *et al* 1981). Recently large filamentous bacteria associated with high-sulphur containing marine and freshwater sediments have been found to sequester nitrate in vacuoles at up to 800mol/L (Sayama 2001). This suggests that this concept should at least be explored in the rumen ecosystem. This is discussed at the end of this manuscript.

It is improbable that a large proportion of the nitrate is absorbed and lost via the urine as nitrate appears to be efficiently used when fed as a sole source of nitrate in purified diets (Tillman *et al* 1965).

The concept of a large proportion of nitrate being flushed to the omasum is equally improbable as this would require a flush of liquid out of the rumen which would also effect the concentrations of other chemicals including ammonia and VFA which is not evident.

The net rate of removal of nitrate from the rumen is a composite of its microbial uptake and metabolism to ammonia, its removal from the gut, and the recycling of absorbed nitrate-N and nitrite-N to the rumen. The rate of reduction of nitrate to nitrite and ammonia in many anaerobic systems is effected extensively by sulphur availability and there are complex interactions of SRB and NRB as discussed in the background section and which is also further discussed below.

It is apparent that the utilization of nitrate in the rumen is dependent on how the nitrate is administered and the role of nitrate will be quite different where it is fed in a diet rather than administered in large dose rates given at a set time. There is a need to establish labeled N studies to examine and compare the dynamics of nitrate-N added as a load to the rumen in cattle and sheep without acclimation to dietary nitrate and also in well-acclimated animals and in sheep fed nitrate as a component of the diet.

3.6 Acclimation of rumen organisms to nitrate in their feed

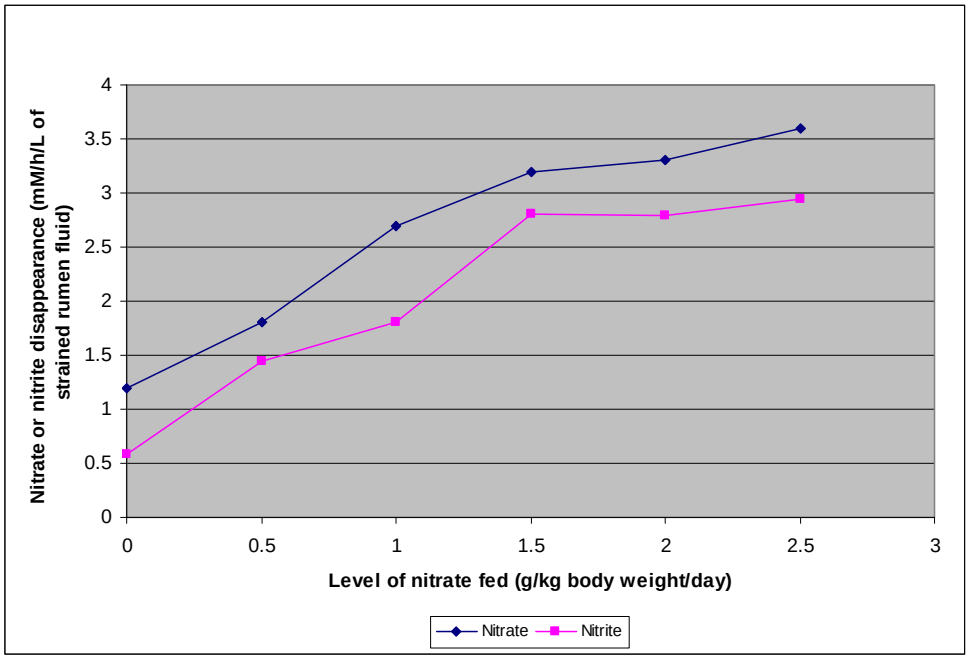
A large number of microbial species found in the rumen have the capacity to reduce nitrate or nitrite to ammonia (see Cheng and Phillips 1988; Cheng *et al* 1985). These organisms are quickly transferred between animals and their population densities respond to the presence of nitrate in a diet (Cheng *et al* 1985; Alaboudi and Jones 1985). As sheep become accustomed to increasing intakes of dietary nitrate, the rates of both nitrate and nitrite reduction in rumen fluid increase by 3-10 fold (Alaboudi and Jones 1985; Allison and Reddy 1984) (see also Figure 11). The increase is associated with marked increase in populations of NRB and selection of different predominant bacterial groups in the nitrate-adapted animals (Allison and Reddy 1984). Other studies of sheep by Allison and Reddy (1984) showed that introducing nitrate for the first time into the rumen, increased the rate of nitrate reduction in strained rumen fluid, in just 4 h, by 15 fold from the low level of activity prior to nitrate introduction. This increase from very low levels to

substantial is further increased on a daily basis with time from nitrate's first inclusion in the diet (Allison and Reddy 1984: Aloubadi and Jones 1985). Thus the accumulation of both nitrate and nitrite in the rumen depends on how long the animal has been accustomed to nitrate in the feed.

In 40kg sheep given a diet of lucerne/ground corn, nitrate and nitrite levels in rumen fluid were undetectable but on day 1 of inclusion of nitrate (0.17g of NO_3^- /kg body weight) at 1 h post feeding, nitrate and nitrite concentration in rumen fluid were 1.7 and 0.26 m-mol/L. respectively. Within 6 days the rumen microbes had adapted and nitrate and nitrite were so rapidly metabolized that their concentrations in rumen fluid were undetectable 1h post feeding (Allison and Reddy 1984).

In a study with sheep Alaboudi and Jones (1985) increased dietary KNO_3 concentration every 2 weeks (from 0 to 2.5g/kg body weight in 0.5g/kg body weight steps). The rate of removal of added nitrate or nitrite in strained rumen fluid taken from those sheep at the end of each adjustment period was measured *in vitro*. Three flasks of strained rumen fluid were incubated with both substrate and residual nitrate, or nitrite was measured after 1, 2 and 3h and the rate of disappearance calculated from the slope of plots relating residual nitrate or nitrite to incubation time (see Figure 11). Similar results were obtained by Allison and Reddy (1984) who tested rumen fluid from sheep receiving higher levels of dietary nitrate concentration but in a less stringent experimental approach.

Figure 11. Effect of level of K-nitrate fed to sheep on the nitrate and nitrite disappearance from strained rumen fluid incubated *in vitro* (Alaboudi and Jones 1985).



Extrapolation of the highest rates of nitrate reducing activity recorded in strained rumen fluid to the rumen *in vivo* indicates that when 150g of KNO_3 was included in the diet of sheep, reduction of the nitrate to ammonia in the rumen could have accounted for 53g or 35% of dietary intake. Strained rumen fluid would have a much lower microbial count than rumen fluid *in vivo*, as the majority (70%) of rumen microbes are attached to feed particles (see Preston and Leng 1985) that would have been largely removed in processing the rumen fluid. It appears reasonable to suggest that, when nitrate is ingested by acclimated sheep, there is sufficient nitrate/nitrite reducing activity to utilize quite high concentrations of nitrate in the feed and any nitrite produced in the rumen. These sheep were ingesting 1200 g of a hay/grain based feed and 150g of KNO_3 could supply approximately 21g of N which is more than adequate to meet the fermentable N requirements of the microorganisms digesting this feed in the rumen. The diet also contained considerable crude protein possibly between 12-15% of the dry matter.

As already discussed, some of the nitrate administered by drenching appears to be rapidly absorbed (Winter 1961) and may be partially excreted in urine (Setchell and Williams 1962) or returned to the GI tract via saliva or other secretions (see Wang *et al* 1961). However, the low peak nitrate concentrations after consuming nitrate in the feed (Alaboudi and Jones 1985) suggests that absorption would be much reduced in acclimated animals. Cheng *et al* (1985) confirmed that the ability of rumen fluid to reduce nitrite and nitrate was enhanced by adapting cattle to increasing levels of nitrate given by drench on a daily basis but also showed that adaptation was a temporary phenomenon. In theses studies, KNO_3 was administered as a drench to cows (800kg live weight) fed lucerne hay and the amount administered was increased every three days (from 0 to 0.16 to 0.32 to 0.48 to 0.54 g KNO_3/kg body weight). The ability of the cows' rumen fluid to reduce nitrite was about 10% of that in the sheep and increased only slowly as the KNO_3 in the diet was step-wise increased. Three day intervals of adjustment of nitrate levels in a diet appear to be too short a period for substantial acclimation, (see Allison and Reddy 1984). At a drench rate of 0.54 g KNO_3/kg live-weight (about 400g/cow), all animals died on the first day following drenching. The difference between the two studies may reside in the diets. In the studies with cattle, lucerne hay was fed, whereas in the studies with sheep the diets where mixed cereal grain and brome-lucerne hay with much lower true protein content and in addition much of the protein would have been highly soluble and therefore rumen degradable on the lucerne diet as compared to the grain/hay diet. The potential effects of a high soluble protein intake on nitrite accumulation in ruminants are discussed in the section dealing with the possibility NR-SOB being actively involved in nitrite accumulation. However, the conclusion must be that drenching once a day did not induce acclimation and/or the 3-day interval was too short to promote acclimation of rumen microbes in cattle given the lucerne hay diet.

The most confusing aspect of past research examining *in vivo* metabolism of nitrate has been the failure (intentional or otherwise) of researchers to prior acclimate their experimental animals to nitrate. This is appropriate when researching the toxic aspects of sudden ingestion of nitrate, particularly as nitrate toxicity occurs in grazing ruminants when nitrate levels in feed suddenly increase. From a nutritionists' viewpoint, good husbandry demands that any diet is introduced to ruminants relatively slowly to enable the rumen microbes and the animal to adapt to the changed feeding conditions.

3.7 Sustained/slow release nitrate

It seems appropriate here to draw attention to the need for slow adjustment to any nutritional changes with any animal. Often the adaptation is better where the dietary additions are slowly available once ingested by the animal. Nitrates for feeding to animals can be prepared by a number of processes to have sustained or slow release rates, as has been developed to extend and enhance fertilizer use in soils. Such products may be better than nitrate solutions or nitrate salts to acclimate the rumen of animals in extensive grazing areas where a supplement intake may only be accessed once a day or even less frequently. Bentonite may also be able to play a role here as it binds numerous compounds that carry a charge. Nitrate has a negative charge that might promote its adsorption onto the surface of clays and zeolites thus delaying its rate of release after entering the rumen.

3.8 Conclusions on the benefits of acclimation

Nitrate added to the diet of sheep rapidly promotes multiplication of microbes that utilize nitrate as a nitrogen source, or induces or increases the activity of nitrate reductase systems in microbes that are already present in the rumen. This increase (up to15 fold) occurs rapidly over the first few hours (Allison and Reddy (1984)) and the capacity to handle nitrate increases with time of exposure and amount of nitrate fed (a further 3-10 fold). The capacity to use nitrate returned to pretreatment levels within 3 weeks of removal of nitrate from the diet of sheep (Aloubadi and Jones 1985).

3.9 Rumen ammonia and nitrate/nitrite reduction to ammonia

In many studies of nitrate utilization *in vivo*, nitrate has been added without consideration of the type of diet or its crude protein content. Clark *et al* (1970) reported that hay with high nitrate content was successfully fed to dairy cows without problems until a urea supplement was introduced into the diet. At this time, numerous deaths occurred apparently from nitrate poisoning. Hoar *et al* (1968) undertook feeding trials with growing lambs that showed that when nitrate was added to balanced concentrate diets there was no effect on lamb growth. However, the quantities of nitrate were very small relative to the other fermentable-N sources. When, Clarke *et al* (1970), added soybean meal in place of urea in a diet, the amount of methaemoglobin formation in cattle fed the same amount of nitrate appeared to increase. However, the results could have been compromised by the short length of the acclimation period. Sokolowski *et al* (1969) and Cline *et al* (1963) showed that sheep were unaffected by levels of nitrate in their diet that had previously been considered lethal. In addition, the sheep achieved good growth rates (170-240g/head per day) when the diet was well balanced. On a high concentrate diet adding 2% nitrate in a dairy cow ration had no effects on milk production or composition (see Jones *et al* 1966;Farra and Satter 1971).

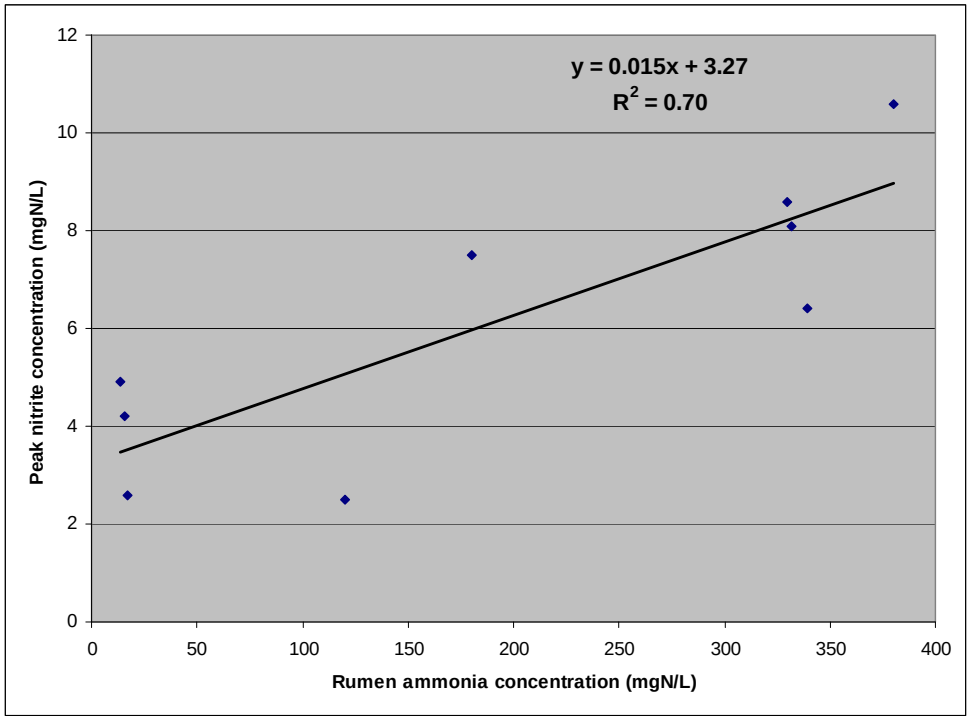
Current knowledge generally does not support the concept that nitrate and nitrite reduction are inhibited by ammonium ions in the rumen. However, research has been limited. In the majority of the studies, nitrate ingested or administered intra-ruminally to sheep and cattle was additional to the other crude protein components in their feed and, in nitrate poisoning situations, both rumen fluid nitrite and ammonia levels have usually been elevated. Moreover, when nitrate is added to the system, ammonia levels have often increased, even though at substantially slower rates then might be expected if the nitrate was rapidly and quantitatively converted to ammonia (see Figure 2 and also Figure 21). Because of the relatively high rate of rumen degradability of grass-protein grazing ruminants on high protein forage would already have substantially elevated rumen ammonia levels and these levels may be increased further and promote ammonia toxicity if high nitrate feed is suddenly introduced into the rumen. These same forages also promote high levels of ruminal fluid hydrogen sulphide which has been demonstrated by measurements of rumen gas space hydrogen sulphide levels (Dewhurst *et al* 2007). As will be discussed later the level of hydrogen sulphide in rumen fluid may have significant implications on the pathways of nitrate utilization by NRB.

From the reports where sheep and cattle have been given an intra-ruminal load of nitrate and measurements made of rumen ammonia, nitrite and nitrate concentrations, there are indications that ammonia levels are critical to the extent that the nitrite levels increase after consuming nitrate (see Figure 12, adapted from the data of Lewis 1951; Burrows *et al* 1987; Sar*et al* 2004a; Wang *et al* 1961). The very low rumen fluid ammonia levels that were associated with a small nitrite peak in the rumen were measured in cows. The researchers suggested that concentrate feeding could reduce ruminal nitrite production from added nitrate. The main effect of adding the concentrate, however, appears to be through promotion of more rapid microbial growth that uses ammonia and reduces rumen ammonia levels to minimal values (Burrows *et al* 1987).

The effects of rumen ammonia concentration on nitrite production in the rumen may be critical in feeding nitrate under field conditions and so is an area for immediate research. Combining data, together results from small numbers of diverse studies as has been done in Figure 12 is subject to potential errors and is only justified because there is almost no information in the ruminant literature. However, there are numerous enzyme studies that indicate that accumulation of ammonia may be a controlling factor of the rates of nitrate and nitrite reduction to ammonia by assimilatory but not dissimilatory pathways in some bacteria that inhabit the fermentative areas in the gut of ruminants (and other herbivores), the lower gut of monogastric animals and also in anoxic water and soil habitats.

Interaction of SRB and NRB in these systems has been implicated in preventing nitrite build up following a nitrate load. There is a possibility that high protein diets induce a high population of a NR-SOB that may be responsible for nitrite accumulation. This is discussed further below.

Figure 12. Relationship between peak nitrite level after an intra-ruminal load of nitrate in sheep and cattle and rumen ammonia concentration at peak nitrite.



3.10 Microbiological/enzyme studies

Bacteria capable of nitrate reduction usually also catalyze nitrite reduction to ammonia in dissimilatory metabolism. Eight electrons are required to form ammonium in both assimilatory and dissimilatory pathways. In the former nitrate reduction is regulated so that ammonia is formed only at a rate required for the synthesis of organic compounds and little accumulates in the culture medium (Hewitt, 1975; Payne 1973). Enzyme synthesis is induced by nitrate or nitrite but repressed by ammonia. These regulatory mechanisms ensure that reduced cofactors are preferentially conserved for ATP generation. The dissimilatory nitrate reductase system in obligate anaerobes, rapidly reduces nitrate and nitrite to ammonia even when ammonia is present in significant concentrations in the medium (Hewitt, 1975; Cole and Brown 1980). On the basis of the rapid disappearance of nitrate from the rumen in studies where un-acclimated sheep were administered a large intra ruminal dose of nitrate and rumen fluid ammonia levels were high (see for example Lewis 1951) the dissimilatory pathway system has been suggested as the likely mechanism in the rumen (Cole and Brown 1980) but it could be equally suggested that because of the high rumen ammonia and high initial rumen concentrations of nitrate that this is the pathway stimulated when these fermentable nitrogen sources (ammonia and nitrate) are excessively high in the culture medium. Organisms that use intracellular ammonia produced from nitrate must be advantaged when rumen fluid ammonia is low and nitrate is supplied in stoichiometric amounts.

The effects of nitrate and ammonia on dissimilatory nitrate reductase have been researched for various organisms in pure culture. *Veillonella alcalescens* isolated from the rumen produces nitrite from nitrate but nitrate-N is also synthesized into cellular proteins as shown by isotope studies using ^{15}N-nitrate (Inderlied and Delwiche 1973). This indicates that it produces both nitrite and ammonia suggesting that the electron transfer systems in this strain have both assimilatory and dissimilatory characteristics.

Although nitrate reduction in V. *alcalescens* C-1 is dissimilatory in most anaerobic environments (Kemp and Atkinson, 1966; Ruoff and Delwiche 1977), evidence has been presented by Inderlied and Delwiche (1973) which suggests that nitrite reduction in this bacterium

functions in a true assimilatory capacity. Whilst in *E. coli,* which is also a dissimilatory nitrate reducer (Kemp and Atkinson 1966), the reduction of nitrite to ammonia appears to be primarily a mechanism for detoxification or a means of dissipating electrons allowing metabolism of the primary substrate to be continuous. In these microorganisms (*E. coli*), nitrite reductase is induced by nitrite, but ammonia exhibits no repressive effects on enzyme activity or synthesis. In *V. alcalescens* C-1, nitrite reductase is a soluble enzyme which is induced by nitrate or nitrite and repressed by ammonia and amino acids (Yordy and Dewiche 1979). Assimilatory nitrate reduction in organisms has been reported in *S. ruminantium* subs *lactilytica* (Iwamoto *et al* 1999), and *W. succinogenes* (Bokranz *et al* 1983). Allison and Reddy (1984) reported that nitrate reductase activity in rumen organisms was membrane bound and is inhibited by azide and hydroxyquinoline-N-oxides, indicating it is a respiratory-type nitrate reductase in which ATP is generated with the production of ammonia . This is also suggestive of the presence of NR-SOB (see later).

In many enteric bacteria, anaerobic reduction of nitrate can use either NADH or formate as electron donors to reduce nitrite rapidly to ammonia (Cole 1988). The two pathways are biochemically and genetically independent. The NADH-dependent enzyme is a soluble, cytoplastic protein and does not generate ATP; the membrane bound enzyme is associated with ATP generated by transfer of electrons from the cytoplasm (see Page *et al* 1990). It is interesting that mutants of *E coli* that are deficient in NADH dependent enzymes convert nitrate to ammonia at 5-20% of the normal rate, indicating that some organisms may use both enzyme systems to reduce nitrate and, as the nitrate metabolism would be energetically more favorable to growth in nitrate limiting conditions, would use nitrate in an assimilatory way. Both enzymes are active only in bacteria grown in anaerobic conditions; both are repressed by oxygen, and induced by nitrite (see Page *et al* 1990). It appears possible that in the rumen bacteria that use nitrate NADH dependent enzyme may be the enzyme that is rapidly induced in rumen microbes in response to high nitrate loads whereas the membrane bound enzyme may be controlled by nitrate or nitrite availability and repressed by ammonia. This is a major research area for study in ruminants using nitrate as a major fermentable N source.

The potential for assimilatory pathway of ammonia production from nitrate or nitrite in the rumen is at least established by the research reported above and therefore it is potentially possible that high ammonia levels in rumen fluid could suppress nitrite to ammonia and release nitrite from nitrate to the medium. Iwamoto *et al* (1999) have previously demonstrated *in vitro* that nitrite accumulation in incubations of ruminal microorganisms when nitrate is supplied can be lowered by supplying fumarate. The latter supplying both a carbon source and energy for NRB that are rumen residents including *S. ruminantium, V. parvula and W. succinogenes* (Stewart *et al* 1997; Asanuma *et al* 1999).

As an example of this possibility studies with *Pseudomonas fluorescens*, a prevalent soil bacterium, showed that this obligate aerobe grown on ammonium sulphate or ammonium nitrate failed to take up nitrate (Betlach *et al* 1981). Cultures grown on nitrate alone took up nitrate and also ^{13}N-labeled nitrate accumulated in cell contents as ammonium and was incorporated into amino acids. These results indicate that, in this bacterium, the nitrate was taken up via active transport (not concentration-dependent diffusion) and that the nitrate assimilation is both inhibited and repressed by ammonium. High rumen ammonia levels (exceeding 10mM) normally present when high protein forages are consumed would lower the rate of nitrate conversion to ammonia but may allow some nitrite production which would accumulate in rumen fluid

Rumen organisms evolved from soil organisms (Hungate 1965) and the milieu that develops in the rumen is dependent on diet and other factors. The question arises, if ruminants were raised on diets in which the majority of the fermentable nitrogen was provided as nitrate, could such organisms adapt to the rumen environment? This seems to be a highly likely scenario because grazing animals are inadvertently ingesting soil organisms associated with the N –cycle and, considering the microbial population diversity that can be supported in animals on different diets it is probable that other organisms that use nitrate may become established in the rumen (see Hungate 1965).

3.11 Synchrony of nitrate metabolism and fermentation rate

It appears that for efficient microbial growth the rate of ammonia accumulation, and therefore availability in the rumen, should be synchronised with the rate of fermentation and the availability of ATP for microbial growth. By definition assimilatory nitrate reduction produces ammonia in the cell at rates that are compatible with its requirement for synthesis. Synchrony of nitrate reduction to ammonia in dissimilatory pathway and electron generation in fermentation is highly significant as carbohydrate energy sources in ruminant feeds are highly variable in the rates that they are converted to volatile fatty acids and therefore the rate at which reduced cofactors are required to be re-oxidised.

Following a large dose of nitrate added into the rumen, the clearance of nitrate appears to be extremely rapid and slows to a much lower clearance rate after about 1h post the administration of the nitrate load (see Figure 3). If at high nitrate concentrations in rumen fluid, the availability of electron donors was limiting, the response in bacteria might be to reduce nitrate to nitrite with accumulation of the latter in rumen fluid. The former enzymic step requires 2 electrons whilst the conversion of nitrite to ammonia requires a further 6 electrons. Jones (1970) showed that for incubations of rumen fluid without added substrate, and therefore a low fermentation rate of organic matter, the product of nitrate metabolism was largely nitrite. Inclusion of hydrogen donors such as formate increased the rate and extent of nitrate and nitrite disappearance from the incubation medium. It appears feasible that nitrite production may be controlled by the relative rates of availability of nitrate and the fermentation rate (or the availability of hydrogen donors) in dissimilatory nitrate reduction. This is supported by research of Barnett and Bowman (1957) that used an artificial rumen approach to study nitrate metabolism (see Figure 18). The rate of production of ammonia for microbial cellular synthesis must also be synchronised to microbial growth and the availability of ATP. The latter also is directly controlled by the availability of fermentable organic and its potential rate of degradation.

In any future feeding systems based on nitrate as major N sources for ruminants, there needs to be a careful consideration of the methods of incorporation of nitrate into diets. For example nitrate supplementation in grazing animals may need to use slow release nitrate preparations or ensure that animals access nitrate at intervals over a day so that synchrony of feed intake (fermentation rate) and availability of ammonia from nitrate is achieved in the rumen. In this respect the presence of a so-called 'futile cycle' (there is a cost in terms of ATP requirements) of alanine synthesis and degradation in rumen bacteria, as a mechanism for smoothing the availability of ammonia from urea or nitrate for microbial growth, requires some considerable research (see Blake *et al* 1984).

The build up of nitrite in the rumen where a nitrate load is given over a few minutes obviously creates artifacts that would perhaps not be expected in feeding trials where the intake of feed and nitrate would be synchronised. It is interesting to speculate here that assimilatory nitrate reduction may be favoured where substrate is slowly fermented. For example, where forage and structural carbohydrates are the major feed biomass and their fermentation rate is slow. Where soluble sugars are a major source of fermentable carbohydrate, the dissimilatory pathway of nitrate conversion would seem to be more compatible with the high rate of fermentation. Carbohydrates that are more readily fermented in the rumen have been associated with better use of nitrate-N by ruminal organisms (Sapiro *et al* 1949; Takahashi *et al* 1980).

3.12 Indirect effects of feeding nitrate to ruminants

Indirect effects of nitrate on the rumen ecosystem may be a critical issue if nitrate is to be used as a major N source for ruminant digestion. There is a great deal of apprehension about feeding nitrate which has largely stemmed from research that attempts to explain the toxicity syndrome in ruminants. Toxicity does represent a risk that is probably manageable in the same way as the risk of urea poisoning is avoided. Marais *et al* (1988) have pointed out that nitrite and nitrate may more subtly affect animal production through detrimental indirect effects on rumen organisms, particularly through toxic effects on cellulolytic organisms (Hall *et al* 1960) that may

lower the apparent digestibility of forage or pure cellulose *in vitro.* Repeating their earlier studies, Marais *et al* (1988) demonstrated a lowering of digestibility of powdered kikuyu grass *in vitro* when nitrate was added to the culture medium. They suggested that the reduction in digestibility could be the result of nitrite accumulation in the *in vitro* cultures inhibiting the electron transport system of certain microbes and limiting ATP generation and growth at nitrite levels as low as 0.29mM. However, even in un-acclimated ruminants given large doses of nitrate, the concentration of nitrite in the rumen fluid is low and fleetingly present (see Figures 2, 4, 5). In contrast, in an acclimated sheep given a large amount of nitrate, twice daily in the ration, nitrite levels in rumen fluid peaked at 0.21mM for about 30 min (Alaboudi and Jones 1985) making it unlikely that such a transient effect would decrease the *in vivo* digestibility of forage. Nitrate in ruminant diets appears to alter the ratio of volatile fatty acids towards more acetic acid production at the expense of butyrate production (Bryant 1965; Farra and Satter 1971, Nakamura *et al* 1981; Alaboudi and Jones 1985). This result is to be expected as the lowering of butyrate production delivers more electrons, but loss of nitrate from the rumen quickly returns the ratio back to pre-nitrate feeding levels (Farra and Satter 1971).

That there are interactions between nitrate and sulphur metabolism in anaerobic environments is clear. In situations where oil organics are fermented, nitrate application apparently blocks hydrogen sulphide production (Greene *et al* 2003) and balance studies in sheep have shown that nitrate feeding decreased sulphur retention (Sokolowski *et al* 1969).

Anaerobic fungi in the rumen require a source of reduced sulphur for growth (Gordon and Phillips 1998). These organisms are essential for adequate comminution of highly lignified plant materials in the rumen and a study of the effects of nitrate supplementation on their sulphur requirements is a priority research area.

3.13 Feeding trials where nitrate is a major source of fermentable nitrogen in a diet

a) Purified diets

Tillman *et al* (1965) studied nitrate metabolism in the rumen of 24 kg sheep in a feeding trial in which the lambs were offered purified diets in which nitrate was the sole source of fermentable nitrogen. The diet consisted of corn starch (24%), corn dextrose (24%) and purified cellulose (31.8%), KNO_3 (6.5%), $NaNO_3$ (6.5%) with the remainder as a complete minerals and vitamin mix and supplied approximately 25gN/kg of calculated digestible organic matter. Feed intake was 340g/day and the sheep grew at about 42 g/day. Nitrite concentrations in blood were apparently well below toxic levels; however, methaemoglobin levels in blood and rumen ammonia levels were not reported.

In a separate trial with mature sheep fasted for 12h but on the same diet with nitrate as the sole source of fermentable N, 500g of the ration was suspended in water and administered directly into the rumen through a fistula. The nitrates may have dissolved in the water and therefore readily available. Rumen fluid ammonia increased from 80mgN/L to 200mgN/L over the next 9 h, indicating that dissimilatory nitrate reduction occurred. This could be an artifact of the excessively high intake (43.7g nitrate or 9.86g of nitrate-N) that would be released over a short interval of time in the rumen and lack of fermentable organic matter for microbial growth (imbalance between nitrate concentrations and rate of generation of reduced coenzymes in fermentation). As in all research where nitrate has been administered as a sudden load, nitrite accumulated in the rumen. When molybdenum was omitted from the diet, nitrite accumulation was significantly lower (this is discussed in the section that provides evidence for the involvement of SRB). On the other hand in the feeding trial with growing lambs fed purified diets with N requirements met by nitrate, nitrite concentrations in blood were insignificant. The level of nitrite in blood tended to increase with time on the diet but never exceeded 0.025mM.

b) Concentrate based diets

Sokolowski *et al* (1960) appears to have been the first to suggest that nitrate in a relatively good quality diet that supported substantial growth was not apparently toxic to sheep. The same

author showed that sheep can tolerate levels of KNO_3 in their diets that had previously been considered lethal and grow normally. In subsequent publications, Sokolowski *et al* (1969) found that adding 3.2% KNO_3 to a concentrate based diet, with or without added sulphur, lowered the overall growth rates of lambs offered the diet over 48d (see Table 5). However, there appears to have been no acclimation period, so the slight drop in live weight gain where nitrate was included in the diet could have been a result of a reduced initial growth when the animals were acclimating to the nitrate. Carver and Pfander (1973) found that 21 d was needed to enable sheep on similar diets to acclimate to KNO_3. N balance appeared to be greater when sulphur was given with the nitrate, but there was a large variation in N balance in the lambs given both nitrate and extra sulphur (see standard deviations in Table 5). It appears that, perhaps for this reason and in order to attempt to confirm whether the trend was an indication of a much better apparent utilisation of absorbed amino acids when nitrate and extra sulphur were included in the diet, a second experiment was undertaken. In the second experiment, lambs (26 kg) were offered the same diet but restricted to an intake of 1200g/d. The addition of 3.2% nitrate had little effect on N retention but N balance increased significantly when sulphur was also included in the diet (Table 5 and Figure 13).

N balance was measured after the lambs had been on the diet for 22 d so they were well acclimated to nitrate in their diet. As will be discussed later, nitrate appears to have a major effect on sulphur metabolism in the rumen. In this study, when nitrate was added to the diet, the apparent sulphur balance in the animal decreased from +0.67 to -0.8 g/day (Table 5). This could have resulted from high nitrate in the diet inhibiting SRB in the rumen (see Nikolic *et al* 1984. This might have been confirmed if hydrogen sulphide measurements had been made during the S balance study.

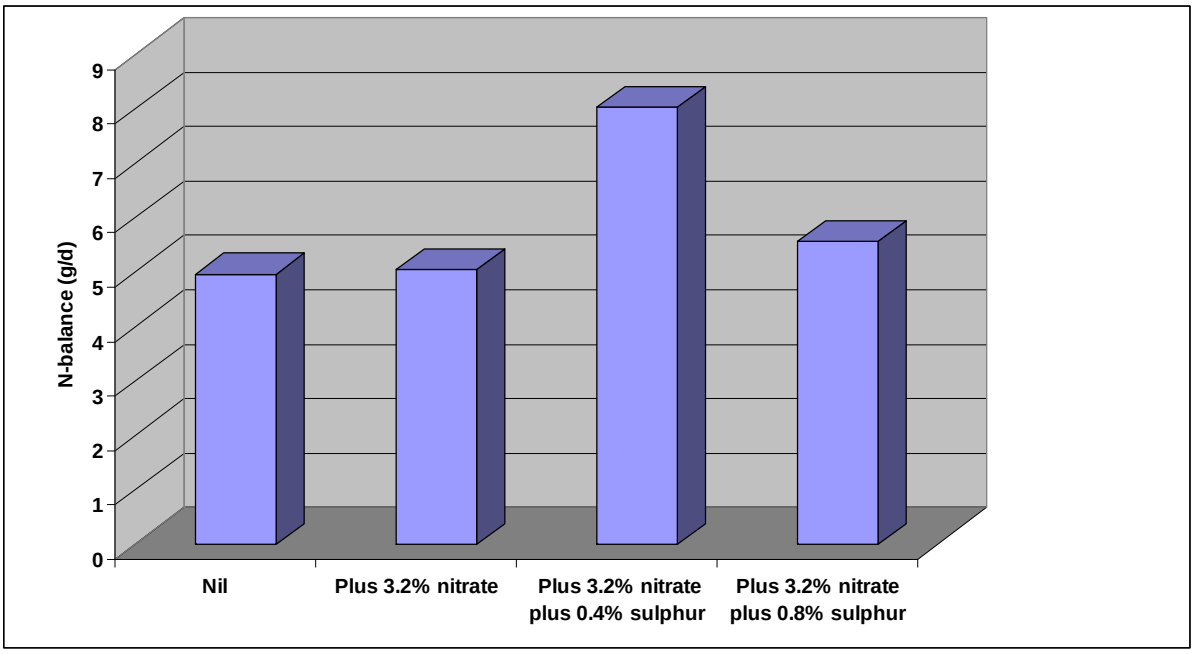
Table 5. The effects of adding KNO3 and /or sulphur to a concentrate based, well balanced diet for sheep (weight 35-39kg). The diet contained maize (30%), soybean meal (10%), corn starch (21-24%), ground corn cob (25%), molasses (6%) and maize oil (2%) and the sheep were also given recommended amounts of minerals and vitamins. There were 5 lambs per treatment.

Supplement	+0.4% sulphur	+3.2% K-nitrate	+3.2%K-nitrate +0.4% sulphur	+3.2%K-nitrate+0.8% sulphur
Dietary crude protein (%)	8.6	10.5	10.5	10.5
Dietary sulphur (%)	0.52	1.2	0.52	0.92
Daily gain (g/day)	220	170	170	200
Feed intake(g/day)	1.4	1.53	1.45	1.51
Feed conversion ratio (g/g)	7.0	8.8	8.6	7.5
Sulphur balance (g/day)	0.67	-0.8	1.22	2.86
N balance (gN)	3.95(1.12)*	3.35(1.58)	5.60(3.18)	3.62(4.94)
Wool growth (g per $100cm^2$/day)	2.3	2.6	2.6	2.5

*SD of the mean

Hydrogen sulphide is produced from S-amino acids in protein and inorganic sulphur in the rumen and passes into the gas space from which it is lost by eructation. Some of the eructated gases are re-inhaled and enter the lungs and most of the sulphide is absorbed into blood (Dougherty *et al* 1965), then oxidized to sulphate and excreted in the urine. Nitrate appears to inhibit SRB that produce hydrogen sulphide in the rumen and it would be interesting to have known the relative losses of sulphur via the faeces, urine and mouth. Potentially the sulphur balance is altered by nitrate through reduced metabolism of sulphur components in the feed and increased losses in the faeces. The interactions between NRB, SRB and NR-SOB in the rumen might provide answers for the results reported by Sokolowski *et al* (1969).

Figure 13 N-balance (g/N/day) in lambs given a high concentrate diet supplemented with nitrate or nitrate and sulphur (after Sokolowski *et al* 1969).



An important conclusion from the study of Sokolowski *et al* (1969) was that nitrate had no ill-effects in lambs on diets that were apparently already adequate in fermentable N. However, with sulphur supplementation, nitrate appeared to enhance the animal's protein nutrition. Wool growth in sheep is highly correlated with the amount of protein absorbed from the intestines. In the sheep in the studies of Sokolowski *et al* 1965) there was a tendency for clean wool production to increase with the addition of nitrate (Table 5). In addition the digestibility of nitrogen was highest when both nitrate and sulphur were added to the diet. In both experiments the standard deviation of the N balance of the lambs given both nitrate and sulphur were high. It seems that the response of individual animals to additional sulphur in the diet is extremely variable (see also Gould et al 1998). Intensive studies are needed to elucidate the reasons for such variability.

Cline *et al* (1963) followed up Sokolowski's Masters Research (Sokolowski 1959) and returned to nitrate research because of conflicting reports that nitrate lowers the efficiency of vitamin A utilisation by ruminants. They showed that nitrate had no effect on liver storage of vitamin A. In addition, replacing urea in the already balanced diet (but high in crude protein) with iso-nitrogenous amounts of nitrate had no effect on growth rates of sheep that were about 240g/d.

The interaction of urea and nitrate within a diet has been examined by Carver and Pfander (1973). They found that, when sheep were offered a typical concentrate-based diet, the addition of 2% KNO_3 and 1% urea decreased average daily gains, feed utilisation and thyroid function. These effects occurred whether KNO_3 was introduced before or after urea. The harsh effect of 2% KNO_3 in the diet can be entirely attributed to a lack of acclimation because these effects disappeared after 21 d.

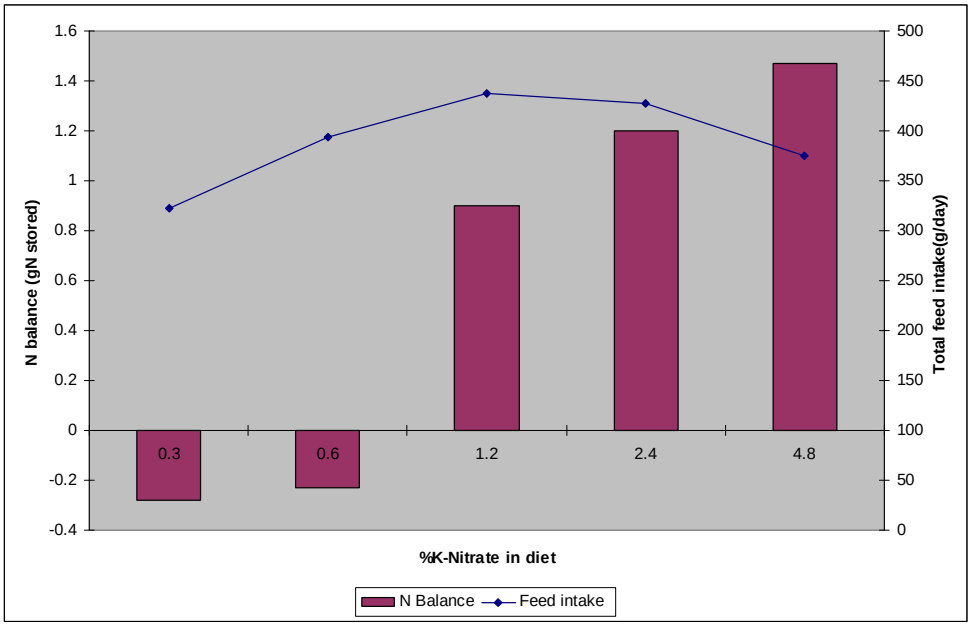
A major point that arise with the studies cited in this section is the fact that true protein levels in the diets fed were low compared to diets based on forages where nitrate poisoning occurs at times (e g on perennial rye grass pastures where forage crude protein is often as high as 24%). This factor will be discussed further in the coming sections.

c) Roughage-based diets

Carver and Pfander (1974) examined the interaction of urea and nitrate in a diet of Timothy hay and molasses (92:8) supplemented with soybean meal that should have been adequate in fermentable N. Supplementing such a diet with 1, 2, 4 or 5% of either urea or KNO_3 had no serious ill-effects on the animals, despite increasing urea and ammonia concentrations in

blood. No nitrite toxicities were observed during nitrate supplementation. These workers tentatively suggested that when urea and K-nitrate were supplemented to the basal diet, the rumen microorganisms utilized the nitrate first, thus decreasing the rate of urea hydrolysis

Figure 14. Average daily feed intake and N-balance in young goats fed molasses/straw (30/60) with K-nitrate. The % K- nitrate in the diet was adjusted upwards every 7th day (Quang Do *et al* 2008).



In young goats, increasing the nitrate concentrations in a low protein feed in steps every 7 d converted a negative N balance at low concentrations (0.3 and 0.6%) to a positive and increasing N balance at 1.2, 2.4 and 4.8% of the diet. N balance (see Figure 14), clearly demonstrating that nitrate was efficiently used as a fermentable nitrogen source for microbial growth in the rumen (Quang Do *et al* 2008). Growth rates or N balance were the same in goats fed the same basal diet but including 1% of body weight as tree foliage when either nitrate or urea was the major fermentable nitrogen source (unpublished).

Nitrite utilization by the assimilatory process should increase the potential cell yield as ATP for growth of microbes is available from nitrate reduction to ammonia. When *Clostridium perfringens* reduced nitrate in cultures rich in ammonia and organic nitrogen, cell yield and ATP yield were increased by 16 to 26% relative to un-supplemented cultures (Zarowny and Sanwall 1963). The transfer of electrons in ammonia in cell synthesis also retains more energy in rumen end products than when the electrons are donated to methane that is excreted. However, the net result must be more oxidized end-products *e.g.* higher proportion of acetate rather than propionate or valerate. Increased microbial growth efficiency plus the additional energy stored in microbes could result in a higher efficiency of feed utilization of nitrate containing feeds than of urea containing feeds.

The conclusion from the feeding trials is that nitrate can be used as a source of fermentable N in the rumen and, provided the animal is acclimated to nitrate, there will be no ill effects and possibly improved efficiency of microbial growth. A cautionary note is that the presence of nitrate probably increases the requirements for sulphur and this is an area for research. There is evidence that, if diets already adequate in crude protein to supply fermentable N, additional nitrate will be reduced to ammonia and in some way reduce the rate of urea conversion to ammonia (Carver and Pfander1974). Therefore substantial mitigation of methane production may be anticipated where nitrate replaces urea in low protein diets given to ruminants but again research is needed to test this concept.

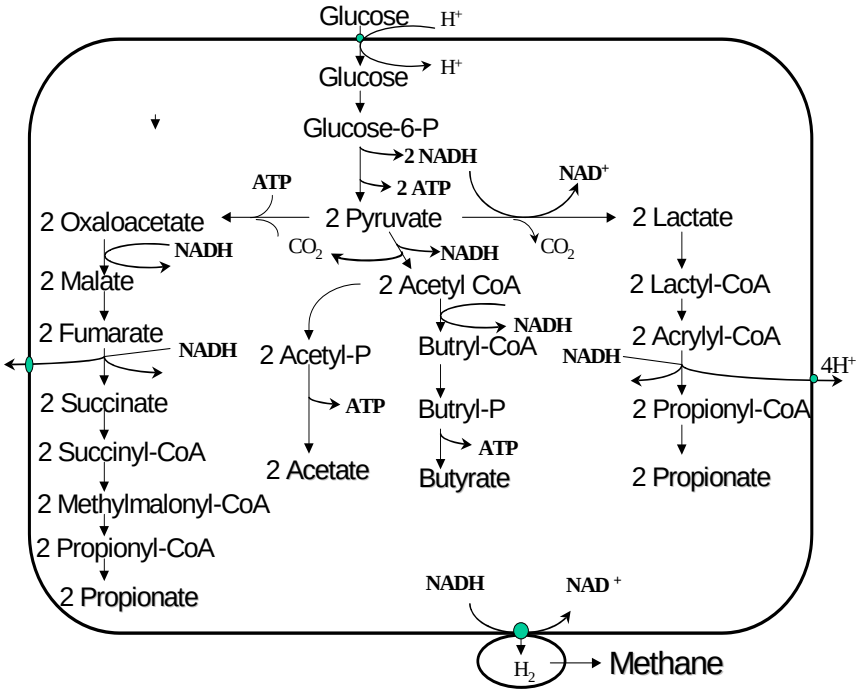
4 OVERVIEW OF TRANSACTIONS IN THE RUMEN AND METHANOGENESIS

Acknowledging that this part is repetitious of the early part of this report, a short description of methanogenesis in the rumen is provided here so as to remind the reader of the basis for much of the discussion of the potential effects of nitrate on methanogenesis in the rumen

The ruminant mode of digestion evolved to utilize the nutrients in plant biomass that are resistant to mammalian digestive enzymes. The major factor enforcing evolutionary change was the need to derive nutrients from plant biomass that required a fermentative digestion and was often mature and had a high proportion of structural carbohydrates. Because fermentation of complex carbohydrates, relative to enzymatic degradation of carbohydrates such as starch is a much slower process, feed had necessarily to be ingested and held for an extended time in an enlarged part of the digestive tract to allow fermentation of a high proportion of the organic matter. In addition, the animal had to maintain microbial activity through removal of the end-products by absorption or flow of digesta to the lower digestive tract and at the same time provide a buffered incubation medium through salivary secretions.

In ruminants forage is initially mechanically shredded in the process of harvesting, chewing and rumination. The particle size of feed in the rumen is further reduced by the growth of the radii of anaerobic fungi through the plant tissues. The mechanical and fungal initiated reduction in particle size increases the surface area of substrate and exposes digestible polysaccharides allowing a high density of microbes, mainly bacteria, to attach and solubilise structural carbohydrates, proteins and other components of the feed with the production of volatile fatty acids (VFA). The energy released in conversion of feed to VFA is partially used in microbial growth. A critical requirement in a continuous fermentation is the oxidation of reduced cofactors that are generated in the conversion of organic matter (largely carbohydrate) via the Embden Meyerhof pathway and additional pathways to VFA (see Figure 15). Under anaerobic conditions prevailing in the rumen, the reduced cofactors must be regenerated by electron transfer to acceptors other than oxygen and the major electron sink in the rumen is methane that is produced by the reduction of carbon dioxide; however, rumen microorganisms can use both sulphate and nitrate as alternative electron acceptors. The generalized outline of methane production from glucose is shown in Figure 15.

Figure 15. Pathways for the anaerobic fermentation of glucose by gut inhabiting microbes (Nolan 1999).

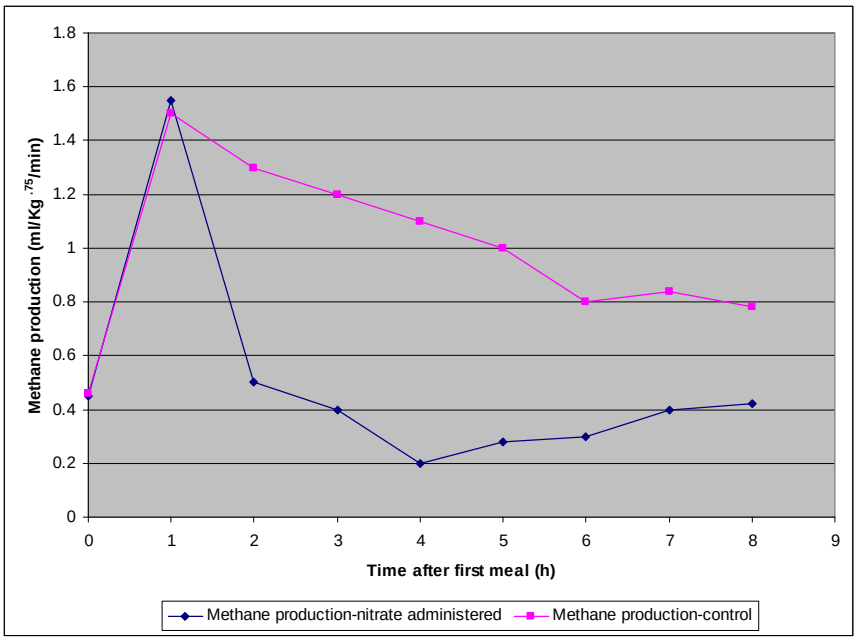


4.1 Overview of nitrate utilization and enteric methane production

Nitrate is a potent inhibitor of methanogenesis in all systems from fermentative digestion in the rumen to secondary fermentation in a wide range of systems from anaerobic biodigestors to sediments (Hungate1965; Allison et al 1981; Akunna *et al* 1994). It appears that respiratory conversion of nitrate to ammonia by anaerobic organisms is highly competitive as an electron sink consuming 8 electrons in the process and out competing methanogens for electrons in agreement with the free energy change in the reactions which are -598 kJ for the reduction of nitrate to ammonium and -131 kJ for the reduction of carbon dioxide to methane (see Allison and Reddy 1984; Allison et al 1981). The inhibition of methanogenesis may be enhanced by small amounts of NO and NO_2 (see Table 3) which are highly toxic to some methanogens (Kluber and Conrad 1998a,b). Nitrous oxide appears to be produced in cultures of rumen organisms incubated with nitrite or nitrate (Jones, 1972) but as a byproduct of dissimilatory nitrite reduction to ammonia, rather than a product of denitrification (Kaspar and Tiedje 1981). Denitrification has never been demonstrated in the rumen. All the intermediates in nitrate metabolism exhibit reversible or irreversible inhibition of methanogenesis depending on the particular organism and the concentration of the added N compounds (Table 3). Thus it is possible that the nitrate reducing organisms are advantaged in the rumen by their ability to compete for electrons produced in fermentation, and also by inhibition of methanogens directly by the oxides of nitrogen produced none enzymatically. This is an area for more research.

Some nitrate is consumed in food by all animals and oxides of nitrogen are produced internally and these could again be enhanced by feeding nitrate. For example NO production is likely to occur when nitrite enters the acidic portions of the gastrointestinal tract. In humans for example, nitrite is none enzymatically converted to NO by the acidic contents of the stomach (see Duncan *et al* 1995). In ruminants receiving high doses of nitrate in their feed or otherwise, it is probable that nitrate absorption and recycling to the GI tract occur and also that nitrate moves with ruminal fluid into the abomasum. It seems that NO production in the abomasum is a possibility and may play a part in inactivating methane producing organisms. The major point made here is that nitrate reducing organisms are able to out-compete methanogens for the electrons generated in fermentation of organic matter, but there could be assistance to this by direct inhibition of methanogens by nitric oxide.

Figure 16. The effects on methane production of administration of a nitrate load into the rumen of fed sheep un-acclimated to nitrate in their diet (Sar *et al* 2004a).



Takahashi and Young (1991) showed that nitrate inhibited methanogenesis in sheep *in vivo* when around 25-30 g sodium nitrate was added via the rumen in un-acclimated animals that were also

fed diets high in fermentable nitrogen. In a similar study by Sar *et al* (2004a) shown in Figure 16, sheep were fed half their ration at zero hour and nitrate or water (control) was administered at 30min after the animals were fed. Inhibition of methane production appeared to be delayed by some 30min Thus there is strong evidence for nitrate addition to the diet of ruminants severely inhibiting methane production and more so in the animal adapted to nitrate (Allison et al 1981; Allison and Reddy 1984). Advancement in the development of such feeding systems has been stymied or discouraged by the potential toxicity associated with nitrite accumulation which appears in rumen fluid some 4-6h after a nitrate injection (see McAllister *et al* 1996). This is illogical considering the strong evidence that this does not occur in animals that are acclimated to nitrate feeding. The accumulation of nitrite in the rumen when nitrate is present in feed appears to be associated with soluble protein content of the diet and indirectly hydrogen sulphide concentrations in rumen fluid which has led to the formulation of a new concept in an attempt to explain nitrate poisoning (see section 5).

To the knowledge of this author, there have been no attempts to study dietary nitrate utilization in ruminants in relation to the effects on methanogenesis where nitrate represents the sole or major source of dietary fermentable nitrogen. However, there is strong research evidence that on low protein diets that urea can be replaced by nitrate with the potential for substantial reduction in enteric methane production. Where urea is a high proportion of the fermentable nitrogen in a diet, its replacement could eliminate methane production entirely for instance where cattle are fed sugar cane or molasses or caustic soda treated straw as the major feed components (see Preston and Leng 1985 for discussion of production systems on these feeds).

Research from the Japanese school has done much to focus international attention on the ability of nitrate to replace carbon dioxide as the electron acceptor in the rumen and reduce methane production. The approach used, however, has been one referred to here as the "toxicological approach". The experiments have followed the approach established by Takahashi and Young (1991). Four sheep are used in a 4x4 Latin square design with a week between change over to the one day administration of nitrate plus additives. With this l design, the rumen microbes are given no opportunity to adjust to the nitrate as a source of fermentable nitrogen. As already discussed, full acclimation to nitrate in a diet requires in excess of 6 d. Also it has been previously discussed that any increased nitrate utilizing ability in the rumen is lost over a period of 21d when nitrate is removed from a diet (Allison and Reddy 1984; Alaboudi and Jones 1985). The results from research using the toxicological approach have direct implications for understanding nitrate toxicity but may be misleading, when applied to the nutritional application of nitrate.

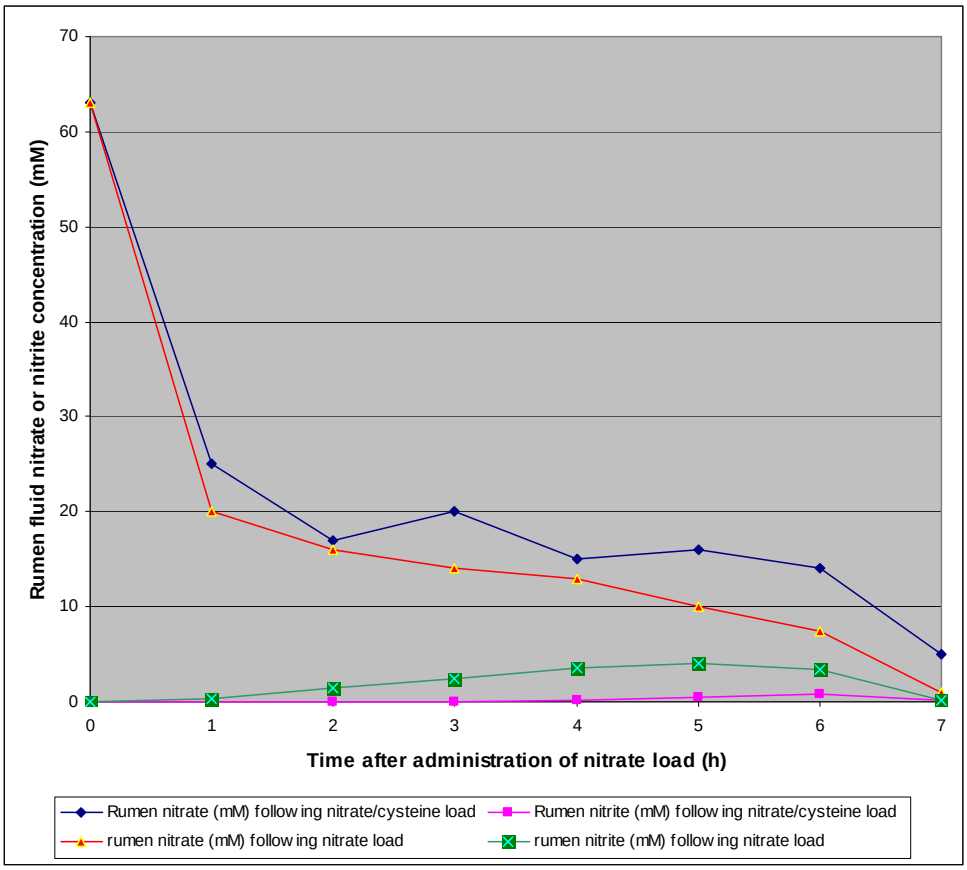
4.2 Role of cysteine in nitrate-induced reduction in enteric methane

Takahashi and Young (1991 and 1994) showed that L-cysteine administration into the rumen of sheep decreased methaemoglobin formation in blood when a nitrate load was imposed on the rumen. More recently, in a news release (that went around the world), it was claimed that cysteine prevented nitrate poisoning in dairy cows ingesting high nitrate pastures. It has been claimed that 0.21 g S/kg $^{0.75}$ body weight as cysteine given orally to lactating dairy cows (about 80g cysteine for a 600kg cow) is effective in preventing nitrate poisoning when feeds high in nitrate are ingested and, at the same time, methane generation is lowered. This is an unusually large dose rate of cysteine as, in studies of Dewhurst *et al* (2007), 8g of cysteine added to the rumen of dairy cows increased the hydrogen sulphide content in rumen gases within 15 min by between 490-957 parts per million (ppm), whereas a similar load of sulphate-S only increased this by 230 ppm.

The studies of Takahashi and colleagues have shown that when cysteine was given with nitrate through the fistula of sheep, there was no or little nitrite accumulation in the rumen and that methane production was much reduced (Figure 17). The zero time concentration of nitrate in the graph in Figure 17 has been calculated from the dose and an estimated rumen volume assuming that the load mixed in the rumen fluid pool. This could, in practice vary by up to 10% if rumen volume is over- or under-estimated. The changes in nitrate and nitrite concentrations in rumen fluid after a single load in these sheep were similar to the other studies presented in a number of

publications by these researchers when nitrate was administered alone. The addition of cysteine potentially allows nitrate to be safely used to limit enteric methane production.

Figure 17. Changes in rumen fluid nitrate and nitrite with time after administration of Na-nitrate alone or a combination of Na-nitrate and cysteine to sheep (Takahashi *et al* 1998).



Although potentially a major breakthrough in control of methane emissions from livestock, the high cost of cysteine is a major constraint to its application. However, if the mode of action cysteine can be elucidated, it may pave the way for future use of nitrate in diets containing modest amounts of true protein.

The difference in metabolism of nitrate to nitrite in the rumen where cysteine availability has been increased substantially together with nitrate is possibly explained through the interaction of the bacteria that have the capacity to metabolize sulphate, nitrate, nitrite and sulphide. NR-SOB are found in a number of anaerobic systems and organisms from the rumen appear to be able to grow on nitrate and sulphide in the presence of fermentable organic matter (e.g. *W. succinogenese,* Bokranz *et al* 1983). A hypothesis to explain the prevention of nitrite accumulation when a nitrate load is administered together with cysteine into the rumen of sheep is presented in section 5.

Cysteine-S in the rumen is rapidly converted to sulphide. Sulphide is the key intermediate in rumen sulphur metabolism and is produced more rapidly from cysteine than sulphate and least rapidly from methionine (Bray and Till 1975; Dewhurst *et al* 2007). *Desulfovibrio* species are widely distributed and a significant proportion of the rumen microbial biomass is responsible for sulphur reduction (see Forsberg 1980). *D. desufuricans* and *D. propionicus* can reduce nitrate (Seitz and Cypionka, 1986) but the majority of the *Desulfovibrio* species have only the capacity to reduce nitrite and do not reduce nitrate (Mitchell *et al* 1986). Administration of a cysteine load into the rumen could overcome the inhibition of SRB from events associated with the high nitrate concentrations in the rumen. If the cysteine-S is readily reduced to hydrogen sulphide, NR-SOB might be stimulated to return to nitrite ammonification thereby reducing their production of nitrite. Similarly, the increased activity/populations of the SRB, e.g. *Desulfovibrio* species are then able to

switch to nitrite utilization as cysteine availability declines, which could increase the microbial biomass that can reduce nitrite to ammonia in the rumen.

Megasphaera elsdinii is also potentially stimulated by a sudden increase in cysteine concentration in the rumen as it is one of the most potent users of cysteine (as indicated by its content of cysteine desulfhydrase activity) (Forsberg 1980) and, at the same time, a potent reducer of nitrite (see Cheng *et al* 1988). *W. succinogenese* is another rumen organism that can use hydrogen sulphide and formate or sulphur for growth (Macy *et al* (1986) and is potentially assigned to the NR-SOB group of rumen organisms.

4.3 In vitro studies

In a long term incubations (24h) of diluted rumen fluid with minimum levels of nitrate (8mM) and other fermentable substrate, Takahashi *et al* (1989) found that increasing concentrations of sulphide (from 1-8 mM) progressively lowered nitrite accumulation. The incubation medium is reported to contain 8mM sodium nitrate and the control incubation (where no sulphide was added) contained 103μg/ml of nitrite-N (7.3mM) when analyzed at 24 h (see Figure 1 in the paper of Takahashi *et al* 1989). Unless there is a mistake in the report, the entire mass of nitrate was converted to nitrite in the control incubation medium without simultaneous or subsequent conversion of nitrite to ammonia. This is atypical of the rumen *in situ* where nitrate is converted to nitrite and ammonia but the extent of nitrite accumulation is 0.001 of peak nitrate concentration (Takahashi and Young 1991). It may be that there was insufficient reducing power in the incubation medium for ammonia production. However, it is difficult to draw conclusions from this work without data on nitrate and ammonia concentrations. If the suppression of percent nitrite production had been accompanied by increased ammonia production, these results would have provided good evidence for the presence of NR-SOB in the incubation medium.

4.4 Artificial rumen studies

An association of nitrate conversion to nitrite without further metabolism to ammonia in anaerobic media low in fermentable substrate (i.e. low in organic electron donors) has been observed in experiments using artificial rumen equipment.

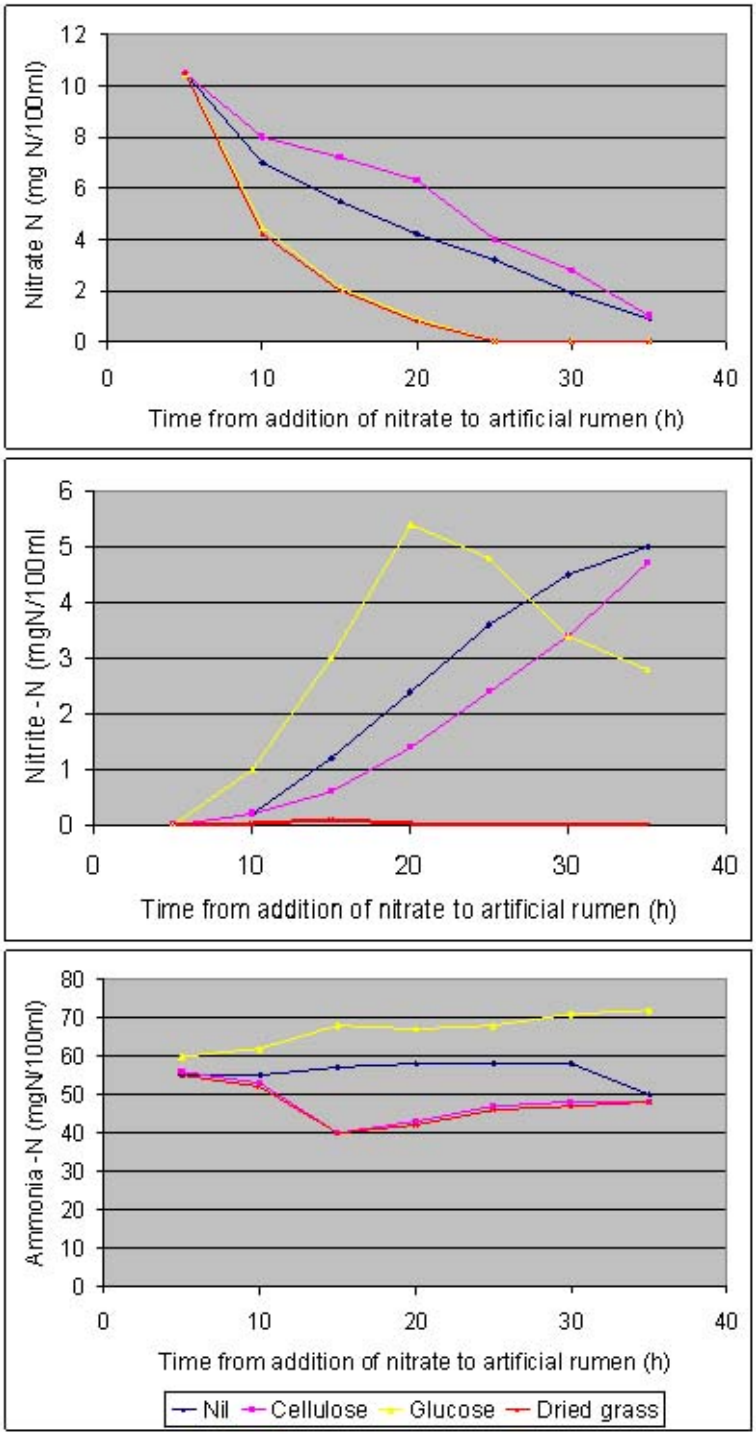
Artificial rumen are a more sophisticated approach to examining reactions of rumen organisms over a longer period of time than simple *in vitro* incubations in flasks. Rumen fluid obtained from donor animals is used as a source of microbes and diluted with buffered artificial saliva and incubated under anaerobic conditions. The incubation medium can be adjusted with time for changes in pH and other essential ingredients and sampled over a long time scale.

Barnett and Bowman (1957) used an artificial rumen to study nitrate metabolism. Their results are very interesting, but the approach used is open to criticism. In their studies rumen fluid for the inoculums was obtained from sheep slaughtered at an abattoir. They made no reference to diet or the period of time that had elapsed since the sheep were last fed. In most abattoirs the animals would have been fasted for a minimum of 24 h. Following collection, the rumen contents were strained through muslin to remove large particles and stored at 39^0C until used on the morning of the next day following collection. Rumen fluid (100ml) was added to vessels containing 100ml artificial saliva and 470ml of water, which is a high dilution rate, particularly of rumen contents that had been deficient in substrate for some time. The point being made is that the rumen fluid was possibly held at 39^0C for 12-24h without substrate before incubation in an artificial rumen. If the donor animals had been fasted for 24h, then the elapse of time between feed availability and commencement of the trials was possibly 48h. It is well established that a high proportion of rumen organisms lyse when substrate is withdrawn for any period of time (Wells and Russell 1996). In addition to lyses of bacteria, the pool of organisms in the fluid would also have been reduced by straining out large particles and by dilution with water and artificial saliva. It is therefore unlikely that the population density or species distribution in the inoculums were representative of those in the rumen of the feeding animal from which they were extracted.

The population of microbes remaining would have been low relative to cell counts in rumen fluid and maybe dominated by different species. Therefore quantitatively any reactions will be much slower in these artificial rumens as compared to the fed rumen *in situ*. Solid conclusions therefore cannot be drawn from this research so far as rumen systems is concerned. However there are six most interesting outcomes (see Figure 18).

1. Nitrate when added to the "rumen" (initial concentration 10.5 mmol/L) alone a considerable amount disappeared over the first 5h of incubation without reciprocal increases in either rumen ammonia or nitrite. This suggests some form of temporary sequestration or binding of nitrate or nitrite in some other pool and is in agreement with *in vivo* studies where nitrate clearance studies were undertaken (Figure 3).
2. More interesting is that the nitrate that disappeared rapidly from the incubation medium in the first 5h appeared to be released as nitrite over the next 30h; the nitrite-N concentration at 35h of incubation increased to almost equal to half the nitrate-N at the start of the incubation period. Supporting the concept of the temporary sequestration in the rumen of nitrate or nitrite.
3. When powdered cellulose was included to the incubation medium there appeared to be little or no effect on the pattern of nitrate disappearance or nitrite accumulation, perhaps indicating a lack of cellulolytic activity and therefore availability of electron donors. A high proportion of cellulolytic bacteria would have been excluded when the rumen fluid was strained and also are the most likely to lyse without substrate and even if they survive their populations would be the least likely to increase in population density in the time of incubation because of their relatively slow growth rate and the requirement of adhesion to the insoluble cellulose.
4. Addition of glucose increased nitrate clearance increased the rate of nitrite accumulation and when nitrate was apparently cleared from the medium, nitrite concentrations declined indicating metabolism of nitrite was occurring. Glucose because it was in solution is capable of supporting exponential growth of bacteria and thus a rapid fermentation could have developed. The pattern of nitrate and nitrite change in the incubation medium with glucose is compatible with an increased availability of electron donors (high fermentation rate) increasing activity of nitrate and nitrite reductases in microbes. It appeared that nitrate may have been first reduced to nitrite that enters the incubation medium and the nitrite is then reduced to ammonia by dissimilatory nitrite reductase since ammonia levels increased in the incubation medium.
5. Supplying nitrate together with dried grass had a major effect on the whole system; nitrate disappearance was extremely rapid (the same as when glucose was added) with insignificant accumulation of nitrite. Dried grass would have supplied readily fermentable carbohydrate to generate electron donors for NRB. Nitrate was not metabolized by a dissimilatory pathway as ammonia in the incubation medium declined with nitrate disappearance. This could be compatible with a component in the dried grass stimulating the growth or activity of a NR-SOB that reduced nitrate to nitrite and nitrite to ammonia by an assimilatory pathway without releasing nitrite or ammonia to the incubation medium. As discussed earlier the NR-SOB would have oxidized sulphide in nitrite ammonification. The incubation medium was low in total sulphur and the soluble protein in dried grass could have supplied a readily available sulphur source as S-amino acids which are rapidly converted to sulphide in rumen fluid (see Dewhurst *et al* 2007).
6. The rate of nitrate disappearance when dried grass was in the incubation medium was the same as when glucose was added. This may indicate that nitrate metabolism was in both cases the result of a single species (or group of species) that converted nitrate to nitrite when sulphide concentrations were low and fermentation of glucose was high(no scarcity of electron donors) and nitrate to ammonia by assimilatory nitrate reduction providing ammonia and ATP for growth when dried grass was present. The latter providing the vital source of electron donors and sulphide. The depression in ammonia being solely the result of other bacteria growing with the dried grass providing ammonia from protein and also substrate for fermentation

Figure 18 Pattern of change of nitrate, nitrite and ammonia in media contained in an artificial rumen after adding nitrate 5h after the start of incubation with nil substrate, powdered cellulose, glucose or dried grass (after Barnett and Bowman 1957).



This research, though fraught with difficulties in relating to the rumen because of the experimental approach, at least provides support for the hypothesis that NR-SOB are involved, and that in an anaerobic ecosystem with organisms originating from the rumen, there is some form of nitrite or nitrate sequestration occurring and both availability of electron donors and the supply of (protein) sulphide are factors that may be implicated in the extent of nitrite accumulation. The organisms that survived the long period without added substrate are either extremely robust or able to survive by spore formation.

Bryant (1964) followed up Barnett and Bowman's research. Rumen fluid obtained from a cow (30 min previously) fed red clover, was placed in flasks with added glucose mixed and then nitrate added. Over a 1 h incubation period, nitrate concentrations dropped from 9mmol/L to 0.5mmol/L with little effect on the rumen fluid ammonia over the same period. Glucose disappearance and lactate accumulation were substantial with a probable excess of reducing power. If microbial growth accounted for 20mgN/g of carbohydrate fermented, then approximately 5 mg N could have been taken up and accounts for the 250g of added glucose that disappeared from the *in vitro* incubation medium. Nitrate metabolism under these conditions could only be by the assimilatory pathway as ammonia concentrations remained constant or rose slightly through the 60min of incubation. Clover would have had a high content of protein and support the production of hydrogen sulphide in the rumen (Dewhurst 2007). Therefore any bacterium, that uses hydrogen sulphide as a substrate would have been present but much greater population density could be expected compared to the density in the artificial rumen medium used by Barnett and Bowman(1957). The important issue is that nitrite accumulation in all incubations was negligible. Even when nitrate concentration in the medium at the start of the incubation was 44 mmol/L accumulation of nitrite did not exceed 0.017mmol/L (Bryant 1964).

Although the concept of temporary sequestration or storage of nitrate appears to be feasible especially in energy limited conditions, the *in vitro* studies suggest that nitrate reduction to ammonia and assimilation may be sufficiently rapid to account for nitrate disappearance in some situations. If nitrate reducing organisms that have the assimilative pathway have the capacity for explosive (exponential) growth in the presence of fermentable carbohydrate, the rapid clearance of nitrate following a nitrate load is a result of the rapid assimilation of ammonia into cell growth. If the NRB capable of this exponential growth conditions are or are induced to become NR-SOB (see Macy *et al* 1986) then the growth rate would be only limited by the availability of hydrogen sulphide.

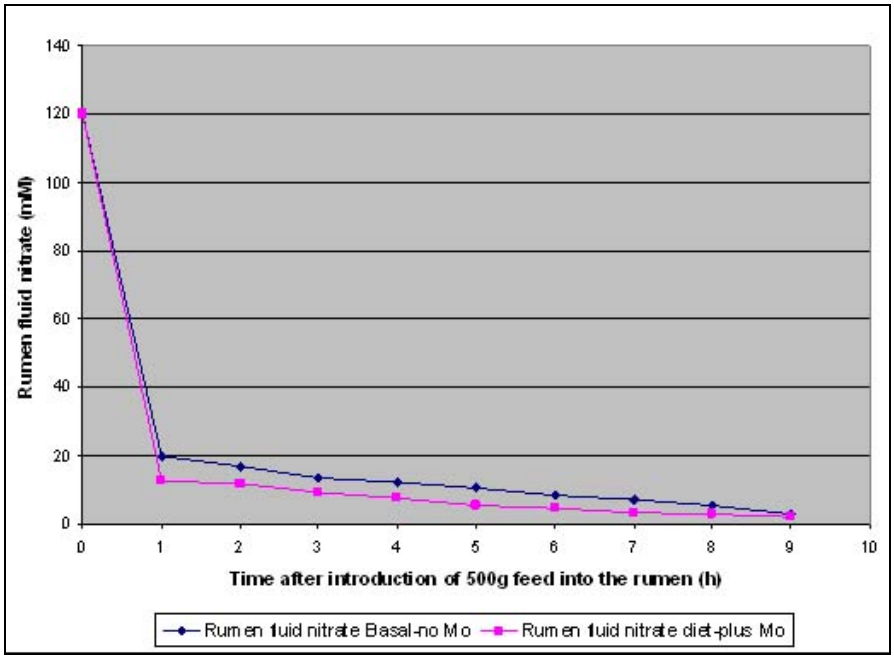
Bryant (1964) obtained rumen fluid from clover fed animals which would be high in true protein and perhaps therefore containing a high population of NR-SOB. Barnett and Bowman (1957) used diluted fluid which would be exhausted in substrate and whilst nitrate was cleared when a source of electron donors was included in the medium the nitrate was mainly converted to nitrite but when a source of protein and electron donors (dried grass) was included in the medium nitrate was cleared rapidly with no detectable nitrite production and ammonia concentrations actually declined.

5 FURTHER EVIDENCE FOR THE INVOLVEMENT OF SULPHUR REDUCING ORGANISMS IN NITRITE ACCUMULATION IN THE RUMEN OF SHEEP FED NITRATE IN THEIR DIET

Tillman *et al* (1965) demonstrated that molybdenum had marked effects on nitrate metabolism in the rumen of sheep fed purified diets with nitrate as the sole source of fermentable N. Two groups of sheep were used and one group had the molybdenum content increased by 1ppm. 500g of each diet were introduced into the rumen of 12 h fasted sheep that had been acclimated to the diets. The additional molybdenum apparently increased nitrite accumulation in rumen fluid 5-6 fold (see Figures 20) but the clearance of nitrate was very similar on both diets (Figure 19).

Molybdenum is essential for microbial growth but also appears to be toxic or inhibitory with some microorganisms, particularly the SRB (Bryden and Bray 1972; Gawthorne and Nader 1976; Spears *et al* 1977). Molybdate (MoO_4) has been proposed as an analog of sulphate that blocks the sulphate activation step (catalyzed by ATP sulphurylase) in bacteria that reduce sulphate (Oremland and Capone, 1988). Taylor and Oremland (1979) showed that MoO_4 specifically inhibits SRB in pure culture and in sediments (Oremland and Silverman, 1979; Sorenson *et al* 1981). Jones *et al* (1982) demonstrated that Mo may have inhibiting effects on other organisms in sediments as it reduced methanogenesis when sulphate was limiting. Bracht and Kung (1997) showed that MoO_4 at concentrations greater than 10 ppm reduced sulphide production in ruminal fermentations lowering sulphide production in both the liquid and gas phase. Under the conditions, MoO_4 appeared to be a specific inhibitor of SRB because there was no effect on methane or hydrogen production (Bracht and Kung 1997). The increased nitrite production (Figure 20) could be explained if an NR-SOB continued reduction of nitrate to nitrite but was restricted in converting nitrite to ammonia because of the inhibition of SRB and lower sulphide production (see Background; "Conditions supporting the nitrate reduction pathway by microorganisms"). In ruminants, molybdenum is a trace mineral with a very narrow margin between the amount needed to fulfill the animal's requirements and toxic levels. Loneragan *et al* (1998) reported that supplementing sodium molybdate reduced hydrogen sulphide concentrations in the gas cap of cattle fed a high sulphur diet.

Figure 19. The effects of dietary molybdenum on the clearance of nitrate from rumen fluid of sheep after placing 500g of feed containing 6.5% K-nitrate and 6.5% Na-nitrate into the rumen (Tillman *et al* 1965)



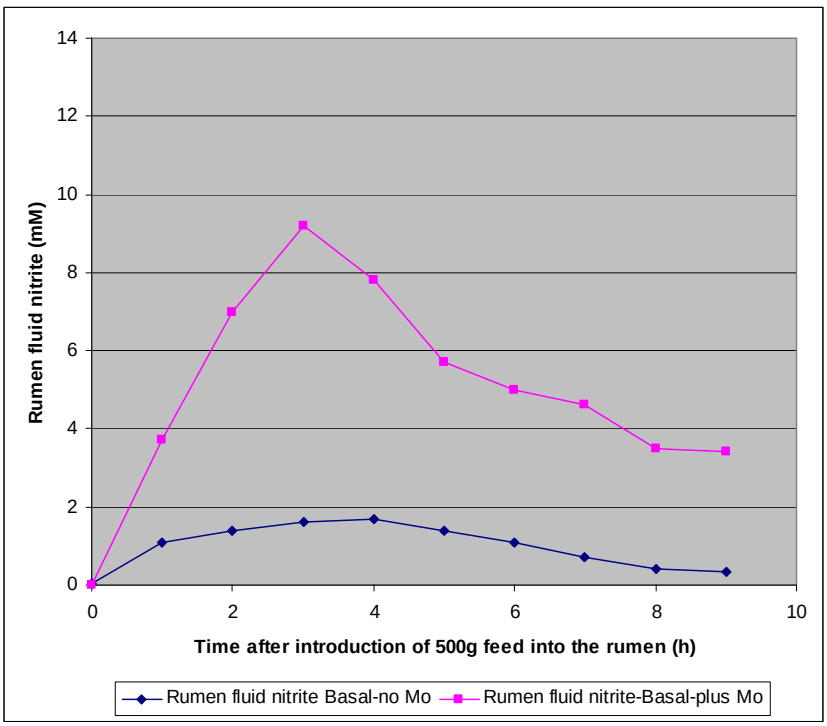
The effects of molybdenum on nitrite production in sheep strengthens the argument that nitrite build up when nitrate is administered to the rumen is associated with an inhibition of SRB that may also reduce nitrite to ammonia. That nitrate supplementation increases sulphur requirements of finishing lambs on well-balanced diets was demonstrated by Sokolowski *et al* (1969). These workers also showed that sulphur balance in lambs was markedly decreased when 3.2% nitrate was added into the diet but this was rectified by additional sulphur supplementation.

Inclusion of nitrate in the feed may synchronize the availability of N to S more closely to microbial requirements, particularly those of the SRB. The nitrogen to sulphur ratio in a diet is of the order of 15:1 (w/w). When nitrate is introduced into the rumen, the N:S ratio inflates enormously, whereas the N:S ratio of a combined cysteine /nitrate injection (as given in the studies of Takahashi and colleagues) the N:S ratio is 1.7:1. A bacterium that has the dual role of nitrate and sulphur reduction is likely to be inhibited when the N:S ratio is heavily imbalanced and highly likely to be switched on by a low N:S ratio. There is, in addition, the possibility of a NR-SOB being present in the rumen particularly in the nitrate supported rumen. If higher levels of molybdenum in this diet inhibit SRB, it could be expected to add to the inhibition arising from the competition of NRB for electrons or electron donors.

Rumen fungi require sulphur in reduced form to meet their growth requirements. It is known that increasing the sulphur content of a diet deficient in sulphur enhances fungal activity significantly which in turn increases the rate of breakdown of feed particles. This in turn enhances the rate of fiber degradation (see Gordon and Phillips 1998). The implications of the effects of nitrate supplementation on sulphur reduction in the rumen and on fungal growth require further research.

A further, more remote explanation for the apparent effect of cysteine on nitrite accumulation would be the inhibition of oral conversion of salivary nitrate to nitrite by hydrogen sulphide which is partially lost through eructation. That nitrite and cysteine react spontaneously to produce a complex compound that exhibits anti microbial activity towards in particular; *Clostridium perfringens* was first demonstrated by Perigo *et al* 1968 and attributed to the production of inhibitory substances when these two compounds are brought together (Rhia and Solberg 1975; Moran *et al* 1975).

Figure 20. The effects of dietary molybdenum on the accumulation of nitrite in rumen fluid of sheep after placing 500g of feed containing 6.5% K-nitrate and 6.5% Na-nitrate into the rumen (Tillman *et al* 1965).



5.1 Assimilatory nitrate reduction appears to dominate in the rumen

Throughout this report the flow of ideas repeatedly returns to the pathway by which nitrate is metabolized in the rumen. Considerable evidence points to the availability of both dissimilatory and assimilatory pathways being dominant under different circumstances. A problem with the literature is a tendency to ascribe the names to different processes. However, the literature tends to refer to nitrate metabolism in the rumen as dissimilatory even though there are a number of experiments that indicate that ammonia is not necessarily excreted by rumen NRB but remains in the cell to be synthesized into the amino groups of cellular protein. The following section risks the criticism that it is repetitive but in a report as compared to a referred article it appears justifiable to bring the evidence together in one section concerning the dominant pathway of metabolism of nitrate in the unique ecosystem of the rumen.

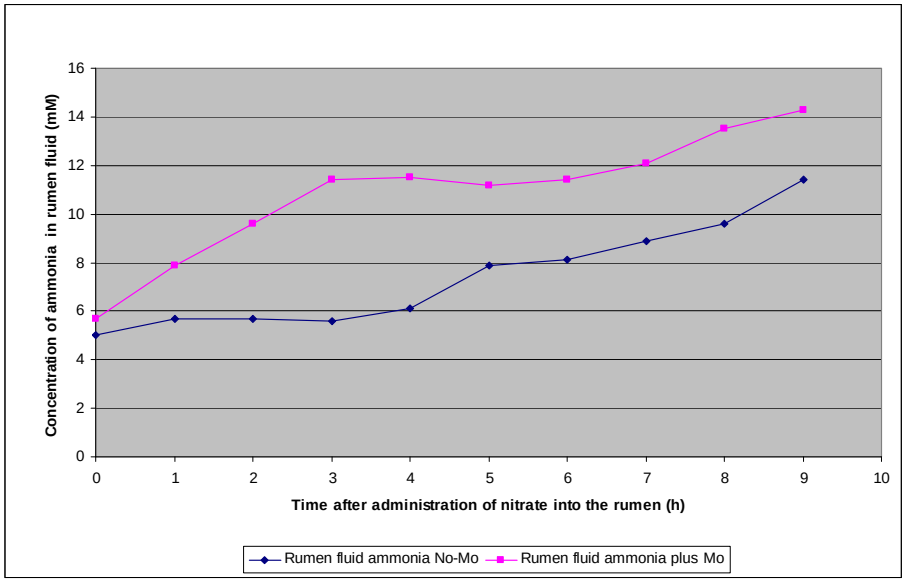
A considerable number of researchers report a low accumulation of ammonia in the rumen or in rumen fluid, when nitrate has been added, that do not appear to be compatible with nitrate disappearance from the rumen being by the dissimilative pathway. This has been discussed earlier but to emphasize this point the research of Tillman *et al* 1965) is further reviewed.

Tillman *et al* (1965) monitored ammonia concentrations in rumen fluid in sheep given 500g of a purified diet with or without additional Mo and, as just discussed, with the higher level of Mo, the accumulation of nitrite peaked at 9.2 mM as compared to 1.7 mM when Mo was omitted; the peaks occurring at approximately 3-4h post administration of the feed into the rumen (Figure20). The pattern of ammonia accumulation was different between sheep with or without Mo (Figure 21), yet nitrate disappearances from rumen fluid was similar. The presence of Mo in higher concentrations in the diet increased nitrite accumulation which may be attributed to suppression of SRB that also reduce nitrite to ammonia or to the presence of a NR-SOB that rapidly reduce nitrate to nitrite when sulphur reduction to hydrogen sulphide is interrupted. The calculated nitrate level in the rumen, assuming instantaneous mixing, would have been 120 mM but had been reduced to below 20 mM after 1h., thus some 80% of the nitrate disappeared in the first hour after administration. The nitrate could not be accounted for by conversion to nitrite or ammonia released into rumen fluid and the blood levels in the sheep indicate that the amount absorbed was small relative to that injected. Fermentation rate in the rumen would have been rapid because of the nature of the purified diet and as evidenced by the rapid decline in pH from 7.4 to 5.6 over the 4h following addition of feed to the rumen. The rumen organisms were dependent on nitrate as the major source of fermentable N but N recycling from urea produced from both absorbed ammonia and catabolism of amino acids in the tissues could have accounted for some of the ammonia present in the rumen. The relatively slow and small increase in rumen ammonia concentration is strong evidence for the assimilatory nitrate reduction pathway being dominant in the rumen in animals fed nitrate as the sole source of N.

The gradual increase in rumen ammonia following the addition of nitrate in the feed may be indicative of a slow release of ammonia from intracellular pools. These pools could be nitrate or amino acids. However, the contribution of urea from the blood could also increase ammonia in the rumen

Ammonia generated intracellular could be temporarily stored as amino N. Blake *et al* (1983) found there was a high accumulation of alanine (intracellular concentration of 2.4 mM) in bacterial cells from the rumen of calves fed largely urea (or ammonia) as the sole N source. Erfle *et al* (1977) using an artificial rumen system inoculated with the contents of the rumen of a cow fed a synthetic diet in which the N was mostly as non-protein nitrogen, showed that, when ammonia concentration in the incubation medium was high, alanine concentration in the bacteria rose rapidly. On the other hand, when ammonia concentration in the incubation medium was low, the accumulated alanine and glycine were metabolized readily by rumen microorganisms providing fermentable N for growth. The overall effects suggest that alanine might serve as an intracellular storage depot in bacteria that retains ammonia-N when the rate of ammonia production from nitrate exceeds its rate of assimilation in growth.

Figure 21. The changes in ammonia in rumen fluid of sheep fasted for 12h and then force fed 500g of a purified diet directly into the rumen (Tillman *et al* 1965).



In the studies of Tillman *et al* (1965) the diet higher in Mo appeared to create an increased rate of ammonia accumulation when compared to the diet lower in Mo when these were force fed at the same level. The rates of ammonia accumulation may be explained by slight changes in assimilatory nitrate reduction relative to dissimilatory pathway which is influenced by the presence or absence of Mo.

6 A THEORY OF THE ETIOLOGY OF NITRATE POISONING IN RUMINANTS BASED ON THE PRESENCE OF NR-SOB IN THE RUMEN

In the section on background information, the roles of NRB, SRB and NR-SOB were discussed in relation to their roles in oil field souring. NR-SOB have only recently been discovered but are now under intensive research. The significance of NR-SOB resident in the rumen has not been researched. It seems likely that they will be identified in the future and this will have major implications for the use of dietary nitrate supplementation, both for the capacity to lower methane production and also to provide a source of nitrogen for organic N synthesis in the rumen. Knowledge of the mechanism by which nitrite is "spilled" by rumen organism will lead to methods to prevent this and reduce the risk of nitrite poisoning and could remove the presently perceived barriers to the use of nitrates as N sources for ruminants, at least replacing urea with nitrate in commercial diets.

Bacteria that have been shown to reduce nitrate to ammonia and oxidize hydrogen sulphide to sulphate in *in vitro* incubations are present in the rumen (e.g. *W. succinogeneses* - see Bokranz *et al* (1983)). By inference from the activity of NR-SOB from other environments, rumen NR-SOB might produce different end-products (nitrite or ammonia) depending on the concentration of hydrogen sulphide in rumen fluid. The ratio of nitrate to sulphide may also be a mediator in the channeling of nitrate to nitrite or to ammonia by the ammonification pathway with the generation of ATP in the latter for growth.

The complexity of the interaction of nitrate and sulphide reducing organisms is indicated by the suggestion that some nitrate reducing and even sulphate reducing bacteria have nitrate reducing –sulphide oxidizing capability (Dannenberg *et al* 1992). If this were the case for some rumen organisms that primarily reduce nitrate or sulphate, it could explain nitrite accumulation and its prevention by provision of cysteine, following administration of high nitrate loads in the rumen (Takahashi *et al* 1998).

From research with *Thiomicrospira* sp and *Sulphurospirillium* species (see Greene *et al* 2003) NR-SOB would tend to produce nitrite when the ratio of nitrate to hydrogen sulphide in the medium is high (e.g. when a nitrate load is given) and produce ammonia when this ratio is lowered. In all the nitrate clearance studies undertaken, the ratio nitrate to sulphide is high immediately following the injection of nitrate. However 1h after administration of the dose, nitrate concentration has decreased precipitously and the ratio of nitrate to sulphide should be relatively low depending on the amount of sulphur in the diet. This is the approximate concentration of nitrate when the rate of loss of nitrate from rumen fluid apparently slows, as shown in Figure 3.

Cysteine is much more rapidly converted to hydrogen sulphide in the rumen than sulphate (Bray and Till 1975; Dewhurst *et al* 2007) and thus the rapid production of sulphide could alter the ratio of nitrate to sulphide in rumen fluid sufficiently to provide sulphide for nitrite ammonification to continue and reduce or prevent nitrite production by an NR-SOB following a nitrate load. The amount of nitrite produced when a nitrate load is administered into the rumen would be largely determined by the population density of the NR-SOB which may then determine the severity of nitrite toxicity (methaemoglobin formation). The population of NR-SOB in the rumen in turn may be governed by the sulphide content of rumen fluid prior to loading which is directly related to the amount and rate of protein fermentation.

Cysteine administration may also provide substrate that boosts the activity in the rumen of the SRB such as *Desulfovibrio* species providing an increased population of bacteria that can metabolize nitrite as sulphide levels decline following the exhaustion of the amount of cysteine remaining for degradation in the rumen. This would increase their population density, meeting the requirement for higher density of cell numbers (Haveman *et al* 2004) which are then able to effectively use nitrite at rates equal to its production rates by NRB.

The availability of hydrogen sulphide in the rumen largely depends on the protein (S-amino acids) content of the diet (see Dewhurst *et al* 2007) and the time since the completion of a meal. Dewhurst *et al* (2007) found that 4 h after cessation of feeding, hydrogen sulphide concentration

in the gas space in the rumen of dairy cows at pasture declined to zero. A NR-SOB would be established at population densities dependent on the amount of protein degraded in the rumen. Accordingly, nitrite production in response to administration of nitrate could be expected to be higher on high protein diets such as early growth phase of forages such as perennial ryegrass. Thus the population density of NR-SOB would substantially increase in the rumen when animals are grazing lush green pastures. The NR-SOB populations established when nitrate is present in only small concentrations in forage will control the amount of nitrite produced when nitrate suddenly increases in the feed consumed. Lovett et al (2004) clearly showed with perennial rye grass re-growth that there was a linear increase in nitrate as protein in the re-growth forage increased with N fertilizer application but the levels of nitrate were quite low. At high protein content in forage the NR-SOB should be at higher population densities in the rumen of animals fed the forage. The concept is that when the levels of nitrate in forage suddenly increase from 'normal' range (0.4-4g/kg dry matter-after Lovett et al 2004) to ten times these values (see Table 1) that the population density of NR-SOB established by the 'normal' nitrate content in the feed will dictate the amount of nitrite produced and therefore the severity of methaemoglobinaemia.

It is necessary to assume that nitrite is not normally released into the rumen medium by NRB. This may be a factor that explains some of the variability in reported dose levels of nitrate that are toxic to ruminants in different countries and different soil fertility and climate conditions that have effects on protein levels in forages (see the dichotomy of results of Kemp *et al* (1977) and Crawford *et al* (1966)).

A dose of nitrate given some time after feeding, when much of the soluble protein has been degraded and hydrogen sulphide production is low (Dewhurst *et al* 2007) is therefore more likely to induce a higher rate of nitrate reduction to nitrite and therefore accumulation of nitrite in the rumen than when given to animals that were feeding. This provides a possible reason for the early morning incidence of nitrate poisoning when animals have fasted overnight: the pool of sulphide would be much reduced and therefore nitrate metabolism to ammonia involving sulphide oxidation would be much reduced.

6.1 Evidence for the presence of NR-SOB in the rumen

The rumen organism *W. succinogenese* has been extensively studied and it has been shown to metabolize nitrate to nitrite by the dissimilatory pathway and nitrite to ammonia by respiratory ammonification coupled to the generation of an electro-chemical proton potential across the membrane and ADP phosphorylation to ATP (Bokranz *et al* 1983; Simon 2002). *W succinogenese* has physiological similarities to the free living NR-SOB, *Sulphurospirillium deleyianum* including almost identical three- dimensional structures, location of heme groups, similar substrate and product channels and similar architecture of the catalytic site (see Simon 2002). These similarities suggest that they have similar metabolic roles; *S deleyianum* having the capacity to reduce nitrate and oxidize sulphide (that is it belongs to the NR-SOB family) suggests that *W succinogenese* can also be considered to belong in the same group. Growth of *W. succinogenese* has been demonstrated with sulphide as an electron donor and fumarate as an electron acceptor (Macy *et al* 1986). Similarly resting cells of *D. desulphuricans* CSN catalyze the oxidation of sulphide to sulphate coupled to the reduction of nitrate to ammonia (Dannenberg *et al* 1992). Intact cells of *W. succinogenese* grown with formate and nitrate catalyze electron transport from formate to nitrate at extremely rapid rates (Simon *et al* 2002). As discussed earlier, *W. succinogenese* and *Desulfovibrio* species are rumen organisms that can be readily isolated from a wide range of animals on different feeds and diverse rumen ecosystems. The available evidence supports the concept that NR-SOB are present in the rumen and the diversity of substrates they are able to use allow them to be permanent members of most rumen ecosystems. It is also necessary to hypothesize that these organisms are capable of rapidly adjusting to nitrate when it is first introduced into the rumen.

It seems likely that the density of NR-SOB in the rumen would be controlled by the low concentration of sulphide (approx 0.1mM). Their presence would be advantageous as they would

maintain pools of various oxidized and reduced forms of sulphur for the use of the mixed microbes in the rumen ecosystem which could be depleted rapidly by the rate at which sulphide is protonated and lost to the gas phase in the rumen. As discussed earlier, accumulation of nitrite in the rumen when a quantity of nitrate is ingested or administered would then be dependent on pool size of the NR-SOB. This in turn would be associated with the availability of protein (S-amino acids) and other sources of sulphur. The nitrate to sulphur ratios in the rumen during experiments where a nitrate load is placed on the rumen are extremely high. The initial nitrate levels vary from 20-120mM (see Table 4) and the hydrogen sulphide levels may be 0.1-0.3mM (cf. up to 2.5mM hydrogen sulphide in oil field's water). The concentration of nitrate in the rumen in the clearance studies are much greater than the levels used to control sulphide production in oil field waters (10mM) but the oil fields medium would be much lower in organic matter and thus in supply of electron donors.

It appears that the amounts of organic electron donors probably affect the interactions of the SRB, NRB and NR-SOB. The concentration of nitrate at which the SRB re-establish sulphate metabolism to sulphide - and in NR-SOB the extent of nitrate reduction to nitrite is curtailed - is probably dependent on the fermentable substrate present.

In the oil fields studies the ratio of nitrite produced to ammonia produced from nitrate was apparently controlled largely by the availability of electron donors which was dependent on the concentration of lactate in the medium (Hubert and Voordouw 2007).

Hubert and Voordouw (2007) showed that complete removal of hydrogen sulphide from a bioreactor medium inoculated with water from an oil well, required increasing the nitrate concentrations to 10mM at concentrations of sulphate varying from 0.75, 2 and 6 mM. In the rumen, the clearance of a nitrate load is extremely rapid initially, but at 30-60 min following administration, when nitrate concentrations have been lowered to 20-30 mM, its clearance appears to slow down. This may be interpreted as the point when SRB activity returns producing sulphide and NR-SOB cease nitrite production and commence nitrite ammonification. The strongest support for this concept is that the transition from a fast to a slow rate of nitrate clearance appeared to occur at the same nitrate concentrations in the rumen in sheep administered intra-ruminally with widely different amounts of nitrate (see Figure 3).

6.2 Summary of possible effects of a nitrate load in the rumen

1. NRB activity is increased
2. NRB inhibit the metabolism of SRB by competing for electrons in the rumen and sulphide production is reduced
3. At high nitrate concentrations relative to sulphide, respiratory ammonification is inhibited but dissimilatory conversion of nitrate to nitrite continues in NR-SOB.
4. SRB may continue to convert nitrite to ammonia but at rates below nitrite production by NR-SOB and so nitrite accumulates. It appears to be more plausible that SRB are inactive and that it is the production of nitrite by NR-SOB population that determines the amount of nitrite that accumulates.
5. Nitrate is cleared from rumen fluid by a combined effect of nitrate reduction to ammonia and assimilation in cell growth and absorption across the rumen wall and possibly temporary storage in bacteria as nitrate or metabolic end-products.
6. When nitrate concentrations in the rumen drop to a certain level, SRB are reactivated and increase the concentration of hydrogen sulphide in rumen fluid and nitrite production by NR-SOB ceases. This appears to coincide with a nitrate concentration of 20-30 m-mol/L.
7. Simultaneous administration of nitrate with cysteine, the latter being converted rapidly to hydrogen sulphide, maintains or increases the hydrogen sulphide pool in the rumen. Ammonification of nitrate/nitrite by NR-SOB is promoted and nitrite production is eliminated or reduced to rates that are readily taken up by the SRB. Lack of, or low

nitrite production results in no inhibition of SRB possibly allowing both increased sulphide production and increased nitrite uptake.

6.3 A possible explanation for the occurrence of nitrate poisoning syndrome in grazing ruminants

Leaf protein content of lush pasture growing under good conditions may reach 20-30%. At times, photosynthesis is compromised by factors such as low temperatures, lowered sunlight irradiation and decreasing water availability, and nitrate levels may build up to 2-4% in a few hours (see section on nitrate poisoning in Background). A possible scenario is as follows. After animals ingest this high-nitrate feed, NRB out compete SRB for the available electrons generated in fermentation and lower hydrogen sulphide production from sulphur compounds. NR-SOB actively metabolize nitrate but at the high nitrate intake and lowered sulphide concentrations, nitrite is preferentially produced. The amount of nitrite produced may be directly related to NR-SOB population in rumen fluid which is determined by protein content and nitrate normally present in the forage. When rumen nitrate levels decline to a critical level, increasing SRB activity increases sulphide concentrations allowing NR-SOB to metabolize nitrite by the respiratory ammonification pathway. Prior to ingestion of excessive nitrate, the level of protein in a feed would determine the population density of SRB and the levels of both protein and nitrate would control NR-SOB and therefore the extent of production and accumulation of nitrite in rumen fluid when nitrate is ingested.

Some lush pastures (e.g. in white clover or sudan grass) contain cyanide in addition to high concentrations of protein. The presence of cyanide appears to limit the amount of hydrogen sulphide lost in the gas space (Dewhurst *et al* 2007) which indicates a lower concentration in rumen fluid (see Gould *et al* 1997). It could be expected on this forage, that the pool size of NR-SOB would be lower than on say a rye grass pasture. The lower population density would tend therefore; to lower nitrite accumulation in the rumen when nitrate is consumed. Bryant (1965) demonstrated that rumen fluid from red clover fed cattle did not produce nitrite from nitrate.

Similar effects may occur when high-nitrate feeds are ingested by animals supplemented with protein concentrates. Takahashi *et al* (1980) examined the clearance of nitrate from the rumen of sheep on diets of different content of soybean meal and corn starch. These were fed at different levels to enable comparisons to be made of nitrate clearance at different energy and protein intakes. The peak nitrite in the rumen following the administration of 30g $NaNO_3$ was significantly related to the crude protein in the diets (Figure 22). At the same nitrate load, nitrite accumulation in rumen fluid was greatest when the protein content of the diet was highest. This supports the concept that the population density of nitrite producing organisms is controlled by the protein content of the feed.

Initially the apparent effects of a high ammonia concentration in the rumen (indicative of high protein in the feed) on nitrite accumulation was suggested as a mechanism whereby nitrate reduction to ammonia could be repressed causing a build up in nitrite in the medium. This remains a possibility. However, if NR-SOB are present in the rumen - which is speculative but supported by indirect evidence of the versatility of organisms such as Desulfovibrio and *W. succinogenese* to use multiple substrates in vitro - it would seem that the effects of high protein diets on nitrite accumulation following a nitrate load in the rumen are likely to be related to the interactions amongst the nitrate and sulphate reducing bacteria.

In the research summarized in Table 6 and Figure 22, it seems that the ratio of fermentable organic matter (and therefore the rate of availability of electrons) in the presence of excess nitrate is a factor causing nitrate to be reduced to nitrite in the rumen. At half the feed intake but the same crude protein intake, nitrite accumulation was higher when the same nitrate load was introduced into the rumen (Table 6). Again Bryant (I965) showed that rumen fluid from cows fed clover did not produce nitrite from nitrate perhaps because of the high level of rapidly fermented substrate in the incubation flasks and the potentially low NR-SOB as postulated earlier.

Further support for this argument comes from Hubert and Voordouw (2007), who demonstrated that it was the ratio of lactate to nitrate in the medium that determined whether nitrite or ammonia was produced by *Sulfurospirillium* species (a NR-SOB isolated from oil reservoir water). However it still appears probable that in the rumen the population density of any NR-SOB is highly dependent on sulphide content of the incubation medium which is directly related to dietary protein. This is further supported supported by the studies of Sapiro *et al* (1947) and Barnett and Bowman (1957).

From the research reported it is speculated that;

- Rumen NRB convert nitrate to ammonia without production of nitrite and mainly by assimilatory pathways.
- A small specialized population of NR-SOB are universally present in the rumen and population density is controlled by the extent and pattern of availability of nitrate and sulphide. .
- NR-SOB are versatile organisms with a wide spectrum of sources of nutrients but when nitrate is available and hydrogen sulphide concentration is adequate they reduce nitrate to ammonia without the intracellular intermediate, nitrite being released to the medium.
- The rapid reduction of nitrate by both NRB and NR-SOB effectively compete with SRB for the electrons generated in fermentation, lowering sulphur reduction to sulphide. Decreased sulphur reduction and increased sulphide oxidation lower sulphide concentrations in the rumen which then inhibits ammonification of nitrite in NR-SOB and nitrite accumulates in the medium.

This then suggests that nitrite is not normally produced by one group of organisms to be used by a second group in the rumen. The second point may be the most contentious as it is hypothesised that in the rumen nitrite is only produced in small quantities and only by the NR-SOB

Figure 22. The effects of crude protein content of a diet on peak nitrate following injection of 30 g Na-nitrate intra-ruminally (Takahashi et al 1980).Each point is the mean of 4 measurements.

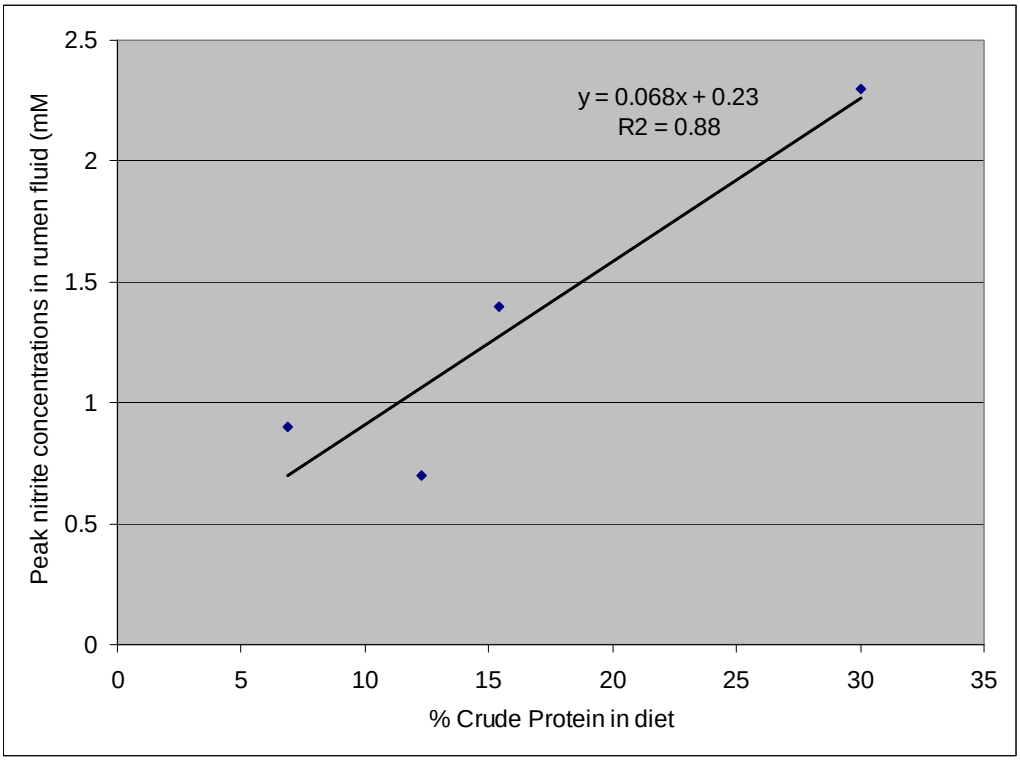


Table 6. The effects of protein intake on the accumulation of nitrite in rumen fluid of sheep following the intra-ruminally administration of 30g Na-nitrate (Takahashi et al 1980). The values in brackets are standard errors.

Treatments	Feed intake (g/day)	Crude protein(% dry matter	N intake (g/day)	Peak nitrite in rumen fluid (mM)	Peak nitrate in rumen fluid (mM)
1	843(86*)	12.3	21.0(0.7)	0.7	25
2	488(26)	30.7	20.4(0.1)	2.3	28
3	784(47)	6.9	11.8(1.8)	0.9	29
4	490(20)	15.4	11.8(1.8)	1.4	28

Speculating, that it is the interaction of electron availability and population density of NR-SOB that determines whether nitrite accumulates it would seem that the following conditions explains the different production rates of nitrite in Table 6.Figure 22 .

- Treatment 1 Low population of NR-SOB; high availability of electrons
- Treatment 2 High population of NRB-SOB; low available reducing capacity high inhibition of SRB
- Treatment 3 Low population of NR-SOB; high available reducing capacity
- Treatment 4 High population of NR-SOB; low reducing capacity with partial inhibition of SRB

The postulated population density of NR-SOB would increase with increasing protein intake but the availability of reduced cofactors would affect the degree of inhibition of SRB and therefore the availability of hydrogen sulphide for nitrite ammonification by NR-SOB

6.4 An unanswered question: Do some rumen bacteria store nitrate-N?

The literature on nitrate metabolism in the rumen has produced a number of conundrums that may be explained if, in ruminants with nitrate in their diet, there are bacteria in the rumen that can temporarily sequester nitrate. This leads to a hypothesis that such organisms can store nitrate-N and mobilize it to provide ammonia when this is depleted in rumen fluid. The reasoning behind the development of this hypothesis is:

1. The initial rapid clearance of nitrate from rumen fluid after a load of nitrate is given over a short time period. This was very evident in adapted sheep on purified diets with nitrate as the sole N source, where more than 80% of the nitrate (560 of the 700mM) administered in 500g of feed disappeared in 1 h (Tillman *et al* 1965) but fermentation in the rumen was continuously high (as indicated by rapidly falling Ph of rumen contents) for up to 9 h.
2. The apparent rapid uptake of nitrate in the rumen of sheep when they are adapted to nitrate and it is included in the feed (Aloubadi and Jones 1985).
3. The continuing accumulation of ammonia in rumen fluid after a nitrate load has been cleared from the rumen (see Figures 19 and 21, Tillman *et al* 1965).
4. The dilution of ^{15}N labelled nitrate following injection into the rumen of cattle (Figure 9, Wang *et al* 1961). In this case the nitrate in rumen fluid would need to equilibrate with nitrate stored as such in rumen microbes

Blake *et al* (1983) postulated that when rumen ammonia levels are high that rumen bacteria may store some ammonia N in intracellular amino acids particularly alanine which may be released to rumen fluid when ammonia levels decline below a critical level. However, the extent of such “storage” may not be sufficient to explain the large quantities of nitrate disappearing from the rumen over a short time interval following an intra-ruminal load of nitrate. However, such

a cycle would be consistent with the rapidity of the nitrate reductase reactions converting nitrate to ammonia but it does not explain the data of Wang *et al* (1961), that appears to demonstrate a continuing entry of nitrate into the rumen from sources other than that provided that day. However Wang *et al* (1961) drenched their cow daily with a nitrate load and the apparent nitrate entry into the rumen could be an artifact of the experimental approach.

Nitrate or nitrate -N accumulating organisms would provide a distinct advantage to using nitrate as a fermentable nitrogen source where intake may be irregular as in extensively grazing animals where nitrate would be accessed maybe only once a day from a supplement. In such situations the availability of nitrate-N in the rumen would be maintained for some time and availability could also be better synchronized with feed intake.

The possibility of nitrate -N accumulation have not been examined in either enteric or rumen microbes. However recent studies of ecosystems in anoxic sediments below both marine and fresh water have demonstrated that some bacteria under anaerobic conditions are capable of accumulating considerable amounts of intracellular nitrate which is potentially conserved until nitrate becomes limiting (see Sayama 2001).

Fossing *et al* (1995) reported a large intracellular pool of nitrate in a filamentous sulphur bacterium, *Thioploca* species in marine ecosystem at a water depth from 40- 280 meters in an upwelling region off the coast of Peru and Chile. Intracellular nitrate concentration in vacuoles in these organisms contained up to 500mM. *Beggiatoa* species from the Bay of Concepcion, Chile, also concentrated nitrate intracellular from 15 to 116 mM (Teske *et al* 1999). A respiratory conversion of nitrate to ammonia driven by oxidation of hydrogen sulphide or elemental sulphur is indicated as the main pathway of metabolism for the intracellular nitrate (see Sayama 2001). These organisms may thus be classified as NR-SOB and have some things in common with the organisms that reduce nitrite and oxidize sulphide as discussed for oil field organic fermentations (see Grigoryan and Voordouw 2008). Recently a new genus of nitrate-accumulating sulphur bacteria, *Thiomargarita*, has been discovered in sediments off the Namibian coast that also oxidizes sulphide with nitrate that is accumulated to a concentration of 100 to 800 mM in a central vacuole (Schulz *et al* 1999). Of major significance is that the intracellular nitrate accumulated in the vacuoles is reduced to ammonia by an assimilatory process and does not enter the denitrification pathway (see Sayama 2001).Such levels of accumulation in rumen bacteria may account for some of the nitrate loss rate from the rumen of 120mmol/L/h calculated from the research of Tillman *et al* (1965).

Nitrate-N accumulation in the rumen bacteria would greatly enhance the efficiency of utilization of nitrate as a fermentable N source. It would therefore improve the potential to feed nitrate to reduce methanogenesis in ruminants. It is fairly easily examined by the use of labeled nitrate and it should have a high research priority.

7 CONCLUSIONS - USING NITRATE AS A NON-PROTEIN NITROGEN SOURCE FOR RUMINANTS

- Nitrate reduction to ammonia can replace the reduction of carbon dioxide to methane as a major sink for disposal of hydrogen in the rumen when included in suitable amounts in ruminant diets.
- Nitrate as a sole fermentable N source in a diet could totally inhibit enteric methane production by ruminants.
- Nitrate can provide a major proportion of the ammonia N for assimilation by rumen organisms as intracellular ammonia or ammonia released to the rumen medium.
- The barrier to the use of nitrate in the diet of ruminants to reduce enteric methane production is the potential for the accumulation of nitrite in the rumen that is anti-nutritional and can cause reduced productivity and even death when absorbed.
- Nitrite production from nitrate in the rumen may be prevented or ameliorated by simple feeding management. There are numerous feeding systems where nitrate may be used to provide fermentable N and reduce enteric methane. These are mostly where the available feed resources are low in true protein and require supplementation with urea which would be replaced with nitrate.
- The use of nitrate in high protein feeds is unlikely to be practical. It appears that excess protein predisposes the generation of nitrite from nitrate. It would also be illogical to add extra nitrogen into such diets that would inevitably increase the excretion of N by the animal, potentially leading to release of nitrogen oxides/methane from the excreta. Nitrogen oxides (NO_x) being considerably more potent green house gas than methane.
- Understanding the metabolism of nitrate in the animal will be the key to enable the use of nitrate as a nutrient and as anti methanogenic compound in the rumen.
- Dissimilatory nitrate reduction and assimilatory nitrate reduction (respiratory nitrate ammonification) pathways appear to be present in numerous microbial species in the rumen and, in sheep with nitrate as the sole source of nitrogen, the assimilatory pathway of nitrate reduction is dominant. As the latter pathway is associated with energy conservation as ATP, feeding nitrate may increase microbial growth yields leading to better protein nutrition than if urea was the N source.
- The efficiency of feed utilisation by ruminants maybe improved since the electron sink-ammonia is incorporated into microbial biomass in the rumen. From 2-15% of the gross energy is lost in methane.
- An important issue is that the microbial species that may occupy a nitrate fed rumen system have received little attention from rumen microbiologists and there are possibilities of new organisms presenting themselves under these conditions.
- In the past scientists have dismissed the use of nitrate as major N source for ruminants because of the risks of toxicity associated with the accumulation of nitrite in the rumen.
- The repeated use in research, of what may be described as the “toxicology approach” appears to have masked the high potential of nitrate as an alternative to urea. From a nutritional viewpoint, nitrite accumulation in the rumen appears to be an artefact of the experimental approach which mostly administers nitrate as a single dose given over a short time interval.
- In sheep acclimated to nitrate their capacity to use nitrate and nitrite increased by up to 10 fold. Nitrite accumulation in the rumen was insignificant and transitory when ruminants were adapted to nitrate feeding, even with dose rates equivalent to those required to supply fermentable nitrogen at sufficient rates to support optimal microbial growth efficiency.

- It appears that only modest increases in the capacity of the rumen organisms to metabolize nitrate/nitrite results from once a day administration of nitrate when the diet is high protein forage. The rate of adaptation of rumen organisms to nitrate on low protein substrate is unknown.
- The capacity to use nitrate by the milieu of rumen microbes in ruminants adjusted to nitrate inclusion in their diets is returned within 21d to that in the unadapted animal once nitrate is removed from the diet.
- Most microbes that reduce nitrite to ammonia by the assimilatory pathway are able to dissimilatory convert nitrate to nitrite. Conversion of nitrite to ammonia by assimilatory nitrite conserves energy as ATP. The latter enzyme is suppressed and inhibited by ammonia. Nitrite would likely be a partial end product of nitrate metabolism at high nitrate inclusions in a culture medium or when ammonia levels are excessively high.
- There is direct evidence for increased requirements for sulphur in a diet supplemented with nitrate but the interrelationships need to be established.
- High levels of Mo in a diet appear to effect SRB and in turn the rate of nitrite accumulation that increase the risk of methaemoglobinaemia.
- Nitrite accumulation in the rumen may be controlled by administration of cysteine a potent source of hydrogen sulphide. However the amounts required make this an impractical solution.
- There is a strong possibility that cysteine applied with nitrate stimulates organisms that have the capacity to oxidise sulphide and reduce nitrate/nitrite directly to ammonia.
- Sulphur nutrition and sulphur metabolism in the rumen are apparently intimately associated with nitrate and nitrite metabolism in the rumen. The rational application of nitrate in feeding ruminants will depend greatly on understanding the interaction that occurs between the various groups of organisms that are involved.
- Newly discovered organisms that reduce nitrate and oxidise sulphide (NR-SOB) from non rumen ecosystems and their peculiarities in metabolism appear to provide possibilities for explaining the patterns of metabolic end product production in the nitrate fed rumen.
- Theoretically, the presence of a NR-SOB could be the major organism responsible for nitrite spilling when nitrate is introduced into a diet.
- It is hypothesised that the population density of a rumen NR-SOB is controlled by the mean sulphide concentration in rumen fluid. Sulphide in rumen content is dependent on the dietary protein content of grazing ruminants. This suggests that low protein diets would support minor populations of these organisms in the rumen providing support for the research that as already demonstrated the ability to effectively replace urea with nitrates in low protein diets.
- A theoretical model is presented to attempt to explain why nitrate is toxic in some feeding systems. Testing the hypotheses should provide strategies for future use of alternative N sources in ruminant production systems.
- There are numerous feeds used throughout the world for ruminant production that require supplementation with a source of fermentable nitrogen to balance the diets for production. These include traditional grain based feeding systems as adopted by the large feedlot industry. Urea has been the traditional source of rumen ammonia but with careful slow adjustment (a requirement which also applies to urea feeding) there seems to be no impediment to replacing urea with nitrate with possibly better results (more efficient microbial growth and less energy losses as methane).
- The application of nitrate as the sole source of fermentable nitrogen in growing sheep fed purified diets and the recent research showing that nitrate can be used to totally to replace urea in a forage/sugar diet given to young goats maintaining them in positive nitrogen balance is evidence that nitrate has a role to play in curbing enteric methane production.

Crop residues and mature pastures represent such diets which are often the major feed of ruminants in developing countries.

- It seems feasible to supply nitrate to well balanced diets and substantially reduce methane production in sheep. The level of nitrate fed and the reduction in methane requires research to establish the relationship but theoretically a 1% increment in K-nitrate in a diet will reduce methane production by 10%.
- Numerous possibilities exist for replacing urea with nitrate in unconventional feeds that are being used for ruminant production in various parts of the world, for example diets based on sugar cane, sugar beet, molasses or cassava. Great possibilities exist to replace urea in block licks for animal grazing stubbles and dry pastures. Supplementation of urea and minerals is a consistent requirement of grazing industries throughout the world.
- Recent developments using straw treated with alkali to improve digestibility of straw has seen ruminant production on these materials with urea and appropriate supplements approaching those obtained on grain based feedlot diets. There seems to be little impediment to replacing the urea with nitrate in these feeding systems. There are 35 million tonnes of straw that are mostly wasted annually in Australia and with a paradigm shift in management this could support 5-10 million cattle.
- With research and successful application of nitrate feeding of ruminants on low protein feeds, a paradigm shift in pasture plant breeding and management may be needed. One aim may be optimising biomass production without regard to high protein content in future pasture grasses. Depending on the interactions of nitrate and ammonia in the rumen, cattle and sheep production may come to depend on low protein forage supplemented with nitrate or forages with a high content of secondary plant compounds that protect protein from degradation in the rumen as a future objective of the pastoral industries.
- The success of any strategy to use nitrate as the major fermentable N source and at the same time secure a significant reduction in methane production from highly productive ruminants on high quality feeds will require feed processing to partially protect the protein in these feeds from ruminal fermentation and enhancement of the energy content of the feed so that nitrate can be added to the diet.
- Achieving high levels of animal production (meat, milk and fibre) on feeds with optimum nitrate for optimum methane inhibition will be possible when there is a better understanding of how to optimise microbial growth at high energy intakes. One issue requiring further work is the requirements of rumen organisms for fermentable N and preformed amino acids and peptides and the animal for dietary bypass protein.

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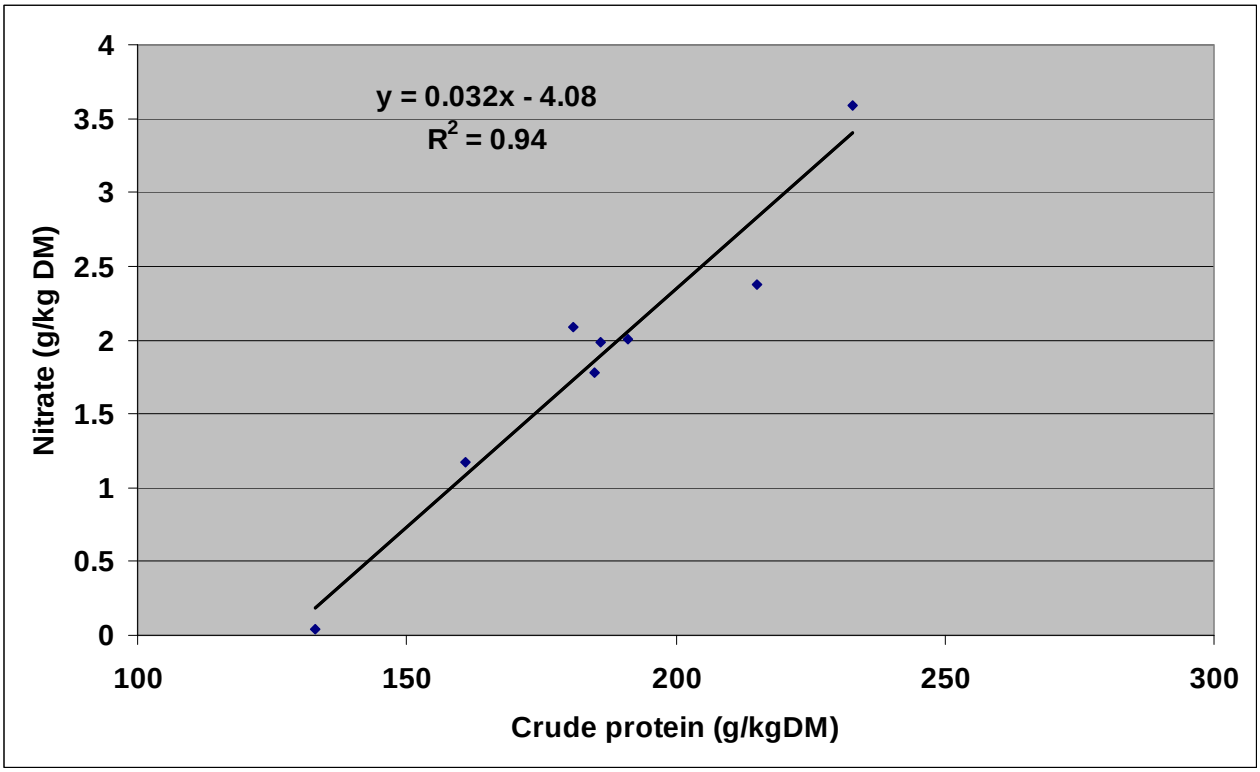
MATERIAL ADDED AFTER SUBMISSION OF THE REPORT TO THE DEPARTMENT OF CLIMATE CHANGE; SUPPORT FOR THE ROLE OF NR-SOB IN NITRATE POISONING.

1) The relationship of protein and nitrate in forage crops

Lovett *et al* (2004) reported on the effects of nitrogenous fertilizer on the protein content of perennial rye grass forage and its regrowth and showed that protein content increased with level of fertilizer application. Nitrate content of the herbage also increased with increasing protein content (see Figure 23) with a surprisingly high linear correlation. Marais (1994) also reported an almost identical response to fertilisation of kikuyu grass and protein and nitrate levels were related although in this case the correlation coefficients could not be calculated from the data presented.

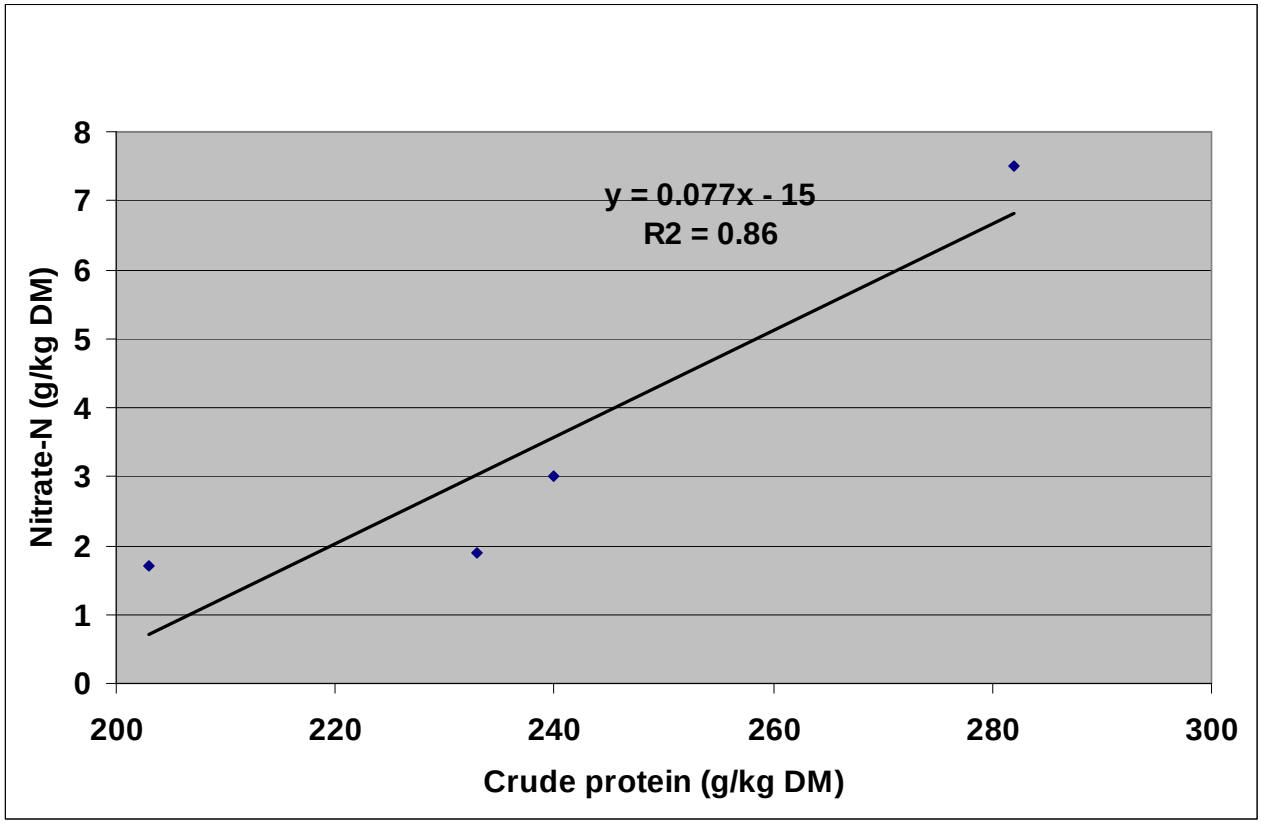
Figure 23 Relationship between nitrate and crude protein content of perennial rye grass (Lovett *et al* 2004)



The relationship between nitrate and crude protein in alfalfa (lucerne) regrowth produced in growth chambers is also of interest since alfalfa appears to have a much greater accumulation of

nitrate at higher crude protein contents (see Figure 24). This may be important as alfalfa has often been the basal diet where nitrate metabolism of ruminants has been studied. For example Cheng *et al* (1985) when stepwise adjusting cattle to nitrate that were fed solely on alfalfa hay, found that at a dose rate of 0.4g nitrate/kg body weight; all cattle succumbed to nitrate toxicity. This could have been related to the level of nitrate in the alfalfa hay that would have influenced the population densities of both NRB and NR-SOB established in the experimental animals and therefore the reaction to excessively high nitrate administration. Unfortunately nitrate in the alfalfa hay was not reported.

Figure 24 Relationship between nitrate and crude protein in alfalfa grown under increasing N fertilizer application (Cherney *et al* 1994)



The two relationships shown in Figures 23 and 24 provide strong evidence that NRB populations would be established in grazing animals under conditions where nitrate poisoning could potentially occur and their population densities would be determined largely by the nitrate in the forage. The proteins in grasses are 60-70% Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase), which is highly soluble and readily fermentable and this leads to a high availability of S –amino acids (mainly cysteine) that are rapidly converted to hydrogen sulphide. The effects on hydrogen sulphide concentration of feeding either white clover or perennial rye grass forage to dairy cows following a 4 hour fast are shown in Figure 25. Fasting for this length of time reduced the hydrogen sulphide to zero in the rumen gas space and as sulphide is readily protonated and released to the gas space, the increase in hydrogen sulphide into the gas space represents the pattern of production of sulphide in the rumen.

Figure 25. Effects of a meal (9.5 kg wet weight) of white clover or perennial rye grass on the concentration of hydrogen sulphide in the rumen head gas-space of dairy cows (Dewhurst *et al* 2007).

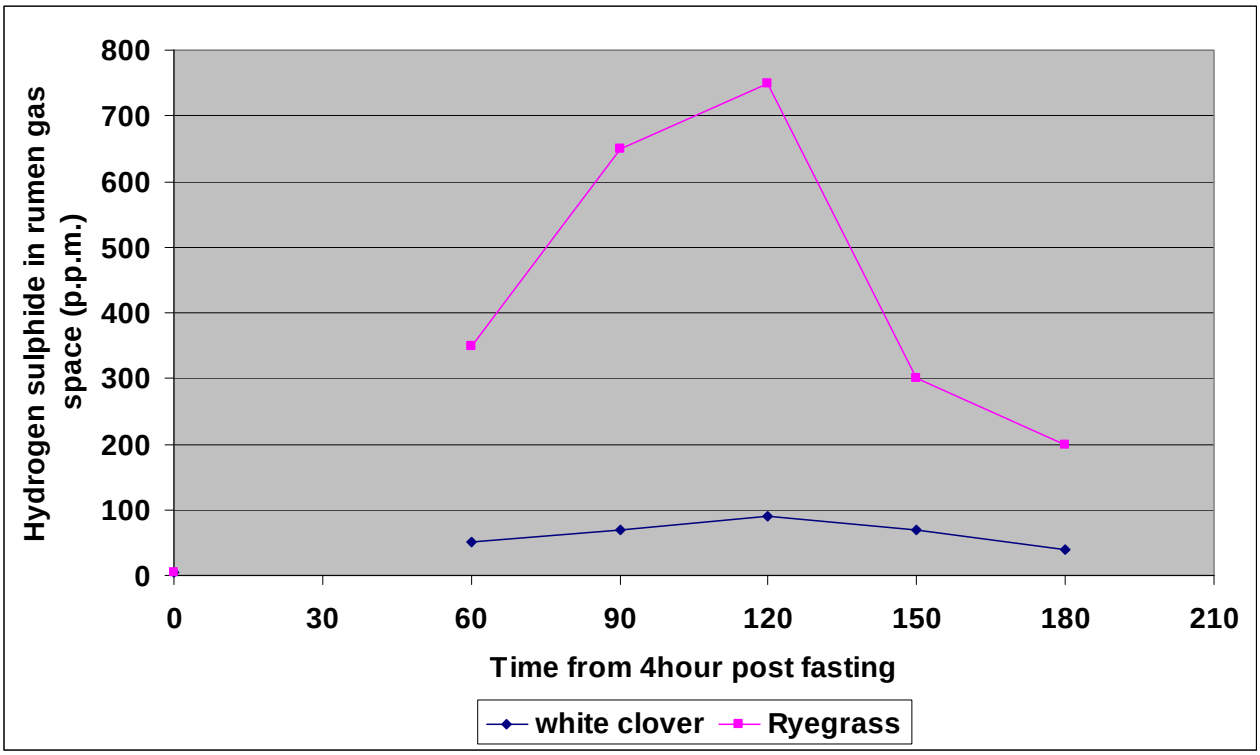
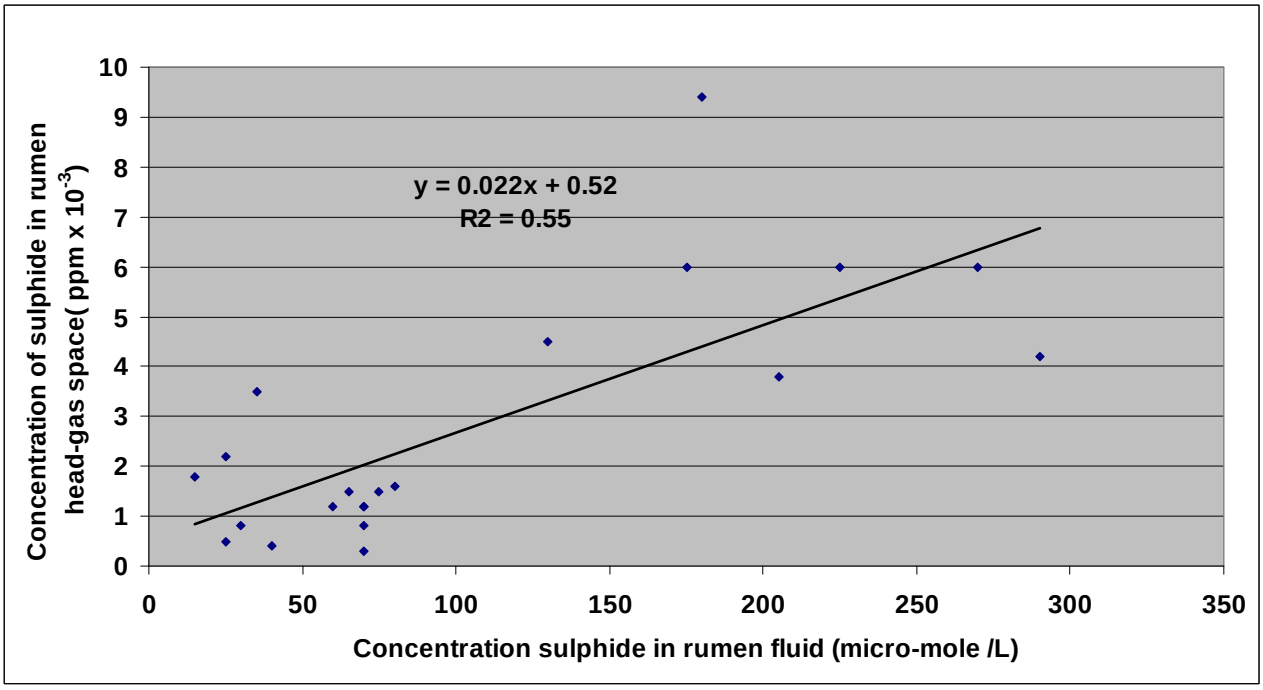


Figure 26. The relationship between hydrogen sulphide in rumen fluid and in the gas space of the rumen of cattle (after Gould *et al* 1997)



There is a reasonable relationship between hydrogen sulphide in rumen fluid and in the rumen gas space in cattle (see Figure 26 after Gould et al 1997)) As the major sources of sulphur in the rumen of cattle on forage based diets are the S amino acids then it is hypothesised that the level

of sulphide in rumen fluid increases with protein content of the forage. This appears to be strong evidence for a population of NR-SOB to be controlled by protein content of the diet which in turn determines the amounts of nitrate available. When photosynthesis is impeded, nitrate levels can increase 10 fold and the degree of inhibition of SRB because of competition for electrons by NRB may result in the lowering of sulphide in rumen fluid and the extent of nitrite accumulation would depend on the population density of NR-SOB that had been established by the protein and nitrate content of the herbage, prior to the accumulation of nitrate brought on by environmental factors.

An interesting aspect of the studies of Dewhurst *et al* (2007) is that white clover fed to fasted cows resulted in much lower sulphide concentrations in the rumen gas space. This suggests that despite having a higher content of protein that is more readily fermented then that of perennial rye grass, that either there is much less hydrogen sulphide produced or more of the hydrogen sulphide was metabolised in the rumen from clover relative to perennial rye grass. This is an important area for research.

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