Some frontiers of research in basic ruminant nutrition

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The primary objective of research scientists working in the field of rumen function is to advance animal production through greater understanding of the mechanisms involved in digestion and metabolism in the rumen. This article covers some of the more recent developments in the field together with some ideas on meeting the challenge of advancing Animal Science. Recent research on energy metabolism covers mechanisms of energy generation in anaerobes and their influence on the efficiency of the ruminal fermentation. Factors affecting microbial protein synthesis and the extent to which dietary protein leaves the rumen undegraded (bypass protein) and which are critical for the efficient use of dietary N are reviewed. Factors limiting digestion of plant material, treatment of low quality roughages to improve digestibility and the use of solid substrate fermentors are discussed. The nutritional dependence of ruminants on their microflora makes them especially suited to genetic manipulation of the rumen ecosystem. Ideas on the genetic modification of rumen bacteria are covered. The importance of technology and methodology in the advancement of science is discussed briefly together with a few examples of recent advances in analytical instrumentation.

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Die hoofdoel van wetenskaplikes wat navorsing doen oor rumenfunksie is om diereproduksie te bevorder deur die prosesse wat plaasvind tydens vertering en metabolisme in die rumen beter te verstaan. Hierdie artikel bevat inligting oor sommige van die jongste ontwikkelings in die veld tesame met idees om die uitdaging van bevordering van Diereproduksie die hoof te bied. Onlangse navorsing oor energie-metabolisme sluit die meganismes van energie-opwekking in anaerobe en die invloed daarvan op die doeltreffendheid van fermentasie in die rumen in. 'n Oorsig word gegee van faktore wat mikrobiese proteïensintese beïnvloed en die mate waartoe die voerproteïen die rumen onverteerd verlaat (verbyvloeiproteïen) en wat kritiek is vir die effektiewe gebruik van voer-N. Faktore wat vertering van plantmateriaal beperk, die behandeling van laekwaliteit-ruvoer om verteerbaarheid te verbeter en die gebruik van onoplosbare-substraat-fermentors word bespreek. Omdat herkouers afhanklik is van hul mikrobiese populasie vir voeding, is hulle veral geskik vir genetiese manipulasie van die rumenekosisteem. Idees in verband met genetiese veranderings van rumenbakterieë word bespreek. Die belangrikheid van tegnologie en metodiek in die vooruitgang van die wetenskap word kortliks bespreek asook 'n paar voorbeelde van onlangse vooruitgang op die gebied van analitiese instrumentasie.

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Introduction

Feedstuffs consumed by ruminants are all initially exposed to the fermentative activity in the rumen prior to gastric and intestinal digestion. Dietary polysaccharides and protein are generally degraded by the ruminal micro-organisms into characteristic end-products which in turn provide nutrients for metabolism by the host animal. The extent and type of transformation of feedstuffs thus determines the productive performance of the host. The anaerobic nature and other characteristics of the ruminal fermentation place an upper limit on the potential nutrient yield. However, the ruminant animal provides a system for providing human food from materials which cannot be utilized directly by man and this casts the ruminant in a central role in the total effective use of the world's nutrient resources. It is anticipated that the world's population will be of the order of 6,5 billion by the turn of the century, and that about 5 billion of these people will be in countries with food scarcity (Phillips, 1981). In South Africa alone the population will have increased from 25 million in 1980 to 40 million by the year 2000, and to some 80 million people in 2020. Thus, as we contemplate the situation in the year 2000, the prospects for achieving either an adequate level of production of animal products in relation to overall world needs, or a reasonable balance in supply of animal products between the developing and developed countries, are not encouraging (Phillips, 1981).

The primary objective of research scientists working in the field of rumen function is to advance animal production through greater understanding of the mechanisms involved in digestion and metabolism in the rumen. Despite a large amount of research which has clearly led to improvements in animal production, full benefits still remain to be realized because our knowledge is incomplete and in many cases qualitative rather than quantitative (Baldwin & Allison, 1983). Recently both the American Society of Animal Science (1983) and the American Dairy Science Association (1981) celebrated 75 years of activity with reviews of progress in the field especially over the last 25 years. Attention is directed towards the papers by Russell & Hespell (1981) and Baldwin & Allison (1983) on rumen metabolism. This article will attempt to cover some of the more recent developments in digestion and metabolism in the rumen together with some ideas on meeting the challenge of advancing Animal Science into the 21st Century — a mere 15 years hence.

Energy metabolism in rumen bacteria

All organisms require a continuous supply of energy to maintain their structure, to grow and to multiply. Nature has

selected adenosine 5'-triphosphate (ATP) as the carrier of this biologically useful energy and all energetic processes in living cells are coupled, directly or indirectly, to the conversion of ATP to ADP or AMP (Gottschalk & Andreesen, 1979). Until recently it was thought that most anaerobes synthesized ATP exclusively by substrate-level phosphorylation (SLP). In fact, the majority of ruminal bacteria which ferment a variety of carbohydrates are dependent on SLP for the major portion of ATP synthesized (Hungate, 1966). However, recent research has demonstrated other mechanisms for generating ATP which could have a profound influence on the overall energetic efficiency of the ruminal fermentation.

Electron transfer linked phosphorylation (ETP) in anaerobes

ETP is a means of generating ATP from the flow of electrons through membranes as a consequence of biological redox reactions. In bacteria, electrogenic proton pumps translocate protons across the cytoplasmic membrane from cytoplasm to the external medium. This translocation results in the generation of an electrical potential ($\Delta\psi$), inside negative, and a pH-gradient (ΔpH), inside alkaline, across the membrane (Figure 1). The sum of the two forces is termed the proton motive force (Δp):

$$\Delta p = \Delta\psi - Z\Delta\text{pH(mV)}$$

in which $Z = 2{,}3\ RT/F$, with R the gas constant, T the absolute temperature and F the Faraday constant. This proton motive force can drive energy-consuming processes in the cytoplasmic membrane by a reversed flow of protons and/or positive charges. In this way the energy of the electrochemical proton gradient can be converted into chemical energy such as the synthesis of ATP or into electrochemical energy (including ion) gradients (Nicholls, 1982). Important proton motive force generating mechanisms in bacteria are the cytochrome-linked electron transfer system and the proton-translocating ATPase complex.

Many rumen bacteria have been shown to possess components of electron transfer chains that are essential for ATP synthesis. Nutritional studies with *Bacteroides ruminicola* demonstrated a requirement for haemin and other metalloporphyrins which constitute the prosthetic groups of cytochromes (White, Bryant & Caldwell, 1962; Caldwell, White, Bryant & Doetsch, 1965; McCall & Caldwell, 1977). Other components of electron transfer chains have also been found in rumen bacteria including cytochromes (*Anaerovibrio lipolytica, B. ruminicola, Bacteroides succinogenes, Selenomonas ruminantium, Veillonella alcalescens, Wolinella succinogenes*), quinones (*B. ruminicola, Succinovibrio dextrinosolvens, W. succinogenes*), flavoproteins (*Megasphaera elsdenii*), ferredoxin (*M. elsdenii, Ruminicoccus albus, W. succinogenes*) and Fe/S proteins (*A. lipolytica, B. ruminicola, M. elsdenii, S. ruminantium*). Dawson, Preziosi & Caldwell (1979) measured $Y_{substrate}$ in the presence and absence of uncouplers of ETP (DNP and DCCD) and found that *B. ruminicola, B. succinogenes* and *Butyrivibrio fibrisolvens* could derive 33,50 and 26% respectively of their total $Y_{substrate}$ from electron transfer reactions. $Y_{substrate}$ is the molar growth yield expressed as gram dry cells per mole of carbohydrate fermented.

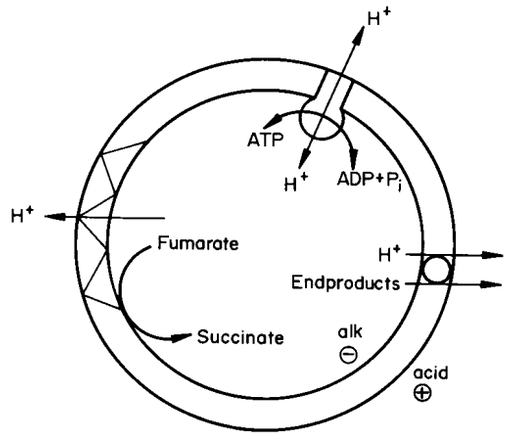

Figure 1 The major systems found in anaerobic bacteria for generating proton motive force: the membrane bound ATP'ase complex, the end-product efflux system and the electron transfer to fumarate as electron acceptor (Konings, Otto & Ten Brink, 1985).

There are four biochemical reactions known to occur in ruminal bacteria which yield ATP via ETP. The most important of these is the reduction of CO_2 to CH_4. The ruminal ecosystem contains at least three species of methanogens, *Methanobrevibacter ruminantium* (Smith & Hungate, 1958), *Methanobacterium formicicum* (Bryant, 1965) and *Methanomicrobium mobile* (Paynter & Hungate, 1968). These H_2-oxidizing bacteria maintain the partial pressure of H_2 at a very low value (Bryant, 1979). From available data, as yet incomplete, it seems that *M. ruminantium* obtains ATP via ETP and that SLP, if present at all, plays a minor role. Uncoupling reagents were found to be potent inhibitors of methane synthesis (Sauer, Bush, Mahadevan & Erfle, 1977; Sauer, Erfle & Mahadevan, 1979). Sulphate-reducing bacteria (*Desulfovibrio* sp.) are known to occur in the rumen (Howard & Hungate, 1976). Intracellular lactate (and pyruvate) oxidation generates H_2 which diffuses across the cell membrane. The hydrogen is oxidized by a periplasmic hydrogenase and the resulting electrons are transferred across the cytoplasmic membrane and utilized in SO_4^{2-} reduction to S^{2-} via an electron transfer chain (Peck & Odom, 1984). From growth yield studies it was calculated that this reduction yields 1 mole ATP per mole sulphate reduced to sulphide. Nitrate reduction to nitrite has been reported in *Veillonella alcalescens* (Inderlied & Delwiche, 1973), *Selenomonas ruminantium* and *Anaerovibrio lipolytica* (De Vries, Van Wijck-Kapteyn & Oosterhuis, 1974). Results showed that for *V. alcalescens* ATP was generated via electron transport from pyruvate to nitrate (De Vries, Rietveld-Struyk & Stouthamer, 1977). *Wolinella succinogenes* isolated from the rumen (Wolin, Wolin & Jacobs, 1961) oxidizes formate (or H_2) and reduces fumarate to succinate. This reaction is coupled to phosphorylation and was uncoupler sensitive (Kroger & Winkler, 1981).

Several other mechanisms exist for the generation of proton motive force although they have not been documented in ruminal bacteria. Important energy-transducing systems in the cytoplasmic membrane of streptococci are the transport systems for solutes (metabolizable substrates and ions). Three distinctly different solute transport systems are found: (i) the PEP-dependent sugar transport system (PTS); (ii) the secondary transport system; (iii) the ATP-dependent transport systems. These systems are responsible for the translocation of sugars (glucose, galactose, sucrose, lactose) for fermentation in the case of the PTS system; sugars, some amino acids, and lactate by secondary transport; and K^+, Na^+, Ca^{2+}, phosphate and some amino acids by the ATP-driven systems (Konings, Otto & Ten Brink, 1985). Proton motive force has been implicated in the transport of peptides by streptococci (Payne & Nisbet, 1981). During fermentation anaerobes continuously excrete metabolic end-products. Additional energy could be obtained if the energy stored in the end-

product gradient (higher internally) could be converted into chemiosmotic energy. This 'energy recycling model' proposed by Michels, Michels, Boonstra & Konings (1979) is the reverse of solute uptake via a secondary transport system. During solute uptake the energy present in Δp is used to drive the accumulation of solute into the bacterial cell, whereas during end-product efflux the energy in the product concentration gradient is used to generate an electrochemical proton gradient (Konings, *et al.*, 1985). A steadily increasing body of experimental evidence in favour of the energy recycling model has been obtained from studies of lactate excretion in streptococci. Homofermentative lactate producing streptococci obtain metabolic energy during sugar metabolism via SLP and lactate excretion (Figure 2).

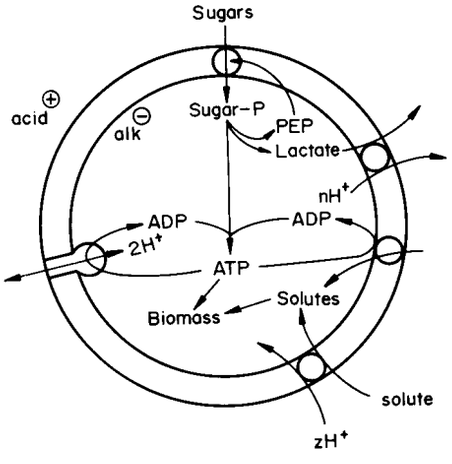

Figure 2 Scheme for the generation and consumption of energy in streptococci during homolactic fermentation of a sugar (Konings, Otto & Ten Brink, 1985).

It has been calculated that lactate efflux could supply an additional energy gain of 50% in streptococci growing on lactose. Continuous culture experiments with *Streptococcus cremoris* showed that both decreasing growth pH and increasing the external lactate concentration resulted in lower $Y_{lactose}$ values (Ten Brink, Otto & Konings, 1984; Otto, Sonnenberg, Veldkamp & Konings, 1980). A beneficial effect of lowering the external lactate concentration (and therefore increasing the lactate gradient) was also demonstrated in continuous cultures of *S. cremoris* where the introduction of a lactate-consuming pseudomonas species reduced lactate concentration from 82 to 33 mM while the molar growth yield of the lactate-producing streptococci increased by 60 – 70% (Otto, *et al.*, 1980). This example of the beneficial interrelationship (mutualism) between lactate-producing and -utilizing organisms helps to explain the relationship which occurs in the rumen between these two metabolic groups of bacteria (Mackie & Gilchrist, 1979). Although there is no information available on the effect of pH on ETP in rumen bacteria, the growth rate of pure cultures (Russell, Sharp & Baldwin, 1979; Russell & Dombrowski, 1980; Therion, Kistner & Kornelius, 1982) and mixed rumen bacteria (Slyter, Bryant & Wolin, 1966; Erfle, Boila, Teather, Mahadevan & Sauer, 1982) is reduced as pH is decreased. Although reduced pH is likely to have multiple effects on metabolism, the translocation of protons across the cell membrane will be disrupted and hence the efficiency of ATP synthesis via ETP will be decreased. Further research on this aspect of the effect of pH on the efficiency of microbial growth would be worthwhile.

Ruminal organisms are subjected to periods of nutritional stress and starvation at different times in the feeding cycle when concentrations of free sugars drop to very low levels and insoluble polymers are being degraded very slowly. The survival capacity of ruminal bacteria is considerably less than that of other bacteria from the soil or aquatic sediments (Mink & Hespell, 1981 a,b). The 50% survival time in *Megasphaera elsdenii* ranged from 3 – 5 h; 0,5 – 2,5 h for *Selenomonas ruminantium* grown under carbon limitation and 3,6 – 8,1 h when grown under nitrogen limitation. Values for *Ruminococcus flavefaciens* ranged from 7 – 25 h (Hespell, 1984). These values can be influenced significantly by growth conditions and growth rate prior to starvation. These results provide an explanation for the considerably higher ratios of viable to direct cell counts on high concentrate and high-quality forage diets when compared to low-quality roughage diets, the beneficial effect obtained from increased frequency of feeding, and the rapid decline in rumen fermentation and function when animals are deprived of feed or go 'off-feed'. It is likely that non-viable, non-growing cells still contribute to metabolism in the rumen. It is also likely that ruminal bacteria would survive longer in the ruminal environment due to the presence of products from lysed cells and fermentation although lysis can contribute substantially to decreased cell yields (see later). These results suggest that ruminal bacteria are unable to reduce their rate of metabolism in an environment with low levels of readily available organic nutrients due to a lack of genetic strategy for such conditions. A proper understanding of the energy status of ruminal bacteria during growth and starvation would need to include measurements of the magnitude of the proton motive force and the adenylate energy charge.

Ionophores

The use of ionophores to increase the efficiency of ruminal fermentation has been the subject of intense research effort since 1976. This article would not be complete without some mention of the effects of these compounds on energy metabolism. The most consistent response to the feeding of ionophores is the increased molar proportion of propionic acid with a concomitant decline in the molar proportion of acetic and butyric acids during VFA production in the rumen (Owens, Weakley & Goetsch, 1984; Bergen & Bates, 1984). A decrease in methane production is often associated with this change in VFA proportions. The increased efficiency of energy metabolism has been ascribed to the lower heat increment of propionate than acetate, an increase in ME available to the host and sparing of amino acids normally used for gluconeogenesis (Bergen & Bates, 1984). Ionophores are also known to influence nitrogen metabolism both in the rumen and the animal. While the animal response to the feeding of ionophores has been well documented their exact mode of action remains largely unknown. Ionophores are compounds that form lipid-soluble complexes with polar cations (Na^+, K^+, Ca^{2+}, Mg^{2+}), serving as carriers by which these ions are transported across cell membranes (Pressman, 1976). Thus, any explanation of the effects of ionophores must focus on the interaction of these compounds with the cell membrane.

Monensin has been shown to affect ruminal fermentation by causing shifts in the ecological balance of organisms (Chen & Wolin, 1979; Wallace, Czerkawski & Breckenridge, 1981). Gram-positive bacteria are the primary acetate, butyrate, H_2 and formate producers and are inhibited by ionophores. Gram-negative bacteria, many of which produce succinate, are resistant because of the complex lipids in their outer

Table 1 Effect of ionophores on ATP pool, proton motive force, and rate of lysis in *Bacillus subtilis**

Ionophore	Mode of action	ATP[a] pools	Rate of lysis ($k \times 10^{-2}$)/min	$\Delta\psi$ mV	$Z\Delta pH$ mV	Δp mV
Control		100	0	90	22	113
Gramicidin	Transports K^+, Na^+, H^+	0	2,6	0	0	0
Valinomycin	Transports K^+	80	3,7	0	20	20
CCCP[b]	Transports H^+	0	3,0	0	0	0
Nigericin	K^+/H^+ antiporter	0	2,1	39	4	43
Monensin	Na^+/H^+ antiporter	0	3,7	85	0	85

*(after Jolliffe, Doyle & Strips, 1981)
[a]Value as a percentage of control
[b]Carbonyl cyanide m-chlorophenyl hydrazone

membrane which protects the inner, cytoplasmic membrane (Kadner & Bassford, 1978). Methanogenesis is probably not directly inhibited by monensin, but is reduced by a diversion of metabolic H_2 from CH_4 production to propionate production (Van Nevel & Demeyer, 1977). These shifts in microbial population are essentially a long-term response or adaptation to monensin. However, a more immediate and direct response of ionophores on the energetics of ruminal bacteria may also be involved. The effects of various ionophores, including monensin, on Δp and ATP levels in *Bacillus subtilis* are presented in the absence of comparable data for ruminal bacteria (Table 1).

Monensin totally dissipated the proton gradient across the cell membrane, causing a decrease in Δp and depletion of the intracellular ATP pool. Thus any ruminal bacteria in which the proton gradient across the membrane is collapsed by the action of ionophores would be at distinct disadvantage unless they could couple this process to electron transport. This is possibly the case with Gram-negative bacteria, whereas Gram-positive bacteria are unable to do so. This is supported by results obtained on the effects of monensin on $Y_{substrate}$ in *Selenomonas ruminantium* and *Bacteroides ruminicola* (which both possess fumarate reductase and are Gram-negative) as compared to *Ruminococcus albus* and *Streptococcus bovis* two common Gram-positive organisms. Monensin addition to pure cultures of *S. ruminantium* and *R. albus* grown in pure culture result in rapid shifts in the acetate: propionate ratio (Table 2).

Table 2 Substrate yield, protein yield and ratio of acetate to propionate in pure cultures of rumen bacteria after adaptation in chemostat culture (D = 0,17h^{-1})

Na^+ conc (mM)	Y_s (mg dry cells/ g glucose)	Y_p (mg protein/ mg glucose)	Ratio (acetate/ propionate)
Selenomonas ruminantium			
10	0,13	0,060	0,25
10+[a]	0,13	0,104	0,44
100	0,24	0,078	0,84
100+[a]	0,24	0,149	0,88
Ruminococcus albus			
10	0,23	0,150	0,79[c]
10+[b]	0,21	0,130	0,93[c]
100	0,24	0,207	0,74[c]
100+[b]	0,18	0,097	0,67[c]

[a]Monensin concentration: 10 µg/ml
[b]Monensin concentration: 0,05 µg/ml
[c]Units: mole acetate/mole glucose

These results (Mackie, Bahrs & Therion, 1984) showed that both Gram-negative and Gram-positive bacteria are able to adapt to the effects of monensin albeit that the concentration of ionophore was 200-fold higher for *S. ruminantium* (10 µg/ml) than for *R. albus* (0,05 µg/ml).

The importance of gradients is demonstrated in Figure 3 which shows that as external pH increases, the transmembrane proton gradient (ΔpH) decreases. Similarly the distribution of Na^+ across the membrane has an influence on ion flux and would be influenced by factors such as the feeding of dietary buffers ($NaHCO_3$) or NaOH-treated roughages.

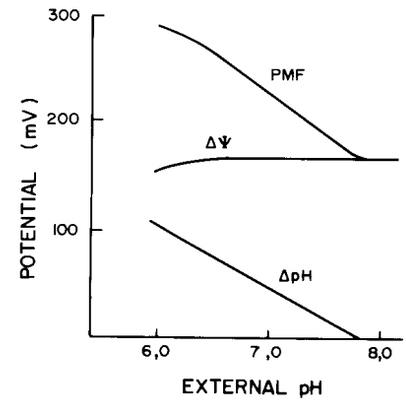

Figure 3 Influence of external pH on ΔpH, electrical potential ($\Delta\psi$) and proton motive force (PMF). When external pH equals internal pH, the ΔpH is negated and PMF is dependent only on $\Delta\psi$ (Booth & Hamilton, 1980).

Flow-microcalorimetry as a tool in the study of microbial maintenance energy requirements

Micro-organisms produced in the fermentation of feed components in the rumen are a major source of protein to the ruminant. Consequently, the availability of protein to the host animal is affected by the efficiency with which the available energy sources and other nutrients in the rumen are utilized in the synthesis of microbial biomass.

On the basis of knowledge of the average composition of the mixed microbial population of the rumen and the ATP expenditure in known biosynthetic pathways of the main classes of macromolecules of microbial cells, expected values of Y_{ATP}, i.e. the yield of cells, expressed as grams of dry mass, per mole of ATP expended, can be calculated. However, experimentally determined values generally fall far short of these predictions. In a review paper on the efficiency of rumen microbial growth, Hespell & Bryant (1979) made a detailed

analysis of factors which contribute to the discrepancy between expected and experimentally determined Y_{ATP} values. Disregard of growth maintenance requirements in the calculation of theoretical Y_{ATP} values could, for the most part, explain this discrepancy. Growth maintenance requirement was defined as the net apparent or real diversion of energy and/or carbon from the growth-limiting or energy-generating substrate to processes not resulting in an increase of cell mass. The maintenance energy requirement not only varies between micro-organisms, but for a given species it is a function of growth rate and it varies considerably depending on growth conditions.

Of a number of probable 'components' of the maintenance energy requirement, Hespell & Bryant (1979) singled out energetic uncoupling as the one which was likely to have the greatest effect on growth yields. This uncoupling is due to the fact that the capacity of most bacteria to produce energy from catabolic processes far exceeds their ability to utilize that energy for biosynthetic purposes. Moreover, the anaerobic bacteria in particular appear to have only coarse control over the rate of energy production whereas ATP levels and ATP production from catabolic processes are subject to fine control mechanisms. Any condition, other than energy limitation, that restricts the growth rate of a population below its potential maximum value will have the effect of impeding the rate of utilization of ATP in biosynthetic processes and thereby decrease the yield of microbial cells per unit of energy substrate utilized. In the rumen ecosystem, the nature of the available nitrogen sources and their concentration appears to play a significant role in this respect. Even though ammonia as the major, and in many cases the sole essential, nitrogen source for bacterial growth occurs in more than adequate pool sizes under most feeding conditions, the provision of small amounts of amino acids or peptides can effect a marked improvement of microbial cell yields. The probable explanation is that such additions may eliminate the necessity of a rate-limiting biosynthetic step for a monomer for protein synthesis and thus increase microbial growth rates and thereby partially relieve energetic uncoupling.

The main method that has been used for the determination of maintenance energy requirements entails lengthy series of measurements of cell dry mass yields in chemostat cultures run at different dilution rates and the computation of maintenance energy coefficients from double reciprocal plots of cell yields versus dilution rates. This very laborious procedure has discouraged systematic studies of the effect of environmental factors such as the nature and concentration of energy and nitrogen sources, pH, osmolality of the medium on the maintenance energy requirements of monocultures, and mixed cultures of rumen micro-organisms. The alternative approach of investigating energetic uncoupling by measuring directly the energy that is dissipated unproductively in the form of heat has received little attention. This is surprising since the studies of Forrest & Walker of the 1960's (see Forrest, 1972, for references) on pure and mixed batch cultures of, amongst others, rumen micro-organisms proved microcalorimetry to be a sensitive tool capable of monitoring closely overall metabolic processes of microbial populations (Figure 4). For the purpose of investigating effects of environmental factors on energetic uncoupling, continuous culture studies would have the distinct advantage that steady-state conditions can be maintained over extended periods and that the effects of step changes in any variable of interest can be followed without interference from associated changes in other variables. Chemostat studies are particularly suited to the study of effects of changes in specific growth rates. Moreover, the commercially available flow-microcalorimeters, the design and performance of which has been refined over the past 15 years, combine well with continuous culture fermentors. Addition of test substances and sampling are performed on the external culture vessel and do not perturb the heat flow recordings of the micro-calorimeter system. Unfortunately, a deterrent to the wider use of this type of system in the study of energetic uncoupling is the high price of the instrumentation.

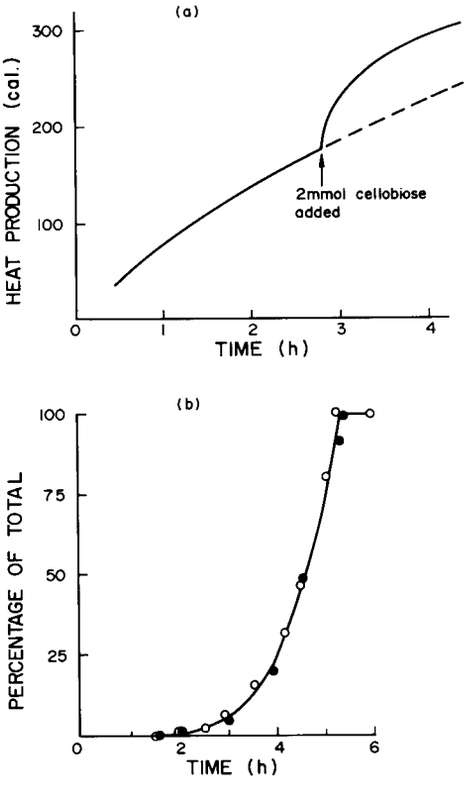

Figure 4 (a) Rate of heat production from ruminal ingesta (b) Relationship between heat production (○) and dry mass of cells (●) in a growing culture of *Streptococcus faecalis* under glucose limitation. (Forrest, 1972).

Nitrogen metabolism in the rumen

In ruminants, the amino acid requirements are provided by microbes synthesized in the rumen and from dietary protein that is not degraded in the rumen but is intestinally digestible (bypass protein). The following discussion will cover some of the factors affecting microbial protein synthesis and the extent to which dietary protein leaves the rumen undegraded which are critical for the efficient use of dietary N.

Protein degradation

A large but variable proportion of the dietary protein is degraded by rumen micro-organisms and it is the rate at which the different proteins can be hydrolyzed which controls the extent of their degradation before they pass out of the rumen. This has an important influence on the proportions of undegraded dietary protein and microbial protein which are presented to the small intestine for digestion by the host animal. Furthermore, this forms the basis of all modern systems for evaluating and predicting protein utilization by ruminants. Although much attention has been focussed on physical and chemical methods of controlling the rate of protein degradation in the rumen, little research has been done

on the factors influencing the proteolytic activity of ruminal bacteria despite the nutritional significance of this activity.

For ruminants receiving diets containing protein, the change in quantity and pattern of amino acids which results from the conversion to microbial protein in the rumen can be an advantage or a disadvantage depending on the composition of the food protein. If the latter is of good quality there is a reduction in value, since the microbial protein is of lower digestibility and is accompanied by nucleic acids. Under these conditions it would be advantageous to limit degradation of dietary protein provided this does not lead to a reduction of the microbial population and their activities such as fibre digestion and VFA production (Smith, 1969). On the other hand, proteolysis during ruminal fermentation may benefit the host animal if the microbial protein synthesized from the products is of higher biological value than the feed proteins (Tamminga, 1979).

Recent work confirms that the proteolytic activity in the rumen is almost entirely associated with bacterial cells and that cell-free rumen fluid and protozoa have little activity towards soluble proteins (Nugent & Mangan, 1981). However, the protozoa play an important role in the engulfment of bacteria and particulate matter and hence degradation of insoluble proteins (Coleman, 1979). Proteolytic acvitity in the rumen is not confined to a single bacterium, but is a variable property possessed by many different bacteria which may be active in the degradation of other feed constituents, mainly carbohydrates. Furthermore, the predominant proteolytic bacteria will differ depending on diet (Mackie, 1982). Although extracellular enzymes are usually produced by Gram-positive bacteria, the most important protease-producing bacteria in the rumen are Gram-negative, including species of *Bacteroides, Selenomonas* and *Butyrivibrio* (Cotta & Hespell, 1985). Other species, probably of less significance are *Megasphaera elsdenii, Streptococcus bovis, Clostridium* spp., *Eubacterium* spp., *Lachnospira multiparus, Succinivibrio dextrinosolvens* and the Spirochaetes (Allison, 1970). Russell, Bottje & Cotta (1981) demonstrated that the Gram-positive *S. bovis* played a predominant role in ruminal proteolysis especially on high concentrate diets.

Proteases have been studied in few rumen bacteria. *Bacteroides ruminicola*, which can utilize peptide nitrogen instead of ammonia, was shown to have proteolytic activity in batch culture that was maximal and mostly (>90%) cell-associated during the mid-exponential phase of growth (Hazelwood & Edwards, 1981; Hazelwood, Jones & Mangan, 1981). The proteolytic activity comprised a mixture of serine, cysteine and aspartic acid proteinases with the possibility that some of the activity is dependent on the presence of metal ions (Mg^{2+}). *Bacteroides amylophilus* produces both cell-bound and cell-free proteolytic activity, which consistently amounts to 80% and 20% respectively of the total activity during exponential growth, but the proportion of cell-free activity increases in stationary phase cultures (Blackburn, 1968). This cell-free activity may be truly extracellular or might indicate that a proportion of bacteria are lysing during growth. In contrast, *Butyrivibrio fibrisolvens* was shown to have proteolytic activity that is essentially all extracellular regardless of stage of growth (Cotta & Hespell, 1985). However, additional strains of these organisms and other proteolytic species require urgent examination before a clear understanding of the proteolytic activity in the rumen will be achieved.

Proteolysis results in oligopeptide production. These oligopeptides then undergo degradation to smaller peptides and amino acids. A comparison of peptide and amino acid use showed that peptides were more effectively incorporated into bacterial protein while a greater proportion of amino acids were fermented to VFA's (Cotta & Hespell, 1985). Thus free amino acids are not incorporated into microbial protein *per se* but undergo rapid deamination providing ammonia for bacterial growth. The deamination and degradation of specific amino acids are of special relevance to bacterial growth in the rumen. The most important of these is the conversion of leucine, isoleucine and valine to isovalerate, 2-methyl-butyrate and isobutyrate respectively. These branched-chain fatty acids are either required or highly stimulatory to the growth of many ruminal bacteria, particularly the fibrolytic species (Bryant & Robinson, 1962, 1963).

Ammonia assimilation in rumen bacteria

Ammonia is an important source of N for the growth of rumen micro-organisms. Depending upon the diet, 60 – 90% of the daily N intake by the ruminant is converted to ammonia and from 50 – 70% of bacterial N can be derived from ammonia (Mathison & Milligan, 1971; Pilgrim, Gray, Weller & Belling, 1970; Nolan & Leng, 1972). Bryant & Robinson (1962) found that 92% of ruminal bacterial isolates could utilize ammonia as the main source of N, while it was essential for growth of 25% of all isolates tested. The fact that many species of rumen bacteria require ammonia and cannot utilize amino acids efficiently is consistent with conditions in the ruminal micro-environment in which little amino acid nitrogen is available for microbial growth and amino acids are rapidly broken down to NH_3, CO_2 and VFA before being utilized for microbial protein synthesis.

Since ammonia is the preferred source of N for most rumen bacteria, enzymes of assimilation are essential to the growth of most rumen organisms. Glutamate dehydrogenase (GDH) and the dual enzyme system glutamine synthetase (GS) and glutamate synthase (GOGAT) are the two most important routes by which ammonia may be assimilated (Hespell, 1984). Joyner & Baldwin (1966) detected GDH activity in nine species of rumen bacteria which were considered representative of about 40% of the rumen microbial population. GS activity has been detected in extracts of mixed rumen bacteria but definitive studies on the second enzyme in this pathway, GOGAT, have only been done on *Selenomonas ruminantium* (Smith, Hespell & Bryant, 1980, 1981). The pathway of ammonia assimilation which operates in the rumen will be influenced by the ammonia level in the rumen although both pathways may be used simultaneously by different organisms and no single pathway operates to the exclusion of the other. When ruminal bacteria are grown under N limitation and intracellular concentrations are $< 0{,}5$ mM NH_3-N the energy-dependent, two-step GS/GOGAT system is utilized. This high-affinity system is well suited to scavenging low levels of ammonia from the environment and these organisms would be at a competitive growth advantage under conditions of NH_3-N limitation. In contrast, the GDH system has a relatively poor affinity for NH_3-N (apparent K_m 2 – 5 mM) and predominates when ruminal ammonia levels are > 1 mM (Hespell, 1984). When comparing data from ammonia-limited and glucose-limited cultures of *S. ruminantium* Y_{ATP} was 30% less in ammonia-limited cultures and about half of this could be attributed to increased ATP utilization by GS during growth under ammonia limitation.

It is possible in the rumen ecosystem that the rate-limiting step in the synthesis of microbial protein is the uptake or assimilation of ammonia rather than the hydrolysis of dietary protein. There is, however, very limited information available

on the transport of ammonia by bacteria. The bacterial cell membrane is basically impermeable and no ions can pass into or out of cells by passive leakage. Furthermore at physiological pH the vast bulk of substrate is present as NH_4^+. Apart from the partial characterization of an uptake system in *Escherichia coli* (Stevenson & Silver, 1977) all other information is based on experiments with fungi or algae (i.e. eukaryotes) and most often indirectly, using the labelled analogue $^{14}CH_3NH_3^+$. These experiments demonstrated energy dependent, concentrative uptake of $^{14}CH_3NH_3^+$ and that NH_4^+ was capable of inhibiting this uptake. A shared permease was thus responsible for transport, with a much greater affinity for NH_4^+. Preliminary experiments with *S. ruminantium* have shown that $^{14}CH_3NH_3^+$ is not transported indicating a highly specific NH_4^+ transport system (Therion & Mackie, unpublished results). Confirmation of this will only be possible using $^{15}NH_4^+$ (see instrumentation). In this respect, the enzymes of ammonia assimilation are likely to be involved in the uptake system since GDH complexes with NH_4^+, and a glutamine-binding protein has been found to function as a binding (transport) protein. Most bacterial active transport systems are secondary and not primary since the transported solutes are species other than protons such as sugars, amino acids, peptides or cations (see energy metabolism). Thus the transport system could either be by uniport, symport or antiport (Rosen & Kashket, 1978).

Influence of ciliate protozoa on nitrogen turnover in the rumen

Ciliate protozoa play an important role in rumen metabolism and considerable attention has been aimed at the measurement of the protozoal contribution to protein flowing from the rumen. In animals fed grain diets at a restricted level of intake where protozoal numbers can exceed 10^6 per ml, protozoa may represent 40 – 60% of the total microbial biomass and up to 80% of the total volume occupied by micro-organisms. Under most dietary conditions the protozoal contribution is less than this. In general, there is an inverse relationship between protozoal and bacterial pool sizes since the protozoa engulf large numbers of bacteria and also compete for available nutrients (Leng & Nolan, 1984). On high concentrate diets, protozoa may have a competitive advantage and also benefit the host by rapidly storing readily fermentable carbohydrate for subsequent fermentation. This may also increase the death rate of bacteria by rapidly reducing the concentration of fermentable substrate in ruminal fluid.

In vitro studies showed that engulfment of bacteria was maximal at pH 6 and that 2×10^6 entodinia per ml could engulf more than 10^8 bacteria per day in the rumen (Coleman & Sandford, 1979). Thus, if the process were continuous, the bacterial pool would be turned over once per day by engulfment alone. Protozoa were also selective in their engulfment of bacteria, preferring mixed ruminal bacterial suspensions. Entodinia species engulfed cellulolytic bacteria more rapidly than other ruminal or non-ruminal bacterial species. This aspect of selective engulfment could have an important influence not only on the microbial ecology of the rumen but also lead to alteration of ruminal fermentation patterns. These findings emphasize that *in vitro* estimates of bacterial engulfment may be too high but it is suggested that 40% of bacteria are engulfed by protozoa daily (Abe & Kandatsu, 1969). Clearly, if this turnover of N within the rumen (intraruminal recycling) could be reduced, the amount of protein leaving the rumen could be increased.

A considerable quantity of N is recycled potentially between the rumen ammonia pool and micro-organisms, both in presence or absence of protozoa, indicating that many organisms grow, metabolize and excrete N compounds, lyse or die and are subsequently fermented in the rumen. The sources of entry into and loss from the ruminal ammonia pool are summarized in Figure 5. Sources of entry into the pool include components soluble in the rumen liquid phase, peptides, and amino acids derived from peptide hydrolysis, influx into the rumen via saliva, protozoal excretion of NH_3, and the turnover of microbial protein and endogenously secreted protein (Leng & Nolan, 1984). Ammonia is irreversibly lost from rumen fluid by incorporation into microbial cells that pass out of the rumen, by absorption through the rumen epithelium, and in fluid passing out of the rumen. Nolan & Leng (1972) found that in sheep fed chaffed alfalfa, 30% of total ammonia flux (4,3 g NH_3-N per day) was turned over in the rumen. In sheep fed chopped wheaten hay with a slower outflow rate, the intraruminal recycling was even higher (50%) (Nolan & Stachiw, 1979). More recently, using a mathematical approach, extensive intraruminal movement between bacterial-N, protozoal-N and ammonia-N pools has been demonstrated *in vivo* (Leng & Nolan, 1984). Using the difference between 'total' growth measured with ^{32}P incorporation and 'net' growth measured using uptake of soluble NPN, Van Nevel & Demeyer (1977) concluded that 50% of microbial protein synthesized was degraded. Defaunation increased net synthesis by 30% without altering total microbial growth (Demeyer & Van Nevel, 1979). This extensive turnover of N in the rumen (30 – 50% of the ammonia flux) is primarily the result of lysis of micro-organisms and secretion of amino acids and ammonia by bacteria and protozoa. Large populations of protozoa and selective retention of them in the rumen increase the recycling of N. Ruminal degradation of protein and amino acids of microbial origin reduces the efficiency of microbial growth and net yield of microbial amino acids to the animal (Chalupa, 1984). It should be remembered that although pathways are well understood, estimates of pool sizes and transfer coefficients are often based on meagre evidence obtained in a single laboratory.

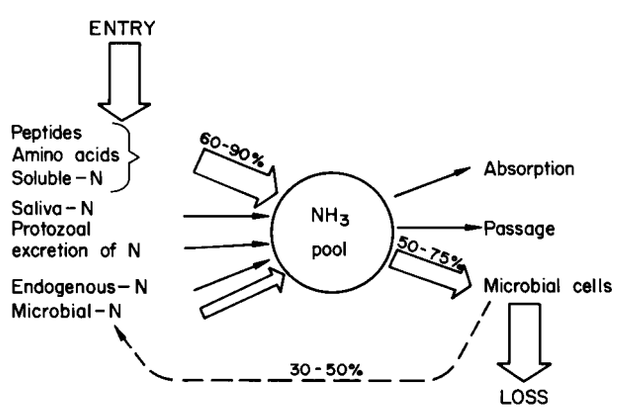

Figure 5 Sources of entry into and loss from the ruminal ammonia pool.

Fibre digestion in the rumen

Factors limiting digestion of plant material

Cellulose accounts for about 40 – 50%, hemicellulose for 20 – 30% and lignin for 15 – 25% of the dry mass of vascular plants (Ward, 1981). The lignin fraction (lignin and other phenolics) and silica are associated with the polysaccharide constituents of the cell walls and contribute to the structural

rigidity of plants. This association accounts for much of the resistance of plant cell walls to microbial digestion with lignin protecting about 1,4 times its own mass of cell wall carbohydrates (Van Soest, 1981). This protection is dependent on covalent linkages between carbohydrates and lignin, so that digestion of other cell constituents that are not part of the cell wall matrix are not affected. On the other hand, there are soluble lignin-hemicellulose complexes that are resistant to ruminant digestion. Thus removal of this limitation of digestion is dependent either on cleavage of the bond between lignin and carbohydrates or hydrolysis of polysaccharides away from the lignified matrix (Van Soest, 1981). A possible mechanism limiting digestion of the polysaccharides is that lignin inhibits attachment of rumen micro-organisms to cell walls (Hartley, 1981). Recent reviews tend to discount the theory that physical encrustation of carbohydrates by lignin limits digestion (Bacon, Chesson & Gordon, 1981; Van Soest, 1981).

Silica is deposited in the walls of epidermal cells, xylem vessels and sclerenchyma deposited in the walls at the same time as the polysaccharides during the process of secondary thickening (Hartley, 1981). Silica is classified as a structural inhibitor forming a barrier to microbial entry and limiting digestion (Harbers, Raiten & Paulsen, 1981). Van Soest (1970) showed that removal of silica from rice straw or tropical grasses by treatment with neutral detergent lead to significnt increases in OM digestibility measured *in vitro*. Dry matter digestibility of shoots obtained from growing cereal plants was 60,4% (12% of dry matter as silica) as compared to 71,1% (0,1% of DM as silica). The digestibility of OM of the shoots containing high silica was also decreased owing to the association of silica with their cell walls.

The lignin molecule is formed by polymerization of phenolic monomers or phenylpropanoid compounds (Hartley, 1981; Jung & Fahey, 1983a). The primary phenolics in roughages are *p*-coumaric and ferulic acids. These acids, termed 'noncore' lignin, may act as cross linkages between lignin and structural carbohydrates. Linkages are of at least three types: cleaved by reduction, cleaved by alkali and resistant to alkali (Morrison, 1974). Ester linkages between 'noncore' lignin and *B*-type (branched) hemicellulose are most prominent in forages. Early work suggested that the phenolic nature of lignin inhibited microbial activity (Crampton & Maynard, 1938). Jung & Fahey (1983b) found that fermentation of common energy sources such as cellulose, starch and protein by rumen bacteria was inhibited by high concentrations of phenolic monomers. The various compounds tested differed in the magnitude of inhibition and substrate affected. Significant metabolism of phenolic monomers appears to occur during fermentation with mixed ruminal micro-organisms (Balba & Evans, 1977; Balba, Clarke & Evans, 1979; Akin, 1980). Although definitive data on the mode of action by which phenolic compounds affect nutrition are not yet available, it is clear that they have a significant effect on nutrient availability. Apart from effects on ruminal fermentation, phenolics form indigestible complexes with proteins and enzymes, bind minerals in the gut, influence lipid metabolism and may have immunosuppressive effects (Jung & Fahey, 1983a).

The digestion of forage fragments in the rumen occurs by adhesion of rumen micro-organisms to plant cell walls. Using electron microscopy, Akin and co-workers (Akin, 1985) have demonstrated that, although many different morphological types of rumen bacteria adhere to forage fragments, coccoid bacteria are most common. Cell wall type or structure has an important influence on digestion rate. Mesophyll cell walls are digested rapidly, followed by walls of phloem and epidermis while the least digestible walls are those of the xylem vessels. Mechanical fracture of the forage material by rumination is a most important factor in exposing cell walls to bacterial attack in the rumen (Latham, Brooker, Pettipher & Harris, 1978a,b). Rumination was found to be responsible for 50 – 75% of the reduction in particle size of forages in the rumen (Ulyatt, Dellow, John, Reid & Waghorn, 1985).

Until recently the major cellulolytic microbes in the rumen were accepted as being anaerobic bacteria. However, fibre-colonizing rumen anaerobic fungi also digest cellulose (Bauchop, 1979) and they are thought to play a significant role in the digestion of fibrous cell walls in the rumen, particularly of vascular tissues such as those present in cereal straws. The anaerobic phycomycetous fungi are well adapted to the gut environment and scanning electron microscopy has shown extensive attachment and colonization of fibrous plant fragments (Bauchop, 1981). Unlike the rumen bacteria and protozoa, the extent of the fungal populations cannot be assessed by enumeration of any stages of their life cycle. It should be possible to assess the quantity of fungal rhizoid tissues using chitin which occurs specifically in these organisms. The relative contributions of the bacteria and fungi to cellulose digestion remain to be measured. Since the fungi heavily colonize vascular tissue and the more refractory plant material it suggests that they may be the microbial agents responsible for the solubilization of lignin in the rumen.

Major advances in the understanding of the biochemical and enzymological mechanisms of lignin biodegradation have been made in the last few years (Crawford & Crawford, 1984). Research has involved two ligninolytic organisms, the white rot fungus (*Phanerochaete chrysosporium*) and the actinomycete (*Streptomyces viridosporus*). Conclusive evidence has been found in *P. chrysosporium* for the involvement of activated oxygen, specifically H_2O_2, in lignin degradation. The activated oxygen is probably held within the active site of an enzyme molecule. The involvement of specific enzymes in lignin degradation by both organisms has also been confirmed including extracellular non-specific peroxidases and oxygenases. They show numerous activities including dehydrogenative, peroxidatic, oxygenative and C_α-C_β cleavages of lignin side chains. The combined activities of these enzymes generate water-soluble polymeric modified lignin fragments. Peroxidases are produced by several ruminal organisms (Holdeman & Moore, 1977; Morris, 1976) but occur generally amongst facultative anaerobes and may be important in breaking key linkages and solubilizing some of the lignin in the rumen.

Treatment of low-quality roughages to increase digestibility

Two recently developed methods for roughage treatment to improve digestibility utilize the principle of activated oxygen to degrade lignin. The Northern Regional Research Centre of the USDA in Peoria has patented a process for treating chopped or ground wheat straw with 1% hydrogen peroxide at pH 11,5. Perhydroxyl radicals are generated which break lignin bonds. At the same time, the crystalline structure of the cellulose is broken down by the treatment. Approximately 50% of the lignin and most of the hemicellulose is solubilized within a few hours and the residue is composed of small, highly water absorbent fibres with a pulplike consistency. The cellulose content of this material is 60 – 65%, compared to 35% in the starting material, and 95% of this is degradable

by enzymes. Feeding trials at the University of Illinois have shown the material to be highly digestible by cattle. At the present stage of development the cost of the cellulosic material is about twice that of maize, which makes its use an uneconomic proposition. However, the position could change if the hemicelluloses in the supernatant from the process could be converted to marketable products. These hemicelluloses are very effectively utilized by *Butyrivibrio fibrisolvens* and a strain obtained from our laboratory was among the most efficient utilizers. The main fermentation products were volatile fatty acids for which there is no great demand, but the pattern of end-products could be changed by genetic manipulation.

The Division of Chemical and Wood Technology of the CSIRO in Australia has developed a different and supposedly cheaper process for upgrading the digestibility of low-quality roughages. It also depends on the breakage of lignin bonds by perhydroxyl radicals, works at ambient temperatures and pressures and generates no pollutants. The treatment with 15% ozone raises *in vitro* organic matter digestibility of wheat straw and bagasse from 30% to nearly 90%. Again, present treatment costs are high, but research is in progress to find uses for the by-products in order to make the process economically viable.

Use of solid substrate fermentors to simulate rumen fermentation

There is currently great interest in the construction of continuous culture systems which allow solid feeding and dual effluent removal designed to simulate the differential flows of liquids and solids found in the rumen (Hoover, Crooker & Sniffen, 1976; Czerkawski & Breckenridge, 1977; Merry, Smith & McAllen, 1983). Some have been quite successful in achieving quasi steady-state conditions and in maintaining concentrations and proportions of fermentation products similar to those in the rumen of the donor animal. However, despite pH control and the differential flow rates for solids (0,05 h^{-1}) and liquids (0,10 h^{-1}) difficulty has been experienced in maintaining a population of protozoa in these fermentors. Little attention has been paid to defining the microbial population which develops in these systems. Furthermore, these systems are too complex for precise quantitative analysis of the experimental variables.

Very little has been published on specific growth rates of ruminal organisms on insoluble substrates since the strong adhesion of the fibrolytic bacteria to their insoluble substrates interferes with the precise determination of numbers, biomass or cultural properties proportional to biomass. Recently Kistner and co-workers have developed a sophisticated system in which the bacteria are grown in pH auxostat or chemostat continuous culture using evenly dispersed ball-milled filter paper cellulose as substrate (Kistner, Kornelius & Miller, 1983). Results obtained at different dilution rates for the extent and rate of solubilization of cellulose by pure cultures of *Ruminococcus flavefaciens* and *Bacteroides succinogenes* are presented in Figure 6.

The extent of solubilization was maximal at low dilution rates and decreased as dilution rate increased. The rate of solubilization, i.e. dilution rate multiplied by the difference in cellulose concentration in the medium reservoir and that in the culture vessel, reached a maximum at a dilution rate of 0,32 h^{-1} for *R. flavefaciens* and 0,18 h^{-1} for *B. succinogenes* (Kistner, Miller & Kornelius, unpublished data). Under the conditions of these experiments and for these particular strains *R. flavefaciens* was a more rapid cellulose digester than *B. succinogenes*. Microscopic examination

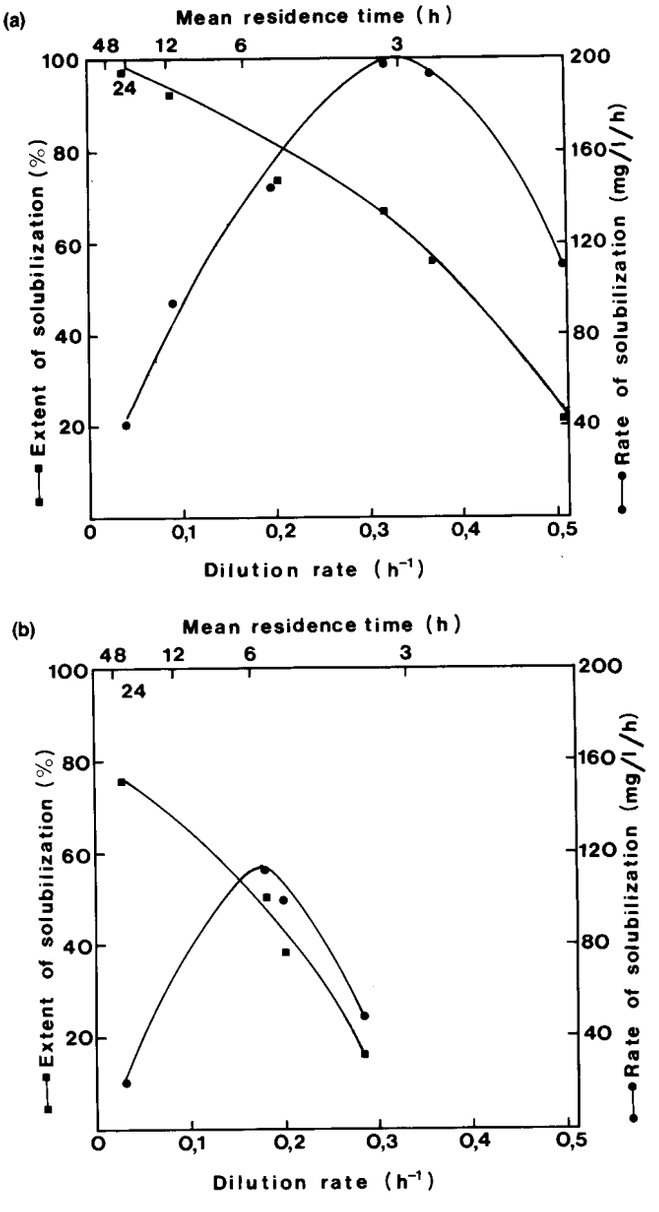

Figure 6 Influence of dilution rate on the rate and extent of solubilization of cellulose by
(a) *Ruminococcus flavefaciens*
(b) *Bacteroides succinogenes*.

showed that at low dilution rates (maximum extent of solubilization) cellulose fibrils were heavily colonized and microbial cells were substrate limited. At the other extreme, cellulose fibrils were lightly colonized although a majority of organisms were attached, with substrate in excess. At a point between these two extremes where the rate of solubilization was maximal, a balance was achieved between attachment of cells and availability of substrate. This work is to be extended to further species of fibrolytic bacteria and other solid substrates more likely to be encountered in their natural habitat such as intact plant cell walls. Further improvement in rates will be dependent on the use of defined mixed cultures or even whole ruminal ingesta as an initial inoculum in order to obtain the beneficial synergistic interactions which are known to occur amongst the ruminal bacteria.

Genetic manipulation of ruminal bacteria

Potential benefits arising from new technology are enormous and agriculture is one of the prime areas where the impact of genetic engineering will be greatest in the future. Most likely

this will be the result of improvement in the quantity and quality of a food crop or animal product through manipulation of micro-organisms associated with them. In this regard, the nutritional dependence of ruminants on their microflora makes them especially suited to this type of improvement.

Manipulation of the rumen ecosystem via genetic modification of rumen bacteria appears to be a reasonable future approach if appropriate genetic systems are developed and adequate biochemical information is available (Smith & Hespell, 1983). Applications for utilizing genetically engineered ruminal bacteria can be divided under the following headings:

(i) Controlled degradation of specific nutrients in feedstuffs, e.g. cellulose, lignin, proteins, and urea.
(ii) Regulation of specific fermentation products, e.g. lactate and ratio of acetate to propionate.
(iii) Controlled growth of specific bacterial species, e.g. *Streptococcus bovis* and methanogens.
(iv) Production of substances beneficial to host metabolism, e.g. specific amino acids, certain vitamins, antibiotics and possibly even hormones.

Any proposed research programme should focus on understanding the mechanisms and utilizing the biosynthetic and fermentative properties of obligate ruminal anaerobes which have been genetically modified for improved energy and protein metabolism in the rumen, and also for industrial fermentation technology.

A recent study tour (Kistner & Mackie, 1984) afforded an opportunity to find out what research is currently being undertaken and is, as yet, unpublished. Most of the information concerned the transfer of genes from rumen bacteria to genetically well characterized species mainly *Escherichia coli*. A multitude of recombinant DNA techniques are available for this. The reverse process, namely the introduction of foreign genetic material into rumen bacteria is not possible at this stage due to lack of knowledge of their genetic constitution and a system for the transfer of genetic material.

At least three groups have succeeded in introducing genes coding for endocellulase production into *E. coli* (Forsberg, University of Guelph, Ontario; Teather, Agriculture Canada, Ottawa; Orpin, ARC, Cambridge, England). In all cases the donor organism was *Bacteroides succinogenes*. This is not merely coincidental but related to the fact that this strongly cellulolytic species is very susceptible to lysis which facilitates DNA isolation. Although expression of endocellulase activity was obtained, this could only be liberated on lysis or rupture of *E. coli* cells. Forsberg was optimistic that exocellulase activity could also be cloned in *E. coli*. Orpin's group appear to have cloned endocellulase genes derived from the anaerobic, cellulolytic, phycomycete fungi isolated from the rumen. There is also a possibility that silage could be inoculated with an engineered organism that would partially degrade lignin and hydrolyse bonds between lignin and hemicellulose, thus exposing more cellulose for degradation.

A new research team under Dr R.B. Hespell at the USDA laboratory in Peoria is planning extensive work on a predominant ruminal organism *Butyrivibrio fibrisolvens* which has a wide range of biochemical capabilities including cellulase, hemicellulase and protease activity. They propose to build up a data bank of the physiological, biochemical and genetic characteristics of this organism over the coming years. Work in the area of proteases would be aimed at reducing the breakdown of feed protein of high biological value or enhancing the biological value of bacterial protein synthesized in the rumen.

A further problem with genetically engineered bacteria is their ability to maintain themselves in the rumen ecosystem in sufficient numbers when competing with naturally selected species and strains. This can be overcome under intensive feeding conditions by repeated re-inoculation with the engineered organism as often as required. Another strategy would be to clone genes for antibiotic resistance (including ionophores) together with the desired biochemical activity into an engineered rumen bacterial strain. The inclusion of a feed antibiotic or ionophore in the diet would then provide a positive selection pressure for the engineered bacterial strain.

Although very little has been published thus far, we can expect a rapidly expanding body of information on genetic manipulation involving rumen bacteria which will not only greatly increase our understanding of the regulation and control of biochemical processes occurring in the rumen but also have an impact on the nutrition of ruminants and the industrial utilization of renewable resources.

New research techniques and instrumentation

The importance of technology and methodology in the advancement of science has been the subject of considerable debate. It has been argued that many conceptual breakthroughs would not have been possible without first developing techniques and instruments to approach basic problems. Indeed, the development of new instruments and methods often leads to unanticipated theoretical innovations (Garfield, 1984). Notwithstanding the comparisons made between the intellectual value of theoretical or conceptual research and that of instrumentation or methodology there is incontrovertible evidence that technology opens up new areas of science.

Rather than an exhaustive review of all advances in analytical instrumentation, the present section presents a sample of a few sophisticated techniques to supplement those already covered in the preceding sections.

Automated ^{15}N Analysis

Nitrogen unfortunately does not have a radioactive isotope of sufficiently long half-life to enable radioactive tracer experiments to be performed. Therefore to carry out tracer experiments with N, the stable isotope ^{15}N must be used. The advantage of using stable isotopes is that there are no health or environmental hazards and there is no radioactive decay. The disadvantage is the more difficult, complicated and more expensive method of detection. The amounts of ^{14}N and ^{15}N, i.e. isotope ratio can be determined by means of mass or emission spectrometric techniques. Compared to mass spectrometry, emission spectrometry is much simpler, since no high vacuum is involved and the instrument can be maintained and operated by any experienced analyst. Although emission spectroscopy is more sensitive requiring much smaller samples ($5-10$ µg N) than mass spectroscopy it is not as accurate (0,01% versus 0,0005% ^{15}N). In our laboratory we have developed, with the assistance of the National Institute of Materials Research, CSIR, an automated system based on a Carlo Erba ANA and a custom-made emission spectrometer capable of analyzing 12 samples containing ^{15}N per hour. A description of the instrument and its performance will be published shortly (Therion, Mackie, Kistner, Human & Claase, 1985).

Intrinsic labelling of plants with stable isotopes

The availability of different nutrients can be assessed by following the fate of the label after digestion and metabolism of endogenously labelled nutrients. The intrinsic labelling

technique is time consuming and specialized and is often done using stable isotopes (^{13}C, ^{15}N) but is particularly well suited to minerals and trace elements (^{65}Zn, ^{75}Se, ^{59}Fe). However, because of the high cost of certain of the stable isotopes efficient labelling of nutrients is imperative (Weaver, 1984). This idea is not new and has been used in the past with radioisotopes (^{14}C) to assess digestion and metabolism in the rumen (Alexander, Bartley & Meyer, 1969).

New spectroscopic techniques for studying metabolic processes

Nuclear magnetic resonance (NMR) spectroscopy has been used extensively in the field of biochemistry in studies of biological macromolecules. Recently high resolution NMR studies have been extended to intact cells, organelles and organs. They provide a rapid and noninvasive technique for the study of metabolism in intact cells, describing and detecting reactions precisely as they occur in the living cells (Shulman, 1983). This technique relies on the fact that atomic nuclei with an odd number of protons and neutrons (^{1}H, ^{13}C, ^{15}N, ^{31}P), have an intrinsic magnetism that makes such a nucleus a magnetic dipole and in an applied magnetic field they have a characteristic resonance. In cells, tissues and organisms the precise frequency of the resonance is also influenced by the chemical environment of the nuclei (Shulman, Brown, Ugurbil, Ogawa, Cohen & Den Hollander, 1979; Shulman, 1983).

It is appropriate to indicate the nature of the metabolic information that can be obtained using NMR spectroscopy. The role of *Megasphaera elsdenii* in the fermentation of [2-^{13}C] lactate in the rumen of dairy cows was investigated using ^{13}C-Fourier transform – NMR measurements eliminating the need for time-consuming VFA separation and degradation studies to determine position labelling (Counotte, Prins, Janssen & deBie, 1981; Counotte, Lankhorst & Prins, 1983). The utilization of and the VFA produced from [1-^{13}C] glucose during anaerobic digestion of manure was investigated using the same technique (Runquist, Abbott, Armold & Robbins, 1981). The combination of ^{13}C-labelled substrates and ^{13}C-NMR has been used to study not only product-precursor relationships but also the activity of metabolic pathways in micro-organisms. Metabolites can be measured repetitively and non-destructively without prior extraction from tissues (Brainard, Hutson, London & Matwiyoff, 1984). This technique is ideal in the study of biosynthesis. Recently the most important biosynthetic pathways in *Methanospirillum hungatei* were followed using ^{13}C-NMR with ^{13}C-labelled acetate and CO_2 as carbon sources (Ekiel, Smith & Sprott, 1983). *In vivo* ^{15}N-NMR was used to study the regulation of N assimilation and amino acid production by *Brevibacterium lactofermentum* (Haran, Kahana & Lapidot, 1983). A recent symposium concerning new spectroscopic techniques for studying metabolic processes includes papers on branched-chain amino acid metabolism (Crout, Lutstorf & Morgan, 1983), *in vivo* kinetic rates involving glycolytic enzymes in *Saccharomyces cerevisiae* and the gluconeogenic pathway in liver and yeast (Den Hollander & Shulman, 1983), and isotope exchange kinetics in enzyme systems (Foxall, Brindle, Campbell & Simpson, 1983). The application of high resolution ^{13}C-NMR of solids using the recently developed technique of cross-polarization and magic angle spinning with proton decoupling has been evaluated for predicting nutritional value of forages (Efloson, Ripmeester, Cyr, Milligan & Mathison, 1984). Solid-state ^{15}N-NMR has also been reported for metabolic studies on intact soybeans and lyophilized seeds, pods and leaves (Skokut, Varner, Schaefer, Stejskal & McKay, 1982).

The application of stable isotopes and NMR to the study of metabolism and its regulation in living systems has unique advantages over traditional biochemical or physiological techniques. It is reasonable to expect that within a few years this technique will revolutionize our understanding of how biochemistry proceeds in living cells including animals and man.

Finally it is obvious that our knowledge of ruminal physiology and digestion is far from complete. The type of research required to make further 'Advances in Animal Science' will need to become more innovative and increasingly sophisticated. It will be most productively attacked by multidisciplinary groups of scientists. The limitations imposed on research in the field of animal science by conventional approaches and techniques will have to be discarded if we hope in any way to meet the challenge of feeding our growing population as we prepare to enter the 21st Century.

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