CHAPTER 4

4. COUPLING OF ATP AVAILABILITY AND MICROBIAL GROWTH

During heterotrophic growth, the yield of microbial cellular materials depends on the potential energy, made available as adenosine triphosphate (ATP), from catabolism of various substrates and on the ATP costs of synthesis of these cellular compounds. The relationship between the supply of ATP and polymer synthesis in anaerobes grown with the fermentable substrate as the limiting nutrient has been termed Y^{ATP} (dry weight of microbes /mol ATP) (Bauchop and Elsden 1960). The approach of Bauchop and Elsden (1960) was the first attempt to show that microbial growth was primarily dependent on the yield of ATP rather than on the availability of substrate ($Y_{substrate}$; dry weight of microbes /mol substrate) *per se* and that differences between organisms in $Y_{substrate}$ arose primarily from the different amounts of ATP derived from fermentation of substrate.

Although the values of Y^{ATP} reported by Bauchop and Elsden (1960) varied both within (9.3–11.5) and between (8.3–11.5) organisms on the same substrate and also between substrates, the authors concluded that under the experimental conditions that were imposed, Y^{ATP} was a constant. This conclusion, supported by other workers such as Payne (1970) and Forrest and Walker (1971), has since been challenged by others such as Isaacson *et al.* (1975), Maeng and Baldwin (1976a) and Stouthamer (1979). Regardless of this debate, a general conclusion throughout the literature is that experimentally determined values of Y^{ATP} are usually less than those based on theoretical ATP requirements for the formation of various cellular materials.

4.1 Maintenance energy requirements

Energy stored in the phosphate bonds of ATP must accommodate the requirements for maintenance (E_m), growth (E_g) and wasteful processes (E_w) (e.g. futile cycles) and *in vivo*, bacterial lysis may further lower the 'true' efficiency of use of ATP for these processes (Nolan and Leng 1972). The energy costs of growth are related to the transport of monomers and the synthetic processes associated with the formation of protein, lipid, polysaccharide, nucleic acids and other cellular materials. Maintenance energy requirements are incurred through such

processes as maintenance of osmotic gradients, stabilisation of internal pH, turnover of cellular materials, motility and intra and extra cellular transport of materials.

Generally Y^{ATP} increases with specific growth rate (h^{-1}) and *in vitro*, the latter is synonymous with dilution rate. This relationship has led to the conclusion that maintenance energy requirements are independent of specific growth rate and are therefore constant (1.63 mmol ATP /g cells /h; Isaacson *et al.* 1975) and progressively consume a smaller proportion of the total energy requirements of the organism as specific growth rate increases. This conclusion does not appear to be valid for all growth conditions with maintenance requirements being at times dependent on specific growth rate (Stouthamer and Bettenhaussen 1973; Neijssel and Tempest 1976; Russell 1986), the nature of the growth limiting material (Nagai and Aiba 1972; Neijssel and Tempest 1976) and the degree of storage polysaccharide accumulation (Russell 1986).

Maintenance energy requirements are generally calculated using the approach of Pirt (1975) where the actual growth yield is related to both growth unencumbered by E_m (theoretical maximum, Y_{max}) and to E_m. In practice this is achieved by a double reciprocal plot of Y^{ATP} or $Y_{glucose}$ versus specific growth rate with the resulting function having slope E_m and the inverse of the intercept Y_{max}. Russell (1986) points out that this approach cannot accommodate compositional changes to cells and if growth conditions favour accumulation of energy storage compounds such as polysaccharides (Dawes and Senior 1973; Isaacson *et al.* 1975), the yield of cells per mole of ATP will increase because of the lesser ATP requirement for synthesis of polysaccharide relative to other macromolecules such as protein and nucleic acid (Stouthamer 1979). The net effect of such compositional changes would be to increase the slope of the double reciprocal plot and hence increase the estimate of E_m. On a similar theme, increases in specific growth rate are often accompanied by an increase in the RNA:protein ratio of the cell and in such instances estimates of E_m would also be affected.

4.2 Uncertainties of Y^{ATP} estimates

Part of the discrepancy between experimentally determined and theoretically based values of Y^{ATP} probably arise because theoretical calculations fail to fully account for the maintenance requirements of the cell. However, correction of Y^{ATP} for this non–productive component does not fully account for the discrepancy (Stouthamer 1979). It has been suggested (Stouthamer 1979) that maintenance of membrane electropotential (Mitchell 1961) may provide an explanation for the discrepancy between experimentally determined and

theoretically based values of Y^{ATP}. During aerobic growth, membrane electropotential is maintained by electron transport systems. However, during anaerobic growth, energy from ATP hydrolysis is predominant. Stouthamer (1979) calculated that under anaerobic conditions, nearly half of the total ATP production is used to maintain membrane electropotential, a figure many times that of the theoretical requirements for transport processes. Stouthamer (1979) also hypothesised that the magnitude of the energy cost of maintaining the membrane electropotential is attributable to the cytoplasmic membranes being somewhat permeable to protons and thus lowering the proton-motive force.

Calculation of Y^{ATP} relies on the estimation of the yield of ATP arising from the breakdown of various materials. This estimate has generally been regarded as being more certain in anaerobic rather than in aerobic systems because the uncertainties regarding the use of reducing equivalents for the formation of biomass and the amount of ATP produced in the respiratory chain in the latter system are almost absent. However this notion can be misleading because ATP can also be produced in anaerobic systems via electrochemical gradients. Anaerobes that produce succinate or propionate can also generate ATP via oxidative phosphorylation through the passing of electrons from NADH + H^+ to cytochrome *b* (De Vries *et al.* 1973; Stouthamer 1979). When these products are formed it is difficult to determine the exact production of ATP and therefore estimates of Y^{ATP} may become misleading.

Other difficulties in determining the amount of ATP produced as a result of fermentation of carbohydrate result from uncertainty about the relative importance of the randomising and acrylate pathways in the formation of propionate. Similarly it is difficult to adequately deal with organisms that are able to switch fermentation products and hence ATP yields /mole of substrate fermented. One such example is *Selenomonas ruminantium* which changes from propionate and acetate production to lactate formation with a concomitant change in the yield of ATP /mol substrate when dilution rate is increased above 0.2 (Scheifinger and Wolin 1973; Russell 1986).

In rumen micro-organisms propionate can be formed by either the randomising or the acrylate pathway (Baldwin *et al.* 1963; Baldwin 1965) with a low energy charge in the cell favouring ATP production by the randomising pathway. Generally the transfer of carbon from hexose to propionate involves interspecies transfer of succinate and in the non-cellulolytic bacteria, *Selenomonas ruminantium*, it appears that the randomising pathway is the dominate path (Paynter and Elsden 1970; Scheifinger and Wolin 1973). However, in this organism, the

acrylate pathway becomes of major importance when starch is in abundance (Scheifinger and Wolin 1973).

McSweeney *et al.* (1994) point out the dangers of considering energy yields in pure cultures by suggesting that the presence of methanogens increases the yield of ATP from propionate producing organisms. As an example of this, the products of glucose fermentation from *Selenomonas ruminantium* change from propionate, acetate and carbon dioxide to acetate, carbon dioxide and methane in the presence of the methanogen, *Methanobacterium ruminantium* (Chen and Wolin 1977). It is therefore apparent that for these organisms estimates of ATP /mole of substrate will be prone to error.

Calculation of Y^{ATP} also relies on the measurement of the grams of cell dry matter that have been produced. Whilst *in vitro* this is a relatively simple task, *in vivo* estimates are fraught with errors (Siddons *et al* 1982) and estimates derived from mathematical models (Baldwin *et al.* 1970) are subject to all the uncertainties associated with assessment of the ATP requirements for synthesis of various cellular materials and E_m. The estimates of energy expenditure associated with polymer synthesis made by Baldwin *et al.* (1970) differ from those calculated by Stouthamer (1979) who indicated that ATP requirements for polymer synthesis are dependent on the nutrient sources available to the organism. To stress this point, the latter author outlined the theoretical requirements of ATP for transport of metabolites and synthesis and turnover of cellular macromolecules in *Escherichia coli* grown with glucose or pyruvate and supplemented with either preformed monomers (amino acids and nucleic acid bases) or inorganic salts. The main conclusions from this exercise were that 1) growth on a glucose medium with preformed monomers resulted in the greatest values of Y^{ATP} and 2) supplementation of the glucose medium with inorganic nitrogen salts rather than amino acids increased total ATP requirements by only 2.6%.

4.3 Uncoupling of fermentation and microbial growth

Values of Y^{ATP} can be significantly reduced when the nutrients for growth are imbalanced or deficient. This situation may occur under nitrogen, sulphur or phosphorus limiting conditions or alternatively these environments may be viewed as having an excess energy source. In these situations production of ATP and its use for polymer synthesis become uncoupled (Senez 1962) resulting in a low yield of cells per mole of ATP produced. Evidence for uncoupling of ATP production and microbial growth is evinced from the data of Hume *et al.* (1970). In this study, sheep were fed a virtually protein-free diet which was supplemented with minerals and

varying amounts of urea (0.9–6.7% DM basis) to provide from 2.6–16.0 gN/d. Increasing the urea content of the diet had no effect on apparent digestibility of DM and OM in the rumen, or on total production or individual acid proportions of VFA suggesting that the availability of ATP would have been unchanged. However urea supplementation stimulated the total microbial yield and the yield per unit OM. These data strongly suggest that fermentative activity and microbial synthesis were 'uncoupled' as a result of a N limitation in the unsupplemented sheep.

4.4 Conclusion

Uncertainties regarding the exact yield of ATP arising from mixed fermentation systems with a variety of substrates in a diverse anaerobic environment make it exceedingly difficult to accurately gauge the amount of ATP available to bacteria for maintenance, growth and the amount that is not coupled to these. Similarly, difficulties are also encountered *in vivo* in quantifying both the yield of cellular dry matter, the composition of the cells being synthesised and the ATP requirements for polymer synthesis. Where microbial dry matter is determined, the cellular composition in terms of protein, nucleic acid, lipid or polysaccharide content of the cell will be uncertain. This has the potential to affect values of Y^{ATP} because of the different ATP requirements for synthesis of these materials (Table 4.1).

Table 4.1. Energy costs for the synthesis and polymerisation of the major cellular materials

Cellular material	ATP requirement [A] (mol/g macromolecule)
Polysaccharide	0.0124
Protein	0.0391
Lipid	0.0015
Nucleic acid	0.0352

Source: Stouthamer (1979)

[A] ATP requirements for growth in a medium of glucose and inorganic salts.

Ruminants receive the majority of their protein and energy-yielding substrates from both digested micro-organisms and from VFAs. Information on the growth yields of microbial biomass /mol ATP is important because of the nutritional significance it has on the productive performance of the animal. In many pastoral situations it is deficiencies in the protein supply

to the animal that most limits feed intake and animal production (Preston and Leng 1987). Given this, it would be useful if 1) microbial yields were expressed in terms of the yield of microbial true protein rather than dry matter and 2) efficiency of microbial growth were expressed in terms of microbial true protein / unit OM truly digested in the rumen. However, for practical reasons, the most wicely used terms describing microbial yield and efficiency of microbial growth are g CP and g CP / unit OM apparently digested in the rumen (often determined by *in sacco* estimates) respectively. Yet estimation of the yield of microbial-N at a given intake is probably of primary importance to production. The advantage of using this less informative but practical parameter is that in the determination of these estimates the need for animal surgery is eliminated (see Chapter 7) and the problems associated with marker-assisted estimates of digesta flow are avoided.

The introduction of the microbial yield term, Y^{ATP}, provides a framework to understand differential microbial growth from various substrates. Experiments conducted with chemostat cultures have elucidated some of the variables that influence growth yields and many of these have been confirmed *in vivo*. However, because of the many uncertainties that are involved with estimates of Y^{ATP}, particularly in the rumen, it is argued here that *in vivo* estimates of Y^{ATP} are inappropriate. The reasons for this conclusion are;

1. Propionate formation can be achieved by either the randomising or the acrylate pathway with differences in the yield of ATP /mol substrate.
2. Organisms that form succinate or propionate as the fermentation end product can increase the yield of ATP by anaerobic electron transfer from NADH + H^+ to cytochrome *b* making precise estimates of ATP yield difficult.
3. Some organisms change fermentation strategies as a result of changes in dilution rate. In these instances calculation of ATP yields are imprecise.
4. Under certain environmental conditions, compositional changes in microbial cells occur and these have implications for ATP costs per gram of cell synthesis and as well for the nutrition of the ruminant host.
5. Models of ruminal fermentation rely on estimates of the energy costs for polymer synthesis which are not precisely known, and vary depending on the source of nutrients that provide building monomers.

CHAPTER 5

5. NITROGEN REQUIREMENTS FOR MICROBIAL GROWTH

5.1 Introduction

Organic matter consumed by ruminants is subject to microbial fermentation in the rumen yielding the products VFA, methane, carbon dioxide, ammonia and the constituents of microbial cells. Generally, a large proportion of the dietary protein is degraded within the rumen to eventually yield ammonia. Ruminal ammonia can also be derived by enzymatic degradation of non-protein N (NPN) sources such as amines, urea and nucleic acids.

The area of nitrogen metabolism and bacterial growth efficiency in the rumen has been subject to extensive research and reviews over the last 30 or so years. It was not the intention to exhaustively cover this research in the following review and for this reason a range of review publications, although not exhaustive, is listed for the reader (Chalupa 1968; McDonald 1968; Waldo 1968; Allison 1969; Tillman and Sidhu 1969; Smith 1975; Hespell and Bryant 1979; Tamminga 1979; Harrison and McAllan 1980; Leng 1982b; Clark and Davis 1983; Leng and Nolan 1984; Nocek and Russell 1988; Russell *et al.* 1990).

5.2 Nitrogen source

5.2.1 Ammonia

The importance of ammonia as a N source for isolated rumen micro-organisms was demonstrated by Bryant and Robinson (1962). These authors isolated 89 strains of culturable ruminal bacteria from a cow whose ration contained nearly 40% grain. Of the isolated strains 81% grew with ammonium salts as the main N source and of these, 31% had an obligatory requirement for ammonium salts; only 6% required casein hydrolysate, a source of amino acids. In agreement with these *in vitro* observations, Virtanen (1966) reported that high milk yields could be obtained when urea was the sole dietary N source, indicating that the N requirement of rumen microbes could be met by ammonia

In many instances, ammonia has been found to be the major N pool from which bacterial protein is eventually formed. Pilgrim *et al.* (1970) and Nolan and Leng (1972)

estimated that *c.* 60 and 80% of the bacterial protein was derived from the rumen ammonia pool in sheep fed a lucerne diet whilst the former authors calculated a value of *c.* 70% for a wheaten hay diet. Kempton *et al.* (1979) reported that on average 66% of the bacterial-N was derived from the rumen ammonia pool when sheep were fed a basal diet of oat hulls and solka floc supplemented with either urea or casein.

Within the rumen, ammonia exists in either the unionised NH_3 or the ionised NH_4^+ form. However, because the dissociation constant (pKa) for the reaction

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$$

is 8.80 at 40°C (pH range in the rumen environment generally 6.0–7.0), ammonia will exist predominately in the ionised form.

There are 2 enzymes that regulate the assimilation of ammonia in bacteria: glutamate dehydrogenase (E.C. 1.4.1.2) and the glutamine synthetase (E.C. 6.3.1.2) glutamate synthase (E.C. 2.6.1.2) system (Erfle *et al.* 1977). Glutamate dehydrogenase is a constitutive enzyme (Baldwin and Denham 1979) and has a low affinity for ammonia. There are 2 types of glutamate dehydrogenase: NADH and NADPH-linked (Chalupa *et al.* 1970). The NADH linked glutamate dehydrogenase is used in the catabolism of glutamate whilst the NADPH linked enzyme serves for glutamate synthesis (Erfle *et al.* 1977). Assimilation of ammonia via this pathway uses NADPH to condense ammonia with α-ketoglutarate yielding the amino acid glutamate without the hydrolysis of ATP. The affinity of glutamate dehydrogenase for ammonia appears to differ between organisms and estimates of K_m include 7–23 mM (19–391 mg/l) for *Selenomonas ruminantium* (Smith *et al.* 1980) and 19mM for a purified glutamate dehydrogenase extracted from *Ruminococcus flavefaciens* (Duncan *et al.* 1992). A lower estimate (5mM) has been suggested by Hespell (1984).

Glutamine synthetase is an inducible enzyme (Erfle *et al.* 1977; Baldwin and Denham 1979) that has a high affinity for ammonia and utilises the free energy of ATP to condense ammonia with glutamate to yield glutamine. Intracellular glutamate is ultimately regenerated by the actions of glutamate synthase. Kleiner (1985) has noted that ammonium carriers generally have a high affinity for substrate with K_m values of 5–50 μM and Schaefer *et al.* (1980) reported saturation constants ranging from 6–50 μM (NH_3-N) for pure cultures of rumen bacteria.

Bacterial ammonia carriers are repressed when grown with high levels of ammonium salts (McSweeney *et al.* 1994) and these authors postulated that a similar situation may occur for glutamine synthetase in ruminal bacteria. Similarly, addition of ammonium salts has been found to repress the synthesis of glutamine synthetase in *Escherichia coli* (Allison 1969). When ammonium concentrations are low, induction of the ammonia carrier occurs and bacteria generally assimilate ammonia by this system. When this occurs it appears that it is the intracellular levels of glutamine tha regulate ammonia transport (McSweeney *et al.* 1994).

Michaelis-Menton relationships were generated to further elucidate the differences between the 2 enzymes used by bacteria for ammonia assimilation (Figure 5.1). The activity of glutamine synthetase (graph B) is 90% saturated at rumen ammonia concentrations of *c.* 25 mg/l whereas glutamate dehydrogenase is unlikely to become saturated within the range of ammonia normally found in the rumen. When the range of saturation constants reported by Schaefer *et al.* (1980) were used (graph A) saturation of ammonia carriers occurred below 10 mg NH_3-N/l.

Two distinct systems for ammonia incorporation in rumen bacteria have been outlined in the preceding discussion. One of these, glutamate dehydrogenase has a low affinity for ammonia, is not readily saturated and utilises intracellular ammonia that appears to enter the cell by passive diffusion (Russell *et al.* 1990). The other, glutamine synthetase, has a high affinity, is saturated by relatively low concentrations of ammonia and incurs an ATP cost. Hence, it is conceivable that the efficiency of microbial growth will be affected by the relative activities of these pathways. The next section will examine this possibility.

5.2.1.1 Ammonia concentration

The intracellular ammonia concentration of mixed ruminal bacteria is greater than that occurring in rumen fluid (Russell and Strobel 1987) and the ratio of intracellular:extracellular ammonia concentration in mixed rumen bacteria has been found to vary from 15:1 at very low concentrations of ammonia to 1.8:1 at an external ammonia concentration of *c.* 400 mg NH_3-N/l. From these studies, Russell and Strobel (1987) concluded that the intracellular ammonia concentration was a linear function of external ammonia concentration and was *c.* 180 mg NH_3-N/l when optimal bacterial protein synthesis occurred: the external ammonia concentration was *c.* 40 mg NH_3-N/l. The greater intracellular concentration of ammonia at low concentrations of extracellular ammonia was presumed to be evidence for the existence of active transport systems driven by a protonmotive force established by ATP hydrolysis (Kleiner 1985; Russell and Strobel 1987).

In support of the notion of active transport systems at low external ammonia concentrations, Schaefer *et al.* (1980) reported that the saturation constants for ammonium in rumen bacteria grown with ammonia-limited cultures were low (*c.* 0.1–0.9 mg NH_3-N/l) and from this calculated that an ammonia concentration (NH_3-N) of approximately 14 mg/l would result in saturation of the ammonia carriers (Figure 5.1) and support near maximal growth rates. However, on the basis that the rumen ammonia concentration (NH_3-N) is rarely below 14 mg/l for prolonged periods, and that the ratio of intra:extracellular ammonia declines exponentially with increasing ammonia in the external medium (Russell and Strobel 1987), Russell and co-workers (1990) concluded that ammonia most probably enters the cell via passive diffusion and not via active transport.

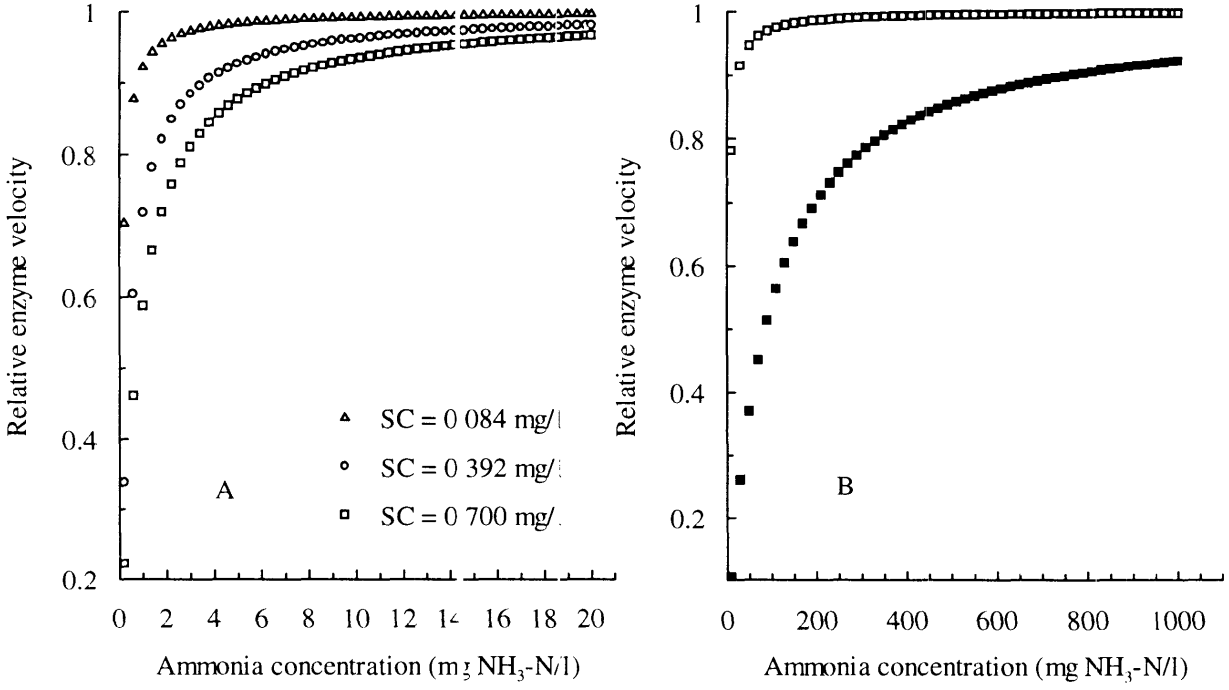

Figure 5.1. Relative activities of glutamine synthetase (unfilled) and glutamate dehydrogenase (filled). Graph A based on the range of saturation constants (SC) of rumen bacteria (Schaefer *et al.* 1980) and graph B on Michaelis-Menton constants of 2.8 and 70 mg NH_3-N/l for glutamine synthetase (unfilled square) and glutamate dehydrogenase (filled square) respectively (Baldwin and Denham 1979).

Ammonia that is provided in excess of microbial requirements is able to be absorbed across the rumen wall (NH_3) and some leaves the rumen in digesta outflow: the balance of these being pH dependent (Smith 1979). Excess ruminal ammonia can contribute significantly

to urea production in ruminants and Nolan and Leng (1972) reported that in sheep on a lucerne diet up to 45% of plasma urea was derived from the ruminal ammonia pool. Ammonia that enters the blood of the animal will be metabolised, predominately in the liver, to yield urea and the overall cost of forming 1 mole of urea via the urea cycle is 4 high energy phosphate bonds (Stryer 1988). In addition to the energy cost of urea synthesis it appears that there is also the cost of utilisation of amino acids that accompany urea synthesis (Lobley *et al.* 1995). This may increase the amino acid requirements of the host. Given these energetic costs, ammonia that is in excess of microbial requirements is undesirable to the ruminant host and the most efficient use of rumen ammonia is when supply to the rumen microbes is matched by ATP availability so that the requirements for optimal microbial growth are met.

In order to determine the optimum level of ammonia in ruminal fluid, Satter and Slyter (1974) examined the effect of infusing graded amounts of urea into a continuous culture system which was initially charged with bovine rumen fluid and fed either 1) a purified, 2) a concentrate (91% maize), or 3) a forage/concentrate (45% maize, 23% hay) based diet. These authors found that increasing the crude protein (CP) content of the three diets (by urea infusion) had little effect on VFA production. In contrast, tungstic acid precipitable-N (TAPN, indirect measure of microbial-N) increased rapidly as the CP content of the diet (g/100g) increased from *c.* 4–12 (diet 1), *c.* 11–15 (diet 2) and 7–13 (diet 3). Further increases in the CP content of the diets had little affect on the yield of TAPN. The corresponding ammonia concentration (NH_3-N) that gave near maximal TAPN for all diets was similar to that later reported by Russell and Strobel (1987) at 50 mg/l. Beyond this value, ammonia accumulated as a linear function of the CP content of the diet. Although TAPN was relatively constant when the ammonia concentration (NH_3-N) exceeded 50 mg/l, Satter and Slyter (1974) found no detrimental effects on the rumen microbiota even when the ammonia concentration (NH_3-N) in the fermentator fluid was 800 mg/l.

To examine the relationship between the CP content of the diet and the efficiency of microbial growth, Satter and Slyter (1974) calculated Y^{ATP} estimates for diet 1. Estimates for the other diets were not calculated because of the possibility that dietary-N was included in the TAPN fraction. Whilst the accuracy of the Y^{ATP} estimates is arguable (see Chapter 4) it was interesting that in the studies reported by Satter and Slyter (1974) the maximum value of Y^{ATP} was achieved at a greater CP content of the diet (*c.* 16 g/100g) than was necessary to optimise the yield of microbial-N (*c.* 12 g/100g). In terms of ammonia concentration (NH_3-N), yield was optimised at *c.* 50 mg/l but efficiency was not maximised until a value of *c.* 170 mg/l was

reached. Presumably this occurred with diet 1 because of a small increase in the outflow of TAPN beyond an ammonia concentration (NH_3-N) of 50 mg/l.

Other authors have suggested that, *in vivo*, the ruminal ammonia concentration that optimises digestion and microbial growth in the rumen is significantly higher than the 40-50 mg NH_3-N/l reported for *in vitro* cultures by Satter and Slyter (1974) and Russell and Strobel (1987). In the classic studies of Hume *et al.* (1970), sheep were fed a virtually protein-free diet (*c.* 50% cellulose, 17% starch, 13% sucrose) which was supplemented with varying amounts of urea (0.9–6.7% DM basis) to provide from 2.6–16.0 gN/d. These authors reported that increasing urea content of the diet increased the ammonia concentration (NH_3-N) of rumen fluid from *c.* 60–300 mg/l, the flow of microbial protein from the rumen and also the microbial yield per unit OM digested in the rumen. In the studies reported by Hume *et al.* (1970) the maximum yield of microbial protein was obtained when urea constituted 3.5% of the diet at which the daily N intake was 9.2 g and the ammonia concentration (NH_3-N) in rumen fluid 133 mg/l. Further increments of urea failed to increase the flow of microbial protein from the rumen.

In the studies of Mehrez *et al.* (1977) the disappearance of rolled barley DM from Dacron bags incubated in the rumen of sheep was maximised when the ammonia concentration (NH_3-N) in rumen fluid was *c.* 190 mg/l. Perdok (1987) was able to determine, that in cattle fed solely on straw and minerals, *in sacco* DM degradation and total VFA concentration were maximised when urea contributed *c.* 2% of the DM intake and rumen ammonia concentrations (NH_3-N) were between 250–300 mg/l. Given the positive association between fermentation and bacterial growth in nitrogen sufficient environments (Hungate 1966) it can be argued that in the studies of Mehrez *et al.* (1977) and Perdok (1987) maximal microbial yields occurred at the rumen ammonia concentration that maximised DM disappearance: but no direct estimates were made.

The concentration of ammonia in the rumen is the balance between production, microbial utilisation, absorption across the rumen epithelium and outflow in rumen liquid. From the preceding discussion it is apparent that the rumen ammonia concentration that is required for maximal rates of microbial growth is not constant. This is understandable given that microbial growth is subject to the influences of factors other than rumen ammonia concentrations (see following sections and also Chapter 4). For example, on the same diet, the yield of microbial cells from the defaunated rumen is higher than that from the faunated rumen even though the concentration of ammonia in the defaunated rumen is lower.

5.2.2 Amino acids and peptides

The importance of amino acids and peptides for growth of ruminal micro-organisms has been a subject of debate over many years. As previously discussed, the data of Bryant and Robinson 1962) suggests that few rumen microbes have an obligatory requirement for amino acids. This strategy seems to be a sensible option for many rumen microbes considering the suggestion of Stouthamer (1979) that when glucose is the predominant carbohydrate source the theoretical ATP requirement for microbial growth is similar regardless of whether amino acids or inorganic salts are used for the formation of microbial protein. However when more oxidised metabolites predominate (e.g. pyruvate or acetate) the growth superiority of adding amino acids becomes apparent (Stouthamer 1979).

Provision of amino acids may at times indirectly stimulate bacterial growth by the formation of branched chain volatile fatty acids (BCVFA) (Van Den Hende *et al.* 1963) with the BCVFA being recognised as important growth factors for many strains of cellulolytic and noncellulolytic micro-organisms (Dehority *et al.* 1967; Bryant 1973).

In support of the notion that addition of amino acids and peptides stimulates microbial growth, Hume (1970a) found that isonitrogenous substitution of *c.* half of the urea-N with casein or zein in a diet of oat hulls (*c.* 0.50), starch (*c.* 0.24) and sucrose (*c.* 0.14) increased the flow of microbial protein from the rumen of sheep and also increased the microbial growth efficiency (g microbial protein/ 100 g OM digested in the rumen). The stimulation of both microbial growth and microbial growth efficiency by addition of zein was of interest because addition of zein did not increase the concentration of rumen ammonia or BCVFA nor did it contribute significantly to the ruminal pool of amino acids or peptides. In fact, it can be calculated from the data of Hume (1970a) that of the 47.2 g/d of zein ingested by the sheep 43.6 g was contained in daily outflow from the rumen.

Maeng *et al.* (1976) also found that, *in vitro*, substitution of *c.* 15% of the urea-N with amino acid-N stimulated bacterial growth. To determine the optimal ratio of urea-N:amino acid-N for bacterial growth, these authors examined, *in vitro*, the effects on bacterial growth of replacing increasing amounts of urea-N with amino acid-N when the predominate carbon source was either glucose, starch or cellobiose. Optimal growth yields were achieved in all cultures when amino acid-N contributed 25% of the total-N supply: a percentage also confirmed by Maeng and Baldwin (1976a). In this experiment, the proportion of amino acid-C that was incorporated into bacterial cells decreased uniformly from *c.* 45% when amino acid-N

contributed 25% of the total-N supply to *c* 13% when the entire N supply was as amino acids. Quantitatively, incorporation of am no acid-C into cells was optimised at 75% amino acid-N.

In the experiments of Maeng *et al.* (1976) it is possible that some of the stimulatory effect of amino acids was due to the presence of BCVFA arising from fermentation of amino acids. However, because additior of amino acids was found to double the yield of bacterial cells per unit carbohydrate fermen ed, it is unlikely that the presence of BCVFA explained a large part of this effect (Hume 197(b).

In a latter study, Maeng and Baldwin (1976b) noted that the stimulatory effect of amino acids on bacterial growth yields wa apparently associated with the level of starch in the rumen. From this association they suggested that the growth of amylolytic rather than cellulolytic bacteria is restricted by the availabi ity of amino acids (Bryant and Robinson 1962). This may be so, however, given the differen e in the rate of fermentation between starch and cellulose (Baldwin and Denham 1979) it is also possible that the responsiveness of rumen bacteria to amino acid supply depends on the synchrony of the supply of ATP (rate and total) and the availability of other growth factors (Sinclair *et al.* 1993).

5.3 Other factors that influence microbial yield from the rumen

5.3.1 Protozoa

No mention of the role that proto zoa play in carbohydrate metabolism in the rumen will be made in this section. Instead the emphasis will be on determining the significance of rumen protozoa on the flow of microbial-N to the host.

Originally the nutritional sig nificance of protozoa to the ruminant host was assessed to be correlated to their ruminal con centration which, depending on diet, can exceed 10^6/ml (Coleman 1975; Schwartz and Gilchrist 1975; Leng and Nolan 1984). This notion implied that protozoa left the rumen at a rate determined by digesta outflow. However, Hungate (1966) speculated that protozoa may attach to larger particles in the rumen and thus leave the rumen at a rate which was slower than that dictated by liquid outflow. Confirmation that protozoa are retained in the rumen was pr sented when Weller and Pilgrim (1974) showed that the concentration of protozoa in rumen outflow, collected at the omasal orifice, was less than 20% that in rumen fluid. This implied that the ruminal concentration of protozoa was a poor guide to the contribution that protozoa make to the outflow of microbial cells from the rumen.

Protozoa can contribute from 13–50% of the microbial nitrogen in the rumen (Pilgrim *et al.* 1970; El Fouly 1983; Punia *et al.* 1992) and even higher estimates have been quoted

(Leng and Nolan 1984). Up to 80% of the protozoal-N can be degraded and recycled within the rumen (Cottle 1980; Leng 1982a; Punia *et al.* 1992) and in general protozoa make a small and variable contribution to the flow of microbial protein from the rumen (references with proportional contribution in parentheses: Pilgrim *et al.* 1970 (0.08); Harrison *et al.* 1979 (0.27); El Fouly 1983 (< 0.01); Ivan *et al.* 1992 (0.06); Punia *et al.* 1992 (0.16)).

Protozoa engulf and partially digest rumen bacteria with bacterial amino acids, purines and pyrimidines being directly incorporated into protozoal macromolecules (Coleman 1975). Approximately half of the bacterial material that is engulfed is retained by the protozoa and the remainder is released into the extracellular environment, predominately in the form of free amino acids (Coleman 1975). The *in vitro* rate of bacterial engulfment by rumen protozoa has been observed to be dependent on both starch and bacterial concentration in the rumen fluid (Coleman 1975). Increasing the starch content of the medium resulted in a slower uptake of bacteria whilst greater bacterial densities provided for a more rapid rate of engulfment, presumably because the need for motility was greatly reduced. Bacterial engulfment by protozoa is quantitatively important and it has been suggested (Abe and Kandatsu 1969 in Leng and Nolan 1984) that 40% of rumen bacteria are engulfed by protozoa. Studies using both ^{15}N (Cottle 1980) and ^{35}S (El Fouly 1983) support this notion and have also shown that from 40–60% of the protozoal-N can be derived from bacterial-N.

Thus, rumen protozoa may at times engulf a large fraction of the bacterial population free in solution, release compounds that can be reused for bacterial synthesis or fermented to yield various end products, are themselves subject to considerable lysis and recycling within the rumen and make a small but variable contribution to microbial-N in rumen digesta outflow. Given these conclusions it seems logical that much research has been conducted to examine the effects on both rumen function and animal production of elimination of protozoa (defaunation) from the rumen.

The general consensus from many of these studies is that defaunation lowers feed digestibility (Ushida *et al.* 1986), though this is not always the case (Soetanto 1985; Romulo *et al.* 1989), and results in lower rumen ammonia concentrations and higher molar proportions of propionate (Ushida *et al.* 1986). Elimination of protozoa also results in an increase in the bacterial density in rumen fluid (Bryant and Small 1960; Eadie and Hobson 1962; Harrison and McAllan 1980) and Ivan *et al.* (1992) reported that the flow of bacterial-N into the duodenum of fauna-free sheep was *c.* 40% greater than their faunated counterparts. However, in the latter studies, sheep were maintained fauna-free from birth and as such are not representative of

'normal' animals. Defaunation of sheep has also been reported to increase the flow of NAN (Ushida *et al.* 1986) and essential and non-essential amino acids into the duodenum (Ivan *et al.* 1991). It has also been reported that defaunation results in an increase in the urinary excretion of purine derivatives (Lawrence 1993) which implies a greater outflow of microbial-N from the rumen.

The practical implications of defaunation in ruminants offered a variety of diets have been well documented (Bird and Leng 1978; Bird *et al.* 1979; Bird 1982; Bird and Leng 1984; Bird 1989). In general it appears that the benefits of defaunation to animal production (e.g. live weight gain and wool growth) are most pronounced when animals are offered poor quality diets low in bypass protein or have an increased requirement for protein (e.g. pregnant, lactating or rapid growth).

5.3.2 Dilution rate

There have been numerous publications illustrating that, in continuous culture systems, there is a positive relationship between specific growth rate (μ, h^{-1}) and bacterial yield (Hobson and Summers 1967; Isaacson *et al.* 1975; Pirt 1975; Stouthamer 1979). In a continuous culture system, specific growth rate is analogous to dilution rate which is defined as the fraction of the culture volume replaced per hour. With regards to the rumen, there are probably at least 2 major dilution rates, these are, the fraction of the rumen fluid volume replaced per hour and the fraction of the rumen particulate matter that is replaced per hour. Generally, when the dilution rate of the rumen is quoted, it is with reference to the fluid volume.

In a much quoted publication, Isaacson *et al.* (1975) demonstrated that increasing the dilution rate of a continuous culture system from 0.02–0.12 h^{-1} increased both propionate production (0.16–0.26) and bacterial mass per mol of glucose fermented (42.2–83.9). The stimulation of bacterial yield by increasing dilution rate is usually explained by suggesting that organisms that have a shorter residence time utilise a lesser proportion of the available ATP for maintenance and hence have a greater proportion of the available ATP available for growth.

Supporting *in vivo* evidence for the positive effect of dilution rate on bacterial yield is less well defined. Harrison *et al.* (1975) increased dilution rate from *c.* 0.04–0.10 h^{-1} by the intraruminal infusion of artificial saliva and stimulated microbial yields. Kennedy *et al.* (1976) and Kennedy and Milligan (1978) found that both fluid dilution rate and microbial yield per unit apparent OMD increased when sheep were held at vastly colder ambient temperatures (*c.* 22 v.'s 2°C). In contrast to these studies increasing digesta flow by varying feed intake appears not to have a marked effect on microbial yields per unit intake from the rumen (Leng 1982b).

CHAPTER 6

6. METHODS TO ESTIMATE THE YIELD OF MICROBIAL CELLS FROM THE RUMEN

6.1 Introduction

Microbial protein in digesta flowing from the rumen to the intestines supplies the majority of the amino acid requirement of ruminants and governs the growth and composition of wool. Various methods have been used to estimate the microbial protein in rumen digesta outflow. The measurement of microbial protein yield from the rumen and the proportion of dietary protein which escapes degradation in the rumen requires the use of techniques which distinguish food protein from microbial and endogenous protein in digesta leaving the stomach. For this purpose, animals are generally prepared with either simple or re-entrant duodenal cannulae (Phillipson 1952; Macrae 1975). The methods used for estimating the yield of microbial protein in rumen digesta outflow are imprecise (Siddons *et al.* 1982), tedious and expensive to implement and consequently no single definitive technique has become established.

Generally, naturally occurring (DAPA, RNA) or isotopic markers (^{15}N, ^{35}S, ^{32}P) are used to identify the contribution of microbial-N to total-N in digesta leaving the rumen. The unaccounted N in digesta is then taken to represent non-microbial-N. When the marker is found to exist in only the microbial fraction, estimation of the proportion of microbial-N in total-N in digesta is possible using the technique of marker dilution (Siddons *et al.* 1982). When total-N flow at the duodenum is also estimated, calculation of the flow of microbial-N and by difference non-microbial-N can be made. In the remainder of this Chapter some of the more commonly used microbial markers are discussed. For further reference in this area see Broderick and Merchen (1992).

6.2 Diaminopimelic acid

Diaminopimelic acid (DAPA) is unique to prokaryotes and is one of numerous aminosugar chains that crosslink and hence stabilise the peptidoglycan envelope present in bacterial cell walls. The cell wall of gram positive organisms contains more than 50% peptidoglycan whilst

in gram negative organisms this value is only 5–20% (VanDemark and Batzing 1987). DAPA is absent from plant and protozoal material but bacterial contamination of feedstuffs and bacterial engulfment by protozoa mean that, in practice, measurable amounts of DAPA are associated with these materials (Broderick and Merchen 1992). Variation in the proportion of peptidoglycan present in the cell wall of bacteria results in variation in the amount of DAPA. Hence, Purser and Buechler (1966) reported that the contribution of DAPA to total amino acids varied from 0–3.4 g/100g total amino acids in 11 rumen bacterial species. However, the ratio of DAPA:protein in mixed rumen bacteria appears to be relatively constant (Broderick and Merchen 1992).

DAPA has been used to estimate the proportion of bacterial nitrogen present in total nitrogen in duodenal digesta (Weller *et al.* 1958). The main limitation to the use of DAPA is that it does not account for the contribution of protozoal protein in digesta leaving the rumen and will consequently underestimate microbial protein yield. A more serious concern is that a variable but significant proportion of the DAPA leaving the rumen is not associated with intact bacterial cells and this proportion varies considerably with time after feeding (Broderick and Merchen 1992). Thus a proportion of the DAPA flowing out of the rumen will not be associated with bacterial protein and use of the DAPA:protein ratio of isolated bacteria will result in overestimation of bacterial protein yields.

6.3 Ribonucleic acid

Smith and McAllan (1971) measured the ribonucleic acid nitrogen (RNA-N):total-N ratio in duodenal digesta as an indicator of microbial yield from the rumen. However, the use of RNA as a marker is complicated by the fact that RNA is abundant in many feedstuffs and can arise from endogenous material. Hence, complete ruminal degradation or microbial incorporation of dietary RNA is required for this technique to be valid.

In support of the use of the RNA method, Smith and McAllan (1970) found the RNA:DNA ratio in rumen digesta to be similar to that in samples of mixed rumen bacteria, indicating that the nucleic acids present in rumen digesta were mainly of microbial origin. However, Smith *et al.* (1978) have shown that up to 15% of the RNA entering the duodenum of young steers receiving equal amounts of hay and concentrate was non-microbial in origin.

A further complication with the use of RNA as a microbial marker is that the proportion of RNA can vary between and within microbial species depending on the metabolic state of the cell (Smith 1979) and time after feeding (Smith and McAllan 1974; Merry and

McAllan 1983; Cecava *et al.* 1990a). In general, the RNA technique has tended to give slightly higher estimates of microbial-N than other methods, giving some support to the notion that the RNA fraction in duodenal digesta is not solely microbial in origin (Ling and Buttery 1978). It should be noted that RNA analysis must be done on fresh (not frozen) digesta as freezing and thawing of samples can result in losses of RNA (Siddons *et al.* 1982).

6.4 Isotopic markers

Intraruminal infusion of ^{35}S, ^{15}N (Siddons *et al.* 1982) and ^{32}P (Van Nevel *et al.* 1975) to label rumen microbes has been used to determine the proportion of microbial protein in total protein in duodenal digesta. This technique compares the ratio of the specific radioactivity in digesta of an isolated microbial fraction with that of an unfractionated sample. In the absence of food and endogenous protein the ratio will be unity.

Siddons *et al.* (1982) suggested that it would be unlikely that ruminal isotopic markers would be incorporated uniformly throughout all microbial species, thus the species sampled and analysed must be representative. The basis for this presumption, was the notion that labelling will vary between species according to the extent to which organisms incorporate preformed amino acids of dietary origin.

Other complications that have been reported for the use of isotopic markers include the possibility of secretion of organic ^{35}S and ^{15}N compounds into the abomasum. The result of such a situation would be the over estimation of microbial-N in duodenal digesta (Siddons *et al.* 1982).

6.5 Amino acid profile

In the approach proposed by Evans *et al.* (1975) the amino acid profiles (AAP) of feed, microbial and endogenous protein are used as markers. The advantage of this method is that it apportions duodenal digesta protein into its three constituent fractions. In contrast, the use of other microbial markers relies on the estimation of feed protein reaching the duodenum as being the difference between total duodenal protein and microbial protein and an assumption about the magnitude of endogenous input. Errors that occur in the estimation of the microbial protein fraction will be compounded in the determination of the degradability of feed protein in the rumen (Siddons *et al.* 1982). In addition, the amino acid profile of the feed may differ from that of the fermented dietary protein in the digesta outflow due to the selective

degradation of some amino acids, whilst the endogenous amino acid profile is uncertain (Siddons *et al.* 1982).

6.6 Urinary purine derivatives

Purine derivatives excreted in the urine of ruminants include mainly allantoin, with smaller amounts of uric acid, xanthine and hypoxanthine. These compounds are the products of purine degradation and can be derived from both endogenous and exogenous sources. In ruminants, exogenous purines are predominantly microbial in origin (McAllan and Smith 1973).

To estimate the quantity of microbial-N leaving the rumen from the urinary excretion of purine derivatives, it is necessary to know the relationship between 1) the urinary excretion of purine derivatives and the absorption of microbial purines from the small intestine, 2) intestinal digestibility of microbial purines, 3 the ratio of purine:total-N in mixed rumen bacteria and 4) the extent to which purine derivatives excreted in urine are derived from endogenous material. Many of these relationships have been the subject of recent research (Chen *et al.* 1990b; Balcells *et al.* 1991) and are more fully discussed in the following text.

The potential attractiveness of this technique in relation to those currently in use is the absence of any surgical intervention on the animal and the fact that it is comparatively less expensive than using markers. The remainder of this literature review will assess the potential for the use of purine derivatives in urine to predict microbial yield from the rumen.