THE EFFECT OF FORM OF NITROGEN ON THE EFFICIENCY OF PROTEIN SYNTHESIS BY RUMEN BACTERIA IN CONTINUOUS CULTURE

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Submitted in partial fulfilment of the

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ABSTRACT

The aim of this study was to examine the effect of the form of nitrogen available to mixed rumen bacteria on the efficiency of microbial protein synthesis. A novel, solid substrate, continuous culture fermentor which proved capable of maintaining representative populations of mixed rumen bacteria under steady state conditions, at predetermined growth rates was developed for the study. A series of experiments wherein maize straw, alkaline hydrogen peroxide-treated wheat straw, rye grass or a mixture of maize straw and maize starch were used as substrates were performed in the fermentor. The effect of supplementing these substrates with different forms of nitrogen, namely peptides in the form of a partial hydrolysate of casein, or ammonium salts alone or ammonium salts plus branched-chain volatile fatty acids, on digestion of the substrate and microbial protein synthesis was investigated. Supplementation of maize straw with peptides increased microbial protein synthesis, however this was only significant ($P<0.05$) when the availability of nitrogen and specific growth rate of the bacteria were not limiting. Organic Matter digestion was not affected by peptide supplementation. Replacement of casein hydrolysate with sunflower oilcake as a source of amino acid nitrogen further increased microbial protein synthesis. The form of nitrogen supplementation did not affect the digestion of constituent cell wall monosaccharides of maize straw. Supplementation of alkaline hydrogen peroxide-treated wheat straw with either branched-chain volatile fatty acids or casein hydrolysate increased the synthesis of microbial protein

significantly (P<0.05) and caused a slight, but insignificant increase in the digestion of cellulose-glucose. The efficiency of microbial protein synthesis on rye grass was high, relative to the other substrates, and unaffected by the supplementation of either branched-chain volatile fatty acids or peptides. On media containing high levels of starch, microbial protein synthesis as well as Organic Matter digestion were increased by peptide supplementation, but these differences were only significant (P<0.05) when the bacteria were growing at a high specific growth rate. In all of these experiments, peptide supplementation was accompanied by extensive degradation and deamination of amino acids which offset any increase in microbial protein synthesis. Peptide supplementation therefore resulted in far less efficient overall utilization of protein.

I hereby declare that this entire thesis and the associated research, unless indicated to the contrary in the text, comprises my own, original work.

B. L. KERNICK

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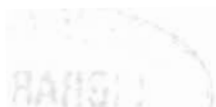

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CHAPTER 1

GENERAL INTRODUCTION

The ability of ruminant animals to convert lignocellulosic plant material, which would otherwise have little value, to high quality meat, milk and fibre, has long been recognised and exploited by man. This ability to digest polymers which are totally resistant to mammalian digestive enzymes, is attributable to the highly specialised and diverse microbial population that exists in the rumens of these animals (Hungate, 1966). Over the years, research by animal nutritionists, microbiologists and biochemists has revealed much about the ecology and metabolism of the microflora inhabiting the rumen, however there are still many aspects about which little is known.

The microbial population of a normal rumen consists of bacteria, protozoa and anaerobic rumen fungi. The anaerobic rumen fungi are a fairly recent discovery and there is still much to be learnt about their role in the rumen ecosystem (Orpin & Joblin, 1988). Ciliate protozoa number approximately 10^6 cells/ml in a normal rumen, however numbers can fluctuate considerably depending on factors such as the species of the host and the diet being consumed (Hungate, 1966; Dehority & Orpin, 1988). Bacteria number approximately 10^{10} to 10^{11} cells/ml and account for by far the majority of metabolic activity in the rumen. Due to their relative importance with respect to the nutrition of the host animal, the majority of research in rumen microbiology has centred around the bacterial population, and it is in this area that most progress has been made in elucidating the processes of microbial digestion in the rumen.

The bacterial population of the rumen is diverse and approximately 200 different species have been isolated (Russell & Hespell, 1981). These species differ in terms of the substrates that they are able to ferment and also in their fermentation end-

products and it is on this basis that rumen bacteria are often classified (Hungate, 1966; Russell & Hespell, 1981; Baldwin & Allison, 1983). Under different dietary conditions, different species of bacteria can be expected to predominate in the rumen. On high-concentrate diets, for example, there is a predominance of starch-digesting and lactate-utilizing bacteria while cellulolytic and hemicellulolytic species predominate when roughage-based diets are fed (Hungate, 1966; Van Gylswyk & Schwartz, 1984; Dehority & Orpin, 1988).

Microbial fermentation in the rumen is of great significance to the nutrition of the host animal. The process of fermentation of the various dietary carbohydrates gives rise to a number of end-products which include volatile fatty acids, CO_2 and methane Russell & Wallace, (1988). While the CO_2 and CH_4 are essentially waste-products, the volatile fatty acids are absorbed and metabolised further by the host and constitute a major source of energy in ruminants. During fermentation, there is a proliferation of microbial cells which eventually pass out of the rumen, into the lower digestive tract where they are hydrolysed by the digestive enzymes of the host. The amino acids and peptides thus released are then absorbed into the bloodstream and form an important source of protein to the host animal. Depending on various factors, including diet and the production of the animal, microbial flow from the rumen can supply the animal with 50 percent or more of its total protein requirements (Ørskov, 1982). The amino acid composition of microbial protein synthesized in the rumen is remarkably constant over a wide range of diets (Storm & Ørskov, 1983) and the nutritive value of this protein to the host animal is high (Storm *et al.*, 1983a; 1983b).

In order to accurately establish the dietary protein requirements of ruminants, it is important that the contribution of microbial protein to the overall requirements of the animal be accounted for. All modern ruminant protein evaluation systems such as those of the ARC (1980) and NRC (1985) have attempted to predict the

flow of microbial protein from the rumen under a variety of conditions. The principal factor governing the flow of microbial protein from the rumen is the quantity of organic matter (OM) which is fermented in the rumen (ARC, 1980; NRC, 1985). The efficiency with which this organic matter is converted to microbial protein however shows large variation, as evidenced by the figures presented by the ARC (1984). In this publication, a review of the pertinent literature covering 262 different diets fed to cattle and sheep revealed microbial yields that varied from 15 to 72 g N/kg OM apparently digested in the rumen. The mean value was found to be 32g N/kg OM apparently digested in the rumen with a coefficient of variation of 0.39. The efficiency with which available energy is utilized for microbial growth therefore has a major influence on the amount of protein synthesized in the rumen and it is important that this aspect of microbial growth and the factors affecting it be clearly understood.

The efficiency of microbial growth is a subject that has drawn attention for some time. Bauchop & Elsden (1960) first introduced the concept of Y_{ATP} which expresses efficiency of microbial biomass synthesis in terms of the yield of microorganisms per mole of ATP supplied by the energy source on which the organisms are growing. These figures are based on theoretical calculations of the amount of ATP that is derived from known biochemical pathways during metabolism of the energy source on which the organisms are growing. Hespell & Bryant (1979) reviewed the observed Y_{ATP} values for a number of pure strains as well as mixed rumen bacteria. The majority of these values ranged between 8 and 16 g of cells/mole of ATP expended. This is considerably lower than the theoretical values of 27 to 32 g cells/mole of ATP which is based on the amount of ATP required for the synthesis of the various polymers which constitute microbial cells. Hespell & Bryant (1979) have reviewed the factors which may contribute towards the low Y_{ATP} values observed in practice, relative to theoretical values. These factors include changes in cell

composition, supply of nutrients for growth, energy requirements for nutrient transport, maintenance energy requirements and energetic uncoupling.

Maintenance energy is defined as that energy which is required by bacteria for purposes other than for producing an increase in cell mass and efficiency of microbial growth is determined to a large degree by the relative proportions of ATP used for either maintenance or growth. The maintenance energy of rumen micro-organisms has been shown to vary widely under different conditions (Harrison & McAllan, 1980; Mathers & Miller, 1981). The specific growth rate of a bacterial population has a major influence on maintenance energy requirements. At low growth rates, proportionally more of the ATP generated during the fermentation process is used for maintenance requirements than at higher growth rates, resulting in less ATP being available for biosynthetic processes and consequently, less efficient growth at low growth rates. This principle was well demonstrated in the study of Isaacson *et al.* (1975) wherein Y_{ATP} values for mixed rumen bacteria of 7.5, 11.6 and 16.7g cells/mole ATP were observed at specific growth rates of 0.02, 0.06 and 0.12 h^{-1} respectively. Another factor cited by Hespell & Bryant (1979) as having a major influence on maintenance energy and hence on growth efficiency of rumen bacteria is energetic uncoupling which refers to the relative degree to which ATP or other energy-rich compounds produced during fermentation are utilized for biosynthetic activities in the cell (Senez, 1962). Energetic uncoupling occurs when nutrients other than energy are inadequately supplied. Under such conditions, fermentation of the substrate continues with the formation of the usual fermentation end-products, however cell growth is reduced in relation to the supply of the limiting nutrient.

In order for growth to occur, rumen bacteria require, in addition to a source of energy, some form of N, various minerals and vitamins, and in some cases, C_4 to C_6 branched- and straight-

chain volatile fatty acids (Hungate, 1966). Bryant & Robinson (1962) provided much information about the nutrient requirements of various species of rumen bacteria. This study elucidated the complex nutritional requirements of rumen bacteria and showed that in many cases, specific nutrients were required in order for growth to occur.

Hespell & Bryant (1979), Stern & Hoover (1979), Bergen *et al.* (1980), Russell & Hespell (1981) and Buttery & Lewis (1982) all cite N metabolism as being one of the major factors affecting energetic uncoupling and growth efficiencies in rumen bacteria. Numerous studies have investigated the effects of both the availability and form of N on the efficiency of protein synthesis by rumen bacteria and it is clear that situations may exist where the efficiency of microbial growth is reduced by the inadequate supply of N to the microbial population.

Ammonia is undoubtedly the principal source of N and is essential for the growth of most rumen bacteria (Allison, 1969). Bryant & Robinson (1962) found that of 89 strains tested, 82% of rumen isolates utilized ammonia as the main source of N, while for 25% of the isolates, ammonia was essential for growth. The ability of rumen bacteria to utilize ammonia as their primary source of N is important in ensuring their survival in the rumen environment where the concentrations of free amino acids are low (Wright & Hungate, 1967) and dietary protein is rapidly degraded to VFA, NH_3 and CO_2 (Prins, 1977; Tamminga, 1979). Estimates of the proportion of the N requirements of mixed rumen bacteria that can be met by ammonia range from 40 to 100% (Pilgrim *et al.*, 1970; Mathison & Milligan, 1971; Al-Rabbat *et al.*, 1971; Nolan & Leng 1972; Nolan *et al.*, 1976; Salter *et al.*, 1979). It is however evident that ruminants are not only able to survive but can also remain productive when fed diets that are devoid of amino acids (Virtanen, 1966). This serves to emphasize the importance of *de novo* synthesis of amino acids by rumen bacteria.

Ammonia concentrations in the rumen vary considerably, and range from approximately 15 to 550 mg/l, depending on factors such as the diet, time in relation to feeding and rumen fluid volume (Wohlt *et al.*, 1976; Wallace & Cotta, 1988). Rumen bacteria, in general, have a high affinity for ammonia and Schaefer *et al.* (1980) have shown that many species of rumen bacteria will achieve 95% of their maximum specific growth rate at ammonia levels as low as 1mM. Although low ammonia levels will support maximum growth rates, the yield of bacteria under such conditions may be reduced due to the energy that is required to scavenge ammonia at low levels.

Assimilation of ammonia by rumen bacteria is thought to occur mainly via glutamate dehydrogenase (GDH) or the dual enzyme system of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Hespell, 1984). The pathway which is used for ammonia assimilation is determined largely by the level of ammonia in the surrounding medium. Due to its high affinity for ammonia (Umbarger, 1978), the dual enzyme GS/GOGAT system is utilized when ammonia concentrations are low (<1mM) whereas the GDH system which has a poorer affinity for ammonia predominates when higher levels of ammonia (>1mM) are present. Both of these pathways may however operate simultaneously, in different organisms. GS and GOGAT have been detected in mixed rumen bacteria growing under conditions of low ammonia (Erfle *et al.*, 1977) but at higher concentrations, its activity is negligible (Wallace, 1979). The GS/GOGAT system is energy dependent and utilizes ATP in the process of ammonia assimilation. This has the effect of reducing Y_{ATP} values of bacteria growing under ammonia-limited conditions (Hespell, 1984). Erfle *et al.* (1977) and Wallace (1979) have detected GDH activity in mixed rumen bacteria growing under conditions of adequate ammonia.

The level of ammonia required to support maximum microbial yields in the rumen has been the subject of a number of studies. Satter & Slyter (1974) found that microbial yields of mixed rumen

bacteria grown in continuous culture were reduced when ammonia levels fell below 50mg/l. This was later confirmed by Russell & Strobel (1987). Kang-Meznarich & Broderick (1981) however found that 85mg/l was the optimum level of ammonia required to maximize microbial protein yields in the rumen of cows fed on a high-concentrate diet while Pisulewski *et al.* (1981) reported values of 40, 84 and 22 mg/l for high concentrate, mixed roughage-concentrate and high roughage diets respectively. The values for rumen ammonia levels reported as optimum for degradation of the substrate are considerably higher than those reported as optimum for microbial growth and range from 61 to 235 mg/l (Meherez *et al.*, 1977; Wallace, 1979; Odle & Schaefer, 1987). It is not clear whether or not the ammonia levels observed as optimal for substrate degradation would also be optimum in terms of synthesis of microbial protein, however the more rapid degradation of substrate achieved at these higher levels would imply more efficient microbial growth. It is evident therefore that considerable variation exists with respect to determined values of optimal rumen ammonia concentrations, however it would appear that efficiency of microbial growth can be expected to decline at levels below 50 mg/l.

An important factor in the utilization of ammonia by rumen bacteria is the supply of S which is essential for microbial growth due to the fact that it contributes to microbial S-containing amino acids. Work done by Hume & Bird, (1970), Elliot & Armstrong (1982) and Kandylis & Bray (1987) has shown that the supply of S has a pronounced effect on microbial protein synthesis in the rumen. The inclusion of adequate S in ruminant diets, particularly those containing a high level of non protein N is therefore important and it is recommended by the ARC (1980) that sufficient sulphur be included in these diets to ensure an N:S ratio of 14.1:1.

In the study of Bryant & Robinson (1962), it was shown that less than 7% of the rumen isolates tested required casein hydrolysate

in order for growth to occur, however inclusion of casein hydrolysate in the medium proved to be moderately to highly stimulatory to many species of rumen bacteria. Although ammonia is the primary source of N for rumen bacteria, preformed amino acids are also incorporated into microbial protein. Based on theoretical calculations, Hespell & Bryant (1979) have shown that the energetic advantage of synthesizing microbial protein from preformed amino acids as opposed to *de novo* synthesis from ammonia is slight. The higher energy cost for amino acid and peptide transport across the cell may even offset this advantage. Numerous studies, both *in vivo* and *in vitro* however provide evidence to show that the efficiency of microbial protein synthesis by rumen bacteria is improved by the availability of amino acid N.

Maeng *et al*. (1976) found that maximum growth of rumen bacteria grown on glucose in batch culture was achieved when 25% of the urea N in the medium was replaced with either mixed amino acids or casein hydrolysates. In a similar study, Argyle & Baldwin (1989) confirmed these findings and also showed that little growth occurred when ammonia was the sole source of N, but that the addition of low levels of amino acids or peptides quadrupled growth. A further finding of this study was that specific amino acid subgroups did not stimulate microbial growth whereas complete amino acid mixtures did. The above mentioned studies provide strong evidence that the growth of mixed rumen bacteria is stimulated by amino acids and even more so when the amino acids are provided in the form of peptides. Also, it is apparent that the absence of specific amino acids or groups of amino acids does not limit microbial growth. Due to the fact that these experiments were performed in batch culture, specific growth rate could not be controlled. Considering the effect of growth rate on microbial growth efficiency, any change in microbial growth rate may have had a considerable effect on the results of the experiment. The extrapolation of these results to rumen conditions, particularly with regard to more practical diets

should be done with caution. The fact that glucose or other soluble sugars were used as a substrate would presumably have selected for organisms such as *Streptococcus bovis* which have a high affinity for these substrates. The bacterial population may not therefore have been totally representative of that found in the rumen. It should also be borne in mind that the washed cell suspensions used for these experiments were prepared from rumen contents of cows which were fed on a diet containing high levels of rumen-degradable protein. This may explain why little growth occurred when ammonia was the only N source, as the bacterial population was preselected and was presumably adapted to conditions in which amino acids and peptides were plentiful. Cotta & Russell (1982) found evidence of rumen bacteria requiring to adapt to conditions of low amino acids. In this study, it was shown that *Bacteroides ruminicola* and *Megasphaera elsdenii* were only able to grow in a medium devoid of amino acids if first placed in a medium containing high levels of amino acids which were gradually replaced with ammonia.

Further evidence of the stimulatory effect of preformed amino acids on growth efficiency of rumen bacteria was obtained in the study of Cotta & Russell (1982) which showed that the growth efficiency of five species of rumen bacteria grown in chemostat was increased substantially by the inclusion of peptides in the medium. An important observation made in this experiment was that the level of amino acids required to maximize microbial protein synthesis did not lead to the most efficient net utilization of the amino acids supplied in the medium.

Peptides, rather than free amino acids appear to be the preferred source of amino acid N for rumen bacteria (Prins, 1977; Cotta & Hespell, 1986). Thomsen (1985) found that peptides of increasing chain length increased cellulose digestion by mixed rumen bacteria *in vitro*. In the study of Pittman & Bryant (1964), it was shown that *Bacteroides ruminicola* was able to utilize peptides and ammonia as N sources, but not free amino acids. This

is consistent with conditions in the rumen where concentrations of free amino acids are low (Wright & Hungate, 1967; Wallace, 1979). During hydrolysis of dietary protein, peptides may accumulate, depending on the nature of the protein being degraded. Russell *et al.* (1983) showed that although casein was hydrolysed rapidly by mixed rumen bacteria *in vitro,* accumulation of peptides in the medium did occur. Broderick & Wallace (1988) showed that feeding sheep casein resulted in a substantial but transient accumulation of peptides in rumen fluid. When more slowly degraded ovalbumin was included in the diet, there was no significant accumulation of peptides in rumen fluid.

The free amino acids released during hydrolysis of dietary protein are rapidly deaminated, producing VFA and ammonia in the process (Chalupa, 1976; Prins, 1977). Of particular importance in the rumen environment is the deamination of valine, isoleucine and leucine which give rise to i-butyrate, 2-methylbutyrate and i-valerate respectively (Dehority *et al.*, 1958). These compounds collectively constitute the branched-chain VFA. The degradation of carbohydrates as well as that of the amino acids proline, arginine, lysine and methionine gives rise to n-valerate. The branched-chain VFA have been shown to be either essential or stimulatory to the growth of many species of rumen bacteria, particularly the fibrolytic species (Bryant & Robinson, 1962). The growth of mixed rumen bacteria both *in vitro* (Russell & Sniffen, 1984; Cummins & Pappas, 1985; Gorosito *et al.*, 1985; Varga *et al.*, 1988) and *in vivo* (Hume, 1970a; Robinson & Sniffen, 1983) has been shown to be stimulated by branched-chain VFA. Stimulation of microbial growth by amino acids may therefore be due to the indirect effect of the supply of branched-chain VFA.

From the foregoing, it would appear that some form of amino acid N would be required in ruminant diets in order to maximize microbial protein synthesis and indeed many studies have examined the effect of the form of N on the efficiency of microbial protein synthesis *in vivo*. The response to the inclusion of

protein in the diet, in terms of microbial protein synthesis, varied considerably in these studies.

In a number of cases, microbial growth efficiency has been increased by the inclusion of a form of amino acid N in purified diets which are low in N. Hume (1970b) found that by partially replacing urea with either casein or zein in a semi-purified, mixed roughage-concentrate diet, the efficiency of microbial protein synthesis in sheep was increased. The same effect was however not observed when urea was replaced with gelatin in these diets. Ben-Ghedalia *et al.* (1978) also showed that on a purified, roughage-concentrate diet, replacement of 10% of urea N with maize gluten improved the efficiency of microbial protein synthesis, however no response to the inclusion of either casein or fishmeal was observed. In the study of Blake *et al.* (1983), microbial protein synthesis was increased by replacing urea in a semi-purified diet with groundnut meal.

A characteristic of all of the basal diets used in the above mentioned experiments is that they were very low in crude protein content. The response to the inclusion of a source of rumen-degradable protein, in terms of increased microbial protein synthesis strongly suggests that amino acid N was limiting to microbial growth on these diets, however other factors may also have played a role in stimulating microbial yields. The fact that only specific sources of protein stimulated growth in the studies of Hume (1970b) and Ben-Ghedalia *et al.* (1978) implies that the positive response may have been due to the slower, more sustained release of ammonia from these protein sources as opposed to urea. This would have resulted in better synchronization between the release of energy from the substrate and the availability of N, which could well have resulted in higher microbial yields (Smith, 1979; Sniffen & Robinson, 1987; Nocek & Russell, 1988). It is unlikely that the increase in microbial efficiency was due to the supply of precursors for branched-chain VFA as the concentration of these compounds in the rumen appeared similar across all

treatments in the study of Ben-Ghedalia *et al.* (1978) and were actually included in all diets by Hume (1970b). The possibility of the protein sources having supplied other growth factors cannot however be ruled out. Although it is probable therefore that microbial growth in these experiments was increased by the incorporation of amino acid N from the protein sources added to the diets, this cannot be concluded with certainty due to the possibility of other factors, such as improved synchronization between N and energy supply, having caused the response.

A number of experiments performed with more practical, mixed diets have shown no increase in microbial yields with the inclusion of a source of rumen-degradable protein. Mercer *et al.* (1980) observed no difference in bacterial N flow from the rumen of sheep fed on a barley-based diet when the N was supplied as either urea, groundnut meal or fishmeal. Microbial yield was also unaffected when either urea, single-cell protein, maize gluten or rapeseed meal was used to supplement diets based on barley and straw (McAllan *et al.*, 1988). These results lend support to the ARC (1984) conclusion that with more practical diets, sufficient degradable protein is normally present to supply any requirements for peptides, amino acids or branched-chain fatty acids.

Varied responses have also been observed with protein supplementation of low-quality roughages. Amos & Evans (1976) found that supplementing low-quality bermudagrass with sunflower meal increased microbial protein synthesis whereas supplementation with urea did not. It is likely that the increase in microbial yields observed with the sunflower meal was attributable to the increased supply of amino acid N, however the possibility still exists that the response was due either to better synchronization of energy and N or to the supply of branched-chain VFA or other growth factors. In the studies of Kropp *et al.* (1970a; 1970b), Leibholz & Kellaway (1979) and Redman *et al.* (1980) however, microbial protein synthesis did not increase when low-quality roughage was supplemented with rumen-

degradable protein. In all of the studies mentioned above where no response to the supplementation of protein was observed, microbial yields were far lower than the ARC (1984) figure of 32g N/kg OM apparently digested in the rumen. It would appear therefore that some factor other than the supply of amino acid N was limiting to microbial growth on these diets and indeed Kropp *et al.* (1977a) found evidence that microbial growth was limited by available energy and rumen turnover time rather than by N availability. This explanation appears to be quite feasible considering the indigestible nature of most low-quality roughages.

Microbial yields on diets based on silage, particularly grass silage, are frequently low in comparison to the ARC (1984) value of 32g N/kg OM apparently digested in the rumen (Thomas *et al.*, 1980; Thomson *et al.*, 1981). In a number of studies, supplementation of silage-based diets with a source of rumen-degradable protein has had the effect of increasing microbial protein synthesis. Cottrill *et al.* (1982) showed that microbial N synthesis on maize silage diets was increased by replacing urea with fishmeal. On grass silage diets, increases in microbial yield have been observed in response to supplementation with soyabean meal (Rooke *et al.*, 1985), fishmeal (Dawson *et al.* 1988) and casein (Rooke & Armstrong, 1989). The high crude protein content of the silages used in these experiments, together with the correspondingly high rumen ammonia levels indicate that N *per se* was not limiting to microbial growth. It could be argued that supplementation with protein resulted in better synchronization between energy and N, however in the study of Rooke & Armstrong (1989), casein and urea were both infused continuously into the rumen. Under these conditions, both sources of N would have been made available to the microbial population at the same rate, therefore the response to casein could not have been due to better synchronization of N and energy supply but rather appears to be attributable to the form of N supplied by the casein. It is most probable therefore that despite the relatively high crude

protein content of silages, insufficient amino acid N is present to sustain maximum microbial growth.

From the discussion above, no firm conclusions can be drawn as to whether peptides or amino acids are stimulatory to microbial growth under practical, *in vivo* conditions. Clearly though, there are numerous factors which complicate the execution as well as the interpretation of *in vivo* experiments designed to investigate this issue. From the results of *in vitro* experiments, it is clear that situations exist where amino acid N and in particular, peptides are required in order to maximize microbial yields. These *in vitro* experiments are however limited and have been done under very specific conditions which are in many respects far removed from the rumen situation and may therefore not always be applicable to *in vivo* conditions. As has been pointed out, the composition of the microbial population may change considerably, depending on the type of substrate being fermented. No definitive work has yet been done under controlled, *in vitro* conditions to investigate the amino acid N requirements of mixed rumen bacteria growing on substrates which commonly occur in ruminant diets. It was also noted in the foregoing that a number of other factors affect microbial growth, most notably specific growth rate and availability of ammonia. The *in vitro* studies which have examined the effect of amino acids or peptides on microbial growth have either been done in batch culture, with no control over specific growth rate, or, in the case of chemostat experiments, at only one particular growth rate. Due to the profound effect of growth rate on microbial growth efficiency, it is quite possible that nutrient requirements may differ at different growth rates. There is however no data available on the effect of growth rate on the requirement of mixed rumen bacteria for amino acids or peptides. Similarly, requirements for amino acid N have not been established under conditions of high and low ammonia.

Before firm conclusions can be drawn about the effect of supplementary degradable protein sources *in vivo*, more definitive

work is required *in vitro* in order to investigate the effect of supplementary amino acids or peptides on mixed rumen bacteria growing on more practical substrates. Such an approach would obviously imply a deviation from traditional techniques and the development of novel methods to facilitate such studies.

The main objective of the present study was to examine the effects of supplementary peptides on the growth of mixed rumen bacteria growing on substrates commonly constituting ruminant diets. The effects of other factors such as growth rate and ammonia levels on the response of the bacterial population to the supply of peptides was also investigated.

CHAPTER 2

THE DEVELOPMENT AND EVALUATION OF A CONTINUOUS CULTURE FERMENTOR FOR THE STUDY OF MIXED RUMEN BACTERIAL POPULATIONS

2.1 INTRODUCTION

Much of the present knowledge about the metabolism and physiology of the rumen microbial population has been obtained from experiments carried out under *in vitro* conditions. In many instances it is essential that studies be performed in this manner in order to avoid the complications and difficulties that occur *in vivo*. The obvious disadvantages of working *in vitro* lie in the fact that it is impossible to exactly simulate rumen conditions, particularly with regard to absorbtion of fermentation end-products, the presence of endogenous secretions and the passage of digesta out of the rumen. Although the results of *in vitro* experiments may therefore not always be directly applicable to the rumen situation, they are nevertheless able to provide accurate answers about some of the more basic aspects of rumen microbial metabolism.

A number of techniques have been developed for culturing mixed rumen bacteria, the most simple being batch culture. Although this technique has the advantage of being simple and relatively easy to run, the major drawbacks of batch cultures are that the microbial population may not fully adapt to the substrate being used, there is no means of controlling specific growth rate and also, conditions in the culture change continuously with time due to the build-up of fermentation end-products and the depletion of substrate. In continuous, or chemostat culture, many of these problems are overcome with continuous addition of substrate and removal of culture effluent. Although originally designed for the study of pure cultures of a particular organism, the conventional chemostat has been adapted to study mixed bacterial populations in some cases. An example of this is the study of Isaacson *et al.*

(1975) where a conventional chemostat was used to study the effect of growth rate on the growth efficiency of mixed rumen bacteria. A major disadvantage of conventional chemostats however is the fact that their medium delivery systems are not designed to handle solid substrates, which is probably the reason why a number of workers have used soluble substrates, mainly glucose, in their growth efficiency studies on mixed rumen microorganisms. The use of such substrates is likely to select for organisms with a high affinity for soluble sugars and the resultant population may therefore not be representative of that found in the rumen.

Various fermentors have been designed with the aim of maintaining a mixed rumen microbial population under continuous culture conditions on substrates commonly constituting ruminant diets (Abe & Kumeno, 1973; Hoover, *et al.*, 1976; Czerkawski & Breckenridge, 1977; Nakamura & Kurihara, 1978; Merry, *et al.*, 1987; Teather & Sauer, 1988; Fuchigami *et al.*, 1989; Miettinen & Setälä, 1989) The characteristics of some of these systems include differential flow of solid and liquid phases, dialysis for the removal of fermentation end-products and specially-designed agitation systems. These fermentors are usually fed with solid feed accompanied by an infusion of a buffer solution. The flow rate of the liquid phase is faster than that of the solid phase, thus simulating the situation in the rumen. In a number of cases, viable populations of rumen ciliate protozoa have been maintained in the fermentors for extended periods. This type of fermentor probably most closely simulates the rumen environment of all the *in vitro* culture methods used to date. The concept of having different flow rates for solids and liquids would result in two populations existing in the fermentor, each growing at it's own specific growth rate. Due to the fact that the liquid phase of these systems runs at a higher dilution rate and also constitutes a much higher proportion of the effluent than does the solid phase, it could be expected that any treatments imposed on the population in the fermentor would manifest themselves to a greater extent on the fluid-associated population.

The object of the present study was to develop and evaluate a continuous culture fermentor based on the principle of the conventional chemostat but able to utilize solid, complex substrates that typically occur in ruminant diets. The aim was not to try and simulate the rumen as a whole but rather to create conditions within which a representative population of rumen bacteria could be maintained on a particular substrate at any predetermined growth rate.

2.2 MATERIALS AND METHODS

2.2.1 *Construction and operation of fermentor*

The design and construction of the fermentor described here was based on that of the continuous culture system of Kistner & Kornelius (1990) which was used for rate studies of cellulose digestion by pure cultures of rumen bacteria. Various adaptations were made to the system which included the use of less expensive materials which was possible due to the fact that sterilization of the fermentor was not necessary. The general layout of the apparatus is shown schematically in Fig 1. The basic unit of the fermentor consisted of a medium reservoir feeding into three identical fermentor vessels. The medium reservoir was a cylindrical, jacketed vessel of approximately 7 l capacity. Refrigerated water (4°C) was circulated through the jacket of the vessel in order to maintain the medium at a low temperature, thus preventing growth of microorganisms present in the unsterilized medium. The contents of the medium reservoir were continuously and vigorously agitated by means of a vibratory stirrer (Vibro-mixer E1; Chemap, Männedorf, Switzerland). This method of agitation ensured that the solid particles in the medium remained in a homogeneous suspension. The medium reservoir was totally sealed and operated on the Mariotte flask principle (Ricica, 1966). A gas line was passed through the lid of the vessel and had its opening close to the floor of the vessel, near the outlets to the fermentor vessels. A low, but constant gas

pressure was maintained in this line at all times. As the level of fluid within the vessel fell, reducing the head of liquid, the drop in pressure at the bottom of the vessel was compensated for by the escape of gas from the gas line. In this manner the net pressure at the vessel outlets remained the same, irrespective of the level of medium in the reservoir.

Four identical outlet ports were placed approximately 4 cm above the base of the medium reservoir. Three of these led to the fermentor vessels while the fourth served as a sampling port. Whereas the sampling port was opened and closed manually, whenever samples were drawn, the opening and closing of the remaining three outlet ports was controlled by a system of pneumatic pinch-valves. These valves operated on the same principle as those described by Kistner & Kornelius (1990). Each outlet port was normally closed by a pneumatically-operated valve which was connected, via a three-way solenoid valve, to an electronic timing device. Upon opening of the valve, medium flowed out of the vessel and into a tube placed vertically in front of the medium reservoir. This tube served as a dosing pipette and due to the constant pressure maintained within the medium reservoir, the medium that flowed into the pipette always reached the same level for all three outlets. Once the medium in the pipette reached it's maximum level, the outlet valve from the medium reservoir closed and a similar valve positioned below the pipette opened simultaneously. The contents of the pipette were thus discharged into the culture vessel. The frequency of this process could be varied infinitely by adjusting the electronic timer. In this manner the three fermentor vessels could be supplied with identical volumes of medium at any particular frequency and could thus be run at any chosen dilution rate. The volume discharged from the dosing pipettes in a single dosing cycle was approximately 3ml. Depending on the dilution rate selected, the frequency of the dosing cycle varied from approximately once every 20 minutes to once every 5 minutes.

The three fermentor vessels associated with the medium reservoir were identical and consisted of 500 ml cylindrical vessels mounted within a common water bath which was maintained at 39°C. Each fermentor vessel had an outlet port situated near the top of the vessel, such that the working volume of the fermentors was 350 ml. Agitation was effected by a mechanically-driven impeller which was operated continuously. Mixing of the contents of the fermentor was further aided by baffles placed vertically on the inner wall of the vessel. The fermentors were continuously gassed with a mixture of 20% CO_2 and 80 % N_2 at a rate of approximately 15 ml/min.

Effluent from the culture vessel flowed out of the overflow port and was collected in a harvest vessel which was maintained in a cooling bath at 2°C.

A bank of four medium reservoirs, each dispensing into three replicate culture vessels was constructed as described above. The physical arrangement of the system is shown in Fig. 2.

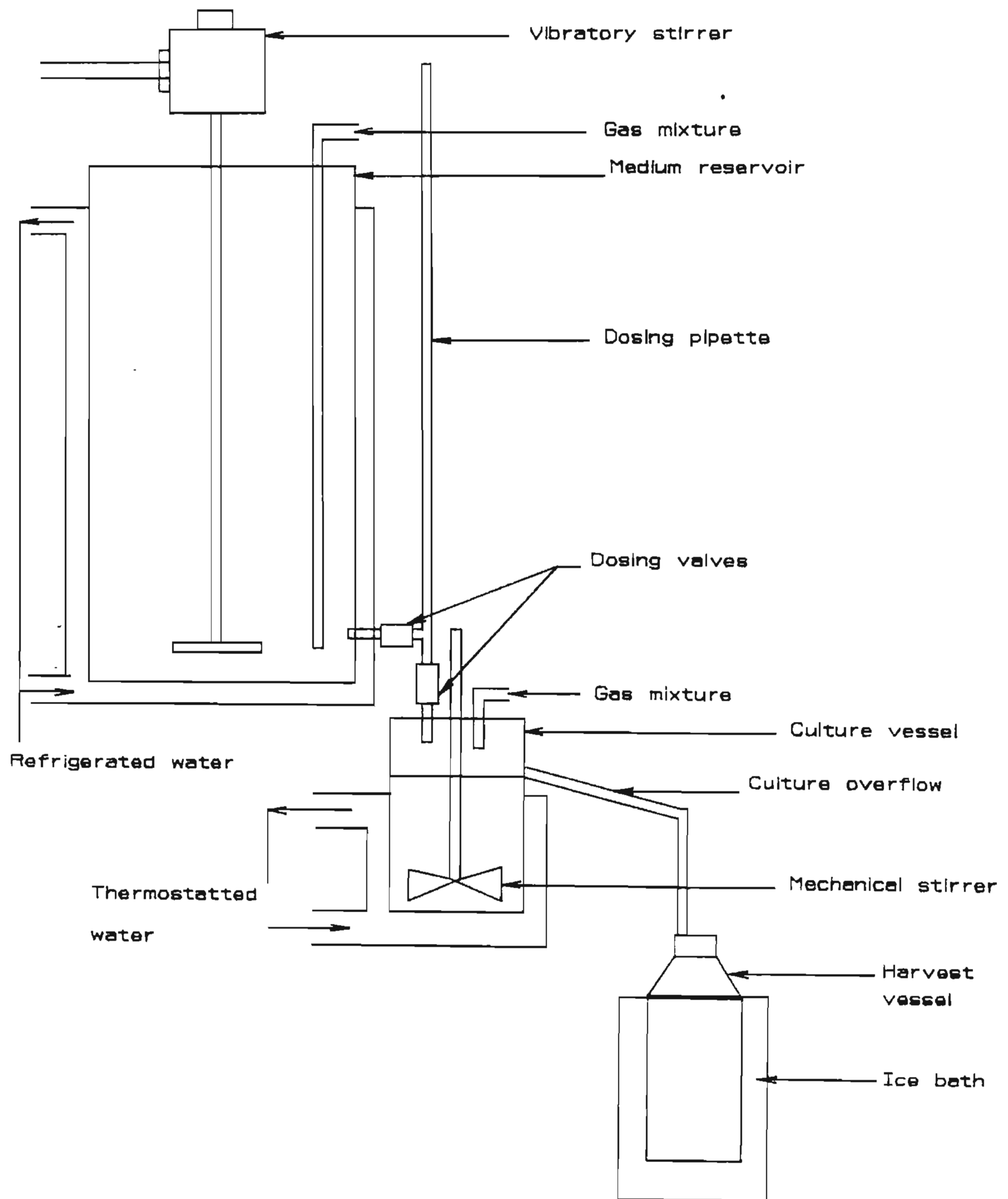

Fig. 1. Schematic layout of the continuous culture fermentor.

Fig. 2. General arrangement of the continuous culture fermentor showing 4 medium reservoirs with 12 culture vessels.
1, Vibratory stirrer; 2, Medium reservoir; 3, Dosing pipette; 4, Culture vessel; 5, Harvest vessel.

2.2.2 *Treatments*

In order to establish whether the fermentor was capable of maintaining a representative population of rumen bacteria in steady state, at different dilution rates, a medium containing maize straw as the major substrate was incubated in one of the fermentor units at dilution rates of 0.03, 0.06 and 0.09 h^{-1}. The composition of this medium is shown in Table 1.

Table 1. The ingredient composition of the maize straw medium incubated in continuous culture at dilution rates of 0.03, 0.06 and 0.09h^{-1}.

Ingredients	Inclusion rate/l
Maize straw (g)	10
$NaHCO_3$ (g)	4
Mineral solution (ml)[1]	50
Pfennigs metal soln. (ml)[2]	10
VFA solution (ml)[3]	10
NH_4Cl (mg)	324
$(NH_4)_2SO_4$ (mg)	126
Casein Hydrolysate (mg)[4]	260

[1] Composition (g/l): $CaCl_2.1H_2O$ 0.53; KH_2PO_4 18; NaCl 18; $MgCl_2.6H_2O$ 0.4
[2] Pfennig & Lippert (1966)
[3] Composition (mmol/l): n-valerate 91.94; i-valerate 91.16; i-butyrate 107.83; 2-methylbutyrate 92.14
[4] Peptone (Merck, Darnstadt. Product No. 7213)

The constituents of the media were weighed out accurately and made up to the appropriate volume in tap water. This procedure applied to all media made up in subsequent experiments in this study.

At the 0.03 and 0.09 h^{-1} dilution rates, VFA and NH_3-N concentrations were measured daily, from the time of inoculation, to determine when steady-state conditions had been attained. In the case of the experiment run at a dilution rate of $0.06h^{-1}$, samples were only taken for analysis once steady-state had been achieved, as evidenced by constant pH, VFA concentrations and NH_3-N levels. After establishing steady-state conditions in the fermentors, the medium reservoir was filled with freshly-prepared medium containing L-[4,5-^{3}H]leucine (1 Ci/mmol, 1 mCi/100ml) added to the medium at a rate of 1ml/l. Another four volume turnovers were then allowed to pass through the fermentors before sampling commenced. Culture effluent was then collected in the harvest vessels until approximately 700 ml (≈ 2 volume turnovers) had been collected. The harvest vessels were removed and the contents immediately frozen at -15°C for later analysis. The medium reservoirs were then refilled and [^{3}H]leucine was added as before. Again four volume turnovers were allowed before sampling commenced. In this manner, two independent samples were obtained from each of the three culture vessels at steady state. This provided a total of six replicate samples per treatment. During the steady-state sampling period, medium was sampled daily and samples thus drawn were frozen immediately for later analysis. Due to the fact that the bank of fermentors could only be operated at one dilution rate over any particular period, the three experiments were run consecutively rather than concurrently.

2.2.3 *Inoculum*

At the start of each experimental period, all of the fermentors were filled to their maximum working volume with inoculum. This inoculum was obtained from two ruminally-cannulated sheep which had been conditioned to a diet of coarsely-milled lucerne hay. The animals were fed 1 kg of lucerne hay each morning at 08h00 and had free access to water as well as a commercial mineral supplement containing phosphorous, calcium, salt and trace

minerals. Rumen digesta were removed via the cannula and filtered through two layers of coarse cheesecloth into a prewarmed container. Preliminary trials showed that this method of straining allowed numerous, small feed particles to be included in the inoculum. Microscopic examination of the inoculum showed large numbers of bacteria adhering to these particles. The strained rumen fluid was taken to the laboratory and immediately transferred to the culture vessels which had been prewarmed to 39°C and flushed with the gas mixture mentioned previously.

2.2.4 *Measurements and analyses*

Samples of culture effluent and medium collected during the steady-state period were thawed carefully to ensure that their temperature did not exceed 5°C. Once thawed, the samples were stirred vigorously on a magnetic stirrer and representative sub-samples drawn by means of a wide-bore pipette. Duplicate 20ml samples of medium were dried and ashed to determine DM and OM respectively. A 50ml sample was freeze-dried and analyzed for N and amino acids. Similar samples of culture effluent were also freeze-dried and analyzed for N, amino acids and [^{3}H]leucine. Duplicate 20 ml samples were also dried to determine DM content. Residual, unfermented OM of culture effluent was determined by first centrifuging 20 ml samples at 20 000 x g for 20 minutes. The supernatant was then removed and the pellet dried and ashed for determination of DM and OM respectively. This method was found to be more accurate for determination of residual, unfermented organic matter than direct drying as it ensured the removal of fermentation end-products, particularly VFA. Preliminary trials showed that directly drying the sample resulted in the majority of VFA being retained in the sample. This led to a serious overestimation (≈ 80%) of unfermented OM. A similar approach to that described here has been adopted by Hoover, *et al.* (1989). The remainder of the culture effluent was centrifuged at 1000 x g to remove feed particles and the supernatant thus obtained centrifuged again at 20 000 x g for 20

minutes to isolate bacteria. The supernatant from this step was removed and analyzed for NH_3-N and VFA. The bacterial pellet was washed by resuspension in water and centrifuging once more at 20 000 x g for 20 minutes before being freeze-dried and analyzed for N, amino acids and [^{3}H]leucine. OM was determined on a composite sample of bacteria.

DM and OM were determined by drying samples at 105°C and ashing at 550°C for 16h respectively. Nitrogen was determined by micro-Kjeldahl and NH_3-N by the phenol-hypochlorite reaction (Chaney & Marbach, 1962) using a Technicon Auto Analyser. VFA were analyzed by gas chromatography on a Packard 432 gas chromatograph with a flame ionization detector and fitted with a 2 m glass column of 2 mm I.D. The column was packed with 60/80 Carbopack C/0.3% Carbowax 20M/0.1% ortho-phosphoric acid and was operated isothermally at 130°C while injector and detector temperatures were set at 170°C and 190°C respectively. Carrier gas was nitrogen, set at a flow rate of 20 ml/min. Specific activity of [^{3}H]leucine in culture effluent and bacterial samples was analyzed as described by Dawson, *et al.* (1989). Samples were first hydrolysed in 6N HCl for 22h at 110°C, washed and dried then resuspended in sodium citrate buffer. Hydrolysates thus obtained were analyzed simultaneously for all amino acids, excluding methionine, cysteine and tryptophan according to the AOAC (1984) method on a Beckman automatic amino acid analyser (Model 7300; Beckman Instruments inc., Palo Alto, California, USA). Radioactivity was determined on the same hydrolysate by liquid scintillation counting on a Packard liquid scintillation counter (Model 2000CA; Packard instrument Co., Downers Grove, Illinois, USA) with external channels ratio quench correction. Specific activity was expressed as dpm/μmol leucine.

Net synthesis or disappearance of various components was calculated from the difference in concentration between medium and culture effluent. The proportion of N present as microbial N in culture effluent was calculated from the specific activity of

$[^3H]$leucine in culture effluent and in the bacterial fractions as described by Dawson *et al.* (1989). The equation used for the calculation of the microbial N content of culture effluent was as follows:

$$Microbial\ N\ (mg/l) = \left(\frac{specific\ activity\ of\ effluent\ leucine}{specific\ activity\ of\ bacterial\ leucine}\right) \times \left(\frac{effluent\ leucine\ (mg/l)}{leucine:total\ N\ for\ bacteria}\right)$$

Apparent OM digestion was calculated as the difference between the OM content of the medium and the residual, unfermented OM in culture effluent. True OM digestion was determined by correcting this value for the amount of bacterial OM in culture effluent.

Methane and hydrogen content of headspace gas were determined qualitatively on a daily basis for all fermentations as an indication of normal fermentation. Due to difficulties experienced in maintaining gas flows through the fermentors at an exact, constant rate as well as problems experienced with integration of peaks, it was not possible to report these results quantitatively. Headspace gas samples were injected into a Perkin Elmer gas chromatograph fitted with a glass column packed with Porapak type Q 50-80 mesh. The carrier gas was nitrogen and detection was by thermal conductivity. Oven and detector temperatures were 80 and 125°C respectively. Minimum detection levels for methane and hydrogen in headspace gas were 0.1 % (v/v).

2.2.5 *Bacterial counts*

In order to determine bacterial numbers in the fermentors, samples were drawn from the cultures, once steady state

conditions had been established at the 0.06 h^{-1} dilution rate, for total culturable counts. The basal medium used for these counts was based on that of the rumen fluid-containing medium of Caldwell & Bryant (1966) with the following exceptions: 0.0005 % indigocarmine replaced resazurin as a redox indicator; 0.251 % $NaHCO_3$, added as a filter-sterilized stock solution to the autoclaved medium after cooling to 50°C, replaced 0.4 % Na_2CO_3; and the medium was equilibrated with an O_2-free gas mixture containing, on a volume basis, 5 % H_2, 30 % CO_2, balance N_2. This was also the gas mixture present in the anaerobic glove box in which the media were dispensed, inoculated and incubated. For most probable number (MPN) counts of total culturable bacteria, 0.2 % of each of cellobiose, maltose, glucose and xylose, added as a combined, filter-sterilized stock solution were after autoclaving, were included in the medium.

Screw-capped bottles of at least 50 ml volume were completely filled with fermentor effluent and introduced to the anaerobic glove box. Five 10 g portions of well-mixed sample were weighed into 250 ml screw-capped polypropylene bottles, 90 ml volumes of sterile anaerobic diluent were added by means of a bottle-top dispenser (Socorex 521-50, Renens, Switzerland) and the diluted samples blended in a high-speed homogenizer (Ultra-Turrax TP 18/2, Janke & Kunkel, Staufeni. Br., Germany) for 1 minute. Further serial tenfold dilutions were made by adding 1 ml volumes of well-mixed samples to 9.0 ml of sterile anaerobic diluent in capped test tubes with the aid of a micropipette (Socorex 831), using sterile Pasteur pipettes as tips. The contents of the test tubes were mixed thoroughly on a vortex mixer after addition of the inoculum and again after withdrawal of aliquots for the next dilution or for the inoculation of media. One ml volumes of dilutions 10^{-6} to 10^{-10} of this 5-replicate dilution series were added to the tubes of the sugar medium which were then incubated at 39°C for 14 days. Growth was detected by increase in turbidity and drop in pH value of $\geq$ 0.3 pH units, compared with uninoculated controls. From the pattern of negative and positive

reactions in the five replicate tubes of dilutions 10^{-8} to 10^{-10}, MPN counts of total culturable bacteria were derived with reference to published tables (de Man, 1977).

2.3 RESULTS

The fermentor functioned reliably and with relatively little supervision over the entire experimental period. At no stage did the pH fall below 6.0 in any of the fermentations. Samples of headspace gas contained considerable quantities of methane (>3% v/v) with hydrogen being virtually undetectable (< 0.1% v/v) throughout all the fermentations. Microscopic examination of samples from the fermentors showed a wide variety of morphological forms of bacteria to be present. Protozoa and zoospores of anaerobic rumen fungi were observed during the earlier stages of the adaptation phase of the various fermentations, however these declined to zero by the time steady-state had been achieved.

2.3.1 *Attainment of steady state conditions*

The time-courses of events measured at the 0.03 and $0.09h^{-1}$ dilution rates are illustrated in Fig.3 and 4.

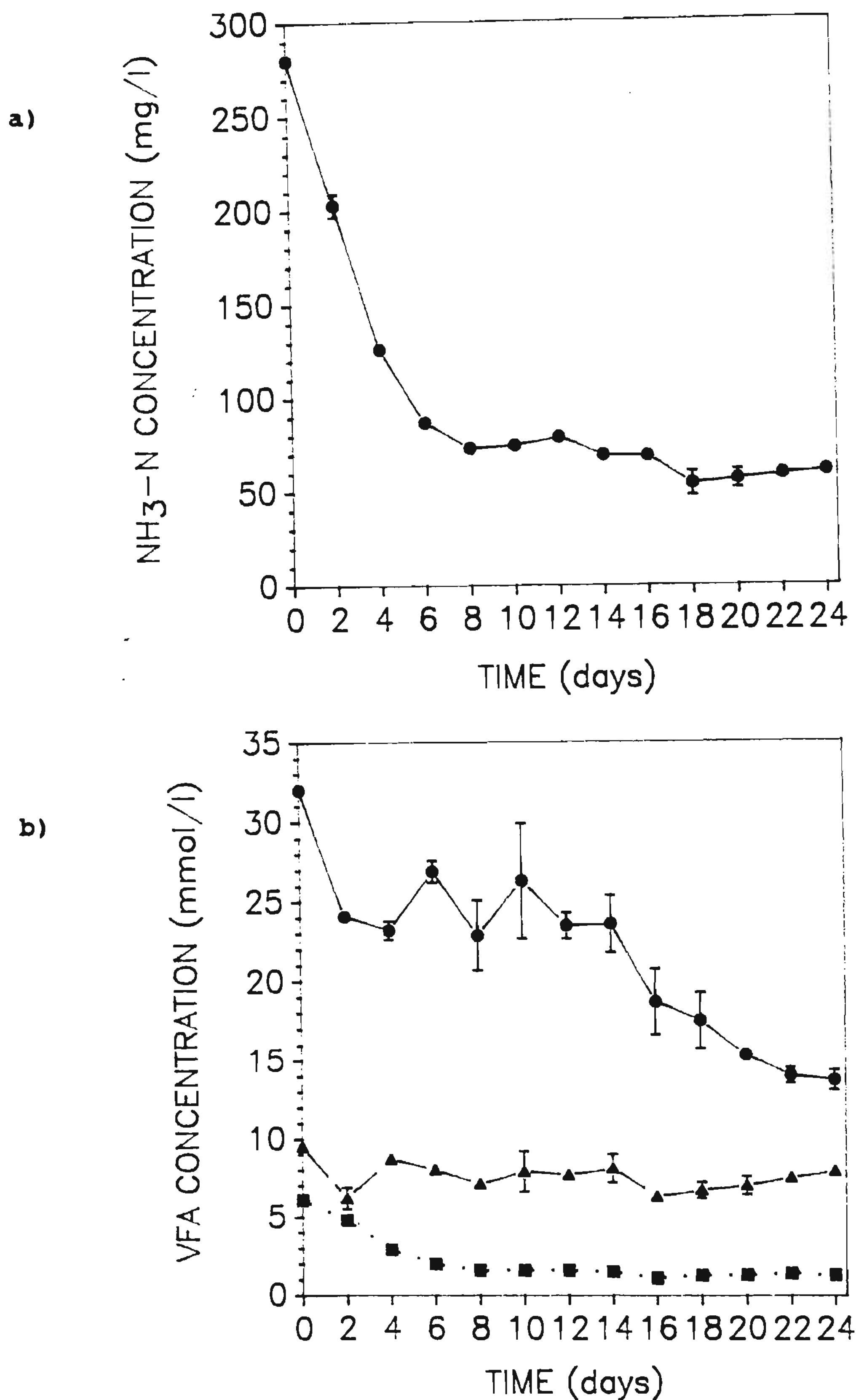

Fig. 3. The concentration of NH_3-N and VFA in fermentors with maize straw media incubated at $D=0.03h^{-1}$. Each point represents the mean of three fermentors. Vertical bars, where discernible, represent S.E. a) NH_3-N concentration. b) VFA concentration. Acetate (●); Propionate (▲); Butyrate (■).

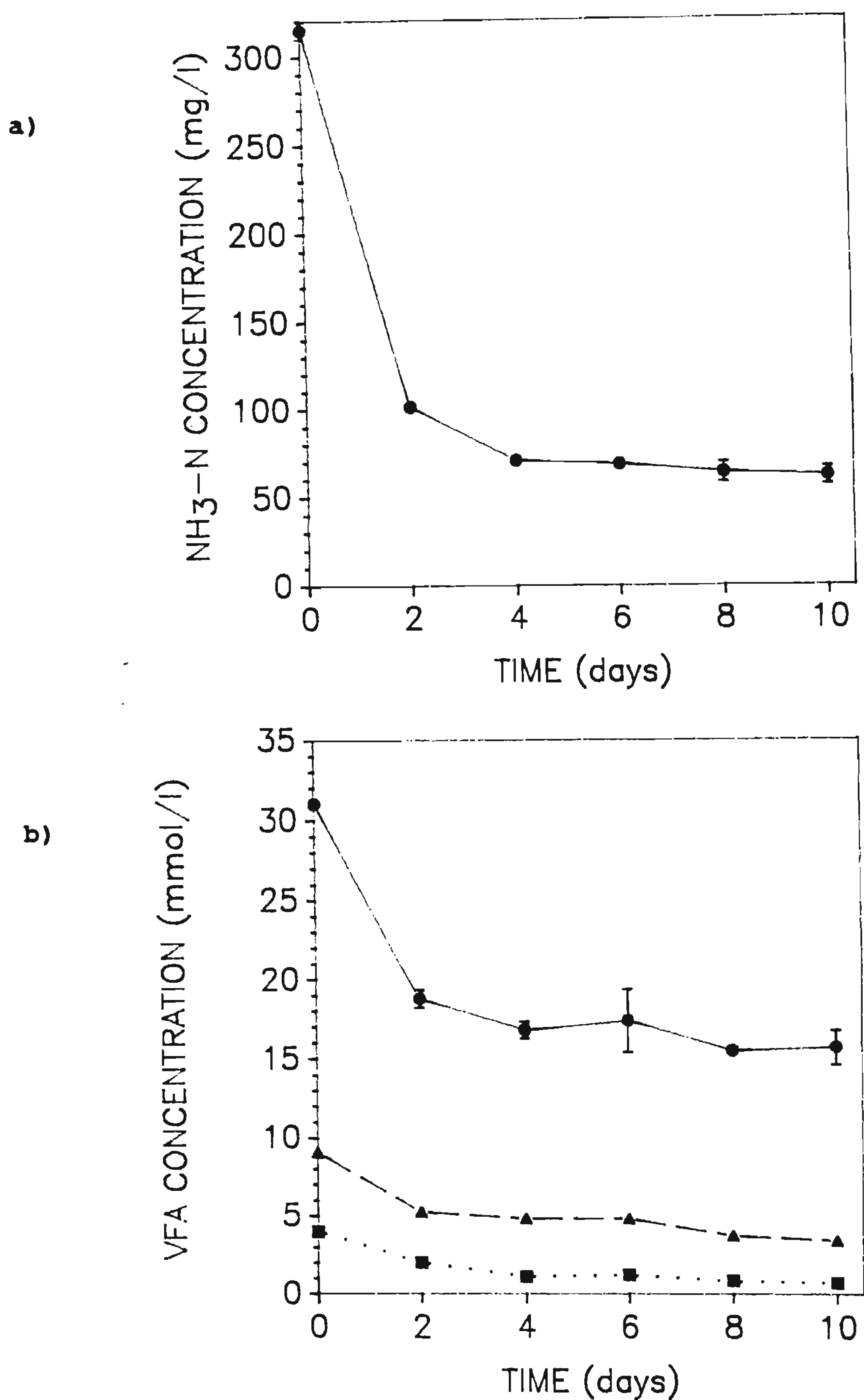

Fig. 4. The concentration of NH_3-N and VFA in fermentors with maize straw media incubated at $D=0.09h^{-1}$. Each point represents the mean of three fermentors. Vertical bars, where discernible, represent S.E. a) NH_3-N concentration. b) VFA concentration. Acetate (●); Propionate (▲); Butyrate (■).

At both dilution rates, the concentrations of VFA and NH_3-N decreased gradually from the level in the initial inoculum. At the $0.03h^{-1}$ dilution rate, total and individual VFA as well as NH_3-N concentrations differed significantly between consecutive days ($P<0.05$) until day 18, whereafter all parameters measured showed no significant differences between subsequent day's samples. A similar trend was observed at the $0.09h^{-1}$ dilution rate with these parameters stabilising as from day 6. This corresponds to approximately 13 volume turnovers in both cases.

2.3.2 *Fermentation parameters at steady state.*

The various fermentation parameters, as measured at steady state are shown in Table 2.

Table 2. Fermentation parameters measured for maize straw media incubated in continuous culture at dilution rates of 0.03, 0.06 and 0.09 h^{-1}.

	Dilution rate (h^{-1})					
Measurement	0.03	SE	0.06	SE	0.09	SE
pH	6.91	0.03	6.38	0.01	6.96	0.02
NH_3-N (mg/l)	57.5	3.53	56.2	0.17	62.7	3.42
App. OM digestion (%)	50.9	1.57	37.7	1.45	41.9	1.91
VFA:						
Total (mmol/l)	24.2	1.21	31.0	0.89	19.9	0.66
Molar %						
Acetate	65.5	1.39	70.9	1.83	78.5	0.55
Propionate	29.2	1.32	20.7	1.55	17.9	0.39
Butyrate	5.3	0.19	4.0	0.55	3.7	0.23
Bacterial N (mg/l)	88.1	4.06	77.4	2.55	116.9	4.85
Efficiency of Bacterial N synthesis (gN/kg OMAD*)	22.2	1.20	24.9	1.71	34.6	1.92

* Organic Matter Apparently Digested.

2.3.3 *Bacterial counts*

The mean value for total culturable bacteria observed in the three fermentor vessels was 2.5×10^9.

2.4 DISCUSSION

Preliminary experiments performed in the fermentor showed that whenever dilution rates exceeded $0.10h^{-1}$, or if purified substrates such as starch and glucose were used, without adding at least 25% of the total volume of the medium as clarified rumen fluid, there was a decline in the level of methane in the headspace and a corresponding increase in the level of hydrogen. Under normal conditions, the concentration of hydrogen in the rumen is very low ($\approx 10\mu M$) due to CO_2 reduction with H_2, yielding CH_4, through the action of methanogenic bacteria (Bryant, 1979). Loss of methane with the corresponding increase in hydrogen in the fermentors was therefore a sensitive means of detecting any abnormalities in the fermentation. The trend observed in the present experiment where hydrogen was not detectable therefore gives a strong indication that a normal, rumen-like fermentation occurred in all cases. The fact that the pH of the cultures never fell below 6.0, which is well within the normal physiological range found in the rumen (Church, 1979), provides further evidence that the fermentation was rumen-like.

The basic principles of chemostat culture as described by Pirt (1975) are that at steady state, the specific growth rate of the organism in the culture is determined by the medium flow or dilution rate and the concentration of biomass in the culture is controlled by the concentration of the growth-limiting substrate. Once steady state conditions have been reached, the concentration of both biomass and substrate remain constant over time, providing that all other factors remain constant. This steady state is self-regulating. In the present experiment, such a situation was achieved, as evidenced by the concentration of bacteria, OM, VFA and NH_3-N remaining constant over time. Concentrations of NH_3-N, being higher than 50 mg/l, were sufficient to sustain optimum microbial growth (Satter & Slyter, 1974) so that some other nutrient, probably energy, was first-limiting to microbial growth. Since a relatively indigestible

form of plant material was used as the substrate, it is reasonable to assume that growth was eventually limited by the rate at which the bacteria could liberate energy through the solubilisation of the substrate. It is evident therefore that the fermentor functioned according to chemostat principles and that the population being studied was growing at a specific growth rate as determined by dilution rate. The effect of specific growth rate on microbial growth efficiency has been well demonstrated by Isaacson *et al.* (1975) so it is important to control the growth rate of the population being studied.

The absence of protozoa from the culture may evoke criticism, however for the purposes of the studies intended with the fermentor, the presence of protozoa may well have complicated the interpretation of the results. The numbers of protozoa in the rumen may fluctuate considerably depending on various factors such as diet, intake and frequency of feeding (Dehority & Orpin, 1988). Because of their predation on bacteria they have a considerable influence on the recycling of N in the rumen and consequently have an effect on the efficiency of protein synthesis by the bacteria (Leng & Nolan, 1984). As the fermentor described here was designed with the object of studying populations of mixed rumen bacteria growing under steady state conditions, and more specifically, the effect of various factors on the efficiency of microbial protein synthesis by these populations, it was assumed that a population of protozoa in the fermentations would have confounded the results observed.

The wide range of dilution rates imposed in this experiment was chosen to represent the range of dilution rates that would be encountered in the rumen. The lower dilution rate of $0.03h^{-1}$ would however probably be most representative of rumen dilution rates for this type of substrate. Kropp, *et al.* (1977a) found rumen dilution rates to be in the region of $0.021h^{-1}$ on low quality roughage. At all three dilution rates, the observed pH and molar proportions of the individual VFA were typical of those

that would be found in vivo with this type of substrate (Hungate, 1966; Krysl *et al.*, 1989). The percentage of OM digested compares well with *in vivo* studies such as that of Redman *et al.* (1980) in which the ruminal digestion of oat chaff OM varied from 40 to 48%.

The efficiency of microbial protein synthesis observed in this study, at all dilution rates is considerably lower than the average value of 32 g N/kg OM apparently digested in the rumen reported by the ARC (1984). These values are also somewhat lower than those observed by Van Nevel & Demeyer (1979) with mixed rumen bacteria grown on glucose at similar dilution rates. The tendency shown for efficiency to increase with increasing dilution rate is however in agreement with the results of Isaacson *et al.* (1975) and Van Nevel & Demeyer (1979). Low values for the efficiency of microbial protein synthesis have however been observed in a number of *in vivo* studies with low-quality roughages (Kropp *et al.*, 1977a; Leibholz & Kellaway, 1979; Krysl *et al.*, 1989).

The number of total culturable bacteria counted in the fermentors was approximately 10 % of that normally found in the rumen (Hungate, 1966). This relatively low value is however not surprising as the level of substrate in the fermentors was approximately 10 % of that of a normal rumen (Church, 1979).

The fact that the different fermentations were not run concurrently precludes any statistical comparison between them. It should however be borne in mind that they were all run under identical, controlled conditions, so a comparison in terms of trends observed with changing dilution rate would be of relevance. The small difference between the 0.03 and 0.09 h^{-1} dilution rates in terms of the percentage of OM digested is rather surprising, as it would be expected that the longer retention time at the slower dilution rate would result in a far larger proportion of the substrate being digested. This implies

that extent, and not rate of digestion would be the first-limiting factor to the utilization of the roughage used, under *in vivo* conditions. The fact that the material was very finely milled may also have contributed to its relatively high digestion at the high dilution rate. The lower figure observed at the 0.06 h^{-1} dilution rate would appear to be as a result of some other, external factor. It might be speculated that the lower pH in this case may have played a role. The lower microbial yield and high VFA production rate in relation to the amount of OM fermented suggests a higher degree of energetic uncoupling (Hespell & Bryant, 1979).

In conclusion, the fermentor fulfilled its function of maintaining a representative population of mixed rumen bacteria under steady-state conditions at preselected growth rates.

CHAPTER 3

THE EFFECT OF FORM OF NITROGEN ON THE EFFICIENCY OF MICROBIAL PROTEIN SYNTHESIS BY RUMEN BACTERIA GROWING ON MAIZE STRAW IN CONTINUOUS CULTURE.

3.1 INTRODUCTION

Many situations exist, particularly in the sub-tropics and tropics, where ruminants are required to survive on dry, low-quality straw or grazing which is usually characterized by a low crude protein content and low digestibility. The low protein content of this material has a severe detrimental effect on its potential digestibility in the rumen and the provision of some form of rumen-available nitrogen is required in order to optimize its degradation in the rumen (Van Gylswyk, 1970; Fick *et al.*, 1973). It has become common practice to supplement low quality roughages with some form of non protein nitrogen (NPN) such as urea which has the effect of providing the rumen bacteria with nitrogen in the form of ammonia. Many studies have shown that the intake, digestion and overall utilization of the roughage source can be greatly improved by the supplementation of nitrogen (Van Gylswyk, 1970).

When low protein roughages form the basal diet of ruminants, the supply of protein to the lower digestive tract will be largely of microbial origin. The efficiency of protein synthesis by the rumen microorganisms is therefore an important determinant of the amount of protein available to the host animal. The supplementation of these diets with a source of rumen-available nitrogen or protein has the effect of not only improving digestion but also increasing the synthesis of protein by the rumen microflora, thereby increasing the amount of protein flowing out of the rumen (Elliot & Armstrong, 1982).

The inherently low protein content of these roughages raises the

question of whether or not supplementation of NPN alone is sufficient to maximize digestion and microbial protein synthesis and whether the supply of true protein may not have a stimulatory effect on the rumen microorganisms. Thomsen (1985) showed that the addition of peptides to cultures of mixed rumen bacteria *in vitro* increased the digestion of cellulose markedly. This suggests that the provision of rumen degradable protein to animals consuming low quality roughages may well have a beneficial effect on microbial fermentation and protein synthesis. A number of studies have examined the effect of replacing NPN with some form of rumen-degradable protein as a supplement to low protein, fibrous diets. Amos & Evans (1976) showed that the supplementation of low quality grass diets with sunflower meal increased microbial protein synthesis whereas supplementation with urea did not. In similar studies by Kropp *et al.* (1977a; 1977b) however, the substitution of urea with soya bean meal had no effect on microbial protein synthesis in steers fed on either low-quality grass hay or cottonseed hulls. Similarly, Leibholz & Kellaway (1979) found that the efficiency of microbial protein synthesis in sheep consuming oat chaff was unchanged as supplementary urea was replaced with increasing quantities of casein. Kempton *et al.* (1979) also found no increase in microbial growth efficiency in lambs when when casein replaced urea as the source of supplementary N in low quality fibrous diets.

The positive response obtained to the supply of protein in the study of Amos & Evans (1976) may well have been a result of the peptides which the protein source would have supplied to the bacteria in the rumen. It is also possible however that the response might have been due either to the supply of branched-chain VFA or to the slower release of N over time, resulting in NH_3-N levels in the rumen being sustained for longer, thereby resulting in better synchronization between N and energy supply. The lack of response to the supplementation of protein in other studies may be attributable to a number of factors which would

prevail in the rumen of animals fed on low-quality, fibrous diets. The indigestible nature of such a diet would imply that the retention time of digesta in the rumen would be relatively long. This slow turnover of rumen contents would have a profound influence on microbial activity. The mean specific growth rate of the bacteria would be low, resulting in a correspondingly low efficiency of microbial protein synthesis (Isaacson *et al.*, 1975). Indeed, Kropp *et al.* (1977a), reported strong indications that available energy and rumen turnover time, rather than N availability limited microbial protein synthesis. A slow turnover in the rumen would also be expected to increase the recycling of N within the rumen between bacterial, protozoal and ammonia pools (Leng & Nolan, 1984). Nolan & Stachiw (1978) found that when animals were fed a diet of wheat hay, in excess of 50% of dietary nitrogen was recycled in the rumen. The implications of this recycling are twofold. On the one hand, the efficiency of protein synthesis could be expected to decrease further as a result of this while on the other hand, the release of various nitrogenous compounds from lysed cells and excretions by protozoa may reduce the reliance of the bacterial population on the dietary supply of compounds such as amino acids, peptides and branched-chain VFA.

The effect of amino acids and/or peptides on rumen bacteria fermenting low-quality roughages *in vivo* is therefore unclear and is confounded by a number of factors. In all of the *in vivo* studies mentioned above, microbial yields were low, and were far below the value of 32 g N/kg OM apparently digested in the rumen observed over a wide range of diets by the ARC (1984). The reason for these low values would appear to be factors other than the level or form of supplementary N supplied in the diets. The results obtained by Thomsen (1985) *in vitro,* together with those of Amos & Evans (1976), would however suggest that conditions do in fact exist where the availability of amino acids or peptides may well have a stimulatory effect on microbial growth efficiency. This is further supported by the numerous studies where positive responses to protein supplementation have been

observed on diets such as maize silage (Cottrill *et al.*, 1982), ryegrass silage (Dawson *et al.*, 1988) and mixed roughage-concentrate diets (Hume, 1970b) as well as the in vitro studies of Maeng *et al.* (1976) and Cotta & Russell (1982).

The purpose of the present study was therefore to examine the effects of various forms of N on the efficiency of microbial protein synthesis on low quality roughage under conditions within which the effect of confounding factors such as nitrogen recycling, turnover rate, rate of N release and the supply of branched-chain VFA or other factors could either be reduced or eliminated completely. A further objective was to investigate the effect of the level of available N as well as specific growth rate on the response of the microbial population to different forms of N.

3.2 MATERIALS AND METHODS

3.2.1 *Fermentors*

Three of the previously-described fermentor units, each consisting of a medium reservoir supplying three identical fermentor vessels, were used for this experiment. At the start of each experimental period the respective media were mixed and immediately transferred to the medium reservoirs which had been pre-cooled. Inoculum was prepared as described previously and was obtained from two ruminally-cannulated sheep that were accustomed to a diet of milled lucerne hay. Rumen digesta from these animals were strained through two layers of cheesecloth into a prewarmed container. The strained rumen fluid was transported to the laboratory within five minutes of withdrawal from the rumen and immediately transferred to the fermentor vessels which were maintained at 39°C and had been flushed with the CO_2:N_2 gas mixture described previously. Each fermentor was filled to maximum working volume, whereafter the flow of medium into the vessel, at the appropriate dilution rate, was initiated.

3.2.2 *Media and Treatments*

Two sets of media were made up as illustrated in Table 1. Maize straw which had been milled through a 0.5mm screen in a Wiley mill formed the basis of the media, each of which contained equal quantities of minerals, trace minerals, VFA and sodium bicarbonate which acted as a buffer. The two sets of media differed in that they were supplemented with N at either a low (75 mg/l) or a high (150 mg/l) level. Each set of media consisted of three isonitrogenous media which differed only in the form of the supplementary N supplied. In each case N was supplied in the form of either ammonium salts or peptides in the form of a partial, tryptic hydrolysate of casein (Peptone, Merck, Darnstadt. Product No. 7213). The reason for including $(NH_4)_2SO_4$ in addition to NH_4Cl as a source of NH_3-N was to ensure that sufficient S was present in the medium so as not to be limiting to microbial growth. In this, as well as the subsequent experiments in this study, $(NH_4)_2SO_4$ was included at a level such that the N:S ratio of the supplementary N was 5:1. This is well in excess of the 14.1:1 ratio proposed by the ARC (1980) as being sufficient for microbial growth. The different N sources were included at levels in the three different media so as to provide ammonia N:peptide N ratios of 100:0, 75:25 and 50:50 respectively. The solution of VFA added to all of the media contained all the branched-chain VFA as well as n-valerate. As mentioned in chapter 1, the branched-chain have been shown to be stimulatory to microbial growth in a number of studies (Bryant & Robinson, 1962; Hume 1970; Russell & Sniffen, 1983). Although n-valerate alone may not always increase microbial growth (Russell & Sniffen, 1983; Gorosito *et al.*, 1985), it has been shown to have a stimulatory effect on rumen bacteria (Cline *et al.*, 1966). To ensure that it did not limit microbial growth therefore, n-valerate was included in the VFA solution. For the purposes of this experiment, as well as the experiments described in the subsequent chapters, the branched-chain VFA, together with n-valerate are referred to collectively as the branched-chain VFA, unless otherwise stated.

Table 1. The ingredient composition of maize straw media supplemented at either a high or a low level of N with peptide N included at a level of 0, 25 or 50% of the total supplementary N.

Component inclusion rate/l	Level and form of of supplementary N: Low N Peptide N (%) 0	25	50	High N Peptide N (%) 0	25	50
Maize straw (g)	10	10	10	10	10	10
$NaHCO_3$ (g)	4	4	4	4	4	4
Mineral soln. (ml)[1]	50	50	50	50	50	50
Pfennigs metal soln. (ml)[2]	10	10	10	10	10	10
VFA solution (ml)[3]	10	10	10	10	10	10
NH_4Cl (mg)	233	162	91	466	324	182
$(NH_4)_2SO_4$ (mg)	63	63	63	126	126	126
Casein Hydrolysate (mg)	0	130	257	0	260	514

[1] Composition (g/l): $CaCl_2.1H_2O$ 0.53; KH_2PO_4 18; NaCl 18; $MgCl_2.6H_2O$ 0.4

[2] Pfennig & Lippert (1966)

[3] Composition (mmol/l): n-Valerate 91.94; i-Valerate 91.16; i-Butyrate 107.83; 2 methylbutyrate 92.14

The two sets of media described above were each incubated at a high ($0.09h^{-1}$) and a low ($0.03h^{-1}$) dilution rate in the continuous culture fermentor. The experiment was divided into four separate experimental periods during which the three different ratios of ammonia N:peptide N were compared at high and low N levels, each at the high and low dilution rates as follows:

Period 1: Low N, $D=0.03h^{-1}$

Period 2: Low N, $D=0.09h^{-1}$

Period 3: High N, $D=0.03h^{-1}$

Period 4: High N, $D=0.09h^{-1}$

Each medium from either the high N or low N set was randomly allocated to a fermentor unit and incubated at the appropriate dilution rate. Fresh inoculum was added to the fermentor vessels at the start of each experimental period.

3.2.3 *Sampling*

Effluent from the fermentors was discarded for the initial adaptation period. Once steady-state conditions were established, as evidenced by constant pH, NH_3-N and VFA concentrations, the medium reservoir was filled with freshly-prepared medium containing L-[4,5-^{3}H]leucine (1 Ci/mmol, 1 mCi/100ml) at a rate of 1ml/l. Four volume turnovers were then allowed to pass through the fermentors before sampling commenced. Culture effluent was collected in the harvest vessels until approximately 700ml (≈ 2 volume turnovers) had accumulated. The harvest vessels were removed and the contents immediately frozen at -15°C for later analysis. The medium reservoirs were then refilled and again [^{3}H]leucine was added. Again four volume turnovers were allowed before sampling commenced. In this manner, two independent samples were obtained from each of the three culture vessels at steady state. This provided a total of six replications per treatment. During the steady state sampling period, medium was sampled daily and samples thus drawn were frozen immediately for later analysis.

3.2.4 *Measurements and analyses*

During the adaptation as well as the sampling phase of the different experimental periods, samples of headspace gas were drawn and analysed qualitatively for methane and hydrogen, as described previously. pH was also monitored on a daily basis during this period. Samples of medium and culture effluent collected during the steady state period were analysed as described in the preceding chapter. The results of these analyses were used to calculate the production of VFA, OM digestion,

microbial N synthesis, and the flow of individual as well as total amino acids out of the fermentors.

3.2.5 *Statistical analysis*

Each experimental period was analysed separately as a randomized blocks design using analysis of variance techniques with the two sampling periods representing blocks. Differences between means were tested for significance by means of least significant differences (Steele & Torrie, 1960).

3.3 RESULTS

3.3.1 *Composition of media*

The chemical composition of the different media is shown in Table 2. As expected, the inclusion of casein hydrolysate at both the 25% and 50% level clearly increased the total amino acid content of the media. Of the individual amino acids, glutamic acid, proline and isoleucine showed the highest increase with the inclusion of casein hydrolysate.

Table 2. Chemical composition of maize straw media supplemented at either a high or a low level of N with peptide N included at a level of 0, 25 or 50 % of the total supplementary N.

	Level and form of of supplementary N					
Component	Low N Peptide N (%)			High N Peptide N (%)		
inclusion rate	0	25	50	0	25	50
DM (g/l)	12.76	12.65	12.91	12.09	11.87	11.81
OM (g/l)	8.25	8.09	8.45	7.66	7.90	7.70
N (mg/l)	153.8	158.4	159.1	227.6	232.8	236.6
Amino acids (mg/100mg total amino acids)						
Aspartic acid	12.2	11.3	10.5	12.3	11.0	10.4
Threonine	5.9	5.2	4.9	5.6	4.8	4.7
Serine	6.4	6.0	5.8	6.6	5.7	5.7
Glutamic acid	14.8	16.7	18.6	14.8	17.8	20.9
Proline	6.4	6.9	7.8	5.6	7.5	8.7
Glycine	7.5	6.7	5.9	7.2	5.9	5.1
Alanine	8.4	7.6	6.8	8.4	7.3	6.6
Valine	6.3	6.7	6.6	6.0	6.3	6.3
Isoleucine	4.2	4.5	4.8	4.0	4.5	4.7
Leucine	9.2	9.1	9.6	8.9	9.2	8.6
Tyrosine	3.6	3.4	3.4	4.1	3.2	3.0
Phenylalanine	5.7	5.8	5.7	5.7	5.3	4.8
Histidine	1.4	1.6	1.5	1.6	1.7	1.6
Lysine	4.4	4.8	5.0	4.9	5.4	4.6
Arginine	3.7	3.9	3.2	4.3	4.4	4.4
TOTAL (mg/l)	203.4	234.4	299.7	201.1	311.3	382.2

3.3.2 *Fermentation parameters*

In all the experiments, significant quantities of methane were present in the headspace gas of all fermentors with hydrogen remaining undetectable. pH in all cases remained above 6.0

throughout the adaptation as well as the steady state phase. The various fermentation parameters measured at steady state for the low N media are displayed in Table 3.

Table 3. Mean values for pH, VFA concentrations and NH_3-N concentration of culture effluent from incubations of maize straw media supplemented with N at the low level and containing either 0, 25 or 50 percent of supplementary N in the form of peptides.

	Dilution rate (h^{-1})							
	0.03				0.09			
	Peptide N (%)				Peptide N (%)			
Measurement	0	25	50	SE	0	25	50	SE
pH	6.82	6.81	6.84	0.01	6.74	6.77	6.90	0.04
NH_3-N (mg/l)	9.8	7.4	8.3	0.85	14.3[a]	8.3[b]	10.6[ab]	1.33
VFA:								
Total (mmol/l)	28.9	31.3	30.7	0.70	22.9	24.2	20.8	1.49
Molar %								
Acetate	59.2	60.0	57.4	0.73	64.9	66.9	63.4	1.63
Propionate	21.1	22.5	23.2	0.82	17.8	15.5	16.9	1.05
n-Butyrate	5.5[a]	4.6[b]	5.2[ab]	0.20	3.1	3.2	3.5	0.26
i-Butyrate	4.1	3.9	4.2	0.11	4.2[a]	4.3[a]	4.9[b]	0.19
2 m-Butyrate	3.3	2.9	3.2	0.16	3.2	3.1	3.4	0.15
n-Valerate	3.4	3.3	3.6	0.12	3.3	3.4	3.9	0.22
i-Valerate	3.3	2.9	3.3	0.21	3.6	3.6	4.0	0.20

a, b Means in the same row with different superscripts differ significantly ($P<0.05$).

In the case of the low N media, pH did not differ significantly between treatments, at either the high or the low dilution rate.

NH_3-N levels in culture effluent were very low (<1mM) for all the fermentations. The inclusion rate of supplementary peptide N had no effect on NH_3-N levels at the low dilution rate, however, at the high dilution rate, significantly higher levels of NH_3-N were observed for the media containing 0% supplementary peptides than for the medium containing 25% supplementary peptide N ($P<0.05$).

Total VFA concentrations in culture effluent for the low N media were unaffected by level of peptide N at both dilution rates. There were no indications of any trends in this regard. The only differences observed in terms of the molar proportions of the individual VFA were that significantly higher quantities ($P<0.05$) of n-butyrate were present in the 0% peptide N medium as opposed to the 25% peptide N medium at the low dilution rate and also, the concentration of i-butyrate in culture effluent was significantly higher ($P<0.05$) in the case of the 50% peptide N medium than the the other two treatments, at the high dilution rate.

The fermentation parameters observed at steady state for the high N media are illustrated in Table 4. As was the case for the low N media, pH did not differ significantly between treatments for the high N media at either the high or the low dilution rate.

In all fermentations of the high N media, NH_3-N levels were in excess of 50mg/l. No significant differences were observed between treatments in this respect at the high dilution rate, however at the low dilution rate significantly higher levels of NH_3-N were present in culture effluent of the 0% peptide N media than in the the effluent from the other two treatments ($P<0.05$). At both the high and low dilution rates, NH_3-N levels in culture effluent showed a tendency to decline with increasing levels of peptide N in the medium.

The concentration of total VFA in culture effluent showed a tendency to increase with increasing peptide N in the medium at

both dilution rates. The only significant difference observed in this regard was in the case of the high dilution rate where a significantly higher value ($P<0.05$) was observed for the 50% than for the 0% peptide N treatment. The molar proportion of individual VFA were all similar between treatments at the low dilution rate except for n-valerate which was significantly lower ($P<0.01$) in the effluent of the 0% peptide N treatment than for the other two treatments. At the high dilution rate a number of significant differences were observed with respect to the molar proportions of individual VFA, as illustrated in Table 4. In general, there was a tendency for a higher proportion of acetate in the case of the 0% peptide N medium whereas the other two treatments resulted in higher concentrations of branched-chain VFA, especially i-butyrate and i-valerate in culture effluent.

Table 4. Mean values for pH, VFA concentrations and NH_3-N concentrations of culture effluent from incubations of maize straw media supplemented with N at the high level and containing either 0, 25 or 50 percent of supplementary N in the form of peptides.

	Dilution rate (h^{-1})							
	0.03				0.09			
	Peptide N (%)				Peptide N (%)			
Measurement	0	25	50	SE	0	25	50	SE
pH	6.91	6.91	6.91	0.01	6.93	6.97	6.96	0.01
NH_3-N (mg/l)	68.3^a	56.6^b	53.2^b	3.01	65.8	62.9	58.4	4.07
VFA:								
Total (mmol/l)	26.1	28.4	30.3	1.71	21.4^a	23.3^{ab}	24.0^b	0.74
Molar %								
Acetate	59.2	55.8	54.9	2.13	66.8^a	67.1^a	62.9^b	0.58
Propionate	24.8	27.0	26.8	1.69	16.0^a	14.9^b	16.5^a	0.38
n-Butyrate	4.1	4.2	4.9	0.33	3.9^a	3.0^b	3.6^{ab}	0.24
i-Butyrate	3.8	3.7	3.9	0.23	4.1^a	4.4^b	4.5^b	0.09
2-m Butyrate	2.5	2.7	2.6	0.14	2.9	3.3	3.5	0.19
n-Valerate	2.9^a	3.9^b	4.1^b	0.14	3.7^a	3.9^a	4.7^b	0.24
i-Valerate	2.7	2.7	2.9	0.17	2.7^a	3.5^b	4.3^b	0.27

a, b Means in the same row with different superscripts differ significantly ($P<0.05$).

3.3.3 *OM digestion and bacterial N synthesis*

The results obtained with regard to OM digestion and bacterial N synthesis on the high N and low N media are depicted in Tables 5 and 6 respectively.

Table 5. Mean values for the extent of OM digestion and efficiency of microbial N synthesis from incubations of maize straw media supplemented with N at the low level and containing either 0, 25 or 50 percent of supplementary N in the form of peptides.

	Dilution rate (h^{-1})							
	0.03				0.09			
	Peptide N (%)				Peptide N (%)			
Measurement	0	25	50	SE	0	25	50	SE
OM Digestion (%)								
Apparent	46.1	49.1	47.0	1.15	37.3	39.9	32.1	2.41
Actual	63.2	61.5	59.4	1.47	46.4	49.9	42.7	2.15
Bacterial N (mg/l)	88.3	94.2	100.8	8.69	62.7	70.2	74.3	5.79
Microbial growth efficiency								
g N/kg OMAD[1]	22.7	23.2	24.9	2.45	21.3	25.7	28.7	3.11
g N/kg OMTD[2]	18.0	18.4	18.6	1.62	17.0	19.1	21.6	1.78

[1] Organic Matter Apparently Digested
[2] Organic Matter Truly Digested

The inclusion of peptides in the media, at all of the three specified levels did not have a significant effect on any of the parameters measured at the low N level. There was however a distinct tendency for bacterial N and microbial growth efficiency to increase witrh increasing levels of peptide N. This applied to both the high and the low dilution rate.

Table 6. Mean values for the extent of OM digestion and efficiency of microbial N synthesis from incubations of maize straw media supplemented with N at the high level and containing either 0, 25 or 50 percent of supplementary N in the form of peptides.

	Dilution rate (h^{-1})							
	0.03				0.09			
	Peptide N (%)				Peptide N (%)			
Measurement	0	25	50	SE	0	25	50	SE
OM Digestion (%)								
Apparent	50.0	52.1	49.5	1.35	38.6^{a}	41.9^{a}	31.9^{b}	1.76
Actual	60.5	62.8	57.1	1.31	47.9^{a}	55.2^{b}	43.9^{a}	1.76
Bacterial N (mg/l)	79.9^{a}	88.1^{a}	64.4^{b}	4.01	74.6^{a}	116.9^{b}	86.6^{a}	4.54
Microbial growth efficiency								
g N/kg OMAD	21.7^{a}	22.2^{a}	17.6^{b}	1.45	25.3^{a}	34.6^{b}	35.7^{b}	2.05
g N/kg OMTD	17.8^{a}	18.4^{a}	14.7^{b}	1.01	20.2^{a}	26.2^{b}	25.8^{b}	1.30

a, b Means in the same row with different superscripts differ significantly ($P<0.05$).

The only significant difference between treatments at the high N level and low dilution rate was that the amount of bacterial N in culture effluent was significantly lower ($P<0.05$) in the case of the 50% peptide N treatment. This difference was reflected in the values obtained for microbial efficiency which showed similar significant differences ($P<0.05$) between treatments at the low dilution rate. At the high dilution rate, apparent OM digestion was significantly lower ($P<0.01$) for the 50% peptide N treatment than for the other two treatments, with the 25 % peptide N

treatment showing the highest value in this respect. A similar trend was noted for actual OM digestion, after correction for bacterial OM. In this instance however, the value observed for the 25% peptide N treatment was significantly higher ($P<0.01$) than for the remaining two treatments. The same pattern was observed for microbial N synthesis with that of the 25% peptide N treatment being significantly higher ($P<0.01$) than the others. Microbial efficiency, expressed as g bacterial N/kg OM apparently fermented (OMAD) was significantly lower ($P<0.01$) for the 0% peptide N treatment than for the 25% and 50% level. Similarly, when expressed in terms of g bacterial N/kg OM truly digested (OMTD), the value observed for the 0% peptide N treatment was significantly lower ($P<0.01$) than for the other two treatments.

3.3.4 *Amino acid utilization and synthesis*

The amino acid profiles and total amino acid content of culture effluent from the low N and high N fermentations are shown in Table 7 and Table 8 respectively.

Table 7. The amino acid composition of culture effluent from incubations of maize straw media supplemented with N at the low level and containing either 0, 25 or 50 percent of the supplementary N in the form of peptides.

	Dilution rate (h^{-1})							
	0.03				0.09			
	Peptide N (%)				Peptide N (%)			
Measurement	0	25	50	SE	0	25	50	SE
Amino acids (mg/100mg total amino acids)								
Aspartic acid	12.5	12.8	12.7	0.11	13.4	13.6	13.1	0.17
Threonine	5.9	5.9	5.9	0.09	6.3	6.3	6.2	0.05
Serine	5.7	5.7	5.6	0.05	5.9	6.0	6.0	0.16
Glutamic acid	14.2	14.3	14.1	0.11	15.0	15.1	15.1	0.16
Proline	4.1[a]	4.2[a]	4.4[b]	0.07	4.9	4.9	5.2	0.11
Glycine	6.5	6.4	6.3	0.09	7.0	6.7	6.6	0.09
Alanine	8.9	9.0	8.7	0.12	8.7	8.7	8.5	0.11
Valine	6.0	5.7	5.8	0.13	5.8	5.6	5.9	0.14
Isoleucine	4.8	4.7	4.7	0.11	4.6	4.4	4.8	0.18
Leucine	8.9	9.0	8.8	0.08	8.8	8.7	8.8	0.06
Tyrosine	3.4	3.3	3.9	0.22	3.9	4.2	3.7	0.21
Phenylalanine	6.2	6.2	6.1	0.08	5.4	5.4	5.4	0.06
Histidine	1.8	1.8	1.8	0.05	1.6	1.7	1.7	0.06
Lysine	7.5	7.4	7.3	0.10	4.7	5.0	5.1	0.07
Arginine	3.5	3.6	3.9	0.14	4.1	3.8	3.9	0.28
TOTAL (mg/l)	410.8	368.5	399.0	11.97	366.1[a]	367.7[a]	412.5[b]	13.41

a, b Means in the same row with different superscripts differ significantly ($P<0.05$).

Table 8. The amino acid composition of culture effluent from incubations of maize straw media supplemented with N at the high level and containing either 0, 25 or 50 percent of the supplementary N in the form of peptides.

	Dilution rate (h^{-1})							
	0.03				0.09			
	Peptide N (%)				Peptide N (%)			
Measurement	0	25	50	SE	0	25	50	SE
Amino acids (mg/100mg total amino acids)								
Aspartic acid	12.8	12.7	13.0	0.37	12.7	13.0	12.6	0.28
Threonine	6.5	6.2	6.1	0.21	6.3	6.3	6.2	0.07
Serine	5.4	5.4	5.4	0.16	6.0	6.0	5.9	0.09
Glutamic acid	13.5	13.8	14.3	0.56	14.8^{a}	15.0^{ab}	15.3^{b}	0.12
Proline	4.9	4.8	4.7	0.17	4.7^{a}	4.9^{ab}	5.0^{b}	0.11
Glycine	6.7^{a}	6.4^{ab}	6.1^{b}	0.11	6.7	6.8	6.7	0.06
Alanine	8.6	8.4	8.4	0.26	8.7	8.8	8.9	0.10
Valine	5.8	5.9	5.8	0.25	5.9	5.7	5.9	0.20
Isoleucine	4.8	4.8	4.9	0.20	4.7	4.4	4.8	0.13
Leucine	9.4	8.6	8.5	0.42	8.9^{ab}	8.7^{a}	9.2^{b}	0.11
Tyrosine	4.6	4.6	4.6	0.31	3.9	4.0	3.5	0.20
Phenylalanine	5.5	5.5	5.5	0.16	6.0	5.9	6.2	0.17
Histidine	1.8	1.8	1.7	0.09	1.6	1.6	1.6	0.03
Lysine	6.3	7.1	7.0	0.51	5.2	5.2	5.2	0.25
Arginine	3.6	3.9	4.1	0.22	3.7^{a}	3.6^{a}	3.1^{b}	0.11
TOTAL (mg/l)	398.7	440.6	447.9	17.83	426.2	441.4	459.1	11.67

a, b Means in the same row with different superscripts differ significantly ($P<0.05$).

On comparing amino acid concentrations of the different media with that of culture effluent from the respective fermentations, it is evident that there was a net synthesis of amino acids in all fermentations. Total amino acids in culture effluent ranged

from 107.9% to 211.9% of that present in the medium. At the low level of N supplementation, total amino acids in culture effluent were unchanged by the inclusion of peptide N at either level at the low dilution rate. The same applied for the proportions of individual amino acids in culture effluent, except for proline which was significantly higher (P<0.05) for the 50% peptide treatment than for the other two treatments. Total amino acids increased in concentration in culture effluent as the level of supplementary peptide N increased in the case of the low N medium when incubated at the high dilution rate. The amino acid concentration in culture effluent was significantly higher. (P<0.05) for the 50% peptide N treatment than the other two treatments in this case. A similar difference was observed between treatments with regard to the proportion of lysine in culture effluent. No further significant differences (P>0.05) were observed between treatments for any of the other amino acids.

At the high level of N supplementation, there was again an increase in total amino acids in culture effluent as the level of supplementary peptide N in the medium increased. This was evident at both the high and the low dilution rates. None of the differences between treatments in this regard were statistically significant however (P>0.05). At the low dilution rate, the proportion of glycine in culture effluent decreased with increasing peptide N in the medium. The 0% and the 50% treatments differed significantly in this respect (P<0.05). Glutamic acid, proline and leucine all increased with increasing peptide N at the high dilution rate. These differences between treatments were statistically significant, as indicated in Table 8. In the same experiment however, there was a decrease in arginine concentration as the level of peptides increased in the medium with that of the 50% peptide N treatment being significantly lower (P<0.01) than the other two.

The total amino acids of bacterial origin in culture effluent as

well as the amino acid profile of bacteria isolated from culture effluent are presented in Table 9 and 10. The data for the high N medium at the low dilution rate are not presented due to problems that ocurred with the amino acid analyses of these samples.

Table 9. The amino acid composition of bacteria isolated from culture effluent as well as total amino acids of bacterial origin from incubations of maize straw media supplemented with N at the low level and containing either 0, 25 or 50 percent of the supplementary N in the form of peptides.

	Dilution rate (h^{-1})							
	0.03				0.09			
	Peptide N (%)				Peptide N (%)			
Measurement	0	25	50	SE	0	25	50	SE
Amino acids (mg/100mg total amino acids)								
Aspartic acid	13.7	13.6	13.6		13.3	12.5	12.9	
Threonine	6.1	6.1	6.1		5.8	5.7	5.7	
Serine	5.5	5.5	5.5		5.7	5.2	5.5	
Glutamic acid	14.1	13.9	13.9		14.1	13.8	14.0	
Proline	3.4	3.2	3.3		3.3	4.0	3.2	
Glycine	5.7	5.7	5.6		5.7	5.7	5.6	
Alanine	8.7	8.6	8.8		9.0	9.2	8.9	
Valine	5.6	5.7	5.7		5.6	5.9	5.5	
Isoleucine	4.8	4.9	4.9		4.7	5.0	4.7	
Leucine	8.4	8.5	8.5		8.6	8.6	9.0	
Tyrosine	4.5	4.6	4.4		4.4	4.7	4.6	
Phenylalanine	5.6	5.6	5.7		5.9	5.4	5.6	
Histidine	1.7	1.8	1.8		1.9	1.8	2.1	
Lysine	7.4	7.5	7.5		7.0	7.2	7.1	
Arginine	4.7	4.8	4.8		5.0	5.4	5.5	
TOTAL (mg/l)	328.4	349.1	372.4	34.29	191.9	248.1	246.6	17.06

Table 10. The amino acid composition of bacteria isolated from culture effluent as well as total amino acids of bacterial origin from incubations of maize straw media supplemented with N at the high level and containing either 0, 25 or 50 percent of the supplementary N in the form of peptides.

	Dilution rate (h^{-1})							
	0.03				0.09			
	Peptide N (%)				Peptide N (%)			
Measurement	0	25	50	SE	0	25	50	SE
Amino acids (mg/100mg total amino acids)								
Aspartic acid					13.0	12.9	13.0	
Threonine					5.9	5.8	5.9	
Serine					5.4	5.4	5.5	
Glutamic acid					14.2	14.1	14.1	
Proline					3.6	3.7	3.6	
Glycine					5.6	5.6	5.6	
Alanine					8.6	8.5	8.6	
Valine					5.5	5.5	5.4	
Isoleucine					4.7	4.7	4.7	
Leucine					8.4	8.4	8.5	
Tyrosine					4.7	5.0	4.9	
Phenylalanine					5.8	5.8	5.8	
Histidine					1.8	1.8	1.8	
Lysine					7.7	7.7	7.6	
Arginine					5.0	5.0	5.1	
TOTAL (mg/l)					248.4[a]	371.5[b]	300.0[c]	14.89

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$).

Bacterial amino acids represented between 52.4 and 94.7 percent of the total amino acids in culture effluent. The only significant differences observed in terms of total bacterial

amino acids was in the case of the high N media incubated at the high dilution rate. In this experiment bacterial amino acids in culture effluent were highest for the treatment containing 25% peptide N in the medium, followed by the 50% then the 0% treatments. All of these differences between treatments were significant ($P<0.05$). It was noticeable that for all the fermentations, the amino acid profile of bacteria closely resembled that of the culture effluent.

Net amino acid biosynthesis for the various treatments was calculated by subtracting total amino acids present in the media from total amino acids in culture effluent. The results of these calculations are presented in Table 11.

Table 11. Net increase in total amino acids (mg/l) for incubations of maize straw supplemented at two levels of N and containing either 0, 25 or 50 % of the supplementary N in the form of peptides.

	Dilution rate (h^{-1})					
	0.03			0.09		
Level of N	Peptide N (%)			Peptide N (%)		
Supplementation	0	25	50	0	25	50
Low N	207.4	134.1	99.3	162.7	133.3	112.8
High N	197.6	129.3	65.7	225.1	130.1	76.9

The net synthesis of amino acids was dramatically reduced by the supplementation of peptides at both levels of N supplementation and at both dilution rates.

3.4 DISCUSSION

As pointed out previously, conditions that prevail in the rumen of animals consuming low quality roughages are such that microbial yield appears to be limited by factors such as growth rate and the availability of energy. The combination of supplementary N levels and dilution rates used in the present experiment was therefore designed to impose different conditions under which either N *per se*, dilution rate, or the availability of energy were limiting so that the effect of these factors on the response, or lack thereof, of rumen bacteria to the provision of supplementry peptides could be accurately evaluated.

Parameters such as methane and hydrogen levels, pH and VFA proportions indicated that in all experiments, a normal, rumen-like fermentation was maintained at all times. At the low level of N supplementation NH_3-N levels remained below 1mM or 14mg/l once steady state had been attained. These values were far below the 50 mg/l proposed by Satter & Slyter (1974) as being the minimum NH_3-N level required in order to maximize microbial protein synthesis and were indeed lower than the value of 12 mg/l which Russell & Strobel (1987) defined as being the minimum level at which rumen bacteria are able to utilize NH_3-N. It would therefore be reasonable to assume that even at the low dilution rate, the availability of N was the first-limiting factor to microbial protein synthesis at the low level of N supplementation. The significantly lower NH_3-N level observed for the 25% peptide N treatment at the high dilution rate would suggest either a more efficient capture of NH_3-N by the bacteria, or a reduction in deamination of amino acids, however considering the low levels of NH_3-N present in these fermentations, this was probably of no real significance. The proportion of individual VFA in culture effluent compares well with *in vivo* values such as those measured by Redman *et al.* (1980) in steers fed on oaten chaff. It is clear though that in the present study, levels of branched-chain VFA were higher than normal due to their inclusion

in the culture media. The tendency shown for branched-chain VFA levels to increase with increasing peptide supplementation, particularly at the high dilution rate, is to be expected as these compounds are deamination products of amino acids (Prins, 1977). At the high level of N supplementation it is noticeable that NH_3-N levels in culture effluent all exceeded 50mg/l, at both the high and the low dilution rate. The availability of N therefore was presumably not a limiting factor at this level of supplementation. Although NH_3-N levels showed a decline with increasing peptide supplementation, the relatively high levels of NH_3-N observed with these treatments indicate that extensive deamination of amino acids occurred. This assumption is further supported by the higher levels of branched chain VFA observed with the peptide-supplemented media.

At the low level of N supplementation, the inclusion of peptides in the medium clearly had no significant effect on microbial activity in terms of digestion of OM and the synthesis of bacterial N, although there was a slight tendency for increased bacterial N synthesis as the level of peptide supplementation increased, at both the high and the low dilution rates. It would appear therefore that at this level of supplementation, the availability of N was the primary factor limiting microbial growth, irrespective of dilution rate.

The fact that an increase in microbial growth efficiency in response to the supply of peptides was observed at only the high dilution rate in the case of the high N treatments indicates that at the low dilution rate, specific growth rate was the major constraint to microbial growth.

The efficiency of bacterial N synthesis observed in this experiment was far lower than the value of 32 g N/kg OM apparently digested reported by the ARC (1984) in all fermentations excepting those where the high N media were incubated at the high dilution rate. These low values are in

agreement with the *in vivo* results of Amos & Evans (1976), Kropp *et al.* (1977a), Leibholz & Kellaway (1979) and Redman *et al.* (1980). At the high level of N supplementation and the high dilution rate however, the values for microbial efficiency were slightly higher than the ARC (1984) figure of 32 g N/kg DOMR in the case of the peptide-supplemented treatments. Considering all the results presented therefore, it is apparent that the efficiency of microbial N synthesis of mixed rumen bacteria growing on maize straw can be improved by partially replacing NH_3-N with peptide N, providing that other factors such as growth rate and the availability of N are not first-limiting. Although there appeared to be a tendency for bacterial N synthesis to increase when the level of supplementary peptide N was increased from 25 to 50% of the total supplementary N, these differences were not statistically significant. These results also strongly support the contention that the low values observed for microbial efficiency *in vivo* when animals are fed on low quality roughage diets are due to low turnover rates in the rumen which in turn result energy limited conditions and low growth rates. This would also explain the lack of response in terms of microbial N synthesis to supplementary protein that is frequently observed *in vivo*.

In work done in chemostat with rumen bacteria growing on glucose at a dilution rate of $0.101h^{-1}$ Van Nevel & Demeyer (1979) found microbial growth efficiency to be 38.2g/kg OM truly fermented. This value is considerably higher than even the highest value of 26.2 observed at the high N level and high dilution rate in the present experiment. This suggests that even under ideal conditions in terms of growth rate and nutrient supply, microbial growth on low quality roughage may be restricted by some other factor. This may well be related to the difference in microbial populations in terms of predominant species that could be expected to exist under these different conditions.

The amino acid profile of culture effluent was very similar for

all the fermentations in the present experiment, irrespective of level or form of N supplementation or dilution rate. This is in agreement with a number of publications, as reviewed by Thomson (1982), which have shown that the composition of duodenal digesta of ruminants remains very similar across a wide range of diets. Similarly, Ben-Ghedalia *et al.* (1978) found that the replacement of 10% of urea N with either casein, maize gluten or fish meal in purified diets fed to sheep had no effect on the amino acid profile of duodenal digesta. In the same study it was shown that the amino acid composition of duodenal digesta closely resembled that of the bacteria isolated from the rumens of the experimental animals. The same principle was demonstrated in the present experiment where very similar amino acid profiles were observed for culture effluent and for bacteria isolated from the culture effluent.

From the results of total amino acids in culture effluent, it is clear that at the low level of N supplementation, it was only at the high dilution rate and the 50% inclusion level of peptide N that there was any increase in the net flow of amino acids out of the fermentors. As total bacterial amino acids for this treatment were similar to the 25% peptide N inclusion treatment, this increase was presumably due to a greater proportion of amino acids supplied in the medium escaping degradation. This however did obviously not occur at the low dilution rate. At the high level of N supplementation, there was an obvious tendency for total amino acids in culture effluent to increase as the level of peptides in the media increased. Lack of data on bacterial amino acids at the low dilution rates prevents any conclusions being drawn about the source of the increase in amino acid concentrations, however the results for bacterial N synthesis would suggest that increased bacterial synthesis may have been the cause. At the high dilution rate, total amino acids in culture effluent appear to directly reflect the increased microbial synthesis observed in the case of the peptide supplemented media. The degradation of casein and its

derivatives by mixed rumen bacteria have been studied by a number of workers (Mangan, 1972; Russell *et al.*, 1983; Broderick & Wallace, 1988; Broderick & Craig, 1989). The overall picture that emerges from these studies is that casein or its hydrolysates are rapidly degraded in the rumen with the result that shortly after being ingested, they give rise to peptides, free amino acids and ammonia which at least transiently accumulate in the rumen. Russell *et al.* (1983) and Broderick & Craig (1989) showed that the elevated level of peptides from casein degradation may persist for some time (in excess of 7h). It would appear therefore that these peptides are metabolised fairly slowly by rumen bacteria. It is quite feasible therefore that at the low level of N supplementation, at the high dilution rate, a substantial quantity of these peptides flowed out of the fermentors before being metabolised by the bacteria. The reason that this trend was not as pronounced at the high level of N supplementation may be due to the fact that NH_3-N was not limiting, thus allowing for more efficient bacterial growth and therefore more rapid utilization of available peptides. Despite the increased flow of amino acids in the case of the peptide-supplemented media, the net gain in amino acids, as calculated from the difference in concentration between medium and culture effluent showed a dramatic decline as the level of supplementary peptide N was increased. Clearly, the addition of peptides in the form of casein hydrolysate in the medium resulted in more amino acids being deaminated which led to a lower overall utilization of amino acids. At the high level of N supplementation, more amino acids were included in the medium than in the case of the low N media, so despite the fact that more bacterial amino acids were synthesised at the high level of N, this was offset by increased deamination of amino acids supplied in the medium. The large gain in amino acids observed in the case of the treatments supplemented with only NH_3-N emphasizes the importance of the *de novo* synthesis of amino acids by rumen bacteria on low-quality roughages.

The ultimate aim of establishing the response of rumen bacteria to various supplements is to be able to maximize microbial digestion of the substrate as well as to make optimum use of protein sources to maximize the supply of protein available to the host animal. From the results presented here, it is evident that the supplementation of maize straw with peptides had no beneficial effect on its digestion by mixed rumen bacteria. The supply of peptides did however improve the synthesis of bacterial N, provided that the constraints of N limitation and low growth rates were eliminated. Where these limitations to microbial growth existed, the provision of peptides was obviously wasteful due to the fact that it did not increase the net synthesis of amino acids and deamination was extensive. Where a positive response to peptide supplementation was observed in terms of increased microbial growth, it was apparent that no further increase in microbial efficiency was facilitated by including more than 25% of the supplementary N as peptide N. Even at the 25% level the positive effects of increased microbial protein synthesis were not sufficient to overcome the wasteful effects of the degradation and deamination of the extra amino acids supplied by this treatment, resulting in a reduction in total synthesis of amino acids as compared to the treatment in which no supplementary peptides were included. This experiment did not however attempt to establish the exact minimum level of supplementary peptides required to maximize microbial growth. This level may well be far below the levels used here and may be such that the positive effect obtained in terms of improved microbial growth would outweigh the effects of increased deamination. The findings of Cotta & Russell (1982) which showed that the levels of peptide N required to maximize microbial growth were in fact such that the net efficiency of protein utilization was reduced, should however be borne in mind when this is considered.

From the results of published work it would appear that factors other than the form of N would be first-limiting to microbial

growth when low quality roughage diets are fed to ruminants. The results of the present experiment suggest therefore that under normal *in vivo* conditions, the provision of any form of N other than NPN would not be of any benefit in improving either the digestion of the roughage or the efficiency of microbial protein synthesis, unless the constraints of low growth rates could be alleviated.

CHAPTER 4

THE EFFECT OF FORM OF NITROGEN ON THE EFFICIENCY OF MICROBIAL PROTEIN SYNTHESIS AND THE DIGESTION OF CELL WALL CARBOHYDRATES BY RUMEN BACTERIA GROWING ON MAIZE STRAW IN CONTINUOUS CULTURE.

4.1 INTRODUCTION

In the experiment described in Chapter 3, maize straw was supplemented with peptides in the form of a partial hydrolysate of casein. The reason that this source of peptide N was used was to ensure that readily-utilizable peptides were available to the bacteria in the cultures. A number of studies have shown that the growth of rumen bacteria is in fact stimulated by peptides supplied in this form (Maeng *et al.* 1976; Cotta & Russell, 1982; Soofi *et al.*, 1982; Argyle & Baldwin, 1989). In practice however, peptides would normally be made available to the rumen population as intermediate products from the hydrolysis of dietary protein. Protein ingested by ruminants is subjected to microbial attack in the rumen and it is well documented that some protein sources are more resistant to degradation than others (Erasmus *et al.*, 1988). As a result of this, both the rate of release as well as the total supply of peptides and other products of proteolysis may vary from one protein source to the next (Mangan, 1972; Broderick & Wallace, 1988).

A number of workers have shown that the during the degradation of dietary protein in the rumen, peptides, amino acids and eventually ammonia are released into the surrounding medium (Mangan, 1972; Broderick *et al.*, 1981; Chen *et al.*, 1987; Broderick & Wallace, 1988). The rate and extent of uptake and metabolism by mixed rumen bacteria of the different peptides thus released has been shown to vary considerably, depending on the specific nature of the peptides involved (Chen *et al.*, 1987; Broderick *et al.*, 1988).

It would appear therefore that based on their susceptibility to degradation and possibly their amino acid composition, different protein sources may differ in their ability to supply the rumen microbial population with the optimum form and level of peptides and/or amino acids required to maximize microbial yields. This may explain why Ben-Ghedalia *et al.* (1978) found that the efficiency of microbial protein production in sheep fed on a purified diet was increased by replacing 10% of dietary urea with maize gluten but not when casein or fish meal were supplemented on the same basis. Also, the study of Zerbini *et al.* (1988) showed that dairy cow diets supplemented with soyabean meal supported a higher efficiency of microbial protein synthesis than when supplemented with fishmeal. In many instances, the positive response in terms of microbial yield obtained with some protein supplements may well be attributable to better synchronization between energy and protein supply and hence reduced energetic uncoupling. This remains a possible confounding factor with *in vivo* experiments.

An important factor to be considered in determining the optimum form of supplementary N for low-quality roughage is the effect that this might have on the microbial digestion of the roughage source. Results from a number of studies have shown that the form of supplementary N may affect the digestion of fibrous substrates by rumen bacteria. The *in vitro* study of Thomsen (1985) clearly demonstrated a marked increase in cellulose digestion in response to the supply of long chain peptides. Kropp *et al.* (1977b) found that DM, OM and cellulose digestion increased when urea was partially replaced with soyabean meal as a supplement for low quality roughage. Similarly, McAllan & Griffith (1987) showed that the digestion of both cellulose and hemicellulose fractions of alkali-treated straw was increased by the addition of either fishmeal or soya bean meal to the diets. This concept was investigated further by McAllan *et al.* (1988). In this study the effect of different protein sources on the degradation of the constituent carbohydrates of cell walls was examined. The results

of the investigation showed that the substitution of urea with either single-cell protein, maize gluten or rapeseed meal in mixed diets fed to steers increased the digestion of galactose, arabinose, xylose and cellulose-glucose. What is of particular relevance in this study is that the digestion of arabinose, xylose and cellulose-glucose was highest in the maize gluten-supplemented diets followed by single-cell protein, then urea. This would support the contention that the positive effect of the maize gluten was attributable to the sustained release of nitrogenous compounds, as maize gluten was the protein source most resistant to degradation in the rumen. As is pointed out by the authors however, the degradation of these same cell wall components was as extensive when the diets were supplemented with rapeseed meal which was degraded to the same extent as urea. This casts some doubt over the hypothesis of the beneficial effects being as a result of the slower release of protein and suggests that the effect is rather due to the difference in the composition of the products released during degradation of the protein source.

It has already been mentioned in previous chapters that the provision of protein in the diets of ruminants may lead to an increase in the levels of branched-chain VFA in the rumen, these being by-products of protein degradation. Due to the fact that these compounds may have a stimulatory effect on rumen bacteria (Hume, 1970a; Russell & Sniffen, 1984; Cummins & Papas, 1985; Varga *et al.*, 1988) it is possible that they may be the reason for increased microbial yields and/or substrate digestion when NPN is replaced with a source of protein in ruminant diets. It is for this reason that these compounds were included in the media in the previous experiment, thus ensuring that any effect observed in response to peptide supplementation was not due to an indirect effect of the provision of branched-chain VFA. Although branched chain VFA were found not to be limiting to microbial growth when maize straw was fed to lambs (Hefner *et al.*, 1985), it is possible that they might be limiting *in vitro*, particularly when viewed in the light of the results referred to above.

The main object of the present experiment was to examine the effect of supplying protein in different forms, namely casein hydrolysate or sunflower oilcake to maize straw-based media on microbial protein synthesis, digestion of cell wall carbohydrates and amino acid utilization. A further objective was to examine the effect, on the same parameters, of supplementing branched-chain VFA when the supplementary N was supplied in the form of NH_3-N alone.

4.2 MATERIALS AND METHODS

4.2.1 *Fermentors*

A bank of four fermentor units was used for this experiment. As described previously, each unit consisted of three identical fermentor vessels which were fed from a common substrate reservoir. At the start of the experiment, all fermentors were filled to maximum working volume with inoculum consisting of strained rumen fluid from lucerne hay-fed sheep and prepared as described in the preceding experiment.

4.2.2 *Media and Treatments*

Four isonitrogenous media containing maize straw as the primary substrate were made up as depicted in Table 1. All media were supplemented with N at the same level as the high N media in the previous experiment. The only difference between treatments was the form in which N was supplemented and the inclusion or omission of branched chain VFA. The media were supplemented on an isonitrogenous basis with either ammonium salts alone (N), ammonium salts plus branched-chain VFA (V), ammonium salts plus casein hydrolysate (C) or ammonium salts plus sunflower oilcake (S). In the case of the casein hydrolysate and the sunflower oilcake, these were included levels so as to replace 50% of the NH_3-N. In the medium containing sunflower oilcake, a portion of the maize straw was replaced with the oilcake. This was done to

ensure that similar levels of digestible OM were present in all media.

Table 1. The ingredient composition of maize straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V), casein hydrolysate (C) or sunflower oilcake (S).

Ingredient inclusion/l	Supplement			
	N	V	C	S
Maize straw (g)	10.0	10.0	10.0	9.3
$NaHCO_3$ (g)	4.0	4.0	4.0	4.0
Mineral soln. (ml)[1]	50.0	50.0	50.0	50.0
Pfennigs metal soln.[2]	10.0	10.0	10.0	10.0
VFA solution[3]	0	10.0	0	0
NH_4Cl	466.0	466.0	182.0	182.0
$(NH_4)_2SO_4$	126.0	126.0	126.0	126.0
Casein Hydrolysate	0	0	514.0	0
Sunflower oilcake	0	0	0	1200.0

[1] Composition (g/l): $CaCl_2.1H_2O$ 0.53; KH_2PO_4 18; NaCl 18; $MgCl_2.6H_2O$ 0.4

[2] Pfennig & Lippert (1966)

[3] Composition (mmol/l): n-Valerate 91.94; i-Valerate 91.16; i-Butyrate 107.83; 2 Methylbutyrate 92.14

These media were incubated simultaneously in the fermentors at a dilution rate of $0.06h^{-1}$.

4.2.3 *Sampling*

The sampling procedure was the same as that described previously with two independent samples per fermentor being drawn once steady state was achieved. As described previously, [^{3}H]leucine was added to all fermentors to serve as a microbial marker.

4.2.4 *Measurements and analyses*

The same parameters measured in the previous experiment were measured in the present experiment, using the same analytical methods.

In addition, dried samples of medium and effluent were analysed for the monosaccharides glucose, xylose, arabinose, galactose and mannose using the method of Englyst & Cummings (1984). After initial acid hydrolysis, the samples were derivatised by treating with 1-methyl imidazole, acetic anhydride and acetic acid. The monosaccharides were then analysed as their alditol acetate derivatives by means of gas chromatography. The difference in concentration between medium and culture effluent was used to calculate the percentage disappearance of the constituent cell wall carbohydrates.

4.2.5 *Statistical analysis*

The experiment was analysed as a randomized blocks design using analysis of variance techniques with the two sampling periods representing blocks. Differences between treatment means were tested for significance by means of least significant differences (Steele & Torrie, 1960).

4.3 RESULTS

4.3.1 *Chemical composition of media*

The chemical composition of the different culture media are shown in Table 2. The replacement of 50% of the NH_3-N with either casein hydrolysate or sunflower oilcake more than doubled the total amino acid content of the media. With the inclusion of casein hydrolysate the level of glutamic acid, proline, isoleucine, leucine and lysine, in particular, were increased whereas sunflower oilcake increased the levels of glutamic acid, histidine and arginine.

Table 2. Chemical composition of maize straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V), casein hydrolysate (C) or sunflower oilcake (S).

Component	Supplement			
	N	V	C	S
DM (g/l)	12.82	12.75	12.11	12.48
OM (g/l)	8.45	8.39	8.36	8.43
N (mg/l)	230.5	227.4	236.9	233.5
Cell wall carbohydrates (mg/100mg DM)				
Glucose	19.1	19.2	19.2	19.9
Xylose	10.9	11.7	11.8	12.0
Arabinose	2.6	2.0	2.0	2.3
Mannose	0.3	0.2	0.2	0.3
Galactose	0.6	0.7	0.8	0.8
Amino acids (mg/100mg total amino acids)				
Aspartic acid	11.2	12.2	9.1	11.4
Threonine	5.3	5.3	3.9	4.6
Serine	5.9	6.9	5.1	5.7
Glutamic acid	17.1	16.0	21.8	21.1
Proline	6.6	6.1	8.8	5.1
Glycine	7.2	7.6	4.8	6.8
Alanine	9.2	9.9	6.9	6.8
Valine	6.6	6.1	6.9	5.4
Isoleucine	3.9	3.8	4.8	4.0
Leucine	9.2	8.4	10.0	8.3
Tyrosine	2.6	3.1	2.7	3.1
Phenylalanine	5.9	5.3	5.4	5.4
Histidine	2.0	1.5	1.8	2.3
Lysine	4.6	4.6	5.7	4.0
Arginine	2.6	3.1	2.1	6.0
TOTAL (mg/l)	218.3	179.4	435.3	439.9

4.3.2 *Fermentation parameters*

Samples of headspace gas from all fermentations showed substantial concentrations of methane with hydrogen remaining undetectable. pH remained above 6 in all fermentors for the duration of the experiment. Mean values for pH, VFA concentrations and ammonia concentrations in culture effluent of the different treatments are displayed in Table 3.

Table 3. Mean values for pH, VFA concentrations and NH_3-N concentration of culture effluent from incubations of maize straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V), casein hydrolysate (C) or sunflower oilcake (S).

	Supplement				
Measurement	N	V	C	S	SE
pH	6.50	6.50	6.38	6.53	0.03
NH_3-N (mg/l)	61.4^{a}	64.9^{a}	56.2^{b}	44.1^{c}	3.46
VFA:					
Total (mmol/l)	29.1	32.8	31.0	30.3	1.87
Molar %					
Acetate	73.5	67.0	70.9	70.1	3.32
Propionate	20.7	19.6	20.7	20.4	0.94
n-Butyrate	5.7	4.1	4.0	7.6	1.82
i-Butyrate	0^{a}	2.9^{b}	1.3^{c}	0.6^{d}	0.40
2 m-Butyrate	0^{a}	2.0^{b}	0.7^{c}	0.4^{c}	0.28
n-Valerate	0.1^{a}	2.4^{b}	1.5^{c}	0.7^{d}	0.44
i-Valerate	0^{a}	2.0^{b}	0.8^{c}	0.3^{d}	0.43

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$).

The NH_3-N concentration of culture effluent from the sunflower oilcake-supplemented medium was significantly lower than that of the remaining three treatments and that of the medium containing casein hydrolysate was significantly lower than in the case of the N and V treatments ($P<0.05$).

The concentration of total VFA in culture effluent showed no significant differences between treatments as did the relative proportions of acetic, propionic and butyric acid. No branched-chain VFA were detectable in the culture effluent of treatment N. Concentrations of branched-chain VFA followed a distinct trend and were highest for treatment V, followed by S, then C with most of these differences being significant ($P<0.05$).

4.3.3 *OM digestion and bacterial N synthesis*

The mean values observed for OM digestion and bacterial N synthesis for the different treatments are presented in Table 4.

Apparent OM digestion was significantly lower ($P<0.05$) for the medium supplemented with NPN alone than for the other three treatments. The same trend was observed with regard to actual OM digestion, however the differences were not quite significant in this case.

Bacterial N synthesis was highest for treatment S, followed by treatment N, C, then V. All of these differences were statistically significant ($P<0.05$). Replacement of casein hydrolysate with sunflower oilcake increased bacterial N synthesis by 23.6%. The efficiency of bacterial N synthesis, expressed in terms of OM either apparently or truly digested was also significantly higher ($P<0.05$) for the sunflower oilcake-supplemented medium than for the treatments V and C.

Table 4. Mean values for the extent of OM digestion and efficiency of microbial N synthesis from incubations of maize straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V), casein hydrolysate (C) or sunflower oilcake (S).

Measurement	Supplement				
	N	V	C	S	SE
OM Digestion (%)					
Apparent	32.5^a	39.9^b	37.7^b	38.8^b	1.75
Actual	44.7	50.5	49.2	47.9	2.23
Bacterial N (mg/l)	83.6^a	71.7^b	77.4^c	95.7^d	3.63
Microbial growth efficiency					
g N/kg OMAD	31.6^a	21.6^b	24.9^b	29.4^a	2.31
g N/kg OMTD	22.4^a	16.9^b	18.9^c	20.9^a	1.19

a, b, c, d Means in the same row with different superscripts differ significantly ($P<0.05$).

4.3.4 *Digestion of cell wall carbohydrates*

The extent of digestion of the different constituent cell wall carbohydrates is displayed in Table 5.

Although no significant differences were observed between treatments, with respect to the extent of digestion of any of the cell wall sugars, disappearance of these compounds tended to be highest in the case of treatments V and C with the values obtained with treatment N being the lowest. Mannose levels in all of the media were very low and only trace amounts were found in culture effluent (<0.1%) for all treatments. Digestion was therefore regarded as being 100% in all cases.

Table 5. Mean values for the extent of digestion of cell wall carbohydrates in incubations of maize straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V), casein hydrolysate (C) or sunflower oilcake (S).

	Supplement				
Measurement	N	V	C	S	SE
Digestion (%)					
Glucose	40.3	51.6	47.4	46.9	4.44
Xylose	32.3	44.9	39.3	37.4	4.77
Arabinose	45.0	50.7	52.0	45.4	2.66
Galactose	27.8	29.9	40.0	24.2	4.40
Mannose	100	100	100	100	

4.3.5 *Amino acid utilization and synthesis*

The amino acid profile and total amino acid content of culture effluent from the different treatments are shown in Table 6. On comparing the total amino acid content of culture effluent with that of the various media, it is evident that there was a net synthesis of amino acids for all treatments except treatment C. The total amino acids in culture effluent were equal to 191.3, 218.5, 94.8 and 109.0 percent of that in the media for treatments N, V, C and S respectively. The total amino acid content of culture effluent was increased above that of the other treatments by the inclusion of sunflower oilcake in the medium, although this was not significant ($P>0.05$). The proportion of histidine and arginine in the culture effluent was increased significantly ($P<0.05$) by the inclusion of sunflower oilcake in the medium.

Table 6. The amino acid composition of culture effluent from incubations of maize straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V), casein hydrolysate (C) or sunflower oilcake (S).

	Supplement				
Measurement	N	V	C	S	SE
Amino acids (mg/100mg total amino acids)					
Aspartic acid	13.3	13.6	13.1	12.3	0.38
Threonine	6.3	6.7	6.8	6.6	0.23
Serine	5.8	6.0	6.1	5.7	0.22
Glutamic acid	15.3	15.2	15.2	15.1	0.23
Proline	4.5	4.6	4.7	4.4	0.10
Glycine	6.9	7.0	7.0	6.7	0.21
Alanine	8.5	8.9	9.0	8.1	0.27
Valine	5.4	5.5	5.9	5.5	0.18
Isoleucine	4.2	4.5	4.8	4.5	0.15
Leucine	8.6	9.0	9.0	8.8	0.24
Tyrosine	3.5	3.8	3.5	3.7	0.18
Phenylalanine	5.8	6.2	6.2	5.9	0.23
Histidine	1.8^{a}	1.8^{a}	1.8^{a}	2.0^{b}	0.03
Lysine	6.9	7.0	7.5	6.9	0.17
Arginine	3.3^{a}	3.2^{a}	2.5^{b}	3.9^{c}	0.20
TOTAL (mg/l)	417.6	392.0	412.6	479.5	21.50

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$).

Total bacterial amino acids and the amino acid profile of bacteria isolated from the fermentors is shown in Table 7. The inclusion of both casein hydrolysate and sunflower oilcake increased the total bacterial amino acid content of culture effluent above that of the other treatments significantly ($P<0.05$). Bacterial amino acids represented 82.5, 71.1, 75.0 and

72.3 percent of the total amino acids in culture effluent for treatments N, V, C and S respectively.

Table 7. Total bacterial amino acids and amino acid composition of bacteria isolated from culture effluent from incubations of maize straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V), casein hydrolysate (C) or sunflower oilcake (S).

	Supplement				
Measurement	N	V	C	S	SE
Amino acids (mg/100mg total amino acids)					
Aspartic acid	12.7	12.9	13.0	12.9	
Threonine	6.0	6.0	5.9	5.3	
Serine	5.3	5.3	5.4	5.3	
Glutamic acid	14.0	13.9	13.8	14.0	
Proline	3.8	3.7	3.7	3.6	
Glycine	5.6	5.7	5.8	5.9	
Alanine	8.4	8.4	8.8	8.0	
Valine	5.6	5.4	5.3	5.5	
Isoleucine	4.9	4.7	4.5	4.7	
Leucine	8.4	8.3	8.2	8.3	
Tyrosine	4.6	4.7	5.0	4.9	
Phenylalanine	5.6	5.7	5.7	5.7	
Histidine	1.7	1.7	1.7	1.8	
Lysine	8.1	8.3	8.1	8.3	
Arginine	5.3	5.3	5.2	4.9	
TOTAL (mg/l)	344.4^{a}	278.9^{b}	309.4^{ab}	347.9^{a}	14.54

a, b Means in the same row with different superscripts differ significantly ($P<0.05$).

4.4 DISCUSSION

The conditions prevailing in the fermentations of this experiment in terms of methane and hydrogen levels, VFA proportions and pH all indicate that a normal, representative population of rumen bacteria existed in all the fermentations. The level of N supplementation chosen for the experiment was such that the availability of N did not limit microbial growth and the dilution rate imposed ensured that the potentially digestible fraction of the substrate was not entirely depleted as a result of being exposed to microbial fermentation for an excessively long period of time. In this manner the effect of the various treatments on microbial activity in terms of extent of digestion of the substrate as well as the efficiency of bacterial N synthesis could be accurately evaluated.

A few unexpected results were observed in the case of the treatment in which NH_3-N was supplemented alone. Firstly, the level of amino acids present in the medium appeared to be too high, as they should have been similar to those in treatment V. Secondly, bacterial N synthesis on this treatment was far higher than expected, when compared with treatment C and V. It appears therefore that either this medium became contaminated with a source of amino acids which elicited a response in terms of microbial yield, or an error occurred during processing and analysis of these samples. It seems likely that a dilution error may have been made. In view of the uncertainty of these results therefore, they have been treated with caution.

NH_3-N appeared adequate for maximum microbial growth in all fermentations (Satter & Slyter, 1974). The lower value observed in the case of treatment S could have been either as a result of increased incorporation into bacterial N or due to less ammonia being released from the deamination of amino acids. The proportion of undegraded NAN in culture effluent calculated as total N less NH_3-N less bacterial N for treatments N, V, C and S

respectively amounted to 37.0, 40.6, 41.9 and 39.2% of total N. This would imply that all N sources were degraded to the same extent. The sunflower oilcake used in this experiment was from the same source as that used by Erasmus *et al.* (1988) in a study to determine the rumen degradability of various protein sources in dairy cows. At a fractional outflow rate of $0.05h^{-1}$, the crude protein degradability of this sunflower oilcake was found to be 86%. Assuming that the degree of degradation of sunflower oilcake in the present experiment was the same as that reported by Erasmus *et al.* (1988) and also that the other sources of supplemental N were entirely degraded, then the flow of undegraded NAN in culture effluent should have been 3 percentage points higher in treatment S as opposed to the other treatments. It is unlikely that such a small difference in would have been clearly reflected in the results presented here. It is apparent however that degradation of the sunflower oilcake, if not complete, was extensive.

The relative proportions of branched-chain VFA reflects to a large degree the extent of deamination of amino acids that occurred with the different treatments. In the case of the medium supplemented with NH_3-N alone, it is clear that the absence of precursors in the form of amino acids resulted in undetectable levels of all branched-chain VFA in culture effluent. The concentration of i-butyrate, 2-methylbutyrate, n-valerate and i-valerate in culture effluent of the medium supplemented with these compounds was 88.2, 71.2, 85.6, and 72.0 percent respectively of that included in the medium. There was therefore a net utilization of these acids, presumably for the synthesis of membrane lipids and amino acids (Dehority *et al.*, 1958). The substantially higher levels of branched-chain VFA in culture effluent of treatment C relative to treatment S suggests a greater degree of deamination in the case of the medium supplemented with casein hydrolysate. This may be at least partially due to the fact that the medium supplemented with casein hydrolysate contained a higher proportion of valine,

isoleucine and leucine which are precursors of i-butyrate, 2-methylbutyrate and i-valerate respectively. Similarly, the higher proportions of lysine and proline in the casein hydrolysate-supplemented treatment may be the reason for the higher concentration of n-valerate in culture effluent of this treatment, despite the high proportion of arginine, also a precursor of n-valerate, in the medium containing sunflower oilcake (Dehority *et al.*, 1958; Amos *et al.*, 1971). It is possible that casein hydrolysate was extensively degraded to amino acids which were rapidly deaminated, giving rise to high levels of NH_3-N and branched-chain VFA, whereas sunflower oilcake was less extensively degraded and yielded more peptides and less amino acids than casein hydrolysate.

The increase in apparent OM digestion in response to the supplementation of branched-chain VFA is in agreement with various *in vitro studies* (Soofi *et al.*, 1982; Gorosito *et al.*, 1985) wherein the digestion of fibrous substrates have been shown to be improved by supplementation with branched-chain VFA. The fact that no further improvement in OM digestion was observed when either casein hydrolysate or sunflower oilcake were included in the medium suggests that the positive response observed with these treatments, in relation to the medium supplemented with NH_3-N alone, could be ascribed to the release of branched chain VFA during degradation of these protein sources.

The increase in bacterial N synthesis achieved by the inclusion of protein in the medium, either as casein hydrolysate or sunflower oilcake, over and above that observed with the branched-chain VFA-supplemented medium provides evidence that the response in terms of bacterial N synthesis to the provision of protein sources is as a result of the supply of amino acids and/or peptides and is not ascribable only to the release of branched-chain VFA from these protein sources. The higher value observed for the sunflower oilcake-supplemented medium in relation to the medium supplemented with casein hydrolysate in

this regard may be due to a number of factors. The possibility that this was due to some undefined growth factor, other than peptides or amino acids cannot be ruled out, as the sunflower oilcake was obviously a far less purified form of protein than the casein hydrolysate. It is however reasonable to assume that the sunflower oilcake would have given rise to peptides of longer chain length than would have been the case with the casein hydrolysate. If this were the case, it could well have contributed to the higher microbial N yields observed with the sunflower oilcake which would be in agreement with Thomsen (1985). It is unlikely that the difference between casein hydrolysate and sunflower oilcake in terms of their effect on bacterial N synthesis was due to the differences in their amino acid profiles. There were no large differences between the respective media in terms of the relative proportion of any particular amino acid and none of the amino acids were present in exceptionally low concentrations. Also, it would appear from the work of Argyle & Baldwin (1989) that bacterial growth is not stimulated by a particular amino acid or group of amino acids but rather by a complete mixture of amino acids.

The results obtained for the digestion of cell wall carbohydrates indicate that the degradation of glucose and xylose was increased, although not significantly, by the inclusion of protein in the medium. This is in agreement with the results of McAllan *et al.* (1988). The source of protein clearly had no effect on the disappearance of these components in the present study. The fact that supplementation of the substrate with branched-chain VFA resulted the same degree of digestion of cell wall carbohydrates as when protein was supplemented, suggests that the positive response observed with the protein sources could well be due to the release of branched-chain VFA which have been shown to be highly stimulatory to fibrolytic bacteria (Bryant & Robinson, 1962). It is also possible however that the cell walls were digested to the maximum possible extent in all

treatments, particularly when viewed in the light of the fact that NH_3-N was not limiting. Under these circumstances, no increase in disappearance of cell wall carbohydrates would be possible, irrespective of the form of supplementary N.

It is perhaps surprising to note that no significant differences were found between treatments in terms of the concentration of total amino acids in culture effluent when viewed in the light of the differences observed between treatments in terms of bacterial N synthesis. The results of total amino acid concentration in culture effluent do however reflect the same trend as those reported for bacterial N synthesis. The high concentrations of histidine and arginine in culture effluent of the treatment supplemented with sunflower oilcake, in relation to the other treatments, reflects the high levels of these amino acids present in the medium of this treatment. It would appear therefore that a portion of the protein from the sunflower oilcake managed to escape microbial degradation, thus altering the amino acid profile of the culture effluent.

Supplementation of maize straw with a form of protein clearly had a beneficial effect on microbial activity. It is most probable that sunflower oilcake was degraded far more slowly than casein hydrolysate which presumably would have resulted in a greater accumulation of peptides in the medium. It is also likely that these peptides were of a longer chain length than those arising from casein hydrolysate. The reason for the higher microbial yield observed with the sunflower oilcake relative to the casein hydrolysate may be attributable to either or both of these factors. The level of protein supplementation applied in this experiment clearly led to inefficient utilization of amino acids and would be most uneconomical in terms of protein utilization in practice. It is possible that a lower level of protein supplementation would have stimulated microbial protein synthesis to the same degree as that reported here. Had this been the case, amino acid utilization would clearly have been far more efficient

than that observed for the treatments in which either casein hydrolysate or sunflower oilcake were supplemented in the present study. Stimulation of microbial protein synthesis would however have needed to occur at a low level of protein supplementation in order to have increased the efficiency of amino acid utilization above that achieved with supplementation of NH_3-N and branched-chain VFA.

CHAPTER 5

THE EFFECT OF SUPPLEMENTARY PEPTIDES OR BRANCHED-CHAIN VOLATILE FATTY ACIDS ON THE EFFICIENCY OF MICROBIAL PROTEIN SYNTHESIS AND THE DIGESTION OF CELL WALL CARBOHYDRATES BY RUMEN BACTERIA GROWING ON ALKALINE HYDROGEN PEROXIDE-TREATED WHEAT STRAW IN CONTINUOUS CULTURE.

5.1 INTRODUCTION

The digestion of plant cell wall carbohydrates by rumen bacteria is frequently retarded by the presence of lignin which is closely associated with the structural carbohydrates in the cell wall matrix (Jung & Fahey, 1983; Van Soest, 1982). The extent of lignification of plant material depends on a number of factors such as plant species and the stage of maturity of the plant (Van Soest, 1982) and the low digestibility of many forms of straw and crop residues is largely attributable to the high degree of lignification of this type of roughage. A number of chemical treatment techniques have been developed which disrupt the bonds between lignin and cell wall carbohydrates, thus exposing the cell wall carbohydrates to the fermentative action of the rumen microbial population (Klopfenstein, 1978; Mora *et al.*, 1979). These techniques involve the treatment of the roughage with a strong alkaline solution of sodium hydroxide or aqueous ammonia. Gould (1984; 1985) developed a technique whereby alkaline hydrogen peroxide was used to delignify straw or other low-quality roughage sources. This treatment has been shown to more than double the DM, ADF and cellulose digestibility of wheat straw by sheep (Kerley *et al.*, 1986). A number of *in vitro* studies such as those of Lewis *et al.* (1988), Bas *et al.* (1989a; 1989b) have further confirmed the susceptibility of alkaline hydrogen peroxide- treated wheat straw to digestion by rumen bacteria.

Low-quality roughages which have been treated with an alkali are often characterised by a high digestibility but low crude protein content. Some form of supplementary N is therefore required to ensure optimum utilization of the treated roughage (Ørskov & Grubb, 1978; Elliot & Armstrong, 1982; McAllan & Smith, 1984; Punia *et al.*, 1988). Due to the low crude protein content in relation to digestibility, it could be expected that rumen bacteria would rely primarily on supplementary N when fermenting these treated roughages. The ability of alkali-treated roughages to supply compounds such as peptides, amino acids and branched-chain VFA as fermentation end-products would presumably also be very limited. The absence of these compounds could well reduce the utilization of the roughage source by rumen bacteria, as demonstrated by the study of Gorosito *et al.* (1985) which showed that the digestion of wheat straw cell walls by rumen bacteria *in vitro* was significantly increased by supplementation with branched-chain VFA and peptides.

Wheat straw that has been treated with alkaline hydrogen peroxide is characterised by an exceptionally high fermentable OM to crude protein ratio. Alkaline hydrogen peroxide treatment not only increases the digestibility of the material, but it also lowers the crude protein content considerably (Lewis *et al.,* 1988). In view of this, together with the observations of Gorosito *et al.* (1985), it could be expected that supplementation of alkaline hydrogen peroxide-treated wheat straw with NH_3-N alone would not be sufficient to ensure maximum microbial protein synthesis and degradation of the substrate. The stimulation of microbial activity achieved by the supplementation of alkaline hydrogen peroxide-treated wheat straw with peptides or branched-chain VFA may not necessarily be expected to follow exactly the same trend as that observed for untreated straw in view of the fact that the more digestible nature of the treated straw may cause a shift in the rumen microbial population. Minato *et al.*, (1989) showed that when untreated rice straw was fed to cows, *Bacteroides* spp. and *Butyrivibrio* spp. were the dominant species in the rumens of

these animals. Replacing the untreated straw with ammonia-treated rice straw however resulted in a proliferation of *Eubacterium* spp., *Fibrobacter succinogenes*, *Ruminococcus albus* and *Succinivibrio* spp. Due to the fact that differences may exist between these species with respect to their requirements for and their ability to utilize NH_3-N, amino acids and VFA (Hespell & Bryant, 1979), it is possible that the stimulation of microbial growth by supplementation with branched-chain VFA or peptides may differ, depending on the composition of the bacterial population in question, which in turn could be affected by alkali-treatment of the particular roughage source.

Little data is available on the effect of form of nitrogen on the utilization of alkali-treated straw by rumen bacteria. The replacement of urea with a source of rumen-degradable protein in mixed diets containing alkali-treated straw and barley has been shown to increase the digestion of the various cell wall components of the treated straw (McAllan & Smith, 1983; McAllan & Griffith, 1987; McAllan *et al.*, 1988). The efficiency of microbial protein synthesis on similar diets was also increased when fishmeal replaced urea as the supplementary N source (McAllan & Smith, 1984). It is not known, however, whether the same effect would be observed with all-roughage diets based on alkali-treated straw.

The object of the present experiment was to examine the effects of supplementing alkaline hydrogen peroxide-treated wheat straw with either branched-chain VFA or peptides on the digestion of cell wall carbohydrates and the synthesis of bacterial protein.

5.2 MATERIALS AND METHODS

5.2.1 *Fermentors*

A total of nine fermentors were used for the experiment. Each of the three treatments was replicated in three fermentors, each of

which were fed from the same medium reservoir. At the start of the experiment, all fermentor vessels were filled to their maximum working volume with inoculum which was prepared by straining rumen digesta drawn from sheep which were accustomed to a diet of lucerne hay, as described in previous chapters.

5.2.2 *Media and treatments*

Three isonitrogenous media, based on wheat straw which had been treated with alkaline hydrogen peroxide (AHP wheat straw) were made up as shown in Table 1. In an adaptation of the method of Gould (1984), wheat straw was treated as follows: A quantity of wheat straw was weighed off together with a quantity of water of 3 times the mass of the wheat straw. Three percent of the mass of the wheat straw of hydrogen peroxide was added to half of the water and this was sprayed onto the wheat straw which was then thoroughly mixed. Ten percent of the mass of the wheat straw was of NaOH was then dissolved in the remaining water and this was mixed in with the wheat straw. The treated straw was then placed in the sun to dry. Once dry, the AHP wheat straw was milled in a Wiley mill fitted with a 0.5mm screen. All media contained equal quantities of AHP wheat straw, $NaHCO_3$, minerals and trace minerals. The only difference between treatments was that they were supplemented on an isonitrogenous basis with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V) or NH_3-N plus casein hydrolysate (C). In treatment C, 50% of the supplementary N was supplied as peptide N. These media were incubated simultaneously in the continuous culture apparatus at a dilution rate of $0.09h^{-1}$.

Table 1. The ingredient composition of alkaline hydrogen peroxide-treated wheat straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V) or NH_3-N plus casein hydrolysate (C).

Ingredient inclusion/l	Supplement		
	N	V	C
AHP Wheat straw (g)	10	10	10
$NaHCO_3$ (g)	4	4	4
Mineral solution (ml)[1]	50	50	50
Pfennigs metal solution (ml)[2]	10	10	10
VFA solution (ml)[3]	0	10	0
NH_4Cl (mg)	466	466	182
$(NH_4)_2SO_4$	125	125	125
Casein Hydrolysate (mg)	0	0	514

[1] Composition (g/l): $CaCl_2.1H_2O$ 0.53; KH_2PO_4 18; NaCl 18; $MgCl_2.6H_2O$ 0.4

[2] Pfennig & Lippert (1966)

[3] Composition (mmol/l): n-Valerate 91.94; i-Valerate 91.16; i-Butyrate 107.83; 2 Methylbutyrate 92.14

5.2.3 *Sampling*

The sampling procedure for the experiment was the same as that described in the preceding chapter with two independent samples per fermentor being drawn once steady state conditions had been established.

5.2.4 *Measurements and analyses*

The same analyses were performed on medium and effluent samples as described in the preceding chapter. These results were then used in the same manner as described previously to calculate OM digestion, bacterial N synthesis and amino acid utilisation or synthesis.

5.2.5 *Statistical analysis*

The experiment was analysed as a randomized blocks design using analysis of variance techniques, with sampling periods representing blocks. Differences between treatment means were tested for statistical significance by means of least significant differences (Steele & Torrie, 1960).

5.3 RESULTS

5.3.1 *Chemical composition of media*

The chemical composition of the various treatment media is presented in Table 2. Inclusion of casein hydrolysate had the effect of almost quadrupling the total amino acid content of the medium. The concentration of glutamic acid, proline, histidine and lysine showed the most marked increase to the addition of casein hydrolysate.

Table 2. Chemical composition of AHP Wheat straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V) or casein hydrolysate (C).

Component	Supplement		
	N	V	C
DM (g/l)	13.7	13.7	13.9
OM (g/l)	9.0	8.8	9.2
N (mg/l)	164.3	170.9	166.2
Cell wall carbohydrates (mg/100mg DM)			
Glucose	20.4	22.5	18.7
Xylose	13.4	14.0	12.6
Arabinose	2.0	2.1	1.9
Mannose	0.3	0.3	0.2
Galactose	0.5	0.5	0.5
Amino acids (mg/100mg total amino acids)			
Aspartic acid	12.4	12.9	10.1
Threonine	4.9	5.8	4.4
Serine	6.2	6.4	5.5
Glutamic acid	19.8	19.4	23.3
Proline	7.4	7.1	10.9
Glycine	7.4	7.1	3.6
Alanine	8.6	9.0	5.1
Valine	6.8	6.4	6.4
Isoleucine	4.3	3.7	4.9
Leucine	9.9	9.0	10.0
Tyrosine	2.5	3.2	2.0
Phenylalanine	6.2	5.8	5.4
Histidine	0	0	1.8
Lysine	1.2	1.9	5.2
Arginine	2.4	2.4	1.5
TOTAL (mg/l)	120.6	116.9	470.3

5.3.2 *Fermentation parameters*

The fermentation parameters measured at steady state are presented in Table 3.

Table 3. Mean values for pH, VFA concentrations and NH_3-N concentration of culture effluent from the incubation of AHP wheat straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V) or casein hydrolysate (C).

	Supplement			
Measurement	N	V	C	SE
pH	6.67	6.81	6.82	0.05
NH_3-N (mg/l)	31.3^{a}	9.8^{b}	9.4^{b}	1.25
VFA:				
Total (mmol/l)	39.6^{a}	49.2^{b}	51.6^{b}	2.12
Molar %				
Acetate	65.5	68.0	67.5	1.72
Propionate	26.1	19.4	24.0	1.23
n-Butyrate	8.2^{a}	5.3^{b}	7.1^{ab}	0.62
i-Butyrate	0^{a}	2.2^{b}	0.3^{c}	0.08
2-m Butyrate	0^{a}	1.4^{b}	0.01^{a}	0.05
n-Valerate	0.1^{a}	2.4^{b}	1.2^{c}	0.11
i-Valerate	0^{a}	1.3^{b}	0^{a}	0.05

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$).

The most striking aspect of these results is the large decrease in NH_3-N concentration of culture effluent associated with the supplementation of the media with either branched-chain VFA or casein hydrolysate. Supplementation with either branched-chain VFA or casein hydrolysate also resulted in a significant ($P<0.05$)

increase in total VFA concentration above that of the treatment supplemented with NH_3-N alone. No branched-chain VFA and only low levels of n-valerate were detectable in the culture effluent of treatment N. The levels of branched-chain VFA and n-valerate in culture effluent were highest in the case of treatment V, followed by treatments C then N with most of these differences being significant ($P<0.05$).

5.3.3 *OM digestion and bacterial N synthesis*

The results pertaining to OM digestion and bacterial N synthesis are presented in Table 4.

Table 4. Mean values for the extent of OM digestion and efficiency of microbial N synthesis from incubations of AHP wheat straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V) or NH_3-N plus casein hydrolysate (C).

	Supplement			
Measurement	N	V	C	SE
OM Digestion (%)				
Apparent	61.8	64.2	62.7	1.44
Actual	76.0	81.1	80.6	1.69
Bacterial N (mg/l)	109.8[a]	128.3[ab]	144.3[b]	7.22
Microbial growth efficiency				
g N/kg OMAD	19.9	22.8	24.7	1.33
g N/kg OMTD	16.2	18.0	19.1	0.83

a, b Means in the same row with different superscripts differ significantly ($P<0.05$).

Little difference between treatments was apparent in terms of either apparent or actual OM digestion. Supplementation with branched-chain VFA and with casein hydrolysate increased bacterial N synthesis over the unsupplemented treatment, however only that of treatment C was increased significantly ($P<0.05$). The efficiency of bacterial protein synthesis showed a tendency to be highest for treatment C, followed by treatment V then N. None of these differences were however statistically significant.

5.3.4 *Digestion of cell wall carbohydrates*

The results observed for the digestion of cell wall carbohydrates are displayed in Table 5.

Table 5. Mean values for the extent of digestion of cell wall carbohydrates from incubations of AHP wheat straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V) or casein hydrolysate (C).

	Supplement			
Measurement	N	V	C	SE
Digestion (%)				
Glucose	55.9	68.1	60.4	3.25
Xylose	73.0	78.5	72.6	2.14
Arabinose	71.9	76.2	72.7	2.41
Galactose	69.3	93.4	94.5	7.50
Mannose	100	100	100	

Digestion of glucose, xylose and arabinose were higher in the case of the treatment supplemented with branched-chain VFA than the remaining two treatments. These differences were however not significant. Digestion of galactose was increased 30 % by the supplementation of either branched-chain VFA or peptides,

although this was not significant due to large variation between replications. Unmeasurably small quantities of mannose were present in culture effluent of all fermentations, so digestion of this compound was calculated as complete for all treatments.

5.3.5 *Amino acid utilization and synthesis*

The concentrations of individual and total amino acids in culture effluent of the different treatments are presented in Table 6. Supplementation of AHP wheat straw media with either branched-chain VFA or peptides significantly increased the concentration of total amino acids in culture effluent over that observed for the treatment supplemented with NH_3-N alone. The concentration of threonine was significantly lower in culture effluent of treatment V than for the remaining two treatments while serine was significantly lower in the case of treatment C ($P<0.05$). Peptide supplementation reduced the concentration of glycine in culture effluent significantly with respect to the other treatments ($P<0.05$). Arginine concentration of culture effluent was highest in the case of treatment C followed by treatments V then N, however only treatments N and C differed significantly ($P<0.05$).

Table 6. The amino acid composition of culture effluent from incubations of AHP wheat straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V) or NH_3-N plus casein hydrolysate (C).

Measurement	Supplement			
	N	V	C	SE
Amino acids (mg/100mg total amino acids)				
Aspartic acid	14.0	14.4	15.1	0.40
Threonine	6.9^a	6.4^b	6.7^a	0.09
Serine	6.3^a	5.7^b	5.6^b	0.08
Glutamic acid	15.4	15.7	15.5	0.16
Proline	3.7	3.8	3.8	0.13
Glycine	6.2^a	6.2^a	5.7^b	0.11
Alanine	8.7	8.5	8.3	0.14
Valine	5.5	5.3	5.3	0.13
Isoleucine	4.7	4.7	4.8	0.10
Leucine	8.4	8.4	8.2	0.15
Tyrosine	3.4	3.4	3.6	0.25
Phenylalanine	5.9	5.7	5.4	0.14
Histidine	1.7	1.6	1.8	0.04
Lysine	6.4	6.9	6.7	0.30
Arginine	2.8^a	3.4^{ab}	3.7^b	0.21
TOTAL (mg/l)	455.5^a	555.6^b	574.7^b	16.33

a, b Means in the same row with different superscripts differ significantly ($P<0.05$).

The amino acid profile of bacteria isolated from culture effluent as well as the total amino acids of bacterial origin in culture effluent are shown in Table 7. Peptide, as well as branched chain VFA supplementation increased the concentration of amino acids of bacterial origin in culture effluent, however this was only

significant in the case of the peptide-supplemented treatment ($P<0.05$).

Table 7. Total bacterial amino acids and amino acid composition of bacteria isolated from culture effluent from incubations of AHP wheat straw media supplemented with either NH_3-N alone (N), NH_3-N plus branched-chain VFA (V) or casein hydrolysate (C).

	Supplement			
Measurement	N	V	C	SE
Amino acids (mg/100mg total amino acids)				
Aspartic acid	12.6	12.8	13.0	
Threonine	6.1	6.1	5.9	
Serine	5.3	5.2	5.3	
Glutamic acid	14.4	14.6	14.5	
Proline	3.4	3.6	3.7	
Glycine	5.6	6.0	5.8	
Alanine	8.2	8.3	8.4	
Valine	5.6	5.4	5.0	
Isoleucine	5.0	4.7	4.3	
Leucine	8.4	8.3	8.3	
Tyrosine	4.6	4.8	5.1	
Phenylalanine	5.7	5.6	5.6	
Histidine	1.6	1.8	1.8	
Lysine	8.3	8.2	8.2	
Arginine	5.0	4.8	5.1	
TOTAL (mg/l)	384.3^{a}	428.5^{ab}	489.8^{b}	21.1

a, b Means in the same row with different superscripts differ significantly ($P<0.05$).

5.4 DISCUSSION

The level at which N was supplemented in the present experiment was based on the level used in the experiments described in chapters 3 and 4 to supplement maize straw. This proved to be sufficient to maintain NH_3-N levels in the region of 50 mg/l in the case of the maize straw incubations, however in the present experiment, this was not the case. The highly digestible nature of the AHP wheat straw resulted in a far greater utilization of NH_3-N, particularly when peptides or branched-chain VFA were included in the medium. Based on the observation of Russell & Strobel (1987), in which rumen bacteria were unable to utilize NH_3-N at levels below 12 mg/l, it is evident that the fermentations which were supplemented with either peptides or branched-chain VFA in this experiment were ammonia-limited. It is also likely that bacterial growth in the fermentation supplemented with NH_3-N alone was negatively affected by the fact that NH_3-N levels in this case were lower than the 50 mg/l proposed by Satter & Slyter (1974) and Russell & Strobel (1987) as being the minimum level required to support maximum microbial growth.

It is clear from the results of this experiment that stimulation of bacterial activity occurred in response to the supplementation of branched-chain VFA and peptides. The most marked evidence of this was the decrease in NH_3-N concentration of culture effluent that was observed when these compounds were included in the media. This decrease in NH_3-N concentration strongly suggests more efficient incorporation of NH_3-N into microbial protein and is in agreement with the results of Gorosito *et al*. (1985) where supplementation of wheat straw cell walls with branched-chain VFA reduced the level of NH_3-N in the culture. Oltjen *et al*. (1971) also observed a decline in rumen NH_3-N levels *in vivo* when branched-chain VFA were added to purified diets in which the N was supplied as urea. The higher concentration of VFA in culture effluent of the branched-chain VFA and peptide supplemented

treatments further suggests increased bacterial activity in relation to the treatment supplemented with NH_3-N alone. The higher levels of n-valerate and branched chain VFA observed in the culture effluent of the treatment supplemented with casein hydrolysate, relative to the treatment supplemented with NH_3-N alone, reflects the increased deamination of amino acids which would have been associated with the bacterial degradation of the casein hydrolysate. The concentration of 2-methylbutyrate and i-valerate in culture effluent was noticeably lower than that of i-butyrate and n-valerate in the case of the treatment supplemented with branched-chain VFA. This was despite similar concentrations of all of these acids being included in the medium. The same trend was apparent in the case of the treatment supplemented with peptides, with i-valerate being absent from culture effluent and 2-methylbutyrate only just detectable, despite an apparent abundance of isoleucine and leucine, which are the precursors for these compounds, being present in the medium. Russell & Sniffen (1984) demonstrated that i-valerate and 2-methylbutyrate, but not n-valerate and i-butyrate, stimulated bacterial protein synthesis *in vitro* when the inoculum used was obtained from cows receiving a diet of low protein grass hay. It would appear therefore that the low concentrations of i-valerate and 2-methylbutyrate in relation to i-butyrate and n-valerate reflect a preferential incorporation of these acids into bacterial protein.

The increased synthesis of bacterial N that was observed with the supplementation of branched-chain VFA and peptides explains the low concentration of NH_3-N remaining in the culture effluent of these two treatments. It is apparent that in the case of the treatment supplemented with NH_3-N alone, some other nutrient was first-limiting to bacterial growth. In the case of the remaining two treatments however, this nutrient or nutrients were clearly in adequate supply, allowing for increased bacterial growth which would have increased the uptake of NH_3-N into bacterial protein. The results of the experiment strongly suggest that i-valerate and 2-methylbutyrate were the limiting nutrients when only NH_3-N

was supplemented. Although peptide supplementation resulted in an additional response over that of the branched-chain VFA, the lack of statistical significance of this response, together with the observation of the low concentrations of i-valerate and 2 methyl-butyrate, provide strong evidence in favour of the hypothesis that peptides had no benefit other than the provision of precursors for branched-chain VFA. It should however be borne in mind that for both the branched-chain VFA as well as the peptide-supplemented treatments, NH_3-N became the limiting nutrient. If NH_3-N had been provided at a higher level so as not to be limiting, it is most probable that an even greater response to branched-chain VFA supplementation would have been observed in terms of bacterial N synthesis, with that of the peptide-supplemented treatment being even higher still. This contention is supported by Gorosito *et al.* (1985) who showed that supplementation with peptides and branched-chain VFA resulted in a higher degree of digestion of wheat straw cell wall than when either of these were supplemented alone.

The slight increase in OM digestion that occurred in response to branched chain VFA and peptide supplementation resulted in no significant differences being observed with respect to the efficiency of bacterial N synthesis. Under the conditions of the present experiment, specific growth rate of the bacteria was controlled by dilution rate of the fermentors, however in a situation where growth rate was not limited in this manner, it is possible that the provision of branched chain VFA and peptides could have increased the specific growth rate of the bacterial population which in turn could have been expected to have significantly increased microbial growth efficiency (Isaacson *et al.*, 1975). This may well occur, at least transiently, in the rumen, providing sufficient substrate is available. The value observed by Bas *et al.* (1989a) for microbial efficiency of rumen bacteria grown on AHP wheat straw in a dual-flow fermentor with liquid and solid dilution rates of $0.10h^{-1}$ and $0.055h^{-1}$ respectively was 46.7g N/kg OM truly digested which is more than

double the value observed in the present experiment. This difference could be attributed to the low value for OM digestion that was measured by these workers. Nonetheless, the microbial efficiency measured in the present experiment is still substantially lower than the ARC (1984) figure of 32g N/kg OM apparently digested in the rumen and is also far lower than the values observed by Isaacson *et al.* (1975) and Van Nevel & Demeyer (1979) for mixed rumen bacteria grown on glucose in chemostat at a similar dilution rate to that used in this experiment. This emphasizes the large degree of energetic uncoupling that occurred due to the fact that nutrients other than energy were limiting to bacterial growth.

From the results of cell wall carbohydrate digestion, it appears that supplementation of AHP wheat straw with either branched-chain VFA or peptides may have had the effect of improving digestion of the cell wall fraction. It is quite probable however, that the lack of any significant positive response in this respect was due to the negative effect of the low NH_3-N levels that presided in the fermentations where branched-chain VFA or peptides were provided. Gorosito *et al.*(1985) showed that the digestion of wheat straw cell walls by rumen bacteria was significantly increased by the addition of either C_4 and C_5 VFA or Trypticase to the medium, with a combination of VFA and Trypticase providing the greatest response. This suggests that a response to branched-chain VFA and peptides in terms of cell wall digestion could have been expected if NH_3-N had not been limiting. The extensive digestion of xylose in relation to glucose is in agreement with the observation of Lewis *et al.* (1989) where the ratio of xylose to glucose in AHP wheat straw declined dramatically after 12h of incubation with mixed rumen bacteria. McAllan & Smith (1983) found a similar trend with xylose showing the greatest response in terms of *in vivo* digestibility to the alkali treatment of barley straw.

The concentration of total amino acids in culture effluent

clearly reflects the increased incorporation of NH_3-N into bacterial protein in the case of the treatments supplemented with either branched-chain VFA or peptides. This is confirmed by the increase in total bacterial amino acids in culture effluent that was observed with these same treatments. Amino acids of bacterial origin accounted for 84.3, 77.1 and 85.2 percent of the total amino acids in culture effluent for treatments N, V and C respectively. This serves to demonstrate the importance of microbial protein synthesis on low protein roughages. The total amino acid content of culture effluent was 3.78, 4.75 and 1.22 times higher than that of the medium for treatments N, V and C respectively. This translates to a net gain in total amino acids, calculated as the difference in amino acid content between medium and effluent, of 334.9, 438.7 and 19.6 mg/l for the three treatments respectively. Supplementation with branched-chain VFA therefore resulted in the most efficient conversion of amino acids in the medium to microbial protein. The increase in bacterial protein synthesis obtained with peptide supplementation was offset by the extensive deamination of amino acids that occurred with this treatment. The function of branched-chain VFA in the process of protein synthesis is to provide precursors for the synthesis of valine, isoleucine, leucine and proline (Dehority *et al.*, 1958). It is clear from the results presented here that in the absence of these precursors, bacterial protein synthesis as a whole was impaired, with no large differences in amino acid profile of either the bacteria or total culture effluent being detectable. The significant differences between treatments that were observed in terms of concentrations of threonine, serine, glycine and arginine are somewhat confusing. The lower concentration of glycine in the case of treatment C may well be related to the relatively low concentration of this amino acid in the respective medium. Erfle *et al.* (1977) reported that glycine may have been synthesised preferentially when NH_3-N was provided in excess to mixed rumen bacteria growing in continuous culture. This could possibly have explained why the concentration of glycine was lower in culture effluent of the treatment

supplemented with peptides relative to the treatment supplemented with NH_3-N alone. If this were the reason however, then the effluent of the branched-chain VFA-supplemented treatment should have also contained a lower level of glycine than that of the treatment receiving NH_3-N alone. It is therefore most likely that the difference was in fact due to a greater proportion of glycine from AHP wheat straw escaping degradation in the case of treatments N and V. The differences between treatments with respect to this, as well as the other amino acids were however small and probably of no major biological significance.

In conclusion it could be said that supplementation of wheat straw with NH_3-N alone is not sufficient to ensure maximum microbial growth. The results of this experiment show a definite positive response to branched-chain VFA supplementation and an even greater (although not significantly so) response to the provision of peptides. The level of N supplemented in the present experiment was inadequate in terms of its ability to sustain sufficient levels of NH_3-N in the cultures. Indications from this experiment as well as from what has been published in the literature indicate that a far greater response to peptide supplementation in terms of digestion of cell walls and synthesis of microbial protein could be achieved if N was supplemented at a level which prevented NH_3-N from becoming the limiting nutrient. The level of peptide supplementation in the present experiment resulted in inefficient utilization of amino acids due to extensive deamination, however it is possible that the same degree of microbial protein synthesis could have been achieved at a far lower level of peptide supplementation, thus improving efficiency of amino acid utilization. AHP wheat straw proved to be a most suitable substrate for the study of N metabolism of rumen bacteria on fibrous substrates. This is attributable to the high digestibility and low protein content of the material, allowing for a large degree of manipulation in terms of the form and level of N available to the bacteria.

CHAPTER 6

THE EFFECT OF SUPPLEMENTARY PEPTIDES OR BRANCHED-CHAIN VOLATILE FATTY ACIDS ON THE EFFICIENCY OF MICROBIAL PROTEIN SYNTHESIS BY RUMEN BACTERIA GROWN ON RYE GRASS IN CONTINUOUS CULTURE

6.1 INTRODUCTION

When ruminants consume fresh forage that is high in N, the supply of protein to the duodenum is generally low and is in fact frequently lower than the total amount of protein ingested (Beever *et al.*, 1976; 1978; 1980; MacRae & Ulyatt, 1974). This loss of N between the mouth and duodenum is commonly ascribed to the rapid degradation of soluble protein and amino acids, resulting in high rumen ammonia concentrations. As little as 42% of the crude protein in fresh forages may be in the form of true protein with the remainder consisting of products of nitrogen assimilation (Mangan, 1982). The soluble protein and various NPN compounds in fresh forage are extensively degraded by the microflora of the rumen and the resultant ammonia used for the synthesis of microbial protein. The contribution of microbial N to the total NAN flow at the duodenum is high. Beever *et al.* (1987) showed that in cattle consuming fresh clover, microbial N constituted 70% of the total NAN flow at the duodenum. In this same study it was found that reducing the solubility of the forage protein by treatment with formaldehyde significantly increased NAN supply to the duodenum, largely as a result of increased efficiency of microbial protein synthesis. This improvement in microbial efficiency was ascribed to a more controlled release of dietary N. Increasing the efficiency of microbial protein synthesis would therefore appear to be a viable and effective means of improving protein supply to the animal from these high N, fresh forages. A number of studies have shown that the supplementation of ryegrass silage with some form of rumen-degradable protein may increase the efficiency of microbial protein synthesis (Rooke *et al.*, 1985; Dawson *et al.*, 1988; Rooke

& Armstrong, 1989). The general conclusion from these studies is that the increase in microbial growth efficiency is due to a more sustained supply of N to the rumen microflora. The possibility however of the response being due to the supply of amino acids and peptides to the rumen microflora cannot be ruled out, particularly when viewed in the light of the findings of Rooke & Armstrong (1989) where the infusion of casein into the rumen caused an increase in microbial N synthesis whereas the infusion of urea had no effect. Little data is available on the effect of supplementing fresh, high-N forages with a form of ruminally-degradable protein. Considering that these forages are often low in true protein, it would be reasonable to assume that microbial protein synthesis might well be stimulated by supplementing some form of rumen-degradable protein. Although any increase in microbial protein synthesis would most likely be attributable to the peptides released from breakdown of protein sources, it is also possible that this stimulation may be as a result of the supply of branched-chain VFA by the protein source. Hume (1970a), Robinson & Sniffen (1983) and Russell & Sniffen (1984) have all showed an increase in the efficiency of microbial protein synthesis in response to branched-chain VFA. Responses to the provision of branched-chain VFA *in vitro* have been observed with a variety of substrates including lucerne hay (Gorosito *et al.*, 1985 which, like many fresh forages such as rye grass, has a high crude protein content.

The purpose of the present study was to determine whether the supplementation of fresh ryegrass with either branched-chain VFA or peptides in the form of a partial hydrolysate of casein would result in an increase in microbial protein synthesis. Steady-state continuous culture techniques were used for the study in order to maintain controlled growth rates, allow for adaptation of the microbial population to the conditions imposed and to eliminate the possible effect of a more even N supply to the microorganisms. Synthesis and utilization of individual amino acids was also studied in order to establish whether the

treatments resulted in a preferential utilization or increased synthesis of any particular amino acid.

6.2 MATERIALS AND METHODS

6.2.1 *Fermentor*

A bank of three such fermentor units, each consisting of a medium reservoir dispensing into three replicate culture vessels was used for this experiment. Inoculum was prepared from rumen ingesta of two ruminally-cannulated sheep which had been accustomed to a diet of coarsely-milled lucerne hay, as described previously. After removal by aspiration, the rumen contents were strained through two layers of cheesecloth into a prewarmed container. The strained rumen fluid was transferred to the culture vessels within 5 min of withdrawal from the rumen. Each culture vessel was filled to maximum working volume with inoculum at the start of the experiment.

6.2.2 *Preparation of ryegrass*

Samples of annual ryegrass (*Lolium multiflorum*) were cut by hand and immediately frozen at -20°C, within 2 h of cutting, before being freeze-dried. This method of preservation was employed in order to ensure minimal denaturation or loss of the protein in the material (Abdalla *et al.*, 1988). After freeze-drying, the sample was ground in a Wiley mill to pass through a 0.5 mm screen and stored at -20°C until required.

6.2.3 *Media and treatments*

Three media containing ryegrass as substrate were made up as depicted in Table 1.

Table 1. Ingredient composition of ryegrass media supplemented with either NH_4Cl alone (C), NH_4Cl plus branched-chain VFA (V) or casein hydrolysate (P).

Ingredient inclusion/l	Supplement		
	N	V	C
Rye Grass (g)	10	10	10
Mineral solution (ml)[1]	50	50	50
Pfennigs metal solution (ml)[2]	10	10	10
$NaHCO_3$ (g)	4	4	4
VFA solution (ml)[3]	0	10	0
NH_4Cl (mg)	200	200	0
Casein Hydrolysate (mg)	0	0	400

[1] Composition (g/l): $CaCl_2.1H_2O$ 0.53; KH_2PO_4 18; NaCl 18; $MgCl_2.6H_2O$ 0.4

[2] Pfennig & Lippert (1966)

[3] Composition (mmol/l): n-valerate 91.94; i-valerate 91.16; i-butyrate 107.83; 2 m-butyrate 92.14

The media were formulated so as to be isonitrogenous and contained identical quantities of ryegrass supplemented with either NH_4Cl alone (N), NH_4Cl plus VFA solution (V), or peptides in the form of casein hydrolysate (C). All ingredients were added to the appropriate quantity of tap water and mixed thoroughly. The suspensions were then immediately transferred to their respective media reservoirs which had been precooled to 4°C. Immediately after transfer to the medium reservoirs the oscillatory mixers were started and run continuously to ensure adequate dispersion of all ingredients. These media were incubated simultaneously at a dilution rate of $0.06h^{-1}$.

6.2.4 *Sampling*

Effluent from the fermentations was discarded until steady-state conditions had been achieved as evidenced by constant pH, VFA concentration and NH_3-N concentration. Once it was established that steady-state conditions existed in all fermentors, sampling proceeded as described previously with two independent samples being drawn from each culture vessel.

6.2.5 *Measurements and analyses*

The same analyses were performed on medium and effluent samples as described in the preceding chapters. These results were then processed in the same manner as described previously to calculate OM digestion, bacterial N synthesis and amino acid utilization.

6.2.6 *Statistical analysis*

The experiment was analysed as a randomized blocks design using standard analysis of variance techniques with sampling periods representing blocks. Differences between treatment means were tested for significance by means of least significant differences. (Steel & Torrie, 1960).

6.3 RESULTS

6.3.1 *Chemical composition of media*

The chemical composition of the three different media are shown in Table 2. The inclusion of casein hydrolysate increased the total amino acid content of the medium in the case of treatment C, with proline, valine, isoleucine, leucine, phenylalanine and lysine showing the most notable increase.

Table 2. Chemical composition of ryegrass media supplemented with either NH_4Cl alone (N), NH_4Cl plus branched-chain VFA (V), or casein hydrolysate (C).

Measurement	Supplement		
	N	V	C
DM (g/l)	12.37	12.41	12.16
OM (g/l)	7.30	7.60	7.12
N (mg/l)	287.12	301.31	284.87
Amino acids (mg/l)			
Aspartic acid	160.5	139.9	165.6
Threonine	74.5	76.6	94.1
Serine	63.1	64.8	76.8
Glutamic acid	180.2	184.1	187.6
Proline	83.1	85.4	121.9
Glycine	96.0	98.7	100.7
Alanine	130.4	134.0	140.4
Valine	90.3	92.8	109.9
Isoleucine	67.4	69.2	86.1
Leucine	139.0	142.9	172.2
Tyrosine	50.1	51.6	57.0
Phenylalanine	93.1	95.7	109.9
Histidine	34.4	35.4	42.4
Lysine	104.6	107.5	133.8
Arginine	73.1	75.1	78.2
TOTAL (mg/l)	1439.7	1453.8	1676.5

6.3.2 *Fermentation parameters*

Nine days after inoculation, steady-state conditions were achieved in all fermentors as evidenced by constant pH, NH_3-N concentration and VFA concentration. Qualitative analysis of headspace gas showed the presence of significant quantities of methane with hydrogen being undetectable throughout the

adaptation period as well as during the sampling period. Microscopic examination of samples of the cultures at this stage showed a diverse bacterial population but no ciliate protozoa or anaerobic rumen fungi. The fermentation parameters measured during the steady state period are presented in Table 3.

Table 3. Fermentation parameters measured for incubations of ryegrass media supplemented with either NH_4Cl (N), NH_4Cl plus branched-chain fatty acids (V) or casein hydrolysate (C).

Measurement	Supplement			
	N	V	C	SE
pH	6.64	6.63	6.74	0.037
NH_3-N (mg/l)	70.0	79.4	79.4	4.992
VFA:				
Total (mmol/l)	34.5	37.3	40.2	1.527
Molar %				
Acetate	71.5	71.6	70.4	0.812
Propionate	16.9	16.9	17.5	0.499
n-Butyrate	6.7	7.0	7.0	0.263
i-Butyrate	1.5	1.2	1.6	0.101
2-methylbutyrate	0.9	0.9	0.9	0.083
n-valerate	1.2	1.2	1.2	0.090
i-valerate	1.3	1.2	1.4	0.074

No significant differences were observed between treatments with respect to either pH or NH_3-N concentration of culture effluent ($P>0.05$). The VFA results for treatment V presented here have been corrected for the branched-chain VFA supplied in the medium. Supplementation of ryegrass with branched-chain VFA resulted in significantly higher concentrations of these VFA being present in culture effluent for this treatment than for the other two media ($P<0.05$), however correction for the amount included in the medium resulted in these values not differing significantly from

the other treatments. Production of total VFA was significantly higher on the medium supplemented with peptides (C) than that of the control (N) ($P<0.05$). No other significant differences in this respect were observed between treatments. There were also no significant differences between treatments with regard to the molar percentages of individual VFA produced.

6.3.3 *OM digestion and bacterial N synthesis*

The values obtained relating to the disappearance of OM, flow of NAN and synthesis of bacterial N are shown in Table 4.

Table 4. The mean extent of OM digestion and flow of NAN, bacterial N and undegraded N in culture effluent as well as the calculated efficiency of bacterial N synthesis for ryegrass media supplemented with either NH_4CL alone (N), NH_4Cl plus branched-chain VFA (V) or casein hydrolysate (C) and incubated in continuous culture at $D = 0.06h^{-1}$.

Measurement	Supplement			SE
	N	V	C	
OM digestion (%)				
Apparent	41.2	41.4	40.8	0.079
Actual	63.5	62.1	66.7	1.906
Mean flow of N (mg/l)				
NAN	205.7	230.1	197.8	12.550
Bacterial N	165.7	169.2	172.0	7.633
Undegraded N	40.0	60.9	48.3	10.655
Efficiency of microbial N synthesis				
g/kg OMAD	55.1	54.4	53.7	2.855
g/kg OMTD	35.7	36.0	34.2	1.208

No significant differences were observed between the treatments for any of the parameters measured. Assuming complete degradation of all supplementary N, whether in the form of NH_4Cl or casein hydrolysate, the degradability of N
in ryegrass was calculated to be 0.82, 0.77 and 0.79 for treatments N, V and C respectively. The proportion of NAN present as bacterial N did not differ significantly between treatments and amounted to 0.81, 0.74 and 0.87 for the three treatments.

6.3.4 *Amino acid utilization and synthesis*

The concentration of individual and total amino acids in culture effluent is presented in Table 5. Supplementation of ryegrass with either branched-chain VFA or casein hydrolysate did not significantly increase the concentration of total amino acids in culture effluent above that of the control. There was however a tendency for lower concentrations of amino acids in culture effluent of the control relative to the other two treatments. This difference was significant ($P<0.05$) with regard to the concentrations of proline, tyrosine and arginine.

Table 5. Mean individual and total amino acid content of culture effluent for ryegrass media supplemented with either NH_4CL alone (N), NH_4Cl plus branched-chain VFA (V) or casein hydrolysate (C) and incubated in continuous culture at $D=0.06h^{-1}$.

	Supplement			
Measurement	N	V	C	SE
Amino acids (mg/100mg total amino acids)				
Aspartic acid	12.1	12.1	12.0	0.16
Threonine	5.5	5.7	5.8	0.09
Serine	5.4	5.4	5.4	0.11
Glutamic acid	14.9	13.8	13.7	0.62
Proline	4.9	5.1	5.2	0.07
Glycine	6.7	6.7	6.4	0.09
Alanine	8.8	8.6	8.4	0.19
Valine	4.9	4.9	5.2	0.18
Isoleucine	4.2	4.1	4.5	0.16
Leucine	9.3	9.4	9.4	0.11
Tyrosine	3.8	4.3	4.4	0.16
Phenylalanine	6.2	6.4	6.5	0.10
Histidine	2.1	2.1	2.0	0.03
Lysine	6.6	6.6	6.3	0.18
Arginine	4.5	4.7	4.8	0.14
TOTAL (mg/l)	1042.1	1130.5	1122.5	43.33

Overall, a net loss of amino acids was observed between medium and culture effluent with total amino acids in culture effluent being 70.5, 77.8 and 68.0 percent of that supplied by the media for treatments N, V and C respectively.

The amino acid content of bacteria isolated from culture effluent and the total bacterial amino acids in culture effluent are presented in Table 6.

Table 6. The amino acid composition of bacteria isolated from culture effluent as well as total amino acids of bacterial origin from incubations of ryegrass supplemented with NH_4Cl (N), NH_4Cl plus branched-chain VFA (V) or casein hydrolysate (C).

	Supplement			
Measurement	N	V	C	SE
Amino acids (mg100mg total amino acids)				
Aspartic acid	12.3	13.8	13.3	
Threonine	6.2	6.0	6.3	
Serine	5.4	5.4	5.3	
Glutamic acid	14.0	13.9	14.2	
Proline	3.9	3.9	3.9	
Glycine	5.7	5.9	5.6	
Alanine	8.6	8.4	8.5	
Valine	5.4	5.1	5.5	
Isoleucine	4.4	4.3	5.0	
Leucine	7.4	8.2	7.8	
Tyrosine	5.3	5.2	5.2	
Phenylalanine	5.5	5.5	5.6	
Histidine	1.7	1.7	1.7	
Lysine	8.5	7.9	8.2	
Arginine	5.0	4.7	4.7	
TOTAL (mg/l)	672.0	678.0	740.1	39.42

Bacterial amino acids constituted 64.4, 59.9 and 65.9 percent of total amino acids in the effluent for treatments N, V and C respectively. Although total bacterial amino acids were slightly higher for the casein-supplemented ryegrass, relative to the other two treatments, this difference was not statistically significant ($P>0.05$).

6.4 DISCUSSION

The results observed for the various fermentation parameters in the present study indicate that conditions in the fermentors closely approximated those found in the rumen. Molar proportions of individual VFA are in very close agreement with those found *in vivo* with perennial ryegrass (Beever *et al.*, 1976; 1987; Waghorn *et al.*, 1989). Similarly, values for OM digestion, N degradability and the proportion of N present as bacterial N all closely resemble those reported *in vivo* (MacRae & Ulyatt, 1974; Ulyatt & MacRae, 1974; Beever *et al.*, 1978). The supplementation of fresh ryegrass with an NPN source would not be expected to have any beneficial effects due to the inherently high soluble N content of the forage. The addition of NH_4Cl to treatments N and V in the present experiment was however done to ensure that all media were isonitrogenous. Despite the addition of the supplementary N, NH_3-N levels in the fermentors were relatively low compared to those normally found *in vivo* (Beever *et al.*, 1987; Waghorn *et al.*, 1989). This serves to illustrate the large contribution of inter- and intra-ruminal recycling of N to the rumen ammonia pool. The addition of the supplementary N served the purpose therefore of ensuring that NH_3-N did not become limiting to bacterial growth. The fact that NH_3-N levels in culture effluent was similar for all treatments indicates that the supplementary peptides included in treatment C were extensively deaminated and further, that efficiency of N capture was similar across all treatments.

The higher total VFA production observed with the peptide-supplemented medium relative to the control was presumably as a result of the fermentation of the supplementary peptides. This further supports the contention of the extensive deamination of these compounds as reflected by NH_3-N concentrations. The fact that molar proportions of all VFA were similar for all treatments indicates that fermentation patterns did not change in response to any of the treatments. The levels of branched-chain VFA

observed in the case of the control (N) suggest that these compounds were not limiting to microbial growth. Russell & Sniffen (1984) demonstrated that little stimulation of microbial growth occurred at concentrations higher than 0.125 mM for isovalerate and 2-methylbutyrate. In the present experiment, all branched-chain VFA concentrations were well in excess of these levels across all treatments.

Supplementation of ryegrass with either branched-chain VFA or peptides clearly had no effect on either the extent of digestion of the ryegrass or the synthesis of bacterial N. It would appear that sufficient amino acids were available in the unsupplemented ryegrass to support maximum microbial growth, despite the fact that extensive degradation and deamination of protein occurred. This supports the findings of Cotta & Russell (1982) and Argyle & Baldwin (1989) which showed that maximum microbial efficiency was attained at very low levels of amino acids in the medium. The supply of additional, fermentable OM in the form of casein hydrolysate would not have been expected to increase microbial yields as a result of more energy being potentially available for microbial growth. Demeyer & van Nevel (1986) showed that microbial yields are low when protein is utilized as an energy source. The mean values observed for microbial growth efficiency in the present study are considerably higher than the mean of 37.8 $\pm$ 10.62 reported by the ARC (1984) for a total of 8 observations of sheep consuming fresh grass or legume forage. This could be attributed to the favourable conditions existing in the fermentors due to substrate being supplied on a continuous basis, thus reducing energetic uncoupling, and also, the absence of protozoa from the system would result in reduced intraruminal N recycling and so increase microbial efficiency (Leng & Nolan, 1984). Microbial efficiency was on average nearly 15 percent higher than that observed by Van Nevel & Demeyer (1979) with mixed rumen bacteria grown in chemostat at a dilution rate of $0.06h^{-1}$ with glucose as substrate. This difference may well be due to different techniques used for determining microbial

yields, however it is in line with the observations of the ARC (1984) that microbial yields on fresh forages are usually high, compared to those measured on either dry roughages or concentrate diets.

Despite having no effect on total amino acid content of culture effluent, it is clear that supplementation of rye grass with either branched-chain VFA or peptides increased the concentration of proline, tyrosine and arginine in culture effluent relevant to the control. It is possible in the case of the medium supplemented with casein hydrolysate, that the higher concentrations of amino acids in the medium resulted in slightly more of these amino acids escaping degradation and remaining intact in the culture effluent. This could however not explain the higher concentrations relative to the control observed with the treatment receiving supplementary branched-chain VFA. Salter *et al.*, (1979) studied the incorporation of [^{15}N]urea into the individual amino acids of rumen bacteria in steers fed on diets containing different levels of rumen-degradable protein. This study showed that the rate of incorporation of N from urea into arginine and proline was low but that this increased as protein in the diet was replaced with NPN. Similarly, phenylalanine and its derivative, tyrosine were poorly labelled with ^{15}N, however this did not change as protein was replaced with NPN. The authors concluded from this study that these amino acids were derived from preformed amino acids and peptides to a greater extent than other amino acids under conditions where an adequate supply of these units was available. The increased concentrations of these amino acids in the effluent of the branched-chain VFA-supplemented medium may be explained by the fact that higher concentrations of amino acid precursors in the form of branched-chain VFA were provided. This would be particularly likely in the case of proline, which is synthesized from valeric acid (Dehority *et al.*, 1958). The fact that the differences in amino acid concentrations between treatments is not reflected in the amino acid content of bacteria implies that these differences were not

due to increased synthesis of the respective amino acids but may rather have been as a result of less extensive degradation.

It can be concluded from this experiment that under the conditions imposed, unsupplemented ryegrass contained sufficient nutrients to ensure optimal bacterial growth. Although, like any other *in vitro* experiment, caution should be exercised in extrapolating the results to *in vivo* situations, it is evident that many of the parameters observed in the present study closely resemble those observed *in vivo*. There appeared to be no advantage to supplementing the ryegrass with either branched-chain VFA or peptides apart from a slight increase in the flow of proline, arginine and tyrosine. It would appear therefore that neither the level nor the form of nitrogen contained in fresh ryegrass is limiting to microbial protein synthesis.

CHAPTER 7

THE EFFECT OF FORM AND LEVEL OF NITROGEN ON THE EFFICIENCY OF MICROBIAL PROTEIN SYNTHESIS BY RUMEN BACTERIA GROWING ON A SUBSTRATE OF STARCH AND MAIZE STRAW IN CONTINUOUS CULTURE

7.1 INTRODUCTION

The requirements for amino acids or peptides of rumen bacteria growing on substrates with a high starch content is unclear. The results obtained in various *in vitro* studies indicate that microbial protein synthesis by bacteria fermenting starch is increased by the supply of amino acids or peptides. In batch culture studies where the substrate was either glucose or soluble starch (Maeng *et al.,* 1976), or a mixture of soluble carbohydrates, including starch (Argyle & Baldwin, 1989), the efficiency of microbial protein synthesis was improved by the inclusion of peptides or amino acids in the medium. In both of these studies the bacterial population was presumably reasonably representative of that which would be found in the rumen of animals consuming a high-concentrate (starch) diet. Furthermore, the study of Cotta & Russell (1982) showed that amino acids stimulated protein synthesis by five different species of rumen bacteria grown on glucose in continuous culture. These five strains, namely *Selenomonas ruminantium, Bacteroides ruminicola, Megasphaera elsdenii, Streptococcus bovis* and *Butyrivibrio fibrisolvens* could all be expected to occur in relatively high numbers in the rumen of concentrate-fed animals (Hungate, 1966). The results of *in vivo* experiments have however yielded conflicting results in this regard. Hume (1970b), Ben-Ghedalia *et al.* (1978) and Salter *et al.* (1979) all found positive responses in terms of microbial protein synthesis when degradable protein was added to diets containing relatively high levels of starch. In contrast to these results, Mercer *et al.* (1980) found that replacement of urea with either groundnut meal or fishmeal in barley-based diets fed to sheep had no effect on the efficiency

of microbial protein synthesis. Similarly, in the study of McAllan *et al.* (1988), no difference in microbial efficiency was observed when either urea, single-cell protein, maize gluten or rapeseed meal was used to supplement a diet consisting of straw and rolled barley. The inconsistency noted in the response to protein supplementation of high-starch diets *in vivo* may well be as a result of differences in the protein content of the basal diets used in these studies. Whereas Hume (1970b), Ben Ghedalia *et al.* (1978) and Salter *et al.* (1979) all fed semi-purified diets which were low in crude protein, Mercer *et al.* (1980) and McAllan *et al.* (1988) fed more complex, practical diets which had a higher protein content. In the case of the complex diets, it quite likely that sufficient peptides were released from degradation of the basal dietary components to support maximum microbial yields, thus explaining why no response was observed with the supplementation of protein. This contention is supported by the *in vitro* studies of Maeng *et al.* (1976), Cotta & Russell (1982) and Argyle & Baldwin (1989) which all demonstrated that maximum stimulation of microbial growth occurred at low levels of peptide or amino acid supplementation.

The optimum level of NH_3-N required to maximize microbial protein synthesis by rumen bacteria has been investigated in a number of studies. Satter & Slyter (1974) found no increase in microbial protein synthesis *in vitro* at NH_3-N concentrations above 50 mg/l. This was later confirmed by the work of Russell & Strobel (1987) using washed cell suspensions grown *in vitro* on soluble carbohydrates. The *in vivo* results of Kang-Meznarich & Broderick (1981) showed that the optimum level of rumen NH_3-N required to sustain maximum microbial protein synthesis was 85 mg/l while Pisulewski *et al.* (1981) reported values of 39, 84 and 22.4 mg/l for iso-energetic diets containing high, medium and low levels of concentrates respectively. In all of the experiments mentioned here, microbial yields were actually measured. From these results it would appear that a minimum level of NH_3-N of between 50 and 90 mg/l is required to maximize microbial efficiency. Various

studies have examined the effect of NH_3-N level on the rate of degradation of the substrate by rumen bacteria and in general, the results reported are substantially higher than those required to maximize microbial protein synthesis. Mehrez *et al.* (1977) found that the maximum rate of degradation of barley was achieved at a rumen NH_3-N level of 235 mg/l. In a similar study by Wallace (1979) the degradation rate of barley was increased by 90% when the NH_3-N level in the rumen was increased from 86 to 187 mg/l. The study of Odle & Schaefer (1987) yielded optimum NH_3-N levels of 125 and 61 mg/l for the degradation of barley and maize respectively.

From the foregoing it is apparent that the optimum level of NH_3-N required for maximum efficiency of microbial protein synthesis may differ from that required to maximize degradation rates of the substrate. Unfortunately, the studies which examined the effect of NH_3-N concentrations on degradation rates did not measure microbial yields so that effect on both parameters could be established simultaneously. Increased degradation rates however imply higher microbial growth rates which in turn would imply more efficient growth and a net increase in microbial protein synthesis. In the study of Odle & Schaefer (1987), it is evident that the degradation rate of barley was substantially higher than that of maize. The higher NH_3-N level required to maximize degradation of barley, relative to that of maize may therefore be related to the growth rate of the bacteria fermenting the respective substrates.

The requirements of rumen bacteria for amino acids or peptides when growing on high-starch substrates may therefore be confounded by ammonia levels or specific growth rate. If either of these factors were limiting to microbial protein synthesis, then little or no response could be expected from the supplementation of amino acids or peptides.

The purpose of the present experiment was to establish whether

NH_3-N levels above 50 mg/l stimulated bacterial N synthesis or the extent of substrate degradation in continuous culture and also, whether these different levels of NH_3-N affected the response of the bacteria to the supply of peptides. A further objective was to determine whether the abovementioned responses to either NH_3-N levels or peptide supplementation were affected by specific growth rate. The effect of these parameters on the utilization and synthesis of total and individual amino acids was also investigated.

7.2 MATERIALS AND METHODS

7.2.1 *Fermentors*

A bank of twelve fermentors was used for this experiment. Each of the four treatments was replicated in three fermentors which were fed from a common medium reservoir. At the start of each experimental period, all fermentor vessels were filled to overflowing with strained rumen fluid drawn from two rumen-cannulated sheep which had been maintained on a diet consisting of 25% *Eragrostis curvula* hay, 25% lucerne hay and 50% of a maize-based concentrate.

7.2.2 *Media and treatments*

A set of four media was made up as illustrated in Table 1. All of these media contained equal amounts of maize starch, maize straw, minerals, VFA and $NaHCO_3$. The two low N media were supplemented with identical levels of N but differed only in that the supplementary N was supplied at NH_3-N:peptide N ratios of 100:0 or 75:25. Peptides were supplied in the form of casein hydrolysate. The high N media contained a higher level of supplementary N also at ratios of NH_3-N:peptide N of 100:0 and 75:25. The experiment was divided into two experimental periods. During the first period, the four media were incubated in continuous culture at a dilution rate of $0.06h^{-1}$. The experiment

was then repeated at a dilution rate of $0.09h^{-1}$ during the second experimental period.

Table 1. The ingredient composition of high starch media supplemented at either a high or a low level of N with peptide N included at a level of 0 or 25 % of the total supplementary N.

Ingredient inclusion/l	Level and form of supplementary N			
	Low N Peptide N (%)		High N Peptide N (%)	
	0	25	0	25
Maize starch (g)	5	5	5	5
Maize straw (g)	5	5	5	5
$NaHCO_3$ (g)	5	5	5	5
Mineral Solution (ml)[1]	50	50	50	50
Pfennigs metal soln. (ml)[2]	10	10	10	10
VFA solution (ml)[3]	10	10	10	10
NH_4CL (mg)	690	480	1165	810
$(NH_4)_2SO_4$ (mg)	200	200	315	315
Casein Hydrolysate	0	400	0	670

[1] Composition (g/l): $CaCl_2.1H_2O$ 0.53; KH_2PO_4 18; NaCl 18; $MgCl_2.6H_2O$ 0.4

[2] Pfennig Lippert (1966)

[3] Composition (mmol/l): n-Valerate 91.94; i-Valerate 91.16; i-Butyrate 107.83; 2 Methylbutyrate 92.14

7.2.3 *Sampling*

All fermentations were run until steady state conditions had been established in the fermentors whereafter sampling took place. As with the previously-described experiments, [^{3}H]leucine was included in all media to serve as a microbial marker. The sampling procedure was as described previously, with two individual samples being taken from each fermentor vessel.

7.2.4 *Measurements and analyses*

Samples of medium and effluent were analysed as described previously and the values thus obtained were used to calculate OM digestion and microbial N synthesis.

7.2.5 *Statistical analysis*

The two experimental periods were analysed separately as randomized blocks designs by analysis of variance techniques with sampling periods representing blocks. Least significant differences were used to test for significant differences between treatment means (Steel & Torrie, 1960).

7.3 RESULTS

7.3.1 *Chemical composition of media*

The chemical composition of the media used in the experiment is shown in Table 2. The addition of casein hydrolysate to these media clearly had the effect of more than doubling the total amino acid content of the medium. It is also noticeable that the profile of individual amino acids was altered by the inclusion of casein hydrolysate. The proportion of glutamic acid and proline and to a lesser extent, isoleucine, histidine and lysine were all increased by supplementation with casein hydrolysate.

Table 2. Chemical composition of high starch media supplemented at either a high or a low level of N with peptide N included at a level of 0 or 25 % of the total supplementary N.

	Level and form of supplementary N			
	Low N Peptide N (%)		High N Peptide N (%)	
Component	0	25	0	25
DM (g/l)	12.8	12.3	12.1	12.4
OM (g/l)	8.3	8.0	7.8	7.7
N (mg/l)	260.3	263.5	410.8	407.8
Amino acids (mg/100mg total amino acids)				
Aspartic acid	12.1	9.9	11.9	9.3
Threonine	4.7	4.7	5.4	4.5
Serine	5.6	5.2	5.6	5.2
Glutamic acid	17.4	22.4	16.7	22.9
Proline	5.3	9.9	5.5	9.8
Glycine	7.3	4.5	6.5	4.0
Alanine	8.6	6.5	9.2	6.1
Valine	6.2	6.3	6.7	6.6
Isoleucine	3.9	4.5	3.9	5.0
Leucine	9.2	9.0	9.6	9.4
Tyrosine	3.9	2.4	3.8	2.3
Phenylalanine	5.9	5.0	5.9	5.1
Histidine	1.4	2.0	1.6	2.0
Lysine	4.5	5.3	4.1	5.8
Arginine	4.1	2.2	3.5	2.0
TOTAL (mg/l)	134.6	398.6	160.7	588.5

7.3.2 *Fermentation parameters*

The fermentation parameters measured at steady state for the different treatments at the low ($0.06h^{-1}$) and high ($0.09h^{-1}$) dilution rates are depicted in Tables 3 and 4 respectively.

Table 3. Mean values for pH, VFA concentrations and NH_3-N concentration of culture effluent from the incubation of high starch media, supplemented with N at the high and low level and containing either 0 or 25 % of supplementary in the form of peptides. $D = 0.06h^{-1}$.

	Level and form of supplementary N				
	Low N Peptide N (%)		High N Peptide N (%)		
Measurement	0	25	0	25	SE
pH	6.46	6.53	6.44	6.54	0.03
NH_3-N (mg/l)	61.2^{a}	53.0^{a}	208.9^{b}	195.0^{b}	7.44
VFA:					
Total (mmol/l)	52.2	50.7	52.9	60.2	2.45
Molar %					
Acetate	64.7^{a}	66.7^{ab}	72.3^{bc}	72.6^{c}	2.02
Propionate	19.7	14.8	13.0	15.8	2.04
n-Butyrate	4.7	6.2	3.8	3.5	0.91
i-Butyrate	2.8	3.7	3.2	2.4	0.42
2 m-Butyrate	2.8	2.7	2.7	2.0	0.23
n-Valerate	2.7	3.5	2.7	1.7	0.55
i-Valerate	2.5	2.4	2.2	2.0	0.14

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$)

Table 4. Mean values for pH, VFA concentrations and NH_3-N concentration of culture effluent from the incubation of high starch media, supplemented with N at the high and low level and containing either 0 or 25 % of supplementary in the form of peptides. D = $0.09h^{-1}$.

	Level and form of supplementary N				
	Low N Peptide N (%)		High N Peptide N (%)		
Measurement	0	25	0	25	SE
pH	6.43	6.61	6.48	6.50	0.05
NH_3-N (mg/l)	47.9^{a}	41.1^{a}	146.6^{b}	150.6^{b}	4.36
VFA:					
Total (mmol/l)	44.7	49.6	45.7	46.9	2.85
Molar %					
Acetate	62.5	60.2	60.6	62.5	1.55
Propionate	27.0	28.9	30.0	24.9	1.76
n-Butyrate	2.0^{a}	1.9^{a}	1.4^{b}	2.7^{c}	0.91
i-Butyrate	2.4	2.5	2.3	2.6	0.79
2-m Butyrate	1.9	1.9	1.8	2.0	0.10
n-Valerate	2.3^{ab}	2.5^{b}	2.0^{a}	3.0^{c}	0.13
i-Valerate	1.9	2.1^{ab}	1.9^{a}	2.3^{b}	0.08

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$)

Supplementation with N at the high level clearly had the effect of increasing the NH_3-N in the fermentors at least three fold at both dilution rates. The only other significant differences observed were in respect of molar proportions of VFA, the most noticeable being the increase in the proportion of acetate in response to the high level of N supplementation that was observed at the lower dilution rate.

7.3.3 *OM digestion and bacterial N synthesis*

The data relating to OM digestion and synthesis of bacterial N is displayed in Tables 5 and 6.

Table 5. Mean values for the extent of OM digestion and efficiency of bacterial N synthesis from the incubation of high starch media supplemented at either the high or low level of N and containing either 0 or 25 % of supplementary N in the form of peptides. $D=0.06h^{-1}$.

	Level and form of supplementary N				
	Low N Peptide N (%)		High N Peptide N (%)		
Measurement	0	25	0	25	SE
OM Digestion (%)					
Apparent	65.7^{a}	58.6^{b}	51.3^{c}	55.6^{b}	1.29
Actual	79.0^{a}	76.6^{a}	69.3^{b}	78.6^{a}	1.82
Bacterial N (mg/l)	108.4	129.0	113.6	123.6	9.38
Microbial growth efficiency					
g N/kg OMAD	18.9	28.1	32.7	31.1	2.59
g N/kg OMTD	15.6	21.4	24.0	23.1	1.48

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$).

At the lower dilution rate, apparent OM digestion at the low level of N supplementation was significantly reduced ($P<0.05$) by the inclusion of peptide N in the medium. This was however not the case with regard to actual OM digestion. At the high level of

N supplementation, peptide supplementation significantly increased both apparent as well as actual OM digestion ($P<0.05$). Although significant differences were observed between the low N and high N treatments with regard to both actual and apparent OM digestion, no logical trend was observed in this respect. Although not significant, there was a tendency for increased bacterial N synthesis in response to peptide supplementation at both the high and low levels of N supplementation. The level of N appeared to have no effect on bacterial N synthesis. Efficiency of bacterial N synthesis, expressed in terms of both actual and apparent OM digestion, was increased insignificantly by peptide supplementation. No further trends or significant differences were noted in this regard.

At the high dilution rate, the inclusion of peptide N in the medium increased both apparent and actual OM digestion significantly ($P<0.05$) at both the high and the low level of N supplementation. There was no significant increase in OM disappearance at the high relative to the low level of N supplementation. Bacterial N synthesis was significantly increased ($P<0.05$) by peptide supplementation at both the high and the low level of N supplementation. Again the level of N had no effect in this regard. No significant differences between any of the treatments were observed in respect of bacterial efficiency expressed either in terms of OM apparently digested or OM actually digested.

Table 6. Mean values for the extent of OM digestion and efficiency of bacterial N synthesis from the incubation of high starch media supplemented at either the high or low level of N and containing either 0 or 25 % of supplementary N in the form of peptides. $D=0.09h^{-1}$.

	Level and form of supplementary N				
	Low N Peptide N (%)		High N Peptide N (%)		
Measurement	0	25	0	25	SE
OM Digestion (%)					
Apparent	53.5^{ab}	60.2^{c}	50.8^{a}	54.9^{b}	1.31
Actual	72.0^{a}	79.5^{b}	66.7^{c}	83.1^{b}	1.43
Bacterial N (mg/l)	186.7^{a}	224.3^{b}	151.2^{c}	220.1^{b}	10.88
Microbial growth efficiency					
g N/kg OMAD	38.7	35.0	35.0	43.0	2.36
g N/kg OMTD	28.6	26.4	26.6	31.3	1.46

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$).

7.3.4 *Amino acid utilization and synthesis*

The concentration of individual as well as total amino acids measured in culture effluent at dilution rates of $0.06h^{-1}$ and $0.09h^{-1}$ are listed in Tables 7 and 8 respectively. Total amino acids in culture effluent were increased significantly ($P<0.05$) by both the level of N as well as by the supplementation of peptides at the lower dilution rate. Lysine was the only

individual amino acid that reflected these same differences between treatments. The only other significant difference observed was that the concentration of leucine in culture effluent of the high N, 25% peptide N treatment was significantly higher ($P<0.05$) than in the remaining three treatments.

At the high dilution rate, the concentration of total amino acids followed the same trend as that observed at the lower dilution rate. None of these differences were however significant. Proline concentration was increased by peptide supplementation at both N levels, but this was only significant ($P<0.05$) at the high N level. Level of N supplementation did not affect proline levels in culture effluent. Tyrosine concentrations in culture effluent were increased significantly ($P<0.05$) by both the level of N supplied as well as by peptide supplementation, at both levels of N.

Table 7. The amino acid content of culture effluent from the incubation of high starch media supplemented at either the high or low level of N and containing either 0 or 25 % of supplementary N in the form of peptides. $D=0.06h^{-1}$.

	Level and form of supplementary N				
	Low N Peptide N (%)		High N Peptide N (%)		
Measurement	0	25	0	25	SE
Amino acids (mg/100mg total amino acids)					
Aspartic acid	12.5	12.8	12.4	12.9	0.17
Threonine	6.2	6.1	6.1	6.1	0.05
Serine	5.3	5.2	5.3	5.1	0.08
Glutamic acid	17.0	16.7	16.7	16.0	0.28
Proline	3.8	3.9	3.7	4.0	0.06
Glycine	5.7	5.9	5.7	5.9	0.07
Alanine	8.6	8.7	8.8	8.6	0.11
Valine	5.6	5.5	5.3	5.6	0.11
Isoleucine	4.8	4.8	4.5	4.8	0.13
Leucine	7.6^{a}	7.6^{a}	7.4^{a}	7.8^{b}	0.07
Tyrosine	4.0	4.0	4.3	3.5	0.23
Phenylalanine	4.8	4.8	4.7	4.8	0.04
Histidine	1.6	1.5	1.5	1.6	0.04
Lysine	6.9^{a}	7.2^{ab}	7.7^{bc}	7.8^{c}	0.21
Arginine	5.6	5.2	6.0	5.4	0.41
TOTAL (mg/l)	624.0^{a}	709.6^{bc}	$649.4a^{b}$	744.6^{c}	27.53

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$).

Table 8. The amino acid content of culture effluent from the incubation of high starch media supplemented at either the high or low level of N and containing either 0 or 25 % of supplementary N in the form of peptides. $D=0.09h^{-1}$.

	Level and form of supplementary N				
	Low N Peptide N (%)		High N Peptide N (%)		
Measurement	0	25	0	25	SE
Amino acids (mg/100mg total amino acids)					
Aspartic acid	13.4	12.8	13.0	12.9	0.18
Threonine	5.9	6.0	6.0	6.0	0.10
Serine	5.5	5.3	5.5	5.4	0.10
Glutamic acid	16.2	15.6	15.4	15.8	0.37
Proline	4.1^{ab}	4.4^{bc}	3.9^{a}	4.7^{c}	0.11
Glycine	5.9	6.0	6.1	6.0	0.09
Alanine	9.4	8.9	9.0	9.0	0.17
Valine	5.4	5.6	5.3	5.3	0.18
Isoleucine	4.6	4.7	4.4	4.4	0.19
Leucine	7.9	7.9	7.9	7.8	0.07
Tyrosine	3.9^{a}	4.4^{bc}	4.8^{c}	4.4^{b}	0.13
Phenylalanine	4.9	4.8	4.8	4.6	0.07
Histidine	1.8	1.8	1.8	1.8	0.05
Lysine	6.9	7.4	7.4	7.5	0.15
Arginine	4.2	4.3	4.7	4.4	0.18
TOTAL (mg/l)	826.7	872.5	822.3	905.6	31.44

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$).

The net efficiency of amino acid synthesis, expressed as mg amino acids in culture effluent per mg amino acids supplied in the medium at the lower dilution rate was 4.64 and 1.78 respectively for the treatments supplemented with 0 and 25% peptide N at the

low N level and 4.04 and 1.27 respectively at the high N level. The same values calculated at the high dilution rate were 6.14 and 2.19 for the low N treatments and 5.12 and 1.54 for the high N treatments for 0 and 25% peptide N media respectively.

Total bacterial amino acids as well as the amino acid profile of bacteria isolated from culture effluent are presented in Tables 9 and 10. Although there was a large increase in bacterial amino acids in response to peptide supplementation, at both the high and low N level, these differences were not quite significant in the case of the experiment run at the lower dilution rate. The same trend was observed at the high dilution rate, however in this case the differences were statistically significant ($P<0.05$).

Table 9. Total bacterial amino acids and amino acid content of bacteria isolated from culture effluent from the incubation of high starch media supplemented at either the high or low level of N and containing either 0 or 25 % of supplementary N in the form of peptides. $D=0.06h^{-1}$.

	Level and form of supplementary N				
	Low N Peptide N (%)		High N Peptide N (%)		
Measurement	0	25	0	25	SE
Amino acids (mg/100mg total amino acids)					
Aspartic acid	12.7	12.4	12.6	12.7	
Threonine	5.8	5.3	5.5	5.7	
Serine	5.4	5.7	5.7	5.2	
Glutamic acid	14.2	14.4	14.5	13.9	
Proline	3.8	3.4	4.1	3.8	
Glycine	5.4	5.6	5.4	5.5	
Alanine	8.7	9.1	9.4	8.7	
Valine	5.5	6.0	5.4	5.7	
Isoleucine	4.7	5.0	4.3	4.7	
Leucine	8.2	8.3	8.1	8.4	
Tyrosine	4.6	4.0	5.0	4.6	
Phenylalanine	5.5	5.5	5.3	5.5	
Histidine	2.3	2.2	1.8	1.8	
Lysine	8.2	8.3	8.0	8.3	
Arginine	5.0	4.8	4.8	5.4	
TOTAL (mg/l)	551.2	637.1	517.7	711.7	49.64

Table 10. Total bacterial amino acids and amino acid content of bacteria isolated from culture effluent from the incubation of high starch media supplemented at either the high or low level of N and containing either 0 or 25 % of supplementary N in the form of peptides. $D=0.09h^{-1}$.

	Level and form of supplementary N				
	Low N Peptide N (%)		High N Peptide N (%)		
Measurement	0	25	0	25	SE
Amino acids (mg/100mg total amino acids)					
Aspartic acid	12.9	12.7	13.0	12.8	
Threonine	5.6	5.7	5.6	5.7	
Serine	5.7	5.3	5.4	5.4	
Glutamic acid	14.4	14.1	13.8	14.1	
Proline	3.7	3.5	3.6	3.6	
Glycine	5.6	5.5	5.6	5.5	
Alanine	8.2	8.4	8.2	8.3	
Valine	5.3	5.7	5.4	5.6	
Isoleucine	4.4	4.8	4.6	4.8	
Leucine	8.2	8.2	8.2	8.2	
Tyrosine	4.6	4.7	4.9	4.5	
Phenylalanine	5.2	5.3	5.2	5.2	
Histidine	1.9	1.9	1.9	2.0	
Lysine	8.5	8.7	8.7	8.8	
Arginine	5.8	5.5	5.8	5.5	
TOTAL (mg/l)	604.9[a]	764.6[b]	501.3[c]	768.4[b]	30.41

a, b, c Means in the same row with different superscripts differ significantly ($P<0.05$).

7.4 DISCUSSION

The media used in this experiment were formulated so as to simulate a high-concentrate diet which would commonly be fed to high-producing ruminants. The reason that a purified form of starch was included was to ensure that as little as possible protein was contributed from the basal ingredients of the medium, thus allowing a large degree of manipulation of the form and level of N by appropriate supplementation. The level of purified starch included in the medium was the maximum that could be used without disrupting the normal fermentation pattern. Preliminary experiments showed that at higher rates of inclusion, a loss of methane occurred in the headspace gas with a corresponding increase in hydrogen, indicating a deviation from a normal rumen-like fermentation.

The different levels of supplementary N used in the experiment were chosen so as to maintain NH_3-N levels in the fermentors in the region of 50 and 200 mg/l respectively. The rationale behind this was to be able to establish whether either microbial protein synthesis or degradation of the substrate was increased when NH_3-N levels exceeded the level of 50 mg/l proposed by Satter & Slyter (1974) and Russell & Strobel (1987) as being the optimum for growth of mixed rumen bacteria.

The higher proportion of acetate observed for the high N relative to the low N treatments at the lower dilution rate is difficult to explain as no other workers have reported a similar trend. The fact that this phenomenon was not observed at the high dilution rate indicates a possible experimental error. The proportions of the individual VFA generally resembled those found under *in vivo* conditions where diets high in concentrates are fed (Hungate, 1966).

No consistent trend with respect to differences in OM digestion between treatments was noticed in the case of the lower dilution

rate. The significant differences that were observed appear to be as a result of the inexplicably low value measured for the 50% peptide medium at the high N level. This low value appears to be as a result of experimental error. Neither apparent nor actual OM digestion appeared to be affected by either the form or level of supplementary N at this dilution rate. The supplementation of peptides clearly had a far greater stimulatory effect on microbial activity, in terms of bacterial N synthesis and OM digestion, at the high, as opposed to the low dilution rate. Growth rate therefore appeared to be first-limiting to microbial yield at the $0.06h^{-1}$ dilution rate and no additional source of peptides was required to sustain the microbial growth imposed by this dilution rate. Despite the significant increase in bacterial N synthesis observed in response to peptide N at the high dilution rate, the efficiency of bacterial N synthesis was unaffected due to the corresponding increase in OM digestion.

It is clear that at both dilution rates, the level of NH_3-N present in the fermentations at the low level of N supplementation was sufficient to support maximum microbial growth, within the limits imposed by other constraints such as growth rate and peptide availability. This is in agreement with the value of 50 mg/l proposed by Satter & Slyter (1974) and Russell & Strobel (1987) as being optimum for microbial growth.

The net flow of amino acids from the fermentors relative to those supplied in the medium provide evidence that extensive degradation of the supplementary peptides occurred. Despite the increase in bacterial synthesis of amino acids that was observed with peptide-supplementation, the net efficiency of amino acid synthesis was clearly more than double in the case of the treatments containing no supplementary peptides. This was due to the extensive degradation of protein that occurred in the case of the peptide-supplemented media. This same principle was demonstrated by Cotta & Russell (1982). The concentration of amino acids of bacterial origin in the culture effluent at both

the lower as well as the high dilution rate followed the same trend as that observed for bacterial N synthesis. The increase in total amino acid content of the culture effluent which accompanied the supply of peptides in the medium was therefore primarily ascribable to the increased synthesis of bacterial amino acids and not to the increased flow of undegraded amino acids in culture effluent. The significantly higher concentration of leucine in culture effluent of the 25% peptide N, high N treatment relative to the other three treatments at the lower dilution rate probably reflects a greater proportion of this amino acid escaping degradation which in turn is related to its higher concentration in the medium. At the lower dilution rate, the net synthesis of lysine clearly responded to the level of N supplementation. The same tendency was noted at the high dilution rate, although this was not statistically significant. This trend was not observed by Erfle *et al.*, (1976) when mixed rumen bacteria were grown in continuous culture at different NH_3-N levels.

The amino acid profile of culture effluent flowing from the fermentors was very similar to that of duodenal digesta of sheep fed on a purified diet containing a high proportion of starch (Ben-Ghedalia *et al.*, 1978). Similarly, the amino acid profile of the bacteria isolated from culture effluent closely resembled that of bacteria isolated from the rumen of sheep (Ben-Ghedalia *et al.*, 1978) and cattle (Salter *et al.*, 1979) fed on high-starch, purified diets.

The findings of the present study when extrapolated to *in vivo* situations with more practical diets may have a number of implications. Probably the most significant aspect of the results was the increase in microbial activity, measured both in terms of OM disappearance and bacterial N synthesis, that occurred in response to the supplementation of peptides. Bacterial growth rate in the present experiment was limited by the dilution rate at which the fermentors were operated. The greater extent of OM

digestion which occurred as a result of the availability of peptides suggests that bacterial growth rate might have increased if it were not directly controlled by dilution rate. The more rapid degradation of the substrate would presumably have led to a more rapid passage of digesta through the rumen in an *in vivo* situation, thus increasing the net flow of bacterial protein out of the rumen.

Whether or not the observations made in this study would be applicable in an *in vivo* situation would depend on a number of factors. For example, a more rapidly degradable form of starch such as barley would probably support a more rapid bacterial growth rate than would a more slowly-degraded form of starch such as maize. Based on the results of this experiment, the bacterial population that was growing at the faster growth rate would have a greater requirement for amino acids or peptides and would therefore be more likely to respond to the supplementation of this form of N. The protein content of the basal diet would undoubtedly also affect the response to peptide supplementation. As pointed out already, stimulation of microbial growth by peptides occurs at very low levels which may well be sustainable by many practical, complex diets (Mercer *et al.*, 1980; McAllan *et al.*, 1988).

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The main objective of this study was to establish whether or not, and to what extent, the growth of rumen bacteria on solid, complex substrates was affected by the supply of peptides, and the effect of this on the overall efficiency of protein utilization. Before final conclusions can be drawn from the results, the methods used for obtaining these results should be evaluated in terms of their ability to answer such questions and also, their applicability to more practical *in vivo* situations. The direct extrapolation of results obtained *in vitro* to *in vivo* situations should be done with caution, however well-planned *in vitro* experiments can reveal much about the functioning of the microbial population in the rumen. The techniques applied in the present study were in many respects a substantial advancement of more conventional *in vitro* techniques. As pointed out previously, continuous culture incubations overcame the problems of end-product accumulation, substrate depletion, lack of control over growth rate and the confounding effects of synchronization between N and energy supply. The technique was also a substantial improvement over conventional chemostat methods due to the fact that solid, complex substrates which are representative of ruminant diets could be incubated in the system. An important criterion of the system developed for these studies was its ability to maintain a representative, rumen-like bacterial population under steady state conditions. The results of the experiment described in chapter 2 showed that this was achieved. The results of all subsequent experiments further confirmed this in terms of the fermentation parameters observed under the different conditions that were imposed. The nature of the study was such that it was essential to ensure that all nutrients were supplied in excess of requirements and that by manipulating the supply of N, incubations could be done under conditions of N limitation or N excess. The results of the various experiments

showed that when N was supplemented at a low level, the concentration of NH_3-N fell to levels which clearly reflected the fact that NH_3-N was fist-limiting to microbial growth (Russell & Strobel, 1985). At higher levels of N supplementation, NH_3-N concentrations increased to levels at which they were no longer limiting to microbial growth. The corresponding increase in OM digestion that occurred under these conditions, which is evident in chapter 3, indicated that the release of energy from the substrate, and not some other nutrient, then became first-limiting to microbial growth. Under these conditions, the effects of the form of N on microbial growth could be accurately determined.

The dilution rates chosen for the various fermentations were not intended to simulate the flow of digesta through the rumen, but were used to impose specific growth rates on the bacterial populations involved. The different growth rates that were applied in the various experiments reported on in this study, fell within the range of those which might be encountered in the rumen (Bergen *et al.*, 1980).

It is evident from the results therefore that in these experiments, populations of rumen bacteria which were representative in terms of growth rates and fermentation patterns of those found in the rumen were maintained in steady state. Although the results from the different experiments are not comparable on a statistical basis, a number of important trends can be observed from a comparison between experiments.

The concentration of the individual VFA in culture effluent appeared to be affected mainly by dilution rate as well as by the type of substrate. The ratio of acetate to propionate appeared to increase with increasing dilution rate on the maize straw media. With the high-starch medium however, the reverse was true, which is in agreement with Isaacson *et al* (1975) and Van Nevel & Demeyer (1979) who found that the ratio of acetate to

propionate decreased with increasing dilution rate when mixed rumen bacteria were incubated in chemostat with glucose as substrate. The change in acetate to propionate ratios observed with the maize straw medium was unexpected and is difficult to explain. This does however serve to demonstrate the different fermentation patterns observed with roughages and high-starch substrates which probably reflects the difference in bacterial populations associated with the different substrates. No major changes were observed in terms of the relative proportions acetate, propionate and butyrate in response to peptide supplementation in any of the experiments. It is unlikely therefore that any significant shift in the microbial population occurred in response to the availability of peptides on any of the substrates tested. This is in agreement with the findings of Slyter *et al.* (1971) where it was shown that the microbial population in the rumen of steers was not affected when dietary N was supplied either as urea, biuret or soya protein.

It is apparent from a comparison between experiments that the efficiency of microbial growth in these experiments was affected by a number of factors. The average values for efficiency of bacterial N synthesis on maize straw, under conditions where N was not limiting, were 22.0, 25.3 and 32.0 g N/kg OM apparently digested at dilution rates of 0.03, 0.06 and 0.09 h^{-1} respectively. Similarly, for the high-starch media, values of 27.7 and 38.0 g N/kg OM apparently digested were observed at the 0.06 and 0.09 h^{-1} dilution rates. This confirms the findings of Isaacson *et al.* (1975) and Van Nevel & Demeyer (1979) which showed that microbial growth efficiency increased at higher growth rates. The efficiency of bacterial N synthesis was also affected by the level of supplementary N. On the maize straw media, microbial growth efficiency declined from an average of 32.0 g N/kg OM apparently digested at the high level of N supplementation to 25.2 g N/kg OM apparently digested when N was supplemented at the low level. Also, the value of 22.5 observed for AHP wheat straw appears very low considering the high

dilution rate at which this experiment was run. This low value was presumably as a result of the fact that N was limiting to microbial growth. The different substrates used in the experiments also appeared to have had an effect on microbial growth efficiency. At the 0.06 h^{-1} dilution rate, the average values observed for microbial growth efficiency were 25.3, 27.7 and 54.4 g N/kg OM apparently digested for the maize straw, high-starch and rye grass media respectively and at the 0.09 h^{-1} dilution rate, the values for the maize straw and high-starch media were 32.0 and 38.0 g N/kg OM apparently digested. The higher efficiency observed with the high-starch medium relative to the maize straw is in agreement with the study of Stern *et al* (1978) which showed that microbial yields in continuous culture increased with increasing non-structural carbohydrates. In the ARC (1984) review of microbial yields determined *in vivo*, it was shown that higher than average microbial yields were observed with fresh forage diets and also that mixed diets supported higher microbial yields than either all roughage or all concentrate diets. The trends observed in the present study are in general agreement with this review.

From the foregoing, it is evident that factors other than the form of N had a significant effect on the efficiency of microbial protein synthesis and that in some cases, the magnitude of these increases in efficiency were large in relation to the increases observed in response to the supplementation of peptides. This emphasises the importance of an adequate N supply to the rumen microorganisms and also, the negative effect of slow turnover times in the rumen on microbial yield.

The response to peptide supplementation in terms of microbial yields was also affected by growth rate, level of N supplementation and the substrate being fermented. The experiment described in chapter 3 clearly demonstrated that supplementation with peptides only elicited a response if the constraints of N limitation and low growth rates were removed or alleviated. With

the high-starch medium, a similar observation was made, namely that at the lower dilution rate, the response to peptide supplementation was slight, and statistically insignificant while at the high dilution rate, there was a large, significant increase in bacterial N synthesis in response to peptide supplementation. A number of factors may be involved in this lack of response to peptide supplementation at low growth rates. It is quite likely that the long exposure of the substrate to microbial attack at the low dilution rate resulted in substrate depletion which in turn led to energy being limiting to microbial growth. The slow turnover may also have increased recycling of N within the bacterial population due to lysis of cells. This would have had the effect of more peptides, amino acids and branched-chain VFA being released into the surrounding medium (Leng & Nolan, 1984) which would have contributed to the requirements of the bacterial population in this regard.

Although there was a tendency for microbial yields to increase slightly in response to peptide supplementation when N was limiting on the maize straw media, these increases were not statistically significant and were smaller in magnitude than the increases observed at the high level of N supplementation. This may have been partially due to the fact that at the higher level of N supplementation, more peptide N was supplied in the case of the treatments supplemented with either 25 or 50 % peptide N than the same treatments at the lower level of N supplementation. It is however most likely that at the low level of N supplementation NH_3-N was first-limiting to microbial growth with the result that no benefit was obtained by supplementing peptides under these conditions.

It could be expected that with the different substrates used in these experiments, different species of bacteria would have predominated in the fermentations, depending on the substrate being fermented. The fact that increases in microbial yield were observed in response to peptide supplementation of maize straw,

high-starch and AHP wheat straw media demonstrates the stimulatory effect of peptides over a range of different microbial populations. The lack of response to peptide supplementation of rye grass however was undoubtedly due to the fact that sufficient protein was present in the material to satisfy the requirements of the bacterial population for amino acids and peptides.

The results of the different experiments provide evidence that increases in microbial yield that were observed when peptides were supplemented were not due only to the supply of branched-chain VFA as products of deamination of the amino acids constituting the peptide sources. Microbial yields were seen to increase with peptide supplementation of maize straw in chapter 3 as well as the high-starch media in chapter 7, even though branched-chain VFA were adequately supplied in all treatments. Also, the results presented in chapter 5 showed that microbial yields were higher when maize straw was supplemented with casein hydrolysate than when branched-chain VFA were supplemented.

The amino acid profile of both culture effluent and isolated bacteria were remarkably similar for all experiments, irrespective of dilution rate, level of N supplementation or substrate used. These values also were also found to agree well with those reported on in the literature and supports the findings made *in vivo*, that the amino acid composition of microbial protein remains constant across a wide range of diets (Storm & Ørskov, 1983). In no experiment did it appear that a particular amino acid or group of amino acids restricted microbial growth. The higher microbial yield observed when maize straw was supplemented with sunflower oilcake as opposed to casein hydrolysate however suggests that the form of peptides available to the bacteria may affect microbial yield.

Inclusion of protein or peptides resulted in a corresponding decrease in the net efficiency of amino acid biosynthesis in all

of the experiments reported on here, irrespective of whether microbial yields were increased or not. Although the minimum requirements of the bacterial population for amino acid N were not accurately established, it would appear from this, as well as the work of Cotta & Russell (1982) that the level of protein required to maximize microbial yields leads to inefficient overall utilization of protein. Apart from situations where supplementary protein appeared to escape degradation, supplementation with peptides or protein did not alter the amino acid profile of effluent flowing from the culture vessels.

An assessment of the results observed throughout this study lead to the general conclusion that, under the conditions presiding in the fermentations, the supplementation of all the substrates excepting for rye grass with peptides caused an increase in microbial yield, providing that growth rate or availability of N did not limit microbial growth. The increase in microbial protein synthesis achieved by the level of peptides supplemented was however offset by the increased deamination of the additional amino acids supplied by the peptides, resulting in a decrease in the overall efficiency of protein utilization. The design of the experiments and the techniques used were such that the response in terms of microbial yield that was observed when casein hydrolysate was supplied in the media, could be attributed, with a large degree of certainty, to the supply of peptides to the bacterial population. Other confounding factors such as the supply of various growth factors and synchronisation between N and energy supply were effectively eliminated.

Although the results of this study may not necessarily be directly applicable to practical, *in vivo* situations, they do have certain implications with regard to the protein nutrition of ruminants. The study clearly demonstrated that the synthesis of microbial protein by mixed rumen bacteria growing on complex substrates commonly occurring in ruminant diets may be reduced if sufficient peptides are not present. This also provides

evidence to support the contention that the increase in microbial protein synthesis commonly observed when a form of rumen-degradable protein is fed to ruminants is attributable to the supply of peptides released during the degradation of the protein source. The increased digestion of the substrate that occurred in response to peptide supplementation could be expected to have substantial benefits *in vivo*, as this would imply a more rapid passage of digesta from the rumen which in turn would result in increased microbial yields and higher intake by the animal. It is however doubtful if these positive effects would offset the degradation of supplementary protein which would occur in the rumen. The study confirms the wasteful degradation of protein that occurs in the rumen and emphasizes the importance of the *de novo* synthesis of amino acids in satisfying the protein requirements of the host animal.

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