Chapter 1

Introduction

Grasses, fibrous residues from processing food for humans and crop residues are of little or no immediate nutritive value to man. The feeding of these fibrous materials to ruminant animals is of greater importance than the feeding of cereal grains which can be used to feed humans. Serious famine in some developing countries together with the likely increase in the environmental cost of fossil fuel used when growing grains crops have prompted an increase in research for alternative ruminant feeding systems. Increasing ruminant production through utilising fibrous materials makes economic, ecological and environmental sense, because it uses the least expensive feed supply to produce meat/milk and more efficiently recycles waste materials.

The structural carbohydrates of fibrous materials are the world's most abundant renewable energy source for ruminants. The advantage of the digestive systems of ruminant animals over monogastrics is in their ability to hydrolyse and utilise the β-1,4-glucosidic bond for which no monogastric enzyme exists. The micro-organisms including bacteria, fungi and protozoa which inhabit the rumen and reticulum of ruminants are of importance in the digestion and fermentation of structural carbohydrates. Their fibrolytic activities and interactions hold the key to the exploitation of these resources. Fermentative reactions, ultimately, provide volatile fatty acids (VFA) and microbial cells as nutrients that provide energy as well as amino acid sources for ruminant animals to produce milk, meat, wool, draught power and so on.

Little can be done to improve the nutritive quality of microbial cells that leave the rumen (Czerkawski, 1976 ; Purser, 1970 ; Storm & Orskov, 1983 ; Kobayashi et

al. 1993) but the quantity of microbial cells can be altered by manipulating the factors that limit microbial growth in the rumen. The availability of the substrates required for microbial growth, the specific growth rate of rumen microbes and the turnover of microbial cells within the rumen appear to be the major factors influencing the efficiency of net microbial cell synthesis in the rumen and therefore microbial cell yields as nutrients entering the intestines.

In developing countries in the tropics and subtropics, fibrous materials are the principal sources of ruminant feeds and are available seasonally. Most of these fibrous materials are low in true protein, non protein nitrogen (NPN) and often low in a number of minerals which are essential and required for microbial cell synthesis in the rumen (see Preston & Leng, 1987). An insufficient supply in the rumen of these substrates for microbial growth will limit microbial growth efficiency and potentially impair digestion. This increases an imbalance in the nutrients absorbed from the fermentative end-products (acetogenic VFA relative to glucogenic and microbial yields relative to VFA production). Consequently ruminant productivity is inefficient due to inefficient utilisation of absorbed nutrients and low voluntary intake. Thus provision of nitrogenous substrates and minerals to meet the requirements of rumen microbes is of primary importance to improve digestibility and microbial growth efficiency and therefore increase the production of ruminants fed on low-nitrogen fibrous material.

Increased microbial growth efficiency in the rumen is generally associated with an increase in microbial yields relative to VFA production (see Baldwin et al. 1970 ; Leng, 1982b). An increase in protein:energy ratio (P:E ratio) in the nutrients available for absorption and utilisation is often associated with an increased efficiency of production of ruminants that are given low nitrogen fibrous materials (see Leng et al. 1993).

The studies reported in this thesis are part of the continuing programme of research being undertaken in this laboratory into methods aimed at increasing the efficiency of net microbial cell synthesis in the rumen of ruminants fed on low nitrogen-forage based diets. The major objective of these studies is to determine the role of nitrogenous compounds (NPN, peptides and amino acids) on the microbial ecosystem,

the digestibility of a low nitrogen forage and the microbial cells synthesised in the rumen and that leave this organ. A spin-off benefit should be to accurately predict the ruminal fluid ammonia concentrations required to optimise the efficiency of net microbial cell synthesis and maximum digestibility in the rumen.

Chapter 2

Literature Review

2.1 Scope of the review

The rumen is one of the most complex ecosystems in nature (see Hungate, 1966). The dynamics of the rumen ecosystem and fermentation are influenced by the chemical and physical properties of feedstuffs ingested by the ruminant and the animal's reaction to its food. The feed entering the rumen is quickly colonised by micro-organisms including fungi, bacteria and protozoa (see Orpin, 1983/84 ; Bauchop, 1989). The polymers of feedstuff are degraded to soluble compounds which are taken up and fermented by the primary attached and/or other micro-organisms dependent on their specific enzymes, substrate affinities and preferences. Rumen microbes have evolved different strategies for growth (see Russell & Hespell, 1981) which include metabolic, physical and nutritional interactions between different microbial populations. These interactions are essential for sustaining the microbial community and its activities. Thus, the kind and extent of microbial interactions regulate the overall activities of individual species, the nature of fermentation end-products and microbial yields or net microbial cell synthesis in the rumen.

In many situations, determination of total microbial cell synthesis in the rumen but not the net microbial cell synthesis that leaves the rumen may not give a straightforward indication of the availability of microbial yields for absorption by the host ruminants. Protozoa in the rumen are known to reduce the net microbial cell synthesis or microbial yields that enter the duodenum (Teather et al. 1984). This is because of their predation of fungi (see Orpin, 1975) and of bacteria (Coleman, 1975) and the sequestration (Abe et al. 1981) and lysis of protozoa (70-90 %) within the rumen (Weller & Pilgrim, 1974 and Leng, 1982a). However, a study of the efficiency

of net microbial cell synthesis requires a precise microbial index. Urinary excretion of purine derivatives is now generally accepted as a good indicator of microbial cells that enter the small intestine (see Chen & Gomes, 1992). Knowledge of the efficiency of net microbial cell synthesis in the rumen is necessary for description of the limits to both qualitative and quantitative aspects of the rumen ecosystem (see section 2.2) and the nutrients available to the animal (see section 2.4.3.1).

This review is aimed at establishing the background for a better understanding of the efficiency of microbial growth or net microbial cell synthesis in the rumen. The availability of substrates and the way of microbial populations and their interactions which influence the molar growth yield to the animal are discussed. The utilisation of urinary excretion of purine derivatives, as an indicator of net microbial cell synthesis is paramount to this study.

2.2 Microbial populations in the rumen

Fungi, bacteria, protozoa, bacteriophages and mycoplasmas are functional micro-organisms in the rumen. Each of the microbial populations has its own niche within the ecosystem. Studies with scanning- and transmission-electron microscopy have shown that rumen microbes are compartmentalised (see Cheng & Costerton, 1980 ; Czerkawski & Cheng, 1988). In general, the microbial populations can be divided into 4 groups due to their locations in the rumen. These are

(i) microbes suspended in the fluid phase which are easily isolated from ruminal fluid by simple differential centrifugation,

(ii) microbes associated with feed particles (particle-associated microbes) which attach firmly to the feed particles. These microbes can be isolated by more complex technology with a combination of chemical and physical treatments,

(iii) microbes associated with the rumen wall, and

(iv) microbes associated with other microbes (bacteriophages, mycoplasmas and methanogenic bacteria which attach to protozoa).

The interactions of microbial populations within and between different groups are dynamic and essential for maximising the amounts of nutrients that can be

extracted from the feedstuffs entering the rumen. Changes in the microbial populations are due primarily to changes in the availability of substrate which can alter with diets and also with time after feeding. Ultimately, the overall microbial interactions will determine the net microbial cell synthesis and the availability of nutrients for digestion and absorption. Thus, an overview of microbial populations and their interactions is important for understanding this dynamic ecosystem.

2.2.1 Bacteria

Diversity within the rumen bacteria is extensive. A number of primary criteria have been used for bacterial identification (see Hungate, 1966). Those are

(i) gram reaction,

(ii) cell morphology,

(iii) substrate utilisation,

(iv) fermentation end-products,

(v) motility,

(vi) sporulation, and

(vii) ability to grow aerobically.

These basic classifications are of the descriptive bacteriology and microbial physiology that are associated with the development of anaerobic culture techniques (see Hungate, 1950).

For further details of the characterisation, the chemotaxonomic methods have been applied to rumen bacteria (see Stewart & Bryant, 1988) including

(i) the guanine and cytosine content of the DNA,

(ii) types of electron carrier,

(iii) nucleic acid homologies, and

(iv) patterns of antibiotic sensitivity.

These detailed criteria are important information for studies at the gene level and therefore the gene technology.

The bacteria inhabiting the rumen contain approximately 10^{10}-10^{11} cells per ml ruminal fluid and about 200 species have been isolated (Bryant, 1959). Most of

bacteria are cocci and short rods and their sizes are generally in the range of 0.4-1.0 μm in diameter and 1-3 μm long (see Hungate, 1966).

Bacteria in the rumen are the principal organisms in fermenting various carbohydrates. The majority of gram-negative bacteria are found in the rumen of ruminants fed forage-based diets and there is an increased proportion of gram-positive bacteria in ruminants given high grain-based diets (see Hungate, 1966). *Bacteroides succinogenes*, *Butyrivibrio fibrisolvens*, *Ruminococcus albus* and *Ruminococcus flavefaciens* are the principal among the cellulolytic bacteria in the rumen (see Schwartz & Gilchrist, 1975 ; Hobson & Wallace, 1982a). However, some strains of *Bacteroides succinogenes* in the rumen and most strains of *Butyrivibrio fibrisolvens* are also amylolytic bacteria (see Hungate, 1966). The most widespread non-cellulolytic bacteria in the rumen are *Streptococcus bovis*, *Bacteroides amylophilus, Bacteroides ruminicola*, *Succinimonas amylolytica* and *Selemonas ruminantium* (see Stewart & Bryant, 1988). *Bacteroides ruminicola* is the most numerous bacteria in ruminants on various diets and *Streptococcus bovis* is known as one of the most active lactic acid producing bacteria (see Stewart & Bryant, 1988).

Other important bacteria in the rumen include the methanogenic bacteria such as *Methanobactrium formicicum*, *Methanobrevibacter ruminantium*, *Methanomicrobium mobilis* and *Methanosarcina barkeri* (see Stewart & Bryant, 1988). The role of these organisms is as hydrogen sinks (see Hungate, 1966). *Anaerovibrio lipolytica* is a lipolytic bacterium which excretes lipase extracellularly (see Stewart & Bryant, 1988). The hydrolysis of lipids and utilisation of lactate are suggested to be the major roles of this organism.

Most species of bacteria in the rumen can utilise ammonia as the sole source of nitrogen for bacterial protein synthesis (see Bryant & Robinson, 1962).

Rumen bacteria are essential to rumen function and the availability of rumen bacteria entering the intestine for absorption is important in determining the protein status of the host ruminants particularly those fed on low protein forage with or without supplements of NPN.

2.2.2 Protozoa

The identification of rumer ciliate protozoa particularly small entodinia is difficult and the classifications into genera are observed to be inconsistent with the original classifications in the literatu e (see Williams & Coleman, 1988). The problems of identification and classification are associated with difficulties in the culturable and classifying techniques.

The important ciliate protozoa in the rumen are entodiniomorphs and holotrichs (see Hungate, 1966). On forage-based diets, entodiniomorphid ciliates represent 86-99.5 % of total protozoal biomass in the rumen (see Jouany, 1989). There is an increased proportion of the holotrichs in the rumen of ruminants given fresh grass or sugar/fibre diets (see Hungate, 1966 ; Coleman, 1975).

More than a 100 species of rumen ciliate protozoa have been reported in the literature (see Williams & Coleman, 1988). They are associated with both the fluid and feed-particle phases (see Orpin, 1985/84 ; Bauchop, 1989). The population densities of rumen protozoa vary from 10^3 to 5×10^6 cells/ml ruminal fluid depending on diets (see Schwartz & Gilchrist, 1975). There s a decrease in protozoal population in the rumen of ruminants fed on low nitrogen forage-based diets (see Bird & Leng, 1985). The role of protozoa on fibre digestion in the rumen is not totally clear (see Jouany, 1989 ; Demeyer, 1989). However, protozoa in the rumen are not vital for ruminants since the animals lacking protozoa remain hea thy (see Hobson & Wallace, 1982a).

The turnover time of protozoa in the rumen has been reported as ranging from 5.5 to 48 h depending on the protozoal species (see Warner, 1962 ; Hungate, 1966 ; Leng, 1982a ; Punia et al. 1992). Studies with ^{14}C-methyl choline have shown that the mean turnover time is 14 h for small entodinia, 23 h for polyplastron, 33 h for dasytricha and 48 h for isotricha (see Leng, 1989). The mean turnover time of protozoa in the rumen is generally 3-5 times longer than that of ruminal fluid (see Leng, 1989). This indicates that most of protozoa are retained in the rumen and therefore the majority of protozoa lyse and are degraded within the rumen and only 10-30 % enter the intestine (Weller & Pilgrim, 1974 ; Leng, 1982a). The protozoal-N pool

can contribute to 20-80 % of total microbial-N pool in the rumen (see Hungate, 1966 ; Pilgrim et al. 1970 ; Cottle et al. 1978 ; Coleman, 1979b ; Punia et al. 1992). The amount of microbial protein entering the intestine for absorption by the host ruminants is likely to be lower when protozoal populations in the rumen are high because of predation of protozoa on bacteria and fungi and because they are retained and lysed within the rumen.

2.2.3 Fungi

Rumen fungi were first recognised in the mid 1970's and are assigned to the class Chytridiomycetes which are zoospore-producing fungi (Orpin, 1975 ; 1976 ; 1977 ; Bauchop, 1979). The classifications of the rumen fungi into genera are defined on the basis of thallus morphology (mono or polycentric), rhizoid type (filamentous or bulbous) and number of flagella per zoospore. The fungal species are classified mainly on details of zoospore ultrastructure (Munn et al. 1988). At present, 5 genera (13 species) of Chytridiomycetes fungi have been isolated from the gastrointestinal tract of herbivores (see Trinci et al. 1994 ; Wubah et al. 1993). Those are Neocallimastix, Piromyces (Piromonas), Caecomyces (Sphaeromonas), Anaeromyces and Orpinomyces. The first three genera are monocentric and the last two are polycentric.

The Embden-Meyerhof pathway appears to be the major pathway of glycolysis for generating ATP in rumen fungi (Bauchop & Mountfort, 1981 ; Yarlett et al. 1986 ; Wubah et al. 1993). This is because of ultrastructural studies indicating that they do not have mitochondria (Munn et al. 1981 ; Heath et al. 1983 ; Munn et al. 1988).

The life cycle of anaerobic fungi in the rumen generally consists of 2 stages (see Bauchop, 1989 ; Orpin, 1989). Fungal zoospores are the motile flagellated stage while fungal sporangia are the non-motile or vegetative or reproductive form. The life cycle of fungi in the rumen (zoospore and sporangia) lasts about 24-32 h (see Orpin & Joblin, 1988 ; Bauchop, 1989). Davies et al. (1993b) and Wubah et al. (1993) have proposed the resistant sporangial form (resting sporangia) which may be a third stage of the life cycle. The resting sporangia can be isolated from the hind gut and faeces (Davies et al. 1993a,b ; Wubah et al. 1993). Thick-walled sporangia containing melanin

have been identified (see Wubah et al. 1993) but the function of the resting sporangia have yet to be identified.

In general, the densities of fungal zoospores and fungal sporangia are in the range of 10^3-10^5/ml ruminal fluid (see Orpin & Joblin, 1988) and 10^3-10^4/cm^2 leaf blades (Romulo, 1986). The fungal biomass, estimated by chitin content, has been shown to contribute approximately 8 % of total microbial biomass (Orpin, 1981). This method of determination of the fungal biomass may not be reliable (Orpin & Joblin, 1988) since the chitin content of rumen fungi varies with growth conditions (Argyle & Douglas, 1989).

Studies with scanning electron microscopy have shown that rumen fungi extensively colonise plant material and the lignin-containing tissues (Bauchop, 1979 ; 1981 ; Wubah et al. 1993). The penetration of plant tissue by rumen fungi leads to a decrease in the textural strength of the tissues (Akin et al. 1983). The weakening of the tissues allows greater colonisation on the tissues by rumen bacteria and protozoa (see Orpin, 1989 ; Bauchop, 1989 ; Wubah et al. 1993) and therefore the rate and extent of fibre degradation in the rumen will be increased. Thus rumen fungi are likely to play an important role in the digestion of low quality forage that are ingested by ruminants.

2.2.4 Bacteriophages

A large and diverse population of bacteriophages has been reported to occur in the rumen of cattle and sheep (see Hoogenraad et al. 1970 : Ritchie et al. 1970 ; Klieve & Bauchop, 1988). Varying from 26 to more than 40 distinct types of the tailed phages in the rumen have been identified (see Ritchie et al. 1970 ; Klieve & Bauchop, 1988). However, rumen bacteriophages, so far, have yet to be classified into species which can be delimited on the basis of tailed phage by a combination of morphology and DNA homology or serology (see Ackermann et al. 1992).

The densities of bacteriophages in the rumen of cattle and sheep have been estimated in the range of $2x10^7$-$1.6x10^{10}$ /ml of ruminal fluid (Paynter et al. 1969 ; Klieve & Bauchop, 1988 ; Klieve & Swain, 1993).

At present, the role of bacteriophage in the rumen, their infectious cycles, their general biology and their effects on the rumen ecosystem are not understood. In view of the large number of bacteriophages present in the rumen, Klieve & Bauchop (1988) extrapolated that their activities may contribute considerably to bacterial lysis and recycling within the rumen.

2.2.5 Other organisms

Anaerobic mycoplasmas have been isolated from the rumen of cattle and sheep and assigned to the genus Anaeroplasma according to the following characteristics; obligatory anaerobic, polar lipids contain plasmalogens, bacteriolytic activities, sterol required for growth and phospholipids required for growth (see Robinson, 1984).

Mycoplasmas were found to be associated with most of the rumen fungi tested but the ecological role of the association is still unknown (Kudo et al. 1990). With the available information on these viruses, they might contribute to microbial lysis in the rumen due to their bacteriolytic and proteolytic activities (see Robinson, 1984).

2.3 Interactions between rumen microbes

Like other microbial habitats several types of interactions are evident between micro-organisms in the rumen (see Wolin, 1975 ; Russell & Hespell, 1981 ; Hobson & Wallace, 1982a). There is antagonism or competition, synergism or mutualism, neutralism, amensalism and predation. Certain species of rumen microbes may be involved in various types of the interactions at any one time depending primarily on rumen conditions. A result of the overall microbial interaction in the rumen is important in determining the quantity of nutrients extracted from feedstuffs during its degradation by rumen microbes. In turn this determines the availability and balance of fermentation end-products including microbial cells available for digestion and absorption by the host animal.

2.3.1 Bacteria-bacteria interactions

Studies based on scanning electron microscopy have shown that most of the colonisers of feedstuffs entering the rumen are bacteria (see Cheng et al. 1990). This suggests that rumen bacteria are the principal organisms in fermenting the substrates in the rumen.

After colonisation, the transformations of substrates to the overall fermentation end-products and bacterial cells are a result of the activities of bacterial consortia where one bacterial species grows on the end-product of metabolism of another. An example is that fibrolytic bacteria produce fibrolytic enzymes, digest and release various intermediates which are utilised by non-fibrolytic bacteria (Russell, 1985 ; Miron et al. 1994). These inter-dependent activities of bacterial populations in the rumen result in the sequential fermentation process which allows and accelerates enzymatic degradation of fibre to VFA (see Cheng et al. 1990).

The metabolic interactions between rumen bacteria appear to increase the availability of ATP and substrates for bacterial growth and therefore increase amount of nutrients that are extracted from the feedstuffs during its degradation and fermentation in the rumen.

2.3.2 Bacteria-fungi interactions

Studies *in vitro* have shown that there are various interactions between bacteria and fungi in a coculture of bacterium and fungus compared with a monoculture of either organism. Synergistic interactions between methanogenic bacteria and *Neocallimastix frontalis* have been demonstrated by Bauchop & Mountfort (1981) and Mountfort et al. (1982). These interactions resulted in an increased cellulose degradation in the coculture relative to the monoculture of fungus as reported by these authors. Interactions between some non-fibrolytic bacteria tested and *Neocallimastix frontalis* appeared to be synergistic, as degradation of xylan in the coculture was greater than in the monoculture of either bacterium or fungus (see Trinci et al. 1994). In contrast, an antagonistic effect of *Ruminococcus flavefaciens* has been shown on

the cellulolytic (Bernalier et al. 1993) and xylanolytic (Williams et al. 1991) activity of *Neocallimastix frontalis* in the coculture. However, neither synergistic nor antagonistic effects of either non-fibrolytic or fibrolytic bacteria tested on xylanolysis of *Piromyces communis* was observed (Williams et al. 1994b). These *in vitro* results indicate that interactions between bacterial and fungal populations in the rumen are possibly complex due to a large diversity of rumen bacteria and fungi.

Although competition for substrates between fibrolytic bacteria and fungi is possibly common in the rumen, this appears to have little effect on the overall fibre digestion in the rumen since the attachment sites between fibrolytic bacteria and fungi are possibly different as shown by scanning electron microscopy studies (see Cheng et al. 1990 and Wubah et al. 1993). The penetration of the lignin-containing tissues of plant material by rumen fungi is important in allowing greater colonisation on the plant tissues by rumen bacteria (see Orpin, 1989 : Bauchop, 1989 ; Wubah et al. 1993). Thus, the overall interactions between bacteria and fungi in the rumen are likely to be beneficial to the overall nutrient extraction from feedstuffs in the rumen.

2.3.3 Protozoa-fungi interactions

The predation on fungal zoospores by protozoa in the rumen has been reported by Orpin (1975). Enzymes able to degrade fungal cell walls in the rumen have been detected mainly in the protozoal population (Morgavi et al. 1994). These organisms contribute to the breakdown and turnover of fungal cells within the rumen and ultimately result in a reduction in fungal biomass in the rumen (Newbold & Hillman, 1990). This is consistent with an apparent increase in fungal numbers in fauna-free animals (Romulo et al. 1989 ; Ushida et al. 1989 ; Newbold & Hillman, 1990 ; Bird et al. 1994).

The extent to which the presence of protozoa influences the contribution of fungi to fibre degradation is not clear. Orpin (1983/84) has observed that numbers of rumen fungi are higher on a high fibre-diet than on a low-fibre or a high concentrate-diet but the opposite is found in numbers of protozoa. Based on this observation, Orpin (1983/83) concluded that interactions between fungi and protozoa in the rumen

may be complementary rather than competitive for substrates. Recently, Widyastuti et al. (1995) studied the effect of protozoa on the degradation of rice straw cell walls *in vitro* by *Neocallimastix patriciarum*. They reported that the fungal carboxymethylcellulase activity was reduced to approximately 50% in the fungal culture incubated with protozoa relative to the pure culture of the fungus but weight losses from the cell walls between the two culture were similar. This is suggested that these two organisms do not exhibit complementary attachment to feed particles and the presence of protozoa may reduce the contribution of fungal enzymes to fibre digestion in the rumen.

2.3.4 Protozoa-bacteria interactions

Under most conditions, the most important inhabitants of the rumen are protozoa and bacteria (see Hungate, 1966). The predatory activity of protozoa in engulfing bacteria, digesting and releasing products which are utilised for growth by the remaining organisms in the rumen is well established (see Coleman, 1975). Some species of rumen protozoa possibly release enzymes which are able to hydrolyse bacterial cell walls without prior engulfment (Coleman & Laurie, 1974 ; Morgavi et al. 1994). These activities of rumen protozoa contribute significantly to the degradation and turnover of bacterial protein within the rumen (Wallace & McPherson, 1987).

The predation by protozoa leads to a decrease in bacterial numbers in ruminal fluid (see Coleman, 1975 ; 1989). This is consistent with an increase in bacterial numbers in ruminal fluid in fauna-free animals (Orpin & Letcher, 1983/84 ; Rowe et al. 1985 ; Veira, 1986 ; Bird et al. 1990 ; Newbold & Hillman, 1990). The extent to which the predation by protozoa influences the size of bacterial population associated with feed particles is not known. Bacteria carboxymethylcellulase associated with barley straw was 38 % less in the bacterial culture incubated with protozoa relative to the bacterial culture without protozoa (Newbold et al. 1989). This suggests that the presence of protozoa in the rumen reduces the fibrolytic activity of bacteria and therefore the size of the bacterial population associated with feed particles.

2.4 Efficiency of microbial growth in the rumen

The rumen displays one of the most complicated ecosystems (see Hungate, 1966). It contains an enormous numbers of organisms which can vary in response to substrates available for growth and to interactions among other micro-organisms. Different microbial populations require different substrates and differ in the nature of their energy-yielding mechanisms. The utilisation of a certain quantity of substrates will give rise to a certain amount of preformed precursors for cellular formation and energy that can be used for maintenance processes as well as the formation of new cell material (Isaacson et al. 1975). The yield of new cell material formed from a substrate will depend on the adenosine triphosphate (ATP) generated during catabolism and the monomers required for the synthesis of new cell material (see Leng, 1982b). Under some growth conditions (see Stouthamer, 1979 ; Hespell & Bryant, 1979), the rate of ATP produced from carbohydrate breakdown can be greater than the rate of ATP required for cell synthesis. This can result in a decrease in growth rate or microbial growth efficiency and consequently a large proportion of ATP is used for maintenance purposes (M_{ATP} ; see Stouthamer, 1979). Knowledge of microbial growth efficiency or yield (Y_{ATP}) is necessary to understand the relationship between substrate utilisation, ATP generation and the formation of microbial cells.

2.4.1 Theoretical relationship between Y_{ATP}, M_{ATP} and the specific growth rate

Bauchop & Elsden (1960) found that the growth yield of a micro-organism was directly proportional to the amount of ATP that could be produced during breakdown of the energy substrate in the medium. The term Y_{ATP} was introduced by these authors and defined as the dry weight of cells produced per moles ATP available. They also found that Y_{ATP} of several organisms was fairly constant at 10.5. However, it is now recognised that Y_{ATP} is a variable due to a variation in a requirement of maintenance energy (M_{ATP}) and the availability of nutrients for cell synthesis. Deficiency of essential nutrients will result in energy spilling reactions by bacteria.

The M_{ATP} would be defined as the amount of ATP needed for the true maintenance processes (not involved in growth) and for the activity during growth. The M_{ATP} will vary with various micro-organisms and environmental conditions (see Stouthamer, 1979 ; Hespell & Bryant, 1979). The components of M_{ATP} listed by these authors are energy used for

(i) motility,

(ii) turnover and resynthesis of cell constituents,

(iii) synthesis of extracellular polymers (mostly enzymes),

(iv) active transport and energetise membrane,

(v) energy spilling, and

(vi) loss of energy from lysed cells.

Hespell & Bryant (1979) concluded that the ATP expenditure for motility, extracellular polymer formation and transport would probably account for no more than 15 % either lowering in theoretical Y_{ATP} values or of the total ATP needed for cell formation. Theoretically, M_{ATP} has commonly been estimated from the method of Pirt (Pirt, 1965) using double reciprocal plot of yield versus dilution rate described by the equation:

$$1/Y = M_S/\mu + 1/Y_G \text{ ---------- (1)}$$

Where Y is the molar growth yield, M_S is the maintenance coefficient (moles substrates per g cells per h), μ is the dilution rate (the specific growth rate), Y_G is the maximum growth yield corrected for maintenance.

The underlying principle of the equation is that a double reciprocal plot of Y versus μ would have a straight line (slope) of M_S. Stouthamer (1979) and Hespell & Bryant (1979) argued that the double reciprocal plot could not give a straight line in a culture where ATP yield was variable. However, when a variation in ATP produced per mole of substrate fermented was taken into account, a double reciprocal plot of Y_{ATP} against μ for the micro-organisms produced a straight line. The following equation which was devised by de Vries et al. (1970) is now generally used in studies on microbial growth yields (see Stouthamer, 1979 ; Hespell & Bryant, 1979):

$$1/Y_{ATP} = M_{ATP}/\mu + 1/Y^{M}_{ATP} \text{ ----------(2)}$$

Where Y_{ATP} is the observed Y_{ATP} (g cells per mole ATP), M_{ATP} is the maintenance coefficient (mmoles ATP per g cells per h), μ is the dilution rate (the specific growth rate) and Y^{M}_{ATP} is the maximum Y_{ATP} attainable, corrected for maintenance.

The estimated M_{ATP} of some bacteria in continuous cultures varied from 0.72 to 6.10 mmoles ATP per g cells per h (see Russell, 1984). However, The M_{ATP} of mixed rumen bacteria from continuous cultures (Isaacson et al. 1975) and of mixed rumen microbes from continuously fed sheep (Kennedy & Milligan, 1978) was in a range of 1 to 2 mmoles ATP per g cells per h. The M_{ATP} of mixed rumen microbes is low when compared with non-rumen bacteria (see Hespell & Bryant, 1979). It is suggested that rumen microbes should have maximum Y_{ATP} value of about 26 which is almost 80 % of the maximum theoretical Y_{ATP}.

There are two major difficulties in the calculation and interpretation of the theoretical Y_{ATP} of micro-organisms as pointed out by Hobson & Wallace (1982b). These problems are associated with

(i) the calculation of the ATP-consuming processes of cellular formation by known biosynthetic pathways, and

(ii) the calculation of the amount of ATP generated per unit of substrate metabolised due to a variation in the nature of energy-yielding mechanisms of micro-organisms.

Determination of the actual M_{ATP} in the rumen is not possible. However, the implication from the second equation is that as the dilution rate (the specific growth rate) increases, relatively less energy may be used for M_{ATP} and more energy would be used for growth and consequently Y_{ATP} is increased (see Stouthamer & Bettenhaussen, 1973 ; Stouthamer, 1979 ; Hespell & Bryant, 1979). An increased Y_{ATP} availability will also be associated with decreased heat production in fermentation (see Leng, 1982b : Russell, 1986). An increase in utilisation of organic matter digested in the rumen for microbial cell synthesis relative to VFA, CO_2 and CH_4 production is associated with increasing Y_{ATP} (see Baldwin et al. 1970 : Leng, 1982b ; Nolan & Leng, 1989). Measurements of the dilution rate and heat production relative to cell yields and the

amount of cell yields in relation to the productions of VFA, CO_2 and CH_4 may provide an indirect measure of a relative change in M_{ATP} in the rumen.

2.4.2 Relationship between Y_{ATP} and the utilisation of substrate fermented in the rumen

Under anaerobic conditions in the rumen, complete degradation of feedstuffs is not possible. The lack of oxygen limits the availability of ATP and most of the energy of the substrate fermented is retained in the end-products which are VFA, CH_4, CO_2 and microbial cells (see Hungate, 1966). Intermediates from the fermentation are utilised as building blocks for synthesis of microbial cells and/or further degraded to give rise to ATP and individual VFA (see Baldwin, 1970). As the yield of microbial cells per unit of substrate fermented in the rumen (organic matter truly digested ; OMTFR) is increased (see Figure 2-1a), a greater proportion of the OMTFR is used as the monomers for synthesis of microbial cells, and relatively less is fermented to VFA, CH_4 and CO_2 (see Baldwin et al. 1970 ; Leng, 1982b ; Nolan & Leng, 1989). A relationship between the yield of microbial cells per unit of ATP (Y_{ATP}) and VFA, CH_4 and CO_2 production is shown in Figure 2-1b .

It is apparent that changes in molar proportions of acetic and propionic acids in the rumen are not consistently related to changes in microbial growth efficiency. Higher ratios of acetic:propionic acids associated with greater efficiency of microbial growth in the rumen have been reported (Harrison et al. 1975 ; 1976). Others have found the opposite relationship (Hume, 1970a ; Jackson et al. 1971 ; Kennedy & Milligan, 1978). Based on the known biochemical pathway and assuming that 4-6 ATP can be generated per mole glucose and 2 ATP are formed per mole acetic acid produced, 3 ATP per mole propionic acid and 2 ATP per mole butyric acid (see Czerkawski, 1986). Leng (1982b) concluded that changes in proportions of VFA to higher propionic acid produced in the rumen relative to acetic and/or butyric acids (beneficial to the animal) have only a minor effect on ATP generation and on the yield of microbial cells. The higher ATP produced by rumen microbes is offset by the increased requirement of monomers for synthesis of microbial cells to a large extent (see Leng, 1982b). However, the effect of producing higher propionic acid on Y_{ATP}

will be small relative to effects of availability of nutrients, maintenance ATP requirement including the growth rate and the turnover of microbial cells within the rumen (see later).

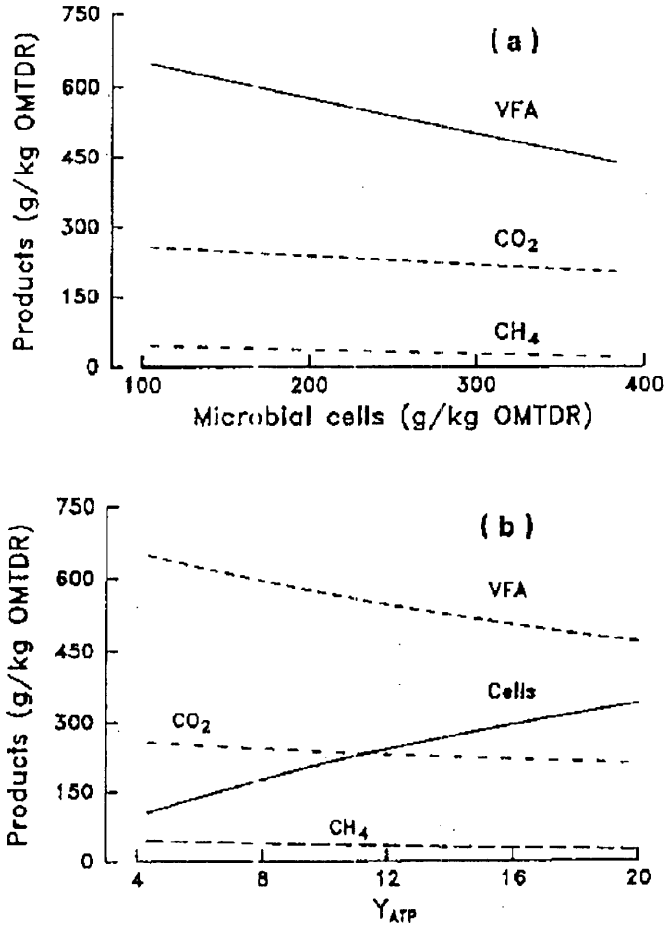

Figure 2-1. Relationship between (a) efficiency of cell synthesis and (b) Y_{ATP} and the fermented organic matter that is partitioned into VFAs and gases (CO_2 and CH_4) and that entering into microbial cells (Nolan & Leng, 1989).

2.4.3 Factors affecting the yield of microbial cells to the animal

The molar growth yield or Y_{ATP} can be calculated from VFA production or experimentally. From the known metabolic pathways, the moles of ATP produced in carbohydrate fermentation together with the known ATP required for synthesis of cells and detail of the macromolecular composition of cell to be form are necessary for calculation of the theoretical Y_{ATP}. Measuring microbial yields relative to the substrate used by rumen microbes from *in vitro* and *in vivo* studies is also important for calculation of Y_{ATP} experimentally (see Hobson & Wallace, 1982b). The theoretical and experimental (organisms growing anaerobically in various media) Y_{ATP} values range from 27 to 32 (g cells/mole ATP) and from 4.7 to 25.4 (g cells/mole ATP), respectively (see Stouthamer, 1979 ; Hespell & Bryant, 1979). These authors have detailed a numbers of factors that lead to the discrepancy such as changes in cell composition, availability of nutrients, growth rate of microbes, transport processes, dissipation of the energised membrane and energetic uncoupling processes. However, the actual Y_{ATP} of organisms in the rumen of cattle and sheep is always less than that measured in continuous cultures of mixed rumen microbes. It is evident that the major factors directly affecting the yield of microbial cells leaving the rumen are

(i) availability of nutrients for biosynthesis,

(ii) the dilution rate or the specific growth rate, and

(iii) the turnover of microbial cells within the rumen.

2.4.3.1 The availability of substrates for microbial growth

Microbial growth in the rumen requires the simultaneous provision of all necessary cell precursors including minerals, branched-chain carbon, growth factors, carbohydrates and nitrogen. Minerals, particularly sulphur (see Bray & Till, 1975) and phosphorus (see Durand & Kawashima, 1980) are also essential for microbial growth efficiency in the rumen (see Hungate 1966) and sulphur is vital for the growth of fungi in the rumen (Akin et al. 1983). Specific substrates, branched-chain carbon compounds and other growth factors, are required by particular groups of organisms in the rumen (see Allison, 1969 ; Bryant, 1973). Small increases in the concentrations of branched-

chain compounds for cellulolytic organisms in the rumen (Russell & Sniffen, 1984) and in nicotinic acid availability for amylolytic organisms (Riddell et al. 1980) can result in an increase in the microbial yields per unit of substrate fermented. Carbohydrate and nitrogen supplies are of paramount importance in providing the energy and nitrogen precursors for microbial growth in the rumen (see section 2.4.3.1.1 and 2.4.3.1.2). Increased efficiency of microbial growth in the rumen requires the continuous supply of all substrates and particularly, the type and rate of availability of dietary carbohydrate and nitrogen are the major factors.

2.4.3.1.1 Carbohydrate fermentation and the availability of ATP

Feed materials entering the rumen are initially colonised by rumen microbes and polysaccharides are hydrolysed and finally fermented to give rise to fermentation end-products. An outline of the pathways of ruminal degradation and fermentation of carbohydrates is shown in Figure 2-2 (see Leng, 1970).

Rates of digestion and fermentation of dietary carbohydrates are markedly different. Digestion (24 h) of starch and the fibrous carbohydrates (cellulose and hemicellulose) in the rumen has been reported ranging from 80 to 95 % and from 30 to 70 %, respectively (Hungate, 1966 ; Topps et al. 1968 ; Drennam et al. 1970 ; Smith et al. 1971 ; Thomson et al. 1972). Rates of digestion of grains vary from 0.6 to 12.1 % per h depending on the method of grain processing (see Nocek & Russell, 1988). Rates of digestion of cellulose and the cell wall component of a range of forages are between 3.2 and 27 % per h depending on the maturity of forages (Gill et al. 1969 ; Smith et al. 1971 ; Nocek & Russell, 1988). The rate of cell wall digestion is positively related to the soluble content of the forage (Hungate, 1966 ; Smith et al. 1971). In general, soluble sugars can be fermented within 1 h in the rumen, while starch and soluble polymers may require 6 h (see Hungate, 1966). The fermentation of the cell wall component can be depressed immediately after feeding and continue to rise gradually depending on rumen conditions (see Baldwin & Denham, 1979). The rate and extent of digestion and fermentation are all influenced by the rate and amount of ATP available for microbial growth in the rumen.

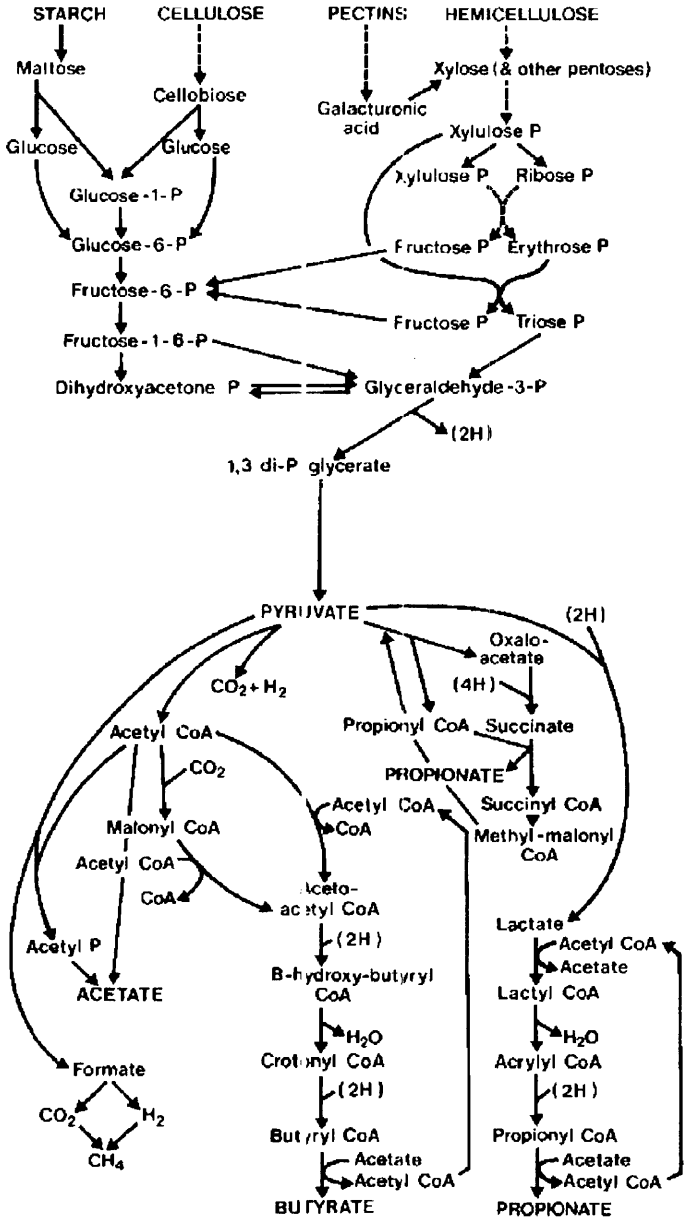

Figure 2-2. An outline of the pathways of fermentation in the rumen (Leng, 1970).

Microbial growth in the rumen depends primarily on the availability of ATP. The difference in microbial growth yields from different sources of fermentable substrates (media and/or diets) may be primarily affected by the difference in the expenditure of ATP for maintenance and growth. Stouthamer (1979) calculated the ATP requirement for the formation of microbial cells from a number of carbon sources

including glucose, pyruvate, lactate, malate, acetate and CO_2, and showed that the amount of cell material formed per mole ATP (Y_{ATP}) was 28.8, 13.5, 13.4, 15.4, 10.0 and 6.5, respectively (see Table 1). It was concluded that the difference in Y_{ATP} is due to the difference in ATP requirement for monomer formation and for transport process (see Table 1). With a continuous culture, Stern et al. (1978) found that Y_{ATP} was increased with increasing proportions of starch in the media. To further increase Y_{ATP}, gelatinisation of starch by cooking was suggested by Smith (1979). However, Offer et al. (1978) suggested that the ATP generated from a mixture of starch and cellulose was utilised more efficiently for microbial growth than the energy released from either starch or cellulose alone. Similarly, maximum microbial yield was observed when forage was offered at about 70 % of feed and the yield was reduced at lower proportions of forage (Chamberlain & Thomas, 1979 ; Mathers & Miller, 1981). The decline in the microbial yield may be due to the imbalance between the rate of ATP generation and the rate of ATP utilisation which results in uncoupled fermentation (see Sniffen & Robinson, 1987). To increase microbial growth yields, the rate of carbohydrate fermentation must be synchronised to the availability and therefore assimilation of ammonia and/or peptides and amino acids (Oldham et al. 1977 ; Smith, 1979 ; Baldwin & Denham, 1979 ; Stern & Hoover, 1979 ; Nocek & Russell, 1988).

2.4.3.1.2 The availability of nitrogenous substrates

A continuous supply in the rumen of nitrogenous substrates (ammonia, peptides and amino acids) is important for microbial growth (Bryant & Robinson, 1962). Virtanen (1966) showed that the production of microbial protein from the rumen of sheep given purified diets containing starch, sugar and cellulose as a source of carbohydrate and urea as a source of nitrogen was sufficient for dairy cows producing 10-12 kg of milk daily. Microbial protein synthesis in the rumen of sheep fed protein-free diets containing oat hulls, starch and sucrose with casein as the nitrogen source was greater than that with urea (Hume, 1970b). A similar result was also observed in sheep given basal diets containing maize starch, molasses, molassed sugar beet pulp and barley (Chen et al. 1992b).

Table 2.1 ATP requirement for the formation of cell material in mineral salts containing various carbon sources[a]

Synthesis of	Cell[b] composition	ATP requirement (10^4 x mole/g cells) for growth on					
		Glucose	Pyruvate	Lactate	Malate	Acetic	CO_2
Polysaccharide	16.6	20.6	71.8	71.0	51.0	92.0	195.0
Protein	52.4	204.9	339.4	339.0	285.0	427.0	907.0
Lipid	9.4	1.4	27.0	85.0	25.0	50.0	172.0
RNA	15.7	43.7	71.2	85.0	70.0	101.0	212.0[d]
DNA	3.2	10.5	15.9	16.0	13.0	19.0	
Transport		52.0	200.0	200.0	200.0	30.6	52.0
Total		333.1	725.3	738.0	644.0	995.0	1538.0
Y_{ATP}[c]		28.8	13.5	13.4	15.4	10.0	6.5

[a] The data are modified from Stouthamer (1979).
[b] The cell composition based on *Escherichia coli* (g/100 g cells).
[c] Gram cells/mole ATP.
[d] The value for RNA plus DNA.

A number of *in vitro* studies have shown that there is a considerable increase in microbial yields in cultures containing urea with small amounts of preformed protein relative to urea alone (Maeng et al. 1976 ; Maeng & Baldwin, 1976a,b ; Cotta & Russell, 1982 ; Russell, 1983 ; Russell & Sniffen, 1984 ; Argyle & Baldwin, 1989 ; May et al. 1993). The increased microbial yield is not due to the relative supply of amino acids or ammonia because the energy cost for the formation of amino acids from ammonia is relatively small (see Stouthamer, 1979 ; Hespell & Bryant, 1979). Decreased energetic uncoupling due to a faster adaptation for growth during transitional growth periods (from the rumen to the flask with preformed protein relative to urea) may be the explanation (see Stouthamer & Bettenhaussen, 1973). However, ammonia is a primary source of nitrogen for microbial protein synthesis in the rumen (Bryant & Robinson, 1962) and therefore *de novo* synthesis of amino acids is an important activity of rumen microbes (see Allison, 1969).

The *de novo* biosynthesis of amino acids of rumen microbes is vital, particularly when ammonia is a major source of nitrogen. Knowledge of the pathways of ammonia assimilation is necessary to determine microbial protein synthesis in the rumen and therefore growth efficiency. A number of enzymes of ammonia assimilation exhibited by rumen microbes have been reported (see Hespell, 1984). Glutamine synthetase and glutamate dehydrogenase are the major enzymes of ammonia assimilation as shown in Figure 2-3.

Glutamine synthetase has a high affinity for ammonia (Km) of 0.2 mM (Baldwin & Denham, 1979). The glutamine synthetase reaction occurs via an acyl phosphate intermediate which requires an ATP to phosphorylate the δ-carbon of glutamate and consequently nitrogen is bound to give glutamine (see Mathews & van Holde, 1990). The amide group of glutamine can be either catalysed by glutamate synthase to form glutamate or utilised in the biosynthesis of some amino acids, purines, pyrimidines and other compounds (see Figure 2-3). This is consistent with the results of Salter et al. (1979) showing that the ^{15}N was incorporated into bacterial amides prior to being transferred to glutamate and other amino acids. The activity of glutamine synthetase is known to increase when concentration of ruminal fluid ammonia is reduced (Smith et al. 1980 ; Hespell, 1984 ; Patterson & Hespell, 1985). However, this activity is subject to control by changing rumen conditions particularly when concentrations of ammonia, glutamate (Smith et al. 1980), alanine and glycine (Patterson & Hespell, 1985) in ruminal fluid are increased.

Glutamate dehydrogenase has a low affinity for ammonia which has been reported ranging from 1 to 23 mM depending on species of rumen microbes (Wallace, 1979 ; Baldwin & Denham, 1979 ; Smith et al. 1980 ; Hespell, 1984 ; Duncan et al. 1992). A reaction of ammonia assimilation catalysed by glutamate dehydrogenase to form glutamate does not require an ATP (see Figure 2-3). The relative activity of glutamate dehydrogenase in a certain species did not vary noticeably with changing concentrations of ruminal fluid ammonia (Wallace, 1979 ; Smith et al. 1980 ; 1981) but it was apparently reduced when amino acids were the source of nitrogen for growth (Smith et al. 1981).

Maximum microbial protein synthesis has been reported to occur over a wide range of ruminal fluid ammonia concentrations from 50 to 238 mgN/l (Hume et al. 1970 ; Miller, 1973 ; Satter & Slyter, 1974 ; Stern & Hoover, 1979 ; Balcells et al. 1993). This possibly suggests that the composition of the experimental diets and microbial populations in the rumen influence the optimum requirement of ammonia. Under a low ammonia concentration, ammonium is actively transported across the microbial membrane (Russell & Strobel, 1987) and may be mainly assimilated via glutamine synthetase relative to via glutamate dehydrogenase as mentioned earlier. This may result in an increased energetic cost and consequently less efficient microbial protein synthesis. On the other hand, at a high ammonia concentration, increasing ammonia assimilation via glutamate dehydrogenase is likely to benefit the energetic expenditure and consequently increase efficiency of microbial protein synthesis in the rumen.

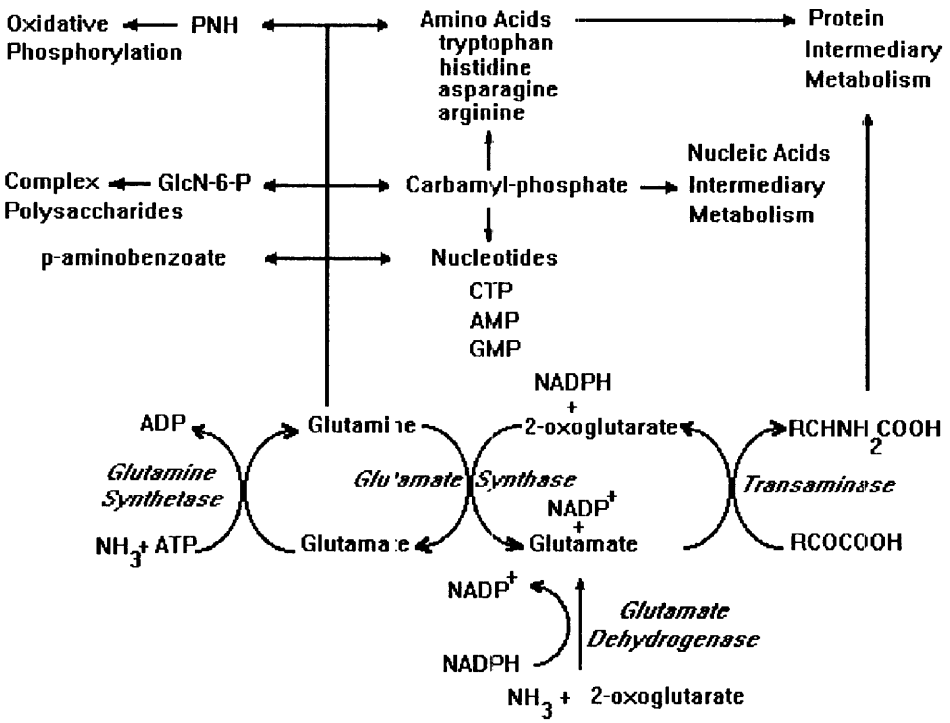

Figure 2-3. Pathways of ammonia assimilation in the rumen bacteria for the production of glutamine and glutamate and some of the roles of these compounds in intermediary metabolism (Tyler, 1978).

2.4.3.1.3 The availability of minerals

Microbial growth is influenced by the availability of minerals (both major and minor minerals) in the rumen. Feedstuffs ingested by ruminants are the major source of several minerals such as calcium, potassium, magnesium, manganese and zinc in ruminal fluid for microbial growth in the rumen whereas saliva provides the bulk of sodium and phosphorus (Joblin & Lee, 1990). The roles of both major and minor minerals in the nutrition of rumen micro-organisms and therefore rumen function have been reviewed by Durand & Kawashima (1980) and Mackie & Therion (1984). The requirements of sulphur, phosphorus and magnesium in relation to the availability of carbohydrates have been reviewed by Durand & Komisarczuk (1988). The effects of the availability of sulphur and phosphorus as related to the availability of nitrogenous substrates on microbial growth efficiency in the rumen is discussed below.

Sulphide in ruminal fluid is an important intermediate required for the *de novo* synthesis of sulphur amino acids of rumen bacteria (Moir, 1970 ; Bray & Till, 1975 ; McMeniman et al. 1976) and fungi (Orpin & Greenwood, 1986 ; Gulati et al. 1989). Relatively less bacterial cysteine was synthesised from the sulphide pool when diets contained higher proportions of the dietary sulphur peptides and amino acids (McMeniman et al. 1976) since the sulphur amino acid in the rumen was incorporated directly into sulphur amino acids of bacteria (Nader & Walker, 1970) and fungi (Gulati et al. 1989). This suggests that dietary sulphur can be a growth-limiting nutrient for rumen microbes in ruminants fed on low protein diets and supplemented with NPN. The precise level at which sulphide concentration in ruminal fluid limits microbial growth has not been established possibly due to the rapid turnover of the sulphide in the rumen (100-600 times/d in sheep) as reported by Hegarty (1989), it has been suggested to be around 1 μgS/ml ruminal fluid (see Bray & Till, 1975). Elliott & Armstrong (1982) and Kandylis & Bray (1987) reported that the efficiency of microbial cell synthesis in the rumen increased by between 30 and 47% with increasing total sulphur intake from 0.3 to 3.21 g/d and urea intake from 20 to 35 g urea/d in sheep. This suggests that the requirements for sulphide in ruminal fluid for optimum microbial growth increase with increasing concentrations of ruminal fluid ammonia

Phosphorus is a constituent of nucleic acids and coenzymes such as flavin and pyridoxal phosphate and is essential for carbohydrate fermentation (see Durand & Kawashima, 1980). Approximately 1.1, 8.6, 18.4 and 71.9% of the total phosphorus in rumen bacteria is contained in inorganic phosphorus, DNA, phospholipid and RNA, respectively (Van Nevel & Demeyer, 1977). This indicates that the available phosphorus in the rumen is mostly required for synthesis of RNA by rumen microbes. The level at which concentration of the soluble phosphorus in ruminal fluid limits microbial growth in the rumen has been suggested to be between 75 and 100 mgP/l (Durand & Kawashima, 1980 ; Komisarczuk et al. 1987b). With a roughage diet but not with a concentrate or mixed diet, the concentration of the soluble phosphorus in ruminal fluid below 50 mgP/l (Bennink et al. 1978) may limit microbial growth in the rumen. An insufficient supply of phosphorus in the rumen may decrease net microbial cell synthesis by between 37 and 177% (Breves et al. 1985 ; Komisarczuk et al. 1987b ; Petri et al. 1988 ; Gunn & Terrouth, 1994a,b) depending on the extent of the depletion and the duration (see Durand & Kawashima, 1980 ; Durand & Komisarczuk, 1988). Reduced assimilation of ammonia into both fluid- and particle-associated bacteria have also been observed when a phosphorus deficiency occurs (Komisarczuk et al, 1987a). Gunn & Ternouth (1994a) reported that there was a 47% increase in net microbial cell synthesis in the rumen, when the amount of dietary phosphorus was increased from 0.23 to 2.3 gP/kgDM with the addition of monosodium phosphate to sheep given a barley straw based diet containing 20gN/kgDM (35% of N derived from urea). This indicates that increasing the availability of phosphorus in the rumen will increase the utilisation of NPN by rumen microbes and therefore increase microbial growth.

2.4.3.2 The dilution rate or the specific growth rate

From batch cultures (see Stouthamer & Bettenhaussen, 1973) and continuous cultures (Isaacson et al. 1975), an increasing dilution rate of media or the specific growth rate of microbes induces an increase in Y_{ATP} (i.e. ATP is used more efficiently in growth). This has been used to investigate in the rumen conditions. Some authors have found a positive effect from an increase in the dilution rate on microbial growth efficiency (Potter et al. 1972 ; Harrison et al. 1975, 1976 ; Kennedy & Milligan, 1978 ;

Bergen et al. 1980 ; Harrison & McAllan, 1980 ; McAllan & Smith, 1983 ; Zinn & Owens, 1983 ; Robinson et al. 1985) Others have not found a relationship between the dilution rate and microbial growth efficiency (Walker et al. 1975 ; Leng, 1982b ; Firkins et al. 1987b). The difference can be explained by several factors including the nature of the diets, animal factors (see Owens et al. 1984), pH in ruminal fluid (see Russell, 1984) and the turnover of microbial cells within the rumen (see Leng, 1982b). Therefore, the effect of the dilution rate on microbial growth efficiency in the rumen depends on rumen conditions.

Recently, in sheep given different levels of feed but with a similar intake of digestible energy and crude protein from a pellet containing different ratios (10:10, 30:70 and 50:50) of barley straw and barley, Dlouvinov & Todorov (1994) found that there was no effect of an increasing dilution rate by increasing ratios of barley straw in the pellets on microbial growth efficiency in the rumen. Despite that their results were not confounded with the level of energy and nitrogen intake, lower pH and therefore protozoal numbers in sheep on the diet with 10 % straw could influence the results. In a subsequent study by replacing barley straw with polyvinylchloride (PVC) and dehydrated alfalfa, they found that there was a substantial increase in microbial growth efficiency with an increasing dilution rate by increasing PVC in the pellets. A close inspection of their results showed that protozoa disappeared almost completely in all dilution rates while pH was similar. These results suggest that recycling of microbial cells within the rumen due to the predatory activity of protozoa (see Coleman, 1975) and/or the sequestration (Abe et al. 1981) and lysis of protozoa in the rumen (Weller & Pilgrim, 1974 ; Leng, 1982a) may be an important component of energetic cost for M_{ATP} in the rumen conditions.

2.4.3.3 Turnover of microbial cells within the rumen

There is evidence that the contents of microbial cells that die in the rumen are substantially degraded in the rumen (Nolan & Leng, 1972 ; Coleman, 1975 ; Cottle et al. 1978 ; Nolan & Stachiw, 1979 ; Demeyer & Van Nevel, 1979b ; Leng & Nolan, 1984 ; Wallace & McPherson, 1987 ; Newbold & Hillman, 1990 ; Firkins et al. 1992). Studies with the tracer ^{15}N have shown that 30-75 % of microbial protein synthesis is

degraded and recycled within the rumen (Nolan & Leng, 1972 ; Cottle et al. 1978 ; Nolan & Stachiw, 1979 ; Firkins et al. 1992). The degraded microbial protein is further fermented to VFA, CO_2, CH_4 and NH_3 and only a small amount of ATP is generated so that microbial cells resynthesised is only 10% of the microbial cells lysed (see Leng, 1982b). This is possibly a major futile cycle that leads to a considerable reduction in Y_{ATP} available to the host animals. Knowledge of manipulation of the rumen to increase microbial protein that enters the intestines and is available for absorption to ruminants is needed to understand the factors that cause the turnover of microbial cells within the rumen.

In general, there are two major phenomena of microbial protein turnover in the rumen including turnover of endogenous protein without lysis (Hobson & Wallace, 1982a ; Wallace & McPherson, 1987) and degradation of microbial cells resulting from several factors. Bacteriophages (Adam et al. 1966 ; Hoogenraad et al. 1970) and mycoplasmas (Robinson & Hungate 1973) infect and break bacterial cells leading to bacterial cell lysis. Protozoa ingest and digest fungi and bacteria as a source of nutrient resulting in a substantial recycling of fungal and bacterial cells within the rumen (Jarvis, 1968 ; Lindsay & Hogan, 1972 ; Coleman, 1975 ; Demeyer & Van Nevel, 1979b ; Wallace & McPherson, 1987 ; Newbold & Hillman, 1990). Autolysis due to the aging of protozoa (Weller & Pilgrim, 1974 ; Leng, 1982a) and depletion of substrates in the rumen (Hespell, 1979) has been reported. Microbial cell lysis caused by undefined lytic factors (Jarvis, 1968) unrelated to the proteolytic activity in ruminal fluid (Wallace & McPherson, 1987). Some protozoa and bacteria in the rumen are killed by traces of O_2 in digesta (Hungate, 1966). The most important cause of the turnover of bacterial cells in the rumen may be the predation by protozoa with all other factors being of minor importance (Wallace & McPherson, 1987).

Quantification of organic matter digested and net microbial cell synthesis in the rumen are essential to differentiate the overall effects of dietary treatments on microbial growth efficiency.

Digestibility of a diet in the rumen may be assessed by *in vivo*, *in sacco* or *in vitro* techniques. However, quantification of microbial cell synthesis in the rumen or

outflow of microbial cells into the lower digestive tract by using markers and cannulated animals at the intestine is time consuming and often of low precision. A number of microbial markers have been studied by Ling & Buttery (1978) to differentiate microbial protein which enters the intestinal digesta of sheep. The ratio of RNA to total N in the digesta appears to be an appropriate marker of microbial-N in digesta-N entering the duodenum. However, the techniques involved are time consuming and animals need to be surgically modified. Alternatively, there appears to be consensus now that urinary excretion of purine derivatives is a good indicator of the quantities of microbial biomass that leave the rumen and are digested in the intestines of ruminants (Topps & Elliott, 1965 ; Antoniewicz et al. 1980 ; Fujihara et al. 1987 ; Lindberg, 1989 ; Verbic et al. 1990 Chen et al. 1990b ; Balcells et al. 1991 ; Puchala & Kulasek, 1992).

2.5 Purine metabolisms in ruminants

Hypoxanthine, xanthine, uric acid and allantoin excreted in urine are metabolic derivatives of both endogenous and exogenous purines (adenine and guanine) that are degraded in the body. There is evidence that purine derivatives excreted in urine originate mostly from microbial nucleic acids which are absorbed in the intestines and further metabolised to their derivatives. The quantities of uric acid and allantoin excreted in the urine have been found to be highly related to the pool of microbial nucleic acids in the rumen (Topps & Elliott, 1965) and to those in the small intestine as well as to the amounts of the purine derivatives in the blood (McAllan, 1980). Thus the utilisation of urinary excretion of purine derivatives as an indicator of net microbial cell synthesis in the rumen requires an understanding of purine metabolisms in ruminants as follows

(i) nucleic acid metabolisms of micro-organisms in the rumen,

(ii) digestion and absorption of nucleic acids entering the intestines, and

(iii) metabolisms of absorbed and endogenous purines involving *de novo* synthesis of nucleic acids in the ruminants host.

These are reviewed below.

2.5.1 Purine metabolism in the rumen

Urinary excretion of purine derivatives increases linearly with incremental duodenal input of microbial nucleic acids (Fujihara et al. 1987 ; Chen et al. 1990b), of microbial RNA (Antoniewicz et al. 1980 ; Balcells et al. 1991). The amounts of nucleic acids in the duodenum digesta are similar to those in the rumen (Smith & McAllan, 1971) but different to those added to the feed (Razzaque & Topps, 1972). Ratios of RNA:DNA of rumen bacteria are not different from those of ruminal fluid but markedly different to those of the natural diets (Smith & McAllan, 1970).

Nucleic acids of the diets (McAllan & Smith, 1973) and added nucleic acids (Smith & McAllan, 1970) in the rumen are rapidly degraded into nucleotides, nucleosides and free bases within the rumen (McAllan & Smith, 1973). These substrates can be utilised as a source of carbon or nitrogen for microbial synthesis in the rumen (Belasco, 1954 ; Jurtshuk et al. 1958) and they may be incorporated as a precursor of nucleic acids by rumen microbes (Smith & Mathur, 1973).

With respect to the former, hypoxanthine and xanthine but not adenine and guanine can be transiently accumulated in the rumen digesta (McAllan & Smith, 1973). Hypoxanthine is degraded partly in the rumen but guanine, xanthine and uric acid (Jurtshuk et al. 1958) as well as allantoin (Belasco, 1954) are degraded completely. In the rumen, acetic acid, CO_2 and ammonia are the major end-products of the degradation of purine bases that undergo fermentation by rumen microbes (Jurtshuk et al. 1958).

In regard to the latter, protozoa in the rumen can incorporate nucleotides, nucleosides and free bases (except thymine) into their nucleic acids (Coleman, 1979a) possibly via salvage pathways (Jaffe & Gutteridge, 1974). Unlike bacteria in the rumen, the majority of bacterial nucleic acids can be synthesised via the *de novo* pathway (see McAllan, 1982). However, rumen bacteria are freely permeable to adenine and uracil and these can be incorporated into bacterial nucleic acids (Smith & Mathur, 1973). At present, there appears to be no information available on metabolisms of nucleic acids of fungi in the rumen. The only indirect evidence is that

Neocallimastix frontalis zoospores do not show chemotaxis to purines and pyrimidines (Orpin & Bountiff, 1978) and the growth of *Neocallimastix patriciarum* is not affected by the absence of the nucleotide bases, adenine and guanine in the media (Orpin & Greenwood, 1986).

The nucleic acid content of bacteria but not that of protozoa in the rumen (Arambel et al. 1982) can vary widely due to different species and types of bacteria, energy sources and the time post-feeding. The nucleic acid-N:total-N of gram-positive bacteria in the rumen appears to be slightly lower than that of gram-negative bacteria (Arambel et al, 1982). The ratios of nucleic acids:total-N (Craig et al. 1987), RNA:total-N (Merry & McAllan, 1983 ; Bates et al. 1985) and purine:total-N (Klusmeyer et al. 1991) are all significantly higher in fluid-associated than in particle-associated organisms. The RNA:total-N ratio of the fluid bacteria but not of the particle-associated bacteria is significantly higher in the rumen of sheep fed a high-concentrate diet than in those given a low-concentrate diet or a roughage-based diet (see Bates et al. 1985 ; Susmel et al. 1993). The ratio of nucleic acids:total-N decreased markedly within 1 h after feeding and gradually increased over 12 h after feeding (Craig et al. 1987). From the study of Craig and colleague, the feed was offered to the animal only 1 h and the remaining feed was removed. However, when the feed was offered ad-libitum, the ratios of RNA:DNA (John, 1984 : Baker & Car, 1992) and of RNA:total-N (Susmel et al. 1993) of the fluid bacteria increased sharply after feeding and reached a maximum from 2 to 4 h post-feeding and declined slowly thereafter.

2.5.2 Intestinal digestion and absorption of microbial nucleic acids

The majority of purine nucleosides and free bases presented in the small intestine of ruminants (McAllan, 1980) are microbial in origin as mentioned earlier and a small proportion of those (relatively less than 15 %) can be contributed from the endogenous purines (see McAllan, 1982) secreted by the small intestine (Berlin & Hawkins, 1968). Microbial nucleic acids entering the small intestine of ruminants are hydrolysed by a series of nuclease enzymes to nucleotides, nucleosides and free bases

(see Barnard, 1969 ; Armstrong & Hutton, 1975 ; McAllan, 1982). The digestibility between the proximal duodenum and distal iliem of sheep and cattle appeared to be higher for microbial RNA than DNA ranging from 87 to 97 % and 75 to 85 %, respectively (see Smith & McAllan, 1971 ; McAllan, 1982 ; Storm et al. 1983). When corrected for the endogenous purine, the true digestibility of microbial nucleic acids was 91 % (Chen et al. 1990b). The digested nucleosides and free bases (except hypoxanthine) are almost entirely absorbed before reaching the terminal iliem of ruminants (McAllan, 1980, 1982).

2.5.3 Metabolism of the absorbed purines in the ruminant host

Purine derivatives but not nucleosides (guanosine and adenosine) and purines (guanine and adenine) are detected in the plasma samples of sheep and steers (Balcells et al. 1992b). The concentrations of purine derivatives do not differ between the portal and jugular blood (see Chen et al. 1990b) and between the portal and peripheral blood (Balcells et al. 1992b). This may indicate that the absorbed nucleosides and bases are metabolised during passage across the intestinal wall.

The absorbed nucleosides are split by nucleoside phosphorylases to deliver their free bases. Guanine is then deaminated by guanase to xanthine (see Zöllner, 1982). Unlike guanine, there appears to be no adenase activity in the small intestine (Berlin & Hawkins, 1968). Adenosine is deaminated by adenosine deaminase to form inosine (see Berlin & Hawkins, 1968) where it is catabolyzed by nucleoside phosphorylase to hypoxanthine (Zöllner, 1982). Hypoxanthine and xanthine can be further oxidised or salvaged depending upon the existence of xanthine oxidase in tissues particularly in the mucosal epithelium of the small intestine as well as in the blood.

In sheep hypoxanthine, xanthine, uric acid and allantoin are consistently detected in the plasma and the concentrations of hypoxanthine and xanthine but not uric acid and allantoin are significantly higher in the portal than peripheral blood (Balcells et al. 1992b). In steers, uric acid and allantoin but not hypoxanthine and

xanthine are consistently found in the plasma and the concentration of uric acid is higher in the portal than peripheral blood (Balcells et al. 1992b). The differences in concentrations of purine derivatives between the portal and peripheral blood as well as between sheep and cattle can be explained by the differences of the activities of xanthine oxidase in different organs and species. The activities of xanthine oxidase in cattle are abundant in the tissues of all 20 organs tested (except the pancreas) including blood and serum whereas those in sheep are rich only in the liver (Al-Khalidi & Chaglassian, 1965). Therefore, in sheep, hypoxanthine and xanthine are available to be reutilised or salvaged by other organs before reaching the liver (Chen et al. 1990b) but, in cattle, the purine derivatives might be salvaged only in tissues of the small intestine (Verbic et al. 1990 ; Balcells et al. 1991).

Tissue nucleic acids in ruminants may originate from the *de novo* synthesis of purines and from absorbed purines that have been salvaged. There is evidence that the absorbed purines are used by various organs in ruminants. Microbial adenine-8-^{14}C (Condon et al. 1970 ; Smith et al. 1974 ; Razzaque et al. 1981) and RNA-^{14}C-UL (Condon et al. 1970) absorbed from the small intestine of lambs were substantially incorporated into the body tissues of lambs. On the other hand, glycine-^{14}C-UL which is a precursor for the *de novo* synthesis of nucleotides is not significantly incorporated into the tissues of lambs (Condon et al. 1970). Condon et al. (1970) concluded that salvage of the absorbed purines is preferable to *de novo* synthesis of purines for the animal to replace nucleic acids losses from tissue turnover. An incremental increase in the amounts of purines absorbed in the small intestine appeared to be associated with a gradual increase in the salvage of the absorbed purines but with a progressive decrease in the *de novo* synthesis of purines in sheep (Chen et al. 1990b). This may be a benefit in reduced amounts of ATP that are required for nucleotides formation. In general, the energetic cost of mononucleotide formation via the salvage pathway is 3.5-4 times less than that via the *de novo* pathway (see Mathews & Van Holde, 1990). Pathways of purine metabolisms in the body involving enzymes of purine biosynthesis and salvage are well described by Zöllner (1982) and Murray (1971) and are summarised in Figure 2-4.

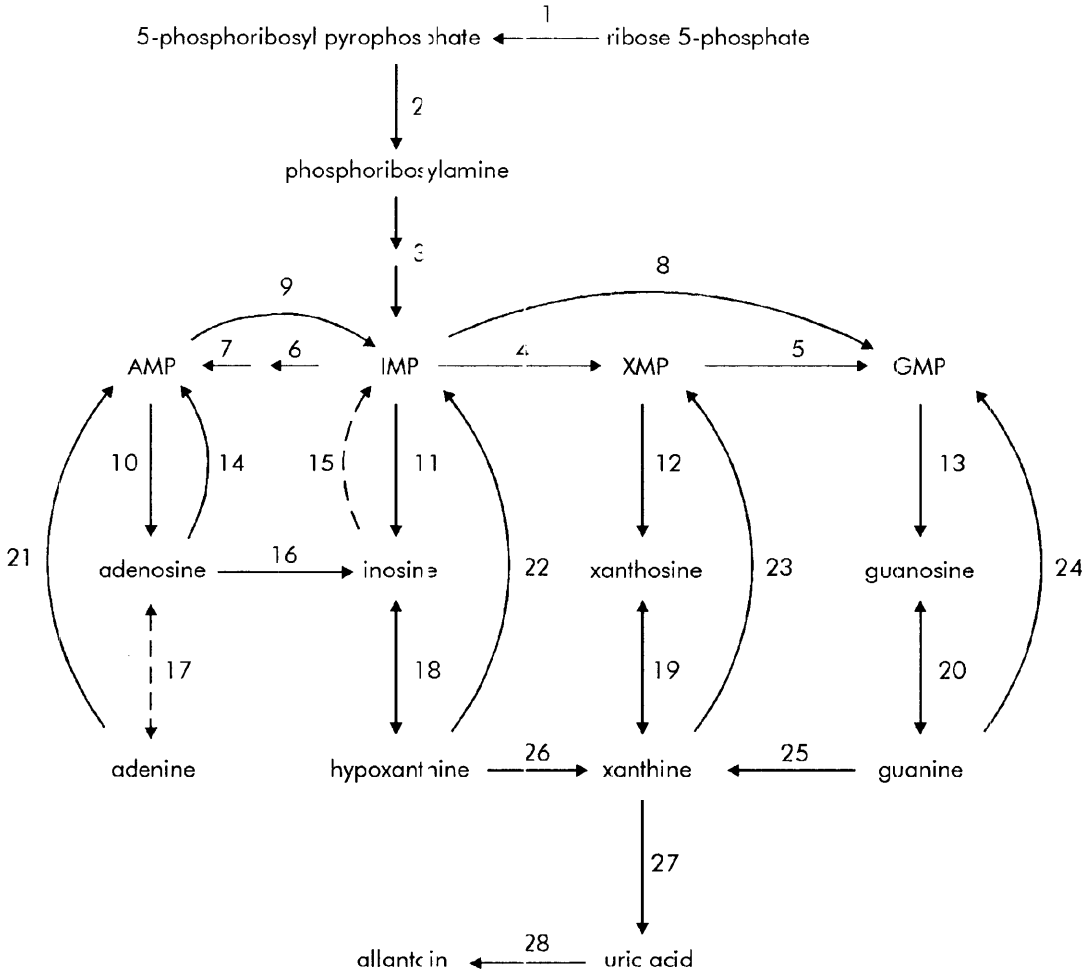

Figure 2-4. Pathways of purine biosynthesis and degradation in ruminant tissues.

Reactions indicated by dashed lines appear to be low activities in animal tissues. The numbers refer to the following enzymes or sequences of enzymes. 1: PRPP synthetase; 2: PRPP amidotransferase; 3: the enzymes of *de novo* purine biosynthesis; 4: IMP dehydrogenase; 5: XMP aminase; 6: adenylosuccinate synthetase; 7: adenylosuccinate lyase; 8: GMP reductase; 9: AMP deaminase; 10, 11, 12, 13: 5'-nucleotidase; 14: adenosine kinase; 15: inosine kinase; 16: adenosine deaminase; 17, 18, 19, 20: purine nucleoside phosphorylase; 21 adenine phosphoribosyltransferase; 22, 23, 24: hypoxanthine/guanine phosphoribosyltransferase; 25: guanine deaminase; 26, 27: xanthine oxidase; 28: uricase (Zöllner, 1982 and Murray, 1971).

2.5.4 The relationship between absorbed and excreted purines

Purine derivatives in the plasma of ruminants can be derived from the turnover of tissue nucleic acids (endogenous purine derivatives) and from absorbed purines which have not been incorporated into tissues (exogenous purine derivatives). Although the plasma purine derivatives can be excreted via the renal route and secreted via non-renal routes i.e. via the salivary (Chen et al. 1989) and gut secretion (Berlin & Hawkins, 1968), the concentrations of purine derivatives in the plasma are highly correlated with those in the urine (McAllan, 1980). Therefore purine derivatives excreted in urine arise from both the endogenous and exogenous purine derivatives.

The amount of endogenous purine derivatives excreted in urine is 3 times higher in cattle than in sheep. In sheep it ranges from 454 to 514 μmol/ kg $BW^{0.75}$ per day (Fujihara et al. 1987 ; Chen et al. 1990a) and in cattle from 164 to 189 μmol/kg $BW^{0.75}$ per day (Giesecke et al. 1984 ; Fujihara et al. 1987 ; Chen et al. 1990a ; Balcells et al. 1991). Similar levels are expected in sheep and goats (Lindberg, 1989). However, as the absorbed purines increase with increasing supplies of microbial nucleic acids, the contribution of endogenous purine derivatives to total purine derivatives that are excreted in urine becomes relatively small (Antoniewicz et al. 1980 ; Giesecke et al. 1984 ; Fujihara et al. 1987 ; Lindberg, 1989 ; Chen et al. 1990a, b ; Balcells et al. 1991).

A linear model has been used by Antoniewicz et al. (1980), Giesecke et al. (1984) and Fujihara et al. (1987) to describes the relationship between absorbed and excreted purines. However, Chen et al. (1990b) and Balcells et al. (1991) have shown that a curvilinear model provides a better explanation. An increase in urinary excretion of purine derivatives (Y; mmol/d) with increasing amounts of absorbed purines (X; mmol/d) is described by Chen & Gomes (1992) using the following equations:

$$Y = bX + (cBW^{0.75} e^{-kX}) \quad \text{---------for sheep}$$

$$Y = bX + (c\,BW^{0.75}) \quad \text{---------for cattle}$$

The slope b of 0.84 and 0.85 for sheep and cattle, respectively, represents the recovery of the absorbed purines that are excreted in urine. This implies values of 0.16

and 0.15 in sheep and cattle respect vely, for the amount of absorbed purines lost via non-renal routes.

The part of the equations wi hin parenthesis represents the contribution of the endogenous purine derivatives excr ted in the urine. Where c (0.150 for sheep and 0.385 for cattle) is the endogenou s purine derivatives excreted in urine when the absorbed purines are zero. $BW^{0.75}$ is the metabolic body weight (kg) of the animal and k is the rate-constant of -0.25 for th e replacement of *de novo* synthesis of purines by salvage of the absorbed purines.

2.6 Conclusion

Protein nutrition of ruminan ts on low nitrogen forage-based diets is largely dependent on the availability of microbial protein from the rumen. Increasing availability of microbial protein by in creasing efficiency of net microbial cell synthesis is beneficial not only in reducing amounts of supplementation of an expensive by-pass protein but also in increasing the effi iency of overall plane metabolisms of the nutrient absorbed. An increase in the efficien cy of net microbial cell synthesis in the rumen is generally associated with a decrease in heat production (see Leng, 1982b ; Russell, 1986) as well as VFA, CO_2 and CH_4 production (see Baldwin et al. 1970 ; Leng, 1982b). Therefore, the efficiency of utilisation of the nutrient absorbed by ruminants on low nitrogen forage-based diets is primarily determined by the efficiency of net microbial cell synthesis in the rumen.

The dynamics of the rumen ecosystem including microbial populations and their interactions and substrates required for their growth need to be better understood to enable manipulation of the rumen to increase the efficiency of net microbial cell synthesis.

Microbial populations in th e rumen will change primarily with changing feedstuffs that are ingested by ruminants while the amounts of nutrients that can be extracted from the feedstuffs will depend largely on the interactions of rumen microbes. Bacterial and fungal carboxymethylcellulase that are associated with the

feedstuff appear to be reduced when there is the presence of protozoa in the milieu (see section 2.3.3 and 2.3.4), whereas rumen digestibility of the feedstuff in the presence of protozoa is generally higher when compared with the absence of protozoa (see Veira, 1986). However, protozoa population appears to be the major factor causing a reduction in the efficiency of net microbial cell synthesis in the rumen (see section 2.4.3.3).

A supply in the rumen of nutrients that are deficient in feedstuffs ingested by ruminants holds a key to improvement in microbial growth efficiency. A concentration of ruminal fluid ammonia below a critical minimum is a major constraint to the growth of bacteria (Bryant & Robinson, 1962). Preformed protein (amino acids and peptides) appears to be required to a certain extent for the growth of rumen microbes (see section 2.4.3.1.2). However, the requirement of ammonia concentration and preformed protein for optimum microbial growth in the rumen reported in the literature is variable. The roles of these nitrogenous substrates in the microbial milieu appear to be lacking and still cause considerable controversy.

The experiments reported in this thesis were aimed at increasing knowledge on the effect of increasing the availability of ammonia and preformed protein (peptides and amino acids) on changes that occur in the microbial milieu, rumen fermentation, digestibility of a forage and efficiency of net microbial cell synthesis as indicated by urinary excretion of purine derivatives.

Chapter 3

General materials and methods.

3.1 Introduction

The materials and methods generally used throughout the research reported in this thesis are described in this chapter. The experiments that require the use of additional or specialised techniques are presented in the relevant chapters.

3.2 Experimental procedures

3.2.1 General animal management

All sheep were surgically fistulated in the rumen and held in metabolism crates in a room controlled at 20^0. The room was well ventilated and continuously illuminated. The sheep had access to clean water.

3.2.2 Diets

3.2.2.1 Preparation of the basal diet

Urea solutions were daily prepared by dissolving the amounts of urea used in 40 ml of tap water and each solution was sprayed onto the daily ration of oaten chaff. After spraying, the diets were left for about 20 min and thoroughly mixed. The feed was then offered to the sheep.

3.2.2.2 Mineral mix

The mineral mix consisted of 1 part of Pfizer vitamin-minerals (Pfizer Agricare, NSW), 1 part of Na_2SO_4, 1 part of NaCl and 2 parts of Ca_2HPO_4. This mineral mix (1 or 2 % of feed intake) was added to the daily ration of oaten chaff before being sprayed with the urea solution.

3.2.3 Feeding

The ration was delivered to each sheep in 24 equal portions at hourly intervals by an overhead automatic-feeding machine.

3.2.4 Ruminal fluid sampling

Ruminal fluid samples were collected from sheep through a probe placed in a caudal position in the ventral sac of the rumen. The probe consisted of a thin stainless steel pipe with a small metal cage at one end which was covered with a double layer of nylon stocking material. A well-mixed portion of each sample of ruminal fluid was removed from the probe using a 50 ml syringe.

Prior to acidification, a ruminal fluid subsample was extracted from each sample of ruminal fluid to be used for pH measurement and enumeration of protozoa. The remaining sample was acidified with 5 drops of concentrated H_2SO_4 and stored at -20^0. Prior to analysis of NH_3-N, VFA and Cr, the sample was thawed and centrifuged at 3000 g at 150 for 10 min. The supernatant was kept for analysis.

3.2.5 Ruminal digesta sampling

A sample of rumen digesta was collected via the rumen fistula with a core sampling probe of 3 cm diameter (Figure 3-1). The sample was processed to isolate protozoa in the ruminal fluid, bacteria in the fluid phase and particle-associated microbes. These microbes were analysed for purine and nitrogen content.

3.2.6 Urine sampling

The daily urine voided by the sheep was quantitatively collected each day into a container containing 500 ml of 2 % (v/v) CH_3COOH and 1 % (v/v) H_2SO_4 over 24 h. A subsample of the urine sample was taken each day and stored at -20^0. The subsamples of each sheep were bulked prior to analysis of purine derivatives.

3.3 Removal of particle-associated microbes from rumen digesta

Techniques for isolation of particle-associated microbes were modified from Forster (1989) as shown in Figure 3-3. Rumen digesta samples were collected and placed in ice at 4^0. Following cooling, the liquid and solid phases were separated with a bodum filter (see Figure 3-2). Bacteria and protozoa from the liquid phase were harvested using differential centrifugation as shown in Figure 3-4. The solid phase was rinsed and mixed at high speed (a hand held mixer ; General Electric Co., USA, Approx. 11000 rpm) in a phosphate buffer containing 0.03 M K_2HPO_4, 0.02 M KH_2PO_4 and 3.57 M $NaHCO_3$. The liquid and solid phases were separated with a bodum filter. The solid phase was retained and re-treated with the buffer once. Subsequently, the solid phase was suspended with the buffer with 0.2 % cellobiose and 0.3 % High-substitution Methyl Cellulose (HMC ; 200-250 centipoises, BDH Chemicals Ltd, Poole, England) and then mixed at high speed according to the methods of Forster (1989). The liquid and solid phases were separated with a bodum filter. The liquid contained the particle-associated microbes and feed particles. The particle-associated microbes and the large feed particles were separated by a differential centrifugation procedure similar to that used for the isolation of bacteria from protozoa in the fluid phase (see Figure 3-4). Subsequently, the particle-associated microbes fraction was treated in the same way as bacteria in the fluid phase as described in Figure 3-4.

3.4 The pH in ruminal fluid

After collecting the ruminal fluid sample, 7 ml of the subsample of the well-mixed ruminal fluid was placed in a vial and the pH was immediately determined on a calibrated pH meter.

3.5 Enumeration of protozoa

A vial containing 16 ml of 4 % formal saline (11.1 % formaldehyde solution and 0.9 % NaCl) plus 4 ml of ruminal fluid, was thoroughly shaken and a sample pipetted into a Hawksley counting chamber (Cristalite B.S. 748 ; Lancing Sussex, England) of 0.2 mm depth and covered with a double thickness coverslide. Protozoa (greater than 200) were then counted and also classified into three groups, namely small and large *Entodinium sp. and Holotrich sp* under a light microscope. The protozoal population per ml was calculated.

3.6 Enumeration of fungal sporangia

A nylon bag containing 10-12 pieces of oat leaf samples was suspended for 24 h in the rumen to allow fungi to colonise the oat leaf blades and produce sporangia. The leaves were then removed and clipped in 4 % formal saline (11.1 % formaldehyde solution and 0.9 % NaCl) in a vial. The technique was similar to that recommended by Bauchop (1979). The leaves were randomly sampled and stained with lactophenol cotton blue (Gurr, 1965) for 2 min and then washed with deionised water to remove the excess stain. Sporangial counts were made under a light microscope at the following sites (1) the areas near the two ends (2) between the middle and the two ends and (3) the middle of the stained leaf. The sporangial population per 1 mm^2 was calculated from the surface area estimated from the microscopic field radius of a specific objective.

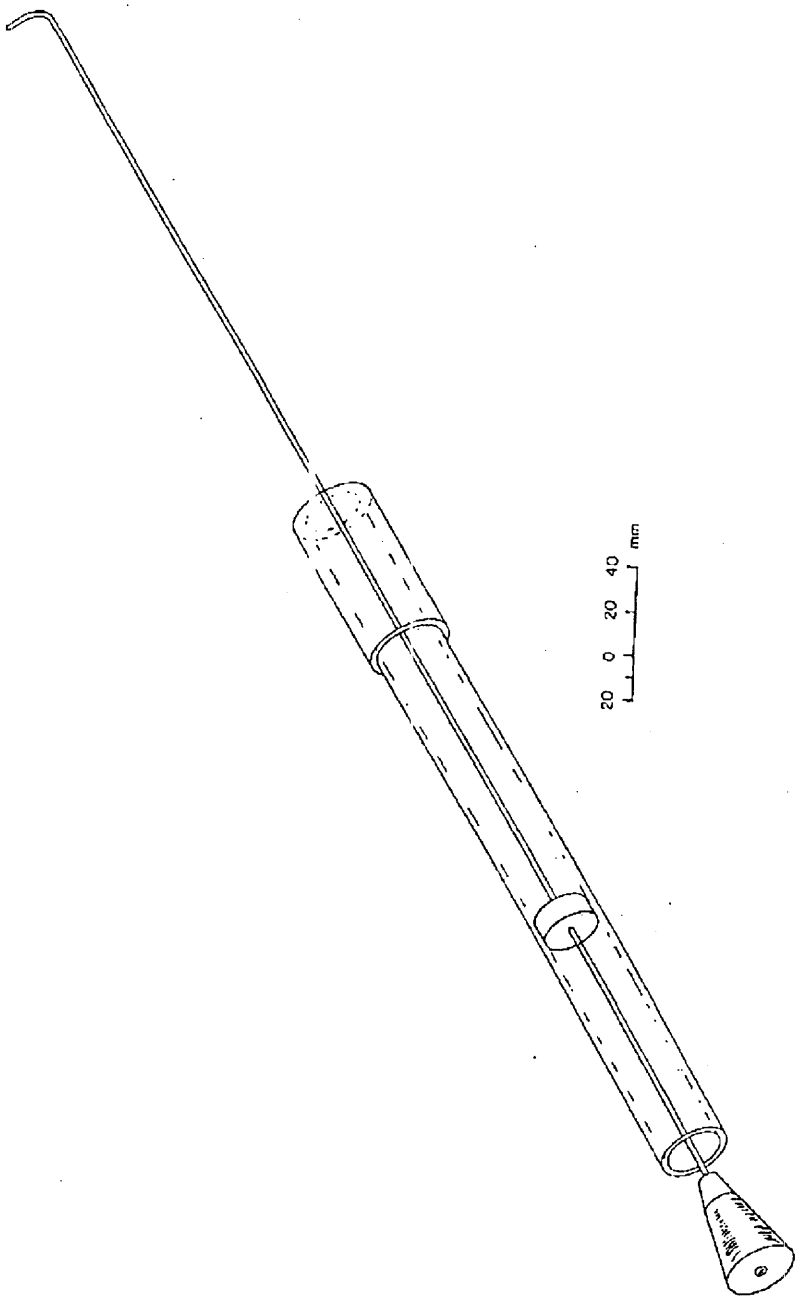

Figure 3-1. Core sampling probe used to collect a sample of rumen digesta.

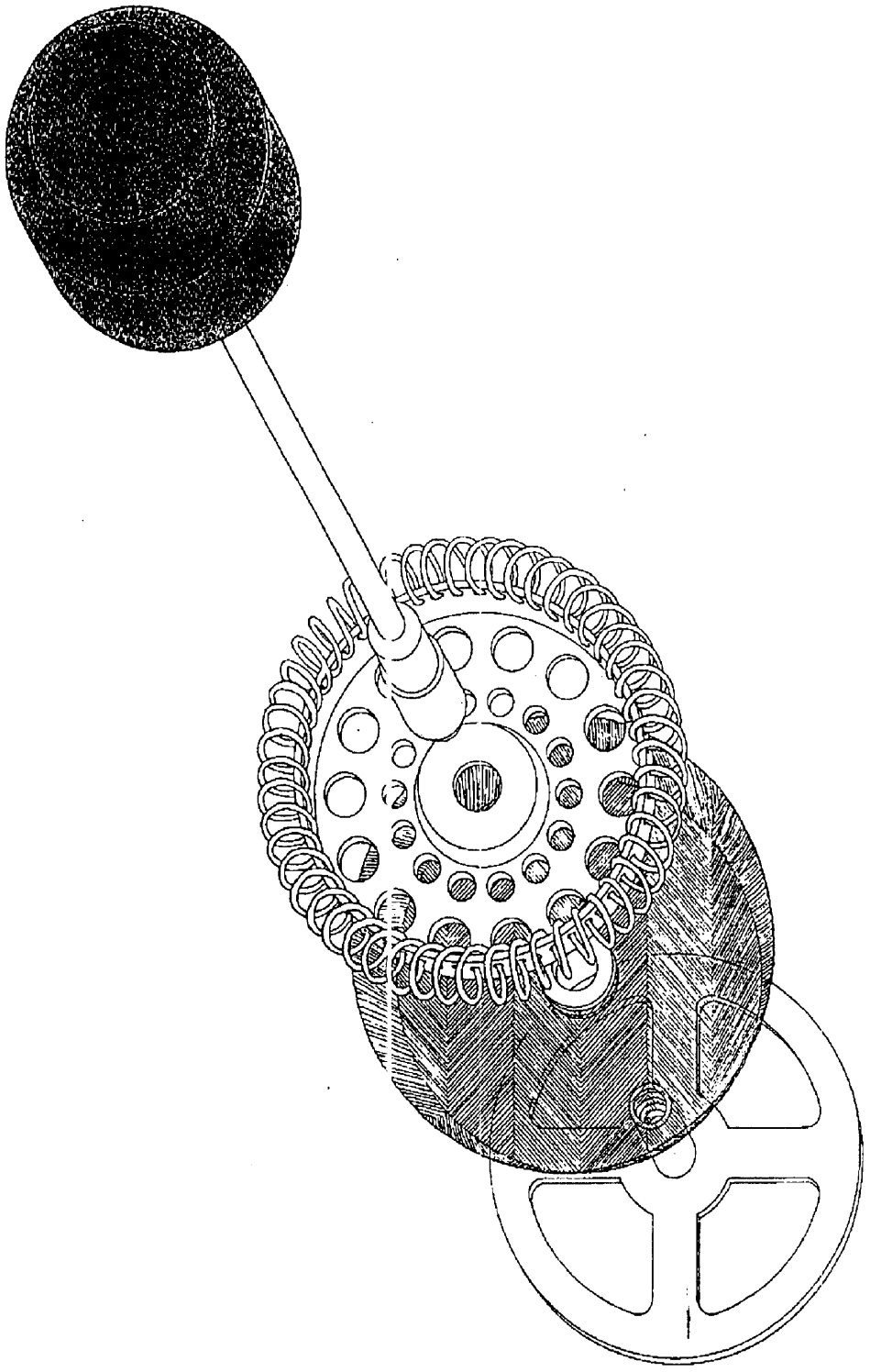

Figure 3-2. Bodum filter used to separate the fluid and particle fractions of rumen digesta.

Rumen digesta
↓
Separate liquid and solid with a bodum filter → liquid retained for free bacteria and protozoa (A)
↓
Rinse and mix the solid with equal volume of the phosphate buffer
↓
Separate liquid and solid → Discard liquid
↓
Repeat the two previous steps once
↓
Suspend the solid in an equal volume of the phosphate buffer containing HMC and cellobiose
↓
Mix at high speed (a hand held mixer) for 2 min let stand 1 min and mix for a further another 2 min
↓
Separate liquid and solid with a bodum filter
↓
The liquid retained for particle-associated microbes (B)

Figure 3-3. Removal of particle-associated microbes from rumen digesta (Modified from Forster, 1989).

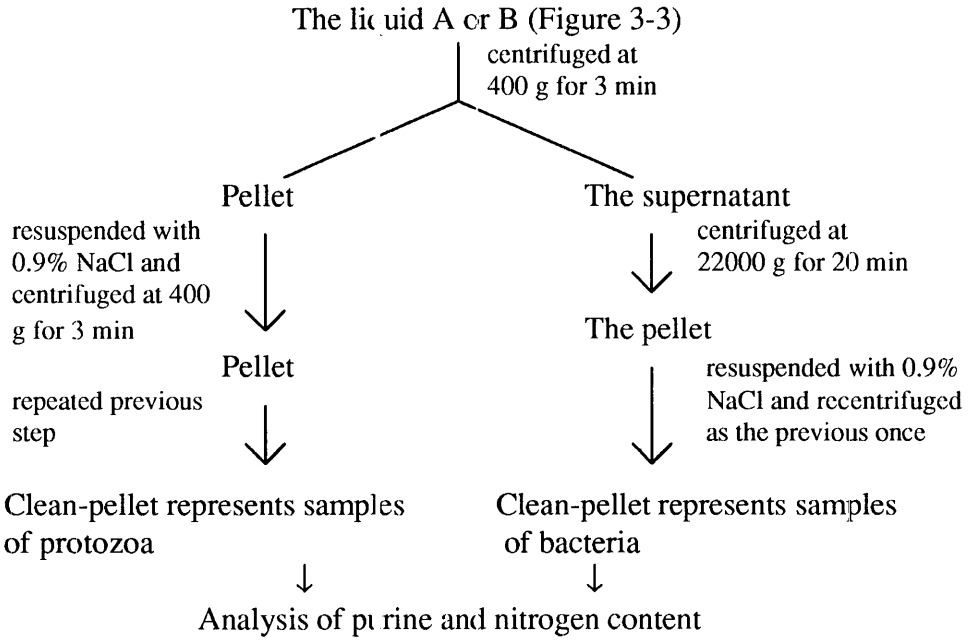

Figure 3-4. Techniques to harvest bacteria in ruminal fluid, particle-associated microbes and protozoa by differential centrifugation.

3.7 *In sacco* digestibility

The technique used to estimate the *in sacco* organic matter digestibility of feed is similar to that reported by Orskov et al. (1980). Nylon bags were prepared from a monofilament polyester with a pore size of 44 µm and measured 7x14 cm. Oat chaff samples were air dried and ground so that they would pass through a 2 mm sieve. A marble was placed inside each bag to facilitate immersion in the ruminal fluid. Each bag and marble were weighed and a 3 g sample of the feed was placed into the bag which was then tightly tied with nylon string.

The bag was wetted with tepid tap water and placed inside the rumen through the rumen cannula for 24 h. The bag was then withdrawn and washed under running tap water until the wash water was clear. The bag was dried in a forced air oven at 60^0 for 48 h. The bag was allowed to attain room temperature in a desiccator. The string was removed and the bag was then weighed.

A crucible was incubated in a forced air oven at 60^0 for 12 h. The crucible was cooled in a desiccator and weighed. The oven-dried sample in the bag was transferred into the crucible and then weighed. The crucible was ignited for 6 h at 550^0 in a muffle furnace. The crucible was cooled in a desiccator. Organic matter was determined from the difference between dry weight and ash weight of the oven-dried sample. The loss of organic matter was then calculated.

3.8 Analytical procedures

3.8.1 Total nitrogen (N)

The concentration of total-N was determined in the relevant sample by the LECO (EP-228, LECO Co., St Joseph, Michigan, USA).

3.8.2 Ruminal fluid ammonia nitrogen (NH_3-N)

Samples of the ruminal fluid supernatant were analysed for the concentration of NH_3-N using an Auto-analyser (Technicon Equipment Co., NJ, USA) according to the method of Crooke & Simpson (1971) modified by Bietz (1974).

3.8.3 Volatile fatty acids (VFA)

The VFA concentration and molar proportions of acetic, propionic, butyric, isobutyric, isovaleric and valeric acids were determined in the ruminal fluid supernatant by gas liquid chromatography (model 427, Packard Instrument Co., USA) according to the method of Erwin et al. (1961). The chromatograph was connected to data processors (model 604, Packard Instrument Co., USA). Iso-caproic acid was used as an internal standard (Geissler et al. 1976).

3.8.4 Chromium (Cr)

The concentration of Cr in the ruminal fluid supernatant was measured by atomic absorption spectrometry (model 360 Perkin-Elmer, Connecticut, USA) according to the method of Downes & McDonald (1964).

3.8.5 Purine derivatives in urine

Purine derivatives in urine were analysed according to the method of Balcells et al. (1992a) modified by Kahn (1993) using a dual-pump HPLC system (Waters Associates, USA) with an automatic injector (WISP model 710B) connected with two C18 reverse-phase columns (Novapak 300 mm * 3.9 mm I. D., Waters Associates) and allopurinol was used as an internal standard (Balcells et al. 1992a).

3.8.6 Purine content in micro-organisms

Purine content in micro-organisms was determined according to the methods of Zinn & Owen (1986). The method was modified by using 60 % guanine (2-amino-6-hydroxypurine, Sigma) and 40 % adenine (6-amino purine, Riedel-de Haen) as a

standard. The maximum absorbance of the standard occurred at 253 nm where it was similar to that of purines isolated from microbial samples. The optical density of a sample was read using a spectrophotometer (150-20 spectrophotometer with the built-in scanning graph and 150-20 data processor, Hitachi) between 251 and 254 nm depending on the maximum absorbance of the sample.

3.9 Statistical analysis

Statistical problems of a study on ruminant nutrition are associated with individual animal and period or time variations due to the changes in rumen function and physiological state. To increase the sensitivity of the statistical test, these variations or effects can be statistically removed by subtracting the degrees of freedom of both effects from the total degrees of freedom and therefore from the residual degrees of freedom. This is the fundamentals of the analysis of variance used in this thesis. The experimental designs reported in this thesis are as described by Steele & Torrie (1980). Statistical significance of the experimental data was analysed using SAS (1989) and the difference between treatment means was measured by the Least Squares Means.