Chapter 1

1. Introduction

The true ruminants, including sheep, goats, cattle, deer and antelope, are distinguished from simple-stomach animals by the fact that their stomach is comprised of four compartments, i.e., the rumen, reticulum, omasum and abomasum. These compartments are pouches between the oesophagus and the small intestine. Only the abomasum functions as the true gastric stomach by secreting hydrochloric acid, pepsin and mucus.

In addition, compared with simple-stomach animals, ruminants have some unique features in their digestive physiology (Hungate 1988). Firstly, regurgitation of rumen ingesta via the oesophagus to the oral cavity (rumination) is triggered by large plant particles in the reticulum after feeding. The regurgitated bolus of rumen contents is mixed with saliva in the mouth, chewed and re-swallowed. Secondly, a large number of microorganisms exists in the rumen and reticulum which conduct extensive microbial fermentation of ingested feeds. The rumen microorganisms constitute a highly integrated ecosystem, in which solubilisation and dissimilation of feeds, microbial growth, absorpt on and passage of the end-product occur. They also have the ability to adapt to changes in the conditions in the rumen.

In terms of digestion, the rumen is the most important part of the ruminant stomach. In adult ruminants, the rumen is the largest compartment, accounting for around two-thirds of the total weight of their stomach. The rumen and reticulum make up a large fermentation vessel with a volume of around 5-10 l in sheep and 100-150 l in cattle (Trinci *et al.* 1994). Nearly all the feed components ingested are subjected to considerable chemical degradation by the microorganisms in the rumen. This unique feature makes digestion, absorption and transport of nutrients in ruminants dramatically different from those in simple-stomach animals. The supply of nutrients to ruminants originates from the end-products of microbial fermentation in the rumen, e.g., volatile fatty acids (VFA), microbial carbohydrates, protein and lipids, as well as dietary components by-passing the rumen. Therefore, an important consequence of microbial fermentation in the rumen is that the post-ruminal supply of nutrients to the host animal is largely different from the type and balance of nutrients ingested. Furthermore, the nutrients absorbed from the gut are extensively transformed and metabolised in the portal-drained viscera and the liver, so that the nutrients ultimately reaching the peripheral tissues of ruminants are also different from those absorbed (Lomax and Baird 1983).

The literature that was reviewed provides information first on the rumen microorganisms, and microbial digestion and metabolism of dietary carbohydrates, nitrogen, lipids in the rumen. Since microbial protein yield from the rumen and dietary protein escaping the rumen are the sources of amino acids available to ruminant tissues, microbial protein yield from the rumen and its estimation by means of measuring urinary excretion of purine derivatives are then discussed. Finally, manipulation of protein degradation in the rumen to increase the intestinal flow of dietary protein is summarised.

Chapter 2

2. Nutrient Transactions in the Rumen

2.1 The rumen

The rumen functions as a continuous fermentation system, the environment in which remains relatively stable (Hungate 1988; Trinci *et al.* 1994). The rumen digesta have a dry matter (DM) content of 10-18% with a retention time ranging from 10-24 h (water, solute, small particles and microbial cells) to 2-3 days (large particles). The oxidation-reduction potential in the rumen is about -250 to -450 mV and the rumen has a constant, nearly neutral pH achieved by the presence of buffers (e.g., phosphate and bicarbonate, in saliva entering the rumen) and the absorption of VFA and ammonia through the rumen wall. In addition, the system is essentially isothermal (around 39°C) and is regulated by the homeothermal mechanism of the host animal. The osmotic pressure, 400 mOsmol/kg is normally lower than the isotonic level.

Typical concentrations of VFA in rumen fluid are: 60 mmol/l of acetate, 20 mmol/l propionate and 10 mmol/l butyrate, although these concentrations vary with diets. The rumen gases include carbon dioxide (65%), methane (27%), nitrogen (7%), oxygen (0.6%), hydrogen (0.2%) and hydrogen sulphide (0.1%).

2.2 Rumen microorganisms

Any microorganisms found on feed or in water, such as bacteria, fungi, bacterial viruses and yeasts, may exist in the rumen (Pond *et al.* 1995). More than 300 species and strains of microorganisms have been identified in the rumen (Trinci *et al.* 1994), but only around 30 species are predominant (Leng 1991). Under most conditions, the most important rumen microorganisms are bacteria, protozoa and fungi.

In the rumen, metabolic activities of the microorganisms, which are generally inversely related to the size of microbial cells, are considerably diversified and highly integrated so that the microbial population density within the rumen is extremely high (Hungate 1988). In addition, the composition and population of the microorganisms are predominantly determined by diets and the turnover time of rumen digesta rather than the species of the host animal (Van Soest 1994). For microorganisms to successfully inhabit the rumen and maintain a stable population, they must

have a generation interval of less than 0.69 of the turnover time of rumen digesta (Hungate 1988).

2.2.1 The bacteria

Bacteria represent 60-90% of the microbial biomass in the rumen. They have a population density around 1×10^{10}/ml of rumen fluid and a mean generation interval of around 20 min (Hungate 1966; Van Soest 1994). Around one-half of rumen bacteria are freely suspended in rumen fluid, and the other attached to the surface of feed particles and the rumen wall.

Typically, rumen bacteria, on a DM basis, consist of about 78% of organic matter (OM) and 7.7% nitrogen (N). In addition, around 67% of bacterial N is amino acids, and methionine and lysine account for 2.6 and 7.9% of the total bacterial amino acids, respectively (Clark *et al.* 1992).

Rumen bacteria are very diverse in their functions and are involved in most metabolic procedures in the rumen. They are specialised in fermenting structural or non-structural carbohydrates, in degrading protein, lipids or organic acids, or utilising hydrogen. The end-products of bacterial fermentation include VFA, carbon dioxide, methane, ammonia, hydrogen and ethanol (Van Soest 1994).

2.2.2 The protozoa

Protozoa account for 10-40% of the microbial biomass in the rumen. Rumen protozoa have a population density of 1×10^{4} to 3×10^{5}/ml of rumen fluid (Hungate 1966; Van Soest 1994). More than 100 species of protozoa are found in the rumen and belong to three groups: the rumen flagellates, the holotrichs and the entodiniomorphs (Theodorou and France 1993). They have a generation interval of around 6-48 h (Hungate 1966).

Enzymes secreted by protozoa are capable of hydrolysing starch, hemicellulose and cellulose (Hoover and Stockes 1991). The end-products of protozoal fermentation *in vitro* on carbohydrates are VFA (acetate and butyrate with a trace amount of propionate), hydrogen, carbon dioxide, formate and lactate (McDonald *et al.* 1988; Dijkstra 1994). Usually 16-37% of total VFA in the rumen may be produced by protozoa (Dijkstra 1994). Moreover, most of protozoa are able to deaminate amino acids and excrete ammonia as an end-product (Nolan 1993). In addition, protozoa are major methane-producing microbes in the rumen (Bird 1991), and methanogenic bacteria attached to their cell surface produce methane from either formate or carbon dioxide and hydrogen - the latter two gases probably originating from hydrogenosomes in the protozoa.

The N content of rumen protozoa is lower than that of rumen bacteria (3.8-7.9% vs 5.0-12.4% DM), but the digestibility of protozoal N is higher (76-85% vs 44-86%) (Van Soest 1994). In addition, protozoal protein is higher in phenylalanine, isoleucine, leucine and lysine (Purser and Buechler 1966).

Protozoa tend to settle in the rumen due to their attachment to large feed particles and on the rumen wall, and because of their tendency to engulf starch particles and sugars (Preston and Leng 1987). Only 20-40% of protozoal biomass may flow out from the rumen, which accounts for only 5-15% of the total microbial biomass flowing out of the rumen (Weller and Pilgrim 1974).

Protozoa obtain nutrients from not only plant materials of dietary origin, but also by engulfing bacterial cells, fungal zoospores and other protozoa. They mainly degrade and utilise dietary carbohydrates, protein and lipids (Williams 1989). The holotrichs utilise soluble sugars and ingest both soluble and particulate proteins and degrade them intracellularly (Nolan 1989; Bird 1991), whereas the entodiniomorphs utilise simple and complex carbohydrates including cellulose, hemicellulose and pectin (McDonald *et al.* 1988; Williams 1989), and degrade insoluble, particulate proteins (e.g., bacterial cells, chloroplasts) extracellularly by secreting peptidases (Nolan 1989; Bird 1991). In addition, protozoa may also control fermentation of starch in the rumen through engulfing and storing starch particles in their cells (Jouany 1989; Mendoza *et al.* 1993).

Protozoa are largely responsible for dietary and bacterial protein turnover in the rumen. Faunated animals have a higher ruminal ammonia concentration than defaunated animals, which probably results from the inefficient utilisation of the bacterial protein they engulf (Veira and Ivan 1983).

Protozoa are unlikely to be essential to rumen fermentation. Eliminating protozoa from the rumen reduces ruminal degradation of dietary protein, increases microbial yield from the rumen, the efficiency of microbial protein synthesis (Merchen and Titgemeyer 1992; Vercoe 1996) and the ratio of protein to energy in the absorbed nutrients (Nolan 1989; Bird 1991). As a result, wool growth, live-weight gain, milk production, daily output of milk fat and milk protein of ruminants fed low quality forages are usually increased by defaunation (Bird 1991). However, the quality of diets, nutritional status and nutrient requirements of the host animal determine whether defaunation is beneficial to the host animal (Veira 1986; Gordon and Phillips 1995). In short, defaunation benefits the animals fed low N forages by increasing microbial yield from the rumen. On the other hand, protozoa are probably important for animals fed high-concentrate diets because of their role in stabilising ruminal pH and reducing the risk of acidosis.

2.2.3 The fungi

Fungi account for 5-10% of the microbial biomass in the rumen, and their population is around 1×10^4/ml of rumen fluid (Theodorou *et al.* 1990). Currently two classes of fungi have been isolated from the rumen, i.e., the monocentric and the polycentric fungi, and twelve species of the former and four of the latter have been identified (Gordon and Phillips 1995). Rumen fungi have a generation interval of around 24-30 h (Bauchop 1981). The amino acids of fungal protein are well balanced (Kemp *et al.* 1985) and digestibility is high (Gulati *et al.* 1989).

Fungi utilise monosaccharides (glucose, fructose, xylose), disaccharides (cellobiose, lactose and maltose) and polysaccharides (starch, pectin and cellulose) as substrates (Trinci *et al.* 1994). The end-products of fungal fermentation on carbohydrates include VFA, formate, lactate, ethanol, carbon dioxide and hydrogen (Orpin and Joblin 1988; Gordon and Phillips 1989).

The importance of rumen fungi is that they are cellulolytic organisms that specifically attach to, colonise and grow on structural plant components. They are able to degrade structural carbohydrates of plant cell walls and utilise the resultant xylan and cellulosic components. The hydrolytic enzymes, including cellulases, hemicellulases, xylanases, amylases, amyloglycosidases, p-coumaroyl esterase, various disaccharidases and pectinases, and proteases (Akin and Borneman 1990; Trinci *et al.* 1994), are secreted from the rhizoids which grow into inter-cellular spaces and through plant cell walls, and the resultant nutrients are transported to the sporangium (Gordon and Phillips 1989; Wubah *et al.* 1993).

Fungi are more efficient in degrading structural polysaccharides than ruminal bacteria. Fungal cellulase is more soluble than bacterial cellulase (Van Soest 1994), and cellulose and hemicellulose are particularly susceptible to fungal enzymes (Akin *et al.* 1983). As a result, fungi may compete with bacteria in digestion of structural polysaccharides of plant cell walls (Gordon and Phillips 1989; Akin and Borneman 1990).

Furthermore, fungi are important facilitators of bacterial fermentation of insoluble plant cell wall components by attacking and weakening lignocellulosic tissues. Although bacteria are the dominant organisms degrading plant cell wall carbohydrates in the rumen, fungi are the first organisms to invade plant cell walls and solubilise lignocellulosic structures, which enables plant cell wall components bound to lignin to be available to bacterial fermentation (Preston and Leng 1987). Fungi are capable of penetrating deeply into plant tissues normally inaccessible to bacteria, and reduce the tensile strength of fibrous feed particles. This increases particle breakdown in the rumen (Bauchop 1981; Akin *et al.* 1983; Akin *et al.* 1990; Akin and Borneman 1990). Fungi may separate 20-30% of lignin from lignin-carbohydrate complexes by dissolving carbohydrates surrounding the lignin (McSweeney *et al.* 1994), but they cannot further degrade

and utilise the lignin (Gordon and Phillips 1989; McSweeney *et al.* 1994). In addition, polycentric fungi are more efficient in solubilising lignified plant tissues than monocentric fungi (Akin *et al.* 1990).

Unlike most cellulolytic bacteria in the rumen which are not usually proteolytic, rumen fungi possess both cellulolytic and proteolytic activities (Wallace and Joblin 1985). Fungal metalloproteinase is secreted extracellularly at the early growth stage but intracellularly at the late growth stage (Wallace and Joblin 1985; Hoover and Stokes 1991).

2.3 Nutrient transactions by rumen microorganisms

2.3.1 Carbohydrate transactions in the rumen

2.3.1.1 Carbohydrates in ruminant diets

From the viewpoint of plant physiology, carbohydrates can be divided into three distinct categories: simple sugars and their conjugates, storage reserve compound (e.g., starch, sucrose and fructans) and structural polysaccharides (e.g., pectins, hemicellulose and cellulose) (Van Soest 1994)

2.3.1.2 Degradation of carbohydrates in the rumen

The degradation of dietary carbohydrates including cellulose, hemicellulose, pectin, starch and soluble sugars, in the rumen can be divided into two stages. The first stage is the breakdown of complex carbohydrates to simple sugars by extracellular microbial enzymes. Polysaccharides are firstly hydrolysed to oligosaccharides by both polysaccharidases and glycoside hydrolases mainly secreted by rumen bacteria and fungi. Starch and dextrins are firstly decomposed by amylases to maltose and isomaltose, then by maltases, maltose phosphorylases or 1, 6-glucosidases to glucose or glucose-1-phosphate, whereas cellulose is converted to cellobiose by a complete array of enzymes comprising endo-1, 4-β-D-glucanase, exo-1, 4-β-D-glucanase and β-D-glucosidase (Wood 1991), and the resultant cellobiose is further hydrolysed to glucose or glucose-1-phophate by phosphorylase. In addition, the breakdown of hemicellulose yields pentoses, xylose and uronic acids by enzymes attacking the β-1-4 linkages. Moreover, pectins are firstly converted by pectinesterase to pectic acid and methanol, which are eventually converted to uronic acids (McDonald *et al.* 1988).

The second stage is the utilisation and metabolism of the resultant sugars by the microorganisms. The pre-formed sugars (e.g., glucose, maltose, sucrose, arabinose, xylose and cellobiose) are firstly transported across the cell membrane into bacterial cells by an active transport system driven by the ionic (Na^+ or Li^+) gradient across the membrane and an energetically efficient

phosphotransferase system (PTS) (Martin 1994; McSweeney *et al.* 1994). The latter involves a specific binding phosphocarrier, the enzyme III, in the membrane which translocates the sugars using the high energy phosphate compound, phosphoenolpyruvate (PEP) rather than adenosine triphosphate (ATP).

Then, the intracellular sugars are catabolised through different pathways all involving pyruvate as a common intermediate (McSweeney *et al.* 1994). Acetate and butyrate are further formed from pyruvate with acetyl CoA as an intermediate, whereas propionate is formed mainly by the succinate pathway or by an alternative pathway involving acrylate.

The concentration of total VFA, i.e., acetic, propionic and butyric acids with small amounts of isobutyric, valeric, isovaleric and 2-methylbutyric, in the rumen is normally 70-130 mM, or 7-15 g/l of rumen fluid (McDonald *et al.* 1988). Although the major VFA produced in the rumen is acetate, the molar proportion of acetate, propionate and butyrate varies with microbial composition which is itself affected by the composition of the diet, the type, availability and interaction of dietary carbohydrates, and the rate of degradation of carbohydrates and protein (Dijkstra 1994).

Acetic, propionic and butyric acids produced in the rumen are absorbed through the rumen wall, at rates which approximate their rates of production, by simple diffusion (France and Siddons 1993). They are then utilised as a major source of metabolisable energy by ruminants which possess nearly the same intermediary metabolism as simple-stomach animals, with the exception of utilisation and conservation of glucose and gluconeogenesis (Van Soest 1994).

The concentration of individual VFA affects the rate of absorption of individual VFA, and the rate of absorption is further modified by ruminal pH (Dijkstra 1994). As entry of VFA increases, the concentration of VFA increases, and this, in turn, increases the rate of absorption. In general, the ruminal concentration of individual VFA is linearly correlated with the effective production rate of individual VFA (Leng 1970). Therefore, the ruminal concentration of VFA generally reflects the production rates of VFA in the rumen.

Volatile fatty acids may account for up to 70% of the total energy supply to ruminants. In general, acetate and butyrate are mainly oxidised via the citric acid cycle to yield energy or are used in tissue growth. Acetate is also an important lipogenic precursor (Weigand *et al.* 1972). In contrast, propionate is the major glucose precursor reserved for gluconeogenesis. The absorbed propionate is predominantly transported to the liver for the synthesis of glucose by way of methylmalonyl CoA, succinate and oxaloacetate and subsequent gluconeogenesis, which may account for approximately 50-90% of the total glucose utilised by the host animal (Hungate 1966; Cridland 1983).

2.3.2 Nitrogen transactions in the rumen

2.3.2.1 Nitrogen in ruminant diets

The N components in ruminant feeds include non-protein nitrogen (NPN, e.g., nucleic acids, amides, amines, amino acids and nitrate) and true protein. These N components can be classified into four categories: soluble NPN, rapidly degradable protein, slowly degradable protein and totally undegradable protein and NPN (Nolan 1993).

2.3.2.2 Degradation of nitrogen in the rumen

Of the dietary protein entering the rumen 60-90% is usually degraded by rumen microorganisms, although a proportion of the protein is resistant to rumen degradation and escapes into the small intestine where it is then exposed to enzymatic digestion (Kempton *et al.* 1977).

Proteolytic activity is associated with rumen bacteria, protozoa and fungi. However, rumen bacteria are the most important proteolytic organisms. 30-50% of bacterial species commonly isolated from the rumen are proteolytic and they are responsible for more than 50% of the proteolytic activity in the rumen (Annison 1956; Warner 1956). Protozoa have a higher proteolytic ability than bacteria, but they only account for around 10-20% of the proteolytic activity (Nugent and Mangan 1981). In addition, rumen fungi are also highly active in proteolysis (Wallace and Joblin 1985).

Cysteine- and serine-type proteases secreted by bacteria and protozoa are predominant in the rumen, with metallo-type proteases secreted by bacteria and fungi and aspartic-type proteases by protozoa only. In addition, bacterial and protozoal peptidases specifically hydrolyse oligopeptides and dipeptides, respectively (Morrison and Mackie 1996).

Dietary protein is degraded anaerobically in the rumen in two stages (Tamminga 1979; Van Straalen and Tamminga 1990). At the first stage, the peptide bond in protein is hydrolysed by microbial proteases and peptidases either on the surface of bacterial cells or inside protozoal cells, consequently the protein is broken down into peptides and amino acids (Tamminga 1979; Leng and Nolan 1984). At the second stage, the resultant peptides and amino acids are transported across the cell membrane into bacterial cells by an active transport system driven by ionic (i.e., Na^+ and H^+) gradients or ATP (Martin 1994). The peptides are then further hydrolysed to amino acids. Intracellular amino acids are either incorporated into bacterial protein or further degraded by decarboxylation and deamination into VFA, branched chain fatty acids, ammonia, carbon dioxide and methane. Within protozoal cells, if the resultant amino acids are not incorporated into protozoal protein, they are often excreted into rumen fluid.

Most amino acids and peptides yielded from the hydrolysis of dietary protein in the rumen are taken up by rumen microbes rapidly. However, some peptides which are slowly utilised may contribute a significant amount of dietary N flowing to the small intestine (Waghorn *et al.* 1987; Clark *et al.* 1992).

Although, for feeds with a low N content, the rumen microbial system provides distinct advantages for the host animal by making it possible for the host to generate protein from dietary NPN and recycled urea derived from the turnover of tissue amino acids and nucleic acids (Nolan 1993), not all the N in feeds, especially those with a high N content, finally reaches the small intestine as true microbial protein (McDonald *et al.* 1988). There is an inevitable wastage of dietary true protein by urinary excretion of urea formed from ammonia that is absorbed after its formation during protein degradation in the rumen.

2.3.3 Lipid transactions in the rumen

As reviewed by Jenkins (1993), the metabolism of lipids in the rumen includes three major processes: lipolysis, biohydrogenation of the resultant unsaturated fatty acids and the synthesis of microbial lipids. During ruminal lipolysis, dietary lipids, which are usually low in ruminant diets and are mainly present as galactosyl diacylglycerols and in unsaturated forms (Pond *et al.* 1995), are hydrolysed extensively by extracellular microbial lipases. Acylglycerols are completely hydrolysed by lipases to unsaturated fatty acids and glycerol with small amounts of mono- or diacylglycerols. Glycerol is then converted rapidly to propionate. Likewise, plant galactolipids and phospholipids are hydrolysed by galactosidases and phospholipases, respectively.

The resultant unsaturated fatty acids from the lipolysis are rapidly hydrogenated first to monoenoic acid and ultimately to stearic acid (McDonald *et al.* 1988), predominantly by rumen bacteria (Jenkins 1993). During this process, the *cis*-12 double bond in the unsaturated fatty acids with a free carboxyl group is converted first to the *trans*-11 bond by the isomerase, and then another double bond, the *cis*-9, is hydrogenated by a microbial reductase. Finally, hydrogenation of all the *trans*-11 bonds is catalysed by the reductase.

Rumen microorganisms are capable of synthesising *de novo* lipids from carbohydrate precursors (Jenkins 1993). Acetate or glucose is utilised for the synthesis of straight-chain, even-numbered fatty acids, whereas propionate or valerate for straight-chain, odd-numbered long-chain fatty acids. Isobutyrate, isovalerate and 2-methylbutyrate are also utilised for the synthesis of branched-chain fatty acids. The efficiency of microbial lipid synthesis in the rumen is estimated to be about 15 g microbial lipids/kg of lipid-free OM digested in the rumen (Jenkins 1993).

The *de novo* synthesis of saturated and monounsaturated fatty acids within microbial cells involves an anaerobic pathway with two directions (Jenkins 1993). The first is that dehydration

takes place in the α, β position of the β-hydroxy C_{10} intermediate to form a *trans*-2 double bond. Then, following a reduction catalysed by the C_{10}-enoyl reductase, and a chain elongation, saturated fatty acids ($C_{18:0}$) are yielded. The second is that the β-hydroxy C_{10} intermediate is dehydrated in the β, γ position to form a *cis*-3 double bond, then only after a chain elongation, monounsaturated fatty acids ($C_{18:1}$) are formed.

The important practical outcome of these chemical changes is that unsaturated fatty acids (such as those in sunflower meal and cottonseed meal) which are present as plant lipids are largely converted to saturated fats which produce harder fat in meat or butter (Nolan, J.V., pers. comm., 1997).

Chapter 3

3. Microbial Protein Yield from the Rumen

3.1 Microbial growth in the rumen

In the rumen, fermentation of dietary OM to monomers and the synthesis of protein, nucleic acids, polysaccharides and lipids from the resultant monomers occur simultaneously within microbial cells. In these processes, energy released in the form of ATP, ammonia from dietary and endogenous protein, carbon fragments from the resultant amino acids, peptides and end-products of carbohydrate fermentation are utilised for the synthesis of microbial cellular materials.

In most cases, 60-80% of the amino acids absorbed from the duodenum are microbial in origin (Miller 1982a; Merchen and Titgemeyer 1992), and mixed microbial protein is mostly bacterial in origin (Weller and Pilgrim 1974). Comparisons over a wide range of basal diets without protein supplements show that except for leucine and lysine, the composition of essential amino acids in duodenal digesta of ruminants is not significantly different from that of microbial protein (Merchen and Titgemeyer 1992).

The composition of microbial biomass is relatively constant: true protein 32%, small molecules 10%, nucleic acids 8%, cell wall 9%, lipid 11%, polysaccharide 17% and ash 13% (Czerkawski 1986). Compared with bacterial protein, protozoal and fungal proteins are higher in intestinal digestibility and have a better balanced amino acid composition (Nolan 1993). Digestibilities of bacterial protein and protozoal protein are 0.74, 0.91, respectively (McDonald *et al.* 1988), and intestinal digestibility of mixed microbial protein is around 0.85 (AFRC 1993). Furthermore, the biological value (BV) of mixed microbial protein is around 0.8 (McDonald *et al.* 1988).

In most situations, microbial protein synthesised in the rumen is the major source of amino acids, including the ten essential amino acids, available to ruminant tissues (Nolan 1993). The amount of microbial protein entering the small intestine may satisfy the protein requirement for maintenance and low production of animals, but may not satisfy that of either fast growing, or pregnant, or lactating or high producing animals.

3.2 Factors affecting microbial protein yield

3.2.1 Energy

Even though some bacterial species utilise peptides or amino acids as their sole energy source for growth, major rumen microbes require ATP derived from fermentation of dietary OM for growth (Tamminga 1982; Hoover and Stokes 1991; Nolan 1993; Russell and Strobel 1993). Around 45% of ATP yielded during fermentation of feed substrates is utilised by microbes for maintenance, whereas only about 55% for growth (Czerkawski 1986; Russell and Strobel 1993). The energy requirement for maintenance of rumen microorganisms varies between species, but is around 1.63 mol ATP/g microbial biomass/h (Isaacson *et al.* 1975). As shown in Table 3.1, ATP requirements for the synthesis of the major cellular components of microorganisms vary (Stouthamer 1979). In general, the synthesis of 100 g microbial biomass requires about 3.9 mol ATP (Beever 1993).

Table 3.1. *ATP requirements for the synthesis of the major cellular materials of microbes growing in a medium of glucose and inorganic salts (adapted from Stouthamer (1979)).*

Cellular material	ATP requirement (mmol/g macromolecule)
Polysaccharide	12.4
Protein	39.1
Lipid	1.5
Nucleic acid	35.2

ATP in rumen microorganisms are predominantly generated by fermentation of carbohydrates. In general, 2.5-3.0 mol ATP is yielded per 100 g carbohydrates fermented. In contrast, only 1.2-1.5 mol ATP is generated per 100 g protein fermented, and the ATP yield from lipid fermentation is negligible since lipids account for only a small fraction of the substrates degraded in the rumen (Tamminga 1982).

ATP is generated through two stages. The first is that ATP is yielded from both 1, 3-diphosphoglyceric acid and acetyl phosphate; the second is that an ATP is yielded when fumarate is reduced to succinate in propionic acid formation (Hungate 1988). During glucose fermentation, little ATP is generated through the Embden-Meyerhof pathway which splits a carbon-carbon bond, and ATP is mainly derived from the oxidation and reduction of glucose to lactate (Russell and Strobel 1993). However, ATP production depends on the relative reaction

rate of the branched pathways involved which are further regulated by the rate of oxidation of NADH to NAD^+ (McSweeney *et al.* 1994). In general, normal mixed rumen microbes may ferment one mole of fermentable hexose to yield around 4.5 mol ATP (Bergen and Yokoyama 1977; Beever 1993), and one mole of acetate, propionate and butyrate produced may yield 2, 1-3 and 3 ATP, respectively (Van Soest 1994).

When N and other growth factors are not limited, the synthesis of microbial protein from rumen-degradable protein is considered to be energy-dependent. Microbial protein yield is thus linearly related to the ATP concentration in the rumen (Bauchop and Elsden 1967).

The efficiency of microbial growth is often expressed as the amount of microbial cells synthesised from one mole of ATP, i.e., Y_{ATP}. The ATP production has a maximum theoretical value of about 28 mol/kg of carbohydrates fermented in the rumen, and Y_{ATP} has a maximum theoretical value of around 30 g cell DM/mol ATP (Nolan 1993). However, the actual cell DM yield is usually between 10 and 20 (Hespell and Bryant 1979; Nolan and Leng 1989; Beever 1993) which is well below the theoretical maximum value because nearly half the ATP generated is used for cell maintenance rather than polymer synthesis. Expressed on the basis of carbohydrates fermented, maximum efficiency of microbial yield in the rumen is about 300 g cell DM/kg of carbohydrates fermented in the rumen (Nolan 1993). *In vitro* studies show that maximum efficiency of cell DM yield of mixed rumen microbes may be as high as 500 g/kg of carbohydrates fermented in the rumen (Russell *et al.* 1992). Although the efficiency of microbial yield varies with different conditions, it was suggested by ARC (1984) to be 200 g cells/kg of OM apparently digested in the rumen (i.e., 130 g cells/kg of digestible OM or 8.25 g cells/MJ of ME) when the efficiency of utilisation of fermented substrates by rumen microorganisms was assumed to be 0.65.

Feed intake directly determines energy supply to rumen microorganisms, thus affects microbial yield from the rumen. Microbial yield from the rumen is usually proportional to DM intake (Russell *et al.* 1992; Chen *et al.* 1992b; Chen *et al.* 1995), digestible OM intake (Russell *et al.* 1992; Belcells *et al.* 1993) or ME intake (Giesecke *et al.* 1994) rather than N intake (Clark *et al.* 1992). Furthermore, increasing DM intake per kg liveweight of animals increases the outflow rate of ruminal digesta, and consequently increases the efficiency of microbial protein synthesis (Chen *et al.* 1995).

Although dietary fats and oils entering the rumen, typically accounting for 5-10% of total ME intake by ruminants, can be taken up by microorganisms, they provide no fermentable energy for microbial growth (Clark *et al.* 1992; Beever and Cottrill 1994). Addition of fats to ruminant diets may depress fibre digestion in the rumen, but may increase microbial yield from the rumen and improve the efficiency of microbial protein synthesis probably due to its defaunating effect (Stern *et al.* 1994).

3.2.2 Nitrogen

3.2.2.1 Ammonia

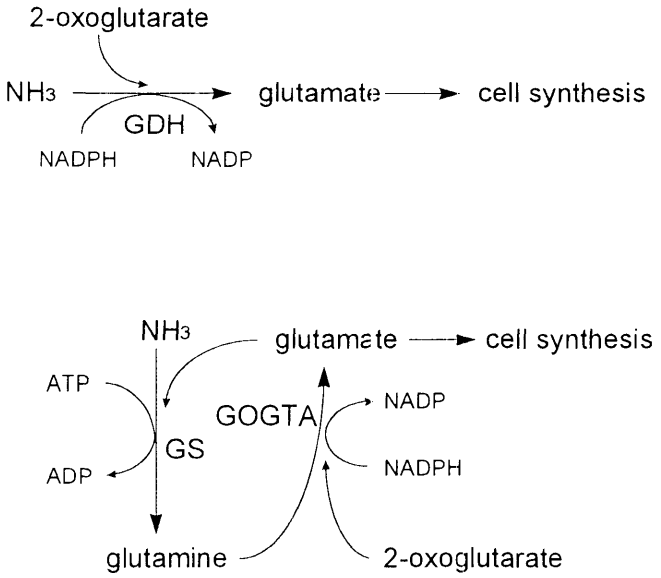
Most rumen microorganisms require ammonia as their major N source for the synthesis of amino acids and nucleic acids (Nolan and Leng 1972). The majority of cellulolytic bacteria species require ammonia as their sole N source (Leng and Nolan 1984; Faichney *et al.* 1994). Ruminal ammonia mainly originates from rumen-degradable dietary protein and endogenous N entering the rumen (salivary urea and protein, sloughed epithelial cells and plasma urea transferred across the rumen wall), lysed microbial cells and N excreted by protozoa (Nolan 1993).

Ammonia is transported across the membrane into microbial cells by passive diffusion, facilitated diffusion and active transport (Martin 1994; McSweeney *et al.* 1994). Ammonia is then assimilated by bacteria through two pathways, namely the low, and the high-affinity pathways (Erfle *et al.* 1977) (Figure 3.1). The former is only switched on at high ammonia concentrations and involves NADP-dependent glutamate dehydrogenase (GDH) to reductively aminate α-ketoglutarate into glutamate, and the latter employs both glutamine synthetase (GS) and glutamate synthase (GOGAT) to yield glutamine from ammonia and glutamate, which requires ATP. However, in some rumen bacteria, ammonia assimilation is conducted predominantly through the GDH pathway regardless of ammonia concentrations (Morrison and Mackie 1996).

Optimum microbial growth in the rumen requires at least 50 (Preston and Leng 1987; Leng 1992) or 60-80 (SCA 1990) mg ammonia-N/l of rumen fluid. However, maximum ruminal fibre digestion and efficiency of microbial fermentation in the rumen requires 150 and 200- 235 mg (NRC 1985; Preston and Leng 1987; SCA 1990) ammonia-N/l of rumen fluid, respectively. Likewise, high ruminal ammonia concentrations are required for optimum digestion of starch in the rumen (Mehrez and Ørskov 1977; Odle and Schaeffer 1987). A high ruminal ammonia concentration is associated with a higher efficiency of microbial growth since microorganisms incorporate ammonia directly into their cellular protein without using ATP through the low affinity pathway. More recently, 20-50 mg ammonia-N/l of rumen fluid was found to be adequate for maximum microbial protein yield, and when ruminal ammonia concentrations were higher than the values above, microbial protein yield was more highly related to truly digested OM in the rumen (Clark *et al.* 1992).

The ammonia concentration required for optimum microbial growth in the rumen can be affected by ruminal pH, which determines the relative ratio of NH_3 to NH_4^+ (Leng 1990). A low ruminal pH may lower the ammonia level required for optimum microbial growth.

Figure 3.1. *Pathways of ammonia assimilation by rumen bacteria (McSweeney et al. 1994).*



3.2.2.2 Peptides and amino acids

Although ammonia, amino acids and peptides may individually serve as N sources for microbial growth in the rumen, optimum microbial yield from the rumen requires the presence of all of them at minimum levels (Hoover and Stokes 1991; Clark *et al.* 1992). The bacteria fermenting structural carbohydrates (i.e., cellulose and hemicellulose) utilise only ammonia as their N source, whereas those fermenting non-structural carbohydrates utilise either ammonia or peptides and amino acids (Leng 1990; Russell *et al.* 1992). Furthermore, amino acids and peptides are also required for optimum growth of protozoa and fungi (Morrison and Mackie 1996).

Peptides and amino acids are transported into microbial cells by active transport (McSweeney *et al.* 1994). Preformed peptides can be taken up by rumen microorganisms at a rate of 0.07 g/g of microbes/h (Sniffen *et al.* 1992). The efficiency of uptake and utilisation of large peptides (from 4 amino acid residues up to 2000 Da) by rumen microbes is higher than that of di-, tri-peptides and free amino acids (Hoover and Stokes 1991; Morrison and Mackie 1996). Therefore, peptides, rather than amino acids, are preferred by some rumen bacteria probably due to a higher energetic efficiency of transport and incorporation of peptides into bacterial protein (Nolan 1993; McSweeney *et al.* 1994). *In vitro* studies also show that some peptides are utilised more selectively and efficiently by mixed rumen bacteria (Ha 1996).

The maximum microbial yield, fibre digestion (Griswold *et al.* 1996) and efficiency of microbial protein synthesis (Clark *et al.* 1992) in the rumen require an adequate supply of ammonia, amino acids or peptides at the same time. Amino acids present in the rumen fluid from lysed microbial cells or excreted by protozoa may improve the efficiency of microbial growth in the rumen (Nolan 1993). *In vitro* studies also show that increasing amino acid availability stimulates microbial yield (Baldwin and Allison 1983). The amino acids directly incorporated into microbial protein at times account for up to 50% of the microbial amino acids synthesised in the rumen of sheep given lucerne chaff (Nolan *et al.* 1976) or more than 20% of microbial N synthesised provided that energy supply is adequate (Baldwin and Allison 1983). Likewise, small peptides are also required for maximum microbial yield from the rumen (Hume 1970). The yield of rumen bacteria fermenting non-structural carbohydrates is proportional to the amount of peptides supplied until peptides account for 14% of total OM in rumen fluid, and 66 and 34% of bacterial N synthesised are derived from peptides or amino acids, and ammonia, respectively (Russell *et al.* 1992).

3.2.3 Synchronisation of the supply of energy and nitrogen

The efficiency of utilisation of N for microbial protein synthesis is determined not only by the availability of fermentable N and energy but also by the synchronisation of the availability of dietary components. For example, the efficiency of microbial protein synthesis in sheep given a diet formulated to be synchronous for the hourly supply of energy and protein to the rumen, was 11-20% higher than that in those give an asynchronous diet (Sinclair *et al.* 1995).

The efficient capture of ammonia by rumen microorganisms largely depends on the availability of fermentable carbohydrates, synchronisation of the rate of ammonia production and the rate of carbohydrate fermentation (Doyle 1987). Likewise, the utilisation of peptides for microbial protein synthesis also depends on the availability of carbohydrates. When fermentable carbohydrates in the rumen are inadequate, peptides are completely converted into ammonia and carbon-skeletons in stead of being directly incorporated into microbial protein (Russell *et al.* 1992).

On the other hand, ruminal digestion of carbohydrates requires a sufficient supply of ammonia. For example, 61 and 235 mg ammonia-N/l of rumen fluid were required for optimum degradation of maize grain (Odle and Schaeffer 1987) and cereal starch (Mehrez and Ørskov 1977), respectively. Similarly, a sufficient supply of degradable dietary protein to the rumen usually improves ruminal digestion of dietary carbohydrates and efficiency of microbial cell synthesis, consequently increases microbial protein yield (Hoover and Stokes 1991). In addition, a high availability of rumen-degradable protein, peptides and amino acids largely improves ruminal digestion of starch, cellulose (Hoover and Stokes 1991) and acid detergent fibre (ADF)

(Griswold *et al.* 1996). Furthermore, branched-chain fatty acids, namely isobutyric, isovaleric and 2-methylbutyric acid yielded from ruminal degradation of valine, leucine and isoleucine, respectively, are essential for optimum growth of some bacterial species (France and Siddons 1993).

Excessive fermentation of carbohydrates in the rumen decreases microbial yield, whereas excessive degradation of dietary N increases N loss from the animal as urinary urea (Russell *et al.* 1992; Nolan 1993). When a large amount of non-structural carbohydrates is fermented extensively in the rumen, ruminal pH declines and eventually growth of cellulolytic microbes is inhibited (Van Soest 1994). On the other hand, if the ammonia supply in the rumen is excessive relative to the energy supply, unused ammonia is absorbed through the rumen wall and finally converted to urea within the liver. A large proportion of this urea is further excreted in the urine, which leads to a loss of dietary N. Also, conversion of ammonia into urea within the liver requires ATP and also increases the catabolism of amino acids and hepatic production of glucose, which eventually brings about a loss of energy.

Many feeding standards can be applied to balance the ruminal supply of energy and nitrogen. For maintenance purpose, the ratio of effective rumen-degradable protein (ERDP) to fermentable metabolisable energy (FME) should be 9.0 g/MJ (AFRC 1993). For maximum microbial yield from the rumen, an optimum balance of nitrogen to energy should be 26.1 ± 1.3 g ruminally available (degraded and recycled) N/kg of rumen-degradable OM (RDOM) (NRC 1985), or 25 g rumen-degradable nitrogen (RDN)/kg of RDOM (Czerkawski 1986), or 27.0 ± 0.7 g RDN/kg of RDOM (Gunter *et al.* 1995).

3.2.4 Minerals

Microbial growth in the rumen requires a sufficient supply of minerals, in particular, sulphur and phosphorus. Sulphur is an essential component of microbial protein, and significantly interacts with copper and molybdenum in the rumen (Price *et al.* 1988). Although rumen microorganisms are able to synthesise sulphur-containing proteins by direct incorporation of pre-formed dietary or salivary amino acids, a sufficient supply of dietary sulphur is required if microbes themselves need to synthesise sulphur-containing amino acids.

As discussed by Gordon and Phillips (1995), rumen fungi, which play a unique role in degradation of the structural components of plant cell walls, are sensitive to dietary sulphur supplies. Low sulphur diets usually support a low fungal population in the rumen, and sulphur-containing supplements, such as methionine and sulphate, rather than elemental sulphur, dramatically increase the fungal population in the rumen.

Phosphorus is required for the synthesis of microbial DNA and RNA, and is a constituent of ATP. Although phosphorus can be recycled via saliva to the rumen and then incorporated into microbial cells, there is always a metabolic loss in faeces since digestion of phosphorus bound to microbial cell walls is incomplete (Van Soest 1994).

3.2.5 Dilution rate

Dilution rate is referred to as the fraction of the rumen fluid volume replaced per hour. Both *in vitro* (Isaacson *et al.* 1975) and *in vivo* (Harrison *et al.* 1975) studies show that microbial yield is increased with increasing dilution rate. The explanation usually given is that the ATP requirement for microbial cell maintenance is negatively related to the dilution rate. Thus microbial species with a high growth rate utilise ATP more efficiently for growth (Van Soest 1994). For example, about 31% of the total ATP generated by fermentation is used by rumen microbes for maintenance when they have a growth rate of 0.05/h, and only 10% when the growth rate is 0.20/h (Russell and Strobel 1993).

Furthermore, a high dilution rate of rumen fluid increases bacteria yield from the rumen by reducing the number of bacteria engulfed by protozoa (Van Soest 1994) and the amount of bacterial protein recycled in the rumen (Vercoe 1996).

3.2.6 Diet composition

The efficiency of microbial protein synthesis for forage diets is higher than that for concentrate diets. A diet containing less than 40% of forages or 20% of neutral detergent fibre (NDF) lowers microbial yield from the rumen (NRC 1985). Some *in vitro* studies indicate that when, on a DM basis, diets contains less than 20% NDF, every 1% decrease in the NDF content results in a 2.5% reduction in microbial yield (Russell *et al.* 1992).

3.2.7 Other factors

The yield of microbial biomass in the rumen can be affected by ruminal gas pressure of hydrogen (Nolan 1993), feeding frequency (Nolan and Leng 1989), nicotinic acid and molar proportion of butyrate (Nolan and Leng 1989). A slightly low ruminal pH may increase bacterial yield due to its defaunating effect, but when ruminal pH is lower than 6.0, both the microbial yield and efficiency of microbial protein synthesis are decreased due to the depressed growth of cellulolytic bacteria (McDonald *et al.* 1988; Russell and Strobel 1993).

3.3 Measurements of microbial protein yield

Microbial biomass flowing into the small intestine is usually the major source of amino acids available for absorption and utilisation by ruminants, i.e., most absorbed amino acids are of

microbial origin. It is therefore necessary to determine the yield of microbial protein from the rumen in order to assess the N status of animals or to make a decision about whether they require supplementation. As reviewed by Broderick (1992), various approaches have been made to quantify microbial protein yield from the rumen. Among these approaches, internal microbial marker methods and external isotopic marker methods are commonly used. Some amino acids and nucleic acids which are assumed to be present only in rumen microorganisms, such as 2, 6-diaminopimelic acid (DAP), D-alanine, 2-aminoethylphophonic acid (AEP) and phosphatidylcholine (PC) have been used as internal microbial markers, whereas ^{15}N, ^{32}P, ^{35}S, ^{14}C have been used as external isotopic makers. These methods require post-ruminally cannulated animals and depend on estimates of digesta flow rate. Their procedures of measurement are complex and subject to considerable errors.

More recently, urinary excretion of purine derivatives has been used for estimating microbial protein yield from the rumen (Chen and Gomes 1992). This approach is detailed in the following Chapter.

Chapter 4

4. Prediction of Microbial Protein Yield from Urinary Excretion of Purine Derivatives

Nucleic acids, i.e., ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), are composed of nitrogenous components (purine or pyrimidine bases), a pentose (ribose or deoxyribose) and phosphoric acid. They contain genetic information and control protein synthesis in living organisms.

The main pyrimidine bases in nucleic acids are cytosine, thymine and uracil, whereas the main purine bases are adenine and guanine. Combining one of the above nitrogenous bases with a pentose forms a nucleoside. The latter can be further esterified with phosphoric acid to yield a nucleotide (McDonald *et al.* 1988).

4.1 Degradation and synthesis of nucleic acids in the rumen

4.1.1 Degradation of nucleic acids in the rumen

Nucleic acids entering the rumen are predominantly in the ingested feeds. Nevertheless, intake of dietary nucleic acids is quite low since the nucleic acid content of common ruminant feeds ranges from 1 to 50 g/kg DM (McAllan 1982). Mucosal secretion and sloughed mucosal cells may also contribute small amounts of purines, allantoin and uric acid to the total ruminal nucleic acids (Ferguson and Terry 1954).

DNA and RNA entering the rumen are degraded rapidly by microorganisms into nucleotides, nucleosides, and purine and pyrimidine bases (Smith 1975). *In vitro* studies show that the degradation of DNA is a little slower than that of RNA. Within 4 h of incubation, RNA is entirely degraded into the bases including xanthine, hypoxanthine and uracil, but DNA is only degraded into oligo- and mononucleotides together with the bases xanthine, hypoxanthine, uracil and thymine. Further catabolism of these nucleic acid derivatives and bases yields acetic acid, ammonia and carbon dioxide from xanthine, guanine, hypoxanthine and uric acid; β-alanine from uracil; β amino-isobutyric acid from thymine (McAllan and Smith 1973).

4.1.2 Synthesis of microbial nucleic acids in the rumen

Rumen bacteria can utilise the pre-formed nucleic acid bases and nucleosides from the degraded nucleic acids for the synthesis of microbial nucleic acids. For example, bacterial nucleic acids are mainly synthesised from guanine, uracil, xanthine, allantoin and uric acid as sources of carbon and nitrogen. Meanwhile, rumen protozoa can incorporate free adenine, guanine, uracil, nucleosides and nucleotides into their nucleic acids (McAllan 1982).

4.2 Metabolism of nucleic acids in ruminants

4.2.1 Digestion and absorption of nucleic acids

Since nucleic acids and their derivatives entering the rumen are rapidly and extensively degraded by rumen microorganisms, the nucleic acids flowing to the small intestine are predominantly microbial in origin (Topps and Elliott 1965; McAllan and Smith 1973). Direct evidence for this is that the ratio of RNA to DNA in rumen digesta is similar to that of mixed rumen bacteria. This ratio is constant over a wide range of diets, which indicates that the dietary contribution to the ruminal nucleic acids is negligible (McAllan 1982). Chen *et al.* (1991) also reported that when a large amount of allantoin was infused into the rumen of sheep and steers, none was recovered in the urine. This was probably because allantoin was rapidly degraded in the rumen.

Microbial nucleic acids leaving the rumen are extensively digested in the small intestine. RNA and DNA are first broken down into 3' or 5'-mononucleotides by pancreatic ribonuclease and deoxyribonuclease together with phosphodiesterases (McAllan 1982). Purine nucleotides are further hydrolysed into purine nucleosides and free bases which are readily absorbed from the small intestine.

The rate of digestion and absorption of nucleic acids in the small intestine is high. The apparent digestibilities of DNA and RNA are around 75-85% and 80-90%, respectively (McAllan 1982), whereas the true digestibilities of microbial nucleic acids and microbial purines are 86% (Storm *et al.* 1983) and 91% (Chen *et al.* 1990b), respectively. McAllan (1982) reported that 97% of the free nucleic acids infused into the small intestine of steers was digested, and most RNA and DNA disappeared in the first one-quarter part of the small intestine of sheep and steers. Chen and Gomes (1992) proposed that the digestibility of microbial purines was 85%.

4.2.2 Synthesis of purines in ruminant tissues

Purines are synthesised in ruminant tissues through two pathways, namely *de novo* (biosynthesis) and via the salvage pathway. The *de novo* pathway begins with 5-phospho-ribosyl-1-pyrophosphate to synthesise purines (Lehninger 1977), whereas the salvage pathway utilises

both absorbed and endogenous purine nucleosides and free bases to synthesise nucleotides and nucleic acids. In cattle, the salvage pathway does not exist due to a high level of xanthine oxidase in most of their body tissues which converts all absorbed purines into uric acid (Chen and Gomes 1992). Precursors required for the synthesis of purines in ruminant tissues include glycine, serine, aspartic acid, glutamine, formate, bicarbonate, dietary and cellular nucleoprotein (Topps and Elliott 1965).

Studies on the salvage pathway show that the synthesis of nucleotides, e.g., adenosine monophosphate (AMP) and guanosine monophosphate (GMP), is through combining 5-phospho-ribosyl-1-pyrophosphate with purine bases catalysed by nucleotide pyrophosphorylases, but the synthesis of nucleosides through the combination of ribose-1-phosphate with purine bases by nucleoside phosphorlyases is less important (Lehninger 1977).

4.2.3 Catabolism of purines in ruminant tissues

Degradation of purines occurs in intestinal mucosal cells, the blood and the liver of ruminants. The concentration of xanthine oxidase in intestinal mucosal cells and the blood of cattle is higher than that of sheep so that the urinary concentration of xanthine and hypoxanthine of cattle is lower than that of sheep (Chen *et al.* 1990a). In contrast, the liver of sheep has high levels of xanthine oxidase and uricase and thus it is the major site for their purine catabolism to allantoin (Chen *et al.* 1990b).

In ruminant tissues, DNA and RNA can be degraded by nucleases and diesterases into oligonucleotides, mononucleotides, nucleosides and free purine bases (Kahn and Nolan 1993). The resultant nucleosides and free purine bases can be utilised through the salvage pathway or further degraded and excreted. Therefore, purine derivatives present in the blood are either of exogenous origin (absorbed from the gut) or of endogenous origin (from the catabolism of tissue nucleic acids).

The exogenous and endogenous purines in excess of requirements for salvage purposes are completely catabolised into their metabolic end-products including hypoxanthine, xanthine, uric acid and allantoin and these are eventually excreted (McAllan 1982; Chen and Gomes 1992) (Figure 4.1 and 4.2).

Figure 4.1. *Catabolism of purines to purine derivatives in ruminant tissues (Chen and Gomes 1992).*

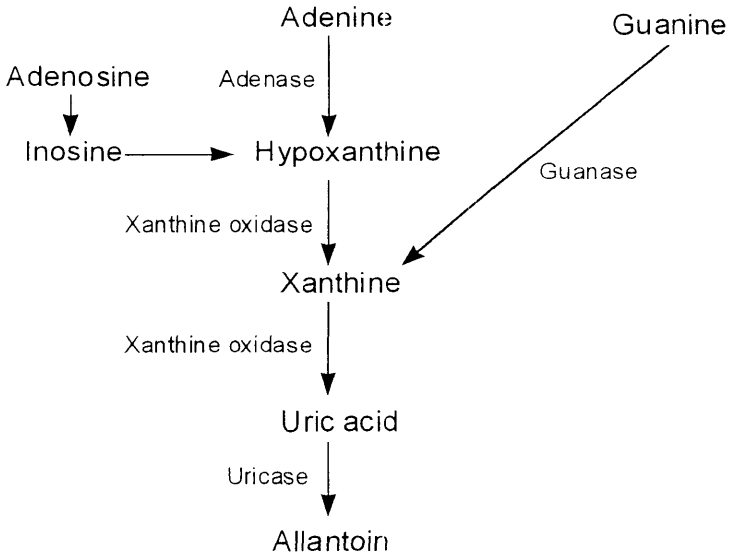
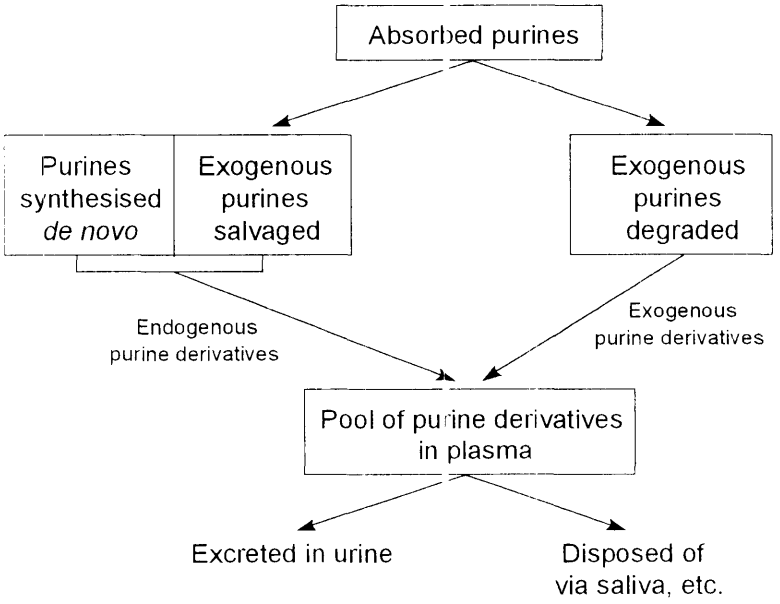


Figure 4.2. *The relationship between urinary excretion of purine derivatives and absorption of exogenous purines in sheep (Chen et al. 1990b)*



Purine derivatives entering the blood are removed rapidly by renal excretion. Plasma allantoin is removed at a fractional rate of 0.3/h and excreted in the urine at 0.23/h (Chen *et al.* 1991). The high rate of plasma clearance of purine derivatives implies that the renal excretion may reflect any changes in purines absorbed.

Major purine end-products are excreted via the kidney, and the rest is disposed of by non-renal routes, e.g., by excretion into the gut via saliva (Chen *et al.* 1990b) (Figure 4.2). Chen *et al.* (1991a) summarised available data and concluded that the recovery of absorbed microbial purines as urinary purine derivatives was 0.84 for sheep, and 0.85 for cattle, which indicated that there was a loss of purine derivatives by non-renal routes.

4.3 Prediction of microbial protein yield from urinary excretion of purine derivatives

Urinary excretion of allantoin and uric acid has been recognised as a possible useful index of microbial biomass synthesised in the rumen since a highly significant correlation exists between the concentration of ruminal nucleic acids and the level of urinary allantoin and uric acid N in sheep (Topps and Elliott 1965).

Purine derivatives excreted in the urine mainly originate from the degradation of absorbed microbial nucleic acids and the excretion of purines is directly related to their absorption (Chen *et al.* 1990a). The relationship between urinary excretion and absorption of purines may be linear if the absorbed purines are not salvaged by body tissues and the endogenous production of purine derivatives remains constant (Chen *et al.* 1991; Chen *et al.* 1992b). However, the relationship is likely to be curvilinear since the *de novo* synthesis of purines decreases with the increasing absorption of purines. Chen *et al.* (1990b) therefore proposed a curvilinear relationship describing urinary excretion of purine derivatives (Y, mmol/d) and absorption of microbial purines (X, mmol/d) in sheep:

$$Y = 0.84\ X + 0.\ 50\ W^{0.75}\ e^{-0.25X}$$

where $W^{0.75}$ is metabolic body weight (kg), the slope of 0.84 is the recovery of absorbed purines as purine derivatives in the urine. The last portion of the equation represents the net contribution of endogenous purine derivatives to total purine excretion after correction for the salvaged microbial purines by the animal, in which the constant of -0.25 is the replacement rate of the *de novo* synthesis of purines by the salvage of absorbed purines, and the coefficient of 0.150 mmol/kg $W^{0.75}$.d is the excretion rate of endogenous purines when no exogenous purine is absorbed.

Estimation of microbial protein yield from the urinary excretion of purine derivatives is based on the assumption that microbial nucleic acid synthesis is correlated with microbial protein production, and the nucleic acids flowing out of the rumen to the small intestine are predominantly of microbial origin. Absorbed purines are degraded in ruminant tissues and excreted in the urine as their derivatives, i.e., allantoin, uric acid, xanthine and hypoxanthine. With knowledge of the ratio of purine N to total N in rumen microorganisms, microbial protein absorption can be calculated from the estimate of purine absorption.

The urinary allantoin can be used as a single parameter for estimating microbial protein yield from the rumen. Puchala and Kulasek (1991) observed that not only urinary allantoin, but also total urinary purine derivatives were highly correlated with microbial nucleic acids entering the duodenum. However, Chen *et al.* (1991a) recommended that, for sheep, it was more accurate to use urinary excretion of all purine derivatives rather than allantoin alone since the proportion of allantoin in total urinary excretion of purine derivatives increased with increasing excretion of total purine derivatives (Balcells *et al.* 1990; Chen *et al.* 1990a; Chen *et al.* 1992b; Fujihara *et al.* 1993) or increasing energy intake (Fujihara *et al.* 1991; Giesecke *et al.* 1994). This possibly results because endogenous purines are mainly catabolised in body tissues into hypoxanthine, xanthine and uric acid, whereas exogenous purines are catabolised in the liver into allantoin due to high levels of hepatic xanthine oxidase and uricase (Chen *et al.* 1990b).

In the way the method is usually carried out, complete collections of urine for several days are required to enable a good estimate of the rate of urinary excretion of purine derivatives. If simpler procedures could be developed, the method would be even more useful. Although urinary excretion of purine derivatives is a function of their plasma concentrations and their glomerular filtration rates (Chen and Gomes 1992; Kahn and Nolan 1993), the plasma concentration of purine derivatives is unlikely to be a reliable marker of microbial protein yield. Chen *et al.* (1995) noted that, although the plasma concentration of purine derivatives showed little diurnal variation, the tubular load of purine derivatives (plasma purine derivative concentration × glomerular filtration rate), rather than the plasma concentration of purine derivatives, was highly correlated with the urinary excretion of purine derivatives. Chen *et al.* (1995) also noted that the ratio of total purine derivatives to creatinine of spot urine was relatively constant and was linearly related to daily excretion of urinary purine derivatives. This ratio, measured from spot urine samples of sheep fed *ad libitum*, may be another practical predictor of microbial protein yield from the rumen.

4.4 Accuracy of estimation of microbial protein yield from urinary purine derivative excretion

An ideal estimation of microbial protein production from urinary excretion of purine derivatives requires that no endogenous purines are excreted as urinary purines (Kahn and Nolan 1993). Purine derivatives excreted in the urine usually originate from endogenous (via biosynthesis and salvage pathways) as well as exogenous (microbial) purines. Therefore, the change in the contribution of endogenous purines to the urinary purine derivative excretion biases the estimate of microbial purines absorbed.

The production of endogenous purine derivatives is almost constant in fasting sheep or goats, or in animals given purine-free diets (Fujihara *et al.* 1987; Fujihara *et al.* 1991). The daily mean excretion of endogenous purine derivatives in sheep ranges from 150 to 190 μmol/kg $W^{0.75}$ (Fujihara *et al.* 1987; Balcells *et al.* 1990; Chen *et al.* 1990b; Chen *et al.* 1991). The *de novo* synthesis of purines diminishes as the supply of exogenous purines increases, and it is completely inhibited by an adequate supply of exogenous purines due to the progressive replacement of the *de novo* purine synthesis by the salvage of exogenous purines (Chen *et al.* 1990b; Chen and Gomes 1992; Kahn and Nolan 1993).

The production of endogenous purine derivatives can be affected by the nutritional and physiological status of animals. The excretion of endogenous purine derivatives increases with liveweight (Chen *et al.* 1992b). However, Antoniewicz and Pisulewski (1982) reported that when the maintenance requirements of animals were satisfied, the endogenous purine derivative production was very low and 92-99% of total allantoin excreted in the urine originated from the absorbed microbial purines. Chen *et al.* (1990b) further indicated that, when animals were fed at a level higher than 0.8 times of their maintenance energy requirement (i.e., total urinary excretion of purine derivative was greater than about 0.6 mmol/kg $W^{0.75}$), the endogenous purine excretion was negligible.

The assumption that the ratio of purine N to total microbial N is constant, i.e., 0.116, has been made for calculation of microbial protein supply from the urinary purine excretion. However, this assumption may not hold under different feeding conditions. McAllan (1982) noted that the nucleic acid content of rumen bacteria was variable, and the ratio of RNA-N to total bacteria N varied with microbial growth rates. Ideally, a sample of rumen microbes should be analysed for purine : DM and N : DM for each treatment during each experiment (Nolan, J.V., pers. comm., 1997).

A constant ratio of renal to non-renal excretion of purine derivatives, i.e., 0.84 : 0.16, has been proposed by Chen and Gomes (1992). However, it is not known whether the loss of purine

derivatives by non-renal routes varies with different conditions. In dairy cows, Giesecke *et al.* (1994) reported that the allantoin excreted in milk accounted for about 1-4% of total allantoin excretion. In sheep, Chen *et al.* (1990a) reported that the saliva contained a significant amount of uric acid and allantoin, and consequently the salivary allantoin was equivalent up to 15% of allantoin excreted in the urine. In contrast, Kahr (1996) reported that 94% of allantoin injected intravenously was recovered in the urine of sheep, and only 1% of injected allantoin entered the rumen via saliva. Kahn (1996) also administered ^{14}C-allantoin intravenously and found only negligible amounts of ^{14}C in rumen carbon dioxide in sheep.

As with other marker methods, the purine derivative technique provides a relative rather than an absolute estimate of microbial protein yield from the rumen. However, estimation of microbial protein yield by this technique can be expected to be more accurate when ruminants are fed at or above the maintenance level compared with animals fed below the maintenance. In addition, this technique provides a non-invasive tool for comparison of microbial yield from the rumen of animals under similar dietary conditions and for evaluation of rumen function.