Chapter 1 Introduction

Microbes (bacteria, protozoa and fungi) within the rumen lead to significant differences in digestive physiology between ruminants and monogastric animals. It is the action of the ruminal microbes that enables ruminants to utilise the cellulosic materials in feeds such as grasses and other fibrous materials which have little or no nutritive value to monogastrics (Van Soest, 1994). Ruminal fermentative digestion of dietary substances carried out by the microbes, converts raw materials into volatile fatty acids (mainly acetate, propionate, and butyrate) and microbial cells (mainly protein), all of which are available for metabolism by the tissues of the host animal (Preston and Leng, 1987).

Attempts to maximise the contribution of microbial protein to the host is one of the most important aspects in nutritional management of ruminants. Eliminating ciliate protozoa from the rumen (defaunation) has been studied for many years as one strategy to achieve this goal. The approach capitalises on the fact that protozoa are predators of other ruminal microbes and competitors for nutrients needed by the other microbes to grow. Due to the sequestration of protozoa in the rumen, however, the post-ruminal flow and contributions of protozoal cells to the host are also not great as expected from measurement of protozoal mass in the rumen. Consequently, there is a general finding in defaunation studies of increased microbial protein synthesis and efficiency (Ushida *et al.*, 1986; 1989) as well as an increase in the availability of protein to be absorbed from the intestines (see Veira, 1986). The impact of this change is most evident on animal performance when the feeds consumed are low in protein content, and animals are in conditions where amino acids are considered as the primary limiting nutrient.

Apart from nitrogen, other nutrients such as lipids are also produced in the rumen. Lipid production in the rumen is known (Harfoot, 1981), but studies on this synthesis have not been as extensively undertaken as those of nitrogen. An increase in ruminal production of lipids is undoubtedly beneficial because through this process, 1) the wastage of energy in the form of methane may be reduced; 2) enhanced long chain fatty acid (LCFA) supply may reduce the need for glucose to be used in LCFA synthesis by the animal; and 3) the absorbed LCFA are utilised more efficiently for growth than are volatile fatty acids (VFA). While it is known that protozoa are higher in lipid content than bacteria, and approximately three-quarters of ruminal lipids is associated with the protozoa (Katz and Keeney, 1967; Keeney, 1970), there has been no study carried out on the effect of defaunation of the ruminal production of LCFA.

This study aims to investigate the effects of the presence or absence of ciliate protozoa in the rumen, and of methionine supplement when protozoa are present, on the ruminal production of LCFA.

Chapter 2 Literature Review

2.1 Protozoa

2.1.1 Types present

The protozoal population in the rumen is composed of flagellate and ciliate species, but the latter group is more numerous and the more important (Williams and Coleman, 1988). The ciliates consist mainly of entodiniomorphs and, to a lesser degree, of holotrichs. Many factors affect the number of protozoa present in the rumen, and these have been discussed extensively by Williams and Coleman (1992).

Entodiniomorphid protozoa are recognised to be more efficient in engulfing particulate matter, even though they are also able to utilise soluble compounds (Williams, 1989). Approximately 90% of the protozoal population in animals given forage diets are entodiniomorphs (Jouany, 1989). On the other hand, the holotrichs are more capable of assimilating the soluble compounds, and their ability to utilise insoluble, particulate matter is inferior to that of the entodiniomorphs. There is evidence that the number of holotrichs increases when animals are fed diets rich in soluble carbohydrates and decreases when animals are fed with low digestibility cellulosic carbohydrates (Jouany, 1989).

The population of mixed rumen protozoa can be high in animals fed a moderate level of concentrate (Eadie *et al.*, 1970), due mainly to the increased number of Entodinia spp. (Dennis *et al.*, 1983). The entodiniomorphs are less sensitive to low ruminal pH compared with the holotrichs, and the last to disappear under

starch-rich feeding (Lyle *et al.*, 198). Van Soest (1994) stated that engulfment of a large quantity of starch may itself cause the lysis of the protozoal cells (predominantly holotrichs) due to overgorgement over a short time-frame. O'Kelly and Spiers (1992) observed that the population of the entidiniomorphs in the rumen fluid decreased between 4 and 8 hours after feeding lucerne hay once daily, while that of holotrichs increased. This was not found when a low-quality diet (*Heteropogon contortus*) was given or diet was offered continuously. These authors argued that by the time of feeding, entodiniomorphs might migrate from fluid to feed particles while holotrichs were evacuated from reticulum to rumen.

2.1.2 Effects on fermentation

2.1.2.1 Volatile fatty acids (VFA)

The main VFA produced by ciliate protozoa are acetate and butyrate, while propionate is produced in only small amounts (Williams and Coleman, 1988). Therefore, most studies have shown that eliminating protozoa from the rumen increases the proportion of propionate and reduces that of butyrate (*e.g.* Whitelaw *et al.*, 1984b; Punia *et al.* 1987; Hsu *et al.*, 1991a) and acetate (Whitelaw *et al.*, 1984b). Results differing from these, however, have been obtained in some other studies. Rowe *et al.* (1985) reported that there was no difference in the proportions of propionate and butyrate between defaunated and faunated animals. The proportion of propionate has been reported to be lower (Habib, 1988) and that of butyrate to be higher (Bird and Leng, 1978) in defaunated than in faunated animals. In the study of Hsu *et al.* (1991a), acetate proportion was also increased following defaunation. Veira (1986) has concluded that there is no consistency in the effect of defaunation on the proportion of VFA.

2.1.2.2 *Methane*

It is well recognised that methanogenic bacteria are closely associated with protozoa. This association may be in ectosymbiotic as well as endosymbiotic forms. Stumm *et al.* (1982) found that 10 - 20 % of ruminal methane-producing bacteria were attached to ciliate protozoa. The endosymbiotic association between the methanogens and the ciliate protozoa has been demonstrated by Finlay *et al.* (1994). These workers found that the methanogens might occupy up to 1 % of the internal volume of *Entodinium spp.* and *Dasytricha ruminantum*. This symbiosis of methanogenic bacteria and protozoa will prevent the flow of the methanogens out of the rumen due to sequestration of their host protozoa.

The attachment of methane-producing bacteria to the protozoal surface is believed to facilitate the uptake of hydrogen required by the methanogens for methane synthesis (Wollin and Miller, 1988). This interspecies hydrogen transfer does not have a clear benefit to protozoa, but Stumm and Zwart (1986) have suggested that the low hydrogen concentrations may favour a more efficient reoxidation of reduced protozoal coenzymes. Coleman (1989) proposed that there might be other reasons apart from obtaining hydrogen for the attachment of the methanogens to the protozoal surface because a massive association between these two still occurred under a culture supplied with 95 % hydrogen.

The defaunated rumen is, therefore, expected to produce less methane than the normal one. Some *in vivo* studies have demonstrated that methane production could be reduced by 30 - 45 % through defaunation (Jouany *et al.*, 1981; Whitelaw *et al.*, 1984a; Finlay *et al.*, 1994). In the defaunated rumen, the hydrogen produced from acetate and butyrate synthesis may be rechanneled toward propionate production rather than into methane. Whitelaw *et al.* (1984a)

observed a close inverse relationship between propionate and methane productions. When the reduced methane production due to defaunation is not followed by an increase in that of propionate, other hydrogen acceptors such as biohydrogenation (Czerkawski, 1972) and microbial-protein synthesis (Czerkawski, 1975) may have been involved in the uptake of the available hydrogen.

2.1.3 Effect on microbial-protein flow

It is generally found that the duodenal flow of microbial non-ammonia nitrogen is higher in defaunated than in faunated animals (*e.g.* Ushida *et al.*, 1990; Meyer *et al.*, 1986). The increased microbial-nitrogen flow following defaunation is mainly associated with the increase in the efficiency of microbial-protein synthesis (Veira *et al.*, 1983; Meyer *et al.*, 1986). When the efficiency is expressed as the amount of microbial nitrogen synthesised per unit organic matter fermented, defaunation can improve the efficiency by 40 - 125 % (see Jouany, 1996 for review). The increased efficiency of microbial-protein synthesis in defaunated animals, and the resulting total increase in duodenal flow, may be caused by the increase in synthesis of net microbial-protein and/or reduction in digestion of organic matter for the same protein production. The improved net microbial-protein synthesis caused by defaunation probably results from the facts that protozoa 1) are bacteria predators, 2) are competitors for nutrients and 3) tend to be retained within the rumen. This has been evident in some studies in which the net bacterial synthesis could be improved by 16 - 39% due to defaunation (Rowe *et al.*, 1985; Kayouli *et al.*, 1986; Ushida *et al.*, 1986). Furthermore, Hegarty (1989) reported that the nitrogen content of fluid-phase bacteria was higher in the rumen of defaunated compared with faunated animals which may give an additional contribution to the increased microbial-nitrogen flow to the duodenum.

Some dietary factors may affect the proportions of microbial nitrogen escaping to the small intestine following defaunation. Ivan *et al.* (1991) observed that the increased microbial-nitrogen flow to the duodenum in the absence of protozoa was greater when supplementing animals with insoluble proteins than soluble ones. This is because protozoa are less able to metabolise soluble proteins than insoluble proteins (Michalowsk , 1989). Therefore, protozoa under this circumstance will satisfy their nitrogen requirements by engulfing bacteria (Michalowski, 1989) and thus reduce the amount of bacterial nitrogen flowing down to the small intestine. The liquid-phase bacteria may be more susceptible compared with the particle-phase bacteria to the predatory activity of protozoa (Orpin and Letcher, 1984). Reduced availability of attachment sites as may occur with low-forage high-concentrate feeding may increase the predation of bacteria by protozoa and reduce the microbial-nitrogen flow (McAllister *et al.*, 1994).

2.1.4. Productive responses to defaunation

It has been shown that defaunation of ruminants results in greater liveweight gain (Bird and Leng, 1978), higher birthweight (Bird, 1989), more wool production (Bird and Leng, 1984) and milk production (Yang and Varga, 1989). These results can be explained by the fact that the amount of both dietary and microbial nitrogen flowing to the small intestine is greater in defaunated animals (Veira *et al.*, 1983; Kayouli *et al.*, 1986), providing more amino acids to be absorbed and utilised by the animals. The effect of defaunation on liveweight gain, however, is sometimes confounded by the changes in ruminal volume and retention time (Faichney and Griffiths, 1978; Orpin and Letcher, 1984). If the rumen fluid volume ~~and retention time~~ ~~are~~ is greater in defaunated animals compared with faunated ones, feed intake and rate of digestion may also be higher in defaunated animals with eventual beneficial effects on animal productivity (Williams and Coleman, 1992).

Nolan (1989) suggested that the consequence of the protozoa-free condition of the rumen was to increase the ratio of protein to energy in the materials absorbed by animals, improving their performance when amino acids were considered as limiting nutrients. Unless the digestibility of fibre has been depressed, the production responses to defaunation will be greatest when the animals are fed a low-protein, poor-quality diet (Williams and Coleman, 1992). The small adverse effect of defaunation on performance of animals receiving diets rich in starch or soluble sugars is likely to result from the positive role of protozoa in maintaining normal ruminal condition and fermentation under such diets. Protozoa are known to take up and store starch, thereby preventing its rapid fermentation and the accumulation of lactate and the drop of ruminal pH (Hobson and Jouany, 1988).

2.2 Microbial Growth and Efficiency

Microbial protein synthesised in the rumen plays a central role in the nutrition of ruminants. Achieving maximal growth of rumen microbes and efficiency of microbial-protein production in the rumen is one of the fundamental objectives of ruminant nutrition. Maximal rumen-microbial growth and efficiency are expected in turn to promote an increased availability of microbial protein for the animal and to reduce the amount of the expensive undegradable protein that needs to be supplied in diets. Preston and Leng (1987) have shown that the calculated ratio of protein to energy to be absorbed in the small intestine corresponds well with the efficiency of microbial growth in the rumen (expressed as g dry cells/mole ATP produced, Y_{ATP}; Table 2.1).

Table 2.1 The theoretical effect of different efficiencies of microbial growth on the P/E ratio available from the rumen of a steer consuming 4 kg of organic matter which is totally fermentable (Preston and Leng, 1987).

	The efficiency of microbial growth (Y_{ATP})			
	8	14	19	25
Microbial protein synthesised (g/d)	500	800	1010	1212
VFA produced (MJ/d)	41	34	30	26
Methane produced (MJ/d)	9.4	8.5	8.0	7.6
Heat (MJ/d)	6.4	5.1	4.3	3.1
P/E ratio (g protein/MJ)	12	25	34	47

2.2.1 Measurement of microbial-protein synthesis

Two measures of microbial-protein synthesis are important in understanding the supply and potential supply of the protein. First is the net microbial-protein synthesis in the rumen which may be measured as the protein leaving the rumen in digesta. This is useful because it defines how much microbial protein is available for absorption but it does not tell how much protein was synthesised then refermented in the rumen. To determine the extent of microbial-protein breakdown in the rumen also requires measurement of the total microbial protein synthesised in the rumen. Techniques for measuring net and total microbial-protein synthesis as well as the markers required for such estimates are described below.

2.2.1.1 *Microbial markers*

Many microbial substances have been studied for use as internal markers in order to differentiate and calculate the proportion of microbial protein in abomasal/duodenal digesta or the amount of microbial protein absorbed from the small intestine. These include diaminopimelic acid (DAPA) (Czerkawski, 1974), D-alanine (Garrett *et al.*, 1986), aminoethyl-phosphonic acid (AEP) and ribonucleic acid (RNA) (Ling and Buttery, 1978), individual purines and pyrimidines (Schelling and Byers, 1984), and total purines (Zinn and Owens, 1986). Amino acid profiles have also been proposed by Evans *et al.* (1975) to be used as an alternative reference to partition digesta protein into its dietary, microbial and endogenous origins.

External microbial markers, generally isotopes, are frequently infused intraruminally and are incorporated into microbial protein during its synthesis within the rumen. The most common ones are ^{35}S (Singh *et al.*, 1973; Walker and Nader, 1975; Kennedy *et al.*, 1980) and ^{15}N (Pilgrim *et al.*, 1970; Mathison and Milligan, 1971).

One method of estimating net microbial-protein synthesis which does not require cannulated animals is based upon the excretion of purine derivatives (allantoin, uric acid, xanthine and hypoxanthine) in the urine. The most important source of urinary purine derivatives excreted by ~~ruminants~~ sheep is the microbial nucleic acids, while the contributions of dietary and endogenous nucleic acids are relatively small (Fujihara *et al.*, 1987; Lindberg, 1989; Lindberg and Jacobsson, 1990). Of various purine derivatives voided in the urine, allantoin is the largest constituent (Lindberg and Jacobsson, 1990) and it has been most frequently used as an estimator of microbial-protein flow. Its use as a single estimator, however, requires further investigation. Balcells *et al.* (1991;

1993) suggested that allantoin was the sole purine derivative responsive to exogenous purine input, but Perez *et al.* (1996) stated that using allantoin as a single compound could oversimplify the method.

Some studies have been conducted to compare various microbial markers for the estimation of microbial nitrogen in digesta (*e.g.* Ling and Buttery, 1978; Siddons *et al.*, 1982; Whitelaw *et al.*, 1984a). Results obtained between markers, however, are variable and the differences are not consistent. Estimates of bacterial-nitrogen flow based on DAPA concentration are highly variable (Ling and Buttery, 1978; Whitelaw *et al.*, 1984a) and microbial-protein synthesis estimates obtained with DAPA are higher than with other markers (Siddons *et al.*, 1982). The concentration of DAPA is different between species of bacteria (Dufva *et al.*, 1982) and unrepresentative sampling of isolated bacteria could have contributed to this variation. The higher microbial-protein synthesis obtained with DAPA compared with other markers (Siddons *et al.*, 1982) may be due to the presence in digesta of dietary DAPA (Rahnema and Theurer, 1986) and/or of cell-unbound DAPA as a result of catabolism in the rumen (Denholm and Ling, 1989). It was suggested that ^{35}S and ^{15}N give more reliable estimates of microbial nitrogen compared with DAPA or amino acid profiles (Ling and Buttery, 1978; Siddons *et al.*, 1982; Whitelaw *et al.*, 1984a). The main source of errors which are often attributed to isotopic markers is the non-uniform distribution of the isotopes among microbial species due to different ability of microbes to incorporate dietary amino acids and peptides (Siddons *et al.*, 1982; Perez *et al.*, 1996).

2.2.1.2 *Total synthesis*

The total synthesis of rumen microbial protein may be tentatively simplified as the sum of microbial protein harvested in the abomasal digesta and that degraded within the rumen. In the tracer study of Nolan and Stachiw (1979), it was predicted that the total amount of microbial protein synthesised in the rumen was at least twice that of net synthesis, suggesting an extensive ruminal degradation of microbial protein.

a. *In vivo* measurement

In vivo microbial-protein synthesis can directly be measured from rumen contents in which microbes have been labelled with external microbial markers. A less than ideal procedure involves feeding animals with an isotope and then measuring the rate of its decline after the cessation of feeding the isotope. Singh *et al.* (1973) utilised $Na^{35}SO_4$ and the relationship between specific radioactivity, SR (μCi/mg bacterial DM [dry matter]) with time (t) after cessation of feeding $Na^{35}SO_4$ was expressed as a single-exponential function:

$$SR_t = SR_o \exp^{-kt}$$

where SR_t = SR at time t
SR_o = SR at time zero
k = rate constant (day^{-1})

This method is flawed by the inability to account for the proportion of dose being incorporated into microbes and by the unsuitability of a single exponential function to describe the changes in the specific radioactivity that occur.

Another direct method of estimating microbial-protein synthesis was developed by Walker and Nader (1975). They assumed that the rate of flow of microbial-

protein from the rumen was equal to the rate of its synthesis under steady state conditions. The ruminal outflow rate of microbial-protein was estimated from the knowledge of microbial N:S ratio and outflow rate of microbial S (estimated from the decline of ^{35}S). Microbial N production was calculated as follows:

$$\frac{\text{Total}^{3}\text{S in rumen digesta protein}}{\text{SR of microbial S}} \times k_1 \times k_2$$

where k_1 = rate of flow of microbial S from the rumen
k_2 = N:S ratio of microbes

The above principles of calculation also apply when using stable isotopes such as ^{15}N. Some of the tracers, however, may not be incorporated and may be lost (Nolan and Leng, 1983) resulting in overestimation of microbial-protein production if correction is not made. In addition, the ^{15}N method is generally based upon the assumption that rumen microbes synthesise all of their proteins from ammonia, while it has been shown that they may also utilise amino acids and peptides as nitrogen sources (Maeng *et al.*, 1976).

Nolan and Leng (1983) proposed the following formula in which the possible losses of ^{15}N to pools other than microbes were taken into account to give higher accuracy of estimation:

$$\frac{\text{Rate of infusion of } {}^{15}NH_3 - \left(\text{absorption of } {}^{15}NH_3 + \text{outflow of } {}^{15}NH_3 \text{ in rumen digesta}\right)}{\text{Enrichment of microbial N}}$$

The above procedures for measuring total microbial-protein synthesis within animals are not routinely used. This is primarily because of the need to have the marker exclusively and instantaneously present in the microbial pool. The most promising *in vivo* technique is that developed in this laboratory by Krebs (1987) in which microbes were labelled by a short high activity infusion into donor

animal before the organisms were relocated into a comparable sheep which had not been infused with the isotope.

b. *In vitro* measurement

Some *in vitro* methods of measuring the total microbial growth have also been proposed. Al-Rabbat *et al.* (1971) stated that the total growth of microbes *in vitro* could be estimated by measuring the total production of VFA after knowing ATP production per mol VFA and yield of cell per ATP. This, however, makes large assumptions about the Y_{ATP} of the microbes. Van Nevel and Demeyer (1977) used ^{32}P to determine the total microbial growth because the incorporation of $^{32}PO_4$ into microbial cells, unlike labels in smaller pools , is independent of degradation of non-growing cells. The latter method assumes that the isotope is not liberated by the non-growing cell or reincorporated by the growing cell during incubation.

2.2.1.3 Net synthesis

The most frequent method employed in measuring net microbial-protein production in the rumen is indirect measurement from abomasal or duodenal digesta. To obtain the fraction of digesta which is microbial in origin, markers are required except when the amino acid profile is employed (Evans *et al.*, 1975). With internal microbial markers, any marker may be used and the method of calculation is similar. Taking DAPA as an example and assuming microbial non-ammonia nitrogen (NAN):DAPA ratio is constant, the quantity of microbial NAN in digesta can be calculated as follows (Nolan and Leng, 1983):

$$\text{Microbial NAN}_{[\text{DIGESTA}]} = \frac{\text{Microbial NAN}}{\text{DAPA}}_{[\text{RUMEN}]} \times \text{DAPA}_{[\text{DIGESTA}]}$$

The quantity of microbial NAN and its passage into the duodenum is calculated from a knowledge of the concentration of microbial NAN in digesta DM and the passage of digesta DM.

A similar calculation is applied when using isotopic markers such as ^{35}S. The isotope will be incorporated by rumen microbes into microbial methionine, and as shown below, the ratio of specific activity (SR) of digesta (D) and microbes (M), D:M, provides the proportion of digesta methionine which is of microbial origin (Ling and Buttery, 1978).

$$\text{Total microbial N} = \frac{\text{SR of D}}{\text{SR of M}} \times \frac{\text{Digesta methionine passage}}{\text{Methionine: total N for M}}$$

The passage of methionine into the site of sampling is calculated from the methionine contents of digesta DM and the passage of digesta DM.

The substantial difference between total microbial synthesis and net microbial synthesis results from intrarumiral recycling of microbial protein. As reviewed by Morrison and Mackie (1996), there are three factors involved in this process, namely, protozoa (via engulfment of bacteria and lysis of protozoal biomass), bacteriophage (known as a parasite of bacteria), and nutrient starvation.

The first quantitative study indicating the intraruminal recycling of microbial protein was conducted by Nolan and Leng (1972) in which approximately 30 % of microbial protein synthesised were indirectly shown to be recycled through the ammonia-N pool. Subsequent studies (Nolan and Stachiw, 1979; Firkins *et al.*, 1992) reported even higher value than this (50 - 75 %). Aharoni *et al.* (1991) fractionated microbial NAN into rapidly and slowly turning over microbial NAN pools. The first one incorporated NH3-N into amide N very rapidly, while the latter converted the amide N into purine N more slowly. These authors

found that 96 and 62 % of the total N entering the rapidly and slowly turning over pools, respectively, were returned to the NH3-N pool.

The basic assumption held in the above studies, that is, all microbial protein N recycling passes through the ammonia pool, may have led to an underestimation of the amount of microbial N being recycled. Direct engulfment of bacteria by protozoa (Coleman and Sandford 1979) does not require ammonia production before reuse of amino acids. Similarly protozoal N may return to the bacterial pool without passing through the ammonia pool (Cottle, 1980). Both bacteria (Blake *et al.*, 1983) and protozoa (Leng and Nolan, 1984) actively excrete amino acids which may directly be reincorporated without being preceded by the deamination process.

2.2.2 Measurement of microbial efficiency

2.2.2.1 Y_{ATP}, $Y_{substrates}$

Microbial catabolism of substrates generates ATP which is used as the energy source for their anabolic processes. From the depletion of the added substrate, the efficiency of microbial-protein synthesis can be expressed as $Y_{substrates}$. If end-products of fermentation are known, the pathway of fermentation and the amount of ATP generated can be predicted, allowing the efficiency to be expressed as Y_{ATP}.

Bauchop and Elsden (1960) introduced the concept of Y_{ATP} (the weight of dry cell [g] synthesised for every mol of ATP generated from substrate fermentation). The theoretical value of Y_{ATP} calculated based on the average chemical composition of microbial cells produced and assuming that all ATP generated

are used for growth is 32 (Stouthamer, 1973), but the observed Y_{ATP} values for mixed rumen bacteria (*e.g* Satter and Slyter, 1974; Isaacson *et al.*, 1975) or many of individual bacteria (Hespell and Bryant, 1979) are far less than this. The discrepancy between the theoretical and observed values of Y_{ATP} is generally attributed to the use of ATP for maintenance purposes, and $Y_{ATP/MAX}$ in which the use of ATP for maintenance is taken into consideration has been proposed by Stouthamer and Bettenhausen (1973). Accounting for the use of ATP for maintenance, however, does not yield an adjusted Y_{ATP} as high as that theoretically possible (DeVries *et al.*, 1970). Russell and Cook (1996) stated that the maintenace energy could not be used solely to explain such discrepancies.

In vivo experiments usually express the efficiency of microbial-protein synthesis in the rumen as microbial DM or nitrogen (protein) produced per unit of dietary organic matter (truly or apparently) digested in the rumen. Nocek and Russell (1988) stated that expressing cell yields as protein rather than DM would give less variation in efficiency at a given amount of energy. This is because the ratio of cellular proteins and carbohydrates is not constant due to possible accumulation of polysaccharides (McAllan and Smith, 1977). Furthermore, the energy cost of synthesising cellular proteins and carbohydrates is not the same. According to Czerkawski (1986) who recalculated 65 individual data sets, the efficiency of microbial-protein synthesis in the rumen was about 121 g crude protein/kg organic matter truly fermented in the rumen.

2.2.2.2 *Factors limiting efficiency of microbial-protein synthesis*

Many factors affect the efficiency of microbial-protein synthesis in the rumen. It is outside the scope of this review, however, to discuss all of them comprehensively, and only a brief discussion on some factors, namely, the availability of nitrogen and energy sources, microbial maintenance energy requirement and rate of passage, will be given. More detailed discussions can be

found in numerous reviews (*e.g.* Hespell and Bryant, 1979; Stern and Hoover, 1979; Sniffen and Robinson, 1987 Nocek and Russell, 1988; Hoover and Stokes, 1991; Firkins, 1996).

a. Available nitrogen sources

Ammonia is the primary form of nitrogen utilised by rumen bacteria and protozoa to synthesise their cellular proteins (Leng and Nolan, 1984). Rumen ammonia concentration affects the efficiency of microbial-protein synthesis because it determines the pathways of ammonia assimilation and the utilisation of energy (Leng and Nolan, 1984). When ammonia is present in high concentrations, ammonia is assimilated without requiring ATP, and more ATP is available to promote a higher efficiency of protein synthesis compared with at low ammonia concentration. Schaefer *et al.* (1980) found that the assimilation of ammonia through the ATP-dependent pathway might lower the Y_{ATP} by about 14 %. Studies on the optimum ammonia concentration required for microbial-protein synthesis, digestion and/or feed intake, however, are variable, and values of between 3 and 20 mmol N/l have been reported (Satter and Slyter, 1974; Mehrez *et al.*, 1977; Boniface *et al.*, 1986, Perdok and Leng, 1989).

Smith (1979) stated that the ammonia concentration for an optimum microbial growth in the rumen is not a constant as it is dependent upon other variables. Microbes differ in their cell permeability to ammonia versus ammonium ions and certain species gain benefit at lower ammonia concentrations (Owens *et al.*, 1984). An indirect effect of ammonia on microbial-protein synthesis through the increase of pH has been suggested by Hespell and Bryant (1979). Ruminal bacteria are able to accumulate a labile nitrogen reserve (Smith, 1979) and the intracellular ammonia concentration may accumulate against a concentration gradient to 15 times the external concentration (Russell and Strobel, 1987). This

intracellular nitrogen reserve may be used for a short period of time when the extracellular ammonia concentration is low.

Another factor contributing to the variability in ammonia-concentration's impact on the efficiency of microbial-protein production is the availability of other nitrogen sources (amino acids and peptides). Ruminal accumulation of peptides and amino acids immediately after feeding is small (Broderick and Wallace, 1988), probably because they are incorporated directly and deaminated rapidly by microbes. The contribution of non-ammonia nitrogen sources to microbial protein synthesis may be up to 34 % (Nolan and Stachiw, 1979) and it has been found that amino acids increase the efficiency of microbial-protein production *in vitro* even though adequate ammonia is provided (Maeng *et al.*, 1976). Clark *et al.* (1992) suggested a deficiency of amino acids might lead to an uncoupled fermentation with degradation of dietary dry matter not being followed by microbial-protein synthesis due to unsynchronised availability of energy and nitrogen. Russell (1993) has found that *Selenomonas bovis* produces less energy as heat when amino acids are included in the growth medium.

The stimulative effect of amino acids and peptides on microbial-protein synthesis when substituted for ammonia, however, seems to be dependent upon the species of bacteria and the source of energy. Several studies have indicated that amino acids and peptides are stimulators for microbial growth when the provided energy sources are rapidly fermented (Maeng and Baldwin, 1976; Russell *et al.*, 1983; Chen *et al.*, 1987; Maeng *et al.*, 1989; Argyle and Baldwin, 1989; Cruz Soto *et al.*, 1994), but not when the energy sources are slowly fermented (Kempton *et al.*, 1979; Redman *et al.*, 1980). It is probable either that when energy is rapidly released from readily fermentable carbohydrates, amino acids and peptides are utilised as such rather than being deaminated to liberate

ammonia, or it is the natural preference of those microbes growing under this condition to utilise non-ammonia nitrogen sources.

b. Available energy sources

The available nitrogen sources will only be incorporated to form microbial protein when there are sufficient energy and other nutrients to support growth. Dietary carbohydrates are the main ruminal energy source, and non-structural carbohydrates (sugars, pectins and starches) which are digested more rapidly than structural carbohydrates (such as cellulose, hemicellulose) control the initial rate of fermentation. The subsequent availability of energy is dependent upon the amount and composition of dietary structural carbohydrates (Williams *et al.*, 1989).

Batch culture studies (Strobel and Russell, 1986) found that monomers of either structural or non-structural carbohydrates promoted a similar value of Y_{ATP}. Based on this and several continuous culture and *in vivo* studies, Hoover and Stokes (1991) concluded that varying the ratio of non-structural to structural carbohydrates in diets would have a marked effect on ruminal digestion of carbohydrates rather than on the efficiency of microbial-protein synthesis. Results from previous *in vivo* studies (Chamberlain and Thomas, 1979; Harrison and McAllan, 1980), however, have suggested that the efficiency is higher in forage feeding compared with concentrate feeding. Owens *et al.* (1984) proposed that the higher efficiency associated with roughage feeding was due to favourable ruminal conditions which allow microbes to grow more efficiently, such as faster dilution rates, higher pH and longer retention time of particulate materials. Feeding concentrate such as starch is associated with slower dilution rates (Feng *et al.* , 1993) and low ruminal pH (Stern *et al.*, 1994) and this could increase maintenance energy cost (Shi and Weimer, 1992). Energetic uncoupling

has also been suggested to occur as dietary starch intake increases (Sniffen and Robinson, 1987).

Some recent studies have been directed to the effects of a synchronised energy-nitrogen supply on microbial-protein synthesis based upon the assumption that rumen microbes may be able to grow more efficiently when their nutrient requirements are provided continuously. The results obtained, however, are equivocal. Positive responses in microbial efficiency as a result of better synchronisation between energy and nitrogen have been found in some studies (Casper and Schingoethe, 1989; Herrera-Saldana *et al.*, 1990; Matras *et al.*, 1991 and Sinclair *et al.*, 1994) but not in others (Henning *et al.*, 1991; Newbold and Rust, 1992 and Stern *et al.*, 1994). McAllister *et al.* (1994) found that certain ruminal microbes may utilise end-products of metabolism by other species (cross-feeding) which may serve to overcome nutrient depletion in unsynchronised energy-protein diets. Some of this discrepancy may also be due to the different forms of dietary nitrogen and energy sources studied and to recycling of N and sulphur.

c. Dilution rate

In vitro studies have indicated that microbial efficiency is closely related to the specific growth rate (dilution rate) (Isaacson *et al.*, 1975; Harrison and McAllan, 1980). In the study of Isaacson *et al.* (1975), the Y_{ATP} for the dilution rates of 0.02, 0.06 and 0.12/h were 7.5, 11.6 and 16.7, respectively. This is because energy in high dilution rates is utilised more efficiently for growth compared with low dilution rates (Hespell and Bryant, 1979). In addition, the mean age of the microbial population will be decreased as dilution rate increases and it is

theorised that this younger population will be less susceptible to death and predation (Van Soest, 1994).

In the rumen, microbes are present in three phases, namely fluid and particle phases, and rumen-wall phase (Cheng and Costerton, 1980), and their outflow from the rumen is associated with the fluid and particle phases. Dilution rates of both liquid and particle phases, and the proportions of microbes associated with them, determine the ruminal outflow and retention time of microbes (Oldham, 1984). Dilution rates of liquid fraction alone sometimes do not correlate positively with the efficiency of microbial-protein synthesis *in vivo* (Stern and Hoover, 1979; Hoover *et al.*, 1984) which may indicate the importance of considering particle-associated microbes. Cheng and Costerton (1980) suggested a higher proportion of particle-phase than fluid-phase bacteria exist in the rumen with roughage feeding, and this was confirmed by Craig *et al.* (1983).

d. Maintenance energy

As in animals, rumen microbes will not utilise energy for productive purposes unless their maintenance energy requirements have been met (Russell and Strobel, 1993). This basal expenditure of energy consists of several non-growth activities requiring ATP, namely a) motility; b) the dynamic state; c) production of extracellular proteins and polysaccharides; and d) active transport (Leng and Nolan, 1984).

Maintenance energy of individual rumen bacteria varies according to species, ranging from 0.12 to 0.83 mmol glucose/g bacteria/h (Russell and Baldwin, 1979; Russell, 1983), while that of mixed rumen bacteria is estimated to be 0.26 mmol glucose/g bacteria/h (Isaacson *et al.*, 1975). Owens and Isaacson (1977) predicted the values of maximum maintenance energy of between 20 and 25 %

of the available ATP, which would lower the theoretical $Y_{ATP/MAX}$ of around 30 to 25. Russell and Strobel (1993) stated that the amount of energy required for maintenance was related to bacterial growth rate: a large proportion of energy would be used for maintenance when the growth was slow, and this proportion would decrease as growth rate increased. Bacterial species with low maintenance energy requirements, and therefore faster growth rate, will dominate bacterial populations in situations where the average growth is expected to be slow, for example under poor-quality roughage feeding (Russell and Hespell, 1981).

2.3 Metabolism of Fatty Acids

2.3.1 Rumen modification of dietary fatty acids

Dietary lipids consumed by ruminants are mainly in the form of galactolipids and phospholipids (roughage-feeding) and triacylglycerols (concentrate-feeding). Upon entering the rumen, all of these are subjected to ruminal hydrolysis (lipolysis) and hydrogenation. Bacteria are primarily responsible for modification of dietary fatty acids (FA), while protozoa are considered to be only of secondary importance (Harfoot and Hazlewood, 1988).

Bacterial hydrolysis of dietary lipids breaks down the acyl-ester linkages releasing unesterified FA and glycerol. There is no accumulation of mono- and diacylglycerols during hydrolysis of triacylglycerols (Dawson *et al.*, 1974) and diacylglycerols are hydrolysed at a faster rate than triacylglycerols (Henderson, 1971; Henderson and Hodgkiss, 1973). Glycerol and galactose (of galactolipids) are further fermented to VFA, *e.g* glycerol gives rise to propionate (Garton *et al.*, 1961). On the other hand, the unesterified long chain fatty acids (LCFA) released

are neither fermented in nor absorbed from the rumen in significant quantities (Wu and Palmquist, 1991; Wood *et al.* 1963).

Unsaturated dietary LCFA released from hydrolysis (mainly C18:3 and C18:2 of roughage and C18:2 and C18:1 of concentrates; Harfoot and Hazlewood, 1988) are then susceptible to extensive bacterial hydrogenation. Because unsaturated LCFA are toxic for many species of rumen microbes, ruminal biohydrogenation has been suggested as a detoxification mechanism (Kemp *et al.*, 1984a). The biohydrogenation process is initiated with isomerisation which can only proceed in the presence of a free carboxyl group, suggesting that lipolysis is a prerequisite for biohydrogenation (Hawke and Silcock, 1969). While hydrogenating capacity of rumen bacteria is unequivocal for chain lengths up to 18, hydrogenation of LCFA with higher chain lengths has not been studied in detail. Ashes and co-workers (1990; 1992) demonstrated that rumen bacteria were unable to hydrogenate eicosapentanoic (C20:5) and docosahexaenoic (C22:6) fatty acids of fish oil, but the reasons for this were not clear.

The extent of ruminal biohydrogenation of LCFA having 18 carbon atoms is generally increased with decreasing level of saturation (Murphy *et al.*, 1987; Enjalbert *et al.*, 1994). Products of biohydrogenation of these acids are mainly stearic acid and to some degree *trans*-vaccenic acid (*trans*-octadecenoic acid, C18:1). Should the *trans*-C18:1 accumulate, this indicates that hydrogenation has proceeded incompletely. This accumulation occurs following an increase in the availability of C18:2 (Kellens *et al.*, 1986; Enjalbert *et al.*, 1994) or when a free form rather than triacylglyceride of oleic acid is utilised as substrate (Noble *et al.*, 1969; Wu and Palmquist, 1991).

As reviewed by Harfoot and Hazlewood (1988), two distinct groups of bacteria are involved in biohydrogenating LCFA, namely groups A and B. Both groups A

(including *Butivibrio fibrisolvens*) and B (*Fusocillus spp.* and a Gram-negative rod, R8/5) hydrogenate substrates up to *trans*-C18:1, but the conversion of this intermediate to stearic acid is carried out only by group B. Group B is probably more susceptible to the toxicity of LCFA, and this could be prevented by the slow release of substrate provided in the form of triacylglycerols.

Food particles are regarded as playing a significant role in ruminal biohydrogenation of LCFA. They are the sites where biohydrogenation takes place (Harfoot *et al.*, 1975), but there is no evidence that biohydrogenating enzymes produced by bacteria are extracellular. Harfoot and Hazlewood (1988) stated: "...there are no reports of soluble fatty acid hydrogenating enzymes produced by rumen bacteria...". Food particles are also found to act as alternative sites for adsorption of LCFA, thereby reducing the toxicity of LCFA to bacteria (Henderson, 1973).

Factors which inhibit the growth of bacteria or reduce the accessibility of bacteria to substrates have been shown to affect lipolysis and thus biohydrogenation. Growth factors such as low ruminal pH due to high grain feeding (Palmquist and Schanbacher, 1991), low dietary nitrogen or increasing forage maturity (Gerson *et al.*, 1983; 1985) reduce the completeness of biohydrogenation. Gerson *et al.* (1988) reported that decreasing the size of food particles reduced biohydrogenation because the number of bacteria adhering to the particles as the site for biohydrogenation was reduced.

2.3.2 Microbial incorporation of dietary fatty acids

Unsaturated and saturated LCFA may be directly incorporated by rumen microbes and utilised in synthesis of their own complex lipid. The proportions of bacterial lipids which are derived from the incorporation of dietary LCFA may

be higher than those synthesised *de novo* from short-chain precursors (Demeyer *et al.*, 1978). Rumen microbes prefer to take up the available LCFA directly when their concentration is high, and *de novo* synthesis of microbial FA from VFA has been shown to be inhibited by high LCFA concentrations (Emmanuel, 1978). An increased lipid concentration in the diet, and therefore in the rumen, appears to enhance LCFA incorporation by some microbes via the formation of cytoplasmic lipid droplets (Bauchart *et al.*, 1990). There are, however, some studies indicating that C18:3 is not easily incorporated into the lipid cell of rumen bacteria (Hawke, 1971; Bauchart *et al.*, 1990).

Direct incorporation of the available LCFA into microbial cells is more energy-efficient than is *de novo* synthesis of cellular LCFA from shorter-chain FA. Zinn (1988) observed an increase in the efficiency of bacterial growth following fat supplementation, and this was attributed to high incorporation of LCFA by bacteria. The known defaunating effect of dietary fat (Ikwuegbu and Sutton, 1982), however, may also contribute to such increase in bacterial efficiency.

2.3.3 *De novo* microbial fatty acid synthesis by rumen microbes

Rumen microbes are able to synthesise straight and branched LCFA *de novo* with carbon chains of both even and odd numbers (*e.g.* C16 as well as C15). Due to this synthesis and the insignificant ruminal absorption and catabolism of LCFA, it is observed that the amounts of LCFA flowing to the small intestine in ruminants are generally higher than those consumed (Sutton *et al.*, 1970; Bickerstaffe *et al.*, 1972; Hogan *et al.*, 1972).

Synthesis of branched LCFA by rumen microbes is of particular importance. These acids are not present in feed materials and therefore can be regarded as

peculiar to microbes. Of the total FA building up bacterial phospholipid, approximately 30 % is branched LCFA (O'Kelly and Spiers, 1991), mainly *anteiso* 15:0 with smaller quantities of *iso* and *anteiso* 14 to 17 (Jenkins, 1994). It is generally accepted that branched, together with unsaturated LCFA, serve to maintain membrane fluidity of bacteria because of their low melting points (Hauser *et al.*, 1979). When a large amount of saturated LCFA is incorporated by rumen bacteria, some authors (Hauser *et al.*, 1979; Bauchart *et al.*, 1990) speculated that rumen bacteria might adjust the FA composition or the repartitioning of polar lipids in their membrane cell for the purpose of ensuring membrane viscosity is maintained.

When polyunsaturated LCFA are identified in the rumen microbial biomass, they are mainly considered to be a result of direct incorporation. It seems that rumen microbes do not synthesise polyunsaturated LCFA, since most desaturases require oxygen. For monounsaturated LCFA, however, they may be synthesised through the anaerobic pathway by rumen microbes as found in most bacteria (Harfoot and Hazlewood, 1988). Monounsaturated LCFA may also be synthesised through the action of anaerobic desaturase which has been observed by Kemp *et al.* (1984b) in one species of rumen fungi (*Piromonas communis*) which desaturates stearic acid.

2.3.3.1 Pathways

The pathway of LCFA synthesis in rumen is believed to be similar to that of plants, involving several individual enzymes (Finnerty and Makula, 1975; Brindley *et al.*, 1969). Synthesis of straight LCFA with even numbers of carbon atoms utilises acetyl-CoA as the common primer, but the principal carbon atom donor is malonyl-CoA (Hadley, 1985). The carbon chain of acetyl-CoA is elongated by the successive condensation of malonyl-CoA, yielding palmitic

acid as the main end-product. Straight LCFA with odd atom carbons are also synthesised through this pathway, but the primers are propionyl-CoA and valeric-CoA in place of acetyl-CoA. LCFA with odd atom C may also be synthesised through α-oxidation of even-numbered LCFA (Emmanuel, 1978), but this mechanism *in vivo* requires further clarification (Bauchart *et al.*, 1990).

The overall reaction of palmitic acid synthesis is as follows:

$$\text{Acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\ \text{NADPH} + 14\ \text{H} = \text{palmitic acid} + 7\ CO_2 + 14\ \text{NADP} + 6\ H_2O + 8\ CO_2$$

For synthesis of branched LCFA by rumen microbes, the pathways involved may be similar to that of *Bacillus*, the genus that is widely studied for its bacterial branched FA synthetase. In this genus of bacteria, the pathways of synthesis of branched and straight LCFA are basically the same in terms of chain extension processes and extender (malonyl-CoA) (Kaneda, 1977). The only difference between these two pathways is that primers for branched LCFA synthesis are branched carboxylic acids and branched-chain α-keto acids rather than acetyl-CoA (Kaneda, 1991). The common pathways of branched LCFA synthesis in bacteria are shown below:

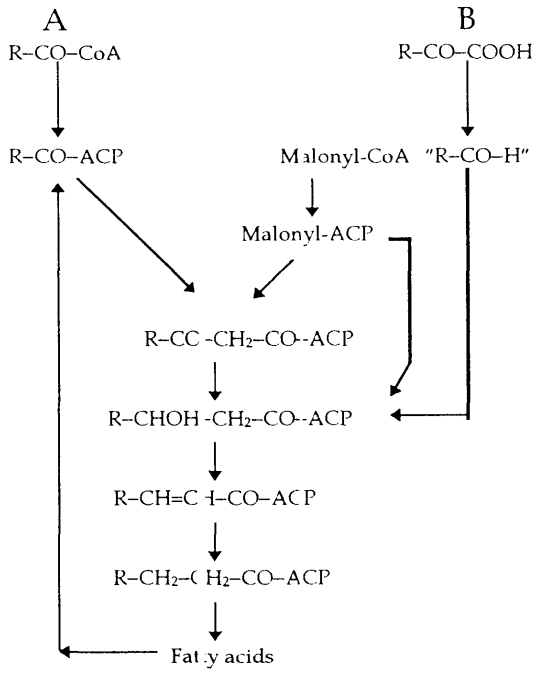

Figure 2.1 Pathways of ba terial branched LCFA synthesis utilising branched chain acyl-Co A ester (A) and branched α-keto acid (B) as primer wher R is either $(CH_3)_2$·CH–, $(CH_3)_2$·CH·CH_2– or $CH_3(C_2H_5)CH$– (Kaneda, 1977; 1991)

2.3.3.2 *Carbon source*

Numerous studies have demonstrated that the labelled carbons of substrates are incorporated by rumen bacteria and protozoa into FA building up their cellular lipids. For example, Patton *et al.* (1968; 1970) showed that the ^{14}C of 1-^{14}C-acetate or U-^{14}C-glucose were utilised by mixed rumen bacteria and protozoa for synthesis of straight LCFA with various chain lengths. In ruminal metabolism, VFA may be activated to form their acyl-CoA esters before being utilised as primers. The common carbon extender, malonyl-CoA, is synthesised from acetyl-CoA through carboxylatio n.

The primers are also produced as intermediary products during the secondary metabolism of pyruvate to VFA. In the pathway of acetate synthesis, pyruvate is

converted to acetyl-CoA (the main primer for even chain LCFA) via pyruvate feredoxin oxidoreductase (Thauer and Kröger, 1984; Melville *et al.*, 1988) and pyruvate formate lyase (Knappe, 1990). Similarly, propionyl-CoA for synthesis of odd LCFA is produced involving several steps through either the succinate (randomising) or acrylate (non-randomising) pathway of propionate production (Czerkawski, 1986).

Synthesis of branched LCFA utilises short chain carboxylic acids as primers, namely isobutyrate, isovalerate and 2-methylbutyrate. These are products of degradation of branched chain amino acids: valine, leucine and isoleucine. It has been shown that the labelled carbons of isobutyrate, valine and isoleucine are incorporated into branched LCFA with odd and even carbon numbers by rumen bacteria and protozoa (Tweedie *et al.*, 1966; Emmanuel, 1974). As in the synthesis of straight LCFA, it is necessary to transform these acids into their related CoA esters before their use. Branched LCFA synthetase enzymes of bacteria can also utilise α-keto acids as primers, and *Bacillus subtilis* utilises these primers with no short-chain carboxylic acid-CoA esters as intermediary products (Kaneda, 1991). Namba *et al.* (1969) found that CoA and NAD were required for the conversion of α-keto acids into the related CoA esters, but according to Kaneda (1977), these were not required by *B. substilis* to synthesise branched FA using α-keto acids as primers; in fact they were inhibitory to the synthesis. Kaneda (1991) concluded that a carboxylase rather than a dehydrogenase was involved in the synthesis of branched FA from α-keto acids in this organism.

2.3.3.3 *Rates of synthesis*

a. Method of measurement

Ruminal (endogenous) synthesis of total LCFA can be determined simply by measuring the difference between the amount of LCFA flowing down to the small intestine and that consumed by the animal. These two values can be regressed, and the intercept of the line becomes the amount of LCFA synthesised for fat-free diet, while the slope is assumed to be the duodenal recovery of dietary LCFA (Wu *et al.*, 1991; Weisbjerg *et al.* 1992a). This method, however, determines the total rather than *de novo* synthesis of LCFA because it does not take into account the proportion of LCFA derived through incorporation.

The result obtained depends, at least, on the suitability of digesta-flow markers being used. Lower concentrations of duodenal LCFA compared to those consumed by the animal (negative balance) are frequently reported (Murphy *et al.*, 1987; Wu *et al.*, 1991) which are mainly associated with the unsuitability of digesta-flow markers. Despite a negative LCFA balance observed in their study, Wu *et al.* (1991) indicated that the actual disappearance of LCFA was lower than that theoretically calculated from the regression equation (1-slope), suggesting the contribution of microbes to duodenal LCFA. On the other hand, the LCFA synthesis may be overestimated due to endogenous supply of FA from bile to duodenal digesta, for which correction is required (Weisbjerg *et al.*,1992a). Reflux of bile lipid into abomasal digesta is unlikely to occur.

b. Published values

Wu *et al.* (1991) found that the intercept and the slope of the regression line of total FA intake against that of duodenal flow were 106 and 0.70, respectively. From these results, the authors assumed that the amount of total FA synthesised in a cow by microbes was 106 g/d, and that duodenal recovery of dietary FA was 70 %. Lower values than these, 68 g/d and 87 % for total FA synthesis and duodenal recovery of dietary FA, respectively, were obtained by Weisbjerg *et al.* (1992a). In the latter study, the duodenal flow of FA was corrected for endogenous bile FA and dry matter intake was lower compared with the former. The lower duodenal recoveries of FA in these studies as indicated by the recovery of dietary FA were attributed to digesta-marker errors, and this may have underestimated the synthesis of FA by microbes. Nevertheless, the *in vitro* synthesis of FA by microbes reported by Wu and Palmquist (1991) was similar, being 109 g/d in cattle.

2.3.3.4 *Factors affecting FA synthesis*

a. Dietary factors

Dietary fat has been shown to inhibit *de novo* synthesis of FA (Demeyer *et al.*, 1978), but the FA contents of bacteria and protozoa are generally increased by feeding fat (Weisbjerg *et al.*, 1992b Bauchart *et al.*,1990). In the study of Bauchart *et al.* (1990), dietary fat increased the FA content of both particle- and liquid-phase bacteria by 150 % without affecting their polar lipid contents. The increased microbial FA content associated with dietary fat is mainly due to the incorporation of dietary FA. Gram-negative bacteria possessing higher lipid (Salton, 1960) content are more resistant than Gram-positive bacteria to fat

toxicity (Galbraith *et al.*, 1971; Henderson, 1973). The increased bacterial FA content, hence, could be due to an increased proportion of Gram-negative relative to Gram-positive bacteria

Methionine is also reported to increase microbial-lipid content (Patton *et al.*, 1968; O'Kelly and Spiers, 1990) as well as the synthesis of LCFA by microbes (Chamberlain and Thomas, 1983). This is due to the increased availability of methyl groups for bacterial lipid synthesis provided by methionine (O'Leary, 1959). Similar to methionine, monensin also increases the lipid synthesis of bacteria (Merry and McAllan, 1983; O'Kelly and Spiers, 1990). O'Kelly and Spiers (1990) and O'Kelly (1987) argued that the increased plasma concentration of cholesterol in cattle following monensin supplementation was due to the increased LCFA supplied by rumen microbes since the circulating level of cholesterol in these animals was apparently dependent upon the absorption of LCFA. Monensin increases the LCFA synthesis because it shifts the proportions of rumen population toward more a Gram-negative than Gram-positive population (Bergen and Bates, 1984), so rechannels carbon and hydrogen from methane to lipid synthesis (O'Kelly and Spiers, 1988). Reduced methanogenesis with monensin is also due to enhanced utilisation of hydrogen in propionate synthesis and an increase in the synthesis of long-chain methylated acids (Wahle and Livesay, 1985). It also alleviates the toxic effect of LCFA to microbes through inhibition of hydrolysis of dietary lipid (Van Soest, 1994).

b. Microbial factors

Particle-phase bacteria are consistently reported to have higher FA content compared with fluid-phase bacteria (Legay-Carmier and Bauchart, 1989; O'Kelly and Spiers, 1988; Bauchart *et al.*, 1990; Hegarty *et al.*, 1994). The concentration of FA around particulate matter is high due to adsorption which may result in a

higher amount of FA being incorporated (Legay-Carmier and Bauchart, 1989). O'Kelly and Spiers (1988) found that the incorporation of FA was positively related to the ruminal FA concentration.

FA content of Gram-negative bacteria is likely to be higher than that of Gram-positive bacteria as Salton (1960) found higher lipid content in the cell of Gram negative compared with that of Gram-positive bacteria. It is also possible, therefore, that the higher FA content of particle-phase bacteria is due to differences in bacterial species. Minato *et al.* (1966) found that the distribution of species of bacteria was different between particle and liquid phases.

2.3.4 Metabolism of LCFA by ruminants

2.3.4.1 *Process of absorption*

It is almost certain that LCFA are not absorbed from the rumen in significant quantities nor catabolised to VFA and CO_2 within the rumen. Ruminal recoveries of the labelled linoleic acid added to the rumen of sheep with a ligated reticulo-omasal orifice after 48 hours are 85 - 96 % (Wood *et al.*, 1963) and minimal radioactivity is detected in blood plasma of sheep equipped with reentrant cannulae (through which all digesta were removed) following a ruminal dose of labelled LCFA (Bickerstaffe *et al.*, 1972). Both *in vitro* (Wu and Palmquist, 1991) and *in vivo* (Wood *et al.*, 1963) studies have indicated that ruminal catabolism of LCFA to VFA and CO_2 is less than 1%. Therefore, essentially all ruminal LCFA, whether dietary or microbial in origin, will pass into the small intestine where absorption takes place.

During preduodenal passage microbial cells and their lipid contents are subjected to disintegration by microbial and abomasal emzymes (Hoogenraad and Hird, 1970). Unesterified LCFA released from disintegration of microbial biomass and those of dietary origin contribute primarily to the LCFA passing to the small intestine, but a small proportion of microbial phospholipids may also be present in digesta (Leat and Harrison, 1975; Noble, 1981; Moore and Christie, 1984). A negligible amount of triacylglycerol is present in intestinal digesta of ruminants fed conventional diets, unless the animals consume rumen-inert fat or oils. All lipid materials are adsorbed onto particulate matter upon entering the small intestine (Moore and Christie, 1984).

In the small intestine, the digestion of lipid proceeds with a biphasic system: an insoluble particulate and a soluble micellar phase. The first phase contains unesterified LCFA, microbial and biliary phospholipids, whereas the second system consists of dissolved LCFA. The insoluble particulate-associated LCFA are not readily available for absorption, and they have to be transferred into the dissolved form of the micellar phase to allow absorption to take place. This process occurring through detergency (Lough and Smith, 1976) is assisted by the presence of bile and pancreatic secretions, and it has been shown that diverting these secretions reduces the flow of FA towards the soluble micellar phase (Leat and Harrison, 1975). Bile salts are the most important of the secretions and play a major role in the solubilisation, while biliary phospholipids, lecithin and lysolecithin as well as pancreatic juice may be only of secondary importance (Scott and Lough, 1971; Leat and Harrison, 1975).

2.3.4.2 *Synthesis of odd/branched LCFA*

Adipose tissue and the mammary glands of lactating animals are the main sites for synthesis of FA in ruminants, including those which are odd and branched

chain. The proportions of odd and branched LCFA in the triacylglycerols of these organs may be 1 - 2 % (Keeney, 1970) or up to 10 % of the total LCFA in sheep and goats fed high grain diets (Christie, 1978; Duncan and Garton, 1978).

Acetate is the main carbon source for synthesis of even, straight LCFA in ruminant tissue (Madsen, 1983; Van der Walt, 1984), but if odd LCFA are to be synthesised, the carbons are derived from propionate. During the carboxylation of propionate to succinate, propionyl-CoA and methylmalonyl-CoA are formed. Propionyl-CoA can replace acetyl-CoA as primer for LCFA synthesis, and similarly methylmalonyl-CoA can be used as carbon extender in place of malonyl-CoA, giving rise to the formation of odd and branched LCFA, respectively (Garton, 1977). Propionate production is greatly enhanced in the rumen under high grain feeding (> 80 %), and it has been shown that odd and branched LCFA are present in high proportions in adipose tissues of sheep and goats fed barley, maize or wheat (Garton *et al.*, 1972; Duncan *et al.*, 1974).

Synthesis of branched LCFA in ruminants is sometimes regarded as incomplete metabolism of propionate in which methylmalonate accumulates and becomes available as a carbon source for chain elongation. "Complete" conversion of methylmalonate to succinate is impaired when methylmalonyl-CoA mutase, the enzyme that catalises the conversion, is overloaded or the quantity of active enzyme is reduced (Elliot, 1980) Because the enzyme is vitamin B_{12} dependent, it is not surprising that the deficiency of either vitamin B_{12} (Duncan *et al.*, 1981) or cobalt which is essential in the synthesis of the vitamin (Kennedy *et al.*, 1990) increases the synthesis of branched LCFA through the reduced activity of methylamalonyl-CoA mutase.

The metabolism of propionate in ruminants, however, especially the utilisation of methylmalonyl-CoA, is not entirely clear. It has been shown by Duncan *et al.*

(1981) that some methylmalonate s lost in the urine and is thus not available for LCFA synthesis. The extent to which methylmalonate is utilised for LCFA synthesis has not been studied in detail. The utilisation of methylmalonyl-CoA for branched LCFA synthesis in cattle, as in sheep and goats, has been demonstrated (Wahle, *et al.*, 1978), with cattle appearing not to accumulate branched LCFA in their tissue to the same extent (Duncan and Garton, 1978).