Chapter 4

The Effects of Dietary Urea on Rumen Function of Sheep.

4.1 Introduction

The contribution of amino acids of microbial origin to ruminants fed low nitrogen roughage-based diets depends on the efficiency of microbial growth and on the lysis and turnover of microbial cells within the rumen. These factors determine the flow of microbial cells, and hence microbial protein into the small intestine. Microbial growth efficiency in the rumen depends on an array of factors including the availability of nutrients critical to their growth and the microbial ecosystem that develops in the rumen which is a function of the nutrients available from the diets. Ruminal fluid ammonia is the major nitrogen source for microbial protein synthesis and growth (Bryant & Robinson, 1962). The optimum concentration of ruminal fluid ammonia for maximum microbial growth and for maximum rate and extent of fermentation has been reported to be in the range of 50-250 mgN/l. From the literature the levels for optimum microbial protein synthesis in the rumen have been reported to vary from 50 mg NH_3-N/l (Satter & Slyter, 1974 ; Russell & Strobel, 1987) to 238 mg NH_3-N/l (Miller, 1973). Recently, Balcells et al. (1993), using urinary excretion of purine derivatives as an indication of the quantity of microbial cells delivered to the intestine, found that this was optimised at an ammonia level of 105 mgN/l.

The maximum rate of digestion of forage dry matter in cattle was observed when concentrations of ruminal fluid ammonia were between 45-60 mgN/l on forage based diets (Perdok et al. 1988 ; Boniface et al. 1986) and between 200-270 mgN/l on starch based diets (Mehrez et al. 1977). Even after forage digestibility in the rumen had been optimised by adjusting ammonia levels to 45-60 mgN/l, forage intake increased

with increasing ammonia concentrations in the rumen above this level and was optimum when ammonia level was in excess of 200 mgN/l in the rumen of cattle fed rice straw (Perdok et al. 1988) or spear grass hay (Boniface et al. 1986). Knowledge of the optimum levels of ruminal fluid ammonia for efficient microbial growth and maximum digestibility of forage based diets is important, as these determine the maximum extraction of nutrients from a forage and the balance of microbial protein relative to energy available for digestion and absorption. It is particularly important to establish the optimum concentration of ruminal fluid ammonia for animals fed low protein forage based diets, where any increase in P/E ratio in the nutrients absorbed could increase the efficiency of forage utilisation for production (Leng, 1990) and an increase in digestibility allows feed intake to increase substantially (Minson, 1982), thus potentially increasing productivity significantly.

The present research was undertaken to examine the effects of incremental increases in ruminal fluid ammonia (arising from urea) on (i) digestibility of a low protein forage (ii) urinary excretion of purine derivatives and, (iii) the microbial mix in the rumen of sheep given oaten chaff

4.2 Experiment 4-1: Effects of Intraruminal Urea Infusion on Rumen Function of Sheep.

4.2.1 Materials and Methods

4.2.1.1 Animals:

Four first-cross Merino x Border Leicester wethers, 2-3 years of age, weighing 35-40 kg and with permanent rumen cannulas were held in metabolism crates (see section 3.2.1).

4.2.1.2 Diets and Feeding:

Oaten chaff (0.6% N) from the same source and batch, was used as the basal diet throughout the study. To this was added 1% of the mineral mix (see section

3.2.2.2). The oaten chaff plus additive was offered at 800 g/d using an automatic-feeding machine which delivered 1/24 of the daily allowance each hour. Each animal received intraruminally infusion of deionised water containing urea (400 ml/d) so that the animals received one of the following rates of intake of urea: 0, 3.47, 6.94, 10.42, 13.89 and 17.36 mg urea/min continuously over a period of 2 w. The animals had clean water at all times.

4.2.1.3 Experimental Procedure:

The sheep were allowed to become accustomed to each diet and the metabolism cages for 2 w prior to commencement of the experiment. Within the 14-d experimental period when infusion were underway for the first 11-d, the animals were left undisturbed and samples were only taken over the last 3 d. On day 12, ruminal fluid was collected at 09.00 h, 13.00 h and 17.00 h through a probe inserted in the rumen. A well-mixed portion of each sample of ruminal fluid was removed and the remaining bulk sample was acidified (see section 3.2.4) and then analysed for NH_3-N (see section 3.8.2) and VFA (see section 3.8.3). The non-acidified sample was used for the enumeration of protozoa (see section 3.5). A blood sample for analysis of plasma urea was taken at 13.00 h (day 12) from the jugular vein using a needle and syringe. The sample was immediately centrifuged and plasma was removed and stored at -20^0 prior to analysis. On day 13, nylon bags containing ground dietary materials (2 mm sieve) were suspended in the rumen in order to assess organic matter digestibility over 24 h. At the same time a nylon bag containing oat leaf samples was suspended for 24 h in the rumen and enumeration of sporangial colonies on the leaf blade was made (see section 3.6). On day 14, urine was collected quantitatively over 24 h for estimation of purine derivatives excretion (see section 3.2.6).

4.2.1.4 Chemical Analysis:

Urea in plasma and urine were analysed using the diacetyl monoxime method of Marsh et al. (1965) on an auto-analyser Technicon Equipment Co., New Jersey, USA).

4.2.1.5 Statistical Analysis

The experimental design was a 4x6 Latin Square. The statistical significance of the data was analysed as indicated in section 3.9.

4.2.2 Results

4.2.2.1 Feed intake

In the animals receiving the oaten chaff diet at hourly intervals, the feed was consumed within a few minutes of its presentation.

4.2.2.2 Effects of urea infusion rate into the rumen on ruminal fluid ammonia, plasma and urinary urea

Continuously fed sheep had constant levels of all rumen metabolites that were measured including ammonia.

The concentrations of plasma and urinary urea and ruminal fluid ammonia were all increased as the urea infusion rate into the rumen of sheep was increased (Table 4-1). The correlation between plasma urea (Y) and ruminal fluid ammonia (X) and between urinary urea (Z) and rumen nal fluid ammonia (X) were highly significantly related as follows:

$$Y = 2.33 + 0.117X - 0.000206X^2 \quad r^2 = 88.7\% \quad (p<0.01)$$

$$Z = -110 + 38.4X - 0.0468X^2 \quad r^2 = 71.7\% \quad (p<0.01)$$

4.2.2.3 Rumen fermentation

In the continuously fed sheep, the profiles of volatile fatty acids (VFA) in ruminal fluid were affected by the different levels of urea fed and therefore ammonia concentrations in ruminal fluid (Table 4-1). Total VFA concentrations in the rumen of sheep on the urea supplemented diets were significantly higher than that of the control

($p<0.01$). The ratio of acetogenic:glucogenic substrates (C2+C4:C3) was significantly lower when ruminal fluid ammonia rose above 200 mgN/l ($p<0.05$).

4.2.2.4 Rumen microbial mix and *in sacco* digestibility

In the sheep given oaten chaff with varying amounts of urea at hourly intervals, the population density of protozoa, the sporangial growth on oat leaf blades over 24 h and purine derivatives excreted in urine were all influenced by the levels of ruminal fluid ammonia ($p<0.01$). The results are shown in Figure 4-1. An initial increment of ammonia levels from 1 to 30 mgN/l induced by infusion of 3.47 mg urea/min increased rumen protozoal numbers in ruminal fluid by 144 % from $1.8x10^5$ to $4.4x10^5$/ml. Fungal growth as indicated by sporangial counts on the leaf blades rose by 173 % from 22 to 60 sporangia per mm^2. Thereafter incremental increases in ruminal fluid ammonia was associated with only slight changes in the indicative fungal population but there was a substantial decrease in protozoal densities (from 4.4 to $1.8x10^5$) as ruminal fluid ammonia was increased to above 200 mgN/l (Figure 4-1).

Urinary excretion of purine derivatives indicated that, when ruminal fluid ammonia was initially increased from 1 to 31 mgN/l, there was an apparent reduction in the microbial biomass flowing out of the rumen. The purine excretion rate then increased with increasing amounts of urea in the diet and appeared to remain constant at ruminal fluid ammonia concentrations between 87 and 179 mgN/l. Further increases appeared to occur when the level of ruminal fluid ammonia increased to above 200 mgN/l (Figure 4-1).

Digestibility of dry matter in nylon bags over 24 h in the rumen of sheep on the urea supplemented diets was significantly higher than that on the control ($p<0.01$: Figure 4-1) and was apparently optimised at concentrations of ruminal fluid ammonia between 30 and 50 mgN/l.

Table 4-1. The effects of increasing levels of urea infusion into the rumen on the profiles of volatile fatty acids (VFA) and the concentrations of ruminal fluid ammonia, plasma urea and urinary urea excretion in sheep fed 800 g/d oaten chaff (Experiment 4-1).

	Urea infusion (mg/min)						
parameters	0	3.47	6.94	10.42	13.89	17.36	S.E.
Rumen NH_3-N (mgN/l)	$1.3^{1,a}$	$31.1^{2,a,b}$	$87.3^{3,b,c}$	$109.0^{3,c}$	178.5^{d}	243.4^{e}	8.42
Plasma urea (mg urea-N/100 ml)	$3.2^{1,a}$	$4.9^{1,a}$	9.9^{b}	11.9^{b}	16.1^{c}	17.6^{c}	0.40
Urinary urea (g urea-N/100 ml)	$0.3^{1,a}$	$0.3^{1,a}$	$2.7^{b,c}$	3.3^{c}	4.1^{c}	6.4^{d}	0.30
Ruminal fluid VFA							
Total VFA (μm/ml)	$62.4^{1,a}$	$83.7^{2,a}$	$80.2^{2,a}$	112.8^{b}	$80.6^{2,a}$	$80.9^{2,a}$	2.60
(C2+C4/C3)	$3.8^{1,2}$	$4.4^{2,3}$	$4.5^{3,a}$	$3.9^{1,2,3}$	$4.3^{3,a}$	$3.2^{1,b}$	0.10
Proportions							
Acetic (%)	68.2^{1}	$69.7^{1,a}$	$70.7^{1,a}$	69.1^{1}	$71.0^{1,a}$	$64.7^{2,b}$	0.50
Propionic (%)	$20.3^{1,2}$	$17.8^{1,a}$	$17.8^{1,a}$	$20.0^{1,2}$	$18.5^{1,a}$	$23.0^{2,b}$	0.60
Isobutyric (%)	0.6	0.6	0.5	0.4	0.9	0.8	0.10
Butyric (%)	8.0	9.4	9.4	9.2	7.8	8.2	0.40
Isovaleric (%)	0.6	0.7	1.1	0.6	0.8	0.7	0.10
Valeric (%)	0.6	0.7	0.6	0.7	1.0	0.8	0.10

Different superscripts in numbers or letters in the same row indicate the difference between treatments at $p<0.05$ or $p<0.01$, respectively.

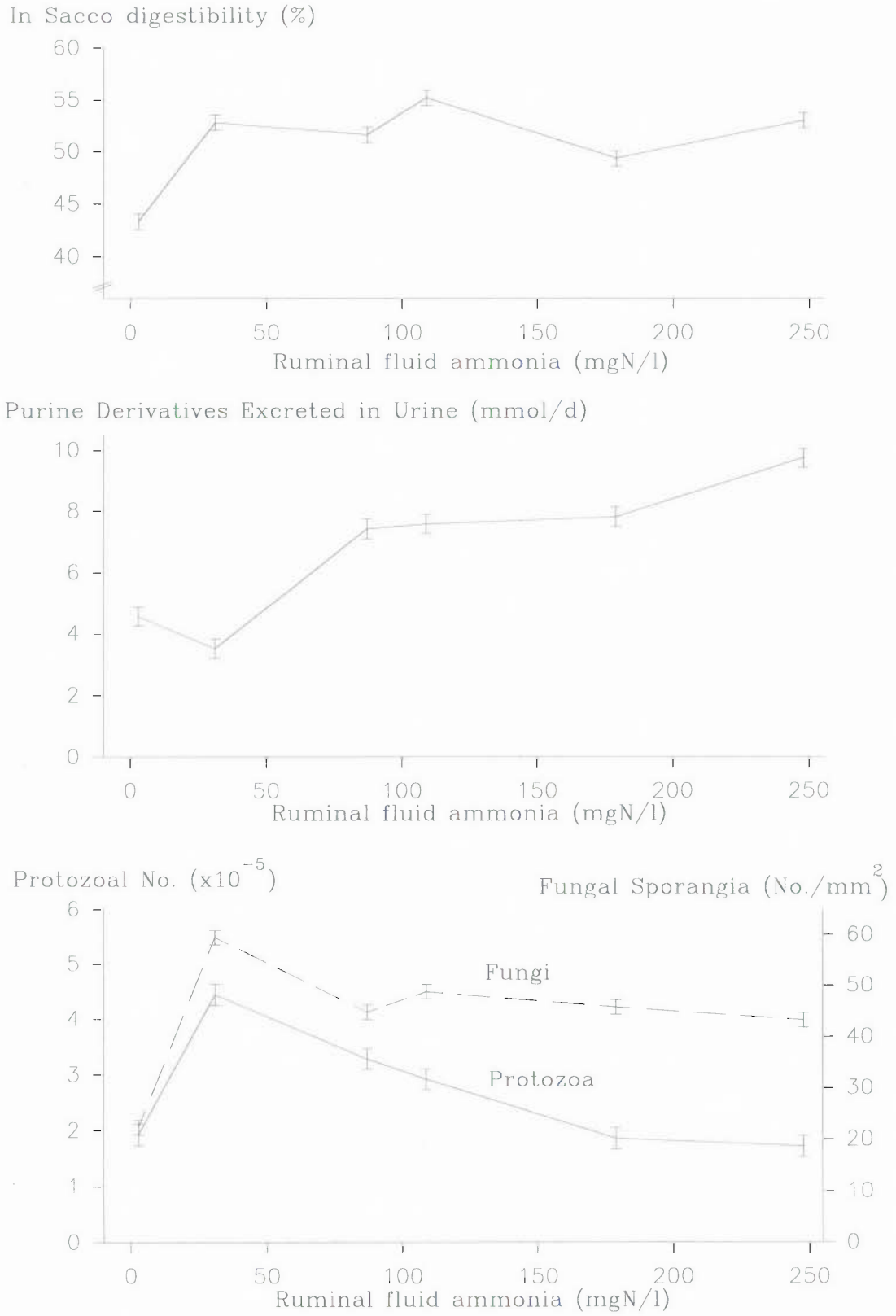

Figure 4-1. Effects of the levels of ruminal fluid ammonia obtained through urea supplementation on microbial mix, urinary excretion of purine derivatives and organic matter digested in the rumen of sheep fed a low protein roughage-based diet (Experiment 4-1).

4.3 Experiment 4-2: Effects of Dietary Urea on Rumen Function of Sheep Fed once Daily.

4.3.1 Materials and Methods

4.3.1.1 Animals:

Eight Merino wethers weighing 25-30 kg about 2-3 years of age and with permanent rumen cannulas were allocated at random to metabolism crates in an animal house (see section 3.2.1).

4.3.1.2 Diets and Feeding:

Oaten chaff (0.8% N) from the same source and batch was used as the basal diet throughout the study. To this was added 2% of the mineral mix (see section 3.2.2.2). Urea solutions were prepared by dissolving 0, 5, 10, 15, 20 g in tap water (see section 3.2.2.1) and the solutions were each sprayed onto a daily ration of 750 g of the chaff. The feed was offered once daily at 09.00 h.

4.3.1.3 Experimental Procedure:

The sheep were allowed to become accustomed to the feeds and cages for 2 w. Within the 21-d experimental period the first 6-d was regarded as a transitional period, the following 10-d allowed for adaptation period, and over the last 5-d intensive sampling was undertaken. On the first 3 sampling days, the daily urine voided by the animal was collected into a container as indicated in section 3.2.6 for later analysis of purine derivatives. On the fourth day, Cr-EDTA (1 mg Cr/kg BW) was injected intraruminally at 06.30 h and the ruminal fluid was collected through a probe inserted in the rumen. Samples were taken before feeding and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 9, 12, 15, 23, 24 h after feeding. Ruminal fluid subsamples were extracted from each sample prior to acidification to be used for enumeration of protozoa (see section 3.5) and for pH (see section 3.4). The rest of the samples were treated as detailed in section 3.2.4 for analysis of NH_3-N (see section 3.8.2), VFA (see section 3.8.3) and Cr (see section

3.8.4). On the last day, 24 h *in sacco* digestibility of the diet was measured and nylon bags containing oat leaf samples were suspended in the rumen over 24 h to allow assessment of fungal activity (see section 3.6).

4.3.1.4 Statistical Analysis:

The experimental design for the once daily fed-group was a double 4x5 Latin Square. Statistical significance of the data was analysed as indicated in section 3.9.

4.3.2 Results

4.3.2.1 Feed intake

In the sheep given their full ration once a day, at 4 h post-feeding, all the sheep had consumed more than 75 % of their feeds excepting the control group (only 61 %). However, all rations were consumed within 6 h after presentation of the feed.

4.3.2.2 Effects of increasing urea in the diets on rumen NH_3-N, pH and fluid kinetics

In the once a day fed sheep, the ammonia pool size in ruminal fluid significantly increased with an increasing intake of urea (Figure 4-2). The maximum ammonia level in ruminal fluid occurred between 1 and 1.5 h after feeding for the sheep on the urea supplemented diets with the highest peak level of 520 mgN/l in sheep given 20 g urea in the ration (Figure 4-2).

The maximum pH in rumina fluid of the sheep on the urea diet was at 0.5 h post feeding. The highest pH in rum nal fluid was 6.9 in the sheep on the highest urea intake. However, all the sheep on diets containing urea had an average ruminal fluid pH that was less than that in the control sheep ($p<0.01$; Table 4-2).

Table 4-2. Concentrations of ruminal fluid ammonia, pH and the kinetics of ruminal fluid in sheep fed 750 g/d oaten chaff supplemented with increasing levels of urea (Experiment 4-2).

	Urea intake (g/d)					
Ruminal fluid	0	5	10	15	20	S.E.
NH_3-N (mgN/l)*	$3.8^{1,a}$	$15.4^{1,a,b}$	$67.4^{2,b}$	$167.8^{3,c}$	$212.9^{4,c}$	14.4
pH*	6.5^{a}	6.2^{b}	6.2^{b}	6.2^{b}	6.3^{b}	0.05
Volume (l)	6.5^{a}	6.3^{a}	6.4^{a}	6.3^{a}	5.6^{b}	0.30
Outflow rates (l/d)	$12.6^{1,2}$	12.9^{1}	$12.5^{1,2}$	$11.6^{1,2}$	11.1^{2}	0.30
Fractional turnover (/d)	1.9	2.1	2.0	1.8	2.0	0.07

Different superscripts in numbers or letters in the same row indicate the difference between treatments at $p<0.05$ or $p<0.01$, respectively.

* A value averaged from samples taken just before feeding and 4 and 9 h after feeding.

Of the kinetics of ruminal fluid studied, only the rumen volume of the sheep on the diet with 20 g/d urea was significantly smaller than the sheep on the other diets ($p<0.05$; Table 4-2).

4.3.2.3 Rumen fermentation

In the sheep fed once daily, the total VFA concentrations in the rumen at different levels of ruminal fluid ammonia are shown in Figure 4-3. Irrespective of the levels of urea supplementation, there was a change of pattern of total VFA concentration indicating a peak VFA production at about 4 h post feeding. The molar proportion of each VFA in ruminal fluid at different levels of ruminal fluid ammonia taken from just before feeding, 4 and 9 h after feeding is given in Table 4-3.

4.3.2.4 Microbial mix

In the sheep fed once per day, the populations of protozoa in ruminal fluid particularly both small and large *Entodinium sp.* ($p<0.05$), the numbers of sporangia appearing on oat leaf blades after 24 h in the rumen ($p<0.01$) and urinary excretion of purine derivatives ($p<0.01$) were all influenced by the levels of urea in the diet and the concentrations of ruminal fluid ammonia (Table 4-4). An initial increment of ammonia levels from 4 to 15 mgN/l was associated with decreasing urinary excretion of purine derivatives but with increasing densities of protozoa and fungal sporangia growth on the leaf blades. As concentrations of ruminal fluid ammonia rose from 67 to 168 mgN/l, the excretion of purine derivatives increased with an apparent plateau at about 7.68 mmol/d. However, when the average concentration of ruminal fluid ammonia was increased above 200 mgN/l, the numbers of protozoa and fungi sharply decreased, whereas the total purine excretion markedly increased. The effects of the concentrations of ruminal fluid ammonia on microbial ecosystem are shown in Figure 4-4.

4.3.2.5 Efficiency of net microbial cell synthesis in the rumen and *in sacco* digestibility

In the once a day fed sheep, when the concentration of ruminal fluid ammonia was above 200 mgN/l, the calculated microbial-N leaving the rumen from purine excretion (Chen et al. 1990b) and the calculated efficiency of net microbial cell synthesis in the rumen were increased by 45 and 47 % ($p<0.01$; Table 4-4). These apparent increases were accompanied by an apparent decrease in the rumen protozoal population.

The 24-h *in sacco* digestibility of oaten chaff in the sheep on urea supplemented diets was significantly higher than the control ($p<0.01$, Table 4-4). However, there were no differences in digestibility of oaten chaff among the sheep on urea supplemented diets ($p>0.05$).

Table 4-3. The profiles of VFA concentration in ruminal fluid at different concentrations of ruminal fluid ammonia (a value averaged from three samples taken just before feeding and 4 and 9 h after feeding ; Experiment 4-2).

	Concentrations of ruminal fluid ammonia (mgN/l).					
Ruminal fluid	3.8	15.4	67.4	167.8	212.9	S.E
Total VFA (μm/ml)	70.6^{a}	$85.1^{1,b}$	$86.4^{1,b}$	$95.0^{2,b}$	$94.6^{2,b}$	2.9
(C2+C4)/C3	$3.6^{1,3}$	$4.2^{2,a}$	$3.7^{1,2,3}$	$4.1^{1,2}$	$3.5^{3,b}$	0.2
Molar proportions						
Acetic (%)	$69.1^{1,a}$	$71.9^{2,b}$	$69.8^{1,2,3}$	$70.9^{3,b}$	$68.4^{1,a}$	0.9
Propionic (%)	22.1^{1}	$19.7^{2,a}$	$21.6^{1,2,3}$	$20.2^{1,2}$	$23.0^{1,3,b}$	1.1
Isobutyric (%)	$0.6^{1,a}$	$0.6^{1,a}$	$0.4^{2,b}$	$0.5^{1,2}$	$0.4^{2,b}$	0.1
Butyric (%)	7.3	6.9	7.2	7.3	6.9	0.4
Isovaleric (%)	$0.4^{1,a}$	0.5^{1}	0.5^{1}	$0.6^{2,b}$	$0.4^{1,a}$	0.5
Valeric (%)	0.4	0.4	0.5	0.5	0.5	0.1

Different superscripts in numbers or letters in the same row indicate the difference between treatments at $p<0.05$ or $p<0.01$, respectively.

Table 4-4. Effects of urea supplementation on the concentrations of ruminal fluid ammonia and microbial mix, purine derivatives excreted in urine, 24 h *in sacco* organic matter digestibility (OMDR) and calculated efficiency of net microbial cell synthesis in the rumen (ENMS ; Experiment 4-2)

	Concentrations of ruminal fluid ammonia (mgN/l)					
Items	3.8	15.4	67.4	167.8	212.9	S.E
Small *Entodinium sp.* (10^{5}/ml)	1.78^{1}	$2.31^{1,2}$	$2.22^{1,2}$	$2.90^{2,a}$	$1.73^{1,b}$	0.44
Large *Entodinium sp.* (10^{2}/ml)	$4.08^{1,2}$	$3.47^{1,2}$	$3.21^{1,2}$	5.99^{2}	$2.95^{1,3}$	0.77
Holotrich sp. (10^{3}/ml)	1.15	3.10	2.56	4.14	2.18	1.40
Total protozoa (10^{5}/ml)	1.80^{1}	$2.35^{1,2}$	$2.25^{1,2}$	$2.94^{2,a}$	$1.76^{1,b}$	0.44
Fungi (Sporangia/mm^{2})	5.4^{a}	68.8^{b}	76.1^{b}	62.5^{b}	39.1^{c}	5.90
Purine excretion (mmol/d)	$6.7^{1,a}$	$6.4^{1,a}$	$7.7^{2,b}$	$7.7^{2,b}$	9.1^{c}	0.52
OMDR 24 h *in sacco* (%)	48.3^{a}	56.8^{b}	58.3^{b}	59.8^{b}	57.3^{b}	1.36
Calculated microbial outflow (gN/d)	$5.5^{1,a}$	$5.2^{1,a}$	$6.4^{2,b}$	$6.4^{2,b}$	7.7^{c}	0.49
ENMS (gN/kg OMDR)	16.7^{1b}	$13.3^{2,a}$	$15.7^{1,2,3,a}$	$15.5^{1,3,a}$	$19.5^{4,b}$	1.02

Different superscripts in numbers or letters in the same row indicate the difference between treatments at $p<0.05$ or $p<0.01$, respectively.

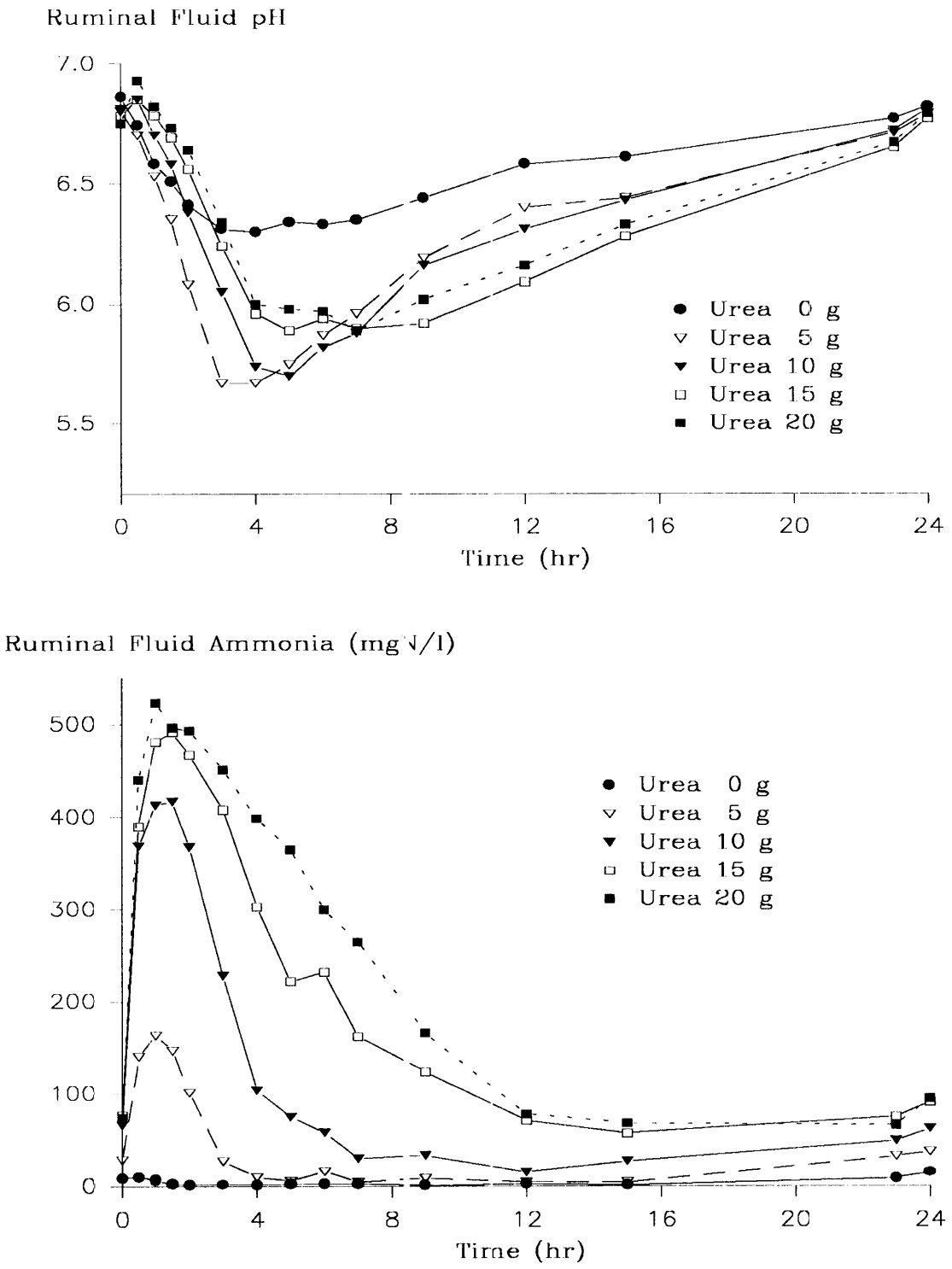

Figure 4-2. Concentrations of ruminal fluid ammonia and pH in the rumen of sheep given low protein forage with increasing levels of urea intake (Experiment 4-2).

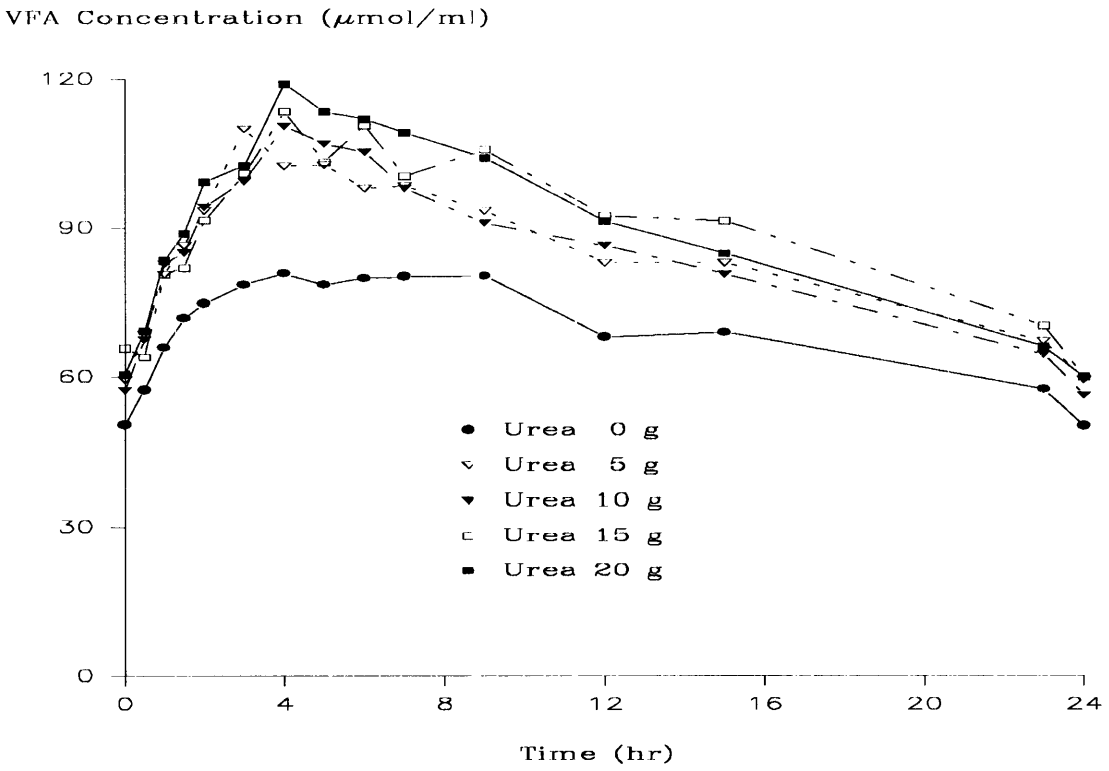

Figure 4-3. Total VFA concentrations in the rumen of sheep given a low protein forage based diet supplemented with increasing levels of urea (Experiment 4-2).

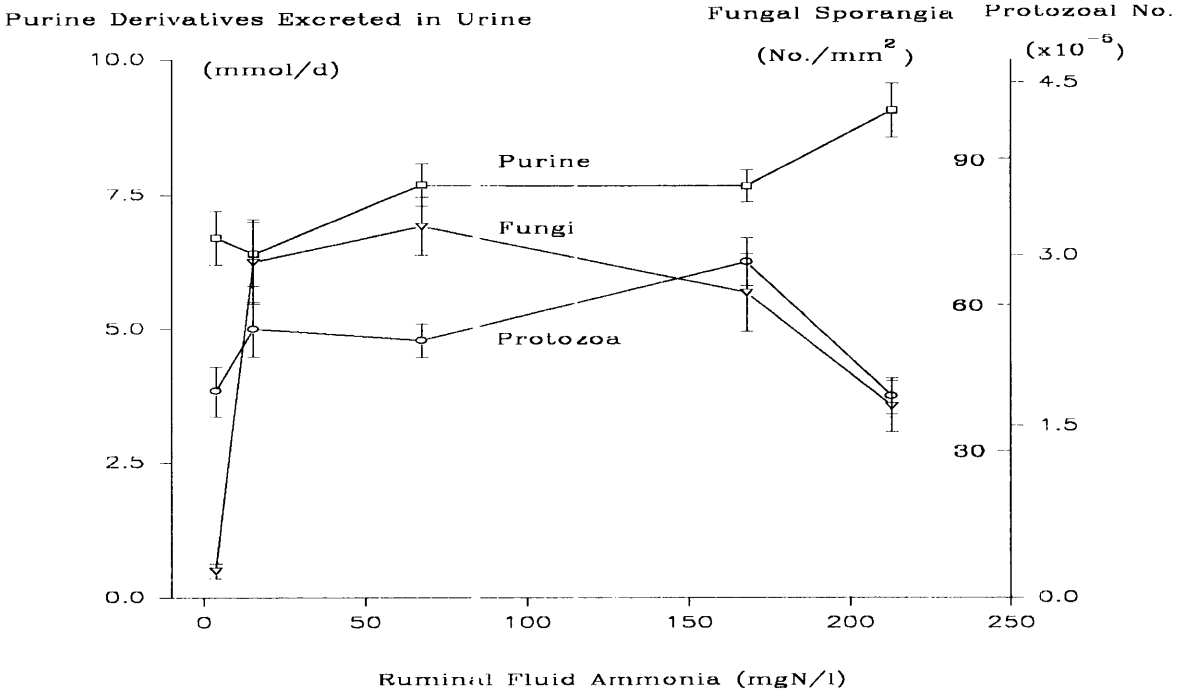

Figure 4-4. Effects of concentrations of ruminal fluid ammonia on the microbial mix in the rumen and urinary excretion of purine derivatives in sheep given increasing amounts of urea in a low protein roughage based diet (Experiment 4-2).

4.4 Discussion

4.4.1 The mode of administration of urea on ruminal fluid ammonia

There appears to be little data on the effects of ruminal ammonia levels on the composition of the microbial ecosystem within the rumen. One of the major objectives of the present study was to determine the effects of increasing ammonia availability in the rumen on the net availability of microbial cells to the host animals intestinal digestive system. A further major objective was to assess the optimum level of ammonia to maximise fibre breakdown in the rumen. As these outcomes are dependent on an interacting mixture of bacteria, protozoa and fungi in the rumen, the changes in these were examined. The contribution of bacterial protein leaving the rumen and therefore digested in the intestine was assumed to follow closely the relative changes in purine derivatives excreted in urine (Chen et al. 1990b).

4.4.2 The relationship between NH_3-N and pH in ruminal fluid

Urea entering the rumen is hydrolysed by microbial ureases to CO_2 and ammonia. The latter is a mixture of unionised or ionised ions depending on pH. The equilibrium constant or pK_a of ammonia solution is 8.8 at 40^o. From the Henderson-Hasselbalch equation, the critical point of ionisation of ammonia (maximum ratio of NH_4^+ to total NH_3) is achieved at pH 6.9. Thus, for every 0.1 pH unit below 6.9, the concentration of NH_4^+ is increased by more than 100 %. On the other hand, for each 0.1 pH unit above 6.9, the concentration of NH_4^+ is decreased by more than 50 %. Unionised ammonia is more readily absorbed across the rumen wall than ammonium ions (Mooney & O'Donovan, 1970) Chalmers et al. (1971) have found that when the pH in ruminal fluid is below 6.9, ammonia concentrations in both peritoneal liquor and jugular blood decrease whereas ammonia concentrations in ruminal fluid remain constant. In the present studies, the maximum pH in the ruminal fluid of sheep

occurred just prior to the peak ammonia concentration (0.5 and 1-1.5 h post feeding, respectively) as shown in Figure 4-2. This suggests that the pool size of ammonia in the rumen, and the rate of ammonia diffusion across the rumen wall is regulated by the pH in the rumen.

4.4.3 Ruminal fluid ammonia and VFA concentrations

The fermentation pattern in the rumen of sheep given restricted amounts of chaff once daily differs markedly from that of sheep fed on an hourly basis. VFA concentrations in ruminal fluid increase rapidly commencing at about 0.5 h post feeding, reach a maximum at 2-4 h after feeding and then they remain high for several hours (see Figure 4-3). Fermentation rate and VFA concentration are correlated (Leng & Leonard, 1965) and thus the changing concentration probably reflects an increasing VFA production and maybe an increase in the pool size of micro-organisms in the rumen.

In the sheep given one twentyfourth of their daily ration at hourly intervals, fermentation and microbial growth rates and pool size are relatively constant and therefore in a steady-state.

Studies both *in vitro* (Russell & Sniffen, 1984) and *in vivo* (Song & Kennelly, 1990 ; Hume, 1970a) have demonstrated that bacteria in the rumen, particularly cellulolytic bacteria, require branched-chain fatty acids for the biosynthesis of branched-chain amino acids (Allison, 1969 ; Bryant, 1973). Isoacids (isobutyric and isovaleric acids) in ruminal fluid are mainly derived from oxidative deamination and decarboxylation of amino acids, i e. valine, leucine and isoleucine (Menahan & Schultz, 1964 ; Allison, 1970) from feed protein or from the bacterial lysis which may be primarily caused by predation of protozoa (Coleman, 1967a,b) and bacteriophage infection (Klieve, 1988). The low concentration of the isoacids in ruminal fluid of the sheep with a high concentration of ruminal fluid ammonia, particularly in sheep fed once-daily, could be a result of a higher rate of microbial growth relative to isoacids production in the rumen or to a lower rate of lysis and turnover of microbial protoplasms in the rumen.

4.4.4 Ruminal fluid ammonia and digestibility in the rumen

The requirement of rumen organisms for ammonia depends largely on the carbohydrate source and the availability of ATP from the reactions involved in its breakdown. Different groups of microbes exhibit different substrate requirements. Mehrez et al. (1977) found that when whole barley containing 2-10 g urea/kg was used as a basal diet for sheep, the concentration of ruminal fluid ammonia for maximum rate of digestion of ground barley in nylon bags in the rumen was about 235 mgN/l. Conversely, Odle & Schaefer (1987) with cattle given two basal diets; rolled-barley or cracked-maize each supplemented with ammonium acetate from 0 to 500 g/steer/day in 100 g increments, found that the minimum concentration of ruminal fluid ammonia required for maximum digestion of grain dry matter was at 125 and 61 mgN/l, respectively for barley and maize. Perdok et al. (1988) showed that the minimum levels of ruminal fluid ammonia for maximum *in vivo* digestion of a fibrous diet was 60 mgN/l for cattle fed rice straw. In the study of Perdok and colleagues, the levels of ruminal fluid ammonia were continuously supplied by urea infused into the rumen. Boniface et al. (1986), gave spear grass hay to cattle as a basal diet with urea continuously infused into the rumen. They showed that the maximum rate of forage digestion occurred at about 45 mgN/l but similarly to the results of Perdok et al. (1988) that forage intake continued to increase up to 200 mgN/l as ammonia in ruminal fluid. The present studies clearly indicate that maximum digestibility of oaten chaff occurred around 30 mgN/l.

4.4.5 Ruminal fluid ammonia and microbial cell synthesis in the rumen

For the last two decades, most of the literature reporting studies of the requirement of ammonia for microbial protein synthesis in the rumen have been influenced by the suggestion from Satter & Slyter (1974) that any increase in concentration of ruminal fluid ammonia above 50 mgN/l has no effects on microbial protein synthesis. This has been supported by an *in vitro* study by Russell & Strobel (1987) who found that the intracellular ammonia in bacteria in the rumen is at least 132

mgN/l greater than the extracellular concentration. The maximum rate of protein synthesis in bacteria appears to be when the intracellular ammonia in bacteria is greater than 181 mgN/l.

The optimum concentration of ruminal fluid ammonia for maximum microbial synthesis in the rumen is almost certainly dependent on the diets which, in turn, depend on the microbial populations in the rumen that develop. Pisulewski et al. (1981), using sheep given semi-purified diets containing three different ratios of concentrate to roughage (88:12, 74:26 and 50:50) and with urea being infused into the rumen from 8 to 293 mgN/l, found that microbial protein production in the rumen was optimised when ruminal fluid ammonia was at 84, 102 and 27 mgN/l, respectively. When protein-free purified diets containing 0.9, 1.8, 3.5 and 6.7 % urea were fed to sheep, microbial protein production reached a peak when ruminal fluid ammonia was 133 mgN/l (Hume et al. 1970). In contrast, when calves were given barley as a basal diet supplemented with 0, 1.75 and 3.5 % urea, net microbial-N synthesis per kg dry matter fermented in the rumen (15.7, 20.7 and 23.1 g) increased with increased levels of ruminal fluid ammonia (24, 101 and 327 mgN/l Leibholz, 1980). These finding were similar to those given by Miller (1973) who found that the highest microbial-N outflow from the rumen of sheep was observed when the concentration of ruminal fluid ammonia was approximately 238 mgN/l.

The rumen ecosystem is complex and diverse (Hungate, 1966), and a shift in the mixture of organisms in the rumen may result in variations in synthesises and also in turnover of microbes within the rumen and therefore microbial biomass leaving the rumen. Protozoa sequester in the rumen and only 10-30 % leave the rumen (Weller & Pilgrim, 1974 ; Leng, 1982a). Regardless of the dietary sources, the outflow of protozoa from the rumen is influenced by the outflow rate of ruminal fluid (Singh & Leng, 1987 ; Leng, 1989). An increase in protozoal pool is likely to decrease the bacterial and fungal pools that would develop in the absence of protozoa and in this way increase protozoa in the rumen which, in turn, may decrease microbial biomass leaving the rumen (see Bird et al. 1990). Thus, a study in which only microbial protein synthesis in the rumen is measured does not take account of microbial biomass lost by

lysis in the rumen. This could lead to different interpretation of the result since the important nutritional information is the microbial biomass that enters the small intestine. In these studies, urinary excretion of purine derivatives was employed as an indicator of the amount of microbial biomass leaving the rumen (Chen et al. 1990b).

4.4.6 Ruminal fluid ammonia and microbial mix in the rumen

The use of urinary excretion of purine derivatives as an indicator of microbial outflow from the rumen has to be done with some caution since feed intake and microbial growth condition may have a direct influence on the RNA and DNA contents of microbes in the rumen and therefore purine derivatives excreted. Regardless of species, on the same diet, the amount of purine derivatives excreted in urine in ruminants is increased with increasing organic matter intake (Vercoe, 1976 ; Chen et al. 1992a ; Dewhurst & Webster, 1992). Although there is a variation in the nucleic acids content of rumen microbes (Arambel et al. 1982), there appears to be a close correlation between specific growth rate and the ratio of RNA to N (Bates et al. 1985). The RNA:N ratio of rumen microbes is significantly different depending on the ratios of roughage to concentrate given to ruminants (Arambel et al. 1982 ; Susmel et al. 1993) and therefore knowledge is necessary on the nucleic acids content of rumen microbes in order to be able to interpret urinary excretion in terms of microbial cells entering the intestine. There appears however, to be no difference of RNA:N ratio in rumen bacteria of ruminants fed on forage based diets (see Bates et al. 1985 ; Susmel et al. 1993). This has been confirmed in studies to be reported (Chapter 5) and therefore results reported here are not confounded by differences in feed intake and microbial purine concentrations relative to protein in rumen microbes.

The results presented in Figure 4-1 and 4-4 showed that protozoal, fungal and bacterial populations in the rumen were influenced by the levels of ruminal fluid ammonia.

When the concentration of ruminal fluid ammonia is between 15 and 30 mgN/l, protozoa and fungi were apparently in high population densities but less microbial

purine derivatives were excreted in urine suggesting that there was a relatively low microbial cell washout to the lower tract. This result conflicts with Balcells et al. (1993) who found that urinary excretion of purine derivatives increased with increasing concentrations of rumen fluid ammonia from 6 to 30 mgN/l. However, their results are confounded with differences in feed consumption which were allowed to increase with increasing ammonia levels. Orpin (1975) found that the numbers of rumen phycomycetes were decreased when they were incubated with high concentrations of protozoa in the rumen. In the present studies, there was an increase in protozoal density at the same time as a significant increase in apparent fungal populations as indicated by the number of colonies observed on leaf materials incubated in nylon bags. The predatory rate of protozoa appears to be insufficient to suppress fungal growth but it is apparent that they reduce the biomass of bacteria entering the intestine.

As the concentration of ruminal fluid ammonia was increased from 67 to 179 mgN/l, urinary excretion of purine derivatives seemed to reach a plateau as shown in Figure 4-1 and 4-4. This is similar to the results of Rihani et al. (1993) who studied rams given citrus pulp with two levels of urea (8.8 and 15.1 g/d) and two methods of supplementation (continuously infused into the rumen or mixed with the feed). They found that concentrations of ruminal fluid ammonia were at about 96 and 176 mgN/l and there were no differences in net microbial protein synthesis at either urea levels or methods of supplementation. At these levels of ruminal fluid ammonia, the bacterial and protozoal pools have been reported to be larger (Teather et al. 1980 ; Purser & Moir, 1966). It is more likely that there was an increase in the turnover rate of bacteria (Firkins et al. 1987a) causing a rise in bacterial-N recycling within the rumen (Nolan & Leng, 1972 ; Firkins et al. 1992) which is possibly a result of engulfment and digestion of bacteria by protozoa (Coleman, 1975 ; Cottle et al. 1978 ; Leng & Nolan, 1984).

In the studies now reported, as the concentration of ruminal fluid ammonia was adjusted upwards above 200 mgN/l there was a marked increase in the excretion of purine derivatives which was also associated with a decrease in protozoal and apparent fungal growth, suggesting that one of the following is involved: (i) there is a decrease in bacterial turnover within the rumen (ii) there is a reduction of rate of ingestion of

bacteria by protozoa in the rumen or, (iii) microbial growth efficiency is markedly increased. It is likely that the increase in urinary excretion of purine derivatives is mainly contributed to by a rise in fractional outflow of bacteria (Elliott & Armstrong, 1982) rather than of protozoa from the rumen (Leng, 1989) since the outflow rate of the fluid phase (see Table 4-2) is not significantly different.

The lowering of protozoal density in ruminal fluid at ammonia concentrations in the rumen above 200 mgN/l, suggests that protozoa may be less competitive for critical nutrients i.e. vitamin B than bacteria (see Briggs et al. 1964b). The possible reasons are discussed below.

The majority of rumen bacteria, particularly cellulolytic bacteria, appear to be able to utilise ammonia as the major source of nitrogen (Hungate, 1966 ; Bryant & Robinson, 1962) but they may require amino acids and peptides to a small extent for maximum efficient biosynthesis (Allison, 1969 ; Maeng et al. 1976). In ruminal fluid there are free amino acids derived from incomplete protein metabolism by bacteria and protozoa (Coleman, 1967b) and from lysis of micro-organisms resulting from a number of factors (Wallace & McPherson, 1987). This may provide all the monomers for efficient bacterial biosynthesis in the rumen under most conditions. Protozoa do not use ammonia as a source of nitrogen (Onodera et al. 1977 ; 1983) and preformed amino acids are required for protozoal growth. At the high concentration of ruminal fluid ammonia, bacteria in the rumen appear to be highly efficient and compete effectively for fermentable energy sources with protozoa. This may effectively decrease protozoal growth.

A high concentration of ruminal fluid ammonia may have specific feed back mechanisms on ammonia diffusion from protozoa which disadvantage protozoal growth (i. e. ammonia can be excreted actively). Sudana & Leng quoted by Leng et al (1993) reported a sudden decrease in protozoal population in sheep given oaten chaff-lucerne with high urea diets. The suggestion is also supported by the studies of Hino et al. (1973) who found that when ammonia in solution was above 420 mgN/l, there was a reduction in protozoal surviving over 24 h. When ammonia was above 700 mgN/l, protozoa were unable to survive for 24 h. Similarly, Setälä & Syrjälä (1980), using

sheep on NaOH-treated wheat or barley straw plus a concentrate mixture containing 50 % barley and 50 % molasses-beet pulp supplemented with 20 g/d urea, illustrated that the maximum concentration of ruminal fluid ammonia achieved was about 510 mgN/l at 1 h post-feeding and protozoal density was only about $1x10^5$/ml ruminal fluid which is extremely low for such a diet

4.4.7 Ruminal fluid ammonia and efficiency of net microbial cell synthesis or microbial growth

Efficiency of microbial growth in the rumen depends on ATP generated in VFA production (i.e. ATP generated/unit VFA production) and on the efficiency of use of the ATP for biosynthesis (Y_{ATP}) and maintenance (M_{ATP}). The net microbial growth (i.e. that leaves the rumen) depends on the extent of death and degradation of cells within the rumen. Fifty-five percent of total ATP generated may be required for maintenance of micro-organisms in the rumen (Isaacson et al. 1975). A high concentration of ruminal fluid ammonia could lead to a change in the normal pathway of ammonia assimilation by micro-organisms to a pathway that is independent of ATP (Tyler, 1978), making more ATP available for cell growth. Heat production from fermentation is the direct consequence of maintenance functions (Leng, 1982b ; Russell, 1986 ; Nolan & Leng, 1989) mostly derived from ion transport (Hespell & Bryant, 1979) but energetic uncoupling processes may be important under some conditions particularly when nutrients are deficient in the diet (Tempest & Neijssel, 1984). A limitation of N availability for biosynthesis relative to ATP production by catabolism results in increasing both maintenance energy and energetic uncoupling (see Hespell & Bryant, 1979). A depression of microbial growth increases VFA, CO_2 and CH_4 production. An increased degradation of microbes within the rumen also increases VFA production. Lysis may be mainly due to predatory activity of protozoa in the rumen as well as lytic factors (Wallace & McPherson, 1987). Thus, an increase in efficiency of net microbial growth in the rumen is associated with decreasing VFA, CO_2, CH_4 and heat production (Baldwin et al. 1970 ; Leng, 1982b). In the *in vitro* study by Satter and Slyter (1974), however, under N-limiting conditions, there was a

decrease in microbial protein production without a concomitant increase in VFA production which is not readily explainable in stoichiometric terms.

4.5 Conclusion

The results from the studies presented here showed that at the concentration of ruminal fluid ammonia from 15 to above 200 mgN/l, microbial biomass entering the duodenum as indicated by purine excretion, was markedly incresaed. Concerrent changes included marginal shifts in VFA concentration, and in digestibility of the basal feed. The net result should be an increase in P/E ratio in the nutrient available for digestion and metabolism. An increase in P/E ratio is often associated with increasing efficiency of ruminant production from low quality roughage based diets (Leng, 1990) and at times with increasing voluntary intake (Leng, 1993). The increase in feed intake as P/E ratios in the nutrients absorbed increase above that at optimum digestibility of a feed is hypothesised to be dependent on heat stress condition. The apparent increase in P/E ratios at increasing concentrations of ruminal fluid ammonia as indicated by increasing urinary excretion of purine derivatives may explain the stimulation of feed intake of cattle on a low nitrogen forage at a concentration of ruminal fluid ammonia in excess of 200 mgN/l (Boniface et al. 1986 ; Perdok et al. 1988). Both the latter studies were carried out in high environmental temperature and humidities. Although the excretion of purine derivatives is increased with decreasing population of protozoa, as shown in Figure 4-1 and 4-4, it can be argued that this may be mainly contributed from a higher ratio of nucleic acids to protein in micro-organisms in the rumen (Smith & McAllan, 1974) rather than a higher net microbial protein flow out of the rumen. This has been disproved for these sheep under the specific conditions in studies to be reported using fauna-free and refaunated sheep.

Chapter 5

The Effects of Increasing Ruminal Fluid Ammonia on the Microbial Ecosystem in Fauna-free and Refaunated Lambs.

5.1 Introduction

Fauna-free ruminants have been studied to elucidate the role of protozoa in the rumen in particular their effects on the rumen ecosystem, fermentative end-products and nutrients entering the duodenum (see Williams & Coleman, 1988 for a review). Regardless of diet, there is an increase in the bacterial density in the fluid phase of the rumen in fauna-free ruminants compared with faunated animals (Orpin & Letcher, 1983/84 ; Rowe et al. 1985 ; Newbold & Hillman, 1990). The proliferation of rumen fungi is also apparently enhanced in the absence of protozoa (Newbold & Hillman, 1990) as indicated by either direct count of sporangia on agar strips suspended in the rumen (Ushida et al. 1989) or zoospore concentrations in media cultures (Romulo et al. 1989). This may be due to the absence of predatory activity of protozoa in the rumen (Coleman, 1975) and/or a lack of competition for nutrients by fungi with protozoa (Bird & Leng, 1985). Although the effect of presence or absence of protozoa on fibre digestion in the rumen is variable (see Demeyer, 1989), it is often lower in the fauna-free animal. However, digestibility in the whole tract in fauna-free animals is generally less than 5 digestibility units lower than in faunated animals (Veira, 1986). It is now generally accepted that there is a greater amount of both dietary and microbial protein entering the post ruminal tract of the fauna-free animal, resulting in an increased availability of amino acids relative to energy for the host animal (Lindsay & Hogan, 1972 ; Bird et al. 1979 ; Bird & Leng, 1984 ; Veira, 1986 ; Meyer et al. 1986 ; Ushida et al. 1991 ; Bird et al. 1994).

The concentration of ruminal fluid ammonia in the fauna-free ruminant is considerably lower than in the faunated animal. Concentration of ammonia-N in ruminal fluid below a critical minimum is a major constraint to the growth of bacteria (Bryant & Robinson, 1962) and possibly fungi (see section 4.2.2.4). In a comparative study (see Chapter 4), increasing ruminal fluid ammonia concentrations from 1 to 30 mgN/l in sheep given feed at hourly intervals resulted in an increase in both protozoal and fungal populations and a reduced microbial yield that leave the rumen. Incremental increases in ruminal fluid ammonia concentration above 30 mgN/l resulted in a gradual decline in protozoal and fungal populations and a concomitant increase in the microbial cells entering the intestines. It appeared that the increased microbial biomass available from the rumen may have been due to a reduced protozoal density and consequently a decreased predatory rate of bacteria and/or to an increased bacterial growth *per se*. These studies all implicated protozoa populations as mediating the effects observed. For this reason, the study presented here was designed to study the role of protozoa in the changes that occur in the efficiency of microbial cell synthesis when ammonia levels are progressively increased.

5.2 Materials and Methods

5.2.1 Animals:

Twenty first-cross Merino x Border Leicester lambs 9-12 months of age and weighing 24-30 kg were surgically fistulated in the rumen. The lambs were held in metabolism crates in an animal house (see section 3.2.1).

5.2.2 Diets and Feeding:

Oaten chaff (0.86 % N) from one source and batch was fed as the basal feed for the lambs throughout the study. The mineral mix (see section 3.2.2.2) was added at the level of 2 % of daily ration. Urea solutions (0, 7, 14 and 21 g urea dissolved in tap water) were prepared daily (see section 3.2.2.1) and sprayed on to the daily ration of

700 g of oaten chaff. The diet was delivered to each lamb in 24 equal portions at hourly intervals by an automatic- feeding machine.

5.2.3 Experimental Procedure:

The experiment was divided into two 28-d periods. The lambs were defaunated in the first period and maintained fauna-free by isolation in single pens (see section 5.2.4) and then refaunated (see section 5.2.5). Sixteen lambs were randomly allocated into four dietary urea treatments and allowed to become accustomed to the diets and metabolism cages for 14 d prior to commencement of the first period. In each of the 28-d experimental periods, the lambs were undisturbed for 21 d and over the last 7-d the experimental procedures were undertaken. On days 22-24, the daily urine voided by the animals was quantitatively collected each day (see section 3.2.6) for analysis of purine derivatives (see section 3.8.5). On day 25, rumen digesta was collected via the rumen fistula (see section 3.2.5). The sample was processed to isolate fluid- and particle-associated bacteria and protozoa in ruminal fluid (see section 3.3) and these harvested microbes were analysed for nitrogen (see section 3.8.1) and purine (see section 3.8.1) content. On day 26, ruminal fluid was taken at 10.00 and 14.00 h via a probe covered with a double layer of nylon stocking material for the determination of the bacterial dry matter. On day 27, Cr-EDTA (1 mgCr/kg BW) was injected intraruminally. Following the injection, 10 samples of ruminal fluid were periodically collected from each lamb over 24 h. Prior to acidification, a sample of ruminal fluid was taken for measurement of pH (see section 3.4) and for either enumeration of protozoa (refaunated period ; see section 3.5) or for checking the fauna-free state (fauna-free period). The remainder was acidified (see section 3.2.4) and then analysed for NH_3-N (see section 3.8.2), VFA (see section 3.8.3) and Cr (see section 3.8.4). On day 28, *in sacco* digestibility of oaten chaff (24 h) was estimated (see section 3.7) and nylon bags containing oat leaf samples were also suspended in the rumen for enumeration of sporangial colonies that developed on the oat leaf blade (see section 3.6).

5.2.4 Defaunation Procedure:

Eighteen lambs were successfully defaunated. Two untreated lambs were used as sources of bacterial and fungal inoculum (see later) and protozoal inoculum (see section 5.2.5). The lambs were defaunated using a procedure similar to that used by Bird & Leng (1984). A 10 % alkanate solution 3SL3 (active ingredient sodium lauryl diethoxy sulphate; ICI Australia Ltd) was delivered directly into the rumen of the lambs via a probe entering through the rumen cannula. Each animal was given a dose on each of 3 consecutive days: 120 ml of the solution for the first two days and 100 ml on the last day. Feed was not given during this 3-d period. The animals were maintained in an isolated area and remained fauna-free throughout the first experimental period. Ruminal fluid was collected from each animal and examined under a light microscope to check for the absence of protozoa. This was carried out once a week for 3 consecutive weeks following the alkanate drenching.

Ruminal fluid from the two untreated lambs was collected via the rumen fistula with a probe covered with a double layer of nylon stocking material and centrifuged at 400 g for 3 min under CO_2. The supernatant fraction was collected with a syringe and recentrifuged twice. The supernatant fraction from the last centrifugation was checked for the absence of protozoa under a light microscope. The fauna-free supernatant was then reinoculated into the rumen of two the fauna-free lambs. Ruminal fluid from the reinoculated lambs was checked and ascertained to be fauna-free for 2 w after reinoculation. A 25 ml sample of ruminal fluid from each of the donor fauna-free lambs was transferred to the rumen of the remaining 16 fauna-free lambs. This procedure was repeated on 3 consecutive days. To enable the rumen populations to reach a new equilibrium, a further 3 w was allowed prior to commencement of the first experimental period.

5.2.5 Refaunation procedure:

Ruminal fluid from the 2 untreated lambs given an oaten chaff plus urea diet was collected and centrifuged at 400g for 3 min under CO_2. Protozoal pellets were

retained and used to reinoculate the rumen of the two donor fauna-free lambs. After 3 w, 25 ml of ruminal fluid from each of the donor lambs was transferred into the rumen of the remaining fauna-free lambs at the end of the first experimental period. Prior to commencement of the second experimental period, the lambs were allowed 3 w for their rumen microbial ecosystem to equilibrate.

5.2.6 Chemical Analysis:

5.2.6.1 Isolation of bacteria in the fluid phase with tungstic acid precipitation

The method described by Shultz & Shultz (1969) for the isolation of bacterial cells in the fluid phase from rumen digesta using tungstic acid precipitation was modified. The large feed particles and rumen protozoa were removed in the precipitate from ruminal fluid following 2 centrifugations at 400 g for 3 min. Fifteen ml of the supernatant from the second centrifugation was decanted into a McCartney bottle and precipitated with 3 ml of 1.07 N H_2SO_4 followed by 3 ml of 10 % Na_2WO_4 and 1 ml of 6 mM EDTA. After standing in ice for 4 h, the mixture was centrifuged at 2000 g for 20 min. The pellet was washed twice (taking care not break the pellet) using 2 ml of a solution containing 10 % (v/v) each of the H_2SO_4 and Na_2WO_4 solutions. The samples were dried in an oven at 105^0 overnight and the dry matter of free bacteria/ml ruminal fluid was calculated and, then, corrected for the neutral detergent fibre (Goering & Van Soest, 1980) present in the pellet (the value of 3% $\pm$ 0.3).

5.2.7 Statistical Analysis:

The analysis of variance was determined according to a Split Plot design. Because the lambs had been allocated at random to dietary urea treatments, each treatment was included as a mainplot. The same treatment were applied in the second period. The faunation status was considered as a subplot in order to increase the sensitivity of the test. Statistical analysis of the data was done as described in section 3.9.

5.3 Results

5.3.1 Defaunation status

All the fauna-free lambs appeared to be in good health following the delivery of the alkanate solution. The animals remained fauna-free throughout the first experimental period.

5.3.2 Concentration and pool size of ruminal fluid ammonia

Concentrations and pool sizes of ruminal fluid ammonia in both fauna-free and refaunated lambs were significantly increased with increasing urea intake ($p<0.01$; Table 5-1). The concentration and pool size of rumina fluid ammonia were 58 and 24%, ($0<0.01$) lower in fauna-free than in refaunated lambs, respectively.

5.3.3 Rumen volume and kinetics of ruminal fluid outflow

The addition of 7 g/d urea to the basal diet resulted in an increase in rumen volume but the volume was decreased thereafter with increasing intake of urea in lambs (Table 5-1). Rumen volume was significantly greater in fauna-free than in refaunated lambs ($p<0.01$). Fluid outflow rates from the rumen in both fauna-free and refaunated lambs were not significantly different with increasing urea intake ($p>0.05$). However, fractional turnover rate in fauna-free lambs was significantly lower than that in refaunated lambs ($p<0.01$).

5.3.4 Fermentation in the rumen

The level of dietary urea had no consistent effect on the molar proportion (%) of individual volatile fatty acids (VFA) in ruminal fluid (Table 5-2). The proportion of acetic, isobutyric, isovaleric and valeric acids were significantly higher in fauna-free than in refaunated lambs but the proportion of propionic and butyric acids were significantly lower ($p<0.05$ or $p<0.01$). Total VFA concentration in fauna-free lambs

was significantly lower than in refaunated lambs ($p<0.01$) but it tended to increase with increasing the levels of urea intake (Table 5-2). However, the total pool size of VFA in ruminal fluid of the fauna-free lambs did not differ from that of refaunated lambs ($p>0.05$).

The pH in ruminal fluid was 0.1 unit higher ($p<0.01$) in fauna-free than in refaunated lambs (Table 5-1).

5.3.5 Microbial mix in the rumen

The density of protozoa in ruminal fluid in refaunated lambs increased with the addition of 7 g/d urea and decreased thereafter with increasing levels of urea intake ($p<0.05$). Fungal sporangia growth on oat leaf blades incubated in the rumen of both fauna-free and refaunated lambs increased, with the initial increase in urea intake (7g/d) and decreased thereafter, with increasing levels of urea intake. Numbers of fungal sporangia on leaf blades in fauna-free lambs tended to be slightly greater ($p>0.05$) than those in refaunated lambs (Table 5-3).

Concentration of bacteria in the fluid phase of the rumen digesta (cell DM/ml) in fauna-free lambs was almost twice that in refaunated lambs ($p<0.01$; Table 5-3). In addition, the bacterial-N pool in the fluid phase of rumen digesta was greater ($p<0.01$) in the fauna-free than in refaunated lambs and tended to increase with increasing levels of urea intake irrespective of faunation status (Figure 5-1)

Urinary excretion of purine derivatives increased with increasing urea intake but it was 32% greater from fauna-free than refaunated lambs ($p<0.01$; Table 5-3). In addition, when compared at the same concentration of ruminal fluid ammonia (160 mgN/l), the excretion of purine derivatives from fauna-free lambs was 47% greater than from refaunated lambs ($p<0.01$)

5.3.6 The purine:total-N ratio of micro-organisms in the rumen

The N content (% DM) of bacteria in the fluid phase and particle-associated microbes did not differ ($p>0.05$) and was 8.53 ± 0.73 and 8.71 ± 0.85 in fauna-free lambs, respectively and 7.98 ± 0.53 and 8.31 ± 0.91 in refaunated lambs, respectively. The mean N content (% DM) of protozoa was 9.02 ± 0.85.

The purine:total-N ratio of bacteria in the fluid phase and particle-associated microbes was not affected by urea intake or faunation status ($p>0.05$). The purine:total-N ratio in the bacteria isolated from the fluid phase was significantly higher than that isolated from the particle phase ($p<0.01$). The purine:total-N ratio of bacteria in the fluid phase and particle-associated microbes was 0.174 ± 0.011 and 0.156 ± 0.01 in fauna-free lambs and 0.184 ± 0.012 and 0.164 ± 0.012 in refaunated lambs, respectively. The ratio for protozoa was 0.149 ± 0.009.

5.3.7 Efficiency of net microbial cell synthesis in the rumen and 24 h OM digestibility *in sacco*

The quantity of bacterial-N in the fluid phase potentially leaving the rumen and entering the intestine was calculated from the outflow rate of ruminal fluid (measured using Cr-EDTA) and the concentration of bacterial dry matter in the fluid phase (see Table 5-3) and N content of these bacteria. The total microbial-N entering the duodenum was calculated from the urinary excretion of purine derivatives using an equation given by Chen & Gomes (1992) modified to take account of the purine and N content of rumen microbes measured in this study. The N content of purine was calculated to be 48.6% assuming a ratio of 60% guanine and 40% adenine (see analysis for purine content in rumen microbes in section 3.8.6). The purine-N:total-N ratio of rumen microbes used in this study was 0.0824 (cf. the value of 0.116 that was used by Chen & Gomes 1992 in their calculations).

Efficiency of net microbial-N synthesis in the rumen increased with increasing urea intake in both fauna-free and in refaunated lambs and was 49 % higher in lambs

on 21 g/d of urea than in lambs on the control diet. It was 34% greater in fauna-free than refaunated lambs ($p<0.01$) and was 46 % greater when compared at the same concentration of ruminal fluid ammonia (Table 5-3).

In sacco organic matter digestibility in the rumen (24 h) was not affected by faunation status ($p>0.05$) when urea was included in the diet, but in the lambs fed the control diet the presence of protozoa apparently increased organic matter digestibility at low ammonia concentration in the rumen (Figure 5-1). However, it tended to increase with increasing concentrations of ruminal fluid ammonia in fauna-free lambs ($p>0.05$).

Table 5-1. Effects of levels of urea intake on concentrations and pool sizes of ruminal fluid ammonia, pH and the kinetics of ruminal fluid of fauna-free (-P) and refaunated (+P) sheep.

NH_3-N	Urea (g/d)					Statistical effects		
(mgN/l)	0	7	14	21	Mean	Urea(U)	Fauna(F)	UxF
-P	3	41	111	161	79	**	**	**
+P	6	77	164	252	125			
Mean	5	59	138	206				
Pool sizes of ruminal fluid ammonia (mgN)								
-P	17	309	722	949	499	**	**	-
+P	35	423	836	1184	619			
Mean	26	366	779	1066				
pH								
-P	6.82	6.80	6.83	6.82	6.82	-	**	-
+P	6.79	6.69	6.7	6.65	6.71			
Mean	6.81	6.74	6.77	6.73				
Rumen volume (l)								
-P	6.16	7.40	6.46	5.88	6.47	*	**	-
+P	5.37	5.49	5.09	4.74	5.17			
Mean	5.76	6.44	5.77	5.31				
Fluid outflow rate (l/d)								
-P	13.36	12.45	11.56	11.27	12.16	-	-	-
+P	14.53	12.51	11.02	10.40	12.12			
Mean	13.95	12.48	11.29	10.84				
Fractional turnover rate (/d)								
-P	2.17	1.72	1.81	1.93	1.91	-	**	-
+P	2.70	2.30	2.18	2.21	2.35			
Mean	2.44	2.01	1.99	2.07				

* or ** indicates statistical difference at $p<0.05$ and $p<0.01$, respectively.

Table 5-2. The profiles of volatile fatty acids in ruminal fluid of fauna-free (-P) and refaunated (+P) sheep with different levels of urea intake.

	Urea (g/d)					Statistical effects		
C2(%)	0	7	14	21	Mean	Urea(U)	Fauna(F)	UxF
-P	71.3	72.1	74.2	71.5	72.3	*	*	-
+P	70.1	71.5	70.6	69.7	70.5			
Mean	70.7	71.8	72.4	70.6				
C3 (%)								
-P	18.5	18.2	16.2	18.3	17.8	*	*	-
+P	20.6	20.9	19.5	20.5	20.4			
Mean	19.6	19.6	17.8	19.4				
IsoC4 (%)								
-P	0.77	0.59	0.69	0.65	0.68	-	**	-
+P	0.54	0.36	0.37	0.25	0.38			
Mean	0.65	0.48	0.53	0.45				
C4 (%)								
-P	6.51	6.44	6.62	6.31	6.50	-	**	-
+P	7.91	7.73	8.61	7.49	7.9			
Mean	7.21	7.09	7.61	6.90				
IsoC5 (%)								
-P	0.86	1.18	1.82	1.95	1.45	-	**	-
+P	0.47	0.43	0.55	0.39	0.47			
Mean	0.67	0.81	1.19	1.16				
C5 (%)								
-P	0.59	0.59	0.51	0.47	0.54	*	**	-
+P	0.39	0.50	0.38	0.33	0.40			
Mean	0.49	0.55	0.44	0.40				
Total VFA concentration (μmoles/ml)								
-P	56.9	62.0	61.4	60.9	60.3	-	**	-
+P	64.1	71.4	78.7	83.4	74.4			
Mean	60.5	66.7	70.0	72.1				
Total VFA pool (mmoles)								
-P	351	459	397	361	390	-	-	-
+P	344	392	401	395	385			
Mean	348	426	399	378				

* or ** indicates statistical difference at $p<0.05$ and $p<0.01$, respectively.

Table 5-3. The effects of different levels of urea intake on the numbers of protozoa and fungi in the rumen, bacterial dry matter in the fluid phase, microbial-N outflow from the rumen (gN/d) and calculated efficiency of net microbial cell synthesis in the rumen (gN/kg.organic matter digested in the rumen : OMDR) of fauna-free (-P) and refaunated (+P) sheep.

Protozoa	Urea (g/d)					Statistical effects		
(10^5/ml)	0	7	14	21	Mean	Urea(U)	Fauna(F)	UxF
-P	-	-	-	-	-	*	-	-
+P	2.0	3.6	2.8	2.4	-			
Fungi (Sporangia/mm^2)								
-P	15	38	28	22	25	*	-	-
+P	12	31	26	17	22			
Mean	14	34	27	20				
Densities of bacteria in the fluid phase (mgDM/ml)								
-P	9.10	12.42	14.43	16.78	13.18	**	**	*
+P	5.26	6.36	7.23	9.00	6.96			
Mean	7.18	9.39	10.83	12.89				
[1]Bacterial N entering the small intestine from the fluid phase (gN/d)								
-P	10.07	12.59	13.63	15.61	12.97	**	**	*
+P	6.13	6.55	6.56	7.73	6.74			
Mean	8.10	9.57	10.10	11.67				
[2]Total microbial N entering the small intestine (gN/d)								
-P	8.11	11.09	12.05	13.02	11.07	**	**	*
+P	6.26	8.07	8.83	10.32	8.37			
Mean	7.19	9.58	10.44	11.67				
Efficiency of net microbial synthesis in the rumen (gN/kg.OMDR)								
-P	29.40	36.69	39.86	41.88	36.96	**	**	*
+P	21.41	26.47	28.76	33.66	27.57			
Mean	25.41	31.58	34.31	37.77				

* or ** indicates statistical difference at $p<0.05$ and $p<0.01$, respectively.

[1] Calculated from concentrations of bacterial-N in the fluid phase and fluid outflow rate.

[2] Calculated from urinary excretion of purine derivatives.

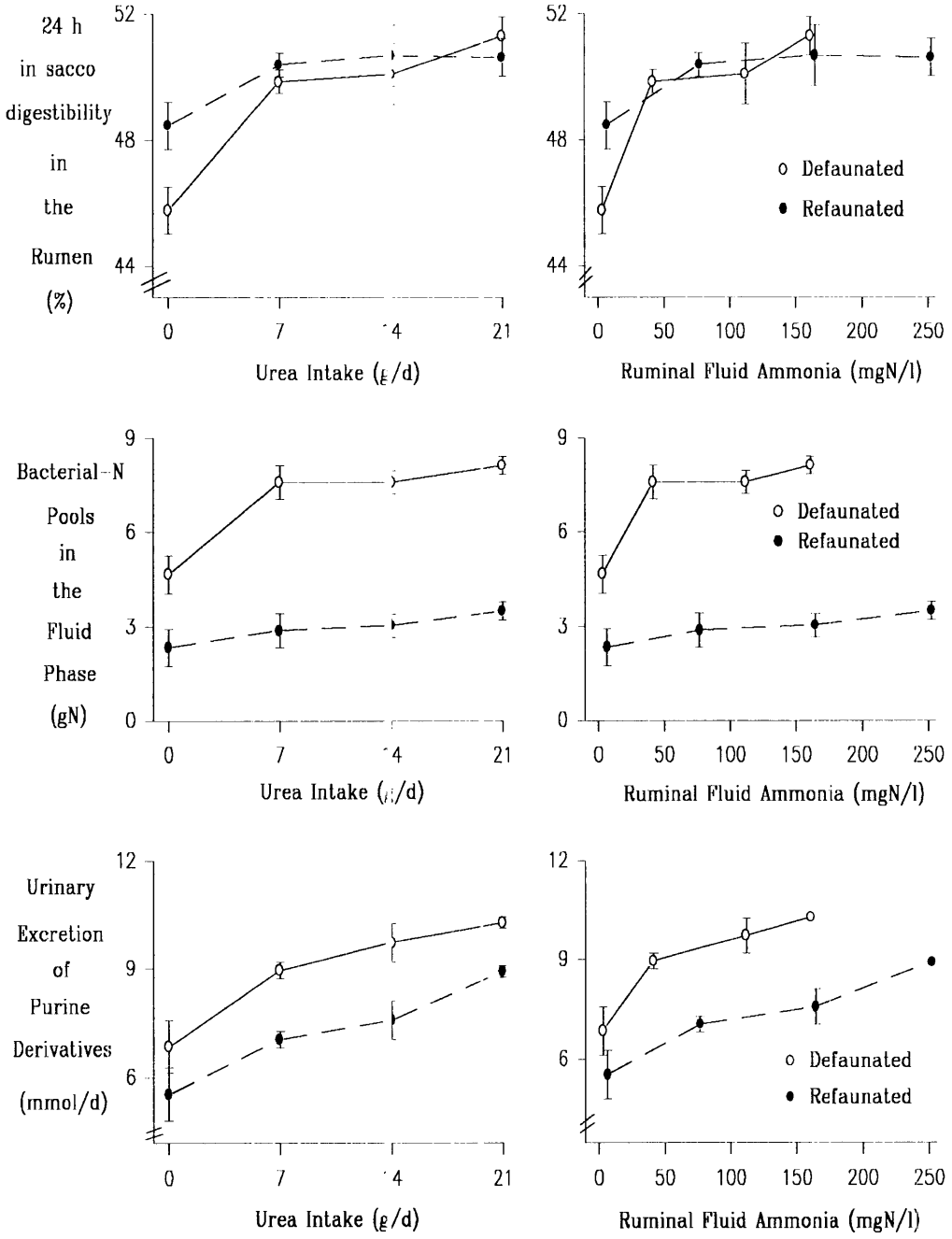

Figure 5-1. Effects of urea intake and concentrations of ruminal fluid ammonia on the pool of bacterial-N in the fluid phase, 24 h *in sacco* organic matter digestibility and urinary excretion of purine derivatives.

5.4 Discussion

The quantities of urea fed to sheep in this study resulted in similar concentrations of ruminal fluid ammonia obtained to similar inputs in sheep in the previous experiment (see Chapter 4). In this study increasing the concentration of ruminal fluid ammonia above 30 mgN/l did not result in any change in the *in sacco* organic matter digestibility. However microbial biomass entering the intestine (estimated by urinary excretion of purine derivatives ; Chen & Gomes, 1992) continued to increase as the concentration of ruminal fluid ammonia increased above 200 mgN/l. The latter may be related to effective competition for other critical nutrients between bacteria and protozoa and fungi in the rumen at high ruminal fluid ammonia concentrations. These interactions may result in either increased total bacterial growth rate as indicated by an increase in RNA:N ratio of bacteria in fauna-free animals (see Smith & McAllan, 1974) or increased net synthesis of bacterial cells as estimated by an increase in incorporation of ammonia-N relative to ^{32}P (Demeyer & Van Nevel, 1979b). The major objective of this study was to compare the relationship between ruminal fluid ammonia concentrations and the specific growth rate of rumen microbes (indicated by the purine:total-N ratio) and the role of protozoa. Changes in these relationships, ultimately, resulted in a change in net microbial cell synthesis that leaves the rumen (calculated using urinary excretion of purine derivatives) of fauna-free and refaunated lambs.

5.4.1 Defaunation of the rumen

The changes in rumen function associated with the fauna-free condition are a balance between protozoal activity (direct and indirect) that is removed and changes in size and activity of bacterial and fungal populations. It was decided to compare the same animals in two experimental periods (fauna-free and refaunated) rather than compare a group of fauna-free and refaunated animals simultaneously as individual animal variation is a problem with the latter design. To minimise the time interaction or increase the sensitivity of the test, the faunation status was regarded as a subplot in the analysis of variance (see section 5.2.7).

The alkanate drench used in this study to defaunate the lambs was also likely to adversely effect other rumen micro-organisms. To minimise this effect, lambs were dosed with ruminal fluid collected from the untreated lambs (protozoa removed) so that these micro-organisms had the opportunity to re-establish themselves in the rumen of the fauna-free lambs.

5.4.2 Ruminal fluid ammonia and rumen volume in fauna-free and refaunated animals

An increase in rumen volume is generally accompanied by an increase in total digesta in the rumen. This is apparently due to an increase in the rate of salivary secretion and the entry rate of water into the rumen via the rumen wall and to an increased retention time of feed particles in the rumen (see Owens & Goetsch, 1986). Compared with faunated animals, rumen volume is generally higher in fauna-free animals, regardless of diet (Pounden & Hibbs, 1950 ; Eadie & Gill, 1971 ; Itabashi et al. 1990 ; Orpin & Letcher, 1984). In fauna-free animals on a concentrate diet, the absence of starch ingestion by protozoa in the rumen has been hypothesised to increase bacterial fermentation of starch, tending to lower pH and to increase the rate of salivary secretion into the rumen in order to buffer the rumen content. However, the amount of starch ingested by protozoa can only be small relative to the feed starch entering the rumen. It is more likely that the increased pool of bacteria in the fluid phase in the milieu allows more rapid colonisation of starch grains. The slower rate of fermentation generally observed in fauna-free animals (see Veira, 1986) fed on low nitrogen forages may increase retention time of the rumen digesta and thus increase rumination which, in turn, increases the rate of salivary secretion (McDougall, 1948).

Ruminal fluid ammonia concentration may also influence rumen volume of sheep (Hume et al. 1970). In the faunated rumen, increasing the ruminal fluid ammonia from 4 to 170 mgN/l had no effect on volume of ruminal fluid but further increases above 200 mgN/l were associated with a decrease in volume of ruminal fluid (see section 4.3.2.2). Similar effects were observed in both fauna-free and refaunated animals in the present study (see Table 5-1). In contrast, the volume of ruminal fluid was unchanged in both fauna-free and refaunated sheep fed a basal diet of wheat straw

supplemented with different sources of protein (Romulo, 1986). The absence of a response in the studies of Romulo (1986) may have been due to the high level of ruminal fluid ammonia concentration (in excess of 200 mgN/l) in these sheep.

5.4.3 Ruminal fluid ammonia in the fauna-free rumen

In this study, the mean concentration of ruminal fluid ammonia in fauna-free and refaunated lambs across treatments was on average 79 and 125 mgN/l, respectively. This result is consistent with the results reported in the literature (see Bird et al. 1990 for a review).

The mean rumen volume in fauna-free and refaunated lambs was 6.5 and 5.2 l, respectively. This difference in rumen volume would account for some of the differences in ruminal fluid ammonia concentration. However, the pool size of ruminal fluid ammonia in fauna-free lambs (510 mgN) was lower than that of refaunated lambs (650 mgN).

Urease activity in the rumen is generally high and as a consequence, urea entering the rumen is rapidly hydrolysed to ammonia (Cheng & Wallace, 1979 ; Norton et al. 1982). In addition, it is apparent that the urease activity in the faunated rumen may be lower than in the fauna-free rumen (Males & Purser, 1970). Therefore, the lower pool size and concentration of ruminal fluid ammonia in fauna-free lambs in this study are unlikely due to a difference in the rate of urea hydrolysis.

The critical pH of ruminal fluid for the diffusion of ammonia across the rumen wall is 6.9 (see section 4.3.2). In this current study, the pH was only 0.1 pH unit higher in fauna-free (6.8) than refaunated (6.7) lambs. Therefore pH of ruminal fluid is unlikely to have a major differential effect on the rate of ammonia transported through the rumen wall.

Bacteria and possibly fungi in the rumen, incorporate ammonia via glutamate dehydrogenase or glutamine synthetase and glutamate synthase (Tyler, 1978) whereas protozoa in the rumen are unable to utilise ammonia as a source of nitrogen (Onodera

et al. 1977 ; 1983). The concentration of bacteria in the fluid phase in the fauna-free lamb (13.2 mgDM/ml) was considerably higher than in the refaunated lamb (7.0 mgDM/ml). This is possibly best explained by the absence of protozoal predation (see Coleman, 1975) with consequently less protozoal degradation of bacterial-N to ammonia within the rumen. At the same ammonia concentration in the rumen, there was a 47 % higher microbial biomass (as indicated by purine excretion) entering the post ruminal tract in fauna-free as compared with refaunated lambs. The reduction in the pool size of ammonia in the absence of protozoa in the rumen may, therefore, result from a major reduction in microbial lysis from predation and a greater capacity for the bacteria to grow and utilise ammonia efficiently.

As evidenced from ^{15}N studies there is a substantial turnover of microbial nitrogen within the rumen (Mathison & Milligan, 1971 ; Nolan & Leng, 1972 ; Nolan & Stachiw, 1979). This is largely due to the predatory activity of protozoa (Coleman, 1975) and possibly bacteriophage invasion of bacteria (Klieve, 1988). The rate of bacterial protein turnover *in vitro* in the absence of protozoa in ruminal fluid was only 6.4 % of that in the presence of protozoa in ruminal fluid (Wallace & McPherson, 1987). Similarly, there was a 10-fold reduction in the rate of the bacterial protein turnover and a 2-fold reduction in the rate of fungal protein turnover *in vitro* in fauna-free ruminal fluid, respectively (Newbold & Hillman, 1990). The predatory activity of the protozoa can be expected to contribute to a high concentration and pool size of ruminal fluid ammonia in the refaunated lambs used in this study.

5.4.4 Ruminal fluid ammonia and VFA concentrations

Total VFA concentration in the rumen of fauna-free lambs (60 µmol/ml) was lower than in refaunated lambs (74 µmol/ml) which is consistent with results reported in the literature (Veira, 1986). An increase in total VFA concentration in the presence of protozoa in the rumen may be partly due to the substantial microbial cell turnover within the rumen and fermentation of the lysed cells to VFA (Kurihara et al. 1968 ; Leng, 1982b). In addition, the inverse relationship between the synthesis of microbial cells (which was lower in faunated animals, see Table 5-3) and VFA production

(Baldwin et al. 1970 ; Leng, 1982b) will contribute to the higher concentration of VFA measured in the refaunated lambs.

5.4.5 Ruminal fluid ammonia and fibre digestion

Fibre digestion in the rumen is a complex process (see Russell & Hespell, 1981) involving a consortium of fungi, bacteria and protozoa. Feed particles entering the rumen are rapidly colonised by fungi, bacteria and protozoa (primary fermentors) particularly in the areas of physical damage and non-cutinised surfaces (Orpin, 1983/84 ; Bauchop, 1989). The primary fermentors digest and release soluble substrates which are further utilised by other organisms (Cheng et al. 1991). While it has been demonstrated *in vitro* that individual species of rumen fungi (Bauchop, 1989 ; Wubah et al. 1993), bacteria (Hungate, 1966) and protozoa (Orpin, 1983/84 ; Coleman, 1985, 1986 ; Bauchop, 1989 ; Jouany, 1989) possess enzymes capable of degrading fibre, the contribution of each group to fibrolytic activity in the rumen is uncertain. As a consequence within a mixed milieu it is difficult to estimate the contribution of individual groups of organisms. Decreased fibre digestion in the rumen of fauna-free animals relative to faunated animals has been observed (see Veira, 1986) which may be due to the loss of specific protozoal cellulolytic enzymes or to the loss of synergetic activity of the protozoa in the microbial ecosystem. However, in the present study significantly lower digestibility in fauna-free lambs was apparently associated with reduced ammonia levels in the rumen.

The attachment of fibrolytic organisms to feed particles and fibrolytic activity in the rumen is apparently influenced by rumen environment. Some recent results indicate that the formation of fibrolytic enzymes by rumen fungi are suppressed by the presence of glucose (Williams et al. 1994a). The quantities of microbes attached to fibre particles (Shriver et al. 1986) and fibrolytic activity (Mould et al. 1983/84 ; Cheng et al. 1991 ; Huhtanen & Khalili, 1992) were shown to be influenced by concentrations of sugars or pH in the rumen. Maximum attachment of bacteria to feed particles occurred at ruminal fluid pH of 6.0 (Bhat et al. 1990) whereas fibrolytic activity decreased with decreasing pH from 7 to 6 (Stewart, 1977). The ruminal fluid pH in fauna-free and

refaunated lambs was similar (6.8 and 6.7, respectively) in this study and was unlikely to have a differential effect on fibre digestion in these two groups of animals.

Fibrolytic bacteria (Bryant & Robinson, 1962) and fungi (see section 4.3.5) appear to require ammonia-N for growth. In this study, the *in sacco* digestibility (24 h) was lower in fauna-free (45.8 5) than in refaunated lambs (48.5 %) given the basal diet. However, when concentrations of ruminal fluid ammonia from urea were above 40 mgN/l, the *in sacco* digestibility was similar in fauna-free and in refaunated lambs. These results suggest that at low ruminal fluid ammonia concentrations the removal of protozoa results in the critical reduction of available ammonia-N in the rumen. A batch culture of ruminal fluid with and without either protozoa incubated with or without added ammonia, showed that cellulose digestion in the protozoa-free culture without added ammonia was significantly lower than the similar culture with added ammonia while the latter culture did not differ from the protozoa culture, regardless of added ammonia (Ushida & Kojima, 1991). Results from this study indicate that maximum *in sacco* organic matter digestion occurred at ruminal fluid ammonia concentration above 40 mgN/l irrespective of faunation status while there was a tendency for the *in sacco* organic matter digestibility in the fauna-free rumen to increase with increasing concentrations of ruminal fluid ammonia (Figure 5-1). With sheep fed on wheat straw plus urea 1.4-1.8% of feed intake as a basal diet supplemented with different sources of dietary true protein, Romulo (1986) found that when the concentration of ruminal fluid ammonia was above 200 mgN/ *in sacco* digestibility was slightly higher in fauna-free than in refaunated animals. These results suggest that the requirement for ammonia-N for maximum fibre digestion in the rumen is higher in fauna-free than in refaunated lambs and is in excess of 160 mgN/l.

5.4.6 Ruminal fluid ammonia and microbial mix in the fauna-free rumen

There is a considerable amount of evidence in the literature indicating a negative correlation between the densities of protozoa and bacteria in ruminal fluid (for a review see Coleman, 1989). A consequence of this relationship is that under the same dietary conditions the pool of microbial biomass may remain similar in fauna-free and

faunated animals (Teather et al. 1984). In the fauna-free rumen, the density of bacteria in ruminal fluid is 3 to 6-fold greater than in the faunated rumen of animals on the same diet (Kurihara et al. 1978 ; Orpin 1983/84 ; Rowe et al. 1985 ; Newbold & Hillman, 1990). In many situations, fungal zoospores or sporangia are also increased in the fauna-free rumen (Romulo et al. 1989 ; Ushida et al. 1989 ; Newbold & Hillman, 1990), as were observed in this study (Table 5-3). In addition to their predatory activity, protozoa compete for substrates with fungi and bacteria. Protozoa secrete chitinases (Morgavi et al. 1994) which may damage fungal cells. Synergistic interactions between some species of bacteria and fungi in the rumen may also contribute to the increased proliferation of fungi in the fauna-free rumen (Williams et al. 1994b).

The relationship between populations of fluid phase and particle-associated bacteria remains unclear. Orpin & Letcher (1983/84) reported that compared with the faunated rumen there was a considerable increase in the fluid phase bacterial numbers in the fauna-free rumen but the densities of particle-associated microbes remained unchanged. The result of Orpin & Letcher (1983/84) indicated that the adhesion of some bacterial species to feed particles may be limited by appropriate attachment sites as was reported in an *in vitro* study (Bhat et al. 1990). However, Argyle & Forster (1989) found that there was a high positive correlation between the numbers of total viable bacteria in the fluid and particle phases in the rumen both with and without protozoa. This may be related to the interchanges of bacteria between the two phases (Leedle et al. 1982). As bacterial-N pools in the fluid phase in fauna-free lambs markedly increased initially with (7 g/d urea) and remained unchanged with (14 g/d urea) and continued to rise thereafter with urea intake (21 g/d), the net microbial-N synthesis in the rumen increased without an apparent plateau with increasing urea intake (Figure 5-1 and Table 5-3). This indicates that the adhesion of rumen microbes to feed particles is unlikely to be limited by attachment sites.

The density and pool size of particle-associated microbes are generally larger than that of microbes in ruminal fluid (Preston & Leng, 1987 ; Cheng et al. 1990). However, the relative contribution of the two microbial pools to net microbial cell

movement out of the rumen is related to the fractional turnover of the two pools. The retention time of digesta particles is 2 to 4-fold longer than that of fluid phase (see Owens & Goetsch, 1986). Using the densities of bacteria associated with digesta particles ($17.4x10^9$ /gDM) and ruminal fluid ($5.3x10^9$ /ml) measured in sheep (Argyle & Forster, 1989) and the flow rate of fluid and digesta phases measured by Forster (1989), the fluid phase bacteria appear to contribute 90 % of the microbial biomass entering the intestine of sheep given a roughage diet. A similar calculation using the data of Legay-Carmier & Bauchart (1989) for particle-associated microbes (70 % of microbial biomass in the rumen) and the flow data of Owens & Goetsch (1986) in cattle given a 50:50 hay:concentrate diet suggests that the fluid phase bacteria contributed approximately 65 % of the microbial biomass leaving the rumen.

In this study, the quantity of bacterial-N in the fluid phase leaving the rumen was calculated using the concentration of bacterial-N in ruminal fluid and the fluid outflow rate (Table 5-3). Total microbial-N entering the small intestine was estimated from the purine derivatives excreted in urine (Chen & Gomes, 1992). In refaunated lambs, the fluid phase bacteria contributed approximately 80 % of the total microbial nitrogen entering the small intestine which is reasonable agreement with the calculated estimate using the Argyle & Forster (1989) data. However, in fauna-free lambs, the outflow of fluid phase bacterial-N was greater than the estimate for total microbial-N entering the small intestine which indicates that this approach may over-estimate the actual value.

The concentrations of bacteria in the fluid phase in both fauna-free and refaunated lambs were increased with increasing concentrations of ruminal fluid ammonia (Figure 5-1). Increasing ruminal fluid ammonia concentrations in fauna-free and in refaunated lambs from 3-151 and 6-252 mgN/l resulted in an increase of approximately 61 and 65 % in the intestinal availability of microbes (indicated by purine excretion), respectively. This indicates that additional ammonia-N is required to maximise net microbial cell synthesis in the rumen of both fauna-free and refaunated lambs. In fauna-free lambs the net microbial cell synthesis in the rumen was 32 %

higher than in refaunated lambs. This indicates that the requirement for ammonia-N will be higher than in refaunated animals.

5.4.7 Ruminal fluid ammonia and net microbial cell synthesis in the fauna-free rumen

The ratio ribonucleic acid to total nitrogen (RNA:total-N) and the specific growth rate of non-rumen and rumen organisms are highly correlated. A high RNA:total-N ratio is associated with a high specific growth rate (Koch, 1970 ; Bates et al. 1985). The RNA:total-N ratio of bacteria in the fluid phase has been found to vary due to the bacterial species (Arambel et al. 1982), the time post-feeding (John, 1984 ; Susmel et al. 1993), levels of feed intake (John, 1984) and diets (Bates et al. 1985 ; Susmel et al. 1993). The RNA:total-N ratio of particle-associated microbes has also been observed to be influenced by diets being lower in organisms isolated from concentrate-fed animals than similar organisms isolated from roughage fed animals (Bates et al. 1985). Regardless of diet, the RNA:total-N ratio of particle-associated microbes was significantly lower than that of bacteria isolated in the fluid phase (Merry & McAllan, 1983 ; Bates et al. 1985 .

The purine:total-N ratio of rumen microbes can be expressed as a function of the nucleic acids:total-N ratio. A change in the purine:total-N ratio is assumed to be due mainly to a change in the RNA: otal-N ratio which, in turn, is used as an index of the specific growth rate of rumen microbes. In the present study, the purine:total-N ratio of organisms in the rumen was not influenced by either faunation status or ammonia concentrations but it was significantly higher in bacteria in the fluid phase than those on the particles. These results suggest that the specific growth rate of bacteria in the rumen was not influenced by either faunation status or ammonia concentration. Therefore the increased net microbial cell synthesis in the rumen associated with fauna-free and urea supplementation was not apparently due to a change in the specific growth rate of bacteria in animals fed roughage based diets. In contrast, Smith & McAllan (1974) reported that the RNA:total-N ratio in the fluid phase bacteria isolated from fauna-free calves fed a high cereal diet was significantly higher than the RNA:total-N ratio in bacteria isolated from faunated calves. The

response in the specific growth rate of bacteria in the fauna-free calves may have been due to higher rate of substrate supply to microbes in the rumen (i.e. concentrate diet vs roughage diet used in this study).

The purine-N:total-N ratio in rumen microbes isolated from lambs used in this study was 0.0824 which is lower than the value of 0.116 quoted by Chen & Gomes (1992). This difference highlights the need to know the purine-N:total-N ratio in rumen microbes when estimating the net microbial cell synthesis or the microbial yields entering the intestines from the urinary excretion of purine derivatives.

5.5 Conclusion

The results presented here clearly show that protozoa appeared to have an important role in fibre digestion in the rumen only when the concentration of ruminal fluid ammonia was below 40 mgN/l. The availability of microbial cells for digestion in the intestines increased as ruminal fluid ammonia levels increased between 3 and 161 mgN/l in the fauna-free lambs and between 6 and 252 mgN/l in the refaunated lambs and it was considerably greater in the fauna-free than refaunated lambs. The results suggest that ammonia requirements for optimum microbial cell synthesis in the rumen are considerably higher than previously reported for forage based diets and that removing protozoa as increasing ammonia concentrations will increase the net microbial cell synthesis. This is in accordance with the result from the previous study (see Chapter 4).