The Effects of Dietary Urea on Microbial Populations in the Rumen of Sheep

J. Kanjanapruthipong[1] and R. A. Leng[2]
Department of Animal Science, Kasetsart University, Nakornpathom, Thailand 73140

ABSTRACT : Two experiments were conducted to examine the effects of a range of concentrations of ruminal fluid ammonia (NH_3-N) on forage digestibility, microbial growth efficiency and the mix of microbial species. Urea was either continuously infused directly into the rumen of sheep fed 33.3 g/h of oaten chaff (Exp. I) or sprayed onto the oaten chaff (750 g/d) given once daily (Exp. 2). Concentrations of NH_3-N increased with incremental addition of urea ($p < 0.01$). Volatile fatty acids (VFA) concentrations and 24 h *in sacco* organic matter digestibility in the rumen were higher when supplemental urea was given ($p < 0.01$). The (C2 + C4) : C3 VFA ratio was lower ($p < 0.05$) when NH_3-N was above 200 mgN/l. The fungal sporangia appearing on oat leaf blades were significantly higher when urea was supplemented, indicating that NH_3-N was a growth-limiting nutrient for fungi at levels of NH_3-N below 30 mgN/l. The density of protozoa was highest when NH_3-N concentrations were adjusted to 30 mgN/l for continuously fed (4.4×10^5/ml) and to 168 mgN/l for once daily feeding (2.9×10^5/ml). Thereafter increasing concentrations of NH_3-N, were associated with a concomitant decline in protozoal densities. At the concentration of NH_3-N above 200 mgN/l, the density of protozoa was similar to the density of protozoa in ruminal fluid of the control sheep (1.8×10^5/ml). The efficiency of net microbial protein synthesis in the rumen calculated from purine excretion was 17-47% higher when the level of NH_3-N was above 200 mgN/l. The possibilities are that 1) there is less bacterial cell lysis in the rumen because of the concomitant decrease in the protozoal pool and/or 2) microbial growth *per se* in the rumen is more efficient with increasing NH_3-N concentrations.

(**Key Words :** Ammonia, Urea, Microbial Growth, Rumen, Purine Derivatives)

INTRODUCTION

The availability of amino acids to ruminants on low nitrogen roughage-based diets depends on the efficiency of microbial growth and the lysis and turnover of microbial cells within the rumen which determine the flow of microbial cells out of the rumen. 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 is a function of the nutrients available. 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 (Russell & Strobel, 1987; Satter & Slyter, 1974) to 238 mg NH_3-N/l (Miller, 1973).

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 (Boniface et al., 1986; Perdok et al., 1988) and between 200-270 mgN/l on starch based diets (Mehrez et al., 1977). 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 for 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

[1] Address reprint requests to J. Kanjanapruthipong.
[2] Department of Animal Science, University of New England-Armidale, Australia 2351

Received January 12, 1998; Accepted April 13, 1998

protein : energy 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 iii) the microbial mix in the rumen of sheep given oaten chaff. This was done with a view to more accurately predicting the rumen ammonia levels required for optimum net microbial growth efficiency, maximum digestibility and intake of forage diets given to ruminants.

MATERIALS AND METHODS

Experiment 1

Animals and housing

Four first-cross Merino × Border Leicester wethers, 2-3 years of age, weighing 35-40 kg and with permanent rumen cannulas were held in metabolism crates in a room controlled at 20℃. The room was well ventilated and illuminated at all times.

Diets and feeding

Oaten chaff containing 0.6% N from the same source and batch, was used as the basal diet throughout the study. To this was added 1% of a mineral mix consisting of 1 part of Pfizer vitamin-minerals (Pfizer Aricare, NSW), 1 part of Na_2SO_4, 1 part of NaCl and 2 parts of Ca_2HPO_4. 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 on 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 wk. The animals had clean water at all times.

Experimental procedure

The sheep were allowed to become accustomed to each diet and metabolism cages for 2 wk prior to commencement of the experiment. Within the 14-d experimental period when infusions 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 placed in a caudal position in the ventral sac of the rumen. The probe consisted of a thin stainless steel pipe with a small metal cage at one end which was covered with a double layer of nylon stocking material. A well-mixed portion of each sample of ruminal fluid was removed and the remaining bulk sample of 15 ml was acidified with 5 drops of concentrate H_2SO_4 and stored at −20℃ prior to analysis of VFA and and NH_3-N. The non-acidified sample was used for the enumeration of protozoa. A blood sample for anlaysis of plasma urea was taken at 13:00 h (day 12) from the jugular vein using a needle and syringe which was immediately centrifuged and plasma was removed and stored at −20℃. On day 13, 3 nylon bags with a pore size of 44 mm and measured 7 × 14 cm containing ground dietary materials (2 mm sieve) were suspended in the rumen in order to assess organic matter digestibility (OMD) over 24 h. The technique used to estimate the *in sacco* OMD of feed is similar to that reported by Orskov et al. (1980). 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 (Bauchop, 1979). On day 14, urine was collected quantitatively over 24 h into a container with 500 ml of 2% (v/v) CH_3COOH and 1% (v/v) H_2SO_4. The urine sample was diluted by 3-4 times just after collection and then stored at −20℃ prior to analysis of purine derivatives.

Experiment 2

Animals and housing

Eight Merino wethers about 2-3 years of age and with permanent rumen cannulas weighing 25-30 kg were allocated at random to metabolism crates in an animal house. The house was well ventilated and lit at all times.

Diets and feeding

Oaten chaff containing 0.8% N from the same source and batch was used at the basal diet throughout the study. To this was added 2% of the mineral mix (see experiment 1). Urea solutions were prepared by dissolving 0, 5, 10, 15 and 20 g in 40 ml of tap water and the solution was each sprayed onto a daily ration of 750 g of the chaff. After spraying, the diets were left for about 20 min and then thoroughly mixed. The feed was offered once daily at 09:00 h. The wethers had free access to clean water.

Experimental procedure

The sheep were allowed to become accustomed to the feeds and cages for 2 wk. 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 above and stored at −20℃ for later analysis of purine derivatives. On the fourth day, Cr-EDTA (1 mg Cr/kg BW) used as a ruminal fluid marker according to the method of Downes and McDonald (1964) was injected intraruminally at 06:30 h and the ruminal fluid was collected through a probe placed 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. A ruminal fluid subsample was extracted from each sample prior to acidification to be used for enumeration of protozoa. The rest of samples were treated and stored at −20℃ as detailed earlier prior to analysis of Cr, VFA and NH_3-N. On the last day, 3 × 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.

Enumeration of protozoa and fungi

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

Ten to twelve pieces of oat leaf blades were incubated for 24 h in the rumen and then removed and dipped in 4% formal saline (mentioned above) in a vial. The leaves were randomly sampled and stained with lactophenol cotton blue (Gurr, 1965) for 2 min and then washed with deionised water to remove the excess stain. Sporangial counts were made under a light microscope at the following sites 1) the near areas of the two ends 2) between the middle and the two ends and 3) the middle of the stained leaf. The sporangial population per 1 mm^2 was calculated from the surface area estimated from the microscopic field radius of a specific objective.

Chemical analysis

Prior to analysis of VFA, NH_3-N and Cr, the samples were thawed and centrifuged at 3,000 g at 15℃ for 10 min. The supernatant fraction was decanted into clean McCartney bottles. VFA was determined by gas chromatography (Erwin et al., 1961; model 427, Packard Instrument Co., U.S.A) using a data processors attachment (model 604, Packard Instrument Co., U.S.A). Isocaproic acid was used as a internal standard (Geissler et al., 1976). NH_3-N was assessed by auto-analyser (Bietz, 1974; Cook & Simpson, 1971; Technicon Equipment Co., New Jersey, U.S.A). Cr was measured by atomic absorption spectrometry (Downes & McDonald, 1964; Perkin-Elmer 360). Urea in plasma and urine were analysed using the diacetyl monoxime method of Marsh et al. (1965) on an autoanalyzer Technicon Equipment Co., New Jersey, U.S.A.

Purine derivatives excreted in urine were analysed according to the method of Balcells et al. (1992) modified by Kahn (1996) by using a dual-pump HPLC system (Waters Associates, U. S. A) with an automatic injector (WISP model 710B) connected with two C18 reverse-phase columns (Novapak 300 mm × 3.9 mm I. D., Waters Associates) and allopurinol was used as a internal standard (Balcells et al., 1992).

Statistical analysis

The experimental design was 4 × 6 Latin Square for the experiment in which sheep were continuously provided with feed and a double 4 × 5 Latin Square for the once daily fed-group. Statistical significance of the data was analysed by SAS (1989) and the difference between treatments means was measured by the Least Squares Means.

RESULTS

Experiment 1

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

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 was in creased into the rumen of sheep (table 1). The correlation between plasma urea (Y; mg urea-N/100 ml) and ruminal fluid ammonia (X; mgN/l) and between urinary urea (Z; g urea-N/100 ml) and

ruminal fluid ammonia (X) were highly significantly related as follows:

$$Y = 2.33 + 0.117X - 0.000206X^2$$
$$r^2 = 88.7\% \quad S.E = 5.43 \quad (p < 0.01)$$
$$Z = -110 + 38.4X - 0.0468X^2$$
$$r^2 = 71.7\% \quad S.E = 7.01 \quad (p < 0.01)$$

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 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 ($p < 0.05$).

Table 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 1)

Parameters	Urea infusion (mg/min)						
	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 (%)	68.9^{1}	$70.0^{1,a}$	$70.7^{1,a}$	69.1^{1}	$71.0^{1,a}$	$65.7^{2,b}$	0.50
Propionic (%)	$20.3^{1,2}$	$18.0^{1,a}$	$17.8^{1,a}$	$20.0^{1,2}$	$18.5^{1,a}$	$23.5^{2,b}$	0.60
Isobutyric (%)	0.6	0.6	0.5	0.4	0.9	0.8	0.10
Butyric (%)	9.0	9.9	9.4	9.2	7.8	8.5	0.40
Isovaleric (%)	0.6	0.7	1.0	0.6	0.8	0.7	0.10
Valeric (%)	0.6	0.8	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.

Rumen microbial populations and *in sacco* digestibility

In the sheep given chaff with varying concentrations 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 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 1.9×10^5 to 4.4×10^5/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 1.8×10^5) as ruminal fluid ammonia was increased to above 200 mgN/l (figure 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 appared to occur when the level of ruminal fluid ammonia increased to above 200 mgN/l (figure 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 1) and was apparently optimised at concentration of ruminal fluid ammonia between 30 and 50 mgN/l.

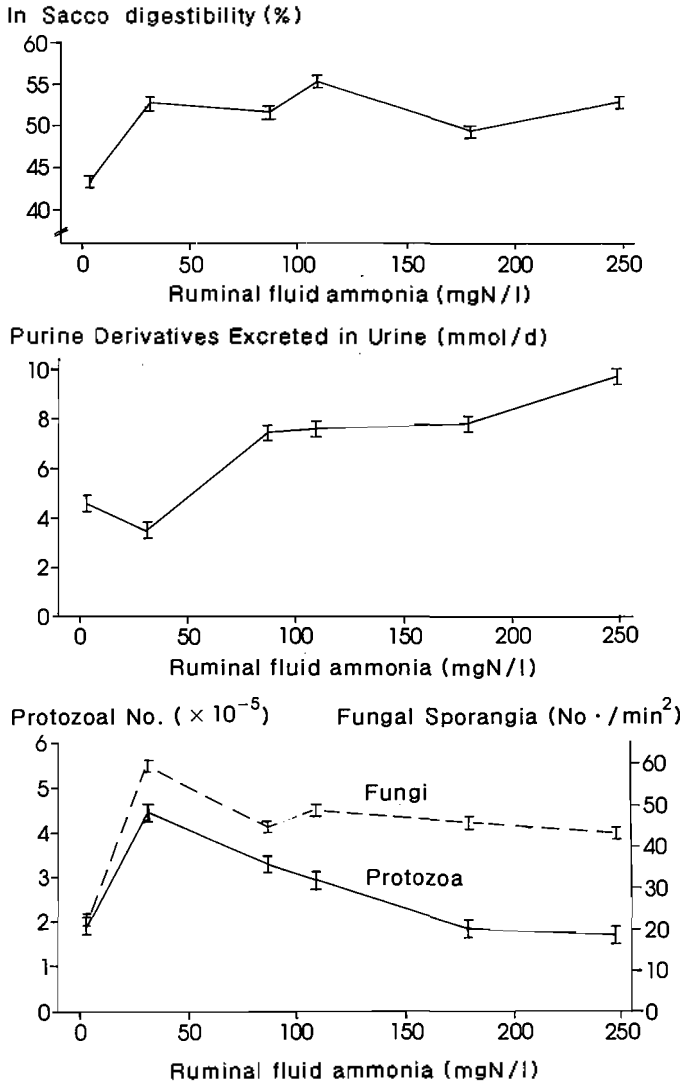

Figure 1. Effects of the levels of ruminal fluid ammonia obtained through urea supplementation on microbial populations, urinary excretion of purine derivatives and organic matter digested in the rumen (OMDR) of sheep fed a low protein roughage based diet (Experiment 1).

Experiment 2

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 artions were consumed within 6 h after presentation of the feed.

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

In the once a day fed sheep, the ammonia pool size in ruminal fluid significantly increased with increasing intake of urea (figure 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 2).

The maximum pH in ruminal fluid of the sheep on the urea diet was at 0.5 h post feeding. The highest pH in ruminal 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 2).

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 2).

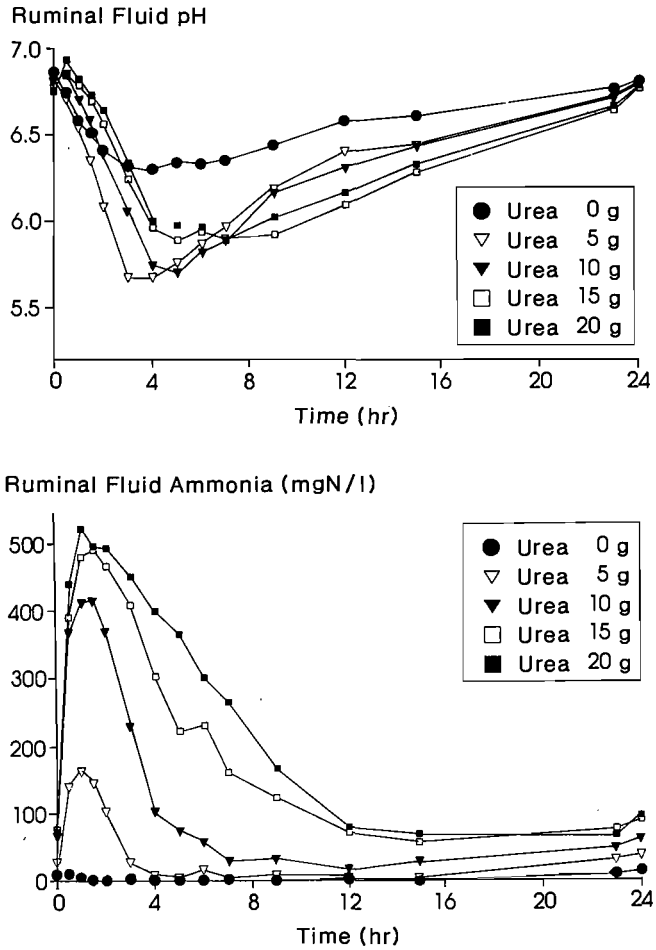

Figure 2. Concentrations of ruminal fluid ammonia and pH in the rumen of sheep given low protein forage with increasing levels of urea intake (Experiment 2).

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

Ruminal fluid	Urea intake (g/d)					
	0	5	10	15	20	S.E.
NH_3-N (mgN/l)*	$3.8^{1,a}$	$15.4^{1,a,b}$	$67.4^{2,b}$	$167.8^{3,c}$	$212.9^{4,c}$	11.4
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.

Rumen fermentation

In the sheep fed once daily, the total VFA concentrations in the rumen at different levels of ruminal fluid ammonia were shown in figure 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 feedging is given in table 3.

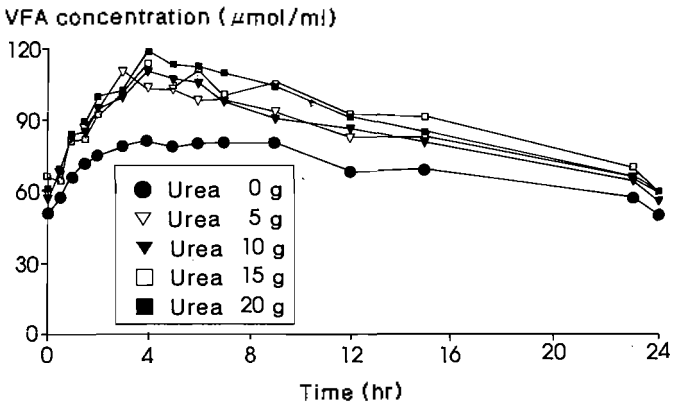

Figure 3. Total VFA concentrations in the rumen of sheep given a low protein forage based diet supplemented with increasing levels of urea (Experiment 2).

Microbial populations

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). 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

Table 3. The profiles of VFA concentration in rumen 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 2)

Ruminal fluid	Concentrations of ruminal fluid ammonia (mgN/l)					
	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 treatmnets at $p < 0.05$ or $p < 0.01$, respectively.

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.

Table 4. Effects of urea supplementation on the concentrations of ruminal fluid ammonia and microbial populations, 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 2)

Items	Concentrations of ruminal fluid ammonia (mgN/l)					
	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* (%)	48.8^{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)	$16.7^{1,b}$	$13.3^{2,a}$	$15.7^{1,2,3,a}$	$15.5^{1,3,a}$	$19.5^{4,b}$	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.

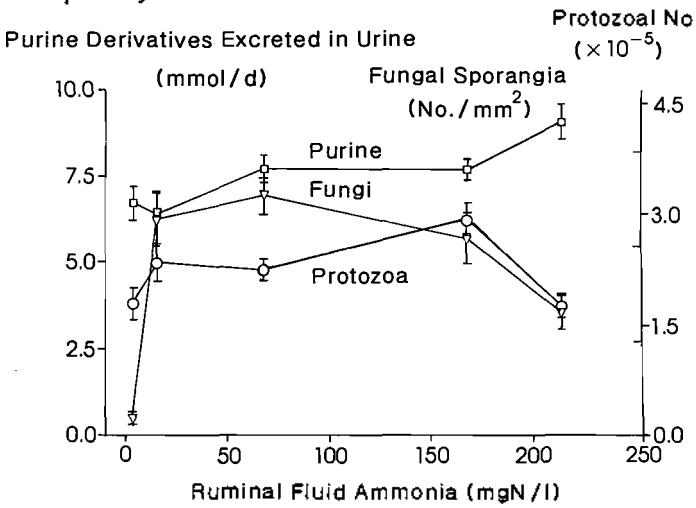

Figure 4. Effects of concentrations of ruminal fluid ammonia on microbial populations in the rumen and urinary excretion of purine derivatives in sheep given increasing amounts of urea in a low protein roughage based diet (Experiment 2).

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

In the once a day fed sheep, when concentration of ruminal fluid ammonia was above 200 mgN/l, the calculated microbial-N leaving the rumen from purine excretion and the calculated efficiency of net microbial cell synthesis in the rumen were increased by 45 and 47% ($p < 0.01$; table 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). However, there were no differences in digestibility of oaten chaff among the sheep on urea supplemented diets ($p > 0.05$).

DISCUSSION

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℃. From 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, each 0.1 pH unit above 6.9 the concentration of NH_4^+ is decreased by more than 50%. Ammonia is readily absorbed across membrane as compared to ammonium ion and appears to be more

readily absorbed (Mooney & O'Donovan, 1970). Chalmers et al. (1970) have found that when pH in ruminal fluid is below 6.9, ammonia concentrations in both peritoneal liquor and jugular blood decrease whereas ammonia concentrations in ruminal fluid remains constant. In the present studies, the maximum pH in 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 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 pH in the rumen.

Ruminal fluid ammonia and VFA concentration

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

Studies both *in vitro* (Russell & Sniffen, 1984) and *in vivo* (Hume, 1970; Song & Kennelly, 1990) 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). Iso-acids (isobutyric and isovaleric acids) in ruminal fluid are mainly derived from oxidative deamination and decarboxylation of amino acids, i. e. valine, leucine and isoleucine (Allison, 1970; Menahan & Schultz, 1964) 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 iso-acids in ruminal fluid of the sheep with a high concentration of ruminal fluid ammonia, particularly in sheep fed once-daily, could be a resulf of a higher rate of microbial growth relative to iso-acids production in the rumen or to a lower rate of lysis and turnover of microbial protoplasms in the rumen.

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 in its breakdown. Different groups of microbes exhibit different substrate requirements. The present studies clearly indicate that maximum digestbility of oaten chaff occurred below 30 mgN/l. While Boniface et al. (1986) and Perdok et al. (1988) showed that the maximum rate of forage digestion for cattle occurred at about 45 and 60 mgN/l, respectively. For sheep given whole barley (Mehrez et al., 1977) and cattle fed rolled-barley or cracked-maize (Odle & Schaefer, 1987) the minimum concentration of ruminal fluid ammonia required for maximum digestion of grain dry matter was 237, 125 and 61 mgN/l, respectively.

Ruminal fluid ammonia and microbial populations in the rumen

In these studies, a correction for the contribution of the endogenous purines to the total purine derivative excretion was estimated according to the model suggested by Chen et al. (1990). However, the use of urinary excretion of purine derivatives as an indicator of the amount of microbial biomass leaving the rumen has to be done with some caution since it may be impossible to get a representative sample of microbes entering the intestine and thus yield estimates from urinary excretion of purine derivatives are relative rather than absolute (Kan-janapruthipong & Leng, 1998).

The results presented in figure 1 and 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. 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 1 and 4. This is similar to the results of Rihani et al. (1993) who 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 (continuously infused into the rumen or mixed with the feed). At these levels of ruminal fluid ammonia, the bacterial and protozoal pools have been reported to be larger (Purser & Moir, 1966; Moir, 1970; Teather et al., 1980). It is more likely that there was an increase in the turnover rate of bacteria (Firkins et al., 1987) causing a rise in bacterial-N recycling within the rumen (Firkins et al., 1992; Nolan & Leng, 1972) 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 1) there is a decrease in bacterial turnover within the rumen 2) there is a reduction of rate of ingestion of bacteria by protozoa in the rumen 3) bacterial 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 2) is not significantly different.

The lowering of protozoal numbers 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 (Briggs et al., 1964). The possible reasons are discussed:

The majority of rumen bacteria, particularly cellulolytic bacteria, appear to be able to utilise ammonia as the major source of nitrogen (Bryant & Robinson, 1962; Hungate, 1966) 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 urea 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 to 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) 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 1×10^5/ml ruminal fluid which is extremely low for a concentrate-based diet.

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

A high concentration of ruminal fluid ammonia could lead to a change in the pathway of ammonia assimilation by micro-organisms to pathway that is independent of ATP (Tyler, 1978), making more ATP available for cell growth. A limitation of N availability for biosynthesis relative to ATP production by catabolism results in increasing both maintenance energy and energetic uncoupling (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, 1982). 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 stochiometric terms. The data from these studies show that the concentration of ruminal fluid ammonia above 200 mgN/l markedly increased microbial biomass entering the duodenum as indicated by purine excretion with a small marginal shift in VFA concentration and in digestibility of the basal feed.

CONCLUSION

It can be concluded that the requirement for ammonia for optimum bacterial growth is above 200 mgN/l which is much higher than that previous recognised on roughage based diets. Although the excretion of purine derivatives is increased with decreasing population of protozoa, as shown in figure 1 and 4, it can be argued that this may be mainly contributed from a higher ratio of nucleic acids to protein in microorganisms 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.

ACKNOWLEDGEMENTS

Jeerachai Kanjanapruthipong acknowledges the receipt of a scholarship from the Equity and Merit Scholarship Scheme on behalf of the Australian International Development Assistance Bureau and the advice and help of Assoc. Prof. Dr John Nolan in the work and the technical assistant from Mr Frank Ball, Mr Simon Stachiw. Mr Lewis Khan assisted considerably in developing the technique for purine analysis.

REFERENCES

Allison, M. J. 1969. Biosynthesis of amino acids by ruminal microorganisms. J. Anim. Sci. 29:797-807.

Allison, M. J. 1970. Nitrogen metabolism of ruminal microorganisms. In: Physiology of Digestion and Metabolism in the Ruminant. Phillison, A. T. Ed. Newcastle-upon-Tyne, Oriel, England. pp. 456-473.

Balcells, J., J. A. Guada and J. M. Peiro. 1992. Simultaneous determination of allantoin and oxpurines in biological fluids by high-performance liquid chromatography. J. Chromatog. 575:153-157.

Balcells, J., J. A. Guada, C. Castrillo and J. Gasa. 1993. Rumen digestion and urinary excretion of purine derivatives in response to urea supplementation of sodium treated straw fed to sheep. Br. J. Nutr. 69:721-732.

Baldwin, R. L., H. L. Lucas and R. Cabera. 1970. Energetic relationships in the formation and utilisation of fermentation end products, In: *Physiology of Digestion and Metabolism in Ruminants*. Phillison, A. T., Ed. Newcastle-upon-Tyne, Oriel, England. pp. 319-334.

Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. Appl. Env. Microbiol. 38(1):148-158.

Bietz, J. A. 1974. Micro-Kjedahl analysis by an improved automated ammonia determination following manual digestion. Anal. Chem. 46(11):1617-1618.

Boniface, A. N., R. M. Murray and J. P. Hogan. 1986. Optimum level of ammonia in the rumen liquor of cattle fed tropical pasture hay. Proc. Aust. Soc. Anim. Prod. 16:151-154.

Briggs, M. H., T. W. Heard, A. Whitcroft and M. L. Hogg. 1964. Studies on urea-fed cattle: 2. Rumen levels of B vitamins and related coenzymes. Life Sci. 3:11-14.

Bryant, M. P. 1973. Nutritional requirements of the predominant rumen cellulolytic bacteria. Fed. Proc. 32(7):1809-1813.

Bryant, M. P. and I. M. Robinson. 1962. Some nutritional characteristics of predominant culturable ruminal bacteria. J. Bacteriol. 84:605-614.

Chalmers, M. I., A. E. Jaffray and F. White. 1971. Movements of ammonia following intraruminal administration of urea or casein. Proc. Nutr. Soc. 30:7-17.

Chen, X. B., F. D. DeB. Hovell, E. R. Orskov and D. S. Brown. 1990. Excretion of purine derivatives by ruminants: effects of exogenous nucleic acid supply on purine derivative excretion by sheep. Br. J. Nutr. 63:131-142.

Coleman, G. S. 1967a. The metabolism of free amino acids by washed suspensions of the rumen ciliate *Entodinium caudatum*. J. Gen. *Microbiol.* 47:433-447.

Coleman, G. S. 1967b. The metabolism of the amino acids of *Escherichia coli* and other bacteria by the rumen ciliate *Entodinium caudatum*. J. Gen. Microbiol. 47:449-464.

Coleman, G. S. 1975. The interrelationship between rumen ciliate protozoa and bacteria In: Digestion and Metabolism in the Ruminant. McDonald, W. I. and A. C. I. Warner, Ed. The University of New England publishing unit, Australia. pp. 149-164.

Cook, W. M. and W. E. Simpson. 1971. Determination of ammonia in Kjedahl digests of crops by an automated procedure. J. Sci. Food Agric. 22:9-10.

Cottle, D. J., J. V. Nolan and R. A. Leng. 1978. Turnover of protozoa and bacteria in the rumen of sheep. Proc. Aust. Soc. Anim. Prod. 12:138.

Downes, A. M. and I. W. McDonald. 1964. The chromium-51 complex of ethylenediamine tetraacetic acid as a soluble rumen marker. Br. J. Nutr. 18:153-162.

Elliott, R. and D. G. Armstrong. 1982. The effect of urea and urea plus sodium sulphate on microbial protein production of in rumen of sheep given diets high in alkalitreated barley straw. J. Agric. Sci. Camb. 99:51-60.

Erwin, E. S., G. J. Macro and E. M. Emesy. 1961. Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. J. Dairy. Sci. 44:1768:1771.

Firkins, J. L., L. L. Berger, N. R. Merchen, G. C. Fahey, Jr. and P. L. Mulvaney. 1987. Ruminal nitrogen metabolism in steers as affected by feed intake and dietary urea concentration. J. Dairy Sci. 70(11):2302-2311.

Firkins, J. L., W. P. Weiss and E. J. Piwonka. 1992. Quantification of intraruminal recycling of microbial nitrogen using nitrogen-15. J. Anim. Sci. 70:3223-3233.

Geissler, C., M. Hoffmann and B. Hickel. 1976. Ein Beitrag zur gaschromatographischen bestimmung. flüchtiger fettsäuren. Arch. Tierernähr. 26:123-129.

Gurr, E. 1965. The Rational Use of Dyes in Biology and General Staining Methods. J. W. Arrowsmith Ltd. Great Britain. p. 422.

Hespell, R. B. and M. P. Bryant. 1979. Efficiency of rumen microbial growth: Influence of some theoretical and experimental factors on Y_{ATP}. J. Anim. Sci. 49(6):1640-1659.

Hino, T. and M. Kametaka. 1973. The cultivation of rumen oligotrich protozoa. I. Factors influencing the life of Entodinia. J. Gen. Appl. Microbiol. 19:305-315.

Hume, I. D. 1970. Synthesis of microbial protein in the rumen. Ⅱ. A response to higher volatile fatty acids. Aust. J. Agric. Res. 21:297-304.

Hungate, R. E. 1966. The Rumen and its Microbes. Academic Press, New York. p. 533.

Kahn, P. L. 1996. Dynamics of allantoin metabolism in sheep: Differences between Merino selection lines in microbial yield from the rumen and utilization of protein for wool growth. Ph. D. Thesis. University of New England, Armidale, Australia.

Kanjanapruthipong J. and R. A. Leng. 1998. Purine derivatives excreted in urine as an indicator estimating microbial yield from the rumen: a review. Asian-Australasian J. Anim. Sci. 11(3):209-216.

Klieve, A. V. 1988. Bacteriophages of rumen bacteria. Ph. D. Thesis. University of New England, Armidale, Australia.

Leng, R. A. and G. J. Leonard. 1965. Measurement of the rates of production of acetic, propionic and butyric acids in the rumen of sheep. Br. J. Nutr. 19:469-484.

Leng, R. A. 1982. Modification of rumen fermentation. In: Nutritional Limits to Animal Production from Pastures. Hacker, J. B. Ed. CAB, Farnham Royal, U. K. pp. 427-453.

Leng, R. A. and J. V. Nolan. 1984. Nitrogen metabolism in the rumen. J. dairy. Sci. 67(5):1072-1089.

Leng, R. A. 1989. Dynamics of protozoa in the rumen. In: The Roles of Protozoa and Fungi in Ruminant Digestion. Nolan, J. V., R. A. Leng and D. I. Demeyer, Ed. Penambul Books, Armidale, Australia. pp. 51-58.

Leng, R. A. 1990. Factors affecting the utilization of 'poor-quality' forages by ruminants particularly under tropical conditions. Nutr. Res. Rev. 3:277-303.

Leng, R. A., N. Jessop and J. Kanjanapruthipong. 1993. Control of feed intake and the efficiency of utilization of feed by ruminants. In: Recent Advances in Animal Nutrition in Australia. Farrell, D. J. Ed. University of New England, Armidale, Australia. pp. 70-88.

Maeng, W. J., C. J. Van Nevel, R. L. Baldwin and J. G. Morris. 1976. Rumen microbial growth rates and yields: Effects of amino acids and protein. J. Dairy Sci. 59(1):68-79.

Marsh, W. H., B. Fingerhut and H. Miller. 1965. Automated and manual direct methods for the determination of blood urea. J. Clin. Chem. 11:624-627.

Mehrez, A. Z., E. R. Orskov and I. McDonald. 1977. Rates of rumen fermentation in relation to ammonia concentration. Br. J. Nutr. 38:437-443.

Menahan, L. A. and L. h. Schultz. 1964. Metabolism of leucine and valine within the rumen. J. Dairy Sci. 47:1080-1085.

Miller, E. L. 1973. Evaluation of foods as sources of nitrogen and amino acids. Proc. Nutr. Soc. 32:79-84.

Minson, D. J. 1982. Effects of chemical and physical composition of herbage eaten upon intake. In: Nutritional Limits to Animal Production from Pastures. Hacker, J. B. Ed. CAB, Farnham Royal, U.K. pp. 167-182.

Moir, R. J. 1970. Implications of the N:S ratio and differential recycling. In: Sulphur in nutrition. D. V. M. Muth and J. L. Oldfield, Ed. The AVI Publishing Company, INC., Westport, Connecticut, USA. pp. 165-181.

Mooney, P. and D. J. O'Donovan. 1970. The permeability of the rumen to simple nitrogenous compounds. Biochem. J. 119:18p-19p.

Nolan, J. V. and R. A. Leng. 1972. Dynamic aspects of ammonia and urea metabolism in sheep. Br. J. Nutr. 27:177-194.

Odle, J. and D. M. Schaefer. 1987. Influence of rumen ammonia concentration on the rumen degradation rates of barley and maize. Br. J. Nutr. 57:127-138.

Onodera, R., Y. Nakagawa and M. Kandatsu. 1977. Ureolytic activity of washed cell suspension of rumen ciliate protozoa. Agric. Biol. Chem. 41(11):2177-2182.

Onodera, R., Y. Yamagachi and S. Morimoto. 1983. Metabolism of arginine, citrulline, ornithine and proline by starved rumen ciliate protozoa. Agric. Biol. Chem. 47(4):821-828.

Orpin, C. G. 1975. Studies on the rumen flagellate *Neocallimastix frontralis*. J. of Gen. Microbiol. 91:249-262.

Orskov, E. R., F. D. Hovel and F. Mould. 1980. The use of nylon bag technique for the evaluation of feedstuffs. Trop. Anim. Prod. 5:195-213.

Perdok, H. B., R. A.. Leng, S. H. Bird, G. Habib and M. F. J. Van Houtert. 1988. Improving livestock production from straw-based diets. In: Increasing Small Ruminant Productivity in Semi-arid Areas. E. F. Thomson and F. S. Thomson, Ed. Kluwer Academic Publishers, Dordrecht, Boston, London. pp. 81-91.

Purser, D. B. and R. J. Moir. 1966. Dietary effects upon concentrations of protozoa in the rumen. J. Anim. Sci. 25:668-674.

Rihani, N., W. N. Garrett and R. A. Zinn. 1993. Influence of level of urea and method of supplementation on characteristics of digestion of high-fibre diets by sheep. J. Anim. Sci. 71:1657-1665.

Russell, J. B. and C. J. Sniffen. 1984. Effect of carbon-4 and carbon-5 volatile fatty acids on growth of mixed rumen bacteria *in vitro*. J. Dairy Sci. 67(5):987-994.

Russell, J. B. and H. J. Strobel. 1987. Concentration of ammonia across cell membranes of mixed rumen bacteria. J. dairy Sci. 70:970-976.

SAS. 1989. SAS/STAT® Uers's Guide, Version 6, 4th Ed, Vol 2, Cary, NC. U.S.A.

Satter, L. D. and L. L. Slyter. 1974. Effect of ammonia concentration on rumen microbial protein production *in vitro*. Br. J. Nutr. 2:199-208.

Setälä, J. and L. Syrjälä. 1980. The effect of formaldehyde treatment on urea utilization. European Association for Animal Production Publication No. 27(2). pp. 582-589.

Smith, R. H. and A. B. McAllan. 1974. Some factors influencing the chemical composition of mixed rumen bacteria. Br. J. Nutr. 31:27-34.

Song, M. K. and J. J. Kennelly. 1990. Ruminal fermentation pattern, bacterial population and rumen degradation of feed ingredients as influenced by ruminal ammonia concentration. J. Anim. Sci. 68:1110-1120.

Teather, R. M., L. D. Erfle, R. J. Boila and F. D. Sauer. 1980. Effect of dietary nitrogen on the rumen microbial population in lactating dairy cattle. J. Appl. Bacteriol. 49:231-238.

Tyler, B. 1978. Regulation of the assimilation of nitrogen compounds. Ann. Rev. Biochem. 47:1127-1162.

Wallace, R. J. and C. A. McPherson. 1987. Factors affecting the rate of breakdown of bacterial protein in rumen fluid. Br. J. Nutr. 58:313-323.