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Effect of feeding level and frequency on microbial protein yield in the rumen of growing lambs

Tsutomu FUJIHARA†, Mayumi HARA-IWAKUNI, David J. KYLE‡, Egil R. ØRSKOV‡§ and Toshiyoshi ICHINOHE

Abstract Two experiments (Expt.I & II) were conducted using growing lambs to investigate the effect of feeding level and frequency on the magnitude of microbes synthesized in the rumen. In Expt. I, the digestion and balance trials were performed on 5-, 7- and 9-month-old of the crossbred lambs fed the ration consisted of mixed hay and concentrate (4 : 6, DM) with 2 and 24 times feeding during consecutive 12 days. The feeding level was 1.2 times of requirement for medium growth in lambs. In Expt. II, the same trials were conducted on 5-, 7- and 10-month-old of crossbred lambs fed the ration as Expt. I with 2 and 8 times feeding at 0.6 times of the feeding level in Expt. I. There was no clear difference ($P>0.05$) in the digestibility of nutrient in both Expts. of I and II. The absorbed nitrogen (N) tended to be more in 24 times feeding than in 2 times feeding at 5-, 7- and 9-month-old, and the retained N also tended to be greater in 24 times feeding at 5- and 9-month-old (Expt. I). In Expt. II, there was no clear difference in absorbed and retained N between 2 feeding frequencies. The constant of passage rate of ingesta in the rumen measured in Expt. II tended to high after 8 times of feeding than after 2 times feeding on 7- and 10-month-old. The plasma level and urinary excretion of purine derivatives (PD) were clearly high in Expt. I than in Expt. II, reflecting the difference in feeding level between 2 Expts. In both Expts., daily microbial N supply tended to clearly high after frequent feeding than after feeding twice a day. As a result of reflection of difference in feeding level, the microbial N supply in Expt. I was about 2-3 times higher than that in Expt. II.

Keywords: feeding frequency, feeding level, growing lambs, N balance, purine derivatives

Introduction

It is the characteristics of protein nutrition in ruminant animals that the most of feed protein ingested is degraded by microbes in the rumen, and so they usually use the microbial protein synthesized in the rumen as protein source. Then, the rumen microbes is obviously the principle protein source for ruminants, and therefore, it is very important point for understanding the protein nutrition in ruminants to measure the magnitude of microbial protein synthesized in the rumen, and further digested in the lower gut, and also to plan for increasing the microbial protein in the rumen.

Allantoin is an end product in metabolism of purine base of nucleic acids in mammals except the primates. In general, nucleic acids in rumen microbes are digested in the lower gut of ruminants, and the absorbed purine base is normally excreted into the urine as allantoin through hypoxanthine, xanthine and uric acid, however, the metabolism of purine base is not always complete, and so, a part of them is excreted into urine as hypoxanthine, xanthine and uric acid, so called purine derivatives (PD). In ruminant animals under an ordinary feeding, the nucleic acids in feed is thought to be almost degraded in the rumen, and then,

† Philippine Carabao Center, Science City of Munoz, Nueva Ecija, Philippines 3132
Tel & Fax: +63−044−456−0731, e-mail: tfuji1943@yahoo.co.jp
‡ Former the Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland, UK
§ James Hutton Institute, Craigiebuckler, Aberdeen AB15 8QH, Scotland, UK

the most of digested nucleic acids in the lower guts should be derived from the microbes synthesized in the rumen, *i. e.,* the nucleic acids flowed out from the rumen mainly originate in microbes synthesized in the rumen (McAllan 1982; Vo TKT & Ørskov 2006; Fujihara & Shem 2011). Then, if there is a positive relationships between absorbed nucleic acids (purine base) and allantoin and/or total PD excreted into the urine, urinary allantoin and/or total PD could reflect the magnitude of microbial protein synthesized in the rumen and further digested in the lower guts. Topps and Elliott (1965) has suggested a close relationship between the magnitude of purine bases in the rumen and the urinary excretion of allantoin in sheep, and since then, it has been already shown that the urinary PD excretion could be able to use as powerful tool for presuming a magnitude of microbial protein synthesized in the rumen (Fujihara *et al.* 1987; Chen *et al.* 1990a, b; 1991).

The magnitude of microbial protein supplied to animals (per some units of ingested feed) is usually calculated based on the urinary PD excretion, *i. e.,* microbial N (g)/ ruminal degradable organic matte (RDOM) (kg), and the values would varied about 4 times (4–16g N/kg RDOM) (ARC 1984). Using the sheep sustained by intragastric nutrition, it has been shown that the changes in dietary energy and/or protein levels have little effect on urinary excretion of endogenous PD in sheep (Fujihara *et al.* 1987; Lindberg & Jacobsson 1990), and furthermore, the variance in urinary excretion of endogenous PD would closely relate to the body weight (BW) of the animals (Chen *et al.* 1992) and also the magnitude of microbial NA entered to the lower guts (Chen *et al.* 1990c; Verbic *et al.* 1990). To increase in rumen microbes, it could be very important to synchronize the production rate of ammonia and energy in the rumen as much as possible, *i. e.,* maintaining more stable and effective microbial fermentation in the rumen. As in an ordinary feeding, the sustainable maintenance of ruminal fermentation will not be easy under the feeding condition that quite large amount of feed is offered to the animal at once or twice a day. When the daily ration given to the animals at 2 times a day, the ruminal levels of ammonia and volatile fatty acids (VFAs) increased after the feeding, and then, the levels declined periodically before next feeding, *i. e.,* the utilization of dietary N by rumen microbes will not be high (Rooke *et al.* 1987). On the other hand, the ruminal pH was relatively stable when the ration is offered frequently after divided a small portion at short time interval (Kaufmann 1976; Braggs *et al.* 1986), and then, under this condition, energy and protein sources for microbial growth will be provided steadily in the rumen. There will be also possibility that the drastic changes in fermentative activity of rumen microbes could improve the utilization of feed, and consequently, there will be an increase of the microbial protein supply to host animal. Furthermore, an increase of feeding frequency had relatively a little effect on microbial fermentation in the rumen, when the dietary level of concentrate was less than 35% in sheep and dairy cows (Ulyatt *et al.* 1984; Robinson & Sniffen 1985). Therefore, when the dietary level of concentrate is relatively high, an increased feeding frequency could have good results in microbial fermentation in the rumen.

In the present study, the effect of feeding level and frequency on ruminal synthesis of microbes was investigated in sheep fed on a high-concentrate diet. The results obtained in this experiment have been partly presented formerly (Fujihara *et al.* 1999).

Materials and Methods

Animals, diet and experimental procedure

Experiment I: Two twins (male & female) of crossbred (Suffolk X Japanese Corriedale) lams at 40–50 days after birth were adopted (see Table 1), and fed the mixed diet (see Table 2) *ad libitum* in the pen. On the 5, 7 and 9

Table 1. Change in body weight of lambs during the experimental period

Month	Animal No.						
	1	2	3	4	5	6	7
1	—	—	3.7#	3.9#	4.5#	4.3#	—
2	18.2	17.6	13.2	13.2	17.0	16.5	—
3	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—
5	28.5	26.3	21.5	19.0	21.3	18.5	32.5
6	35.0	31.2	31.5	28.0	22.0	19.3	35.0
7	34.0	31.5	30.8	26.8	26.8	21.5	33.6
8	41.0	36.5	36.5	32.5	28.5	22.2	38.8
9	42.5	41.3	36.3	33.5	—	—	—
10	48.2	43.5	42.5	36.5	29.3	23.5	43.0

Birth weight.

Table 2. Ingredients and chemical composition of experimental diet

	Mixed hay*	Rolled barley	Soybean meal
	40.2**	41.9	17.9
Chemical composition			
Dry matter (%)	90.5	89.4	91.0
Organic matter	90.1#	97.7	92.1
Crude protein	12.6	13.1	34.6
Ether extract	1.6	2.6	1.7

* Predominantly Italian ryegrass. ** % of w/w. # % per dry matter.

months after birth, they were individually kept in the metabolism crates for digestion/balance trial during 12 days in each treatment; 2 and 24 times feeding a day. In the 2 times feeding a day, ration was equally divided and fed 09 : 00 and 21 : 00hrs and the ration was also equally divided 24 portions and fed hourly in 24 times feeding a day. The feeding level was calculated as based on NRC standard (1985); 1.2 times of requirement (medium level) for early weaned lambs. The fresh water and salt licks contained minerals were available at all the time.

Experiment II: Two crossbred (Suffolk X Japanese Corriedale) female lambs at 40-50 days after birth and a female lamb of same crossbred at 3 month after birth were adopted (see Table 1), and fed the mixed diet (see Table 2) *ad libitum* in the pen. On the 5, 7 and 10 month after birth, they were individually kept in the metabolism crates for digestion/balance trial during 12 days in each treatment; 2 and 8 times feeding a day. In the 2 times feeding a day, the procedure was same as that in Experiment I, and in 8 times feeding a day the ration was equally divided 8 portions and fed 09 : 00, 12 : 00, 15 : 00, 18 : 00, 21 : 00, 00 : 00, 03 : 00 and 06 : 00hrs, respectively. The feeding level was calculated as 0.6 times of that in Experiment I. Using ytterbium (Yb) (Mader *et al.* 1984) and cobalt ethylene diamine tetra acetic acid (Co-EDTA) (Uden *et al.* 1980) as a marker, during the digestion/balance trial the flow out rate of digesta from the rumen was measured on each treatment; 2 and 8 times feeding. The other procedure was the same as that in Experiment I.

Sample collection and measurements

During the consecutive 5 days for each digestion/balance trial of 12 days, daily samples of faeces and feed refusal were taken at 09 : 00. The urine was collected hourly using automatic collector into plastic bottle with 15 ml of 3N H_2SO_4 solution (final pH of urine <3.0). The collected urine was sampled and stored at -20 °C.

On the final day of each trial, blood samples (10 ml) were collected into heparinized tubes at 0, 2, 3, 5, 7 and 12 hours after morning feed by vein puncture. The samples were centrifuged at 1,600 *g* for 20 min and plasma was kept at -40℃ for analysis of N and PD.

Chemical analysis

Nitrogen in the diets, faeces and urine was analyzed by the Kjeldahl method, and crude fat, crude fibre and crude ash in the diets and faeces were determined by AOAC method (Hoitz 1960). Allantoin in urine and plasma was determined by the methods of Young and Conway (1942) and Chen *et al.* (1993). Hypoxanthine, xanthine and uric acid were determined by the method described by Fujihara *et al.* (1987). Yetterbium (Yb) and Cobalt (Co) in the faeces sampled for measuring ruminal out flow rate of ingesta were determined using Inductively Coupled Plasma Emission Spectrometer (ICPS 2000, Shimadzu Co., Kyoto, Japan) after wet ashing with concentrate nitric acid.

Results and Discussion

Feed intake and growth of animals

As shown in Table 3, dry matter intake (DMI) of lambs

Table 3. Dry matter intake by lambs on 5, 7 and 9 (10) month after birth (g/$BW^{0.75}$/d)

Experiment I						
Age*	5 Month		7 Month		9 Month	
Frequency**	2X	24X	2X	24X	2X	24X
	92.9±2.4#	96.8±0.4	85.3±1.4	89.0±2.4	79.6±1.9	88.2±2.0
Experiment II						
	5 Month		7 Month		10 Month	
	2X	8X	2X	8X	2X	8X
	55.6±2.8	56.1±2.3	57.3±1.8	56.1±1.4	58.5±0.4	58.4±0.2

* Month after birth. ** Feeding frequency (times per day). # Mean ± S.E. of 3 or 4 lambs.

Table 4. Daily gain in lambs during the experiment (g)

Experiment I			
Age*	5 month	7 month	9 month
	274.1±17.3#	125.8±22.4	170.6±45.5
Experiment II			
	5 month	7 month	10 monrh
	43.2±29.4	103.7±31.6	116.6±20.1

* Month after birth. # Mean ± S. E. of 3 or 4 lambs.

on 5, 7 and 9 month after birth, and the values in Experiment I was a little higher after 24 X feeding than after 2 X feeding. In Experiment II, the DMI of lambs was almost the same in both feeding frequency of 2 X and 8 X, and then the value was roughly equal to 60% of that in Experiment I.

As shown in Table 1, the growth rate of all the animals (nos.1-4) was almost favorable as same as a standard of crossbred lambs. There was a little difference individually in average daily gain (ADG) measured at 7 and 9 months after birth (Table 4). This would be due to the differences in nutrient digestibility and/or N balance of animals as described later (Tables 5 & 6).

In Experiment II (1b), No. 8 lambs adopted at 3 months after birth, and then the body weight (BW) at was relatively high as compared with the other lambs; probably due to the difference of feeding level before adoption (not early weaned). The growth rate of lambs in Experiment II was relatively slow as compared to that in Experiment I, due to the difference in feeding level in both Experiments.

Apparent digestibility and N balance

As shown in Table 5, apparent digestibility of DM, organic matter (OM), crude protein (CP) and crude fat (CF), and also total digestible nutrients (TDN) were almost the same after 2 and 24 times feeding a day in Experiment I. At 7-month-old, the values of apparent digestibility were relatively low as compared with that at 5- and 9-month-old in Experiment I. The digestion trial at 7-month-old has been done during from the middle to the late of August, and so, a hot climate thought to affect not a little to the animals, although the trial has been done in the temperature-controlled room. This would be supported by increased water consumption (see table 7) at that time as compared with other times (5&9 months). Consequently, increased water consumption resulted in a stimulation of out-flow rate of ingesta through digestive tracts, which will lead to decrease apparent digestibility (Christopherson & Kennedy 1983).

In Experiment II, there was no clear difference in apparent digestibility between 2 feeding frequencies in Experiment I. According to Charmley *et al.* (1991), there was no clear difference in OM digestibility in sheep fed on *ad libitum* or restricted feeding of alfalfa silage supplemented with sucrose after 2 and 8 times feeding a day. Cecava *et*

Table 5. Digestibility of nutrients and TDN in lambs during the experimental period (%)

Experiment I						
Age*	5 Month		7 Month		9 Month	
Frequency**	2X	24X	2X	24X	2X	24X
DM	75.3±0.9#	77.4±0.8	72.7±0.6	71.6±0.7	72.2±1.4	75.1±1.6
OM	77.1±1.0	79.0±0.7	75.1±0.9	74.0±0.8	74.3±1.5	77.1±1.6
CP	74.3±1.8	76.7±0.4	70.5±1.4	71.2±1.6	72.9±1.6	76.5±2.4
CF	74.4±1.5	76.5±3.5	64.4±1.3	61.5±1.6	64.2±2.5	72.7±2.3
TDN	68.9±0.7	70.5±0.8	66.6±0.1	65.7±0.5	66.6±0.5	68.6±2.7
Experiment II						
	5 Month		7 Month		10 Month	
	2X	8X	2X	8X	2X	8X
DM	71.9±6.1	73.0±5.5	78.1±4.7	76.7±5.5	81.4±3.5	81.4±2.8
OM	73.9±5.6	75.1±5.1	79.8±4.5	78.4±5.2	83.1±3.3	82.8±2.6
CP	62.8±8.5	67.2±6.1	72.8±7.3	71.7±8.2	77.8±3.4	77.9±3.3
DF	68.8±9.3	67.4±11.5	74.8±7.0	69.1±12.8	76.0±5.7	75.8±6.2
TDN	67.4±0.6	68.2±1.7	72.1±8.8	71.9±2.0	74.4±1.3	75.4±1.3

* Month after birth. ** Feeding frequency (times per day). # Mean ± S.E. of 3 or 4 lambs.

al. (1990) has also reported that the OM digestibility was almost the same in cattle when they were fed on alfalfa hay and maize after 2 and 12 times feeding a day. On the other hand, Bunting *et al.* (1987) has demonstrated that apparent CP digestibility tended to decrease with an increase of feeding frequency in sheep fed on tall fescue hay after 2, 4 8 and 16 times feeding a day, though there was no effect of feeding frequency on DM and OM digestibility. The digestibility of DM and OM in the present study was in good agreement with that reported by Bunting *et al.* (1987), but the result with CP digestibility in this study is a little different to that their result, and this probably due to the diets in both experiment, *i. e.,* only roughage in the former and high concentrate diet in the latter. In Experiment II, apparent digestibility tended to high as compared to that in Experiment I reflecting the low feeding level, though the values at 5–month–old was relatively low as compared with that in Experiment I.

As shown in Table 6, N intake and absorption tended to high after 24 times feeding than that after 2 times feeding at 5, 7 and 9 month of age in Experiment I. N retention at 5 and 9 month tended to high after 24 times feeding than that after 2 times feeding, although at 7 month that was almost the same regardless the changes in feeding frequency. At 7 month of age, N intake and absorption tended to high after 24 times feeding than that after 2 times feeding, but urinary N excretion was a little higher after 24 times feeding than after 2 times feeding, though the volume of urine was similar after 2 and 24 times feedings. Then, retained N was similar after both feeding frequencies, and consequently, the ratio of retained N per absorbed N was 44.2 and 40.8% after 2 times feeding and 24 times feeding, respectively.

The retained N at 7th month was a little lower than those at 5th and 9th months in both feeding frequencies, and consequently the ratios of retained N to intake N, and to absorbed N were also slightly higher at 7th month than at 5th and 9th months. As mentioned above, the digestion trial at 7th month has been done on the middle of summer (August), and then an increase of water intake and also urinary N excretion would induce a decrease in N retention. The daily urinary excretion of allantoin, however, was a little higher at 7th month than at 5th and 9th months, *i.e.,* the digested and absorbed MP (metabolized protein) was thought to be increased in the former than in the latter.

In Experiment II, there was no great difference in absorbed and retained N between the 2 feeding frequencies. On the 5th month, absorbed N was relatively high after 8 times feeding, but urinary N excretion was high after 8 times feeding, and consequently, the retained N were simi-

Table 6. Nitrogen balance in lambs on 5, 7 and 9 (10) month after birth (g/BW$^{0.75}$/d)

Experiment I						
Age*	5 Month		7 Month		9 Month	
Frequency**	2X	24X	2X	24X	2X	24X
Intake	2.17±0.05#	2.36±0.15	2.06±0.03	2.15±0.05	1.93±0.07	2.12±0.03
Fecal	0.50±0.05	0.53±0.09	0.61±0.02	0.62±0.04	0.60±0.02	0.55±0.04
Urinary	0.79±0.03	0.77±0.03	0.81±0.02	0.90±0.09	0.55±0.06	0.69±0.06
A##	1.66±0.07	1.83±0.07	1.45±0.05	1.53±0.05	1.33±0.08	1.54±0.03
R$^+$	0.88±0.05	1.04±0.16	0.65±0.06	0.63±0.11	0.79±0.09	0.86±0.05
R/A(%)	52.7±1.2	57.1±3.0	44.2±2.5	40.8±7.0	58.9±4.6	55.5±3.6
Experiment II						
	5 Month		7 Month		10 Month	
	2X	8X	2X	8X	2X	8X
Intake	1.29±0.02	1.30±0.03	1.28±0.02	1.26±0.03	1.26±0.04	1.25±0.04
Fecal	0.46±0.12	0.41±0.09	0.33±0.10	0.34±0.11	0.27±0.05	0.27±0.05
Urinary	0.28±0.11	0.34±0.13	0.23±0.06	0.21±0.05	0.34±0.04	0.35±0.03
A##	0.83±0.10	0.89±0.06	0.95±0.09	0.92±0.09	0.98±0.03	0.98±0.02
R$^+$	0.56±0.20	0.55±0.19	0.72±0.12	0.71±0.11	0.64±0.06	0.63±0.05
R/A(%)	66.7±16.5	62.0±17.1	75.9±7.3	76.8±5.8	65.0±4.8	64.5±3.9

* Month after birth. # Mean ± S.E. of 3 or 4 lambs. ## Absorbed. ** Feeding frequency (times per day).

lar after 2 and 8 times feedings. Bunting *et al.* (1987) also reported that feeding frequency did not have any effect on retained N in sheep. On the other hand, there are some opposite results, which indicate a decreased urinary N excretion and an increased retained N with an increase of feeding frequency (Satter & Baumgardt 1962; Ulyatt *et al.* 1984; Ruiz *et al.* 1989). These findings also support that as mentioned above, the retained N in Experiment I tended to increase a little after 24 times feeding than after 2 times feeding. The increased retained N after 24 times feeding however resulted in an increase of N intake rather than a decrease of urinary N excretion.

In Experiment II, there was a quite big variation of N balance of lambs among individuals at 5th and 7th months, and this would be mainly due to a variation of faecal N excretion. The N intake in Experiment II was lower than that in Experiment I, and faecal and urinary N excretions also decreased in Experiment II. The utilizability of dietary N, however tended to high in Experiment II than in Experiment I, and this would be due to decrease of urinary N excretion in the former than in the latter. In ruminant animals, it is well known that they will control the urinary urea excretion under the low dietary N supply, and the saved urea will be re-used for microbial protein synthesis after secretion into the rumen (Obara 1987). Obara *et al.* (1980) presumed that the rumen will be main site to use endogenous urea after feeding low protein diet, however when dietary protein is enough to meet their requirement, an importance of the reticulo-rumen to use urea will decrease, and so the endogenous urea will be secreted into the lower gut. In comparison with N balance of both Experiments in the present study, it is also thought to be that urinary N excretion decreased, and consequently the utilization of N increased could resulted in high utilization of endogenous urea in Experiment II, which the dietary N level was lower than that in Experiment I.

Water consumption and digesta flow rate in the rumen

As shown in Table 7, water consumption by lambs tended to increase with an increase of feeding frequency in both experiment I and II on each month except 9 month in Experiment I, although there was a little variations among animals individually. The increase of water consumption

Table 7. Water intake in lambs on 5, 7 and 9 (10) month after birth (L/d)

Experiment I						
Age*	5 Month		7 Month		9 Month	
Frequency**	2X	24X	2X	24X	2X	24X
	3.72±0.46#	3.77±0.24	4.06±0.29	4.35±0.47	3.79±0.15	3.52±0.24
Experiment II						
	5 Month		7 Month		10 Month	
	2X	8X	2X	8X	2X	8X
	1.92±0.33	2.09±0.20	1.78±0.30	2.16±0.58	1.63±0.24	1.78±0.35

* Month after birth. ** Feeding frequency (times per day). # Mean ± S. E. of 3 or 4 lambs.

Table 8. The passage rate of ingesta in the rumen of lambs during on 5, 7 and 9 (10) month after birth (%/hour)

Rate constant of solid phase						
Age*	5 Month		7 Month		9 Month	
Frequency**	2X	24X	2X	24X	2X	24X
	3.92±0.32#	3.24±0.39	2.61±0.44	2.83±0.17	3.11±0.40	3.92±0.32
Rate constant of liquid phase						
	5 Month		7 Month		10 Month	
	2X	8X	2X	8X	2X	8X
	4.97±0.23	4.24±0.57	4.06±0.49	4.12±0.20	4.33±0.39	5.03±0.29

* Month after birth. ** Feeding frequency (times per day). # Mean ± S. E. of 3 lambs.

after frequent feeding have been shown in sheep fed roughage alone (Ulyatt *et al.* 1984; Bunting *et al.* 1987) or mixed diet (roughage+concentrate) (Malestein *et al.* 1981). Furthermore, Ruiz *et al.* (1989) also reported that frequent feeding increased water consumption and salivary secretion due to increase of plasma osmotic pressure in sheep fed alfalfa silage supplemented with soybean meal.

In Experiment II, the measurement of digesta flow rate in the rumen has been done on 5th, 7th and 10th months, and the obtained results were shown in Table 8. On 7th and 10th months, the constant of passage rate after 8 times feeding tended to high as compared with that after 2 times feeding. This would be due to an increase in water consumption after 8 times feeding (see Table 7). After 8 times feeding, the volume of feed entered the rumen per one time will be smaller than that after 2 times feeding, and then, the digesta volume in the rumen is thought to be smaller than that after 2 times feeding. Consequently there could be a possibility that the retention time of digesta in the rumen was shorter in the former than in the latter. If so, this would reflect an extent of microbial fermentation in the rumen, *i. e.,* an increase of MP entered the lower gut resulted in an increase of urinary PD excretion (see Table 10). Then, the out flow rate of liquid phase was a little faster than that of solid phase in the present study, and this surely support the theory supposed by Faichney (1980), which the outflow rate of digesta from the rumen is surely influenced by a difference in the particle size of digesta.

Plasma allantoin concentration

The time course changes in plasma allantoin level after morning feed was shown in Figure 3. There was no great change in time course changes of plasma allantoin in both feeding frequency on 5th month in Experiment I. On 7th and 9th months, the diurnal pattern of time course changes in plasma allantoin was almost the same in both feeding frequency in Experiment I. After 2 times feeding, the plasma level of allantoin declined at 3 hours, and then it increased until at 12 hours after morning feed (09 : 00hr), and finally the level recovered to the same level at just before morning feed. The plasma allantoin level decreased at 3 hours and then increased at 5 hours after morning feed after 24 times feeding, and finally the level at 12 hours recovered to the same level at just before morning feed. In Experiment II, there was no time course change in plasma allantoin level until 12 hours after morning feed after 2 times feeding on 5th, 7th and 9th months. After 8 times feeding, the plasma allantoin level tended to increase at 2 hours (10th month) and 5 hours (5th month) after morning feed, and on 7th month, the level was relatively steady until 12 hours after morning feed. From these findings, it was presumed that the out flow rate of digesta through the rumen was relatively fast, and absorbed nucleoside was also metabolized quickly after 8 or 24 times feeding than after 2 times feeding.

As shown in Table 9, the plasma allantoin level tended to increase on 5th and 7th month after 24 times feeding in Experiment I, and also on 7th and 10th month after 8 times feeding in Experiment II. The plasma allantoin concentration was relatively lower in Experiment II than that in Experiment I, and this could be due to the difference of feeding level in both Experiments.

The urinary PD excretion

In time course changes in urinary PD excretion in both Experiments, there are no definite pattern in hourly PD excretion into urine regardless the changes in feeding frequency on each month, when the digestion trials has been

Table 9. Plasma allantoin level in lambs on 5, 7 and 9 (10) month after birth (μ mol/L)

Experiment I						
Age*	5 Month		7 Month		9 Month	
Frequency**	2X	24X	2X	24X	2X	24X
	35.58±4.17#	39.78±2.11	36.78±4.00	37.15±1.87	43.54±1.03	39.71±3.54
Experiment II						
	5 Month		7 Month		10 Month	
	2X	8X	2X	8X	2X	8X
	22.88±2.33	17.73±3.30	21.88±2.42	30.43±2.13	21.57±2.10	31.14±1.47

* Month after birth. ** Feeding frequency (times per day). # Mean ± S. E. of 3 or 4 lambs.

done.

Table 10 shows the urinary PD excretion and also the microbial N supplied to animal, which calculated by the equation (Chen & Gomes 1995). In Experiment I, urinary allantoin excretion at 5, 7 and 9 months tended to high after 24 times feeding as compared with that after 2 times feeding, although there was a similar value in urinary excretion of hypoxanthine + xanthine after both feeding frequencies. The microbial N supply (g N/d) tended to increase after 24 times feeding than after 2 times feeding. Urinary PD excretion is mainly controlled by the amounts of absorbed microbial purines, but there is no linear regression between the former and the latter, *i. e.,* an endogenous PD excretion could have clear effect on the total urinary PD excretion (Verbic *et al.* 1990). In Experiment II, the urinary allantoin excretion, microbial N supply and microbial N supply per digested organic matter in the rumen (DOMR) tended to high after 8 times feeding than after 2 times feeding, although there was no clear difference in hypoxanthine + xanthine excretion in both feeding frequencies as same as in Experiment I. In Experiment I, microbial N supply tended to increase after 24 times feeding than after 2 times feeding, and in Experiment II too, it also tended to high after 8 times feeding than 2 times feeding. This clearly shows some changes in the extent of microbial fermentation in the rumen, *i. e.,* frequent feeding should maintain a relatively stable fermentation in the rumen through introducing a small portion of feed with short interval during a day. As shown in Figure 4, diurnal variation of urinary PD excretion was relatively small after frequent feeding than after 2 times feeding, and this could reflect relatively small diurnal variation in microbes digested in the lower gut presuming a stable microbial synthesis in the reticulo-rumen. Furthermore, it is thought to that as shown in the change of flow rate constant of digesta in the rumen, the flow rate of both solid and liquid phase in the rumen clearly increased. At the same time, these could be thought to be related to increase in an outflow of microbes from the rumen to lower gut. There are similar findings in sheep fed hay alone (Bunting *et al.* 1987) or concentrate alone (Al-Attar *et al.* 1976), and in dairy cattle fed hay and concentrate (Tamminga 1981).

In comparison of the results obtained in Experiment I and II, urinary PD excretion at 5th and 7th months in experiment I was mostly 3 times of the values at same month in Experiment II, and also the figure at 9 month in Experiment

Table 10. Urinary PD excretion (mmol/$BW^{0.75}$/d) and microbial N supply (g/d) in lambs on 5, 7 and 9 (10) month after Birth.

Experiment I

Age*	5 Month		7 Month		9 Month	
Frequency**	2X	24X	2X	24X	2X	24X
Allantoin	1.20±0.20#	1.28±0.16	1.50±0.22	1.56±0.20	1.26±0.23	1.51±0.22
Uric acid	0.21±0.02	0.19±0.01	0.19±0.02	0.19±0.01	0.15±0.01	0.16±0.01
X+Hx##	0.13±0.06	0.13±0.04	0.07±0.01	0.08±0.01	0.04±0.00	0.07±0.01
Total PD	1.54±0.23	1.60±0.13	1.76±0.25	1.83±0.20	1.45±0.24	1.74±0.23
MP supply (gN/d)	15.69±2.22	16.23±0.95	21.68±3.02	27.58±1.89	20.12±3.16	23.83±2.89
(gN/kgDOMR)	30.66±4.69	29.78±2.68	39.09±5.67	39.31±4.58	34.81±5.81	36.23±5.29

Experiment II

	5 Month		7 Month		10 Month	
	2X	8X	2X	8X	2X	8X
Allantoin	0.29±0.10	0.32±0.06	0.27±0.04	0.30±0.07	0.43±0.01	0.50±0.03
Uric acid	0.12±0.03	0.12±0.02	0.13±0.01	0.11±0.01	0.16±0.02	0.16±0.01
X+Hx	0.06±0.00	0.07±0.01	0.03±0.00	0.06±0.03	0.04±0.01	0.05±0.00
Total PD	0.47±0.11	0.51±0.07	0.43±0.54	0.47±0.05	0.63±0.02	0.71±0.03
MP supply (gN/d)	4.11±0.80	4.59±0.29	4.04±0.21	4.53±0.71	7.23±1.09	8.14±0.77
(gN/Kg DOMR)	14.98±5.23	16.20±3.65	12.08±2.48	14.12±1.46	18.20±1.47	20.56±1.24

* Month after birth. ** Feeding frequency (times per day).
# Mean ± S. E. of 3 or 4 lambs. ## Xanthine+Hypoxanthine.

I was almost double of that at 10 month in Experiment II. These findings is clearly thought to reflect a large amount of DMI, resulted in an increased microbial synthesis in the rumen, and further microbial protein absorbed in lower gut in Experiment I than in Experiment II.

About the ratios of hypoxanthine + xanthine, uric acid and allantoin to total PD in urine of both Experiments, in Experiment I, the ratio of allantoin to total PD in the urine were 78-87%, although that in Experiment II was 62-70% on each month after birth. These differences could be due to the difference of DM intake in both Experiments, reflecting a decreased urinary allantoin excretion in Experiment II. There was little difference in urinary excretions of hypoxanthine + xanthine and uric acid, *i. e.,* the ratios of hypoxanthine + xanthine and uric acid to total PD in the urine tended to high along with a decrease in urinary excretion of total PD. These are in good agreement with that reported previously by Chen and Gomes (1992). Then, it seems that some changes in exogenous purine supply clearly influenced to the activity of xanthineoxidase and/or uricase. In the present study, however, some effects of exogenous purine supply on enzyme activity are not clear yet.

Usefulness of spot urine sample to estimate the daily total PD excretion

Urinary PD excretion can be used to assume the microbial protein supply in ruminant animals, although there are some problems to be dissolved for practice in the field, because in the method it is necessary to collect daily whole urine at least during consecutive 5 days for reducing an error due to the variation of daily urine volume. Therefore, it is thought to use a spot urine sample to presume daily PD excretion through its PD concentration. Then, in the present study, the relationships between PD concentration of hourly-collected urine and daily total PD excretion after 2 times and frequent feeding on 5, 7, 9 and 10 months after birth were estimated and the values with relatively high coefficient of correlation. There were relatively high coefficients(r) of correlation=0.842, 0.814 and 0.866 after 13, 17 and 20 hours after morning feed (09 : 00) in 2 times feeding, respectively. These findings are a little different from our previous result that total daily PD excretion can be estimated using the PD concentration in spot urine samples collected at 7-8 hour after morning feed in sheep fed twice daily (Fujihara *et al.* 2005). Chen *et al.* (1995) has also reported that the ratio of PD: creatinine in the urine collected between 15.00 and 20.00 hours tended to give a better representation of the daily mean in steers fed once daily (08 : 00 hours) or twice daily (08.00 and 16.00 hours). In 24 times feeding, the coefficients (r) of correlation were 0.841, 0.823, 0.902, and 0.834 after 4, 12, 15 and 24 hours after morning feed (09 : 00), respectively. In 8 times feeding, at only 20 hours after morning feed (09 : 00) a relatively high coefficient of correlation (r= 0.802) was found, and as a whole, there was relatively high correlation after 24 times feeding than after 2 times and/or 8 times feedings. There is tendency that a high correlation between PD levels in spot urine sample and daily total PD excretion into urine was found after 24 times feeding. These findings suggested that the PD level in spot urine sample after frequent feeding will show a high possibility to be more practical index for calculating total daily PD excretion.

Implication

In the present study, for changing the fermentation profile in the rumen of sheep the frequent feeding (8 and 24 times/day) has been done, and then, a relatively good result is obtained in supply of microbial protein as compared to those in ordinary feeding as twice a day, although it will be a little difficult to introduce the method directly to practical feeding in farmer' level. However, the fact that frequent feeding has obviously given good effect on ruminant production could be useful as a fundamental knowledge for improvement in practical feeding system with relative consideration to reduce the labor and/or feed cost, *i. e.,* introducing an auto-feeding system with mechanization in future.

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