INTRACELLULAR AMINO ACID PROFILE OF RUMEN BACTERIA AS INFLUENCED BY UREA FEEDING AND ITS DURATION

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Summary

Rumen bacterial amino acids in sheep on urea diet were monitored to assess a possible change in amino acid synthesis as a long term response to high rumen ammonia environment. A sheep was fed a semipurified diet with soybean meal, followed by a diet with urea as a main nitrogen source. Mixed rumen bacteria were harvested from ruminal fluid taken 3 h after feeding (twice in soybean meal feeding and 6 times in urea feeding) and fractionated as cell wall, proteins and protein-free cell supernatant to monitor amino acids in each fraction. Ruminal ammonia concentration at the sampling ranged from 5.7 to 39.5 mgN/dl. Cell wall and protein fractions of mixed rumen bacteria were stable in their amino acid composition regardless of nitrogen sources of diet and the feeding duration. However, protein-free cell supernatant fraction showed a higher alanine proportion with urea feeding (18.6 and 28.2 molar % of alanine for samples from sheep fed soybean meal and urea, respectively) and its duration (20.6 and 32.9 molar % for samples from sheep on urea diet for 1 and 65 days, respectively). Total free amino acid level of bacteria was depressed in the initial period of urea feeding but restored on 65th day of the feeding. These results suggest that an alanine synthesizing system may develop in rumen bacteria as urea feeding becomes longer.

(**Key Words**: Urea, Ammonia Assimilation, Amino Acid, Alanine, Rumen Bacteria)

Introduction

Rumen bacterial responses to long term feeding of urea were characterized as a decreased ureolysis and an increased cell number with the feeding duration in the previous experiment (Kobayashi et al., 1993). However, ammonia-assimilating pattern has been little described. Activity of ammonia-assimilating enzymes and synthesis of amino acids in the rumen greatly depend on ruminal ammonia concentrations (Erfle et al., 1977; Wallace, 1979). Erfle et al. (1977) reported an increased alanine synthesis by rumen microorganisms in a high ammonia environment. The same has been recently evidenced in rumen wall-attaching bacteria (Legath, 1992). These led us to investigate a possible change in amino acid synthesis by rumen bacteria of sheep with long term feeding of a urea diet. This note describes the influence of urea feeding and its duration on amino acid profiles of cell wall, protein and protein-free supernatant fractions of mixed rumen bacteria of sheep.

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Materials and Methods

Management and feeding of experimental animal and sampling were the same as described previously (Kobayashi et al., 1993), i.e., a wether with ruminal cannula, 43 kg in weight, was housed in a metabolic cage and fed a control diet (C) for 10 d (period C) followed by a urea diet (U) for 65 d (period U) each containing 19% of soybean meal or 3% of urea as a main nitrogen (N) source (75% of total N). Both of the diets were mainly composed by rice straw (40%), corn starch (20 and 30% for C and U, respectively), cane molasses (14 and 20% as above), minerals (7%), vitamins (trace) and the appropriate N source. All the ingredients except rice straw was mixed well and offered with the straw. These semipurified formula were almost isonitrogenous (10.8 and 11.5% of curde protein for C and U, respectively) and isoenergetic (1.94 and 1.84 Mcal/kg of ME for C and U, respectively). Each diet was fed twice daily (450 g/feeding, 09:00 and 21:00).

Ruminal mixed bacteria were obtained by a differential centrifugation procedure (Baldwin and Palmquist, 1965), using ruminal fluid withdrawn 3 h after feeding from the sheep on the 9th and 10th d of period C and on the 1st, 2nd, 8th, 17th, 38th and 65th d of period U (U1, U2, U8, U17, U38 and U65, respectively). On the sampling days of period U, urea was removed from the diet, dissolved in water and infused through the ruminal cannula via a falling drop equipment for 20 min to equalize ingestion rate of urea. The bacteria were fractionated as cell wall, protein and protein-free cell supernatant according to the method of Blake et al. (1983). In brief, the mixed rumen bacteria were disrupted ultrasonically and centrifuged. The precipitate (cell wall) was washed and lyophilized. The supernatant was treated by sulphosalicylic acid to precipitate protein (protein) and the residue was neutralized with sodium hydroxide and filtered (protein-free cell supernatant). The former 2 fractions were hydrolyzed by 6N HCl for 20 h and employed for amino acid analysis with ninhydrin reaction (HPLC system 655-A, Hitachi, Tokyo), the last being employed without the hydrolysis. Amino acids were eluted with sodium citrate buffer (pH 2.0, 3.2, 4.35 and 4.7), employing 2 buffer gradient system. The acids were absolutely quantified with separately chromatographed standard mixture. The recovery of each acid in known standard was between 96.5 and 104.4%. Ruminal ammonia was measured by a phenol-hypochrolite method (Weatherburn, 1967). Student's t test was adopted to examine differences between C and U (U17-U65).

Results and Discussion

Amino acids in all 3 fractions are given in table 1. Most notable trend is an apparent increase in intracellular free alanine proportion with urea feeding (18.6 and 28.2% for C and U17-U65, respectively, $p < 0.05$) and its duration (20.6 and 32.9% for U1 and U65, respectively). Amino acid compositions of bacteria proteins and cell wall fractions, however, were relatively stable (table 1). An ammonia-assimilating pattern by rumen bacteria is more characteristic in an intracellular free amino acid proportion than those in the other 2 fractions (Blake et al., 1983). Thus, the main route of ammonia assimilation in rumen bacteria is alanine formation, which may serve as a temporary reservoir for ammonia. The same finding was reported by Blake et al. (1983), who found that ^{15}N-enrichment in bacterial free alanine with $^{15}NH_4Cl$ administration was highest among all the amino acids.

A high ruminal free alanine level under high ammonia environment has been reported both *in vitro* (Erfle et al., 1977) and *in vivo* (Wallace, 1979). The same was found in hydrolyzates of bacteria adhered to ruminal epithelium with a high N diet (Legath, 1992). This also coincides with the present results (table 1). As shown previously, at the initial period of urea feeding (U2-U17) an extremely high ammonia production (25-50 mgN/dl) was recorded (Kobayashi et al., 1993). Such high ammonia accumulation and probably less developed assimilation system might cause a lower total amino acid level in cell free supernatant fraction (U2-U17, table 1). Ruminal bacterial number was also reduced in the period of U2-U17 (Kobayashi et al., 1993). However, the reduction in total free amino acid level (table 1) as well as in bacterial number (Kobayashi et al., 1993) was recovered in the last sample (U65). These facts suggest that rumen bacteria may develop a certain ammonia-assimilating system, responding to long term exposure to high ammonia environment.

The present data do not allow any conclusion to be drawn as to the pathway by which much alanine is formed. One of the possible ways is through aminotransferases, the amino group coming from glutamate and aspartate. Decreases in glutamate and aspartate and increases in alanine in the cell free supernatant fraction (U65, table 1) implies this possibility. However, several workers have reported low activity of alanine and aspartate aminotransferases in rumen bacteria with high alanine accumulation under the condition similar to the present experiment (Blake et al., 1983; Wallace, 1979). Alanine dehydrogenase, another candidate activating alanine synthesis, has been investigated but evidence in favor of the hypothesis was not obtained (Wallace, 1979). These experiments might not be enough in length of preliminary period before sampling (3-4 wk) to assess enzymatic adaptation to urea in rumen bacteria.

Ruminobacter amylophilus, an amylolytic rumen bacterium, has a very high intracellular alanine

TABLE 1. REPRESENTATIVE AMINO ACIDS IN CELL FREE SUPERNATANT, PROTEINS AND CELL WALL FRACTIONS OF MIXED RUMEN BACTERIA FROM SHEEP ON A DIET WITH SOYBEAN MEAL OR UREA

	Sampling days*							Difference
	C	U1	U2	U8	U17	U38	U65	(C vs U17-65)
Cell free sup.	..... (molar %)							
Aspartate	6.6	5.9	6.0	5.5	4.9	6.2	3.5	NS
Threonine	5.8	4.6	5.6	5.4	4.9	4.2	4.0	NS
Serine	6.2	5.7	7.0	6.0	6.0	6.7	6.9	NS
Glutamate	13.6	17.3	19.9	23.5	20.3	18.0	14.9	NS
Glycine	6.6	6.5	7.5	7.3	6.3	4.9	5.6	NS
Alanine	18.6	20.6	25.7	21.3	27.3	24.4	32.9	$p < 0.05$
Valine	5.0	5.3	4.9	4.4	4.1	4.7	4.1	NS
Methionine	2.8	3.3	2.1	1.3	2.1	2.0	2.4	NS
Isoleucine	5.3	4.4	2.1	3.5	2.9	3.6	5.2	NS
Leucine	8.2	6.2	2.1	3.2	3.0	5.7	4.6	NS
Lysine	13.4	14.5	15.7	16.0	14.3	13.0	10.7	NS
Others	6.9	5.7	1.4	2.6	3.9	6.6	5.2	
	..... (μM/g bacteria)							
Total	65.7	65.0	38.2	30.2	34.1	48.1	61.5	NS
Proteins	..... (molar %)							
Aspartate	11.3	11.8	12.3	11.3	11.0	11.6	12.0	NS
Threonine	6.3	6.0	6.4	5.9	5.8	6.0	6.3	NS
Serine	5.1	5.6	5.9	5.1	4.9	5.3	5.5	NS
Glutamate	6.9	9.9	9.6	8.5	7.8	7.7	9.0	NS
Proline	6.8	4.7	4.7	5.4	7.2	5.6	4.9	NS
Glycine	9.0	9.0	9.0	9.2	8.9	9.2	9.5	NS
Alanine	11.8	11.2	11.1	11.4	11.2	11.5	11.5	NS
Valine	7.8	7.8	7.8	7.6	7.6	7.7	7.6	NS
Methionine	2.2	2.3	2.1	2.1	2.2	2.1	2.0	NS
Isoleucine	5.6	6.1	6.1	6.6	6.5	6.7	6.2	NS
Leucine	10.4	10.0	9.6	10.1	7.9	10.2	9.7	NS
Lysine	7.1	6.8	6.9	7.6	7.2	7.2	7.1	NS
Arginine	3.5	3.4	3.1	3.6	3.5	3.5	3.5	NS
Others	5.2	5.4	5.4	5.6	8.3	5.7	5.2	
	..... (mM/g bacteria)							
Total	0.66	1.06	0.56	0.85	0.98	1.03	0.95	NS
Cell wall	..... (molar %)							
Aspartate	9.7	10.7	9.3	11.2	—	11.1	9.2	NS
Threonine	7.4	6.1	7.3	5.7	—	6.0	7.2	NS
Serine	5.9	5.7	5.7	5.6	—	5.3	5.6	NS
Glutamate	6.2	8.3	5.0	10.3	—	8.2	4.7	NS
Glycine	8.8	9.0	8.3	9.0	—	8.0	8.6	NS
Alanine	13.7	15.1	15.3	12.1	—	16.7	14.4	NS
Valine	7.3	7.4	7.7	7.8	—	7.6	7.7	NS
Methionine	2.5	2.3	2.5	2.3	—	1.9	2.5	NS
Isoleucine	8.2	8.3	8.6	7.2	—	8.4	8.5	NS
Leucine	9.8	10.4	9.8	9.0	—	10.1	10.1	NS
Lysine	7.1	7.4	7.4	7.2	—	7.6	7.3	NS
Arginine	4.2	3.8	4.2	3.4	—	3.8	4.5	NS
Others	9.2	5.8	8.9	9.2	—	5.3	9.7	
	..... (mM/g bacteria)							
Total	1.31	0.69	1.00	1.12	—	0.75	0.89	NS
Ruminal NH_3 (3 h after feeding)	..... (mgN/dl)							
	5.7	16.8	24.3	14.7	13.7	39.5	33.1	$p < 0.05$

* Mixed rumen bacteria were obtained from sheep on the 9th and 10th days of soybean meal feeding period (C) and on the 1st, 2nd, 8th, 17th, 38th and 65th days of urea feeding period (U1, U2, U8, U17, U38 and U65, respectively), i.e. values for period C were presented as averages of the 2 samples, while those for period U were from a single determination on each sampling day.

pool (Jenkinson et al., 1979). A possible dominance of this bacterium in the rumen of sheep on starchy diet like the present diets could partly explain the high free alanine level in mixed bacteria.

In summary, when a urea diet was provided for a long time, it is probable that a high rumen ammonia environment may induce ruminal bacteria to develop alanine-synthesizing system.

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