Studies on Protein Digestion in Ruminants

V. Effect of Carbohydrates and Antibiotics on the Ammonia Concentration

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In previous paper⁹⁾, it was shown that addition of glucose to casein diet markedly decreased the ammonia concentration in rumen of sheep. The experiments described here were undertaken to find whether the same results would be obtained by addition of the other carbohydrates and antibiotics in vitro.

Materials and Methods

An adult female Corriedale sheep, weighing 75 kg was used. The animal was fitted a permanent rumen fistula.

The daily ration was fed to the animal at 9:00 am and 5:00 pm, and consisted of 2 kg green soybean hay and 300 g concentrate (rice bran 2: wheat bran 1), containing 3% of calcium carbonate and 1% of salt. Water was supplied ad libitum.

Rumen samples were taken immediately prior to morning feeding through the fistula. The samples were strained through four layers of gauze and immediately added to the fermentation flasks (50 ml conical flask).

The composition of standard flasks is shown in Table 1. Casein and carbohydrates were

Table 1. Composition of fermentation flasks

	Blank	Control	Test
Strained whole rumen liquor	5 ml	5 ml	5 ml
Casein solution (1%)	—	5 "	5 "
Carbohydrate	0.5 M solution a	—	5 "
	0.25 M " b	—	5 "
	0.15 M " c	—	5 "
	10% " d	—	5 "
	5% " e	—	5 "
	add directly f	—	0.25 g
Antibiotic	200~6,000 IU/ml h	—	5 ml
	20~40 mg/ml i	—	5 "
	0.6~2.5 mg/ml j	—	5 "
	add directly k	—	10~300 mg
Phosphate buffer (M/15, pH 6.80)	20"	15"	10~15 ml

a): xylose, glucose, fructose, galactose, mannose, sucrose, lactose, and maltose.

b): cellobiose. c): raffinose. d): dextrin. e): starch and inulin. f): xylan and cellulose.

h): penicillin G. i): streptomycin. j): tetracycline. k): kanamycin.

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dissolved in M/15 phosphate buffer at pH 6.80 unless otherwise provided. Xylan and cellulose were added directly to the medium. Penicillin (potassium penicillin G, by Takeda Chemical Industries Ltd.) was dissolved in water. Streptomycin (dihydrostreptomycin sulfas DAIICHI, by Daiichi Seiyaku Co., Ltd.) and tetracycline (chlortetracycline, by Meiji Seika Co., Ltd.) were also dissolved in water and neutralized with NaOH. Kanamycin (kanamycin sulfate, by Takeda Chemical Industries Ltd.) was added directly to the medium. The apparent endogenous ammonia production was determined by the analysis of blank flask. The apparent ammonia production in the presence of casein was measured by control flask. The test flask was consisted of strained whole rumen liquor, casein, carbohydrate or antibiotic and made the same total volume (25 ml) as control and blank flask with phosphate buffer. The effect of added carbohydrates and antibiotics was calculated by next equation:

$$\text{Decrease (\%)} = 100 - (T - B) / (C - B) \times 100$$

where B is ammonia-N $\mu\text{g/ml}$ of reaction mixture in blank flask, C is that in control flask and T is that in test flask.

After air phase of the flasks was replaced by carbon dioxide, the flasks were closed tightly by rubber stoppers and incubated in waterbath at 39°C for two hours. Then, the flasks were placed in crushed ice and the reaction mixture were submitted to nitrogen analysis. The nitrogen fractions were analysed by the procedures employed by KANDATSU and TAKAHASHI⁽¹⁰⁾: ammonia nitrogen; the method of VAN SLYKE-CULLEN⁽²³⁾. Microbial nitrogen; 10 ml of reaction mixture was centrifuged for 30 minutes at 12,000 r.p.m. (10,000 g). The sediment was washed twice with water and the nitrogen was analysed by Kjeldahl method. Protein (casein) nitrogen; 5 ml of 20% trichloroacetic acid solution was added to 5 ml of the above supernatant and was allowed to stand for overnight in refrigerator. The sediment was collected and analysed by Kjeldahl method. Soluble nitrogen; the above deproteinized supernatant was analysed by Kjeldahl method. Amino nitrogen; the deproteinized supernatant was neutralized with NaOH and analysed by VAN-SLYKE method⁽¹⁾.

The pH values were determined by use of a glass electrode pH meter (Model, M-4, by Hitachi-Horiba).

Statistical procedures were applied as suggested by SNEDECOR⁽²³⁾.

Results

The effect of carbohydrates and antibiotics on the ammonia-N concentration is shown in Table 2. As shown in last column in Table 2, ammonia-N concentration was markedly decreased (74~141%) by addition of xylose, glucose, fructose, galactose, sucrose, maltose, lactose, cellobiose and raffinose respectively. Decrease (34~55%) in ammonia-N concentration was also observed by addition of mannose, dextrin, starch, inulin and xylan respectively, but the effect was rather small than the above group. Addition of cellulose, however, gave no effect within 2 hours.

Addition of 4 mg/ml of streptomycin or 0.5 mg/ml of tetracycline depressed the apparent ammonia production very much. However, there was a little effect at the other concentration (8 mg/ml and 0.12 mg/ml) of these antibiotics (Table 2). Penicillin had no effect even in high unit. Addition of kanamycin showed increase in ammonia-N concentration.

The changes of nitrogen distribution and pH caused by addition of 0.1 M fructose were measured and results are given in Table 3 and 4. As shown in Table 3, microbial nitrogen

Table 2. Effect of carbohydrates and antibiotics on ammonia-N concentration

	Blank (B)	Control (C)	Test (T)	Derease (%)
	(NH ₃ -N μ g/ml of reaction mixture)			$(100 - \frac{T-B}{C-B} \times 100)$
Xylose (0.1 M) a	52 $\pm$ 4 b	70 $\pm$ 5 b	56 $\pm$ 2 b	80 $\pm$ 11 b
Glucose (")	33 7	46 9	35 10	82 9
Fructose (")	51 6	75 10	51 6	101 4
Galactose (")	37 8	57 4	43 2	74 15
Mannose (")	46 5	82 1	71 3	34 7
Sucrose (")	54 4	73 2	46 2	141 8
Lactose (")	47 8	65 11	50 7	77 18
Maltose (")	35 3	55 3	36 3	93 7
Raffinose (0.03 M)	44 4	62 7	47 4	84 5
Dextrin (2%)	34 4	55 8	45 1	48 6
Starch (1%)	49 5	75 2	64 5	42 10
Xylan (")	40 5	51 8	43 6	55 20
Inulin (")	36 7	57 8	48 7	44 1
Cellobiose (0.05 M)	34 2	54 1	33 5	104 20
Cellulose (1%)	60 7	78 9	76 8	11 2
Penicillin G (40 IU/ml)	40	48	47	12 c
" (400 IU/ml)	38	48	44	40 c
" (1,200 IU/ml)	80	121	117	10 c
Streptomycin (4 mg/ml)	99	111	88	192 c
" (8 mg/ml)	92 2	125 9	118 6	22 4
Kanamycin (0.4 mg/ml)	115	141	136	19 c
" (12 mg/ml)	59 4	82 1	99 6	-85 29
Tetracycline (0.12 mg/ml)	79	100	96	19 c
" (0.50 mg/ml)	81 7	122 11	92 7	84 11

a): final concentration. b): standard error. c : single determination.

considerably increased by addition of fructose than control and the difference from it was significant ($p < .01$). The blank flask showed decrease in microbial nitrogen. Ammonia-N concentration of the test flask showed same level as blank and difference from control was significant ($p < .01$). After two hours incubation the pH dropped by addition of fructose and difference from control was significant ($p < .01$). Protein and soluble nitrogen decreased by addition of fructose, however, the differences from control being not significant. Amino nitrogen gave high concentration by addition of fructose than control, but the difference from it was not significant.

As shown in Table 3 and 4, when fructose was added to medium, the significant drop in pH values was accompanied with significant decrease in ammonia concentration. So, the relationship between pH and ammonia concentration was investigated. Result is presented in Fig. 1. From this figure, the optimum pH for apparent ammonia production was around 7.0 and below pH 6.0 or above pH 8.0, the ammonia-N concentration was rather low. However, apparent ammonia production was observed in relatively wide pH ranges (pH 5.25~8.0) employed.

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Table 3. Effect of fructose on nitrogen distribution and pH

	Blank (B)	Control (C)	Test (T)	
	(N μ g/ml of reaction mixture)			
Microbial-N				
Before incubation	346 $\pm$ 41 b	346 $\pm$ 39 b	365 $\pm$ 46 b	
After incubation	336 43	359 43	399 45	B-C, T**
Increase	- 10 6	13 4	34 4	C-T**
Protein-N				
Before incubation	—	357 12	330 28	
After incubation	—	136 23	133 32	
Increase	—	-221 25	-197 25	
Soluble-N				
Before incubation	103 9	122 14	121 28	
After incubation	109 8	341 38	277 45	B-C, T**
Increase	6 3	219 29	156 28	
Ammonia-N				
Before incubation	39 5	40 4	37 3	
After incubation	44 3	63 3	43 0	B-C, T**
Increase	5 3	23 1	6 2	C-T**
pH				
Before incubation	6.80 0.00	6.80 0.00	6.80 0.00	
After incubation	6.31 0.01	6.30 0.02	5.88 0.09	C-T**

b): standard error.

B-C, T**: Differences in nitrogen increase between blank and control or test are significant at the 1% level.

C-T** : Difference in nitrogen increase or pH values after incubation between control and test is significant at the 1% level.

Table 4. Effect of fructose on amino-N, ammonia-N and pH

	Blank (B)	Control (C)	Test (T)	
	(N μ g/ml of reaction mixture)			
Ammonia-N				
Before incubation	70 $\pm$ 4 ^b	75 $\pm$ 4 ^b	74 $\pm$ 4 ^b	
After incubation	91 7	156 9	105 5	B-C, T**
Increase	21 3	81 6	31 2	C-T**
Amino-N				
Before incubation	0 0	0 0	0 0	
After incubation	0 0	36 7	43 5	
Increase	0 0	36 7	43 5	B-C, T**
pH				
Before incubation	6.80 0.00	6.80 0.00	6.80 0.00	
After incubation	6.36 0.05	6.33 0.05	5.61 0.15	C-T**

b): standard error.

B-C, T**, C-T**: See table 3!

Fig. 1. Effect of pH on the ammonia-N concentration

Discussion

By addition of carbohydrates there is considerable decrease in ammonia-N concentration. McDONALD¹⁵⁾ reported that when starch alone was added to the rumen of sheep during the late post-feeding stage there was a steady decline in the concentration of ammonia. ANNISON³⁾ showed that the rates of disappearance of ammonia and amino-acids from the rumen, after feeding casein or casein hydrolysate were increased by the additional presence of carbohydrate supplements (starch and sucrose). EL-SHAZLY⁶⁾ demonstrated that the addition of starch to the bean diet distinctly depressed ammonia production. LEWIS and McDONALD¹³⁾ investigated effect of added carbohydrate on the rate of disappearance of ammonia from the rumen in detail. Levan, starch, xylan and glucose were much effective, while in the presence of cellulose there was no significant decrease in ammonia concentration until 8 hours after feeding. These results are in accord with that obtained by the author's *in vitro* experiments concerning the decrease in ammonia-N concentration.

When fructose was added to the medium, significant increase in microbial nitrogen was accompanied with decrease in ammonia-N concentration. PEARSON and SMITH¹⁸⁾ showed that the degree of protein synthesis was increased when starch was also included in the diet. The similar results were obtained by many workers in the studies on urea utilization both *in vitro* and *in vivo*^{3,4,16)}. PHILLIPSON et al.¹⁹⁾, demonstrated that number of micro-organism increased by addition of starch. Furthermore, they²⁰⁾ postulated that starch fermenting bacteria might possess the property of using ammonia nitrogen for growth.

By addition of fructose there was a fall in pH value and in ammonia-N concentration. REIS and REID²¹⁾ found that low pH and increased conversion of ammonia into bacterial protein occurred *in vitro* systems as the result of the addition of glucose. MINATO et al.¹⁷⁾ reported the fall in pH by the addition of starch. ELSDEN⁷⁾ demonstrated that when glucose was fermented by rumen organisms, substantial amounts of volatile fatty acids (VFAs) identified as acetic, propionic, and butyric acids were formed. Using washed suspension technique, DOERSCH et al.⁵⁾ studied the action of rumen bacteria on maltose, glucose, and xylose. Acetic,

propionic and unidentified higher acids were formed. HEALD and OXFORD⁸⁾ showed that carbohydrate was fermented by rumen protozoa to VFAs, carbon dioxide and hydrogen. KUMENO and KANDATSU¹²⁾ found that when starch was added to the rumen, there was an increase in VFAs concentration. The decline in pH value, therefore, might occur as the result of carbohydrate fermentation, namely by the production of VFAs.

From the above discussion, the effect of carbohydrate may be concluded as follows: Addition of carbohydrate stimulates the uptake of ammonia by rumen organisms and increases the microbial protein synthesis. Added carbohydrate is fermented to VFAs, so that the pH value of the medium goes down. The fall in pH value depresses the ammonia production from casein. From these results, the low ammonia concentration is observed.

In this experiment, penicillin had little effect for wide range of international unit (IU) on the ammonia concentration. Tetracycline and streptomycin decreased ammonia-N concentration and kanamycin had opposite effect. LEWIS and EMERY¹⁴⁾ showed that three IU of penicilline G per milliliter of reaction mixture consisting of washed rumen bacteria had no effect on amino acid dissimilation, whereas a penicillin concentration of 30 IU per milliliter markedly inhibited amino acid dissimilation. PHILLIPSON et al.²⁰⁾ reported that in very low concentrations (0.1 µg/mL), penicillin and chlortetracycline caused a greater decrease in the concentration of ammonia nitrogen. KLOFFENSTEIN et al.¹¹⁾ found that addition of 20 mg of aureomycin increased protozoal concentration in vivo. The effect of antibiotics on the rumen micro-organisms is very complicated and should be investigated in detail in future.

Summary

- 1) The effect of carbohydrate and antibiotics on the ammonia-N concentration was studied in vitro on the rumen liquor taken from a sheep fitted rumen fistula.
- 2) The marked low ammonia-N concentration was observed by addition of xylose, glucose, fructose, galactose, sucrose, maltose, lactose, cellobiose or raffinose.
- 3) The ammonia-N concentration also dropped by addition of mannose, dextrin, starch, inulin or xylan but the effect was rather smaller than the above group.
- 4) By addition of cellulose there was no significant change in ammonia-N concentration within 2 hours.
- 5) When fructose was present, significant decrease in ammonia-N concentration was accompanied with significant increase in microbial nitrogen and fall in pH value of the medium. Amino nitrogen also increased, while the difference from control was not significant.
- 6) The pH optimum in apparent ammonia production was around pH 7.0. And below pH 6.0 or above 8.0, ammonia-N concentration decreased.
- 7) From these results it is suggested that the addition of carbohydrate stimulates the uptake of ammonia by micro-organisms and their growth. Meanwhile, the carbohydrate is fermented to VFAs and the pH goes down. The low pH depresses the ammonia production. In these ways, the apparent ammonia-N concentration might be reduced.
- 8) Addition of penicillin had little effect on ammonia-N concentration. Tetracycline and streptomycin were effective. In the presence of kanamycin there was an increase in ammonia-N concentration.

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反芻動物の蛋白質消化に関する研究

V. アンモニア濃度に及ぼす炭水化物と抗生物質の影響

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1. 第一胃にフィスチュラを装着した綿羊から採取したルーメンジュースについて *in vitro* でアンモニア濃度に及ぼす炭水化物と抗生物質の影響を研究した。

2. キシロース, グルコース, フラクトース, ガラクトース, 蔗糖, 麦芽糖, 乳糖, セロビオース, ラフィノースの添加でアンモニア濃度は著しく減少を示した。

3. マンノース, デキストリン, デンプン, イヌリン, キシランの添加でもアンモニア濃度の減少が観察されたが, 前記の炭水化物を添加した時より効果は小さかった。

4. セルロースの添加には2時間以内にはアンモニア濃度は殆ど変化を示さなかった。

5. フラクトースの添加時には有意なアンモニア濃度の減少が有意な微生物態窒素の増加と培養液の pH の低下と同時に観察された。アミノ態窒素の増加もみられる

が有意差はなかった。

6. 見かけのアンモニア生産の至適 pH は 7.0 周辺にあり, pH 6.0 以下, 又は pH 8.0 以上ではアンモニア濃度は低かった。

7. 以上の結果から第一胃汁によるカゼインの発酵の際炭水化物を添加すると微生物によるアンモニアの取込みを刺激し, 更にその成長を促進する。他方, 添加された炭水化物は揮発性脂肪酸に発酵されるので培養液の pH は低下する。pH の低下はアンモニアの生産を抑制する。こうして, 炭水化物の添加の際にアンモニア濃度の低下が見られるものと推論される。

8. ペニシリンの添加はアンモニア濃度に殆ど影響しなかった。テトラサイクリン及びストレプトマイシンでは効果がみられた。カナマイシンの添加ではアンモニア濃度の増加が観察された。