SOME FACTORS INFLUENCING TRI-L-ALANINE DISAPPEARANCE AND RUMEN BACTERIAL GROWTH YIELD *IN VITRO*

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Summary

A series of *in vitro* incubation studies with washed rumen bacteria were conducted to determine the influence of incubation time and concentrations of peptides, alanine, ammonia nitrogen and carbohydrate on the rate of peptide disappearance and on bacterial growth.

Disappearance rate of tri-l alanine (ala3) under various conditions was between 30.6 and 58.2 mg hr^{-1} per gram bacterial dry matter. Ala3 was removed from the incubation medium in an almost linear fashion as incubation time and ala3 concentration was increased. Washed rumen bacteria utilized ala3 faster than di l-alanine (ala2) at all concentrations. Adding 9mM carbohydrate significantly increased ala3 disappearance, but level of ammonia nitrogen had no influence on ala3 disappearance. The presence of alanine in the medium significantly lowered ala3 utilization by rumen bacteria.

Bacterial dry matter and nitrogen growth yield were not influenced by alanine and peptides when incubation medium already contained a sufficient level of ammonia nitrogen. Increased ammonia nitrogen in the presence of ala3 did not stimulate bacterial growth. Carbohydrate significantly increased bacterial dry matter and nitrogen growth as expected.

Results indicate that the rate of peptide utilization by rumen bacteria may be altered by type and concentration of peptides, and energy supply, and this may be mediated through changes in numbers and type of bacteria.

(**Key Words**: Peptide Disappearance, Bacterial Growth, Washed Rumen Bacteria)

Introduction

It was assumed that peptides arising from protein breakdown in the rumen have no, or limited, nutritional significance to ruminants until recent studies (Chen et al., 1987a; Broderick and Wallace, 1988) showed a transient accumulation of peptide in the rumen fluid immediately after feeding rapidly degradable proteins. This view was supported by the fact that amino acid concentrations in ruminal fluid were extremely low (Wright and Hungate, 1967) and therefore, it is believed that intermediates of protein digestion, including peptides, are unlikely to make a substantial contribution to microbial nitrogen requirements.

Substantial quantities of peptides, ranging from 54 to 225 mg nitrogen L^{-1}, were detected by Chen et al. (1987a) in ruminal fluid of cows fed various levels and type of proteins. However, values determined in the ruminal fluid of sheep (Broderick and Wallace, 1988) and cows (Ha and Kennelly, Unpublished data) were substantially lower. Although analytical methods contribute to differences in the estimation of peptides (Wallace and Mckain, 1989), factors involved in protein degradation and utilization of degradation products may also influence the results.

Ruminal microbes have been shown to preferentially utilize peptides over free amino acids (Wright, 1967; Chen et al., 1987b; Argyle and Baldwin, 1989), but peptide utilization is not complete. *Bacteroides ruminocola*, for instance, used only 40% of available peptides (Cotta and Russell, 1982) and mixed ruminal bacteria only used 70% nitrogen from trypticase, even when the incubation period was as long as 96 hr (Russell et al., 1989). Effects of structural characteristics of peptides on their utilization have also been reported for mixed rumen microbes (Chen et al., 1987b; Broderick et al., 1988).

The purpose of this study was to investigate factors influencing the rate of peptide disappear-

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ance by washed rumen bacteria *in vitro*. Incubation time, type and concentration of peptides, and level of alanine, ammonia nitrogen, and carbohydrate were factors studied.

Materials and Methods

Inoculum preparation

Rumen contents were obtained at 2 hr after morning feeding from mature Holstein cows fitted with a permanent rumen cannula. Animals were maintained on a diet consisting of 50% rye silage and 50% grain mixture. Rumen contents were strained through 4 layers of cheesecloth and purged with O_2-free carbon dioxide. Large feed particles and protozoa were removed by slow centrifugation (250 × g, 10 min, 4℃). Bacteria cells were then harvested by high centrifugation (15,000 × g, 15 min, 4℃) and washed in the incubation medium described later. Washed bacteria pellets were added to the incubation medium until optical density (A 600 nm) reached 1.5. Oxygen-free CO_2 gas was applied to each tube before and after centrifugation.

Incubation medium

A defined medium was prepared anaerobically with macro-and micro-minerals, vitamins, VFA and carbohydrates. One liter of medium contained 240 mg $KH_2PO_4 \cdot 3H_2O$, 240 mg K_2HPO_4, 480 mg $(NH_4)_2 \cdot SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot H_2O$ and 10 ml of Pfenning's micro mineral solution (Schaefer et al., 1980). Vitamin and volatile fatty acid composition was as described by Russell et al. (1983). A carbohydrate solution containing equimolar glucose, maltose and cellobiose was prepared to have a concentration of 9mM in the final incubation medium. A solution of Na_2S (0.5 g $Na_2S \cdot 9H_2O$ per litter final incubation meidum) was kept at 4℃ until required. The medium was prepared without $(NH_4)_2SO_4$ and the ammonia level was adjusted by adding concentrated $(NH_4)_2SO_4$ solution directly to final incubation tubes.

Incubation procedure

Twenty ml of inoculum were anaerobically transfered to 50 ml culture tubes, to which peptide, carbohydrate and Na_2S solution were added. The tubes containing the final medium mixture were capped and incubated at a constant temperature (39℃). The duration of incubation was 2 hr except in the time course study where incubation lasted 0, 1, 2, 4 and 6 hr. The concentration of di-*l*-alanine (ala2) and *tri*-*l*-alanine (ala3) in final medium of the concentration study was 0, 1, 2, and 4 mM and that in all other studies was 1 mM. The incubation was terminated by putting the tubes in ice-cold water and subsequent centrifugation (27,000 × g, 15 min, 4℃). Incubations were repeated at least 4 times in all experiments. To quantitate initial concentrations of peptides, incubation mixtures without inoculm were prepared and held on ice. Cold inoculum was then added to the tubes and they were mixed and centrifuged immediately.

Analysis

Bacteria dry matter (DM) was estimated by centrifuging the medium (27,000 × g, 15 min, 4℃) and drying the pellet at 60℃ to constant weight (24 hr). The nitrogen content of the bacteria pellet was determined by digesting with sulfuric acid followed by ammonia nitrogen determination (Chaney and Marbach, 1962) with Tris (hydroxymethyl) aminomethane and $(NH_4)_2SO_4$ as standards. The same method was used for the estimation of ammonia nitrogen content in the supernatant portion of the incubation medium. Peptide concentrations were assayed by a fluorimetric method similar to that proposed by Perrett et al. (1975). This method is based on the preferential reaction of fluorescamine with peptides over free amino acids at a certain pH. Briefly, supernatant (75μl) from incubation medium was added to 2.25 ml sodium citrate buffer (0.2 mM, pH 6.2) and then fluorescamine solution (0.28 g/L-acetone) was added as the solution was being vortexed. The fluorescence was measured using a Nova spectrofluorimeter with excitation at 390 nm and emission at 485 nm. Net peptide disappearance was determined in each study relative to 0 hr values.

Statistical analysis

Data obtained were subjected to analysis of variance. Linear and quadratic contrast in response to treatments were tested at probability levels of 0.05 and 0.01 as described by Steel and Torrie (1980) using the Statistical Analysis System (SAS, 1982).

Results

Time-course study

Bacterial dry matter yield was linearly increased ($p < 0.01$) with incubation time but the rate of increase was reduced after 4 hr (table 1). A similar trend in bacteria nitrogen yield was observed, although the magnitude of increase with incubation time was less than that of bacteria dry matter (45.1% vs 63.5% increase after 6 hr incubation) presumably as a result of decreased nitrogen concentration in rumen bacteria associated with prolonged incubation. The response in bacterial nitrogen concentration to incubation time was linear and quadratic ($p < 0.01$)

TABLE 1. BACTERIAL DRY MATTER AND NITROGEN, TRI-L-ALANINE UPTAKE AND NH_3-N CONCENTRATION AS INFLUENCED BY INCUBATION TIME *IN VITRO*

Items	Incubation time (hr)					SEM[1]	Contrast[2]	
	0	1	2	4	6		L	Q
Bacteria dry matter (mg L^{-1})	1475	1768	1989	2342	2412	84.2	0.01	0.26
Bacteria nitrogen (%)	10.4	9.1	8.9	9.4	9.3	0.1	0.01	0.01
yield (mg L^{-1})	153	163	176	218	222	4.8	0.01	0.15
Ala3 uptake (mg g bact DM^{-1})[3]	0.0	33.9	62.6	108.9	123.2	6.1	0.01	0.19
NH_3-N concentration (mg L^{-1})	84.6	80.7	72.0	52.7	42.3	1.3	0.01	0.20

[1] Standard error of the means.
[2] Probability of orthogonal contrasts where L=linear, Q=quadratic effect.
[3] Expressed in mg tri-l-alanine per gram of 0 hr bacterial dry matter.

Disappearance of ala3 from the medium, expressed as mg ala3 per gram bacteria dry matter at zero hr. increased linearly with increasing incubation time ($p < 0.01$). As observed for bacteria dry matter and nitrogen, the extent of peptide disappearance with incubation time declined after 4 hr. Ammonia nitrogen concentration decreased to almost half of zero hr value after 6 hr incubation and the response to incubation time was linear ($p < 0.01$).

Peptide form and concentration

Bacteria dry matter and nitrogen yield, peptide disappearance and ammonia nitrogen as influenced by the concentration of ala2 and ala3 in the

TABLE 2. BACTERIAL DRY MATTER AND NITROGEN, PEPTIDE UPTAKE AND NH_3-N CONCENTRATION AS NFLUENCED BY PEPTIDE CONCENTRATION AFTER 2 HR INCUBATION *IN VITRO*

Items	Peptide	Peptide concentration (mM)				SEM[1]	Contrast[2]	
		0	1	2	4		L	Q
Bacteria dry matter	Ala2	2019	1965	1922	1931	90.5		
(mg L^{-1})	Ala3	2055	2011	2008	1986	98.0	0.74	0.81
Bacteria nitrogen	Ala2	176	175	174	176	7.8		
(mg L^{-1})	Ala3	185	187	179	184	6.1	0.88	0.85
Peptide uptake	Ala2	0.0	30.6	51.6	77.3	7.4		
(mg hr^{-1} g bact. DM^{-1})[3]	Ala3	0.0	40.9	61.8	91.5	8.6	0.01	0.01
NH_3-N concentration	Ala2	60.7	54.7	64.5	69.3	2.5		
(mg L^{-1})	Ala3	54.6	63.0	68.5	69.6	2.7	0.02	0.54

[1] Standard error of the means.
[2] Probability of orthogonal contrasts where L=linear, Q=quadratic effect.
[3] The difference between Ala2 and Ala3 are significant ($p < 0.01$).
Peptide uptake was calculated per gram of 0 hr bacterial dry matter (1471.4±18.1 mg L^{-1})

incubation medium are summarized in table 2.

Neither peptide concentration or type of peptide influenced ($p > 0.05$) bacteria dry matter and nitrogen yield after 2 hr incubation with washed rumen bacteria. Disappearance of ala2 and ala3 from the incubation medium per hr per gram bacteria increased to 77.3 and 91.5 mg, respectively when petide concentration in the medium increased from 0 to 4 mM, and the response to peptide concentration was linear and quadratic ($p < 0.01$). The disappearance rate of ala3 was higher ($p < 0.01$) than that of ala2. Ammonia nitrogen concentration increased linearly ($p < 0.05$) as increasing amount of ala2 and ala3 was added, but type of peptide did not influence ($p > 0.05$) ammonia concentration

Alanine concentrations

The influence of alanine on ala3 disappearance rate was studied with various concentration of l-alanine (table 3). The concentration of alanine did not affect ($p > 0.05$) bacteria dry matter and nitrogen yield after 2 hr incubation. Adding increasing amounts of alanine (0, 1, 5 and 10 mmol L^{-}) reduced ala3 disappearance ($p < 0.01$), indicating an inhibitory or competitive effect from alanine. The reduction in peptide disappearance at the highest alanine concentration (10 mM) was about 22.4%. There was also a tendency of decreased ammonia nitrogen concentration with increased alanine in the medium.

Ammonia and carbohydrate concentration

Carbohydrate (9 mM) increased bacteria dry matter yield and decreased the concentration of nitrogen in bacteria dry matter ($p < 0.01$) regardless of the level of ammonia nitrogen in the incubation medium (table 4). Bacteria nitrogen yield was also increased by additions of carbohydrate ($p < 0.01$). The average yield increase in bacteria dry matter and nitrogen from added carbohydrate was 66.3% and 27.7%, resectively.

TABLE 3. EFFECT OF ALANINE CONCENTRATION ON BACTERIAL DRY MATTER AND NITROGEN YIELD, TRI L ALANINE UPTAKE AND NH_3 N CONCENTRATION AFTER 2 HR INCUBATION WITH WASHED RUMEN BACTERIA *IN VITRO*

Items	Alanine concentration (mM)				SEM[1]	Contrast[2]	
	0	1	2	4		L	Q
Bacteria dry matter (mg L^{-})	1893	1885	1893	1895	143.2	0.99	0.99
Bacteria nitrogen (mg L^{-})	193	182	182	183	12.6	0.86	0.84
Ala3 uptake (mg hr g bact. DM)[3]	40.6	38.5	37.4	31.5	0.7	0.01	0.31
NH_3-N concentration (mg L^{-})	72.5	64.0	66.1	62.9	2.9	0.41	0.80

[1] Standard error of the means.
[2] *Probability of orthogonal contrasts* where L=linear, effect.
[3] Ala3 uptake was colculated per gram of 0 hr bacterial dry matter (1403.7 ± 24.4 mg L^{-})

The average ala3 disappearance with and without added carbohydrate was 47.1 and 56.6 mg per hr per gram bacteria dry matter, indicating carbohydrate increased ala3 utilization ($p < 0.01$).

The concentration of ammonia nitrogen in the incubation medium did not affect bacteria dry matter and nitrogen yield and the rate of ala3 disappearance. A trend to increased dry matter yield, and possibly in nitrogen yield, was observed when ammonia nitrogen was added to the medium in the presence of carbohydrate source. When the carbohydrate source was excluded from the medium this trend was not observed.

Discussion

Early studies indicated that mixed rumen bacteria utilized peptides more efficiently for growth than amino acids (Wright, 1967; Chen et al., 1987b). This view was also supported by studies in which an enzymatic hydrolyzate of casein was used faster than an acid hydrolyzate which should contein mostly free amino acids (Pittman and Bryant, 1964; Chen et al., 1987b). However, other data with pure culture or mixed rumen bacteria showed that the utilization of peptides is not complete, and hence peptide from proteolysis of dietary protein can accumulate in

ruminal fluid (Russell, 1983).

Our data (table 1) show that washed rumen bacteria used 78% of 1 mmole L ala3 after 6 hr incubation. Although the rate of ala3 disappearance was almost linear during the first 4 hr of incubation, the disappearance rate declined considerably by 6 hr, thus incomplete utilization of ala3 within the normal range of liquid turn-over in the ruminal fluid pool is possible. However, present utilization rates are higher than that reported by Russell et al. (1989) who observed only 70% trypticase nitrogen utilization by mixed rumen bacteria after 96 hr incubation. Broderick et al. (1988) reported uptake of 1.1 nmole ala3 per min per mg of mixed rumen microbial dry matter, which is equivalent to 15.2 mg hr^{-} g^{-} microbial dry matter. The disappearance rate of ala3 in our studies ranged from 30.6 to 58.2 mg. The difference between our uptake values and those of Broderick et al. (1988) is likely due to the concentration of ala3 initially added to the incubation medium. In the latter study strained rumen fluid was used as a microbial source and ala3 uptake was expressed per unit of pelleted strained rumen content. As this material is likely to have contained non-bacterial dry matter, underestimation of ala3 uptake per unit bacterial dry matter was possible. *Saccharomyces cerevisiae* have been reported to use ala3 and ala2 in the range of 13.8 and 69.0 mg hr^{-} $gram^{-}$ dry matter (Becker and Naider, 1980).

Proteolysis of dietary protein in the rumen would result in peptides which would vary in both size and amino acid composition. It is difficult to quantify and qualify all the peptide fractions and predict the extent of utilization. Hydrophobicity has been proposed as a factor influencing the transport of peptides to microbial cells (Chen et al., 1987b), and molecular weight of peptides may regulate the transport and hence utilization because many gram negative bacteria such as *E. coli* have pore size barrier (Alves et al., 1985). Difference between disappearance rates of ala2 and ala3 were observed at all concentrations in the present study (table 2). However, no clear explanation can be given for these differences. Perhaps peptides are transported in a molar basis, and therefore, ala3 would be transported at a greater rate than ala2. This could explain the more efficient utilization of peptides than amino acid observed with mixed rumen bacteria in previous reports (Wright, 1967; Chen et al., 1987b). More rapid absortion of peptides over amino acids from the small intestine of animals has been explained in a similar manner

TABLE 4. EFFECT OF LEVEL OF CARBOHYDRATE AND NH_3-N ON BACTERIAL GROWTH AND TRI-L ALANINE UPTAKE BY WASHED RUMEN BACTERIA

Items	Carbohydrate[1] (mM)	NH_3-N concentration (mg L^{-})				SEM[2]	Contrast[3]	
		0	70	140	280		L	Q
Bacteria dry matter	0	1367	1354	1369	1371	11.4		
(mg L^{-})	9	2165	2296	2306	2316	24.3	0.07	0.16
Bacteria nitrogen	0	12.1	11.9	12.6	12.6	0.3		
(%)	9	9.4	9.4	9.6	9.3	0.1	0.55	0.74
Bacteria nitrogen	0	165	161	172	173	3.7		
(mg L^{-})	9	203	216	222	216	3.1	0.20	0.11
Ala3 uptake	0	49.8	45.8	47.0	45.9	1.4		
(mg hr^{-} g bact. DM^{-})[4]	9	55.8	58.2	57.9	54.5	1.5	0.43	0.73
NH_3 N concentration	0	0.0	67.7	132.2	235.2	20.5		
(mg L^{-})	9	0.0	50.0	116.0	266.9	23.1	0.01	0.70

[1] 9 mM carbohydrate mixture contained equimolar (3m mole L^{-}) maltose, cellobiose and glucose. The effect of carbohydrate is significatn ($p < 0.01$) except in NH_3-N.

[2] Standard error of the means.

[3] Probability of orthogonal contrasts where L=linear effect, Q=Quadratic effect.

[4] Ala3 uptake was calculated per gram of 0 hr bacterial dry matter (1461 ± 14.2 mg L^{-}).

(Lis et al., 1971). Broderick et al. (1988) compared the uptake of ala2, ala3, ala4 and ala5, but the uptake rate of ala4 and ala5 was lower than ala3, and therefore factors other than molecular weight influence absorption rate.

Early reports indicated that various bacteria possessed separate transport system for peptides and amino acids (Leach and Snell, 1960; Kessel and Lubin, 1963; Brock and Wooley, 1964). The mechanisms for peptide transport in ruminal bacteria has not been studied in detail, and no concrete evidence of competition in transport between peptide and amino acid is available, although reduced peptide uptake has been reported when constituent amino acids were added to the medium at a very high level (8 times peptide) (Wright, 1967). Our data (table 3) also indicate a reduced ala3 disappearance when ala3 was co-incubated with alanine. The effect was more evident at the highest alanine concentration (approximately 22.4% reduction at 10 mM alanine). Whether the same relationship will hold with different peptides and amino acids remains to be seen.

Since ammonia is the final product of protein degradation and fermentation it was anticipated that ammonia would influence the rate of peptide utilization by washed rumen bacteria. However, it is apparent from the results (table 4) that ammonia did not influence peptide disappearance under the experimental conditions of our study.

Carbohydrate source added to the incubation medium may have increased bacterial cell numbers, which in turn increased peptide disappearance from the medium (table 4). The population shift in favor of higher peptide uptake by added carbohydrate may have been a contributing factor (Russell et al., 1981). Starvation of *Bacteroids ruminicola* almost eliminated peptide uptake (Pittman et al., 1967), indicating the importance of energy supply for peptide utilization.

Growth of rumen microbes in almost a linear function of carbohydrate fermentation *in vitro* (Russell et al., 1981; 1983; Argyle and Baldwin, 1989). It appears that by 6 hr after the initiation of incubation in the present study (table 1) energy source was depleted to such a low level that bacterial growth was slowed down. Our results indicate that bacterial growth was not stimulated by additional peptides (table 2) or amino acid (table 3). The medium used contained 70 mg l. ammonia nitrogen, which is regarded as sufficient for bacterial growth. These results support those of Maeng et al. (1976) and Argyle and Baldwin (1989), who indicated that a single amino acid or peptide would not improve bacterial growth. As expected the addition of carbohydrate source to incubation medium increased bacterial dry matter and nitrogen yield.

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