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Research Article

Influence of Ammonia Concentration on ^{15}N-ammonia Incorporation and *de novo* Amino Acid Synthesis by the Non-cellulolytic Ruminal Bacteria, *Prevotella bryantii* $B_1$4, *Selenomonas ruminantium* HD4 and *Streptococcus bovis* ES1

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Abstract: The influence of ammonia concentration on ^{15}N-ammonia incorporation and *de novo* synthesis of amino acids by three predominant non-cellulolytic species of ruminal bacteria, *Prevotella bryantii* $B_1$4, *Selenomonas ruminantium* HD4 and *Streptococcus bovis* ES1, was investigated. The medium contained pancreatic casein hydrolysate (comprising mainly peptides with some amino acids) at a concentration of 1 g/litre and additions of graded concentrations of $^{15}NH_4Cl$. When the initial concentration of ammonia increased from 0.045 to 0.436 g N/L in the growth medium, the proportion of cellular nitrogen and amino acid nitrogen derived from ammonia by *P. bryantii* and *S. ruminantium* increased (ranging from 0.33 to 0.84 for cellular-nitrogen and from 0.23 to 0.67 for amino acid-nitrogen) ($P<0.001$), but *S. bovis* incorporated a fixed proportion of ammonia and peptides in all media except for the lowest ammonia containing medium ($P>0.05$). Glutamate and aspartate were the most highly labelled amino acids with ^{15}N, whereas ^{15}N enrichment in proline was lower than that in other amino acids in all species, followed by phenylalanine in *P. bryantii*, lysine in *S. ruminantium* and phenylalanine, valine and lysine in *S. bovis*, indicating preferential incorporation of these amino acids from pancreatic casein hydrolysate. The results of the present study, thus, suggest that the concentration of ammonia has an important effect on *de novo* synthesis of bacterial cellular-nitrogen and amino acids in the non-cellulolytic ruminal bacteria and this effect depends on bacterial species.

Key Words: Ammonia, Protein Synthesis, Ruminal Bacteria, Amino Acids

Amonyak Konsantrasyonunun *Prevotella bryantii* $B_1$4, *Selenomonas ruminantium* HD4 ve *Streptococcus bovis* ES1 Gibi Rumen Bakterilerinde Amino Asit Sentezinde Amonyak Nitrojeninden Yararlanım Oranı Üzerine Etkisi

Özet: Bu çalışmada, ortamdaki amonyak konsantrasyonunun *de novo* amino asit sentezinde amonyak nitrojeninden (amonyak-^{15}N) yararlanım oranı üzerindeki etkisi *Prevotella bryantii* $B_1$4, *Streptococcus bovis* ES1 ve *Selenomonas ruminantium* HD4 gibi rumen populasyonunda baskın nitelikte yer alan üç sellülolitik olmayan bakteri türünde incelenmiştir. Çalışmada besi ortamı olarak pankreas enzimleri ile hidrolize edilmiş kazein kullanılmış, ve ortama artan düzeylerde $^{15}NH_4Cl$ ilave edilmiştir. Büyüme ortamında başlangıç amonyak konsantrasyonunun 0.045 g N/L'den 0.436 g N/L'ye yükseltilmesine paralel olarak, *P. bryantii* ve *S. ruminantium* bakterilerinde amonyaktan kaynağını alan hücresel nitrojen ve amino asit nitrojeni oranları tüm besi ortamlarında yükselmiş (hücresel nitrojende 0.33 ten 0.84'e; amino asit nitrojeninde 0.23 ten 0.67'ye), ancak en düşük amonyak konsantrasyonuna sahip olan besi ortamı hariç, *S. bovis* grubunda amonyak ve peptitlerden yararlanım oranı oldukça sabit bir değer göstermiştir ($P>0.05$). ^{15}N içeriği bakımından glutamat ve aspartat diğer amino asitlere oranla daha zengin içeriğe sahip bulunurken, prolin tüm mikroorganizma gruplarında diğer amino asitlere oranla daha düşük ^{15}N içeriği gösterdiği saptanmıştır. Prolini, *S. ruminantium*'da lisin, *S. bovis*'te fenilalanin, valin ve lisin izlemiştir. ^{15}N içeriği açısından gözlenen eğilimler, söz konusu amino asitlerin sentezi açısından kazeinin tercih edildiğini ortaya koymuştur. Çalışmadan elde edilen bulgular, ortamdaki amonyak konsantrasyonunun sellülolitik olmayan rumen bakterilerinde amino asitlerin *de novo* sentezi üzerinde önemli etkilere sahip olduğu ve söz konusu etkinin bakteri türüne bağımlılık gösterdiği görüşünü destekler nitelikte bulunmuştur.

Anahtar Sözcükler: Amonyak, Protein Sentezi, Ruminal Bakteri, Amino Asitler

Introduction

Ammonia is an important source of nitrogen for protein synthesis by ruminal micro-organisms, and fluctuates markedly, depending on the type of diet of the rumen (1). It is also known to affect the activities of the enzymes responsible for assimilation of ammonia (2). In the mixed rumen microbial population, the extent to which microbial protein is synthesised *de novo* from ammonia varies enormously, ranging from 18 to 100% (3-8). The relative amounts of energy and nitrogen available for microbial growth are partly responsible for this variation (9,10), but other factors are thought to be involved (11). In pure culture studies with ruminal bacteria, for example, the concentrations of peptides and amino acids have been reported to be crucial in determining the proportion of bacterial protein formed *de novo* (11,12).

Although ammonia concentration plays a crucial role in the regulation of ammonia assimilation enzymes (1), there has been no report with regard to the effect of ammonia concentration on the proportion of cellular nitrogen which was derived *de novo* from ammonia. The present study was therefore undertaken to investigate the influence of ammonia concentration on the *de novo* synthesis of bacterial nitrogen and amino acid nitrogen by three predominant species of non-cellulolytic ruminal bacteria.

Materials and Methods

Bacterial strains: The bacteria used in this study were *Prevotella bryantii* $B_1$4 (13) (a gift from J. B. Russell), *Selenomonas ruminantium* HD4 (ATCC 35018), and *Streptococcus bovis* ES1 (a strain prototrophic for amino acids, isolated from a sheep at the Rowett Research Institute). The cultures were maintained on medium M2 (14).

Growth conditions: The basal medium for non-cellulolytic bacteria, whose composition was described before by Atasoglu *et al.* (11), was used. The medium contained pancreatic casein hydrolysate (Trypticase®; BBL, Becton Dickinson, Cockeysville, MD, USA) at an initial concentration of 1 g/L and graded concentrations of (0.014 (A), 0.070 (B), 0.210 (C) and 0.350 (D) g N/L) of NH4Cl, where 40% of the NH_4Cl was replaced by 99% labelled $^{15}NH_4Cl$ (Sigma, USA). Inoculation, growth and harvesting conditions were the same as those described by Atasoglu *et al.* (11).

Isotopic and chemical analyses: Determinations of ^{15}N enrichment in total cellular nitrogen (15), total cellular nitrogen (16), ^{15}N enrichment in individual amino acids (17), ammonia concentration (18) and ^{15}N enrichment in ammonia (19) were carried out as described by Atasoglu *et al.* (11).

Calculation of ^{15}N incorporation: The proportion of cellular nitrogen and amino acid nitrogen derived from ammonia was calculated from the equation used by Atasoglu *et al.* (11).

Statistical analysis: The results are all means derived from the analysis of triplicate cultures. The data were compared by analysis of variance, with different cultures used as a blocking factor. All analysis was carried out by using the GENSTAT 5 Statistical Program (20).

Results

The ^{15}N enrichment in ammonia in the spent medium decreased for all species (Tables 1-3). The decrease in medium A was apparent in the cultures of *P. bryantii* and *S. bovis*, indicating that substantial breakdown of peptides and amino acids occurred with these species.

When the basal medium contained graded concentrations of ammonia, the proportion of cellular nitrogen and amino acid nitrogen derived from ammonia by *P. bryantii* and *S. ruminantium* increased as the concentration of ammonia increased in the medium (Tables 1-3), but *S. bovis* incorporated a fixed proportion of ammonia and peptides in all media, except for medium A (Table 3). With all species, the proportion of total cellular nitrogen formed from ammonia was greater than the average proportion of amino acids derived from ammonia; this difference was most pronounced for *S. bovis* (Table 3).

The patterns of *de novo* synthesis among individual amino acids were similar for all media and for all species in that glutamate and aspartate were always the most highly enriched, followed by serine and lysine for *P. bryantii*, serine and threonine for *S. ruminantium* and serine and alanine for *S. bovis*. Proline was the least enriched amino acid for all species in all media, and its biosynthesis was almost shut off in *S. bovis* (Table 3). It

Table 1. Influence of ammonia concentration on incorporation of $^{15}NH_3$ by *P. bryantii* B_14[a]

	Ammonia added (g N/L)				
Parameter/Medium	A 0.014	B 0.070	C 0.210	D 0.350	SED
NH_3 concentration (g of N/L)					
Initial	0.045	0.104	0.254	0.436	0.003***
Final	0.023	0.022	0.135	0.292	0.004***
Enrichment in NH_3 (atom%)					
Initial	29.2	35.1	36.9	37.0	0.6***
Final	15.7	24.5	34.2	36.6	0.9***
Microbial nitrogen formed (g/L)	0.021	0.088	0.091	0.095	0.002***
Enrichment in microbial nitrogen (atom%)	8.6	21.5	29.6	30.9	0.6***
Proportion of microbial nitrogen derived from ammonia	0.38	0.72	0.83	0.84	0.02***
Proportion of microbial amino acids derived from ammonia					
Ala	0.35	0.65	0.63	0.64	0.01***
Gly	0.35	0.64	0.63	0.63	0.01***
Val	0.27	0.56	0.56	0.56	0.01***
Leu	0.40	0.62	0.59	0.59	0.01***
Ile	0.40	0.64	0.62	0.62	0.01***
Pro	0.03	0.09	0.10	0.09	0.01***
Ser	0.49	0.78	0.74	0.74	0.01***
Thr	0.28	0.63	0.58	0.59	0.02***
Phe	0.04	0.09	0.11	0.14	0.01***
Asp	0.50	0.79	0.76	0.76	0.01***
Glu	0.59	0.88	0.82	0.81	0.01***
Lys	0.52	0.72	0.77	0.81	0.02***
Tyr	0.38	0.68	0.66	0.65	0.01***
Mean proportion of amino acid nitrogen derived from ammonia	0.35	0.60	0.58	0.59	0.01***

Results are the means of triplicate cultures; ***P<0.001

was followed by phenylalanine for *P. bryantii* (Table 1), lysine for *S. ruminantium* (Table 2) and phenylalanine, lysine and isoleucine for *S. bovis* (Table 3).

Discussion

The effect of peptide and amino acid concentrations on the *de novo* synthesis of cellular nitrogen and amino acid nitrogen by non-cellulolytic ruminal bacteria was investigated by Atasoglu *et al.* (11), and it was found that the proportion of cellular nitrogen and amino acid nitrogen derived from ammonia decreased as the concentration of peptides and amino acids increased in the growth medium in a concentration-dependent manner. They suggested that the concentration of the intermediate products of protein breakdown (peptides and amino acids) is crucial in determining how much cellular nitrogen and amino acid nitrogen is formed from ammonia and how much is derived from peptides and amino acids. Likewise, this study was undertaken to

Table 2. Influence of ammonia concentration on incorporation of $^{15}NH_3$ by *S. ruminantium* HD4[a]

	Ammonia added (g N/L)				
Parameter/Medium	A 0.014	B 0.070	C 0.210	D 0.350	SED
NH_3 concentration (g of N/L)					
Initial	0.045	0.104	0.254	0.436	0.003***
Final	0.018	0.028	0.160	0.300	0.010***
Initial	29.2	35.1	36.9	37.0	0.6***
Final	28.7	33.4	34.3	35.8	0.9***
Microbial nitrogen formed (g/L)	0.039	0.071	0.069	0.063	0.02***
Enrichment in microbial nitrogen (atom%)	9.6	22.4	26.1	29.4	0.8***
Proportion of microbial nitrogen derived from ammonia	0.33	0.65	0.73	0.81	0.02***
Proportion of microbial amino acids derived from ammonia					
Ala	0.27	0.58	0.67	0.69	0.02***
Gly	0.20	0.58	0.66	0.67	0.02***
Val	0.20	0.52	0.58	0.60	0.01***
Leu	0.24	0.57	0.64	0.66	0.01***
Ile	0.25	0.58	0.65	0.68	0.01***
Pro	0.11	0.46	0.52	0.55	0.01***
Ser	0.20	0.60	0.68	0.71	0.02***
Thr	0.24	0.61	0.69	0.70	0.01***
Phe	0.21	0.49	0.55	0.60	0.02***
Asp	0.31	0.70	0.81	0.83	0.01***
Glu	0.31	0.70	0.80	0.82	0.01***
Lys	0.13	0.42	0.44	0.51	0.03***
Tyr	0.28	0.54	0.60	0.64	0.01***
Mean proportion of amino acid nitrogen derived from ammonia	0.23	0.56	0.64	0.67	0.01***

Results are the means of triplicate cultures; ***P<0.001

investigate the influence of ammonia concentration on the *de novo* synthesis of cellular nitrogen and amino acid nitrogen by non-cellulolytic ruminal bacteria. The choice of bacteria in the present study was made in order to represent the main members of a typical rumen flora. *P. bryantii* and *S. bovis* are major proteolytic bacteria (21, 22) and, together with *S. ruminantium*, are known to be peptide and amino acid utilisers (23). They are classified as non-structural carbohydrate fermenting bacteria in nutritional models (24).

In the present study, when the initial concentration of ammonia in the growth medium was increased from 0.045 to 0.436 g N/L, the proportion of cellular nitrogen and amino acid nitrogen derived from ammonia by *P. bryantii* and *S. ruminantium* increased (Tables 1-2), but *S. bovis* incorporated nearly a fixed proportion of ammonia and peptides in all media, except for medium A (Table 3). This finding could partly explain why the extent to which ammonia is used by ruminal micro-organisms is highly variable in previously published studies, because

Table 3. Influence of ammonia concentration on incorporation of $^{15}NH_3$ by *S. bovis* ESI[a]

	Ammonia added (g N/L)				
Parameter/Medium	A 0.014	B 0.070	C 0.210	D 0.350	SED
NH_3 concentration (g of N/L)					
Initial	0.045	0.104	0.254	0.436	0.003***
Final	0.018	0.061	0.207	0.321	0.018***
Enrichment in NH_3 (atom%)					
Initial	29.2	35.1	36.9	37.0	0.6***
Final	21.7	32.4	36.3	36.8	0.6***
Microbial nitrogen formed (g/L)	0.060	0.069	0.072	0.076	0.004*
Enrichment in microbial nitrogen (atom%)	7.9	19.7	20.7	20.1	1.0***
Proportion of microbial nitrogen derived from ammonia	0.31	0.58	0.57	0.55	0.03***
Proportion of microbial amino acids derived from ammonia					
Ala	0.17	0.44	0.45	0.45	0.01***
Gly	0.16	0.36	0.36	0.36	0.01***
Val	0.07	0.13	0.14	0.14	0.00***
Leu	0.17	0.25	0.25	0.25	0.01***
Ile	0.13	0.19	0.19	0.19	0.01***
Pro	0.00	0.02	0.01	0.01	0.00*
Ser	0.09	0.24	0.25	0.25	0.01***
Thr	0.21	0.44	0.45	0.45	0.01***
Phe	0.08	0.11	0.09	0.09	0.01***
Asp	0.24	0.47	0.46	0.46	0.01***
Glu	0.34	0.61	0.64	0.64	0.01***
Lys	0.12	0.11	0.13	0.13	0.01
Tyr	0.21	0.34	0.34	0.34	0.02***
Mean proportion of amino acid nitrogen derived from ammonia	0.15	0.29	0.29	0.29	0.01***

Results are the means of triplicate cultures; ***P<0.001; *P<0.05

various studies reported that ruminal bacteria could be present at different proportions. *Prevotella* spp., for example, can comprise 60% of a microbial flora (25), whereas under different circumstances, *S. ruminantium* can account for up to 51% of rumen flora (26). *S. bovis* is known to be an amylolytic bacterium (27) and can be present in high numbers in the rumen of starchy feed-fed animals (1). The reasons for this difference in the utilisation of different nitrogen sources may be the presence of different transport systems and the influence of nitrogen source and concentration on biosynthetic pathways, such as the activities of ammonia assimilation enzymes in rumen bacteria (28-30). Transport systems of ammonia, peptides and amino acids should be characterised and the effect of nitrogen source on these transport systems should be investigated in order to support this statement.

The results of the present study are not consistent with those of Russell *et al.* (31), who used a medium containing casein at 1 g/L for the growth of mixed ruminal bacteria on soluble carbohydrates, suggesting that bacteria which ferment non-structural carbohydrates such as starch and sugars always form 0.66 of their cellular nitrogen from peptides and 0.34 from ammonia when both peptides and ammonia are available in the medium. They also indicated that this ratio is not influenced by the growth rate of the microbes. It should, however, be noted that pure cultures of non-cellulolytic ruminal bacteria rather than mixed bacteria growing on starch and sugars were used in the present study. In addition, Trypticase®, a pancreatic digest of casein, as the source of peptides and amino acids rather than casein, was provided to the bacterial species. These factors may cause such differences between these two studies.

The present study clearly demonstrated that glutamate and aspartate were the most highly enriched amino acids in all bacterial species, irrespective of ammonia concentration present, consistent with glutamate dehydrogenase being the main route of ammonia assimilation in most ruminal bacteria (1,2,32). Transamination with oxaloacetate would account for subsequent enrichment of aspartate in all species, and with pyruvate for the enrichment of alanine in *S. bovis*. Similarly, the enrichment of serine indicates an active synthesis from 3-phosphoglycerate, involving transamination from glutamate. The *de novo* synthesis of proline displayed a different pattern in that its biosynthesis was switched off completely in *S. bovis* (Table 3), irrespective of ammonia concentration, but increased substantially in *S. ruminantium* (Table 2), with increases in ammonia concentration in the growth medium. This variation clearly illustrates the diversity of ruminal micro-organisms in a biosynthetic pathway, such as the *de novo* synthesis of proline, and their response to nutrient changes in the ruminal microbial ecosystem. This phenomenon indicates the presence of different regulatory mechanisms in *S. ruminantium* and *S. bovis*, which are classified as peptide and amino acid utilisers and non-celluloytic ruminal bacteria (21-24). Why the biosynthesis of proline decreases more than that of other amino acids is not known. Proline can be formed from glutamate or ornithine, and its synthesis is not linked to the synthesis of other amino acids (33). A similar phenomenon occurs in the mixed ruminal population when pre-formed proline is available (8,10). One possible reason is that pyrroline 5-carboxylate synthetase, which catalyses the conversion of glutamate to glutamate γ-semialdehyde, may be subject to repression type control in the presence of pre-formed proline in the medium.

The proportion of ammonia incorporation into amino acids was lower than that into total cellular nitrogen, indicating that non-amino-cellular nitrogen was derived predominantly from ammonia. This presumably reflects the nature of the biosynthesis of different precursors of nucleic acids such as purines (adenine and quanine) and pyrimidines (thymine and cytosine), and peptidoglycan biosynthesis compared with protein.

In conclusion, the concentration of ammonia has an important effect on the *de novo* synthesis of bacterial protein and amino acids from ammonia, and this effect depends on bacterial species. Provision of non-protein nitrogen as a supplement may suppress the incorporation of amino acids from true protein in the feed under circumstances where certain bacterial species are present in high numbers in the rumen of the animals.

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