Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an *in vitro* semi-continuous culture system (RUSITEC)

M. D. Carro[1]* and E. L. Miller[2]

[1]*Departamento de Producción Animal I, Universidad de León, 24071 León, Spain*
[2]*Department of Clinical Veterinary Medicine, University of Cambridge, Nutrition Laboratory, 307 Huntingdon Road, Cambridge CB3 0JQ, UK*

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Incubation trials were carried out with the rumen simulation technique (RUSITEC) to study the effects of four forms of N on the growth of ruminal micro-organisms and the fermentation variables when an all-fibre basal diet was incubated. The basal diet consisted of 10 g neutral-detergent fibre (NDF) from grass hay plus 2 g NDF from sugarbeet pulp. N forms were isolated soyabean protein, soyabean peptides, amino acids blended to profile soyabean protein and NH_3 as NH_4Cl. Half of the daily N supply was infused as NH_4Cl and the other half was infused as each of the four treatments described. Non-NH_3 N (NAN) forms increased NDF ($P = 0{\cdot}006$), acid-detergent fibre ($P = 0{\cdot}003$) and cellulose ($P = 0{\cdot}015$) disappearance after 48 h incubation, CO_2 ($P < 0{\cdot}001$), CH_4 ($P = 0{\cdot}002$) and total volatile fatty acids production ($P < 0{\cdot}001$), as well as the molar percentages of isobutyrate, isovalerate and valerate, which reflected the fermentation of amino acid C skeletons. NAN treatments also increased microbial N flow ($P < 0{\cdot}001$) compared with NH_3, with peptides and protein supporting more ($P = 0{\cdot}036$) than amino acids. The proportion of microbial N derived from NH_3 decreased successively ($P < 0{\cdot}05$) with $NH_3 >$ amino acids $>$ peptides $>$ protein treatments, indicating preferential uptake of peptides without passage through the NH_3 pool. Microbial efficiency (g microbial N/kg organic matter apparent disappearance) was greater ($P = 0{\cdot}002$) for the NAN forms than for the NH_3 treatment, with peptides and protein treatments supporting higher ($P = 0{\cdot}009$) values than amino acid treatment. These results indicate that N forms other than NH_3 are required for optimal fibre digestion and microbial growth.

Nitrogen: Microbial protein synthesis: RUSITEC

In spite of the amount of research conducted to characterize the N metabolism of rumen bacteria, the N requirements for optimum growth of the mixed bacterial population remain unclear. Several studies (Nolan & Leng, 1972; Russell *et al*. 1983; Argyle & Baldwin, 1987) have shown that although the mixed bacterial population uses NH_3 as its main source of N, it may also use pre-formed amino acids or peptides if these are available. Thus, providing non-NH_3 N (NAN; amino acids, peptides, protein) in addition to NH_3 has been shown to stimulate the growth of rumen bacteria *in vivo* (Chikunya *et al*. 1996) and *in vitro* (Cruz Soto *et al*. 1994), and to enhance digestion of fibre *in vivo* (McAllan, 1991) and in continuous culture (Merry *et al*. 1990; Griswold *et al*. 1996). In contrast, no difference either in digestion or in growth of rumen microbes was found in other *in vivo* (Fujimaki *et al*. 1989) and *in vitro* (Kernick, 1991) studies.

These contrasting results could be explained by differences in the composition of the diet. In most of the studies from which a positive response to pre-formed amino acids and peptides was reported, diets contained a substantial proportion of rapidly fermented carbohydrates, suggesting that rumen fermentation would be stimulated only when the rate of provision of energy permitted (Cruz Soto *et al*. 1994). Thus, Cruz Soto *et al*. (1994) found that addition of peptides and amino acids had no effect on the *in vitro* rate of hydrolysis of cellulose when bacteria were grown on cellulose, although the growth rate of bacteria was stimulated when they were grown on a medium containing cellobiose or glucose.

Because cellulose is the most abundant component of plant cell walls, ruminal cellulolytic bacteria play a central role in the nutrition of ruminant animals fed on diets based

Abbreviations: ADF, acid-detergent fibre; LAB, liquid-associated bacteria; NAN, non-ammonia nitrogen; NDF, neutral-detergent fibre; RUSITEC, rumen simulation technique; SAB, solid-associated bacteria; TB, total bacteria; VFA, volatile fatty acids.

*Corresponding author: Dr Dolores Carro, fax +34 987 291311, email DP1MCT@UNILEON.ES

on forages. However, in spite of the conflicting results reported in the literature, the Cornell net carbohydrate and protein system (Russell *et al*. 1992) assumes that the bacteria fermenting structural carbohydrates (cellulolytic) use only NH_3 as the source of N. This point should be clarified, since it is of importance for the accuracy of prediction models of feed evaluation and animal response, as well as for the good use of non-protein N compounds in the practical feeding of ruminants. The objective of the present study was to investigate the effects of protein, peptides, amino acids and NH_3 on microbial growth and fermentation of an all-fibre basal diet in a semi-continuous culture system (RUSITEC; Czerkawski & Breckenridge, 1977). Some of this work has been briefly published elsewhere (Carro & Miller, 1998).

Materials and methods

Apparatus

The study was carried out using the rumen simulation technique RUSITEC (Czerkawski & Breckenridge, 1977). The complete unit consisted of eight vessels with an effective volume of 700 ml each and the general incubation procedure was as described by Czerkawski & Breckenridge (1977). The inoculum was obtained from three ruminally fistulated sheep fed on a good-quality hay (175 g crude protein/kg DM and 476 g neutral-detergent fibre (NDF)/kg DM) diet at maintenance level (Agricultural and Food Research Council, 1993). Rumen content was collected through the rumen fistula immediately before feeding in the morning and transferred to the *in vitro* system within 30 min as previously described (Carro *et al*. 1992). The flow through the vessels was maintained by continuous infusion of McDougall (1948) artificial saliva (pH 8·4) at a rate of 480 ml/d (dilution rate of 0·029/h). S (Na_2SO_3) was added to the artificial saliva at a N : S ratio of 10 : 1 to prevent a limitation of S for the synthesis of S-containing amino acids. In order to stimulate the growth of cellulolytic bacteria, isobutyric, isovaleric and valeric acids were also added to the artificial saliva to achieve concentrations of 0·3, 0·9 and 0·7 mmol/l respectively (Hume, 1970). On day 9, a dose of 1·8 mg ^{15}N (95 % enriched $(^{15}NH_4)_2SO_4$; Sigma Chemical Co., Poole, Dorset, UK) was added into each vessel to label instantaneously the NH_3 N pool. Then a solution of $(^{15}NH_4)_2SO_4$ was added to the artificial saliva at a daily rate of 6·30 μg ^{15}N/mg NH_3-N.

Diets, experimental procedure and sampling

The diet consisted of NDF (Goering & Van Soest, 1970) from grass hay and sugarbeet pulp and a commercial mineral–vitamin mixture. The diet was supplied to the incubation vessels in nylon bags which contained 10 g (DM) hay NDF, 2 g (DM) sugarbeet pulp NDF and 20 mg mineral–vitamin mixture. The amount of N supplied by the supplements in all treatments was fixed at a rate of 280 mg N/d. This amount was considered adequate as judged by the concentration of NH_3 in the liquid phase of the vessels. In addition the fibre sources in the diet supplied 110 mg N/d. Four forms of N were evaluated: (1) protein (commercial isolated soyabean protein; SUPRO®500E, Protein Technologies International, Corby, Northants, UK), (2) peptides (enzyme hydrolysate of soyabean protein; Peptone, Sigma-Aldrich Química, Sociedad Anónima, Spain), (3) amino acids (individual amino acids blended to profile soyabean protein) and (4) NH_3 as NH_4Cl. Solutions of each N treatment were prepared and mixed every day with the artificial saliva. Half (50 %) of the daily N supply was infused as NH_4Cl and the other half (50 %) was infused as one of the four treatments (protein, peptides, amino acids and NH_4Cl). In order to prevent microbial growth in the infusion solutions, the bottles containing the solutions were held in an ice-water bath.

Two identical incubation runs were carried out independently and treatments were assigned randomly within each experimental run so that two vessels received each of the treatments. Therefore each treatment was conducted in quadruplicate. Each incubation run consisted of 14 d and every day one liquid sample from each vessel was taken daily before feeding and the pH was measured immediately.

On days 9, 10 and 11 the following samples were collected. The gas produced was collected daily in hermetic bags to determine the gas production and the concentrations of CO_2 and CH_4. Liquid effluent was collected daily in containers containing a solution of H_2SO_4 (200 ml/l) to maintain pH values below 2. Effluent (1 ml) was added to 1 ml deproteinizing solution (100 g/l metaphosphoric acid and 0·6 g/l crotonic acid) for determination of volatile fatty acids (VFA) and 5 ml effluent was diluted with 5 ml 0·5 M-HCl for NH_3-N determination. One nylon bag from each vessel was collected daily, washed twice with 40 ml artificial saliva and then washed in the cold rinse cycle (20 min) of a washing machine. DM disappearance after 48 h incubation was calculated from the loss in weight after oven-drying at 60° for 48 h, and the residues were analysed for N, NDF, acid-detergent fibre (ADF) and acid-detergent lignin. On day 12 fluid vessel contents were sampled (about 1·5 ml) at 0, 4, 8 and 12 h after feeding for VFA and NH_3-N concentrations.

On days 12 and 13 saturated $HgCl_2$ (5 ml) was added (replacing the H_2SO_4 solution which could cause bacterial lysis) to the overflow containers, which were held in an ice-water bath to impede microbial growth. For each vessel the total effluent for the 2 d sampling period (days 12 and 13) and the contents of nylon bags which had been removed at the end of these 2 d were mixed and homogenized in a blender at low speed for 1 min to reconstitute total digesta. One portion (300 g) was stored at −20° and lyophilized to determine NAN and ^{15}N enrichment. About 200 g of the mix was acidified and preserved at −20° until NH_3-N and ^{15}N enrichment determination, and about 500 g total digesta was used to isolate bacterial pellets (total bacteria, TB).

On the last day of each incubation trial effluent and fluid vessel contents from the two vessels belonging to the same treatment was collected, mixed with the artificial saliva resulting from washing the nylon bags, and the liquid-associated bacteria (LAB) were harvested. The nylon bag residues (four bags from two vessels) were incubated with 240 ml of a saline solution of methylcellulose (1 g methylcellulose + 9 g NaCl in 1 litre distilled water) at 39° for 30 min to elute attached bacteria (Minato & Suto, 1978). Then, 500 ml cold (4°) methylcellulose solution was added, the mixture was homogenized in a blender and stored frozen until isolation of solid-associated bacteria (SAB) pellets.

LAB and SAB pellets were isolated to study possible differences between bacterial fractions in their incorporation of NH_3-N for the synthesis of protein.

Analytical procedures

Samples of total digesta (about 1·5 g) were wetted with distilled water, adjusted with 1 M-NaOH to pH > 10, and dried at 90° for 16 h to remove NH_3-N (Firkins *et al*. 1992). The resulting residue was analysed for NAN and for N insoluble in acid-detergent solution.

DM, ash and N were determined according to the methods of the Association of Official Analytical Chemists (1995). NDF, ADF and acid-detergent lignin analyses were carried out according to Goering & Van Soest (1970). Acid-detergent-insoluble N was determined by Kjeldahl analysis of the ADF residues. NH_3 concentration was determined by a modified colourimetric method (Wheatherburn, 1967). VFA were determined in centrifuged samples (1 ml) by GC as previously described (Carro *et al*. 1992). The volume of gas produced was measured with a drum-type gas meter (model TG1; Ritter Apparatebau GmbH, Bochum, Germany) and the concentrations of CO_2 and CH_4 were analysed by chromatography as described by Carro *et al*. (1992). The volume of gas (litres/d) produced was corrected for ambient conditions (pressure 1 atm; temperature 273°K) and the amount of each gas produced (mmol/d) was calculated.

Isolation of microbial pellets

The portion of total digesta for isolation of TB was strained through four layers of cheesecloth to remove particulate material. The strained fluid was centrifuged at 500 ***g*** for 10 min at 4°. The supernatant fraction was then centrifuged at 18 000 ***g*** for 25 min at 4° to obtain a bacterial pellet. This was washed by resuspension in saline solution (9 g NaCl/l) and the centrifugation was repeated. Finally the bacterial pellet was washed by resuspension in distilled water followed by centrifugation. The isolation of LAB followed the same procedure. Samples for isolation of SAB were thawed, centrifuged at 500 ***g*** for 10 min at 4° and the supernatant fraction was removed and retained. Then, 100 ml cold methylcellulose was added to the residue, mixed well and then recentrifuged (500 ***g*** for 10 min at 4°). This operation was repeated twice, pooling the supernatant fractions each time. The final mixture of pooled supernatant fractions was centrifuged three times at 18 000 ***g*** for 25 min at 4° as described earlier for TB and LAB isolation. Microscopic examination of final bacterial pellets (TB, LAB and SAB) showed that they were essentially free from feed particles. Bacterial pellets were freeze-dried and analysed for N and ^{15}N enrichment.

Preparation of samples for ^{15}N analysis

The portion of total digesta for NH_3-N analysis was centrifuged at 20 000 ***g*** for 20 min and the supernatant fraction was analysed for concentrations of NH_3-N and ^{15}N enrichment. NH_3-N concentrations were determined by steam distillation and to prevent cross-contamination a solution of distilled water–ethanol (2 : 1, v/v) was distilled between samples. The distillate was collected in 5 ml boric acid (30 ml/l), the boric acid acidified with excess H_2SO_4 and evaporated down to about 1·5 ml volume on a hot plate at 55°. The dried residue was redissolved in distilled water to give a solution containing an appropriate amount of N for ^{15}N analyses. Analyses of ^{15}N enrichment of the distilled NH_4^+ were performed by isotope-ratio mass spectrometry as described by Barrie & Workman (1984).

Lyophilized samples of total digesta (about 2 g) were treated to remove NH_3-N (Firkins *et al*. 1992) and the resulting NAN was collected by micro-Kjeldahl method, distilled as described earlier and prepared for ^{15}N-enrichment determination. Samples of each N treatment (NH_3, amino acids, peptides and protein) were analysed for their ^{15}N content and the natural abundance level of ^{15}N for each treatment was calculated.

Calculations and statistical analyses

The proportion of total digesta NAN of microbial origin was estimated by dividing the ^{15}N enrichment (atom % excess) of the NAN portion of digesta from each vessel by the enrichment of bacterial pellets (TB). Daily microbial N production (mg/d) was estimated by multiplying total NAN production by the proportion attributed to the microbes. The proportion of bacterial N derived from NH_3-N was estimated by dividing the ^{15}N enrichment of bacterial pellets by the enrichment of NH_3-N. The amount of microbial N in the nylon bag residues was estimated as the difference between the NAN content of residues and their acid-detergent-insoluble N content, assuming that this represents the only N fraction of feed origin. This value was considered as an estimate of the amount of the SAB. The proportion of SAB in the daily microbial N production was calculated by dividing the amount of SAB (estimated as previously described) by the amount of microbial N (determined using TB as reference pellets) and the amount of LAB was calculated by difference.

Data relative to fermentation variables were analysed as a split-plot design with N form and incubation trial as the main-plot treatments and day of sampling as the subplot treatment. Effect of treatment (N form) on any of the considered variables was tested using the variance between vessels within treatment and incubation trial as the error term. Microbial flow and efficiency of synthesis data were subjected to ANOVA, with treatment and trial of incubation as main effects. Time-sequence data of VFA and NH_3 concentrations in the liquid phase of the vessel were analysed within each time of sampling. The sums of squares were further partitioned by orthogonal contrasts to analyse differences due to N forms. The contrasts were distributed as follows: C1, NH_3 *v*. NAN forms; C2, amino acids *v*. peptide bound amino acids and C3, peptides *v*. protein. All analyses were conducted using the general linear models procedure of the Statistical Analysis Systems program (1985; Statistical Analysis Systems Inc., Cary, NC, USA).

Results

The analytical compositions of the two NDF sources and the calculated values for the mixed diet used in RUSITEC are

Table 1. Chemical composition (g/kg DM) of neutral-detergent fibre from grass hay and sugarbeet pulp and the calculated composition of the incubated basal diet used in the present study

Feed component	Acid-detergent fibre	Hemicellulose	Cellulose	Acid-detergent lignin	N	Acid-detergent-insoluble N
Grass hay	563	437	487	75·8	9·2	4·06
Sugarbeet pulp	498	502	462	36·2	15·3	5·88
Incubated diet	552	448	483	69·2	10·2	4·36

shown in Table 1. There were no incidents, during the RUSITEC trials, except that in the second run one of the vessels presented a small leak and was eliminated. Therefore, mean values for NH_3 treatment are the mean of three vessels, whereas data for the other treatments are the mean of four vessels. The effects of N form on the daily amount of effluent, pH, degradability of the diet and daily gas production are shown in Table 2. There were no differences ($P > 0·05$) between treatments either in pH or in the daily amount of effluent, and therefore in the N supply to the vessels.

NAN forms increased NDF ($P = 0·006$), ADF ($P = 0·003$) and cellulose ($P = 0·015$) degradability, with no significant differences ($P > 0·05$) between amino acids and peptide-bound amino acid forms (peptides and protein) in any of the considered fractions. Degradabilities of ADF ($P = 0·050$) and cellulose ($P = 0·026$) were greater for protein than for peptides treatment.

The daily productions of CO_2 and CH_4 were greater ($P < 0·001$ and $P = 0·002$ for CO_2 and CH_4 respectively) for the NAN forms (51·1 and 12·5 mmol/d respectively) than for the NH_3 treatment (45·1 and 11·2 mmol/d respectively). There was no difference ($P > 0·05$) between peptides and protein treatments in the daily production of CO_2 and CH_4, but these two treatments produced greater amounts of CO_2 ($P = 0·038$) and CH_4 ($P = 0·007$) than the amino acid treatment.

The effects of N form on the daily production of VFA, NH_3-N and NAN are shown in Table 3. NAN treatments increased ($P < 0·001$) total VFA daily production compared with NH_3, although there were no differences ($P > 0·05$) among amino acids, peptides and protein treatments. NAN treatments also increased ($P < 0·001$) the daily production of acetate, propionate, butyrate, valerate and isoacids compared with NH_3.

There were no statistically significant differences ($P > 0·05$) among the a priori selected treatment contrasts in the daily output of NH_3-N although values declined in the sequence NH_3 > amino acids > peptides > protein. In contrast, the NH_3 treatment resulted in a smaller ($P < 0·001$) daily output of NAN than NAN treatments and the amino acid treatment also resulted in a smaller ($P < 0·001$) output of NAN than the peptide and protein treatments. Mean recoveries of N were 1·01, 1·02, 0·99 and 1·00 of N input for NH_3, amino acid, peptides and protein treatments respectively.

The values of the ^{15}N enrichment of the pellets, NH_3 and NAN fraction of the digesta are given in Table 4. In all treatments the microbial pellets had substantially less ^{15}N enrichment than that of NH_3, indicating incorporation of N from sources other than the NH_3 pool. SAB had less ^{15}N enrichment than LAB, with TB having intermediate values. As shown in Table 5, NAN forms increased total microbial N flow ($P < 0·001$) compared with NH_3, with peptides and protein supporting more ($P = 0·036$) than amino acids. However, estimates of SAB flow (mg/d) were greater ($P = 0·005$) for NH_3 than for the pooled mean of the other three treatments, with no differences ($P > 0·05$) among them. The proportion of total bacteria in the SAB fraction was greater ($P < 0·001$) for the NH_3 treatment than for the amino acid, peptides and protein treatments. Microbial efficiency, expressed as g microbial N/kg organic matter apparent disappearance, was greater ($P = 0·002$) for the NAN forms

Table 2. Effects of nitrogen form on the amount of effluent, pH, fibre disappearance in 48 h and daily gas production in the rumen simulation technique (RUSITEC) system

(Treatment values are the mean of three observations in each of four vessels; *n* 12)

Variable	Ammonia	Amino acids	Peptides	Protein	SED	Statistcal significance of contrast (*P*=) C1	C2	C3
Effluent (ml/d)	561	554	574	551	2·2	NS	NS	NS
pH	6·84	6·83	6·86	6·84	0·027	NS	NS	NS
Disappearance (g/kg)								
NDF	427	462	441	457	8·0	0·006	NS	NS
ADF	364	408	383	403	9·1	0·003	NS	0·050
CEL	396	430	409	437	10·9	0·015	NS	0·026
HCEL	503	527	511	522	8·6	NS	NS	NS
Gas production (mmol/d)								
CO_2	45·1	53·0	50·2	50·2	1·35	0·001	0·038	NS
CH_4	11·2	13·1	12·0	12·3	0·32	0·002	0·007	NS

C1, NH_3 *v.* non-NH_3 N forms; C2, amino acids *v.* peptides and protein; C3, peptides *v.* protein; NDF, neutral-detergent fibre; ADF, acid-detergent fibre; CEL, cellulose; HCEL, hemicellulose.

Table 3. Effects of nitrogen form on the daily production of volatile fatty acids (VFA), ammonia nitrogen and non-ammonia nitrogen (NAN) in the rumen simulation technique (RUSITEC) system

(Treatment values are the mean of three observations in each of four vessels; *n* 12)

Variable	Ammonia	Amino acids	Peptides	Protein	SED	Statistcal significance of contrast (*P*=) C1	C2	C3
VFA production (mmol/d)								
Acetate	14·7	20·1	19·3	19·3	0·42	0·001	NS	NS
Propionate	15·7	19·4	18·2	19·8	0·63	0·001	NS	0·033
Butyrate	1·10	2·38	1·94	1·81	0·178	0·001	0·008	NS
Isobutyrate	0·46	0·96	0·87	0·81	0·044	0·001	0·011	NS
Valerate	0·64	1·26	1·16	0·97	0·095	0·001	0·035	NS
Isovalerate	0·46	1·40	1·23	1·10	0·119	0·001	0·047	NS
Total	33·1	45·5	42·7	43·8	1·29	0·001	NS	NS
Acetate : propionate	0·93	1·04	1·06	0·98	0·025	0·003	NS	0·005
Ammonia-N (mg/d)	219	216	195	190	13·4	NS	NS	NS
NAN (mg/d)	175	183	205	200	2·9	0·001	0·001	NS

C1, NH_3 *v.* non-NH_3 N forms; C2, amino acids *v.* peptides and protein; C3, peptides *v.* protein.

than for the NH_3 treatment, while peptides and protein treatments resulted in greater ($P = 0{\cdot}009$) values than the amino acid treatment (18·7 and 16·5 g microbial N/kg organic matter apparent disappearance respectively).

There were also differences ($P = 0{\cdot}002$) among treatments in the proportion of bacterial N derived from NH_3 (Table 5). When total bacteria were considered, NH_3 treatment resulted in the greatest value ($P = 0{\cdot}007$), while the infusion of amino acids, peptides or protein resulted in decreased values indicating reduced uptake of NH_3 and direct uptake of amino acids or peptides. For LAB, the NH_3 treatment resulted in the greatest ($P < 0{\cdot}001$) proportion of N derived from NH_3 while the amino acids, peptides and protein treatments produced successively smaller values ($P < 0{\cdot}001$). There

Table 4. Values of ^{15}N enrichment (atoms % excess) of bacterial pellets (total bacteria, solid-associated bacteria (SAB) and liquid-associated bacteria (LAB)), the ammonia fraction and the non-ammonia fraction (NAN) of digesta measured in the rumen simulation technique (RUSITEC) system after the infusion of $(^{15}NH_4)_2SO_4$ as a microbial marker

(Mean values with their standard errors)

	n	Ammonia Mean	Ammonia SE	Amino acids Mean	Amino acids SE	Peptides Mean	Peptides SE	Protein Mean	Protein SE
Bacterial pellets									
Total bacteria	4	0·415	0·0255	0·185	0·0088	0·197	0·0081	0·184	0·0021
SAB	2	0·329	0·0282	0·156	0·0084	0·158	0·0007	0·179	0·0020
LAB	2	0·507	0·0166	0·227	0·0025	0·255	0·0073	0·217	0·0054
NH_3-N	4	0·618	0·0031	0·286	0·0073	0·330	0·0023	0·334	0·0042
NAN digesta	4	0·172	0·0036	0·087	0·0040	0·090	0·0009	0·090	0·0029

Table 5. Effects of nitrogen form on microbial nitrogen flow and efficiency of microbial synthesis (EMS; g microbial nitrogen/kg organic matter apparent disappearance) in the rumen simulation technique (RUSITEC) system

(Mean values for four vessels, with the standard error of difference between means)

Variable	Ammonia	Amino acids	Peptides	Protein	SED	Statistical significance of contrast (*P*=) C1	C2	C3
Microbial N flow								
Total (mg/d)	72·8	86·4	93·7	98·1	4·54	0·001	0·036	NS
SAB (mg/d)	35·5	32·0	32·5	32·3	0·99	0·005	NS	NS
SAB (proportion of total)	0·490	0·370	0·347	0·330	0·0227	0·001	NS	NS
LAB (mg/d)	37·3	54·4	61·2	65·8	4·28	0·001	0·033	NS
EMS	14·9	16·5	18·7	18·7	0·77	0·002	0·009	NS
Proportion of microbial N derived from NH_3-N								
Total bacteria	0·672	0·646	0·596	0·550	0·0213	0·007	0·003	NS
SAB	0·533	0·545	0·478	0·537	0·0200	NS	NS	0·015
LAB	0·821	0·794	0·773	0·651	0·0208	0·001	0·001	0·001

C1, NH_3 *v.* non-NH_3 N forms; C2, amino acids *v.* peptides and protein; C3, peptides *v.* protein; SAB, solid-associated bacteria; LAB, liquid-associated bacteria.

were no differences ($P > 0{\cdot}05$) among amino acids and peptides and protein treatments in the proportion of SAB-N derived from NH_3 (mean value of 0·533), but the proportion was smaller ($P = 0{\cdot}015$) for peptides (0·478) compared with protein (0·537).

Data presented in Figs. 1 and 2 show the changes in relation to feeding time in the NH_3 and VFA concentrations in the liquid phase respectively. The pattern of change in NH_3 concentrations was similar in all treatments, with the greatest values being recorded just before feeding. The NH_3 treatment resulted in greater ($P < 0{\cdot}01$) NH_3 concentrations than the pooled NAN treatments at all sampling times. The amino acid treatment had greater ($P < 0{\cdot}001$) values at all sampling times compared with the peptide and protein treatments, while there was no difference ($P > 0{\cdot}05$) between peptide and protein treatments. The change in VFA concentrations was also similar in all treatments, but the concentrations were less ($P < 0{\cdot}001$) for the NH_3 treatment than for the other three treatments at all sampling times, with no differences ($P > 0{\cdot}05$) among amino acids, peptide and protein treatments.

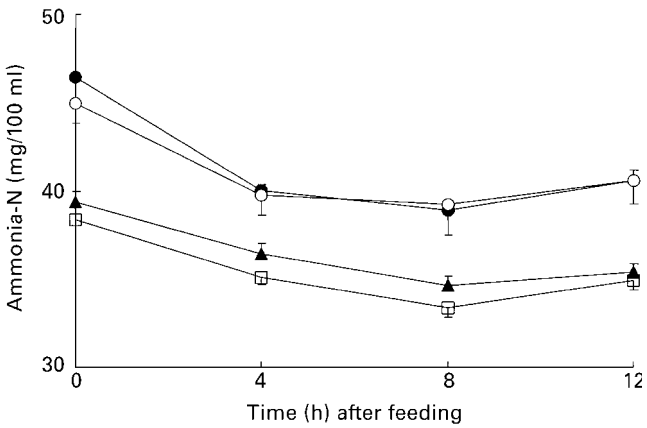

Fig. 1. Effects of nitrogen form (ammonia (●), amino acids (○), peptides (▲) and protein (□)) on ammonia-N concentrations in the vessel fluid in the rumen simulation technique (RUSITEC) system. Values are means for four vessels with their standard errors represented by vertical bars.

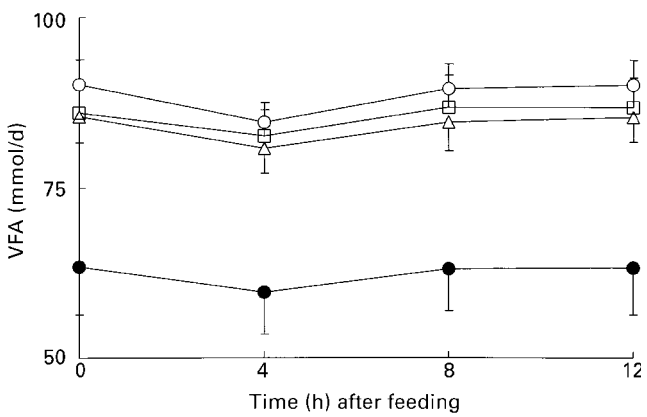

Fig. 2. Effects of nitrogen form (ammonia (●), amino acids (○), peptides (▲) and protein (□)) on volatile fatty acids (VFA) concentrations in the vessel fluid in the rumen simulation technique (RUSITEC) system. Values are means for four vessels with their standard errors represented by vertical bars.

Discussion

Cruz Soto *et al*. (1994) discussed the fact that stimulation of microbial growth by peptides and amino acids would not always occur: the effects may vary depending on the type of carbohydrate (energy substrate) available to the rumen microbes. If bacterial growth rate is limited by the energy source, as on cellulose, the benefits of NAN cannot be realized. On the contrary, if the maximum growth rate is higher, as with rapidly fermented carbohydrates, and is limited by the rate of synthesis of amino acids, stimulation will occur (Cruz Soto *et al*. 1994). The diet used in our study was intended to contain fibre, with a low, if any, content of non-structural carbohydrates (starches and sugars) in order to stimulate the growth of cellulolytic (structural carbohydrate-fermenting) bacteria. The diet combined rapidly degraded (sugarbeet pulp) and slowly degraded (grass hay) sources of fibre, and therefore a sustainable energy supply was expected.

The Cornell net carbohydrate and protein system (Russell *et al*. 1992) assumes that bacteria fermenting structural carbohydrates do not utilize peptides or amino acids, but all their N must come from NH_3. This assumption is in conflict with the results of the present study, where disappearance (degradability) of NDF, ADF and cellulose were all increased by the NAN forms. The greater degradability values achieved by the NAN forms are consistent with the greater VFA production values observed for these treatments. The replacement of NH_3 by amino acids, peptides and protein increased the daily production of VFA 1·4, 1·3 and 1·3 times respectively, indicating a stimulating effect on the fermentation produced by the NAN forms. Similar results have been reported by Griswold *et al*. (1996) in an experiment conducted with continuous cultures given a diet consisting of oat straw and maize grain when non-urea N forms (amino acids, peptides and protein) replaced urea, and by Merry *et al*. (1990) in a continuous culture system when fishmeal replaced urea in supplementing a barley–straw (50 : 50, w/w) diet. Molina-Alcaide *et al*. (1996) reported an increased VFA production when fishmeal replaced urea in supplementing a shrub (*Ulex parviflorus*) which was fed to continuous fermenters, although the degradability of carbohydrates was not improved.

Part of the increase in VFA production may be due to fermentation of the NAN test substrates. This was probably the major source of the increase in branched-chain fatty acids but, when allowance is made for the additional microbial N flow, fermentation of the remaining test NAN source is unlikely to account for more than 0·3 of the increase in straight-chain VFA. Consequently, it is concluded that the greater VFA production and increased acetate : propionate ratio (Czerkawski, 1986) found for NAN treatments reflects the improved fibre degradability. Furthermore, the concentrations of isobutyrate, valerate and isovalerate for all treatments (results not shown) were in the range of those reported as adequate for the normal growth of cellulolytic bacteria (Hume, 1970) and the stimulation in fibre degradability and VFA production is ascribed to the

direct use of amino acids or peptides. However, molar proportions of propionate were greater and those of acetate lower than the ones expected for the fermentation of a fibrous diet. In order to check this unusual VFA pattern a separate trial with batch cultures was carried out. When the hay-NDF was incubated *in vitro* with buffered ruminal liquid for 48 h a normal VFA pattern (Czerkawski, 1986) was obtained, but when the sugarbeet pulp-NDF was incubated *in vitro* under the same conditions the VFA pattern was similar to that found in RUSITEC in the current experiment. Therefore, we concluded that the fermentation of the sugarbeet pulp-NDF prepared for this experiment produced unusually high amounts of propionate and low amounts of acetate. This fact, together with the normal amount of CH_4 produced, led to a lack of H balance, with H utilization in excess of the theoretical production when calculated according to Czerkawski (1986).

The greater CO_2 and CH_4 productions observed for the NAN forms are consistent with the improved fibre digestion for these treatments. Gas arises directly from microbial metabolism and indirectly from the reaction of acid end-products with carbonate salts, an important component of the buffering system, and is related to the amount of feed fermented. The daily CH_4 production values, expressed as mol/mol VFA, were 0·34, 0·29, 0·28 and 0·28 for the NH_3, amino acids, peptides and protein treatments respectively, which are similar to values of 0·27 (Czerkawski & Breckenridge, 1977), 0·23–0·25 (Czerkawski & Breckenridge, 1979) and 0·27–0·28 (Czerkawski & Breckenridge, 1982) reported for the fermentation of hay as the only substrate in RUSITEC despite the marked difference in VFA molar ratio in the present experiment.

The determination of bacterial N flow requires knowledge of a marker : N ratio in isolated bacteria. However, LAB and SAB not only differ in chemical composition (Merry & McAllan, 1983; Martín-Orúe *et al*. 1998) affecting endogenous markers such as diaminopimelic acid, RNA and purine bases but also may take up isotopic markers differentially. In accordance with our results, a greater ^{15}N enrichment of LAB compared with SAB from infused $^{15}NH_4$ salts has been reported from *in vivo* (Pérez *et al*. 1996; Martín-Orúe *et al*. 1998) and *in vitro* (Komisarczuk *et al*. 1987) studies. This difference in ^{15}N enrichment indicates that SAB take up more of their N from NAN sources than do LAB. In order to estimate microbial protein synthesis in the rumen an ideal bacterial pellet should be representative of both fractions (SAB and LAB). In our experiment the TB pellet was used to estimate microbial production. This pellet was isolated after homogenizing and freezing the digesta, methods which have been used to remove bacteria adhering to feed particles. The mixed TB pellet had an intermediate enrichment between those of SAB and LAB (see Table 4) and appeared to be reasonably representative of the total microbial pool. In agreement with the results reported by other authors (Cruz Soto *et al*. 1994; Chikunya *et al*. 1996; Molina-Alcaide *et al*. 1996; S Chukunya & EL Miller, unpublished results), total microbial N flow was significantly increased by all the NAN forms, with the protein treatment producing the greatest values. Values of efficiency of microbial synthesis were in the range reported in the literature for the fermentation of structural carbohydrates (Czerkawski, 1986; Molina-Alcaide *et al*. 1996). The greater values for the NAN forms indicate a more efficient microbial growth in the presence of pre-formed amino acids. Furthermore, protein and peptide treatments resulted in a better efficiency of microbial synthesis than amino acid treatment. The amino acid composition of the mixtures (amino acids, peptides and protein) was the same, so presumably the improved growth with peptides and protein reflects more efficient transport into the cell.

The lower uptake of NH_3 by SAB compared with LAB may be explained by the substantial residual N in the feed fibre being used by the SAB in preference to amino acids and peptides in solution. The enrichment of SAB from the NH_3 treatment indicates the uptake of 16·6 mg NAN/d which could only have come from the feed fibre. In addition a further 5·6 mg N/d was degraded to NH_3 (see below) to give a total of 22·2 mg N/d or a degradability value of 0·201. In contrast, the amino acids, peptides and protein infused in the solution may have been degraded by the LAB so rapidly and completely that little actually reached the bacteria in the solid phase. The lack of differences between amino acids and NH_3 treatment in the daily production of both NH_3 and NAN (Table 3) would indicate an extensive degradation of amino acids, supporting this hypothesis. The NH_3 entry rate, calculated from the ^{15}N enrichment of NH_3 (Table 4) by isotope dilution, minus the infused amount of NH_3 gives an independent estimate of the degradation of feed NAN to NH_3. These estimates are 5·6, 168·9, 127·4 and 118·7 mg N/d for NH_3, amino acids, peptides and protein treatments respectively which can be compared with the input of 110 and 140 mg N from the NDF sources and test NAN sources respectively. The contribution to the NH_3 entry rate from the NDF sources is unlikely to be the same for all treatments in view of the enhanced NDF digestion with NAN supplements (Table 2) but assuming a constant contribution of 5·6 mg N/d gives NH_3 entry to amino acids, peptides or protein supply values of 1·16, 0·87 and 0·85 respectively, indicating complete degradation of amino acids and extensive but smaller degradation of peptides and protein supplements to NH_3.

The lack of differences between NH_3 and NAN forms in the incorporation of NH_3-N by SAB seems to indicate that the supply of supplementary amino acids or protein did not reduce NH_3 uptake. However, the proportion of SAB derived from NH_3-N was significantly lower for the peptide treatment compared with protein, indicating a possible uptake of peptides by the SAB. Nevertheless, the total amount of SAB was not increased by the presence of peptides. On the contrary, NH_3-N was the treatment which presented the greatest amount of SAB, which could be due to the greater amount of feed residues after 48 h of incubation with the associated adherent bacteria.

The proportion of LAB-N derived from NH_3-N was greater for the NH_3 treatment than all the others, suggesting that LAB incorporated amino acids and peptides when they were available. The greater LAB-N flow for NAN forms compared with the NH_3 treatment (Table 5) also indicates that the growth of these bacteria was stimulated by the presence of pre-formed amino acids. As bacteria fermenting non-structural carbohydrate are usually located in free suspension or loosely associated with feed particles, our

results agree in this point with the Cornell net carbohydrate and protein system (Russell *et al*. 1992), which accepted that bacteria fermenting non-structural carbohydrate use either NH_3 or peptides and amino acids as an N form. Russell *et al*. (1983) reported that when non-structural carbohydrate availability allows growth, 0·66 of the bacterial protein comes from peptides and 0·34 comes from NH_3, but when non-structural carbohydrate is limiting the excess peptide N is converted to NH_3. However, the diets used in the present study contained no non-structural carbohydrate. The LAB in this case must be cellulolytic bacteria or bacteria fermenting secondary products released from NDF by the cellulolytic bacteria. In the Cornell net carbohydrate and protein system the growth of bacteria fermenting structural carbohydrate is dependent on the amount of structural carbohydrate digested including all secondary products. Likewise the growth of bacteria fermenting non-structural carbohydrate is dependent on the amount of starches, sugars and pectin digested with a further adjustment for the stimulation of microbial growth by the availability of peptides. No account is taken of possible stimulation by peptides of microbial growth resulting from the fermentation of secondary products (sugars and organic acids) derived from structural carbohydrates and entering the liquid pool.

In conclusion, where NDF provided the only carbohydrate source, the replacement of NH_3 by non-NH_3 sources of N resulted in an increased fibre digestibility and VFA production, as well as an increased flow of microbial N and efficiency of microbial protein synthesis. These results indicate that N forms other than NH_3 are needed not only for maximum growth of fibre-digesting ruminal micro-organisms, but also for maximum fibre digestion.

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