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Influence of the pattern of peptide supply on microbial activity in the rumen simulating fermenter (RUSITEC)

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The source and pattern of N supply was varied in the rumen simulation technique (RUSITEC) in order to determine if continuous, rather than transient, availability of peptides was required for optimum ruminal fermentation. The energy source was fibre prepared from sugar-beet pulp. N was added as NH_3 continuously infused (AC) or peptides (Bacto® Casitone, a pancreatic hydrolysate of casein; Difco Laboratories, Detroit, MI, USA) continuously infused (PC) or added as a single dose at the time of feeding (PS). Free peptides were detected in the fermenter liquid for 4 h after feeding in the AC treatment, for 10 h in the PS treatment, and at all times with the PC treatment. Treatments had no effect on DM degradation. Approximately 40 % of the degradation occurred during the time no peptides were detected in the PS treatment. Microbial N flow tended to be higher with the peptide additions ($P<0{\cdot}061$), with no significant difference between the two peptides treatments. The production of liquid-associated micro-organisms (LAM) was higher in the PC treatment ($P<0{\cdot}05$) and the proportion of LAM derived from NH_3 lower ($P<0{\cdot}05$). However, LAM only accounted for 20–30 % total microbial population. Our main conclusion was that peptides had a small stimulatory effect on the fermentation, but there was no indication that synchrony of supply of energy and amino acid-N in the fermenter promoted a more efficient fermentation than non-synchronous supply. This conclusion must be qualified, however, because some N remained in the fibre and may have become available progressively as the fibre was digested by the micro-organisms.

Synchrony: Microbial protein synthesis: RUSITEC

It has been suggested that matching or synchronizing the supply of energy and N supply in the rumen may improve microbial growth (Johnson, 1976; Sinclair *et al.* 1993). However, experiments which have attempted to synchronize the release of NH_3-N with energy supply to rumen micro-organisms by the infusion of energy or urea into the rumen have generally found little or no benefit in terms of microbial growth (Henning *et al.* 1993; Kim *et al.* 1999). Chamberlain & Choung (1995) concluded that there was little benefit in maintaining a synchronized supply of NH_3-N and energy release in the rumen, although they did suggest that more results were required on the synchronization of energy with other nitrogenous substrates, including peptides and amino acids.

Peptides and, to a lesser extent, amino acids accumulate in rumen contents in the early post-feeding period, the extent of the accumulation depending at least in part on the degradability of the dietary protein, and rapidly decline thereafter (Chen *et al.* 1987; Broderick & Wallace, 1988). The peptides which persist for longer periods are probably not degraded readily by rumen micro-organisms (Wallace & McKain, 1990). Thus, when ruminant animals are meal-fed, little or no peptides will be available in rumen contents for microbial growth for much of the diurnal cycle. Numerous studies have demonstrated benefits to feeding pre-formed amino acids, either as free amino acids, peptides or proteins, in terms of increased microbial growth and/or fibre breakdown in the rumen (Hume, 1970; Maeng & Baldwin, 1976; Maeng *et al.* 1976; Cotta & Russell, 1982; Rooke & Armstrong, 1989; Merry *et al.* 1990; McAllan, 1991). Cruz Soto *et al.* (1994) presented evidence which suggested that stimulation by peptides and amino acids will not always occur; peptides and amino acids benefited cellulolytic bacteria, but only when they were growing on cellobiose, not cellulose. In a later experiment, Chikunya *et al.* (1996) confirmed that the benefit of peptides would only be evident if the energy source supported a growth rate which enabled the organisms to

Abbreviations: AC, ammonia continuously infused; LAM, liquid-associated micro-organisms; PC, peptides continuously infused; PS, peptides as a single dose; RUSITEC, rumen simulating fermenter; SAM, solid-associated micro-organisms.

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respond to added peptides. However, given that peptides will only be available in the rumen for a short time after feeding, it may also be necessary to match the time at which peptides and energy are supplied to the rumen to maximize the stimulation in microbial activity.

The objective of the present study was to utilize the stable conditions of a fermenter to investigate how microbial protein synthesis responds to continuous, synchronous and asynchronous provision of peptides. Carro & Miller (1999) recently used the rumen-simulating fermenter, RUSITEC, to investigate the effects of protein, peptides, amino acids and NH_3 on rumen fermentation. The present study also used RUSITEC, and, given that the response of the rumen micro-organisms to peptides appears to be diet-dependent (Chikunya *et al.* 1996), the diet and conditions were held as closely as possible to those used by Carro & Miller (1999).

Materials and methods

Apparatus

The fermenter was the semi-continuous system developed by Czerkawski & Breckenridge (1977), known as the rumen simulation technique (RUSITEC). The nominal volume in each reaction vessel was 780 ml and the dilution rate was set at 0·80 d^{-1}, the infused buffer being artificial saliva (McDougall, 1948) adjusted to pH 8·4 and supplemented with 0·3, 0·9 and 0·7 mmol isobutyric, isovaleric and *n*-valeric acids/l. Inocula for the fermentation vessels were obtained from a pooled sample of strained rumen contents and rumen solids removed 2 h after the morning feed from five rumen-cannulated sheep receiving 1·4 kg mixed diet/d (g/kg DM: grass hay 500·0, barley 299·5, molasses 100·0, white fishmeal 91·0, vitamin and mineral mixture 9·5) in two equal meals. Twelve vessels were set up as described previously (López *et al.* 1999). The basal diet used in RUSITEC consisted of supplemented fibre from sugar-beet pulp prepared as described later. The food for the fermentation vessels (15 g/d) was provided in nylon bags (110 × 60 mm, mean pore size 50 μm), which were gently agitated in the liquid phase. Normally, two bags were present at any time and one bag was replaced each day to give a 48 h incubation. In order to estimate the degradability of the diet additional bags containing 1 g basal diet (maximum of two additional bags per vessel at any time) were incubated for 3, 9, 12, 24 or 48 h as described later. While the bag was being changed, the vessels were flushed with CO_2 to help maintain anaerobiosis. The experiment lasted for 22 d, with all samples taken during the last 5 d.

Experimental diet and treatments

Fibre from sugar-beet pulp was obtained by washing sugar-beet pulp (placed inside a cloth bag) in a washing machine with commercial detergent at 90°C for 2 h. This procedure was repeated three times and the material obtained was washed again three times in the washing machine at 90°C for 2 h in the absence of detergent. Finally, the material was rinsed for 2 h in cold water and spun for 10 min before drying at 60°C for 2 d. Fibre prepared as described was supplemented with a commercial vitamins and trace elements mix (1·3 g/kg; Norvite Feed Supplements, Insch, Aberdeenshire, UK)

Treatments were allocated at random to four vessels each and were: (1) NH_3 continuously infused (AC): 1·712 g NH_4Cl/l added to the infusion buffer; (2) peptides added as a single dose (PS): 612 mg NH_4Cl/l added to the infusion buffer plus 1·285 g Bacto® Casitone (a pancreatic hydrolysate of casein; Difco Laboratories, Detroit, MI, USA) added in 5 ml water to the vessels at the time of feeding; (3) peptides continuously infused (PC): 612 mg NH_4Cl/l plus 2·06 g Bacto® Casitone (Difco Laboratories)/l added to the infusion buffer.

Infusion buffer was maintained at 4°C in iced water to avoid microbial growth. All treatments were designed to be isonitrogenous and to supply 280 mg N per vessel per d. Na_2SO_4 (177 mg/l) was added to all treatment buffers (N:S ratio 10:1) to prevent growth limitation by S. Based on the study of Carro & Miller (1999) 1·8 mg $^{15}NH_4Cl$ (99 % enriched; Sigma Chemical Co., Poole, Dorset, UK) was added to each vessel after 7 d of the experiment and thereafter $^{15}NH_4Cl$ (3·67 mg/l) was added to all infusion buffers.

Experimental measurements

During the last 5 d of the experiment, fermentation products were determined on samples taken from the liquid overflow. Saturated $HgCl_2$ (10 ml/d) was added to the overflow to prevent fermentation. Volatile fatty acids were determined by GLC using ethylbutyric acid as the internal standard as described by Stewart & Duncan (1985). NH_3 was measured by the phenol–hyprochlorite method of Whitehead *et al.* (1967). The volume of the overflow liquid multiplied by the concentration of fermentation products was used to calculate the daily output of fermentation products. Culture pH was measured using a pH electrode connected to a Russell 660 pH meter (Russell pH, Auchtermuchty, Fife, Scotland, UK) in samples of fermentation fluid withdrawn at the time of feeding. The digestibility of the diet was estimated from the DM remaining in the bags after 3, 9, 12, 24 or 48 h incubation in the RUSITEC vessels. Bags were washed in a domestic washing machine for 20 min in cold water, in the absence of detergent, and allowed to dry to a constant weight at 60–70°C for 48 h. The estimations of DM disappearance at different times were used to derive values describing the shape of the degradation curve according to the equation: $p = a + b(1 - e^{-ct})$, where p is the degradation after time t, a is the intercept of the degradation curve at time zero; b is the portion of the feed that will be degraded; c is the rate constant of degradation of b (Ørskov & McDonald, 1979). Data were fitted to the curve using the program fcurve (Macaulay Land Use Research Institute, 2001)

On two consecutive days, samples (10 ml) of fluid from each vessel were taken 1 h before feeding (changing the 48 h bag for a new one), at the time of feeding (0 h) and 1, 2, 4, 6, 8, 10 and 24 h after feeding. Samples were centrifuged at 120 ***g*** for 5 min at 39°C and the resultant supernatant fraction was centrifuged at 28 000 ***g*** for 20 min at

4°C. To remove protein from the sample, 1·25 ml perchloric acid (250 g/l) was added to the resultant supernatant fraction and the centrifugation was repeated. To neutralize the excess acid, 1·25 ml 2 M-potassium carbonate was added to 5 ml supernatant fraction and the sample was centrifuged again. Peptide concentrations were determined using fluorescamine (Broderick & Wallace, 1988) in a fluorimeter (excitation 365 nm, emission 450 nm; Dynatec Fluorolite 1000; Dynex Labsystems, Ashfield, Middlesex, UK).

Samples for microbial counts were taken on two consecutive days at the time of feeding during the sampling period. Anaerobic methods (Hungate, 1966) were used to prepare media and to cultivate bacteria. Media were dispensed into Hungate tubes sealed with butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ, USA). A sample of fermentation fluid (20 ml) taken directly from the reaction vessel together with a sample of the digesta (1 g) remaining in the nylon bags incubated for the last 48 h were homogenized, under O_2-free CO_2, for 1 min using an MSE top-bladed homogenizer at full speed (MSE, London, UK). Serial 10-fold dilutions were prepared under O_2-free CO_2 for each sample by the anaerobic method of Bryant (1972) using an anaerobic diluent (Mann, 1968). Total viable bacteria were enumerated in roll tubes with a complex rumen contents plus sugars medium to which agar (20 g/l) was added (medium M2; Hobson, 1969), and these tubes were incubated for 72 h at 39°C. Cellulolytic bacterial numbers were determined by a most-probable-number method based on the degradation of filter paper strips (Mann, 1968). Counts of ciliate protozoa were carried out microscopically in a counting chamber (Newbold *et al.* 1987).

Preparation of fractions to measure ^{15}N enrichment

On two consecutive days, 400 ml from the effluent of each vessel were mixed and homogenized in a blender at low speed for 1 min with their corresponding 48 h bag contents to provide total digesta. A subsample was stored at −20°C and lyophilized to provide a total digesta pellet for N and ^{15}N determinations.

Total digesta were used to isolate a total microbial pellet. Total digesta were strained through four layers of muslin. The strained liquid was then centrifuged at 500 ***g*** for 10 min at 4°C, and the supernatant fraction was centrifuged again at 18 000 ***g*** for 25 min at 4°C. The pellet obtained was resuspended with 10 ml distilled water and lyophilized.

Total digesta were also used to estimate the enrichment of ^{15}N in NH_3. Total digesta were acidified by the addition of TCA to a final concentration of 50 g/l and centrifuged at 14 000 ***g*** for 30 min at 4°C. The ^{15}N enrichment in NH_3 was determined by recovering NH_3 from acidified samples of rumen contents by adjusting the pH to >10 with 2 M-NaOH and distilling into 0·1 M-H_2SO_4 in Conway units at 60°C for 2 h in the presence of $KMnO_4$ (Conway, 1957). The acid solution was freeze-dried and analysed for ^{15}N. The NH_3 concentration was determined in a separate assay as described earlier.

On the last day of the experiment, the nylon bag residues (two bags from each vessel, 24 and 48 h) were incubated with 240 ml methylcellulose in saline (1 g methylcellulose +9 g NaCl in 1 litre distilled water) at 39°C for 30 min to elute attached micro-organisms (Minato & Suto, 1978). Then 500 ml cold (4°C) methylcellulose solution were added. The mixture was homogenized in a blender at low speed for 30 s and stored frozen until isolation of solids-attached microbial pellets. After 1 week, samples for isolation of solid-attached micro-organisms (SAM) were thawed and centrifuged twice, the first time at 500 ***g*** for 10 min at 4°C, and then the supernatant fraction was centrifuged at 18 000 ***g*** for 25 min at 4°C. The pellet obtained was resuspended in 10 ml distilled water and lyophilized.

In addition on the last day, the effluent and the contents of each vessel were collected and mixed. A subsample was centrifuged at 500 ***g*** for 10 min at 4°C, and the supernatant fraction was centrifuged again at 18 000 ***g*** for 25 min at 4°C to produce a liquid-associated microbial pellet. The pellet obtained was resuspended in 10 ml distilled water and lyophilized.

In all cases, lyophilized pellets were weighed and N determined by the Dumas procedure (Foss-Heraeus, 1990) and ^{15}N enrichment by flash combustion with isotope ratio MS (VG Isotech, Middlewich, Ches., UK). The atom % excess enrichment in samples was calculated from the enrichment in samples minus the enrichment in standard background samples analysed at the same time. Ash and DM in the diet were analysed by the methods of the Association of Analytical Chemists (1975). Neutral-detergent fibre was determined by the methods of Van Soest & Robertson (1985).

Calculations and statistics

The proportion of the total digesta N of microbial origin was estimated by dividing the ^{15}N enrichment (atom % excess) of the total digesta from each vessel by the enrichment of the total microbial pellet. Daily microbial N production (mg/d) was estimated by multiplying total N production in total digesta by the proportion attributed to the micro-organisms. Daily production of liquid-associated micro-organisms (LAM) was calculated by multiplying the content of N in the LAM pellet by total weight of LAM recovered. The proportion of bacterial N in the total microbial pellet, SAM and LAM derived from NH_3-N was estimated by dividing the ^{15}N enrichment of bacterial pellets by the enrichment in NH_3-N. Each vessel was considered as an experimental unit. Differences between treatments were compared by an ANOVA table using the Genstat computer program (Lawes Agricultural Trust, Rothamsted Herts, UK; Genstat 5 Committee, 1987).

Results

Analysis of the chemical composition of the diet (986 g DM/kg fresh weight, 16·9 g total N/kg DM, 50·6 g ash/kg DM, 645 g neutral-detergent fibre/kg DM) supplied to the RUSITEC indicated that, despite repeated washing in the presence of detergent, the neutral-detergent fibre content of the sugar-beet pulp fibre used in the experiment was <650 g/kg and almost 20 g/kg of the material was N.

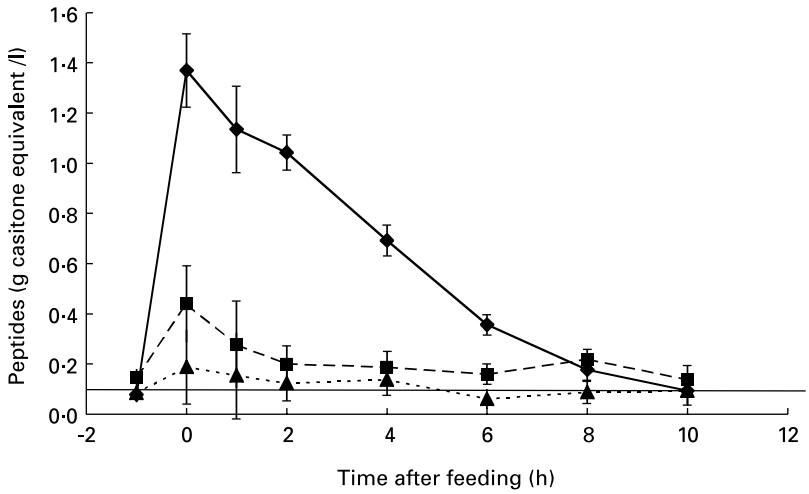

Fig. 1. Influence of different forms and pattern of nitrogen supply on peptide concentrations in RUSITEC. The treatments were: continuous infusion of ammonia (---▲---); peptides added as a single dose at feeding (—◆—); peptides infused continuously (– –■– –). Vessels were sampled 1 h before feeding (−1), at the time of feeding (0), and various intervals thereafter. For details of procedures, see p. 74. Values are means for four vessels per treatment with standard errors shown by vertical bars.

Significantly different patterns of peptide release were obtained with the different treatments (Fig. 1). A peak of peptide concentration occurred in all vessels immediately after feeding. The peptide concentration in vessels with PS treatment was about ten times that in the vessels with AC treatment and triple that in the vessels with PC treatment. Thereafter, the concentration fell in all treatments. The limit of detection of peptides was 0·1 g/l, determined by spiking samples with Bacto® Casitone (Difco Laboratories). In the AC treatment, peptides were detectable for 4 h after feeding, then fell below the limits of detection. The high concentrations in the PS treatment vessels fell rapidly and were apparently below the limit of detection at 10 h after feeding and immediately prior to feeding (Fig. 1). Peptide concentrations for the PC treatment never fell below the limit of detection (Fig. 1).

Measured pH values for PS treatment vessels were higher ($P<0·05$) than in the other vessels (Table 1), but numerically the effect was very small and all pH values were close to 6·7. Peptides treatments had significantly greater daily production of butyrate ($P<0·05$), isobutyrate ($P<0·01$), isovalerate ($P<0·01$) and valerate ($P<0·01$) (Table 1). The NH_3-N outflow was significantly higher with the AC treatment ($P<0·01$, Table 1). There were no significant treatment effects on the numbers of culturable total or cellulolytic bacteria (Table 1).

DM disappearance from nylon bags and its resulting degradation equation variables (Table 2) indicated that there were no significant differences between treatments.

^{15}N enrichment in microbial pellets was lower than that of NH_3 (Table 3), indicating that the micro-organisms incorporated N sources other than NH_3-N. LAM had a greater ^{15}N enrichment than total micro-organisms or SAM, while the total digesta had an enrichment which was less than half of the extracted total microbial fraction (Table 3). When the proportion of microbial N derived

Table 1. Effects of different forms and pattern of nitrogen supply on the daily output of volatile fatty acids (VFA) and ammonia nitrogen, the pH and the numbers of culturable total and cellulolytic bacteria in the rumen simulation technique (RUSITEC)†

(Mean values for four vessels per treatment)

Treatment...	Ammonia continuously infused	Peptides continuously infused	Peptides added as a single dose	SED
pH	6·63	6·70	6·76	0·048*
VFA output (mmol/d)				
Acetate	15·4	16·3	17·9	1·38
Propionate	8·1	8·0	8·6	0·66
Butyrate	2·9	3·5	3·8	0·28*
Isobutyrate	0·2	0·7	0·6	0·04*
Isovalerate	1·0	2·0	1·7	0·12*
Valerate	1·5	2·4	2·2	0·23*
Ammonia-N (mg/d)	180	151	127	11·0*
Total bacteria ($\times 10^8$/ml)	2·6	4·0	2·7	1·45
Cellulolytic bacteria ($\times 10^7$/ml)	2·5	3·9	3·2	0·66

* $P<0·05$.
† For details of procedures, see p. 74.

Table 2. Effects of different forms and pattern of nitrogen on the DM disappearance from nylon bags incubated in the rumen simulation technique (RUSITEC)*

(Mean values for four vessels per treatment)

Treatment...	Ammonia continuously infused	Peptides continuously infused	Peptides added as a single dose	
Length of incubation (h)	DM disappearance (%)			SED
3	13·7	13·3	13·8	1·02
9	16·8	23·1	22·6	3·03
12	33·9	29·7	31·5	4·35
24	37·2	41·3	41·6	5·96
48	53·0	49·9	52·6	1·58
a†	6·2	5·0	3·7	2·61
b†	51·3	48·7	55·8	3·23
c†	0·0506	0·0602	0·0564	0·0091

* For details of procedures, see p. 74.

† The estimations of DM disappearance at different times were used to derive values describing the shape of the degradation curve: $p = a + b(1 - e^{-ct})$, where p is the degradation after time t, a is the intercept of the degradation curve at time zero, b is the portion of the feed that will be degraded, c is the rate constant of degradation of b (Ørskov & McDonald, 1979).

from NH_3 in the different fractions was calculated from the enrichments in microbial fractions and the final ^{15}N enrichment in NH_3, it emerged that the proportion of microbial N derived from NH_3 was highest in LAM, particularly in the AC and PS treatments (Table 4). Total microbial N flow (g/d) did not differ between the treatments (Table 4). The flow of N in LAM was higher ($P<0·05$) for the PC treatment.

Discussion

The objective of the present experiment was to create different conditions for microbial growth in respect of the availability and timing of supply of pre-formed amino acids. The pre-formed amino acids were supplied in the form of a pancreatic casein hydrolysate, which comprises predominantly peptides of average molecular mass 530 Da with only a small quantity of free amino acids (Wallace, 1992). In accordance with the original method described by Czerkawski & Breckenridge (1977), bags removed from the RUSITEC after 48 h incubation were rinsed in a small amount (about 100 ml) of buffer and the washing returned to the vessel. This procedure is necessary to maintain an active fibre-degrading population in the vessel by transferring attached organisms back to the vessel. This procedure was not carried out on days on which the contents of the bags themselves were used in analysis. The rinsing and return of the washings to the vessel probably explains the small increase in peptide observed after feeding in the AC treatment as peptides from cells lysed during the washing were returned to the vessel. It also explains the larger peak observed in the PC treatment, as the bags for these vessels were rinsed in the PC buffer and thus approximately 0·2 g Bacto® Casitone (Difco Laboratories) was returned in the wash buffer. Thus the treatments were not isonitrogenous with the PC and AC treatments supplying approximately 308 mg N compared with 280 mg in the PS treatment.

Peptides were present, albeit at very different concentrations, during the first 10 h of the day in all vessels. No peptides were detected in AC and PS vessels 10 h after feeding or 1 h before the next feeding, suggesting that despite the unintentional addition of peptides via the washing the intended experimental design was achieved for 14 h during the daily cycle. Approximately 2·3 g from a total of 6 g DM digested over 24 h can be calculated to have been degraded in that period, representing almost 40 % of the total DM degradation over 24 h. In spite of the availability of peptides in the PC treatment vessels during this time, no

Table 3. Effects of different forms and pattern of nitrogen supply on different ^{15}N enrichment (atom % excess) of microbial pellets (total microbes, solid-associated micro-organisms (SAM) and liquid-associated microbes (LAM)) the ammonia fraction and the total digesta (TD) after the infusion of $^{15}NH_3Cl$ in the rumen simulation technique (RUSITEC)*

(Mean values with the standard errors for four vessels per treatment)

Treatment...	Ammonia continuously infused		Peptides continuously infused		Peptides added as a single dose	
	Mean	SE	Mean	SE	Mean	SE
Microbial pellets						
TM	0·0909	0·0015	0·0945	0·0015	0·1207	0·0043
SAM	0·0926	0·0029	0·0931	0·0018	0·1212	0·0163
LAM	0·1392	0·0014	0·1045	0·0103	0·1674	0·0096
Ammonia	0·2018	0·0017	0·2131	0·0027	0·2719	0·0052
TD	0·0413	0·0008	0·0429	0·0013	0·0569	0·002

* For details of procedures, see p. 74.

Table 4. Influence of different forms and pattern of nitrogen supply on the flow of microbial nitrogen and on $^{15}NH_3$ assimilation in the rumen simulation technique (RUSITEC)‡

(Mean values for vessels per treatment)

Treatment...	Ammonia continuously infused	Peptides continuously infused	Peptides added as a single dose	SED
Microbial N flow (g/d)				
Total	0·144	0·151	0·157	0·005†
LAM	0·030	0·059	0·039	0·005*
Proportion of microbial N derived from NH_3-N				
Total	0·445	0·444	0·444	0·012
SAM	0·459	0·437	0·443	0·041
LAM	0·690	0·490	0·615	0·049*

LAM, liquid-associated micro-organisms; SAM, solid-associated micro-organisms.
* $P<0·05$.
† $P<0·061$.
‡ For details of procedures, see p. 74.

difference was observed in DM degradation between the vessels. Thus, the availability of peptides did not enhance the digestion of fibre.

These observations are relevant to ideas about synchronizing energy and N supply to the rumen, which have been studied extensively *in vitro* (Henning *et al.* 1991; Newbold & Rust, 1992) and *in vivo* (Rooke *et al.* 1985; Rooke & Armstrong, 1989; Sinclair *et al.* 1993; Henderson *et al.* 1998). It is important to distinguish between N sources in this discussion. Chamberlain & Choung (1995) in their review questioned the need for close synchrony between NH_3-N and energy and proposed that peptides and amino acids should be synchronized with energy, arguing that the peptides can only be detected for a short period of time in the rumen contents (Broderick & Wallace, 1988), while NH_3 is usually available throughout the feeding cycle. In the present study, there was no difference between the PC and PS treatments, indicating that synchronizing energy and peptide supply in these experiments had no benefit.

The counter-argument is, however, that a continuous supply of protein may have been available from the fibre source. In spite of efforts to prepare fibre from the sugar-beet-pulp-feed, a substantial amount of N remained in the material supplied to the fermenter and thus, some N was supplied from the diet. Assuming that N is released from the diet at the same rate as DM loss (and this is almost bound to be an over estimate, given the extensive neutral-detergent fibre washing) then from 12 to 24 h a maximum of 30 mg N could be released, that compares to an infusion of about 90 mg N in the PC treatment over the same time period. However, if microbial synthesis was related to DM degradability then the potential N released from the plant material would be sufficient to account for much of the microbial N synthesized between 12 and 24 h (approximately 38 mg microbial N if proportional to DM loss). Thus, the possibility that at least some of the micro-organisms received a synchronized supply of amino acid-N from the feed can not be ruled out.

Other changes which resulted from the peptides treatments were an increase in certain volatile fatty acids, which would be expected from the fermentation of amino acids (Wallace *et al.* 1997), and changes in LAM in the PC treatment which resulted in an increased flow of microbial protein and a decreased incorporation of $^{15}NH_3$. Salter *et al.* (1979), summarizing data from the literature, found that between 18 and 100 % microbial protein was derived from NH_3. Hristov & Broderick (1996) *in vivo* and Carro & Miller (1999) *in vitro* divided the microbial population into LAM and SAM, and measured the N coming from the NH_3 pool. In the study of Hristov & Broderick (1996) the LAM accounted for 12 % total bacteria, and the synthesis of microbial N from NH_3 was 10–15 % higher in the LAM than in the SAM. Carro & Miller (1999) found that LAM accounted for 51–67 % of the microbial population, and the proportion of microbial N derived from NH_3 was between 10 and 30 % higher than in the SAM. Furthermore, Carro & Miller (1999) found that there was a smaller proportion of microbial N derived from NH_3 in the LAM when peptides and proteins were infused into the RUSITEC vessels, While SAM were unaffected. In the present study, LAM accounted for 20, 39 and 24 % of the total bacterial pool for AC, PC and PS treatments respectively. It was also evident that there was very little change in the proportion of microbial N derived from NH_3 in the SAM in all treatments. On the other hand, although the LAM proportion of microbial N derived from NH_3-N in all treatments was bigger than the SAM proportion of microbial N derived from NH_3-N, there were differences within the treatments: the PC (synchronous) treatment had less uptake of N from the NH_3 pool. These results suggest that perhaps the infused peptides were incorporated by LAM and therefore not available to SAM, or that SAM already had a sufficient supply of peptides from the fibre. This question might be resolved by improved chemical extraction of the fibre, or by using another source of fibre. It may well be the case that micro-organisms digesting certain fibre sources, which have protein closely associated with the fibre, have no need for additional soluble peptides. The effects of micro-environments and nutrient gradients therefore have an important impact on optimizing nutrients for optimal rumen fermentation.

It should be noted, that although ciliate protozoa were present in the initial inoculum, no protozoa were detected in the vessels during the sampling period. The results of

this present study should therefore be considered entirely in terms of bacterial and possibly fungal metabolism, although the latter were not enumerated.

In conclusion, despite the accidental addition of peptides during the washing of the bags and the possible release of N from the feed during the incubation, it was obvious that there was no stimulation in any of the fermentation characteristics measured when peptides were added as a continuous infusion compared with a single dose, suggesting little benefit of synchronizing the supply exogenous peptides with energy release.

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