Uptake of Small Neutral Peptides by Mixed Rumen Microorganisms In Vitro*

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ABSTRACT

The metabolism of a range of neutral di- and oligopeptides and peptide p-nitroanilides was investigated in strained ruminal fluid diluted with anaerobic buffer. Peptide uptake was assayed by decrease in fluorescamine-reactive material in extracellular fluid, and peptide p-nitroanilide hydrolysis by diazotisation of the released p-nitroaniline. Addition of glucose and dithiothreitol did not alter uptake of di- or trialanine. Calculated \( V_{\text{max}} \) for di- and trialanine uptake were 1.4 and 1.9 nmol min\(^{-1}\) mg DM\(^{-1}\), corresponding \( K_{\text{m}} \) were 0.30 and 0.14 mmol litre\(^{-1}\). Dipeptide uptake was 0.72 to 0.90 nmol min\(^{-1}\) mg DM\(^{-1}\), except for glycylproline, which was taken up at 0.43 nmol min\(^{-1}\) mg DM\(^{-1}\). There was a wider range for tripeptide uptake (0.5 to 1.6 nmol min\(^{-1}\) mg DM\(^{-1}\)), with trialanine being taken up most rapidly. Tetra- and pentalanine were removed at 0.93 and 0.71 nmol min\(^{-1}\) mg DM\(^{-1}\). Uptake of amino acid residues as alanine oligopeptides was two to three times more rapid than uptake as dialanine. These data suggest that peptides would accumulate in rumen fluid during hydrolysis of rapidly degraded proteins, but peptide uptake would exceed rate of release from more slowly degraded proteins. Rates of hydrolysis of eight peptide p-nitroanilides were 0.56 to 1.53 nmol min\(^{-1}\) mg DM\(^{-1}\), and

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were of similar magnitude to uptake of di- and tripeptides; proline-containing p-nitroanilides were also more slowly metabolised.

Key words: Peptide metabolism, rumen microorganisms.

1 INTRODUCTION

Protein breakdown by rumen microorganisms leads to the production of ammonia concentrations in rumen contents which frequently exceed microbial growth requirements. The excess ammonia is absorbed through the rumen wall and eventually excreted as urea, thus decreasing the efficiency of nitrogen utilisation by the animal.1,2 Peptides are intermediates in the conversion of protein to ammonia, and there is some in vitro3,4 and in vivo5 evidence that peptide hydrolysis may be the rate-limiting step in the process, at least for rapidly degraded proteins. It is possible that catabolism of protein may be effectively arrested at this step.

Rumen bacteria show a preference for peptides over free amino acids as growth substrates,6-8 and amino acids in peptides are more rapidly metabolised than the corresponding free amino acids.7,9-11 Wright12 demonstrated that ruminal peptidase activity was cell-associated, and Bladen et al.13 concluded from their survey of rumen bacteria that Bacteroides ruminicola was the most significant producer of ammonia from mixed peptides. This organism can apparently assimilate peptides of molecular weight (MW) up to 2000.10 Other bacteria involved in producing ammonia from peptides were Selenomonas ruminantium, Megasphaera elsdenii and some Butyrivibrio isolates.15 The mechanisms of peptide uptake and hydrolysis are fairly well understood in many organisms.14-17 For example, Escherichia coli is known to have three permeases for peptide transport across the cell membrane.16 However, the mechanisms of peptide uptake and hydrolysis have been little studied in rumen organisms.

The purpose of the research reported here was to investigate the rates of uptake of small neutral peptides by mixed rumen microorganisms. A brief report of some of this work has been made.18

2 EXPERIMENTAL

2.1 Animal, diet and sample preparation

A mature wether sheep, fitted with a permanent rumen cannula, was fed 450 g diet each at 0800 and 1600 h. The diet consisted of 67% grass hay and 33% concentrate.19 Strained rumen fluid (SRF) was prepared about 2 h after the morning feeding by straining rumen contents through four layers of muslin. Cell-free supernatant was prepared by centrifuging SRF at 27200×g for 15 min.

2.2 Incubation procedure

SRF was diluted with 0.33 vol of 100 mmol litre⁻¹ potassium phosphate buffer, pH 7.0, previously made anaerobic by boiling and gassing with O₂-free CO₂ and at
Rumen peptide metabolism

39°C. Tubes were capped, mixed by inversion, and incubated at 39°C for 15 to 20 min. Peptide incubations were terminated by centrifuging tubes (12000×g, 2 min). Supernatants were stored on ice until analysed for remaining peptide; peptide disappearance was negligible under these conditions. Separate 0-h samples were prepared in a similar manner except that blanks and peptide-containing tubes were held on ice; iced inoculum was added to tubes before mixing and centrifuging as described. p-Nitroanilide incubations were terminated by the addition of 0.25 ml of 250 g litre⁻¹ trichloroacetic acid and centrifuging (12000×g, 2 min). Duplicates of each blank and sample type were run; results are the means from incubations with three or four separate samples of SRF taken on different days.

2.3 Analyses

The dry matter (DM) content of inocula was estimated in quadruplicate by centrifuging (27200×g, 4°C, 15 min) and drying the pellet at 105°C to constant weight (about 6 h). This was assumed to be DM from mixed rumen microorganisms. The protein content of the pellet was determined using a modified Lowry procedure with bovine serum albumin as standard. Peptides were assayed by a fluorimetric procedure similar to those described by Perrett et al. and Nisbet and Payne, in which fluorescamine reacts preferentially with peptides rather than free amino acids. The response of individual amino acids in the procedure is only 1 to 5% that of peptides. Supernatant (75 µl) was added to 2-25 ml of 0.2 mol litre⁻¹ sodium citrate buffer, pH 6.2, at room temperature. Fluorescamine solution (0.28 g litre⁻¹ in acetone) was added while this solution was vortexed. After mixing for a further 3-4 s, fluorescence was measured within 2 to 30 min using a Baird Nova spectrofluorimeter (Baird-Atomic Ltd, Braintree, UK), with excitation at 390 nm and emission at 485 nm. Although the fluorescence response differed markedly between peptides, each gave a linear response. Net peptide disappearance (i.e. blank-corrected) was determined in each experiment relative to net 0-h fluorescence.

Hydrolysis of peptide p-nitroanilides was measured from the rate of appearance of p-nitroaniline using the diazotisation procedure of Appel.

2.4 Materials

Peptides and peptide p-nitroanilides were obtained from Sigma Chemical Co. Amino acids present in peptides were all of the L-configuration.

2.5 Statistical procedures

Data were analysed by one-way analysis of variance. Where significant treatment effects were detected (P<0.05), mean separation was by least significant difference.

3 RESULTS

3.1 Factors influencing peptide uptake

The rates of uptake of different peptides varied from day to day in inocula prepared from SRF taken from the same sheep; however, several general proper-
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TABLE 1
Effect of Glucose and Dithiothreitol (DTT) on Uptake of Di- and Tri-L-alanine by Mixed Rumen Organismsa

<table>
<thead>
<tr>
<th>Addition</th>
<th>Rate of uptake (nmol min⁻¹ mg DM⁻¹) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ala₂</td>
</tr>
<tr>
<td>Control</td>
<td>0.594</td>
</tr>
<tr>
<td>5 mg litre⁻¹ glucose</td>
<td>0.654</td>
</tr>
<tr>
<td>SEM</td>
<td>0.086</td>
</tr>
<tr>
<td>Control</td>
<td>0.746</td>
</tr>
<tr>
<td>1 mmol litre⁻¹ DTT</td>
<td>0.624</td>
</tr>
<tr>
<td>2 mmol litre⁻¹ DTT</td>
<td>0.555</td>
</tr>
<tr>
<td>3 mmol litre⁻¹ DTT</td>
<td>0.574</td>
</tr>
<tr>
<td>SEM</td>
<td>0.178</td>
</tr>
</tbody>
</table>

aData are means from three incubations. Microbial DM averaged 4.96 g litre⁻¹. Initial peptide concentration was 0.25 mmol litre⁻¹.

Ties could be clearly distinguished. With an initial incubation concentration of 0.25 mmol litre⁻¹, trialanine was always removed more rapidly than dialanine, at rates of 1.1 and 0.6 nmol min⁻¹ mg DM⁻¹, respectively, in one series of experiments (Table 1). Glucose had a minor stimulatory effect which was not statistically significant (P>0.10) for either peptide (Table 1). The addition of dithiothreitol (DTT) to the incubation mixture tended to inhibit the removal of both peptides, but this effect again was not statistically significant (P>0.10; Table 1). It was decided, therefore, to carry out subsequent experiments without added energy sources or reducing agent.

3.2 Michaelis–Menton kinetics

Initial rates of uptake were determined for di- and trialanine at concentrations of 0.1 to 0.5 mmol litre⁻¹, and values of $K_m$ and $V_{max}$ were calculated using the integrated form of the Michaelis–Menton equation²⁵ (Table 2). $V_{max}$ was slightly

TABLE 2
Michaelis–Menton Kinetics of Uptake of Di- and Tri-L-alanine by Mixed Rumen Organismsa

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$V_{max}$ (nmol min⁻¹ mg DM⁻¹)</th>
<th>$K_m$ (µmol litre⁻¹)</th>
<th>$k$ (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala₂f</td>
<td>1.48</td>
<td>324</td>
<td>1.48</td>
</tr>
<tr>
<td>Ala₃f</td>
<td>2.03</td>
<td>165</td>
<td>4.42</td>
</tr>
</tbody>
</table>

aCalculated using means from incubations with four separate samples of SRF for each peptide and using the integrated Michaelis–Menton equation.²⁵
bFractional rate of uptake, $k=(V_{max}/K_m)\times(\text{DM ml}^{-1})\times(60 \text{ min h}^{-1})$.²⁵
cMicrobial DM averaged 4.71 g litre⁻¹. Rates of uptake of di- and tri-L-alanine at 0.25 mmol litre⁻¹ were 0.665 and 0.811 nmol min⁻¹ mg DM⁻¹, respectively.
dMicrobial DM averaged 5.99 g litre⁻¹. Rates of uptake of tri-L-alanine at 0.25 mmol litre⁻¹ were 1.066 nmol min⁻¹ mg DM⁻¹.
TABLE 3
Uptake of Various Neutral Di- and Tripeptides by Mixed Rumen Organisms

| Group      | Peptideb | Nc | Rate of uptake | CVc (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(nmol min⁻¹ mg DM⁻¹)</td>
<td></td>
</tr>
<tr>
<td>Dipeptides</td>
<td>Ala2</td>
<td>6</td>
<td>0.904d</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Gly3</td>
<td>6</td>
<td>0.771d</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Gly Ala</td>
<td>3</td>
<td>0.809d</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Gly Pro</td>
<td>3</td>
<td>0.427e</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Val Ala</td>
<td>3</td>
<td>0.724d</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td></td>
<td>0.120</td>
<td></td>
</tr>
<tr>
<td>Tripeptides</td>
<td>Ala3</td>
<td>9</td>
<td>1.241d</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Gly3</td>
<td>3</td>
<td>0.537f</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Ala Gly2</td>
<td>9</td>
<td>0.484e</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Gly Ala3</td>
<td>3</td>
<td>0.707e, f</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Gly2 Ala</td>
<td>6</td>
<td>0.554f</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Leu Gly2</td>
<td>6</td>
<td>0.863e</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Gly2 Leu</td>
<td>3</td>
<td>0.624e</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Phe Gly2</td>
<td>9</td>
<td>0.530f</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Gly2 Phe</td>
<td>6</td>
<td>0.569f</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td></td>
<td>0.117</td>
<td></td>
</tr>
</tbody>
</table>

aData are summarised from 12 incubations with mean DM and protein concentrations of 5.94 and 2.37 g litre⁻¹, respectively. Initial peptide concentration was 0.25 mmol litre⁻¹.

bAll optically active amino acid residues were of the L-configuration.

CN, number of separate incubations; CV, coefficient of variation.

d,e,fMeans within peptide groups having different superscripts are significantly different (P<0.05).

greater for trialanine, but the much lower $K_m$ resulted in a fractional rate of uptake (computed from the ratio $V_{max}/K_m$) which was 3.0 times greater than that of dialanine.

3.3 Uptake of different neutral peptides

The rates of uptake of four dipeptides were similar, at 0.72 to 0.90 nmol min⁻¹ mg DM⁻¹, whereas glycylylproline was removed more slowly (Table 3). Trialanine was taken up more rapidly than any other di- or tripeptide, and intermediate rates were observed for leucylglycine and glycylglycine, and slower rates (0.48 to 0.62 nmol min⁻¹ mg DM⁻¹) for the other tripeptides (Table 3).

Four alanine peptides, dialanine to pentalaalanine, were compared (Table 4). Again, trialanine was most rapidly taken up, followed by tetraalanine, pentalaalanine and finally dialanine. In terms of the uptake of alanine residues, the higher homologues were similar but the dimer gave a significantly slower rate of amino acid uptake.

3.4 Hydrolysis of peptide $p$-nitroanilides

Three dipeptide $p$-nitroanilides were hydrolysed at similar rates, of about 1.4 to 1.5 nmol min⁻¹ mg DM⁻¹, whereas proline-containing dipeptides were broken
TABLE 4  
Uptake of L-alanine peptides by mixed rumen organisms*

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Rate of uptake (nmol min⁻¹ mg DM⁻¹) of peptide</th>
<th>Alanine residuesᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Ala₂</td>
<td>0.772</td>
<td>0.209</td>
</tr>
<tr>
<td>Ala₃</td>
<td>1.572</td>
<td>0.239</td>
</tr>
<tr>
<td>Ala₄</td>
<td>0.933</td>
<td>0.079</td>
</tr>
<tr>
<td>Ala₅</td>
<td>0.711</td>
<td>0.148</td>
</tr>
</tbody>
</table>

aData are means from four incubations which contained DM and protein concentrations of 5.31 and 2.08 g litre⁻¹, respectively. Initial peptide concentrations was 0.25 mmol litre⁻¹.  
bComputed peptide uptake rate times alanine residues per peptide.

down more slowly (Table 5). Two p-nitroanilides containing three amino acid residues were hydrolysed 20% more slowly than the former dipeptides.

3.5 Location of activities

When rates of disappearance of di-, tri- and tetraalanine in cell-free supernatant were compared with those of SRF, the supernatant had no activity against tetraalanine and only 14% of the total activity against trialanine. In contrast, 46% of the activity against dialanine occurred in the cell-free supernatant. With the peptide p-nitroanilides, 8% and 6% of the total hydrolysis activities were cell-free

TABLE 5  
Hydrolysis of Peptide p-Nitroanilides by Mixed Rumen Organisms*  

<table>
<thead>
<tr>
<th>p-Nitroanilideᵇ</th>
<th>Rate of hydrolysis (nmol min⁻¹ mg DM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu pNA</td>
<td>1.122ᵈ, ᵗ</td>
</tr>
<tr>
<td>Ala₂ pNA</td>
<td>1.375ᶜ, ᵗ</td>
</tr>
<tr>
<td>Arg Pro pNA</td>
<td>0.559ᶠ</td>
</tr>
<tr>
<td>Gly Arg pNA</td>
<td>1.529ᶜ</td>
</tr>
<tr>
<td>Gly Pro pNA</td>
<td>0.980ᶜ</td>
</tr>
<tr>
<td>Val Ala pNA</td>
<td>1.457ᶜ</td>
</tr>
<tr>
<td>Ala₃ pNA</td>
<td>1.155ᵈ, ᵗ</td>
</tr>
<tr>
<td>Ala₅ Phe pNA</td>
<td>1.142ᵈ, ᵗ</td>
</tr>
<tr>
<td>SEM</td>
<td>0.139</td>
</tr>
</tbody>
</table>

aData are means from three incubations with average DM and protein concentrations of 8.32 and 3.32 g litre⁻¹, respectively. Initial p-nitroanilide concentration was 0.25 mmol litre⁻¹.  
bAll optically active amino residues were of the L-configuration.  
c, d, e, f, g, h, i, j Means with different superscripts are significantly different (P<0.05).
with the di- and the trialanine p-nitroanilides, respectively. The monoalanine
derivative was also incubated with both preparations, and was hydrolysed at a rate
of 0.74 nmol min⁻¹ mg DM⁻¹, of which 0.01 nmol min⁻¹ mg DM⁻¹ equivalent
was cell-free.

4 DISCUSSION

Until now, only relatively poorly defined mixed peptides have been used to study
peptide metabolism in rumen microorganisms. The main feature of the present
paper is the description of the metabolism of single, defined peptides by the mixed
rumen population, an area of metabolism which has received considerable atten-
tion in other microbial systems. 14, 15-17, 25 Although the results have not proved to
be unusual or surprising in the light of this existing knowledge, some interesting
features have emerged.

As was found by Wright 12 with 14C-labelled peptides from a Chlorella hydroly-
sate, peptides were metabolised predominantly by cell-bound activities. The
exception was dialanine, which was metabolised by cell-free rumen liquor at
nearly half the rate of intact SRF. Therefore, peptidase activity appears to be
more closely cell-associated than protease activity. 27, 28 The measurements
reported here were from net removal of fluorescamine-reactive peptide from the
extracellular fluid, so they include intracellular accumulation as well as hydro-
lysis. Furthermore, no distinction can be made between hydrolysis by cell wall
associated enzymes, as occurs with some streptococci, 16, 26 and the more common
accumulation followed by intracellular hydrolysis. 15, 16, 26 Nevertheless, the kine-
tics of di- and trialanine uptake (Table 2) indicated that $V_{\text{max}}$ of the mixed rumen
population for di- and trialanine uptake was 1.3% and 2.9%, respectively, of
those of wild-type E. coli; 31 corresponding $K_m$ in E. coli 31 was 20% and 23% of
those for mixed rumen microbes. The activities in rumen contents were more
similar to those of Saccharomyces cerevisiae, which ranged from 1 to
5 nmol min⁻¹ mg DM⁻¹ for di- and tripeptides. 14

In E. coli, the composition of the amino acid side chains has little influence on
peptide uptake, 15, 16 whereas S. cerevisiae is more selective. 14 Here, various
tripeptides were taken up at only slightly different rates (Table 3), possibly
reflecting the complex mixture of organisms present. A proline-containing dipep-
tide (Table 3) and two peptide p-nitroanilides (Table 5) were more slowly
metabolised, presumably because of the secondary amino acid. However, all of
the observed differences were not great, and it may be concluded that the amino
acid content of small neutral peptides has only a minor influence on their rate of
uptake by a complex mixture of rumen microorganisms. The same may not be
true for other mixtures of rumen microbes or for particular species of rumen
bacteria.

Peptide metabolism should not be considered solely in terms of rumen bacteria.
Rumen contents from faunated sheep had a significantly higher rate of trialanine
uptake than that from ciliate-free animals fed the same diet, whereas dialanine
uptake was not different (R. J. Wallace, G. A. Broderick and M. L. Brammall,
unpublished). Dipeptide metabolism therefore appears to be distinct in character from tripeptide metabolism, and associated primarily with the rumen bacteria. Both protozoa and bacteria appear to have a role in the metabolism of oligopeptides, and their relative importance is dependent upon relative numbers.

The lack of effect of glucose and DTT on the observed rates of peptide uptake (Table 1) should not be interpreted to mean that peptide uptake is not energy dependent or O$_2$-sensitive, but rather that the assay conditions were adequate without further additions. Rumen microorganisms usually have an ample supply of reserve polysaccharide to provide energy during the frequent periods of energy starvation that occur in the rumen, and these reserves may have been used to drive peptide transport in these incubations. Whether or not peptide uptake may be O$_2$-sensitive can only be speculated on, since the high cell density and the anaerobic buffer used in the assay would result in the rapid reduction of any O$_2$ entering the incubation mixture.

In view of the reservations on the value of model compounds (such as peptide p-nitroanilides) as substrates for transport systems and peptidase assays, the correspondence of results obtained with SRF is surprising. Rates of metabolism were similar, at 0·6 to 1·5 nmol min$^{-1}$ mg DM$^{-1}$ for peptide p-nitroanilides (Table 5) and 0·4 to 1·5 nmol min$^{-1}$ mg DM$^{-1}$ for unmodified peptides (Tables 3, 4), and proline-containing compounds were metabolised more slowly (Tables 3, 5). Peptide p-nitroanilide hydrolysis activity was also predominantly cell-associated, as was oligopeptide metabolism. The only difference was in the finding of extracellular dipeptidase activity, which may be difficult to detect using p-nitroanilides. Therefore, p-nitroanilides may be useful in the study of rumen oligopeptide metabolism.

The assay conditions used here gave apparent rates of peptide uptake of about 4 nmol alanine residues min$^{-1}$ mg DM$^{-1}$ for the higher homologues at a concentration of 0·25 mmol litre$^{-1}$ (Table 4), and a maximum value of 5·6 nmol alanine min$^{-1}$ mg DM$^{-1}$ for the peptide most rapidly taken up, trialanine (Table 2). The corresponding rate of casein degradation can be calculated to be greater than these rates. For example, if casein was present at 0·1 mg N ml$^{-1}$ and its fractional rate of hydrolysis was 0·4 h$^{-1}$, its rate of breakdown would be about 0·7 mg N min$^{-1}$ litre$^{-1}$, or, based on the average DM content of SRF measured here at 5 g litre$^{-1}$, 0·14 mg N min$^{-1}$ g DM$^{-1}$. Since the amino acid content of casein is 54 μmol mg$^{-1}$ N, this is equivalent to a rate of release of amino acids of 7·6 nmol min$^{-1}$ mg DM$^{-1}$. Thus, for rapidly degraded proteins like casein, the rate of proteolysis may exceed the rate at which peptides are removed, and peptides may accumulate transiently in rumen fluid. However, if protein were present at lower concentration, or if it were less rapidly degraded than casein, peptides would not accumulate. In vivo experiments tend to support this argument. Heat treatment of soya bean meal decreased its degradability and reduced peptide flow from the rumen of cattle by 80%.

In sheep fed dietary supplements of 6% casein, peptides accumulated to a concentration of 3·8 μmol trialanine equivalents ml$^{-1}$ in rumen fluid 1 h after feeding, whereas with the more resistant albumin only 0·3 μmol min$^{-1}$ was found (R. J. Wallace and G. A. Broderick, unpublished).
ACKNOWLEDGEMENTS

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