Effect of Hydrophobicity on Utilization of Peptides by Ruminal Bacteria In Vitro

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When mixed ruminal bacteria were incubated with a pancreatic casein hydrolysate and free amino acids of a similar composition, rates of ammonia production were much greater for peptides than for amino acids. The pancreatic digest of casein was then fractionated with 90% isopropyl alcohol. Hydrophobic peptides which dissolved in alcohol contained an abundance of phenolic and aliphatic amino acids, while the hydrophilic peptides which were precipitated by alcohol contained a large proportion of the highly charged amino acids. The $K_m$ values of the mixed ruminal bacteria for each fraction were similar (0.88 vs. 0.98 g/liter), but the $V_{max}$ of the hydrophobic peptides was more than twice that of the hydrophilic peptides (18 vs. 39 mg of NH$_3$ per g of bacterial protein per h). Pure cultures of ruminal bacteria had a similar preference for hydrophobic peptides and likewise utilized peptides at a faster rate than free amino acids. Since peptide degradation rates differed greatly, hydrophobicity is likely to influence the composition of amino acids passing unfermented to the lower gut of ruminant animals.

Most procaryotes are able to use ammonia as a nitrogen source (20), but amino acid sources can also play a major role in the nutrition of bacteria (24). Enteric bacteria such as Escherichia coli have a multiplicity of amino acid transport systems (18), and for many years it was assumed that amino acid sources were transported as free amino acids. However, in the 1960s it became apparent that peptides could also penetrate the bacterial membrane (11, 22). Peptide transport systems are widely distributed in bacteria (5, 13, 22, 27).

The ruminal bacterium Bacteroides ruminicola grew poorly with free amino acids as a nitrogen source (25), but was able to take up peptides with molecular masses as large as 2,000 daltons (26). Amino acids are present at very low concentrations (<70 mg/liter) in ruminal fluid (34), but recent work indicated that peptide concentrations were as high as 1,500 mg/liter in vivo (8, 9). The accumulation of small peptides in ruminal fluid indicated that extracellular peptide activity and peptide uptake were limiting.

The following experiments were designed to compare the relative uptake rate of peptides and amino acids by ruminal bacteria and ascertain the effect of hydrophobicity on peptide uptake. Results indicated that (i) peptide nitrogen was taken up more than twice as fast as amino acid nitrogen by mixed ruminal bacteria, (ii) hydrophilic peptides were catabolized twice as fast as hydrophobic peptides by mixed ruminal bacteria, and (iii) pure cultures also preferred hydrophilic peptides to hydrophobic peptides or free amino acids, with B. ruminicola most closely mimicking the mixed cultures.

MATERIALS AND METHODS

Mixed-culture incubation and sampling. Ruminal contents were obtained from a 600-kg, nonlactating, ruminally fistulated dairy cow which was fed 2.5 kg of timothy hay and 2.5 kg of commercial concentrate mix twice daily. At 1.5 h after feeding, ruminal contents were squeezed through eight layers of cheesecloth and purged with O$_2$-free CO$_2$ for 5 min. Protozoa and feed particles were removed by slow-speed centrifugation (143 × g, 10 min, 15°C). The supernatant was centrifuged at 10,300 × g for 15 min (15°C) to collect bacteria. The supernatant was anaerobically (under CO$_2$ gas) removed, and pellets were suspended with anaerobic basal medium containing (per liter) 292 mg of K$_2$HPO$_4$, 292 mg of KH$_2$PO$_4$, 400 mg of Na$_2$SO$_4$, 400 mg of NaCl, 100 mg of MgSO$_4$ · 7H$_2$O, 64 mg of CaCl$_2$ · 2H$_2$O, 4,000 mg of NaHCO$_3$, 1 mg of hemin, 0.1 mg of mercaptoethanesulfonic acid (cocoenzyme M), 0.5 g of Na$_2$S · 9H$_2$O, vitamins (32), and volatile fatty acids (32). The mixed cultures were adjusted to a starting cell density of 1.0 optical density unit (Gilford 260 spectrophotometer, 600 nm, 1-cm light path), anaerobically transferred (9 ml) to 18-mm tubes, and sealed with butyl rubber stoppers. Various nitrogen sources (on an ash-free basis) which had been prepared anaerobically were then added (1 ml) to each tube (see text for type and amount). Incubation temperature was 39°C. Samples were taken at 0, 4, 8, and 12 h, except for kinetic experiments (Lineweaver-Burk plots) in which samples were taken every 30 min for 4 h. All samples were centrifuged at 13,000 × g for 5 min (22°C), and supernatants were stored at −20°C until analysis.

Pure-culture incubations. B. ruminicola B4, Selenomonas ruminantium HD$_2$, and Megasphaera elsdenii B159 were obtained from M. P. Bryant, University of Illinois, Urbana, and Streptococcus bovis JB1 was isolated at the University of California, Davis (30). The basal medium used was the same as described above except that hemin and coenzyme M were omitted. Cells were incubated with Trypticase (BBL Microbiology Systems, Cockeysville, Md.), Casamino Acids (Difco Laboratories, Detroit, Mich.), or hydrophilic or hydrophobic peptides (see description of preparation below) as the sole source of nitrogen (1.5 g of N per liter) and glucose (6 g/liter) as the energy source. Growth rate was monitored by measuring optical density every 15 min until optical density unit had been reached.

Hydrophobic and hydrophilic peptides. Trypticase (100 g) was added to 90% isopropyl alcohol (1,000 ml) and stirred for
2 h. The mixture was then centrifuged at 8,430 × g for 15 min (15°C). Supernatant containing hydrophobic peptides was evaporated on a rotary apparatus at 50°C to remove isopropyl alcohol. After evaporation, the concentrated hydrophobic peptides were diluted to 90 g/liter with distilled water. The hydrophilic pellets resulting from isopropyl alcohol treatment and centrifugation (see above) were also dissolved in distilled water (90 g/liter).

**Peptide analyses.** Hydrophobic and hydrophilic peptides were hydrolyzed with 6 N HCl (110°C, 24 h) under N₂. After cooling, HCl was removed by rotary evaporation (50°C). Since acid hydrolysis destroys tryptophan, the peptide fractions were also hydrolyzed with 4 N Ba(OH)₂ under N₂ (21). Resulting amino acids were reacted directly with ninhydrin (28) or diluted to 100 μg/ml with citrate buffer (50 mM; pH 2.0). Amino acid derivatives were analyzed by high-pressure liquid chromatography, using a Beckman 344 liquid chromatograph, a model 157 fluorescence detector, and an Interaction AA503 column (lithium form). The sample size was 250 μl, the column temperature was 38°C, and solvent was a gradient of 0.2 M lithium citrate (pH 2.8) and 0.2 M lithium borate (pH 10.0) buffers. The flow rate was 0.5 ml/min, and amino acids leaving the column were derivatized with o-phthalaldehyde (3). The hydrophobic and hydrophilic fractions were also diluted in phosphate buffer (100 mM; pH 7.0) and analyzed directly for UV absorption at 215 and 280 nm (Gilbert model 260 spectrophotometer, 1-cm quartz cuvettes).

**Peptide or amino acid utilization.** Previous work has indicated that there is little growth when ruminal bacteria are provided with peptides or amino acids as the sole carbon and energy sources (4, 29, 32). Since growth is dependent on the presence of carbohydrates, ammonia was the predominant end product of peptide or amino acid uptake and metabolism. Ammonia production by mixed cultures of ruminal bacteria was measured by the colorimetric method of Chaney and Marbach (7). The Kᵣ and Vₘₐₓ of the ruminal bacteria for hydrophilic and hydrophobic peptides were determined under substrate-limiting conditions, but in each case <10% of the peptide nitrogen was converted to ammonia. Since <10% of the substrate was converted to product, the velocity should have been similar to the initial rate of catabolism.

Peptide metabolism by pure cultures was estimated from the growth of the bacteria on carbohydrate (6 g of glucose per liter) with peptides or amino acids as the sole source of nitrogen (1.5 g of N per liter). Since the basal medium contained little if any nitrogen (no ammonia), there was little growth in the absence of added peptides or amino acids and growth provided a reliable estimate of metabolism.

All experiments were performed several times, and the results were reproducible. Much of the potential variation in mixed-culture incubations was alleviated by standardizing the starting cell density to 1.0 optical density unit (see above). The coefficient of variation (standard deviation divided by the mean) was <10%.

**RESULTS**

**Ammonia production.** Since enzyme kinetics are usually related to product formation rather than substrate disappearance, we sought to use ammonia production as an index of peptide metabolism. When an excess of Trypticase (15 g/liter) was provided to mixed ruminal bacteria, ammonia production continued at a nearly linear rate for 36 h (Fig. 1). After 36 h the rate of ammonia production decreased, indicating that by this time substrate had become limiting. Carbohydrates were not added to the incubation medium, and little growth occurred during the 48-h period. Because the amount of nitrogen being deposited as cell protein was insignificant compared to ammonia, ammonia production at 12 h provided a means of assessing peptide uptake and deamination under substrate excess conditions. Preliminary experiments (data not shown) also indicated that ammonia production was proportional to a disappearance (uptake) of ninhydrin-positive material from the cell-free medium.

**Peptides versus free amino acids.** When mixed ruminal bacteria were incubated with Trypticase (a casein hydrolysate containing peptides; 15 g/liter), 391 mg of NH₃ per liter was produced during the 12-h incubation period (Fig. 2). However, when Casamino Acids, an acid digest of casein (15 g/liter), was provided, only 170 mg of NH₃ per liter per 12 h was produced. Since both Trypticase and Casamino Acids are derived from casein, the amino acid profiles should have been approximately the same. Thus, differences in ammonia production must have been due to faster catabolism of peptides than amino acids per se.

**Hydrophobic versus hydrophilic peptides.** When Trypticase was fractionated with isopropyl alcohol and standardized to 90 g of glycine equivalent per liter, using a ninhydrin assay, there was little difference in Aₛ₂₁₅ (Table 1). Since peptide
bonds absorb strongly at 215 nm, it appeared that the two fractions had peptides of similar length. The similarity of peptide size was further supported by comparison of ninhydrin reactions with and without HCl hydrolysis. The hydrophobic fraction (soluble in 90% isopropyl alcohol) gave 3.3 times as much color after hydrolysis, while the hydrophilic fraction gave 3.2 times as much color. Hydrophobic peptides, however, had 2.73 times as much $A_{280}$ as the hydrophilic peptides. These results suggested that the hydrophobic fraction had a greater proportion of the phenolic amino acids, and this conclusion was corroborated by amino acid analyses. The hydrophobic peptides contained large amounts of tryptophan, tyrosine, phenylalanine, and proline as well as the branched-chain amino acids leucine and valine. The hydrophilic peptides contained a larger proportion of the highly charged amino acids arginine, lysine, glutamate, and aspartate.

Ammonia production from the hydrophobic preparation was 289 mg/liter per 12 h, much less than the 646 mg/liter per 12 h from hydrophilic peptides (Fig. 2). Kinetic experiments (Fig. 3) showed that the $V_{\text{max}}$ of the hydrophilic peptides was more than twice that of the hydrophobic peptides, but there was little difference in the affinity of the bacteria for either fraction. These results indicated that ruminal bacteria were able to take up and deaminate hydrophilic peptides at a much faster rate than peptides containing high amounts of phenolic or aliphatic amino acids.

**Peptides versus amino acid mixtures.** Because ammonia production was used as the index of peptide metabolism, deamination, hydrolysis, or uptake could have contributed to the difference between hydrophobic and hydrophilic peptides. To further examine the importance of deaminases in peptide utilization, mixed ruminal bacteria were incubated with free amino acid mixtures that had the same amino acid content as the peptides. In this case, ruminal bacteria produced more (not less) ammonia from the hydrophobic amino acids than the hydrophilic amino acids (Fig. 4). Since peptides and amino acids of the same amino acid content differed greatly in their relative rates of ammonia production, intracellular deamination was probably not the primary factor dictating specificity and the rate of metabolism.

**Pure cultures.** Most ruminal bacteria are unable to grow with peptides or amino acids as the sole energy source (4, 15, 29), and only a small amount of growth was detected in the mixed-culture incubations (Fig. 1). Since growth was minimal, there should have been little enrichment or selection during the incubation period and the microbial composition in vivo should have been similar to the in vitro distribution. To see if individual species differed in their preference for hydrophobic and hydrophilic peptides, pure-culture experiments were also performed (Table 2). When pure cultures of ruminal bacteria were incubated with peptides or amino acids as the sole nitrogen source, growth rate on carbohydrate was dependent on the type of amino acid source

### TABLE 1. Properties of the peptide fractions

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hydrophobic peptides</th>
<th>Hydrophilic peptides</th>
<th>Hydrophobic/ hydrophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{280}^a$</td>
<td>2.35</td>
<td>1.78</td>
<td>1.32</td>
</tr>
<tr>
<td>$A_{280}^b$</td>
<td>0.60</td>
<td>0.22</td>
<td>2.73</td>
</tr>
<tr>
<td>Approx chain length$^c$</td>
<td>3.3</td>
<td>3.2</td>
<td>1.03</td>
</tr>
<tr>
<td>Amino acid composition$^d$</td>
<td>Arg 1.8</td>
<td>5.4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>His 2.7</td>
<td>2.7</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Lys 2.9</td>
<td>11.6</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Tyr 5.9</td>
<td>5.0</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Trp 2.2</td>
<td>0</td>
<td>U$^e$</td>
</tr>
<tr>
<td></td>
<td>Phe 8.0</td>
<td>2.5</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Cys 2.9</td>
<td>2.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Met 4.1</td>
<td>3.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Ser 5.5</td>
<td>6.0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Leu 10.0</td>
<td>6.8</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Ile 6.5</td>
<td>6.2</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Val 8.2</td>
<td>4.8</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Glu 15.3</td>
<td>23.7</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Asp 3.3</td>
<td>8.7</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Gly 1.8</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Ala 2.7</td>
<td>2.9</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Pro 16.2</td>
<td>5.6</td>
<td>2.9</td>
</tr>
</tbody>
</table>

$^a$ Values based on 400 mg of peptide per liter.

$^b$ Values based on 95 mg of peptide per liter.

$^c$ Ratio of ninhydrin reaction after HCl hydrolysis to ninhydrin reaction before HCl hydrolysis.

$^d$ The amino acid values in columns 2 and 3 are percentages.

$^e$ U, Mathematically undefined.

[**FIG. 3.** Lineweaver-Burk plot for uptake and deamination of hydrophobic (●) and hydrophilic (○) peptides. The $V_{\text{max}}$ values of the hydrophobic and hydrophilic peptides were 18.1 and 39.2 μg of NH$_3$ per mg of protein per h, respectively, while the $K_{\text{m}}$s were 0.83 and 0.98 g/liter, respectively.]

[**FIG. 4.** Ammonia production from the two peptide fractions and free amino acids having the same amino acid composition by mixed ruminal bacteria (270 mg of protein per liter) in vitro. The concentration of amino acid or peptides in the culture was 15 g/liter.]
provided. In all cases, the response of the pure cultures was similar to that observed with the mixed cultures. Peptides were metabolized faster than amino acids, and hydrophilic peptides were utilized faster than hydrophobic peptides.

**DISCUSSION**

For many years, it was assumed that free amino acids arose as intermediates in the breakdown of proteins by ruminal microorganisms (6). Free amino acids were always found at low concentrations in ruminal fluid (34), and these low concentrations were hypothetically explained by a rapid uptake of amino acids by ruminal microorganisms (6, 34). Peptides resulting from the initial action of proteases were usually ignored as transient products in the complete degradation of proteins to amino acids.

Recent studies showed that high concentrations of peptides (not amino acids) could accumulate in ruminal fluid in vitro (34) and in vivo (8, 9). These observations indicated that ruminal bacteria degraded dietary protein extracellularly to peptides, and it appeared that peptides rather than free amino acids were the end products of protein degradation (16). This assumption was consistent with previous experiments which detected little peptidase activity in cell-free ruminal fluid (33).

Early studies indicated that a variety of bacteria had separate uptake systems for amino acids and peptides (5, 22, 23). Using radioactive peptides, Kessel and Lubin (19) demonstrated that E. coli concentrated peptides and amino acids at different rates and that the two processes were not competitive. Pittman et al. (25, 26) found that a predominant ruminal bacterium, B. ruminicola, utilized peptide or ammonia nitrogen but that growth was poor when amino acids were the nitrogen source. Our results showed that mixed ruminal bacteria utilized peptides more than twice as fast as free amino acids, even though the overall composition (both from casein) was the same (Fig. 2). These results contradicted the assumption of rapid amino acid uptake by ruminal organisms and supported our hypothesis that ruminal peptidases yielded peptides but not necessarily free amino acids.

Even though the rate of peptide metabolism was much faster than amino acids, peptide uptake and deamination was itself not very rapid. Hino and Russell addressed the rate of peptide metabolism by ruminal microorganisms by comparing the deamination rate of intact and cell-free extracts (15). Because the overall capacity of cell-free extracts was greater than that of intact cells, they suggested that transport rather than deaminating enzymes was limiting. While it is impossible to exclude intracellular hydrolysis as another limiting step, the present results are consistent with the hypothesis that amino acid uptake is even more limiting than peptide uptake.

To gain further insight into the factors limiting peptide uptake, casein hydrolysate was fractionated with isopropyl alcohol. The hydrophobic fraction which dissolved in 90% isopropyl alcohol contained an abundance of phenolic and aliphatic amino acids, while the hydrophilic fraction which was precipitated contained a greater proportion of highly charged amino acids. Both fractions were primarily composed of small peptides, and the chain length was not significantly different. The hydrophilic fraction was deaminated at twice the rate of the hydrophobic fraction, and this comparison indicated that hydrophobicity was a more important factor than chain length in the regulation of peptide metabolism.

Kinetic studies showed that the difference between hydrophilic and hydrophobic peptide uptake was due to a difference in V_max but not K_m (Fig. 3). The K_m for peptides was approximately 1.0 g/liter, an amount nearly 1,000-fold greater than the K_m for some ruminal bacteria for simple sugars (31). On the basis of this comparison, it is no surprise that the ruminal bacterium B. ruminicola B4 was able to grow rapidly on sugar but was unable to take up peptides at a fast enough rate to meet its maintenance energy requirement or growth (29). The V_max values for the hydrophobic and hydrophilic fractions were 0.018 and 0.039 mg of NH_3 per mg of protein per h. Assuming that ammonia production was indicative of peptide nitrogen uptake, 82% nitrogen in NH_3 and 5.13 (6.25 x 0.82) g of protein per g of NH_3, the specific rates of nitrogen uptake would have been 0.09 and 0.20 h⁻¹ for hydrophobic and hydrophilic peptides, respectively. These latter values are less than the maximum specific growth rates of many ruminal bacteria but do not consider the fact that some ruminal bacteria can use ammonia but not peptide nitrogen for growth (1). The inability of some ruminal bacteria to use peptide nitrogen would lead to an underestimation of the maximum specific rate of nitrogen uptake.

Alves et al. (2) recently pointed out that the outer membrane of gram-negative bacteria such as E. coli can form a barrier to hydrophobic molecules while allowing the passage of small (<700-dalton) hydrophilic molecules. However, in our studies S. bovis, a gram-positive bacterium lacking an outer membrane, showed the same sort of specificity for hydrophilic versus hydrophobic peptides as the mixed cultures or gram-negative ruminal bacteria (Table 2). Based on this observation, the outer membrane was probably not the major barrier to hydrophobic peptides. In E. coli and Salmonella typhimurium, peptide permeases have periplasmic binding proteins which facilitate transport (12, 14). "Shock sensitive" peptide transport systems have not been demonstrated in ruminal bacteria, but it is possible that these periplasmic proteins could be involved in peptide specificity. Whether hydrophobic and hydrophilic peptides are transported by the same uptake system is still unclear, but the similarity of the K_m values may be more than a coincidence.

Mixed ruminal bacteria had a specific activity of 31 nmol of NH_3 per mg of protein per min (15), but the rate of ammonia production by pure cultures of ruminal bacteria is

<table>
<thead>
<tr>
<th>Strain</th>
<th>Trypticase</th>
<th>Casamino Acids</th>
<th>Hydrophilic peptides</th>
<th>Hydrophobic peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. bovis JB1</td>
<td>1.54 ± 0.05</td>
<td>0.82 ± 0.03</td>
<td>1.49 ± 0.04</td>
<td>1.15 ± 0.04</td>
</tr>
<tr>
<td>B. ruminicola B4</td>
<td>0.82 ± 0.08</td>
<td>0.31 ± 0.02</td>
<td>0.83 ± 0.01</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>S. ruminantium HD4</td>
<td>0.63 ± 0.00</td>
<td>0.46 ± 0.00</td>
<td>0.66 ± 0.00</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>M. elsdenii B159</td>
<td>0.41 ± 0.02</td>
<td>0.26 ± 0.04</td>
<td>0.38 ± 0.01</td>
<td>0.29 ± 0.00</td>
</tr>
</tbody>
</table>

* Maximum specific growth rate during exponential growth (per hour).
* All incubations contained glucose (6 g/liter) and either peptides or amino acids (15 g/liter) as the only nitrogen source.
much less (4). Indeed, B. ruminicola B14, one of the most active ammonia-producing bacteria isolated from the rumen (4), produced <3 nmol of NH₃ per mg of protein per min (29). The failure of pure cultures to produce more ammonia has been explained (4, 29). Since pure cultures were unable to deaminate amino acids at a rapid rate, growth with peptides or amino acids as the sole nitrogen source was used as an index of peptide metabolism. All of the pure cultures metabolized peptides faster than free amino acids and hydrophilic peptides faster than hydrophobic peptides, but results for B. ruminicola most closely mimicked the differences observed with mixed cultures (Fig. 2; Table 2). All of these bacteria are important peptide utilizers (10), but Bladen et al. concluded that “on the basis of number of strains and amount of ammonia produced, Bacteroides ruminicola is usually the most important ammonia-producing bacterium in the rumen of mature cattle” (4).

Rates of ammonia production are often very great in vivo (17), but the specific activity of mixed cultures of ruminal microorganisms is not very great (4, 15, 16, 29). It seems apparent that ammonia accumulation in the rumen is due to the high density of microorganisms rather than the high capacity of individual cells to deaminate amino acid sources. Because the specific uptake rate is not very fast, ammonia is only produced in large amounts when carbohydrate, and hence ATP, limits growth. If carbohydrates allowed a growth rate of 0.07 h⁻¹ or greater, most of the transported peptides were incorporated into microbial protein and did not enter the ammonia pool (32). The influence of hydrophobicity on peptide uptake provides a new insight on ruminal nitrogen metabolism, and this specificity could influence the composition of amino acids passing to the lower gut.

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LITERATURE CITED