Effects of Amino Acids and Peptides on Rumen Microbial Growth Yields

J. L. ARGYLE¹ and R. L. BALDWIN²
Department of Animal Science
University of California
Davis 95616

ABSTRACT
Experiments were conducted using mixed rumen bacterial cultures to determine which amino acids limited growth. Complete amino acid mixtures stimulated microbial growth alone and when added to casein. Amino acid subgroups did not stimulate growth alone or when added to casein or casein hydrolysates. Results were similar whether growth was limited by periodic addition of low amounts of carbohydrate or when higher amounts were added to batch cultures. Little growth occurred with ammonia as sole N source. Addition of 100 mg/L of amino acids and peptides quadrupled growth. Peptides at 10 mg/L resulted in higher growth than the corresponding amount of free amino acids. Apparent saturation of growth occurred when 10 mg/L of a complete amino acid mixture or trypicase was added to cultures. The Michaelis constant values for amino acids and trypicase were determined to be .5 and 1.0 mg/L, respectively. Growth was a linear function of amount of carbohydrate fermented with the coefficient of slope increasing with increasing amino acid concentrations. These experiments demonstrate that growth stimulation from amino acids and proteins is due to the number of amino acids provided in a given mixture rather than specific growth limiting amino acids. Rumen bacterial growth is greatly stimulated by amino acids and peptides, with low affinity constant values, allowing good growth in the concentrations of amino acids and peptides found in vivo.

INTRODUCTION
Dietary proteins are partially degraded in the rumen producing peptides, amino acids, VFA, and ammonia (3, 11). This process has been considered wasteful to the animal (3), a view overlooking the role degradation products play in the nutrition of ruminal microorganisms. Quantitation of ammonia requirements of rumen bacteria has received considerable attention (27, 35, 36), but stimulation in cell yields resulting from amino acids and peptides has been more qualitative (4, 7, 17, 25, 26). The observation that 82% of bacterial strains isolated from one animal were capable of growth with ammonia as a sole N source has often been quoted without recognition that two-thirds of those strains could also use trypicase as a sole N source (7). Amino acids and casein hydrolysates often stimulate growth rates and yields even when ammonia and VFA exceed requirements (7, 12, 25, 26, 32, 33, 34). Many species do not utilize ammonia when trypicase is provided (8). In vivo, 20 to 50% of microbial N arises from feed or endogenous N without passing through the rumen ammonia pool (1, 20). Increased microbial yields were observed in vivo when protein was added to purified diets (4, 17). Protein added to poor quality diets has increased microbial protein synthesis and protein flow from the rumen (2).

Work in vivo, in vitro, and with pure cultures has shown that some amino acids were rapidly removed from culture or rumen fluid while others accumulated (9, 22, 37), implying that a preference for certain amino acids exists. Several species of rumen bacteria require specific amino acids for growth (15, 16, 18). Work

¹Submitted in partial fulfillment of requirements for Ph.D. degree. Present address: Department of Biochemistry and Nutrition, University of New England, Armidale, NSW 2351, Australia.
²Corresponding author.
in vitro with mixed cultures indicated that certain amino acids or groups of amino acids stimulated microbial growth more than others (26). Some bacterial strains require amino acids in peptide form (30, 31), and work in vitro has shown that peptide carbon is more efficiently utilized than amino acid carbon (38). Work with purified diets has shown that protein stimulation of microbial yields in vivo varies with protein source (4, 17), implying that amino acid composition is important. The present experiments were designed 1) to determine which amino acids stimulated microbial growth in cultures containing ammonia, 2) to determine if synergism existed between amino acid stimulation and peptide stimulation, and 3) to determine amounts of amino acids and peptides that limited mixed culture growth.

MATERIALS AND METHODS

Animals and Management

Mature lactating Holstein cows were fed a diet consisting of 50% alfalfa hay cubes, 14% whole cottonseed, and 35% of a grain mixture consisting of 35% rolled barley, 35% rolled corn, 20% cottonseed meal, 3% fat, and minerals. Fresh feed was offered for ad libitum intake twice daily following milking.

Inoculum Preparation

One liter of medium contained 240 mg K2HPO4·3H2O, 240 mg KH2PO4, 240 mg (NH4)2SO4, 480 mg NaCl, 100 mg MgSO4·7H2O, 64 mg CaCl2·2H2O, 1 mg resazurin, 1 mg hemin, 2.5 mg 1,4-napthoquinone, 5 mmol acetate, and .2 mmol each of propionate, isobutyrate, isovalerate, 2-methyl butyrate, and valerate, vitamins, and minerals. Hemin and 1,4-napthoquinone were dissolved in separate solutions by slow addition of NaOH while stirring. A solution containing vitamins in concentrations as described (23) without 1,4-napthoquinone was prepared in advance without sterilization and kept frozen until use. Trace mineral concentrations were as described (23) without Na2CO3. The medium was neutralized, brought to a boil under O2-free CO2, and cooled on ice. Then 4 g/L of Na2CO3 were added and the solution bubbled with CO2 until the Na2CO3 dissolved. A solution of Na2S was then added (.5 g Na2S·9H2O/L, final concentration).

Rumen contents were collected via permanent rumen fistulas just before the afternoon milking (10 h after feeding). Contents were filtered through four layers of cheesecloth and the filtrate was returned to the laboratory. All subsequent steps were performed under a stream of O2-free CO2 gas. Anaerobic centrifuge tubes (Ivan Sorval) were used. Filtrate was centrifuged to remove feed particles and protozoa (268 x g, 10 min, 4°C). Bacterial cells were harvested (5856 x g, 10 min, 4°C) and washed in the medium described. Washed bacterial pellets were added to inoculum buffer until an optical density (A600nm) of .2 was attained.

Experiment 1

The objectives of Experiment 1 were 1) to determine if the growth response observed with casein hydrolysates could be replaced with groups of free amino acids at similar concentrations, and 2) to determine if any groups of amino acids stimulated growth when added to casein hydrolysates at concentrations similar to their respective concentrations in casein. Amino acid mixtures were prepared such that each group contained amino acids equal to the amount (moles) of those amino acids present in a 25 g/L casein solution. The compositions of the groups were (mg/L) basic: Lys·HCl, 918.5, Arg·HCl, 453.9, Glu, 734.8, Asn·H2O, 431.0; usable nitrogen: Asp·5H2O, 390.3, Asn·H2O, 431, Glu·Na, 1320.9, Glu·H2O, 374.8; aromatic: Tyr, 651.2, Phe, 474.5, His·HCl·H2O, 376.7, Trp, 146.5; Neutral: Thr, 213.7, Ser, 605, Pro, 702.6, Gly, 243.0, Ala, 288.1, Val, 462.5, Ile, 518.2, Leu, 801.3; Sulfur-met, 268.0; Carboxylic-Asp·5H2O, 390.3, Glu·Na, 1320.9. Roller culture tubes (Bellco, Fineland, NJ) and 00 butyl rubber stoppers with center wells (A. H. Thomas, Swedesboro, NJ) were used. To each culture tube, 1 mL of a carbohydrate mixture containing 1 mg each of glucose, cellobiose, pectin, and soluble starch was added (.1 mg/L each, final concentration). Culture tubes containing only amino acids received .5 mL of the respective stock solution. The complete mixture received .5 mL from all amino acid group stock solutions except the usable N group (final con-
centrations in cultures containing only amino acids are 1/20 of stock values). Culture tubes containing amino acids and peptides or protein received .25 ml of respective amino acid stocks (final concentrations of amino acids are 1/40 of stock values) and .25 ml (2.5 mg) of one of casamino acids, trypticase, or casein (final concentrations of 25 mg/L). Volumes were adjusted to 2.6 ml with H2O, and 7.4 ml of inoculum were added anaerobically. Cultures were stoppered and incubated in a 37°C water bath. Optical density (A600nm) was recorded hourly using a Bausch & Lomb Spectronic 20 spectrophotometer (Rochester, NY). Carbohydrate solution (.1 ml) was injected through the stoppers each hour following optical density readings. Experimental treatments were run on triplicate cultures. Limitations in numbers of samples that could be conveniently handled resulted in one batch of inoculum being used for amino acids and for amino acids plus casamino acids, whereas amino acids plus trypticase or plus casein were run 2 d later using fresh inoculum.

Experiment 2

The objectives of Experiment 2 were to determine if groups of amino acids were stimulatory when alone and when with casein hydrolysates by adding equal amounts (weights were used for convenience) of each amino acid and equal numbers of amino acids in each subgroup, except for the sulfur group, which contained only two amino acids. The basic, usable N, and aromatic groups contained the same amino acids as in Experiment 1. The neutral group contained only Thr, Ser, Pro, and Ala. The sulfur group contained Met and Cys. A branched chain group contained Val, Ile, Leu, and Gly (to make four amino acids in this group). Stock solutions contained 6.25 g/L of each amino acid except for the complete mixture, which contained 1.2 g/L of each of the 20 amino acids. Solutions of casamino acids, trypticase, and casein contained 25 g/L. The carbohydrate mixture contained 2.5 g glucose, 5 g cellobiose, 20 g starch, and 2.5 g pectin/L. Working solutions were prepared by combining carbohydrate mixture, appropriate amino acid stocks, peptide solution, or protein solution, and water, resulting in concentrations of 15 mg carbohydrate, 6.25 mg amino acids, and 6.25 mg of peptides or protein (if present) per ml. Each culture received 10 ml of inoculum. Optical density was recorded on triplicate cultures as in Experiment 1, except that after each hourly reading stoppers were removed and 200 μl of working solution were added anaerobically (producing final concentrations of .3 g/L carbohydrate and .125 g/L amino acids and peptides or protein).

Experiment 3

The objective of Experiment 3 was to determine if differences in growth responses to groups of amino acids could be observed if the cultures had lower concentrations of amino acids and more carbohydrate for fermentation than had been used in the two previous experiments. Amino acid groups contained 62.5 mg/L of each amino acid (to produce approximately 5 x 10^-6 M final concentrations). The groups were basic: Lys-HCl and Arg-HCl; neutral: Thr, Ser, Pro, Gly; usable N: Asp .5H2O, Asn-H2O, Glu -Na, Gln, Ala; sulfur: Met and Cys; branched-chain: Val, Ile, Leu; aromatic: Phe, Tyr, Trp, His-HCl-H2O. Casein solution contained 1.25 g/L and was neutralized to pH 7.5 with KOH. The carbohydrate solution contained 1 g pectin, 3 g xylan, 1.3 g starch, and 3 g cellulose (as 2% cellulose slurry pebble milled for 24 h) per L. Each culture tube received .1 ml of amino acid stock except that the complete mixture received .1 ml of each solution (final concentration of each amino acid was .625 mg/L), 1.0 ml of casein (if added; final concentration of 125 mg/L), and either .25, .5, .75, or 1.0 ml of rapidly stirring carbohydrate solution (to produce final concentrations of .5, 1, 1.5, or 2 g/L carbohydrate, respectively). Culture volumes were adjusted to 2.7 ml with H2O, flushed with CO2, stoppered, and refrigerated overnight. On the day of the experiment, 7.3 ml of inoculum were added. Cultures were incubated at 37 °C for 8 h and then frozen until analyzed for RNAase as described. Cultures containing only amino acids were run using one batch of inoculum, while cultures containing casein were run with a second batch of inoculum the following day.
**Experiment 4**

The objective of Experiment 4 was to determine concentrations of amino acids and peptides that limited bacterial growth. The carbohydrate mixture contained 4 g/L each of glucose, maltose, sucrose, cellobiose, and xylose. The amino acid mixture contained 50 mg/L each of all 20 amino acids. The trypticase solution contained 1 g/L. Dilutions of amino acid and trypticase solutions were made to produce solutions containing 1 (stock), .1, and .01 g/L. Culture tubes received as needed, an amino acid solution, trypticase solution, and a variable amount of carbohydrate solution. The volumes were adjusted to 3.0 ml with H2O. The experimental design was a three-dimensional block containing four amino acid mixtures (final concentrations of 0, 1, 10, and 100 mg/L), four amounts of trypticase (final concentrations of 0, 1, 10, and 100 mg/L), and seven amounts of carbohydrate (final concentrations of 0, .25, .5, .75, 1.0, 1.5, and 2.0 g/L) for a total of 112 individual cells. Each cell contained duplicate cultures. Culture tubes were flushed with CO2, stoppered, and refrigerated overnight. On the morning of the experiment, 3.5 ml of medium were added to each tube, and after resazurin was reduced, 3.5 ml of inoculum were added. Cultures were incubated at 37°C for 6 h and then frozen until analyzed for RNA.

**Ribonucleic Acid Analysis**

Frozen culture tubes containing bacteria were thawed at 25°C, stoppers were removed, and 1 ml of 3.3 N HClO4 was added with mixing. Acidified samples were transferred to centrifuge tubes and centrifuged (5000 x g, 4°C, 20 min). Spent culture medium was discarded and 1.0 ml of .3 N KOH was added to pellets. Pellets were incubated at room temperature with occasional mixing until dispersed, and then incubated in a 37°C waterbath for 60 min with gentle agitation. Tubes were placed in an ice slush and .4 ml of 1.2 N HClO4 was added. The tubes were kept on ice for 10 min and then centrifuged (5000 x g, 4°C, 20 min). The supernatant was withdrawn and saved. Pellets were washed once with 2 ml of ice cold .2 N HClO4, and supernatants were combined with previous supernatants. Absorbance at 260 nm was measured on combined supernatants using a Gilford 240 spectrophotometer (Oberlin, OH).

**TABLE 1. Bacterial growth1 after 5 h of incubation with amino acid groups equaling their respective concentrations in casein hydrolysates and casein (Experiment 1).**

<table>
<thead>
<tr>
<th>Peptide source2</th>
<th>Amino acids only</th>
<th>Casimino acids</th>
<th>Trypticase</th>
<th>Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic</td>
<td>.54 ab</td>
<td>.65 a</td>
<td>.56 ab</td>
<td>.50 a</td>
</tr>
<tr>
<td>Usable</td>
<td>.55 ab</td>
<td>.65 a</td>
<td>.56 ab</td>
<td>.53 abc</td>
</tr>
<tr>
<td>Aromatic</td>
<td>.56 b</td>
<td>.64 ab</td>
<td>.57 a</td>
<td>.52 c</td>
</tr>
<tr>
<td>Neutral</td>
<td>.56 b</td>
<td>.64 ab</td>
<td>.56 ab</td>
<td>.56 bc</td>
</tr>
<tr>
<td>Sulfur</td>
<td>.55 ab</td>
<td>.63 ab</td>
<td>.55 ab</td>
<td>.54 bc</td>
</tr>
<tr>
<td>Complete</td>
<td>.62 c</td>
<td>.62 b</td>
<td>.56 ab</td>
<td>.59 d</td>
</tr>
<tr>
<td>Peptides only</td>
<td>. . .</td>
<td>.64 ab</td>
<td>.54 b</td>
<td>.55 bc</td>
</tr>
<tr>
<td>Ammonia only4</td>
<td>. . .</td>
<td>.55 c</td>
<td>.42 c</td>
<td>.40 d</td>
</tr>
</tbody>
</table>

* Means in the same column not sharing a common superscript differ (P<.05). Columns with the same superscript shared the same batch of inoculum. Because two batches of inoculum were used, comparisons across rows are not valid.

1Growth was limited by hourly addition of a carbohydrate solution resulting in (.1 g/L each, final concentration) glucose, cellobiose, starch, and pectin.

2 Means of triplicate cultures.

3 Amino acid groups contained basic: Lys, Arg, Gln, Asn; usable N: Asp, Asn, Glu, Gln; aromatic: Tyr, Phe, Trp, His; neutral: Thr, Ser, Pro, Gly, Ala, Val, Ile, Leu; sulfur: Met. See text for concentrations. All amino acids were added at the beginning of incubation.

4 All samples contained ammonia. The ammonia only sampled did not contain added amino acids or peptides.
Statistical Analysis

One-way ANOVA for Experiments 1 and 2 was performed using BMDP1V (BMDP Statistical Software, Inc., Los Angeles, CA). Two way ANOVA on Experiment 3 was performed using BMDP2V. Treatments means were compared using Tukey's multiple comparisons (29). The RNA yields in Experiment 4 were regressed on carbohydrate added, and the resulting slopes compared by analysis of regression (29). Estimates of Ymax maximum growth rate and affinity constant from Experiment 4 were obtained using the Simplex routine (28).

RESULTS

Experiment 1

Addition of the complete amino acid group stimulated microbial growth more than controls containing only ammonia and to subgroups (P<.05; Table 1). There were no differences between amino acid subgroups. Only the aromatic and neutral groups showed increases (P<.05) in growth over that caused by ammonia. Although the increases were statistically significant, they were small and probably not physiologically significant. Casein and casein hydrolysates stimulated growth when compared with ammonia (P<.0001). When casamino acids were added, no combinations of additional amino acids, including the complete mixture, were different from casamino acids alone (Table 1). The neutral amino acid group proved more stimulatory than only trypticase (Table 1). Again the difference was small and probably not physiologically significant. When casein was present, the complete mixture was more stimulatory than protein alone or protein plus any other mixture of amino acids (Table 1). No subgroup added any stimulation to that produced by casein alone.

Experiment 2

When only amino acids were added, only the complete mixture was different from ammonia (P<.01). No other groups of amino acids were different from ammonia alone (Table 2). When casamino acids were present, addition of the complete mixture provided added growth stimulation (P<.05), but no amino acid subgroup was different from casamino acids alone. Trypticase and casein stimulated growth when compared with that from ammonia (P<.05; Ta-

### TABLE 2. Bacterial growth1 after 5 h with equal weights of amino acids in each group (Experiment 2).

<table>
<thead>
<tr>
<th>Peptide source</th>
<th>Amino acids only</th>
<th>Casimino acids</th>
<th>Trypticase</th>
<th>Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic</td>
<td>.49a</td>
<td>.50bc</td>
<td>.74a</td>
<td>.84a</td>
</tr>
<tr>
<td>Usable</td>
<td>.48a</td>
<td>.50bc</td>
<td>.79ab</td>
<td>.80ab</td>
</tr>
<tr>
<td>Aromatic</td>
<td>.40a</td>
<td>.51b</td>
<td>.79a</td>
<td>.71c</td>
</tr>
<tr>
<td>Neutral</td>
<td>.48a</td>
<td>.49bc</td>
<td>.77bc</td>
<td>.83a</td>
</tr>
<tr>
<td>Branched</td>
<td>.46a</td>
<td>.48bc</td>
<td>.79ab</td>
<td>.74bc</td>
</tr>
<tr>
<td>Sulfur</td>
<td>.46a</td>
<td>.50bc</td>
<td>.75bc</td>
<td>.73bc</td>
</tr>
<tr>
<td>Complete</td>
<td>.55b</td>
<td>.56a</td>
<td>.75bc</td>
<td>.84a</td>
</tr>
<tr>
<td>Peptides only</td>
<td>...</td>
<td>.51b</td>
<td>.75bc</td>
<td>.83a</td>
</tr>
<tr>
<td>Ammonia only</td>
<td>.47a</td>
<td>.47c</td>
<td>.47d</td>
<td>.47d</td>
</tr>
</tbody>
</table>

a,b,c,dMeans in the same column not sharing a common superscript differ (P<.05). Columns sharing the same superscript shared common inoculum. Because two batches of inoculum were used, comparisons across rows are not valid.

1Growth was limited by hourly addition of a carbohydrate solution producing (g/L final concentration) .05 glucose, .1 cellobiose, .05 pectin, and .4 starch. 2Means of triplicate cultures.

3Amino acid groups contained basic: Lys, Arg, Glu, Asn; usable N: Asp, Asn, Glu, Gln; aromatic: Tyr, Phe, Trp, His; neutral: Thr, Ser, Pro, Ala; branched chain: Gly, Ile, Val, Leu; sulfur: Met, Cys. Amino acids were added each hour to produce 124 mg/L (final concentration) of each amino acid in sub groups and 6.25 mg/L (final concentration) of each amino acid in the complete mixture. Peptides were added hourly at 125 mg/L (final concentration).

4All samples contained ammonia. The ammonia only sampled did not contain added amino acids or peptides.

Journal of Dairy Science Vol. 72, No. 8, 1989
### TABLE 3. Batch culture growth\(^1\) after 8-h incubations with amino acids (Experiment 3).

<table>
<thead>
<tr>
<th>Amino acid group(^2)</th>
<th>Carbohydrate, g/L.(^3) (µg RNA/culture)</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic</td>
<td></td>
<td>291</td>
<td>345</td>
<td>541</td>
<td>526</td>
<td>425.9(^a)</td>
</tr>
<tr>
<td>Neutral</td>
<td></td>
<td>258</td>
<td>343</td>
<td>534</td>
<td>460</td>
<td>398.6(^a)</td>
</tr>
<tr>
<td>Usable</td>
<td></td>
<td>293</td>
<td>387</td>
<td>551</td>
<td>541</td>
<td>443.0(^a)</td>
</tr>
<tr>
<td>Sulfur</td>
<td></td>
<td>279</td>
<td>380</td>
<td>528</td>
<td>482</td>
<td>417.3(^a)</td>
</tr>
<tr>
<td>Branched</td>
<td></td>
<td>288</td>
<td>381</td>
<td>544</td>
<td>550</td>
<td>440.6(^a)</td>
</tr>
<tr>
<td>Aromatic</td>
<td></td>
<td>289</td>
<td>339</td>
<td>562</td>
<td>569</td>
<td>439.8(^a)</td>
</tr>
<tr>
<td>Complete</td>
<td></td>
<td>295</td>
<td>472</td>
<td>703</td>
<td>652</td>
<td>530.5(^b)</td>
</tr>
<tr>
<td>NH(_4) Only(^4)</td>
<td></td>
<td>279</td>
<td>343</td>
<td>556</td>
<td>517</td>
<td>423.8(^a)</td>
</tr>
<tr>
<td>Means</td>
<td></td>
<td>284.1(^c)</td>
<td>373.7(^d)</td>
<td>564.9(^e)</td>
<td>471.2(^e)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a,b,c,d,e\) Means in the same column not sharing a common superscript differ (\(P<.05\)). Means in the same row not sharing a common superscript differ (\(P<.01\)).

\(^1\)Growth was measured as micrograms RNA per culture.

\(^2\)Amino acid groups contained: basic: Lys and Arg; neutral: Thr, Ser, Pro, Gly; usable N: Asp, Asn, Glu, Gln, Ala; sulfur: Met, Cys; branched chain: Val, Leu, Ile; aromatic: Phe, Tyr, Trp, His. Final concentration of each amino acid was \(0.625 \text{ mg/L}\).

\(^3\)Carbohydrate mixture present at the beginning of the incubation consisted of 12\% pectin, 36\% xylan, 36\% cellulose, and 16\% starch.

\(^4\)All samples contained ammonia. The ammonia only samples did not contain added amino acids or peptides.

### TABLE 4. Microbial growth\(^1\) after 8-h incubations with amino acids plus casein (Experiment 3).

<table>
<thead>
<tr>
<th>Amino acid group(^2)</th>
<th>Carbohydrate, g/L.(^3) (µg RNA/culture)</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic</td>
<td></td>
<td>202</td>
<td>376</td>
<td>341</td>
<td>409</td>
<td>332.1(^a)</td>
</tr>
<tr>
<td>Neutral</td>
<td></td>
<td>211</td>
<td>350</td>
<td>340</td>
<td>405</td>
<td>326.7(^a)</td>
</tr>
<tr>
<td>Usable</td>
<td></td>
<td>204</td>
<td>407</td>
<td>353</td>
<td>474</td>
<td>359.4(^a)</td>
</tr>
<tr>
<td>Sulfur</td>
<td></td>
<td>250</td>
<td>384</td>
<td>365</td>
<td>443</td>
<td>360.7(^a)</td>
</tr>
<tr>
<td>Branched</td>
<td></td>
<td>213</td>
<td>390</td>
<td>331</td>
<td>457</td>
<td>351.9(^a)</td>
</tr>
<tr>
<td>Aromatic</td>
<td></td>
<td>240</td>
<td>395</td>
<td>378</td>
<td>442</td>
<td>363.7(^a)</td>
</tr>
<tr>
<td>Complete</td>
<td></td>
<td>202</td>
<td>416</td>
<td>474</td>
<td>563</td>
<td>413.9(^c)</td>
</tr>
<tr>
<td>Peptide only(^4)</td>
<td></td>
<td>221</td>
<td>382</td>
<td>379</td>
<td>445</td>
<td>356.9(^a)</td>
</tr>
<tr>
<td>NH(_4) Only(^4)</td>
<td></td>
<td>198</td>
<td>301</td>
<td>296</td>
<td>342</td>
<td>284.3(^b)</td>
</tr>
<tr>
<td>Means</td>
<td></td>
<td>215.6(^d)</td>
<td>377.9(^e)</td>
<td>363.9(^e)</td>
<td>442.4(^f)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a,b,c,d,e,f\) Means in the same column not sharing a common superscript differ (\(P<.05\)). Means in the same row not sharing a common superscript differ (\(P<.01\)).

\(^1\)Growth was measured as micrograms RNA per culture.

\(^2\)Amino acid groups contained: basic: Lys and Arg; neutral: Thr, Ser, Pro, Gly; usable N: Asp, Asn, Glu, Gln, Ala; sulfur: Met, Cys; branched chain: Val, Leu, Ile; aromatic: Phe, Tyr, Trp, His. Final concentration of each amino acid was \(0.625 \text{ mg/L}\). Casein final concentration was 125 mg/L.

\(^3\)Carbohydrate mixture present at the beginning of the incubation consisted of 12\% pectin, 36\% xylan, 36\% cellulose, and 16\% starch.

\(^4\)All samples contained ammonia. The ammonia only samples did not contain added amino acids or peptides.
Figure 1. Microbial growth (μg RNA/culture) after 8-h incubations with casein and amino acid groups at different carbohydrate concentrations (Experiment 3). Ammonia as sole N source (X); ammonia plus casein (C); ammonia, casein, and amino acid subgroups (O); ammonia, casein, and a complete mixture of amino acids (Δ).

Table 1. Amino acids did not provide additional stimulation to growth when trypsinase or casein were present.

Experiment 3

There was a significant effect of carbohydrates on microbial growth (Tables 3 and 4). When only amino acid groups were added, .5, 1.0, and 1.5 g/L of carbohydrate differed from one another (P<.01), but 2.0 g/L did not differ from 1.5 g/L (Table 3). When casein plus amino acid groups were added, 1.0 and 1.5 g/L did not differ, but both differed from the .5 and 2.0 g/L treatments (Table 4). Visual inspection of incubated cultures revealed that cellulose hydrolysis varied from complete at .5 g/L of carbohydrate to minimal at 2.0 g/L.

When only amino acid groups were added, the complete mixture stimulated growth when compared with that from ammonia (Table 3). No subgroup stimulated growth more than ammonia. There were no interactions between carbohydrates and amino acid groups. Casein stimulated growth with growth from ammonia whether amino acid groups were present or not (Table 4). Only the complete mixture stimulated growth further when casein was present. A significant interaction was detected between amino acid groups and carbohydrate concentrations (Figure 1). Cultures containing .5 g/L carbohydrate showed little response to added amino acids or protein. Casein stimulated growth at higher concentrations of carbohydrate. Subgroups of amino acids provided no additional stimulation, but the complete mixture of amino acids further stimulated growth when added to cultures containing casein (Figure 1).

Experiment 4

Amino acids and peptides increased microbial growth when compared with that from ammonia controls (Table 5, Figure 2). The highest amounts of amino acids and peptides produced the greatest total microbial growth, but greatest relative increases occurred at low amounts. When compared with ammonia controls, addition of 1 mg/L each of amino acids and peptides increased microbial growth over 2-fold, whereas 10 and 100 mg/L of each improved microbial growth over ammonia 3-fold and 4-fold, respectively (Figure 2).

When growth of cultures (RNA) was regressed on carbohydrate added, there were no differences between peptides and amino acids at 1 or at 100 mg/L, but differences in growth and yield (μg RNA/mg carbohydrate) were found at 10 mg/L. Addition of either 1 mg/L amino acids or 1 mg/L peptides resulted in similar mean growth (Table 5) and similar growth at higher concentrations of carbohydrate. Subgroups of amino acids provided no additional stimulation, but the complete mixture of amino acids further stimulated growth when added to cultures containing casein (Figure 1).
TABLE 5. Microbial growth after 6-h incubations with varying concentrations of amino acids and peptides.

<table>
<thead>
<tr>
<th>Amino acid concentrations (mg/L)</th>
<th>Trypticase concentrations, mg/L (μg RNA/culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 1 10 100</td>
</tr>
<tr>
<td>0</td>
<td>110.1 170.9 219.5 267.0</td>
</tr>
<tr>
<td>1</td>
<td>169.8 185.7 216.3 262.5</td>
</tr>
<tr>
<td>10</td>
<td>197.4 199.4 225.0 267.6</td>
</tr>
<tr>
<td>100</td>
<td>262.8 268.8 272.8 296.8</td>
</tr>
</tbody>
</table>

1Growth was measured as micrograms RNA per culture.
2Data are means of all carbohydrate concentrations.
3All samples contained ammonia as a nitrogen source.

slopes and intercepts when regressed on carbohydrate concentration (data not shown). Addition of 100 mg/L of amino acids (plus 0, 1, or 10 mg/L peptides) resulted in similar mean growth and identical regressions of carbohydrate to RNA values to those obtained with addition of 100 mg/L peptides (plus 0, 1, or 10 mg/L amino acids; Table 5; regressions not shown). However, 10 mg/L peptides (plus 0 or 1 mg/L amino acids) produced higher mean growth than 10 mg/L amino acids (plus 0 or 1 mg/L peptides; Table 5), and peptides at 10 mg/L plus 0 or 1 mg/L amino acids improved microbial growth yields more (21.0 μg RNA/mg carbohydrate) than 10 mg/L amino acids plus 0 or 1 mg/L peptides (18.3 μg RNA/mg carbohydrate) although the significance was marginal (P = .105).

Growth at low concentrations of amino acids plus peptides appears to follow Michaelis-Menten type kinetics (Figure 3). Although growth appears to be maximal when 10 mg/L of peptides were added (Figure 3), a 10-fold increase produced further growth (Table 5). The concentrations at which growth was determined to be one-half maximal were .547 and 1.03 mg/L of amino acids and peptides, respectively. The actual one-half maximal value determined depended on amount of carbohydrate added (Figure 4).

Microbial growth was directly related to amount of carbohydrate added (Figure 2).

Figure 3. Mean growth response over all carbohydrate concentrations to initial amounts of amino acids and trypticase (Experiment 4). Equal amounts of amino acids plus trypticase (○); amino acids only (□); 10 mg/L amino acids plus 1 mg/L trypticase (■); trypticase only (▲); 10 mg/L trypticase plus 1 mg/L amino acids (▲).

Figure 4. Concentrations of amino acids plus peptides that were determined to produce one-half of the maximal growth response obtained by 10 mg/L each of amino acids plus peptides (Experiment 4).
Amino acids and peptides acted as multipliers of growth, improving growth by a given factor at all concentrations of carbohydrate without affecting intercept values.

**DISCUSSION**

Experiments 1, 2, and 3 were assigned to explain increased growth seen when peptides are added to mixed cultures in terms of specific growth-limiting amino acids. In experiment 1, amounts of individual groups of amino acids in casein were doubled by addition of free amino acids. Experiment 2 used equal amounts of all amino acids so that the growth response would be equal for each amino acid. Experiment 3 used higher carbohydrate concentrations to increase growth and therefore demand for amino acids and used lower concentrations of amino acids. In spite of the different growth conditions used in the three experiments, subgroups of free amino acids did not stimulate microbial growth when added alone, nor when added to peptides or protein. Only a complete mixture of free amino acids stimulated growth. The kinetic data obtained in Experiment 4 established that the amino acid concentrations used in Experiments 1 to 3 were not limiting. Together with previous work (26), these results indicate that degree of stimulation depends more on number of amino acids or completeness of a mixture than on specific amino acids or amino acid groups. These results also compare well with results obtained in vivo where gelatin did not stimulate growth while casein and zein did (17).

Experiment 4 clearly demonstrates that availability of amino acids and peptides is an extremely important factor affecting rumen microbial growth. Very small quantities of amino acids or peptides greatly increased microbial growth over that obtained with ammonia as sole N source. Previous work has shown that small amounts of (31 mg/L) of trypsin greatly stimulate microbial yields (12, 33), but we are not aware of any work using concentrations as low as 1 mg/L.

Amino acids, especially valine, leucine, isoleucine, and proline, or peptides containing these amino acids, can be fermented, forming VFA (acetate, isobutyrate, isovalerate, 2-methyl butyrate, and valerate) and ammonia, which are stimulatory to bacterial growth (7, 14, 32, 33). In these experiments, VFA and ammonia were provided in excess of requirements (33, 35, 36). Amino acids can also be fermented as energy sources, but growth of rumen microorganisms on amino acids is negligible (5, 32, 34). Amino acids improved efficiency of microbial growth without altering intercept values (Figure 2), whereas an additional energy source would have increased intercept values. Thus, amino acids and peptides stimulated microbial growth as amino acid sources rather than as sources of energy, ammonia, or VFA.

Estimates of one-half maximal growth stimulation for the amino acid mixture (.5 mg/L, equals 3 μM) are lower than values obtained for ammonia (36). Amino acid and peptide concentrations in vivo vary with diet and time after feeding, but usually exceed 10 mg/L (3, 6, 10, 11, 21, 39). Although these values are considered low, they are high enough to saturate microbial growth based on the kinetic data obtained in these experiments. This could explain why at least 20% of microbial N does not arise from the rumen ammonia pool even on poor quality diets (20). Amino acid concentrations at which growth stimulation was determined to be one half-maximal increased as carbohydrate increased (Figure 4). This effect could be due to differences between initial concentration and average concentration. Growth after 6 h was measured, not growth rates. As the organisms grew they would have utilized the amino acids, resulting in decreased concentrations of amino acids. Higher carbohydrate concentrations would result in longer growth periods and greater decreases in amino acid concentrations.

Comparisons with in vivo and other in vitro information require simplifying assumptions to be made. Composition of bacteria varies in similar in vitro and in vivo experiments (13, 19, 24). However, for purposes of comparison, average values of 10.7% RNA (19), 8% N (13), and a protein:RNA ratio of 3.5 (33) were assumed. From the regressions presented in Figure 2, when 100 mg/L each of amino acids and peptides were added, yields were 255 g cells/kg carbohydrate (27.3 μg RNA/mg carbohydrate × 1 μg cells/107 μg RNA = 255 μg cells/mg carbohydrate) and 20.4 g N/kg carbohydrate (255 g cells/kg carbohydrate × .08 (N) = 20.4 g N/kg carbohydrate). Estimated cell yield is very close to values typically observed in vitro when...
amino acids are provided (24, 25) and to the value of 19.2 g N/kg truly digested organic matter calculated for microbial growth in vivo (13). When ammonia served as sole N source, yields were 64.5 g cells and 5.2 g N/kg carbohydrate, respectively. In order to attain the yields seen in vivo, rumen bacteria must be utilizing high concentrations of amino acids and peptides. Removal of these growth factors will reduce microbial yields. When ammonia served as sole N source, 24.2 μg of protein were synthesized per milligram of carbohydrate (6.9 μg RNA/mg carbohydrate × 3.5 = 24.2 μg protein/mg carbohydrate). Addition of amino acids and peptides at 1 mg/L each, 10 mg/L each, and 100 mg/L each increased microbial yields to 54.6, 73.5, and 95.6 μg protein/mg carbohydrate, respectively, or respective increases over ammonia of 30.4 (54.6 - 24.2), 49.3, and 71.4 μg protein/mg carbohydrate. Low concentrations of amino acids would easily stimulate microbial growth enough to produce more protein than was added to the cultures (20 μg for 1 mg/L amino acids plus 1 mg/L tryppticase), whereas higher concentrations of amino acids and peptides would produce higher microbial yields but would be inefficient as less microbial protein would be synthesized than was added. Other authors have noted that concentrations of amino acids and peptides that produced maximal microbial growth may not result in efficient conversion of amino acids to microbial protein (12, 33).

Growth responses to amino acids and peptides appear to saturate at low concentrations (10 mg/L). However, addition of higher concentrations further stimulates growth (Figure 3, Table 5). Previous work indicated that the growth response saturates at much higher values (12) or not at all (33). Peptides appeared to stimulate growth more than amino acids only at 10 mg/L. This effect may be because peptide amino acids are more efficiently converted to cell protein than free amino acids (10, 38). At 1 mg/L of amino acids, most amino acid carbon may have been used for protein synthesis instead of fermentation, but at 100 mg/L of amino acids, there could have been enough amino acids to support both protein synthesis and fermentation. At 10 mg/L, the availability of amino acids may have been limited due to fermentation of the amino acids, allowing the greater efficiency of conversion of peptide carbon to cell carbon to become apparent. Recent work has indicated that peptide transport may be the limiting step in fermentation of peptide amino acids (10). Further research is required to elucidate the kinetics of peptide and amino acid stimulation of microbial growth.

CONCLUSIONS

Growth of mixed ruminal bacteria is a linear function of carbohydrate fermented, but is greatly stimulated by peptides and amino acids which act as multiplying factors to microbial growth. Bacterial affinity for peptides and amino acids is such that these organisms use these substrates very efficiently at the low concentrations normally found in rumen fluid. Stimulation of growth by amino acids is a general phenomenon, which depends more on how many different amino acids are available to the bacteria in a given mixture than on specific growth limiting amino acids.

ACKNOWLEDGMENTS

Thanks are expressed to J. V. Nolan, University of New England, Armidale, for critical discussion during the preparation of this manuscript.

REFERENCES

7 Bryant, M. P., and I. M. Robinson. 1962. Some nutritional