PEPTIDES AND OTHER NITROGEN SOURCES FOR GROWTH OF BACTEROIDES RUMINICOLA

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ABSTRACT

PITTMAN, K. A. (Agricultural Research Service, Beltsville, Md.), AND M. P. BRYANT. Peptides and other nitrogen sources for growth of Bacteroides ruminicola. J. Bacteriol. 88:401-410. 1964. —Representative strains of Bacteroides ruminicola were found to utilize peptide nitrogen or ammonia nitrogen, but not to utilize significant amounts of free amino acid nitrogen or the nitrogen from a variety of other low molecular weight compounds for growth. All strains grew well in a defined medium containing glucose, minerals, B-vitamins, heme, volatile fatty acids, methionine, and cysteine, with ammonia as the main nitrogen source. Methionine and cysteine were required by some strains. The only compounds found to replace ammonia as the main nitrogen source were a few proteins; tryptic digests of protein; peptide-rich fractions of Sephadex G-25 fractionated tryptic digests of casein; and the octapeptides, oxytocin and vasopressin. Most of the nitrogen present in these compounds was utilized. However, the organism did not utilize nitrogen from any of 12 dipeptides, triglycylglycine, glutathione, or mixtures of free amino acids. Possible reasons for the inability of B. ruminicola to utilize low molecular weight nitrogen compounds are discussed.

Previous studies on the nutrition of Bacteroides ruminicola (Bryant et al., 1958a) indicate that most strains can be grown in media containing minerals, ammonia, carbonic acid-bicarbonate buffer, glucose, B-vitamins, heme, and an enzymatic hydrolysate of casein (Bryant and Robinson, 1962). All strains either require or are stimulated by the casein hydrolysate. A mixture of acetate and one or more of the compounds, isobutyrate, \( \alpha \)-methylbutyrate, isovalerate, and \( n \)-valerate, replaces the casein hydrolysate requirement of some strains and improves the growth of most strains when added to a medium containing casein hydrolysate. An earlier study showed that compounds, possibly peptides, present in an enzymatic hydrolysate of casein but not present in an acid hydrolysate of casein or in mixtures of free amino acids, greatly stimulated growth of a strain grown in media without added volatile fatty acids (Bryant et al., 1958a).

Other studies indicate that, compared with Escherichia coli, B. ruminicola and many other species of rumen bacteria are very inefficient in incorporating the carbon of exogenous free amino acids during growth in media containing ammonia and protein hydrolysates as the main possible sources of cell nitrogen (Bryant and Robinson, 1963). However, most of these species, in addition to inefficient incorporation of amino acid carbon, require an amount of exogenous ammonia approximately equal to the amount of cell nitrogen produced when grown in media containing an enzymatic hydrolysate of casein, whereas B. ruminicola grows very well in the same media containing only traces of ammonia.

The present work was initiated to determine the main sources of nitrogen that are utilized for growth of B. ruminicola in media containing a volatile fatty acid mixture similar to that found in the rumen.

MATERIALS AND METHODS

Organisms and culture methods. The strains studied were previously isolated and described by Bryant et al. (1958a, b) or by Bladen, Bryant, and Doetsch (1961). The bulk of the work was done with type strain 23 B. ruminicola subsp. ruminicola and type strain GA33 B. ruminicola subsp. brevis. Strain 23, which is representative of most strains of the species isolated from the rumen on a nonselective medium (Bryant and Robinson, 1962), requires heme but does not require casein hydrolysate, though stimulated by it. Strain GA33 does not require heme but requires casein hydrolysate.

We used the anaerobic, culture maintenance, and growth estimation methods indicated by Bryant and Robinson (1962).
TABLE 1. Composition of the inoculum and basal media

<table>
<thead>
<tr>
<th>Inoculum medium</th>
<th>Basal medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>Conc (w/v)</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.3</td>
</tr>
<tr>
<td>Minerals</td>
<td>0.0002</td>
</tr>
<tr>
<td>Resazurin</td>
<td>0.0001</td>
</tr>
<tr>
<td>Clarified rumen fluid</td>
<td>20</td>
</tr>
<tr>
<td>Trypticase</td>
<td>0.2</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>0.045</td>
</tr>
<tr>
<td>Cysteine·HCl</td>
<td>0.050</td>
</tr>
<tr>
<td>H₂O</td>
<td>0.40</td>
</tr>
<tr>
<td>CO₂ gas phase</td>
<td></td>
</tr>
</tbody>
</table>

* The pH in both the inoculum and the basal medium was 6.7.

* The composition was 0.05% each of KH₂PO₄ and NaCl, 0.002% each of CaCl₂ and MgCl₂·6H₂O, 0.001% of MnCl₂·4H₂O, and 0.1 mg/100 ml of CoCl₂·6H₂O.

* Bryant, Robinson, and Chu (1959).

* The composition was 2.8 × 10⁻² M acetic, 9 × 10⁻³ M propionic, 4.5 × 10⁻⁴ M n-butyric, and 9 × 10⁻⁵ M each of isobutyric, n-valeric, isovaleric, and DL-2-methylbutyric acids.

* The composition was 0.2 mg/100 ml each of thiamine·HCl, Ca-d-pantothenate, nicotinamide, riboflavin, and pyridoxal; 0.01 mg/100 ml of p-aminobenzoic acid; 0.005 mg/100 ml each of biotin, folie acid, and DL-thioctic acid; 0.002 mg/100 ml of B₁₂.

Inocula for experimental media were prepared as follows. A stab culture, grown on rumen fluid-glucose-cellobiose-starch-agar slants and stored in a refrigerator for 1 day to 2 weeks, was stab-inoculated into a slant of the same medium. After about 24 hr of incubation, the fresh culture was transferred by loop into 5 ml of the inoculum medium shown in Table 1. After 10 to 24 hr of growth, one 4-mm platinum loop of this culture (about 0.01 ml) was inoculated into each 5-ml tube of experimental medium.

Culture purity was checked periodically by observation of wet mounts and Gram stains, and was checked occasionally by inoculation into tubes of Trypticase Soy Agar, 0.5% glucose added, under aerobic conditions. This medium will support the growth of the usual contaminant but not of the strict anaerobe B. ruminicola.

**Experimental media.** The basal medium shown in Table 1 was used in all experimental media. It was prepared by methods similar to those of Bryant and Robinson (1962), being adjusted to pH 6.5 with NaOH before autoclaving and adding sterile, CO₂-equilibrated Na₂CO₃ and cysteine solutions. Two methods were used in making additions of test compounds to the basal medium. (i) The basal medium was prepared as a concentrate and tubed, after autoclaving and addition of sterile, CO₂-equilibrated Na₂CO₃ solution, in such amounts that later addition of sterile, CO₂-equilibrated test and cysteine solutions brought the components of the medium to the desired concentrations in a total 5-ml volume per tube. (ii) The basal medium was prepared and samples were made; test compounds were added to the samples before pH adjustment, autoclaving, and addition of Na₂CO₃ and cysteine solutions. Experimental media used in the first three sections of Results, and those media containing glutathione, egg albumin, edestin, and β-lactoglobulin, B, were prepared by the first method. Salmine (Sigma Chemical Co., St Louis, Mo.) and poly-L-lysine, types I and II (Sigma), were tested in media prepared by both methods. All other media in the fourth and fifth sections of Results were prepared by the second method.

Test compounds not added to the basal before autoclaving were sterilized by autoclaving or by filtering (Millipore type HA filters). The following compounds were filter-sterilized: ascorbic acid, glutamine, glutathione, hydroxylamine hydrochloride, Na₂S, and NaNO₃.

**Chemicals and chemical methods.** E. coli protein was prepared from E. coli strain B by the methods of Roberts et al. (1957). It was dissolved in 0.050 M phosphate buffer at pH 8; samples were hydrolyzed by adding 1.0 ml of 0.0010% (w/v) trypsin (Sigma) in 10⁻³ M HCl to 10.0 ml of 0.50% (w/v) protein in 0.050 M phosphate buffer (pH 8) and incubating the mixture overnight at 37 C.

Enzymatic hydrolysate of casein was fractionated as follows. Sephadex G-25 (90 g; medium grain; Pharmacia Fine Chemicals, Uppsala, Sweden) was suspended in 0.050 M NaCl and packed as described by Flodin (1961) to give a column 3.65 cm in diameter by 37.0 cm in height; 0.50 g of an enzymatic hydrolysate of casein...
(Vitamin-Free Casitone, Difco) was dissolved in 10 ml of water and placed on the column; the hydrolysate was eluted with 0.050 M NaCl; 4.5- to 5.0-ml fractions were collected at rates between 30 and 45 ml/hr. Fractions were autoclaved for 5 min at 120 C to preserve them until use. Total nitrogen and α-amino nitrogen were determined on each fraction. Nine test solutions were then prepared by aseptically combining fractions 33 to 34, 35 to 39, 40 to 44, 45 to 49, 50 to 54, 55 to 59, 60 to 64, 65 to 69, and 70 to 74. The solutions were diluted with sterile water to make each 1.0 × 10⁻² M in total nitrogen, so that later addition to basal media would result in a final concentration of 2.0 × 10⁻³ M nitrogen from each test solution.

A Sephadex column separates compounds because of differences in their molecular size. The largest molecules appear first and the smallest appear last. Figure 1 shows one fractionation of an enzymatic hydrolysate of casein (Casitone). The percentage of α-amino nitrogen is a measure of the amount of amino acid bound in peptides, and shows that the average size of peptides decreases with increasing fraction number. The sharp peak in the curve marks the position of free amino acids. Most of the nitrogen in the hydrolysate appears in fractions containing fairly large peptides.

Total nitrogen was determined by the method of Umbreit, Burris, and Stauffer (1957); α-amino nitrogen, by the method of Rosen (1957); and ammonia nitrogen, by diffusion in plastic Conway dishes from saturated K₂CO₃ into 0.01 M H₄SO₄ for 2 hr, followed by nesslerization. Ammonia was removed from casein hydrolysates and from mixed amino acid solutions by adjusting the pH to 12 with NaOH, aerating for 6 hr in a simple aeration train of large test tubes, and neutralizing with H₂SO₄.

**RESULTS**

**Defined medium containing ammonia.** To determine the compounds in casein that greatly stimulated growth of strain 23 and that were essential for growth of strain GA33 in media containing ammonia (Bryant and Robinson, 1962), we included 7 × 10⁻⁴ M (NH₄)₂SO₄ in the basal medium (Table 1) and compared the effect of various combinations of amino acids with that of 0.2% (w/v) enzymatic hydrolysate of casein. Since a mixture of amino acids, made up to resemble that present in casein, replaced the casein hydrolysate, it was concluded that one or more amino acids were required. Experiments involving single deletions of amino acids from the mixture showed that methionine was essential for growth of strain GA33 and highly stimulatory.
TABLE 2. Effect of various levels of cysteine and ascorbic acid on growth of strain GA33 in basal medium without cysteine but with $3 \times 10^{-4}$ M methionine and $7 \times 10^{-3}$ M $(\text{NH}_4)_2\text{SO}_4$ added*

<table>
<thead>
<tr>
<th>Ascorbic acid (molarity $\times 10^4$)</th>
<th>Cysteine (molarity $\times 10^4$)</th>
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</thead>
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<td>OD $\times 100$</td>
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<tr>
<td>1.2</td>
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</tr>
<tr>
<td>2.5</td>
<td>7</td>
</tr>
<tr>
<td>5.0</td>
<td>8</td>
</tr>
</tbody>
</table>

* The average of four tubes is presented in each case.
† Represents number of hours to reach maximal optical density.

FIG. 3. Growth response of Bacteroides ruminicolae, strains 23 and GA33, in defined basal medium plus $3 \times 10^{-4}$ M methionine to varying ammonium ion concentrations.

for growth of strain 23, since both strains grew rapidly in all media except the one without methionine. It was then found that both strains grew as well in the basal medium plus $7 \times 10^{-3}$ M $(\text{NH}_4)_2\text{SO}_4$ and a minimum of $3 \times 10^{-4}$ M methionine as in the medium containing the amino acid mixture. Figure 2 shows some of the results obtained with strain 23.

Cysteine is necessary for growth of strain GA33 in the basal medium plus $(\text{NH}_4)_2\text{SO}_4$ and methionine; ascorbic acid will not replace it (Table 2). It was noted that ascorbic acid would not efficiently reduce the medium as measured by reduction of resazurin to the colorless state. In this experiment, the inocula slowly reduced the media not already reduced by the higher cysteine levels, and then growth was initiated. Similar results were obtained with strain 23, except that growth was not initiated in most tubes of medium not containing cysteine or containing only $10^{-2}$ M cysteine. Attempts to replace cysteine with other reducing agents or sulfur sources were inconclusive, and further study is needed. In some experiments, good growth was obtained when $10^{-3}$ M Na$_2$S replaced cysteine; but, generally, growth was erratic and very poor.

Since a series of media containing the basal medium plus $3 \times 10^{-4}$ M methionine and graded levels of ammonia (Fig. 3) showed that neither strain was able to utilize significant amounts of cysteine as a nitrogen source, no further attempt was made to replace the cysteine with other compounds. It is evident that ammonia can serve as the main source of cell nitrogen.

Single, low molecular weight nitrogen sources. Because a defined basal medium low in utilisable nitrogen was now available, we were able to test many low molecular weight nitrogen compounds for their ability to serve as the main nitrogen source for the growth of B. ruminicolae in place of ammonia. The compounds were tested at $2.0 \times 10^{-3}$ M in the basal medium plus $3 \times 10^{-4}$ M methionine and a low level of ammonia. The small amount of ammonia present was enough to allow slight, predictable growth, if the added compound was inactive, whereas markedly better or poorer growth would indicate utilization or inhibition by the compound.

Very few compounds, including several small peptides, were active (Table 3). Inactive and most inhibitory compounds were not further tested. Active compounds were tested at several concentrations and checked for ammonia contamination. Though glutamine and KCN supported growth of both strains, it is doubtful that the
TABLE 3. Growth response of two strains of Bacteroides ruminicola in the basal medium plus
$3 \times 10^{-4}$ M methionine to low levels of added ammonia and $2.0 \times 10^{-3}$ M low molecular
weight nitrogen compounds∗

<table>
<thead>
<tr>
<th>Expt</th>
<th>Compounds added to medium</th>
<th>Strain 23</th>
<th></th>
<th></th>
<th>Strain GA33</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD X 100</td>
<td>Time†</td>
<td>OD X 100</td>
<td>Time†</td>
<td></td>
</tr>
</tbody>
</table>
| 1    | None                      | 2         | 15    | 2         | 24
|      | $7.0 \times 10^{-4}$ (NH₄)₂SO₄ | 130       | 22    | 128       | 174
|      | $2.5 \times 10^{-4}$ (NH₄)₂SO₄ | 23        | 15    | 44        | 24
|      | $2.5 \times 10^{-4}$ (NH₄)₂SO₄ plus: | | | | |
|      | L-Asparagine              | 12        | 40    | 32        | 40
|      | L-Glutamine               | 56        | 24    | 72        | 40
|      | Na₃            | 13        | 23    | 20        | 45
|      | NaN₃                     | 0         | 162   | 0         | 162
|      | KCN                      | 55        | 143   | 57        | 143
|      | Hydroxyamine hydrochloride | 0         | 162   | 1         | 162
|      | Spermidine phosphate      | 10        | 48    | 17        | 42
|      | Spermine phosphate        | 0         | 162   | 0         | 162
|      | Tryptamine hydrochloride  | 8         | 90    | 9         | 63
| 2    | None                      | 2         | 19    | 5         | 24
|      | $7 \times 10^{-4}$ (NH₄)₂SO₄ | 124       | 23    | 126       | 24
|      | $1.2 \times 10^{-4}$ (NH₄)₂SO₄ | 7         | 20    | 14        | 24
|      | $1.2 \times 10^{-4}$ (NH₄)₂SO₄ plus: | | | | |
|      | Canavanine                | 0         | 232   | 16        | 64
|      | Guanine                   | 36        | 208   | 25        | 113

∗ The following compounds, not listed by experiment, neither supported nor inhibited growth: L-alanine, β-alanine, L-α-aminoacidic acid, DL-α-amino-n-butyric acid, γ-aminoobutyric acid, α-aminoiso-
butyric acid, β-aminoisobutyric acid, β-amino-n-valeric acid, L-arginine, L-aspartic acid, betaine, L-
citrulline, L-cysteic acid, L-cysteine, L(+)-meso-α, ε-diaminopimelic acid, L-glutamic acid, glycine, L-
histidine, L-homoserine, hydroxy-L-proline, L-isoleucine, L-leucine, L-lysine, L-methionine, DL-nor-
leucine, DL-norvaline, L-ornithine, L-phenylalanine, L-proline, sarcosine, L-serine, taurine, L-threonine, L-
tryptophan, L-valine, DL-alanyl-DL-alanine, DL-alanyl-DL-valine, glycylglycyl-
glycylglycine, glycyl-DL-isoleucine, glycyl-L-lysine, glycyl-DL-methionine, glycyl-DL-serine, glycyl-DL-
valine, DL-leucylglycine, DL-leucyl-DL-isoleucine, L-valyl-L-valine, alamine, cadaverine, glycine, glucosamine, histamine, leucamine, phenylalanine, O-phosphoethanolamine, prolamine, putrescine, tyramine, adenyline, cytokine, thymine, uracil, xanthine, biuret, guanidine, thiourea, urea, hippuric acid, creatine, indole, and NaN₃.
† Represents number of hours to reach maximal optical density.

Compounds were utilized directly, because, in uninoculated media, ammonia was produced from each compound at a rate sufficient to account for all growth observed in inoculated media. Also, $2.0 \times 10^{-3}$ M KCN caused a long lag before growth initiation, and $1.0 \times 10^{-3}$ M KCN prevented growth. Both strains utilized guanine nitrogen very inefficiently as compared with ammonia nitrogen, and further studies showed that $2.0 \times 10^{-3}$ M guanine was inhibitory when compared to $1.0 \times 10^{-3}$ M guanine.

MIXTURES OF AMINO ACIDS AND PEPTIDES. Results in Fig. 4 show the growth response of strain 23 to enzymatic hydrolysate of casein (Vitamin-Free Casitone), to acid hydrolysate of casein (Vitamin Free Cassamino Acid, Difco), and to a similar mixture of L-amino acids; all were tested in the basal medium plus $3 \times 10^{-4}$ M methionine with and without added ammonia. All hydrolysates were previously treated to remove ammonia. The growth response of strain GA33 in these media was very similar to that of strain 23. Free
FIG. 4. Growth response of Bacteroides ruminicola, strain 83, in defined basal medium plus 8 X 10⁻⁴ M methionine to additions of various nitrogen compounds. No additions, 1; 2.5 X 10⁻⁴ M (NH₄)₂SO₄ added, 2; 7 X 10⁻⁴ M (NH₄)₂SO₄ added, 3; 0.2% ammonia-free enzymatic hydrolysate of casein added, 4; 2.5 X 10⁻⁴ M (NH₄)₂SO₄ plus 0.2% ammonia-free enzymatic hydrolysate of casein added, 5; 0.2% ammonia-free acid hydrolysate of casein added, 6; 2.5 X 10⁻⁴ M (NH₄)₂SO₄ plus 0.2% ammonia-free acid hydrolysate of casein added, 7; ammonia-free mixture of 18 pure amino acids equivalent to 0.2% acid-hydrolyzed casein added, 8; and 2.5 X 10⁻⁴ M (NH₄)₂SO₄ plus ammonia-free mixture of 18 amino acids equivalent to 0.2% acid hydrolysate of casein added, 9.

amino acid nitrogen was utilized very inefficiently by the organisms, whereas nitrogen from a source rich in peptides was utilized efficiently.

Since Kihara and Snell (1960) were able to replace peptide stimulation of the growth of Lactobacillus casei in acid-hydrolyzed casein media by making further additions to the basal medium, we decided to test some similar additions to the acid-hydrolyzed casein medium (Fig. 4) for growth of B. ruminicola. Accordingly, 0.0005% (w/v) each of adenine sulfate, guanine hydrochloride, uracil, and xanthine; 0.010% (w/v) each of glucosamine hydrochloride, glutamic acid, isoleucine, tryptophan, and valine; 0.00001% (w/v) spermine phosphate; and varying concentrations of a mixture of 5.0 parts of Tween 80 to 0.50 parts of sodium oleate were added to media containing the basal plus 3 X 10⁻⁴ M methionine and 0.20% (w/v) Casamino Acids. No significant growth of either strain occurred in any of the media unless ammonia was added, and growth was then proportional to the amount of ammonia added.

Further evidence of the nature of the nitrogen compounds in enzymatic hydrolysate of casein that are efficiently utilized for growth was obtained by determining the response of strain 23 to fractions of Casitone from a Sephadex column (Fig. 1 and 5). The results show that strain 23 efficiently utilizes the nitrogen in fractions containing 4 or more moles of total nitrogen per mole of α-amino nitrogen (that is, fractions containing fairly large peptides), but does not efficiently utilize the nitrogen in fractions containing less than 4 moles of total nitrogen per mole of α-amino nitrogen (that is, small peptides and free amino acids).

Proteins, peptides, and poly-amino acids. The growth of strains 23 and GA33 on various proteins and peptides utilized as nitrogen sources is shown in Table 4. It appears that strain 23 uses the nitrogen from these sources more efficiently than does strain GA33, though strain GA33 consistently grew to higher optical densities with limiting but equimolar concentrations of
ammonia as the nitrogen source (Fig. 3). Of the other compounds tested, salmine and poly-L-lysine, type I and type II, did not support growth and completely prevented growth in media with added ammonia, whereas glutathione, cytochrome c, egg albumin, edestin, gelatin, insulin, poly-L-α-aspartic acid, and poly-L-α-glutamic acid were neither utilized nor inhibitory. Figure 6 shows the growth response of strain 23 to varying levels of the total nitrogen in casein, an enzymatic hydrolysate of casein (Casitone), vasopressin (Mann Research Laboratory, New York, N.Y.), and ammonia.

**Growth of other strains in selected media.** Table 5 shows the growth of all available strains of *B. ruminicola* in media designed to test the general applicability of results obtained with the type strains 23 and GA33. The amino acid medium (C) used in experiment 2 contained sufficient ammonia (4.5 × 10^{-4} M) to account for much of the observed growth.

It is evident that in all media most of the strains exhibited growth patterns similar to those of strain 23 and GA33. All strains grew well in the basal medium plus methionine and ammonia, and none utilized free amino acid nitrogen efficiently in place of ammonia. However, several strains, including strain B4 as previously noted by Bryant and Robinson (1962), did not require and were not stimulated by methionine; three strains, representing unusual biotypes from calves (Bryant et al., 1958a), failed to utilize peptide nitrogen. Three strains of *Bacteroides* sp., B127, B40, and B107, isolated from calves and similar to *B. ruminicola* (Bryant et al., 1958b), were also tested. Strain B127 exhibited growth responses similar to those of strain B932-1, except that methionine was more stimulatory, but strains B40 and B107 did not grow or grew very poorly on the media.

**Table 4. Growth response of two strains of *Bacteroides ruminicola* in the basal medium plus 3 × 10^{-4} M methionine to 2.0 × 10^{-3} M nitrogen in various compounds**

<table>
<thead>
<tr>
<th>Compounds added to medium</th>
<th>Growth response</th>
<th>Strain 23</th>
<th>Strain GA33</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD × 100</td>
<td>Time*</td>
<td>OD × 100</td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Casein*</td>
<td>44</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Casitone*</td>
<td>50</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>Pepticase*</td>
<td>46</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td>Escherichia coli protein</td>
<td>58</td>
<td>27</td>
<td>33</td>
</tr>
<tr>
<td>Tryptic digest of E. coli protein</td>
<td>64</td>
<td>16</td>
<td>47</td>
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<tr>
<td>β-Lactoglobulin, B</td>
<td>40</td>
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<td>20</td>
</tr>
<tr>
<td>Oxytocin*</td>
<td>49</td>
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<td>53</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>43</td>
<td>18</td>
<td>32</td>
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</table>

* Represents number of hours to reach maximal optical density.

DISCUSSION

With few exceptions, the growth response of *B. ruminicola* strains 23 and GA33 to nitrogen compounds should be characteristic of the species in general. Previous work (Bryant et al., 1958a; Bryant and Robinson, 1962) showed that most strains of the species are quite similar to strain 23 in nutritional and other characteristics; results reported here show that strains representing many biotypes respond similarly when grown on selected nitrogen sources.

The present study shows that the species can be grown in a chemically defined medium con-
TABLE 5. Growth response of strains of Bacteroides ruminicola in selected media

<table>
<thead>
<tr>
<th>Expt</th>
<th>Strain</th>
<th>Media*</th>
<th>OD × 100</th>
<th>Time†</th>
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* Medium A, basal plus 7 × 10⁻⁴ M (NH₄)₂SO₄; medium B, basal plus 7 × 10⁻³ M (NH₄)₂SO₄ plus 3 × 10⁻⁴ M methionine; medium C, basal plus 3 × 10⁻⁴ M methionine plus 0.010% each of 17 amino acids; medium D, basal plus 3 × 10⁻⁴ M methionine plus 0.010% each of 17 amino acids plus 10⁻⁴ M (NH₄)₂SO₄; medium E, basal plus 3 × 10⁻⁴ M methionine plus 0.20% Casitone; medium F, basal plus 3 × 10⁻⁴ M methionine plus 0.20% Casitone plus 7 × 10⁻⁴ M (NH₄)₂SO₄.

† Represents number of hours to reach maximal optical density.
studies on ammonia production from single amino acids and from mixtures of a few amino acids. Also, there was net uptake of ammonia instead of production during active growth in media containing ammonia and casein hydrolysates, particularly when acid-hydrolyzed casein rather than enzymatically hydrolyzed casein was present (Bryant and Robinson, 1963). One can speculate that the apparent contradiction between the present study and other work arises because many nitrogen compounds cannot penetrate into the cell, but intracellular amino acid deaminases are released from old cultures and cell suspensions.

The reasons for the lack of efficient utilization of single low molecular weight nitrogen compounds and mixtures of free amino acids are not explained by the present data. Though some possibilities are discussed below, further research is required for a satisfactory explanation.

That B. ruminicola can utilize the nitrogen of certain peptides efficiently in place of ammonia, even though it cannot efficiently utilize free amino acid nitrogen, is very interesting. However, the present data allow only a few conclusions as to the effect that the size and structure of a peptide have on its utilization by B. ruminicola. Most striking is that apparently a certain minimal number of amino acid residues, possibly as many as five and almost certainly more than two, are required before a peptide can be utilized. For example, the efficiency of utilization of peptide nitrogen, compared with ammonia as 100%, in Casein fractions from the Sephadex column drops markedly from 60 to about 30% when the average peptide size in the fractions drops from five to three amino acid residues, and, again, to about 20% when the peptide size drops to two amino acid residues (Fig. 5). Also, 60 to 70% of the nitrogen of the octapeptides, oxytocin and vasopressin, is utilized (Table 4), whereas none of the nitrogen of the tripeptide glutathione nor of the dipeptide tested is utilized. Since the data indicate that most of the total nitrogen available in active peptides is utilized, we suggest that peptide nitrogen utilization is not restricted to amide nitrogen nor to the nitrogen of a particular amino acid or few amino acids.

Comparison of the results of this study with results of other studies involving peptides (Gale, 1962; Guirard and Snell, 1962) is difficult because of several important differences. Peptide studies have usually been associated with bacterial species requiring many amino acids. In many of these studies, and in others in which the bacterium required only one or a few amino acids, peptide function was associated with specific, essential amino acids. Such is not the case in this study, because ammonia replaces peptides without slowing growth rate or reducing maximal growth obtained. The present study shows that peptides serve as the main nitrogen source, whereas other peptide studies have not dealt with quantitative nitrogen relationships. Also, our limited data indicate that peptides containing two or three amino acid residues are relatively inactive compared with larger peptides, whereas many other studies have shown dipeptides to be very active.

Guirard and Snell (1962) emphasized that in most peptide studies the reason a peptide is more active than its constituent amino acids is that the peptide obviates some difficulty in the transport of amino acids into the cell or prevents destruction of an amino acid by the organism before it can be used. That destruction is the reason amino acids cannot be utilized by B. ruminicola does not seem likely, because enough ammonia to support some growth would surely be produced from a mixture of 18 amino acids containing several times the amount of nitrogen necessary for maximal growth, if many amino acids were destroyed. There is no evidence to show whether the observed differences in utilization of free amino acid nitrogen and peptide nitrogen by B. ruminicola can be explained by differences in the organism’s ability to transport these compounds. However, if a difference in transport is the explanation, then it is obvious that an efficient transport mechanism(s) for a wide variety of peptides is present and that transport mechanisms for many free amino acids are missing.

In several ways, our results are similar to those obtained by Woolley and Merrifield (1963) with strepogenin and the L. bulgaricus growth factor. Strepogenin activity does not seem to reside in any particular amino acid sequence, no single amino acid residue is essential, and a minimal number of amino acid residues (about five) appears to be required for activity. The results obtained with L. bulgaricus growth factor are quite similar. Strepogenin activity seems to pass through a maximum when peptides are of intermediate size, falling off as peptide size increases.
Our results do not indicate that B. ruminicola is restricted in the utilization of very large peptides; however, several proteins are not efficiently used. Undoubtedly, the action of proteolytic enzymes influences the ability of B. ruminicola to utilize higher molecular weight peptides and proteins. Our results, and those of Woolley and Merrifield (1963), suggest that peptides containing more than two or three amino acids have a special function in bacterial nutrition.

Similar results were also obtained by Phillips and Gibbs (1961), who found maximal stimulation of the growth of Streptococcus equisimalis to be associated with the higher molecular weight peptides obtained by fractionation of trypsinized casein on a Sephadex column.

Studies to determine the mechanism(s) involved in the utilization of peptide nitrogen by B. ruminicola and the reasons for that organism’s inability to utilize free amino acid nitrogen have been initiated.

ACKNOWLEDGMENTS

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LITERATURE CITED


