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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., 1958*a*) 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, α-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., 1958*a*).

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. (1958*a*, *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*[a]

Inoculum medium		Basal medium	
Compound	Concn (w/v)	Compound	Concn (w/v)
	%		%
Glucose	0.3	Glucose	0.5
Minerals[b]		Minerals[b]	
Hemin	0.0002	Hemin	0.0002
Resazurin	0.0001	Resazurin	0.0001
Clarified rumen fluid[c]	20	Volatile fatty acids[d]	
Trypticase	0.2	Vitamins[e]	
$(NH_4)_2SO_4$	0.045	$FeSO_4$	0.0010
Cysteine·HCl·H_2O	0.050	Cysteine·HCl·H_2O	0.025
Na CO_3	0.40	Na_2CO_3	0.40
CO_2 gas phase		CO_2 gas phase	

[a] The pH in both the inoculum and the basal medium was 6.7.

[b] The composition was 0.09% each of KH_2PO_4 and NaCl, 0.002% each of $CaCl_2$ and $MgCl_2 \cdot 6H_2O$, 0.001% of $MnCl_2 \cdot 4H_2O$, and 0.1 mg/100 ml of $CoCl_2 \cdot 6H_2O$.

[c] Bryant, Robinson, and Chu (1959).

[d] The composition was 2.8×10^{-2} M acetic, 9×10^{-3} M propionic, 4.5×10^{-3} M *n*-butyric, and 9×10^{-4} M each of isobutyric, *n*-valeric, isovaleric, and DL-2-methylbutyric acids.

[e] The composition was 0.2 mg/100 ml each of thiamine-HCl, Ca-D-pantothenate, nicotinamide, riboflavine, and pyridoxal; 0.01 mg/100 ml of *p*-aminobenzoic acid; 0.005 mg/100 ml each of biotin, folic acid, and DL-thioctic acid; 0.002 mg/100 ml of B_{12}.

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, CO_2-equilibrated Na_2CO_3 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, CO_2-equilibrated Na_2CO_3 solution, in such amounts that later addition of sterile, CO_2-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 Na_2CO_3 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, NaN_3, and $NaNO_2$.

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 K_2CO_3 into 0.01 N H_2SO_4 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 H_2SO_4.

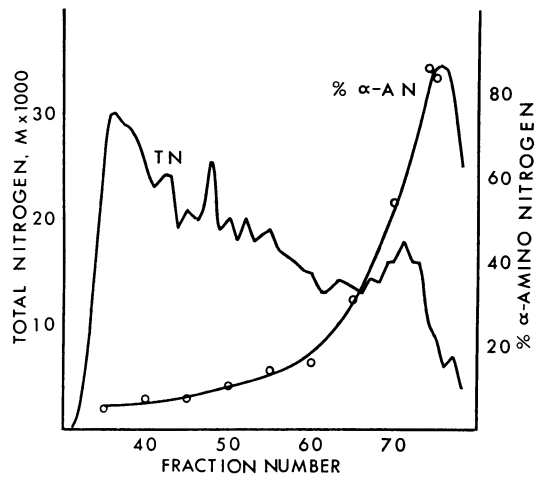

FIG. 1. *Relation of free amino acid nitrogen to total nitrogen throughout the fractionation of an enzymatic hydrolysate of casein (Vitamin-Free Casitone, Difco) on a Sephadex G-25, medium grain column. The abbreviation, % α-A N = μmoles ml^{-1} of α-amino nitrogen/μmoles ml^{-1} of total nitrogen $\times$ 100.*

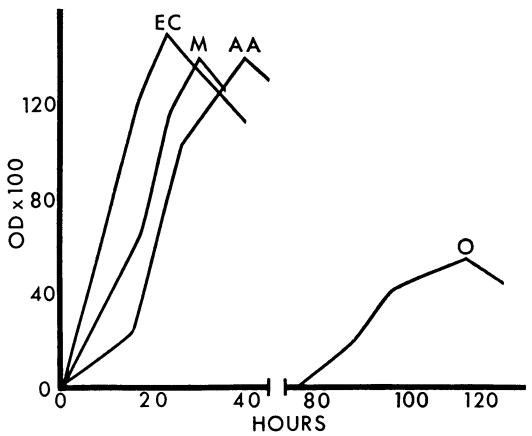

FIG. 2. *Growth response of Bacteroides ruminicola strain 23 in defined basal medium plus 7 $\times$ 10^{-3} M $(NH_4)_2SO_4$ to various additions. No additions, 0; 0.2% enzymatic hydrolysate of casein added, EC; 18 pure L-amino acids equivalent to 0.2% acid hydrolysate of casein added, AA; and 3 $\times$ 10^{-4} M methionine added, M.*

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^{-3} M $(NH_4)_2SO_4$ 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 × 10⁻⁴ M methionine and 7 × 10⁻³ M $(NH_4)_2SO_4$ added**

Ascorbic acid (molarity × 10³)	Cysteine (molarity × 10³)							
	0.00		0.010		0.050		0.25	
	OD × 100	Time†	OD × 100	Time†	OD × 100	Time†	OD × 100	Time†
		hr		*hr*		*hr*		*hrs*
0.00	4	75	11	110	112	60	128	17
1.2	5	170	20	120	106	55	134	18
2.5	7	140	24	95	99	60	129	18
5.0	8	150	22	115	81	60	137	17

* The average of four tubes is presented in each case.

† Represents number of hours to reach maximal optical density.

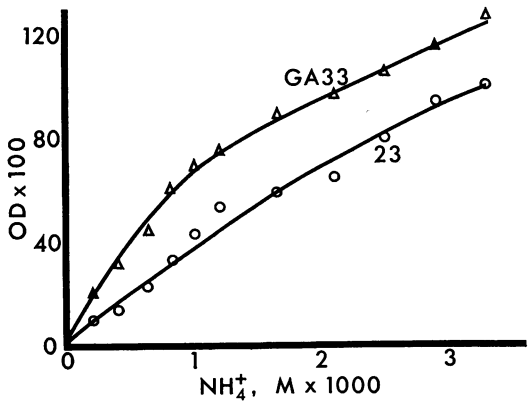

FIG. 3. *Growth response of Bacteroides ruminicola, strains 23 and GA33, in defined basal medium plus 3 × 10⁻⁴ M methionine to varying ammonium ion concentrations.*

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 × 10^{-3} M $(NH_4)_2SO_4$ and a minimum of 3 × 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 $(NH_4)_2SO_4$ 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^{-5} 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_2S replaced cysteine; but, generally, growth was erratic and very poor.

Since a series of media containing the basal medium plus 3 × 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 utilizable 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. ruminicola* in place of ammonia. The compounds were tested at 2.0 × 10^{-3} M in the basal medium plus 3 × 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 were inactive, whereas markedly better or poorer growth would indicate utilization of 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 3×10^{-4} M methionine to low levels of added ammonia and 2.0×10^{-3} M low molecular weight nitrogen compounds**

Expt	Compounds added to medium	Growth response			
		Strain 23		Strain GA33	
		OD × 100	Time†	OD × 100	Time†
			hr		*hr*
1	None	2	15	2	24
	7.0×10^{-3} M $(NH_4)_2SO_4$	130	22	128	18
	2.5×10^{-4} M $(NH_4)_2SO_4$	23	15	44	24
	2.5×10^{-4} M $(NH_4)_2SO_4$ plus:				
	L-Asparagine	12	40	32	40
	L-Glutamine	56	24	72	40
	NaN_3	13	23	20	45
	$NaNO_2$	0	162	0	162
	KCN	55	143	57	143
	Hydroxylamine 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
	7×10^{-3} M $(NH_4)_2SO_4$	124	23	126	24
	1.2×10^{-4} M $(NH_4)_2SO_4$	7	20	14	24
	1.2×10^{-4} M $(NH_4)_2SO_4$ 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-aminoadipic acid, DL-α-amino-*n*-butyric acid, γ-aminobutyric acid, α-aminoisobutyric acid, β-aminoisobutyric acid, δ-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-norleucine, 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-leucine, DL-alanyl-DL-valine, glycylglycylglycylglycine, 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, glycamine, glucosamine, histamine, leucamine, phenylalamine, *O*-phosphoethanolamine, prolamine, putrescine, tyramine, adenine, cytosine, thymine, uracil, xanthine, biuret, guanidine, thiourea, urea, hippuric acid, creatine, indole, and $NaNO_3$.

† 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, 2.0×10^{-3} M KCN caused a long lag before growth initiation, and 1.0×10^{-2} M KCN prevented growth. Both strains utilized guanine nitrogen very inefficiently as compared with ammonia nitrogen, and further studies showed that 2.0×10^{-3} M guanine was inhibitory when compared to 1.0×10^{-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 Casamino Acid, Difco), and to a similar mixture of L-amino acids; all were tested in the basal medium plus 3×10^{-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

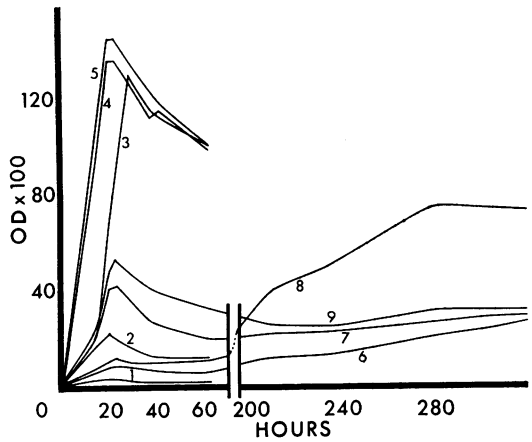

FIG. 4. *Growth response of Bacteroides ruminicola, strain 23, in defined basal medium plus 3 × 10⁻⁴ M methionine to additions of various nitrogen compounds. No additions, 1; 2.5 × 10⁻⁴ M $(NH_4)_2SO_4$ added, 2; 7 × 10⁻³ M $(NH_4)_2SO_4$ added, 3; 0.2% ammonia-free enzymatic hydrolysate of casein added, 4; 2.5 × 10⁻⁴ M $(NH_4)_2SO_4$ plus 0.2% ammonia-free enzymatic hydrolysate of casein added, 5; 0.2% ammonia-free acid hydrolysate of casein added, 6; 2.5 × 10⁻⁴ M $(NH_4)_2SO_4$ 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 × 10⁻⁴ M $(NH_4)_2SO_4$ 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 × 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

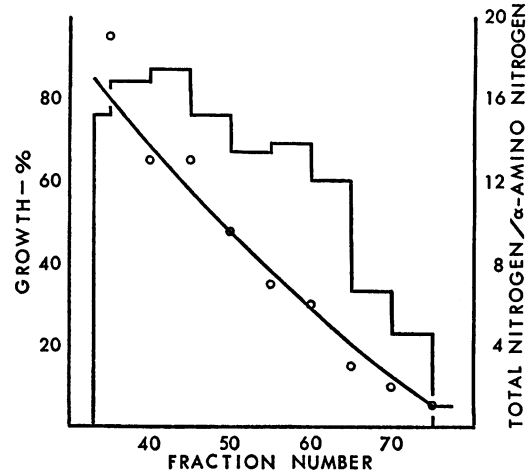

FIG. 5. *Growth response of Bacteroides ruminicola strain 23 in defined basal medium plus 3 × 10⁻⁴ M methionine to the nitrogen of compounds in fractions of an enzymatic hydrolysate of casein from a Sephadex G-25 column. Growth % (bar graph) = 100 × OD at maximal growth on 2 × 10⁻³ M nitrogen of fraction/OD at maximal growth on 2 × 10⁻³ M ammonia nitrogen. Total nitrogen/α-amino nitrogen (open circles) represents the average number of amino acid residues per peptide in the various fractions.*

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 B_14 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., 1958*a*), 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., 1958*b*), 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*

Compounds added to medium	Growth response			
	Strain 23		Strain GA33	
	OD × 100	Time[a]	OD × 100	Time[a]
		hr		*hr*
None	3	15	5	20
Casein[b]	44	40	30	43
Casitone[c]	50	17	29	17
Pepticase[d]	46	18	23	21
Escherichia coli protein	58	27	33	45
Tryptic digest of *E. coli* protein	64	16	47	27
β-Lactoglobulin, B	40	24	20	48
Oxytocin	49	19	53	24
Vasopressin	43	18	32	40

[a] Represents number of hours to reach maximal optical density.

[b] Nutritional Biochemicals Corp., Cleveland, Ohio.

[c] Difco.

[d] Sheffield Chemical, Norwich, N. Y.

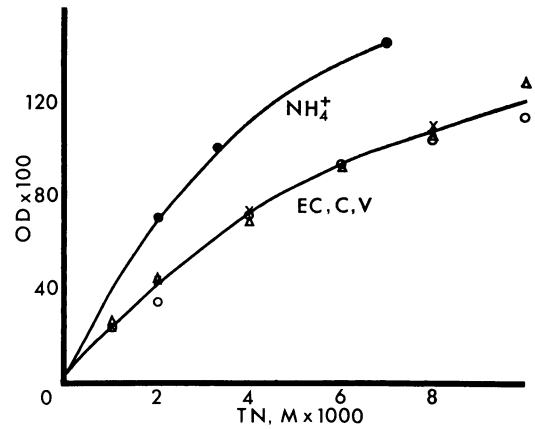

FIG. 6. *Growth response of Bacteroides ruminicola strain 23 in defined basal medium plus* 3×10^{-4} M *methionine to varying concentrations of casein* (○), *enzymatic hydrolysate of casein* (△), *vasopressin* (×), *and ammonium ion* (●); *all were added on the basis of total nitrogen content.*

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., 1958*a*; 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*

Expt	Strain	Media*											
		A		B		C		D		E		F	
		OD × 100	Time†	OD × 100	Time†	OD × 100	Time†	OD × 100	Time†	OD × 100	Time†	OD × 100	Time†
			hr		*hr*		*hr*		*hr*		*hr*		*hr*
1	B 610-1	120	12	120	12	95	70	115	12	130	14	110	12
	B 742-1	115	36	120	20	60	65	115	18	110	22	130	16
	B_1 4	115	12	115	12	28	22	110	12	80	14	110	12
	GA 20	110	130	115	36	80	65	130	20	145	18	130	14
	B 888-1	125	22	120	18	5	36	130	20	27	22	120	16
	B 932-1	120	22	115	18	5	36	125	22	25	22	120	14
	B 747-1	100	60	115	36	5	60	100	36	18	22	115	22
	23	100	130	125	22	45	150	140	20	130	20	140	14
2	23	140	108	137	23	39	21	140	27	145	20	—	—
	B_1 18	140	83	140	27	38	19	133	37	145	18	—	—
	GA33	3	16	140	18	44	21	140	27	130	27	—	—
	GA 103	3	18	135	23	36	27	130	23	125	35	—	—
	118 B	2	14	125	27	45	23	130	35	125	19	—	—

* Medium A, basal plus 7×10^{-3} M $(NH_4)_2SO_4$; medium B, basal plus 7×10^{-3} M $(NH_4)_2SO_4$ 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 7×10^{-3} M $(NH_4)_2SO_4$; 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^{-3} M $(NH_4)_2SO_4$.

† Represents number of hours to reach maximal optical density.

taining glucose, minerals, H_2CO_3-HCO^-_3 buffer, B-vitamins, heme, volatile fatty acids, ammonia, cysteine, and methionine. Methionine replaces the casein hydrolysate previously shown to be essential or highly stimulatory to growth of many strains (Bryant and Robinson, 1962), and ammonia serves efficiently as the main source of cell nitrogen. Further study is needed to determine whether other compounds can replace the requirements for cysteine and methionine.

The results indicate that ammonia is probably the only low molecular weight compound used efficiently as a nitrogen source for growth by *B. ruminicola*. Very many such compounds were tested; yet, with the exception of inefficient and slow utilization of guanine, none would support growth. Since all the low molecular weight compounds were tested on an equimolar basis, each nitrogen of a compound containing more than one bound nitrogen was supplied in high enough concentration to support good growth if it were utilized. Lack of utilization of most single nitrogen compounds and mixtures of free amino acids could not be due to toxicity, because most of the compounds did not prevent rapid growth when ammonia was also present in the medium. Studies showing that *B. ruminicola* incorporates very little free amino acid C^{14} during growth support the present nutritional studies (Bryant and Robinson, 1963). Though such restricted ability to utilize nitrogen compounds is unusual for heterotrophs, other species of rumen bacteria with similar restrictions have been studied (Bryant and Robinson, 1962, 1963); however, most other species differ from *B. ruminicola* in that they will not utilize peptide nitrogen.

Earlier studies indicating that *B. ruminicola* is capable of producing much ammonia from acid as well as from enzymatic hydrolysates of casein seem to contradict the present results, which indicate that ammonia but not acid hydrolysate of casein serves effectively as a nitrogen source. However, ammonia production was demonstrated only after prolonged incubation of cultures (Bryant et al., 1958*a*; Bladen et al., 1961; Abou Akkada and Blackburn, 1963) or with dense resting-cell suspensions (Bladen, 1962). The cell suspensions did not give consistent results in

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 Casitone 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 dipeptides 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.

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