A Study of Bacterial Species from the Rumen Which Produce Ammonia from Protein Hydrolyzate

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It has been well established that the extent of ammonia production in the rumen is an important factor to be considered in the nitrogen economy of the ruminant. The source of ammonia is largely the deamination of amino acids (El-Shazly, 1952b) although some is produced from materials such as urea and nitrate. The concentration of free amino acids in the rumen is usually low (Lewis, 1955). However, breakdown products of amino acids, including volatile fatty acids (El-Shazly, 1952a) in addition to ammonia, are found in high concentrations.

A complete understanding of the catabolism of amino acids in the rumen rests not only on determining over-all reactions carried out by the mixed flora and fauna but on the reactions carried out and the factors affecting the growth and metabolism of the microbial species involved. Most previous work has been conducted with mixed suspensions of rumen bacteria or rumen fluid itself, and data concerning the specific microorganisms actively engaged in the deamination of amino acids is meager. A few bacterial species have been implicated. *Selenomonas ruminantium* (Bryant, 1956) and *Bacteroides ruminicola* (Bryant *et al.*, 1958b) have been shown to catabolize cysteine and casein hydrolyzates, respectively. Lewis and Elsden (1955) showed that *Peptostreptococcus elsdenii* (LC coccus) fermented L-serine, L-threonine, and L-cysteine with the production of ammonia, volatile fatty acids, hydrogen, and carbon dioxide.

The work reported herein was undertaken to characterize and identify some of the more numerous ruminal bacteria attacking protein hydrolyzate with the liberation of ammonia.

MATERIALS AND METHODS

Isolation and identification of ammonia-producing bacteria from the rumen. The three dry Holstein cows used in these experiments were maintained on different diets listed below which were fed at the recommended maintenance levels for total digestible nutrients and were calculated to contain a range of crude protein of 7, 15, and 21 per cent for animals A, B, and C, respectively. Animal A received 20.6 lb per day of timothy hay. Animal B received 4.4 lb per day of grain mixture (15.4 per cent crude protein) and 9.2 lb per day of alfalfa hay. Animal C received 1.1 lb per day of grain mixture (15.4 per cent crude protein), 10.2 lb per day of alfalfa hay and 3.3 lb per day of soybean oil meal (44 per cent protein). Animals were fed at 5 a.m. and 2 p.m. daily with sampling at 11 a.m. They were maintained on the diets for a minimum of 3 weeks before initial sampling.

The anaerobic cultural technique used was that described by Hungate (1950). With this technique, culture media were maintained in sterile rubber-stoppered tubes using oxygen-free carbon dioxide. The pH of all media was adjusted to 6.7 by the addition of Na_2CO_3 and equilibration with the gaseous phase. Cysteine-HCl was used as reducing agent and resazurin was added as an oxidation-reduction indicator.

Rumen content samples were collected, bacteria were enumerated, and strains were isolated using the methods and rumen fluid-glucose-cellobiose agar (RGCA) roll tubes as described by Bryant and Burkey (1953), except that sugars added to the isolation medium included glucose, maltose, and cellobiose each at a 0.1 per cent (w/v) final concentration. After isolation strains were stored as RGCA slant cultures in a Dry Ice box. All strains were designated either A, B, or C depending on the animal from which it was isolated.

About 50 bacterial strains were isolated from each sample and were studied for colony type, morphology, motility, Gram reaction, final pH and appearance of growth in glucose liquid medium, relations to oxygen, H_2S production, gas production, and cellulose digestion as previously indicated (Bryant and Small, 1960). These characteristics were used to presumptively identify the strains as indicated in table 1. References to detailed descriptions of the characteristics of the species are given by Bryant (1959).

Later more detailed studies were performed on strains of the group considered to be of most importance in ammonia production (presumptively identified as *B.*

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ruminicola) in order that positive identification could be made. The methods utilized were previously described (Bryant *et al.*, 1958b).

For the determination of ammonia production, strains were transferred from the RGCA slants to 5 ml of inoculum medium (see below). After 18 hr incubation, about 0.02 ml were inoculated into 5 ml of the ammonia test medium. After 96 hr incubation, 20 per cent (w/v) trichloroacetic acid was added to a final concentration of 5 per cent (w/v). The culture was centrifuged and ammonia was determined by nesslerization (Umbreit, Burris, and Stauffer, 1957) of a 1:10 dilution of the supernatant fluid.

The amount of growth obtained in the ammonia test medium was roughly estimated by optical density (OD) determinations at frequent intervals. A colorimeter with 600 mμ light and culture tubes (13 by 100 mm) for cuvettes was used.

The ammonia test medium used to determine ammonia production of 75 previously described strains of ruminal bacteria contained mineral solutions 1 and 2 of Bryant and Burkey (1953) with $(NH_4)_2SO_4$ deleted; resazurin, 0.0001 per cent (w/v); Trypticase, 1.5 per cent (w/v); a solution of the sodium salts of certain volatile fatty acids (acetate, *n*-valerate, isovalerate, DL-α-methyl-*n*-butyrate, and isobutyrate at concentrations of 420, 60, 30, 30, and 30 μM per ml, respectively), 1 per cent (v/v); clarified rumen fluid, 5 per cent (v/v); glucose, maltose, and cellobiose, 0.066 per cent (w/v) of each; cysteine·HCl·H_2O, 0.05 per cent (w/v); Na_2CO_3, 0.4 per cent (w/v); and a gaseous phase of carbon dioxide. The medium was prepared in a manner similar to that of Bryant and Burkey (1953) with cysteine, Na_2CO_3, and rumen fluid added as sterile solutions equilibrated with carbon dioxide after other ingredients of the medium were autoclaved. The clarified rumen fluid was prepared by centrifugation (12,500 × *g* for 10 min) of rumen fluid obtained by filtration through cheese cloth of rumen contents obtained from animal B at 11 a.m. The fluid was then boiled, again centrifuged, adjusted to pH 7, autoclaved at 15 lb for 15 min under carbon dioxide, and held in the refrigerator.

The ammonia test medium was modified to contain 20 per cent (v/v) clarified rumen fluid and no added salts of volatile fatty acids for the determination of ammonia production by the freshly isolated strains. This was done after tests on many strains (table 2) indicated that some did not grow well in the 5 per cent clarified rumen fluid medium.

The inoculum medium was similar to RGCA medium (Bryant and Burkey, 1953) but contained 0.5 per cent (w/v) Trypticase, no agar, and half as much rumen fluid.

RESULTS

Determination of ammonia production by previously described strains of ruminal bacteria. Table 2 shows that strains of most species produced little or no ammonia from Trypticase. Strains of *S. ruminantium*, *P. elsdenii*, and *B. ruminicola*, species isolated from mature animals, produced relatively large amounts of ammonia. Other strains producing large amounts of ammonia were representatives of species found only in young calves.

It is of interest to note that some strains such as 23 and GA20 of *B. ruminicola* which produced little or no ammonia, but which were included in species in which strains generally did produce a large amount of ammonia, showed poor growth. Both of these strains were shown to produce more ammonia when better growth was obtained by using the 20 per cent instead of the 5 per cent rumen fluid test medium.

TABLE 1

*Some characteristics used in the presumptive identification of bacterial groups**

Presumptive Identification	Morphology	Gram Stain	Motility	H_2S	Cellulose	Gas	Final pH
Bacteroides ruminicola	Pleomorphic rods	−	−	±	−	−	5.0–6.0
Selenomonas sp.	Crescentic rods	−	+	+	−	−	4.3–4.5
Butyrivibrio sp.	Medium or small-sized curved rods	−	+	∓	∓	±	4.8–6.5
Ruminococcus sp.	Cocci, singles, pairs, or chains	±†	−	−	+	∓	5.0–7.0
Bacteroides succinogenes	Pleomorphic rods	−	−	−	+	−	5.0–6.0
Lachnospira sp.‡	Medium-sized curved rods	±	+	−	−	+	4.8–5.2
Eubacterium ruminantium	Medium-sized rod to coccus	±	−	−	−	±	5.0–5.5
Streptococcus sp.	Cocci, singles, pairs and short chains	+	−	−	−	−	4.0–4.5
Succinivibrio sp.	Small, helicoidal, rods with pointed ends	−	+	−	−	−	5.0–6.0
Borrelia sp.	Small, unevenly helicoidal spirochete	−	+	∓	−	−	5.2–6.0

* All strains isolated were strict anaerobes except for the *Streptococcus* sp. which were facultative anaerobes.

† The ± under "Gram stain" refers to variability within a strain and/or between strains. In other cases, ± refers to variability between strains, most strains being positive; ∓ means that most strains are negative.

‡ A filamentous, woolly, colony is very typical of this group.

Predominant bacteria cultured from rumen of animals fed rations of varying protein content. The average number of bacteria isolated from animal A was 645 × 10^6, with a range between 232 × 10^6 to 804 × 10^6 bacteria per g wet weight in 4 samples. Animal B yielded a mean of 578 × 10^6 with a range of 500 × 10^6 to 616 × 10^6 bacteria per g in 5 samples, whereas with animal C the mean was 642 × 10^6 with a range of 456 × 10^6 to 1,108 × 10^6 bacteria per g in 4 samples.

Results in table 3 show the occurrence of presumptively identified bacterial groups in the three animals. These data, where the two samples from each animal were not separated, suggest differences in the numbers of *B. ruminicola*, *Selenomonas*, *Streptococcus*, and *Lachnospira*. However, the differences between samples from the same animal varied as much as the differences between animals.

No significant differences were noted in either the numbers or kinds of ammonia-producing species from any of the test animals (table 3).

Of a total of 271 bacterial strains isolated and presumptively identified, 75 (28 per cent) produced 1 μM or more of ammonia from the Trypticase test medium. The most numerous group (37 strains) was presumptively identified as *B. ruminicola*. This group also contained the organisms which produced the greatest amount of ammonia. *Selenomonas* and *Butyrivibrio* were the only other groups presumptively identified that appeared to contain a significant number of ammonia-producing strains. However, only a few of the many strains presumptively identified as *Butyrivibrio* produced ammonia.

The unknown group (table 3) contained several apparently different kinds of ammonia-producing bacteria. Some of these from each animal appeared to be atypical *B. ruminocola*, whereas 3 strains from the medium level protein-fed animal were anaerobic, motile, lancet-shaped organisms producing a small amount of ammonia.

Further characterizations of the presumptively

TABLE 2

Production of ammonia from Trypticase by predominant bacterial species isolated from rumen of calves and mature cattle

Species	NH_3 Production*	Growth, OD × 100	Strains	Reference
Bacteroides succinogenes	0	37–59	S85, M13, S121	Bryant and Doetsch, 1954
B. succinogenes	0	3	S61, CB40	Bryant and Doetsch, 1954
Lachnospira multiparus	0	50–51	D32, 40	Bryant and Small, 1956a
Borrelia sp.	0	4–44	PC45, 88, B_25	Bryant, 1952
Succinimonas amylolytica	0	10–35	N6, B_24, B_212	Bryant *et al.*, 1958b
Ruminococcus albus	0	4–30	20, D89, 7, B_1C7, B_336, B_337	Bryant *et al.*, 1958c
R. albus (noncellulolytic)	0	33–44	B199, B210	Bryant *et al.*, 1958a
Ruminicoccus flavefaciens	0	12–28	D101, FD1, B_1C45, B_146, C94	Bryant *et al.*, 1958c
Eubacterium sp. (+SR−gGXC)	0	43–46	B17, T162	Bryant *et al.*, 1958a
Eubacterium ruminantium	0	35–44	B4, B_1C26, GA195, B_134	Bryant, 1959
E. ruminantium	1.8	42	B_1C23	Bryant, 1959
Succinivibrio dextrinosolvens	0	35	24	Bryant and Small, 1956a
Lactobacillus sp.†	0.7	63	GA1, GA19	Bryant, 1959
Lactobacillus sp. (+R4)	0.0–0.7	30–34	T112, R15, B62	Bryant *et al.*, 1958a
Lactobacillus sp. (+R3)	0.0–0.6	44–64	R62, B180, T185	Bryant *et al.*, 1958a
Butyrivibrio fibrisolvens	0.0–0.7	24–70	28, D1, A38	Bryant and Small, 1956b
B. fibrisolvens	1.9–2.3	17–48	1, PC4, 49	Bryant and Small, 1956b
Butyrivibrio sp.‡	0.0–0.7	64–71	21C, 9C, 4C	Wilson, 1953
Selenomonas ruminantium	2.4–7.5	27–69	GA192, GA31, PC18, HD_1, HD Long	Bryant, 1956
Peptostreptococcus sp. (C_1)	7.0–7.3	81–82	B43, B116	Bryant *et al.*, 1958a
Peptostreptococcus elsdenii (C_2)	9.0–19.9	50–68	B159, T81, L38	Bryant *et al.*, 1958a
Ramibacterium sp.§	5.5	36	L34	Bryant *et al.*, 1958a
Bacteroides ruminicola	0.8–2.3	5–10	23, GA20	Bryant *et al.*, 1958b
B. ruminicola	11.0	59	B_118	Bryant *et al.*, 1958b
B. ruminicola subsp. *brevis*	11.0–19.4	90–93	GA33, B_14, GA103	Bryant *et al.*, 1958b
B. ruminicola subsp. *brevis*	4.0	8	7D	Wilson, 1953‡
Bacteroides sp. (R1a)	9.0	48	B40	Bryant *et al.*, 1958a
Bacteroides sp. (R1b)	9.0	18	B107	Bryant *et al.*, 1958a
Bacteroides sp. (R1c)	1.9	5	B127	Bryant *et al.*, 1958a
Bacteroides sp. (R2)	30–34.4	53–80	B85, L6	Bryant *et al.*, 1958a

* Ammonia production reported as μmoles per ml test medium.

† Strains from cattle fed fresh alfalfa.

‡ Obtained through the courtesy of Dr. S. R. Elsden.

§ Lactate-fermenting species.

identified strains of *B. ruminicola* including fermentation end products of selected strains were made to positively identify this group (tables 4 and 5). Biotypes 1 and 5 (table 4) correspond to *B. ruminicola* subsp. *brevis* (Bryant *et al.*, 1958b). Biotype 1 corresponds exactly to biotype 3 previously described. Biotypes 2, 3, and 4 belong to *B. ruminicola* subsp. *ruminicola* with 2 and 3 corresponding exactly to the previous biotypes 1 and 3.

Biotypes 6 to 10 appear to be closely related to *B. ruminicola*. However, biotype 6 produced a substantial amount of propionic acid and biotype 7 reduced nitrate and produced indole. Some strains of succinic acid-producing bacteroides from calves that were considered to be closely related to *B. ruminicola* also produced indole and propionic acid (Bryant *et al.*, 1958a). Gutierrez, Davis, and Lindahl (1959a) isolated saponin-fermenting strains similar to *B. ruminicola* that reduced nitrate.

TABLE 3

Occurrence and ammonia production of presumptively identified groups of ruminal bacteria isolated from animals on rations containing low, medium, and high levels of protein

Presumptive Identification	A Low Protein			B Medium Protein			C High Protein		
	No. of strains*	NH_3 production		No. of strains	NH_3 production		No. of strains	NH_3 production	
		Mean†	Range		Mean	Range		Mean	Range
Bacteroides ruminicola	19 (18)	7.4	2.2–23	10 (8)	5.3	1.0–17	14 (11)	7.9	2.6–22
Selenomonas sp.	6 (6)	4.8	2.0–6.8	0	0	0	3 (1)	3.4	0
Butyrivibrio sp.	28 (0)	0	0	33 (2)	4.7	1.8–7.7	32 (5)	4.2	1.8–6.4
Ruminococcus sp.	10 (1)	2.6	0	2 (0)	0	0	10 (0)	0	0
Bacteroides succinogenes	0	0	0	1 (0)	0	0	1 (0)	0	0
Lachnospira sp.	0	0	0	5 (5)	0	0	1 (0)	0	0
Eubacterium ruminantium	3 (0)	0	0	1 (0)	0	0	3 (1)	2.2	0
Streptococcus sp.	3 (0)	0	0	0	0	0	0	0	0
Succinivibrio sp.	0	0	0	0	0	0	1 (0)	0	0
Borrelia sp.	0	0	0	1 (0)	0	0	0	0	0
Unknown‡	28 (9)	3.5	1.4–7.2	33 (7)	3.1	1.0–7.7	23 (6)	4.1	2.1–9.0
Totals	97 (34)	5.8	1.4–23	86 (17)	4.3	1.0–17	88 (24)	5.8	1.8–22

* The number in parentheses is the number of strains that produced 1 μmole or more of ammonia per ml test medium.
† The mean of those that produced 1 μmole or more of ammonia.
‡ Unknown and other organisms that have not previously been adequately described.

TABLE 4

*Some characteristics of presumptively identified Bacteroides ruminicola from the rumen of cows fed different levels of protein**

Characteristic	Biotype									
	1	2	3	4	5	6	7	8	9	10
H_2S production	+	−	−	+	+	−	−	−	−	+
Gelatin liquefaction	+	+	−	−	−	+	−	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	−
Indole production	−	−	−	−	−	−	+	−	−	−
NO_3 reduction	−	−	−	−	−	−	+	−	−	−
Final pH—glucose	5.0–5.2	4.6–6.4	4.7–5.3	4.8–5.2	5.1	5.0	5.3–5.6	5.1	5.0	5.2
Growth in yeast-Trypticase medium	+	−	−	−	+	−	−	+	+	−
Acid from:										
Xylose	+	+	+	+	+	+	+	+	+	−
Maltose	+	+	+	+	+	+	+	+	+	−
Mannitol	−	−	−	−	−	−	−	+	−	−
Inulin	+	+	+	+	+	+	+	−	+	+
Gum arabic	−	−	−	−	−	+	−	−	−	−
No. of strains (high protein)	2	0	4	4	0	0	1	0	0	1
No. of strains (medium protein)	1	1	3	2	0	0	0	1	1	0
No. of strains (low protein)	0	10	5	1	1	1	1	0	0	0

* All strains have the following characteristics: gram negative; anaerobic; nonmotile; no gas from glucose; acid from glucose, lactose, and xylan.

Discussion

In work preliminary to the present study, considerable time was spent in developing a growth medium that would be suitable for screening large numbers and a variety of species of ruminal bacteria for ammonia production from a protein hydrolyzate. Ammonia producing strains of *P. elsdenii* (B159), *S. ruminantium* (GA192), and *B. ruminicola* (GA33, B_14, and 23) as well as strains of *Ruminococcus albus* (20), *Butyrivibrio fibrisolvens* (D1) and *Borrelia* sp. (88) which did not produce ammonia were included. It was found that ammonia production often increased through 72 hr incubation so that a standard time of 96 hr was selected. Even with volatile fatty acids and 1.5 per cent of Trypticase in the medium, rumen fluid was essential to growth of *B. ruminicola* subsp. *ruminicola*. Experiments with media with and without the nitrogen source, Trypticase, indicated that rumen fluid contained little, if any, nitrogen precursors of ammonia. A carbohydrate energy source was necessary for growth and ammonia production of ammonia producing species except *P. elsdenii* and the level of sugars used in the final medium did not inhibit ammonia production of any of the strains. The mixture of three sugars was used because some strains of ruminal bacteria utilize cellobiose or maltose but not glucose. Trypticase was among the best protein hydrolyzates tested for both growth and ammonia production and was selected for use in the final medium because it contained less initial ammonia than other materials that allowed good growth and ammonia production.

The detailed characterization of the presumptively identified strains of *B. ruminicola* (tables 4 and 5) revealed that most strains (biotypes 1 to 5, table 4) were *B. ruminicola* and the other strains were at least very closely related to this species. With the exception of *Eubacterium ruminantium* and *Butyrivibrio* sp., the other groups are more positively identified by the characteristics used for presumptive identification than was *B. ruminicola*. It appears that the presumptive identification of the bacterial groups on the basis of a few characteristics was well founded. The time and expense involved in detailed studies of each strain isolated in the study would have been large as compared with the benefits obtained.

TABLE 5

Fermentation acids produced in rumen fluid glucose medium by selected strains of presumptively identified Bacteroides ruminicola

Strain No.	Biotype	Total Acid mEq per 100 ml	mEq Per Cent of Total Acid*					
			Butyric	Propionic	Acetic	Formic	Succinic	Lactic
118B	1	14.1	0	1.4	20.7	10.1	67.6	0
57A	2	14.6	0	13.2	23.6	3.1	58.8	1.0
119A	2	12.9	0	6.4	24.5	11.9	55.9	0
71C	3	17.9	0	4.0	25.1	13.0	54.1	2.6
152A	3	12.8	0	1.9	21.1	6.1	70.7	0
74C	4	21.4	1.9	2.0	25.6	12.0	56.5	1.6
86C	4	18.8	0	2.0	24.3	14.2	57.0	2.5
105A	5	15.1	0	1.1	19.8	7.0	71.2	0
127A	6	12.0	0	29.9	29.6	5.6	34.3	1.3
65A	7	13.9	0	6.2	23.6	11.8	55.5	2.3

* Values of 3 per cent or less are considered of doubtful significance.

A comparison of the studies on well described species of ruminal bacteria maintained in pure culture for long periods of time (table 2) and on fresh isolates (table 3) led to a similar conclusion, i.e., based on numbers found in the rumen of animals on a variety of rations and the amount of ammonia produced in pure culture *B. ruminicola* is of primary importance in ammonia production from hydrolyzed protein in the rumen. *Selenomonas* also is of significance and *P. elsdenii* may be of importance under certain conditions of high grain feeding (Gutierrez *et al.*, 1959b). Results also suggest that some strains of the genus *Butyrivibrio* may be involved in ammonia production.

The results in table 2 show that many of the species isolated from young calves (Bryant *et al.*, 1958a) produce considerable amounts of ammonia.

It is evident that further assessment of the importance of various ruminal microbial species in ammonia production from hydrolyzed protein rests, to a considerable extent, in gaining further knowledge of the identity of amino acids and/or peptides catabolized, the products produced, and the mechanisms involved in the individual reactions. Some studies along these lines are in progress.

It has been shown that the potential rate of ammonia production from hydrolyzed protein by the ruminal flora correlates with the amount of protein fed to the animal (El-Shazly, 1952b; Warner, 1956). The present results (table 3) suggest that this phenomenon is not due to a large increase in numbers of predominant species of ammonia-producing bacteria. It might be that the increased ammonia production is due to the synthesis of more deaminases without a change in species. Also, it seems evident that ruminal protozoa are of considerable significance in ammonia production from amino acids (Warner, 1956; Williams, Gutierrez, and Doetsch, 1960) and differences in numbers or species of protozoa might be involved.

It is possible that other bacteria, not among the predominant bacteria, would be of importance in ammonia production and that these could be demonstrated by the use of culture media containing protein hydrolyzates as the main source of energy for growth. It is likewise possible that this type of medium would show differences in the ruminal flora of animals on rations of differing protein content. A preliminary study indicated that addition of Trypticase to the

present culture medium resulted in depressed total colony counts. This substantiates the results of McNeill, Doetsch, and Shaw (1954).

SUMMARY

Data on ammonia production from casein hydrolyzate by 74 strains of previously well described ruminal bacterial species and by 271 strains freshly isolated from three cows fed rations of timothy hay, alfalfa hay and grain and the latter with extra soybean oil meal added (7, 15, and 21 per cent of crude protein, respectively) indicate that, on the basis of numbers of strains and amount of ammonia produced, *Bacteroides ruminicola* is usually the most important ammonia-producing bacterium in the rumen of mature cattle. Other species of probable significance include *Selenomonas ruminantium*, *Peptostreptococcus elsdenii*, and some strains of the genus *Butyrivibrio*. Many ammonia-producing species are among the predominant bacteria of young calves.

There appeared to be no significant differences in the occurrence of presumptively identified predominant species, or numbers or species of predominant ammonia producers in the rumens of the animals studied.

Detailed characterization of the strains of presumptively identified *B. ruminicola* indicated that 34 strains did belong to this species and the remainder (6 strains) were very closely related to this species.

ADDENDUM

Unpublished studies of M. P. Bryant and I. M. Robinson indicate that *Bacteroides ruminicola* subsp. *ruminicola* can be grown in media of known composition with hemin replacing the "rumen fluid" requirement. This work was done after the present studies were completed.

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