ISOLATION, ENUMERATION, AND CHARACTERISTICS OF PROTEOLYTIC RUMINAL BACTERIA[1]

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ABSTRACT

FULGHUM, ROBERT S. (Virginia Polytechnic Institute, Blacksburg) AND W. E. C. MOORE. Isolation, enumeration, and characteristics of proteolytic ruminal bacteria. J. Bacteriol. **85**:808–815. 1963.—Colony counts of proteolytic ruminal bacteria in the order of 10^9 organisms per g of whole rumen contents, and total colony counts in the order of 2 to 3 $\times$ 10^9 organisms per g, were obtained from rumen contents of cattle fed a maintenance ration of hay and grain. The proteolytic counts averaged 38% of the total counts. An anaerobic, differential medium characterizing proteolytic colonies by clear zones in an opaque skim-milk suspension was utilized. Proteolytic isolates were assigned to the following taxa: *Butyrivibrio* sp., *Succinivibrio* sp., *Selenomonas ruminantium* var. *lactilytica*, *Borrelia* sp., *Bacteroides* sp., and selenomonadlike organisms similar to the B-385 group of Bryant.

Proteolysis in ruminal fermentation may benefit the host animal, if the resulting products are later synthesized to digestible microbial proteins of higher biological value than the feed proteins; or, conversely, this activity may be detrimental because of net protein loss. In spite of the nutritional significance of this activity, the proteolytic ruminal bacteria have received relatively little attention as a physiological group. Studies have largely been restricted to observations of the degradation of gelatin or casein incidental to other studies of ruminal organisms. Bryant (1959) revealed the paucity of information on the proteolytic flora of the rumen.

Gelatin proteolysis (liquefaction) was reported among isolates from the rumen by Bryant (1951), Bryant and Burkey (1953*a*, *b*, *c*), Bryant and Small (1956*a*), Bryant et al. (1958*a*), Hamlin and Hungate (1956), Huhtanen and Gall (1953), Hungate (1957), and Mann, Masson, and Oxford (1954). Bryant and Doetsch (1954) also reported isolates which attacked casein but not gelatin, and Bryant (1956) reported a strain of *Selenomonas ruminantium* which digested casein but not gelatin. These differences were possibly due to the use of a more sensitive test for casein digestion.

A casein medium designed for the isolation of proteolytic ruminal bacteria was described by Appleby (1955). Blackburn and Hobson (1960*a*) found proteolytic activity in all fractions of rumen contents (protozoa and large and small bacteria), and they initiated isolation of proteolytic bacteria from the ovine rumen (Blackburn and Hobson, 1960*b*).

Fulghum (1958) described the development of two anaerobic, differential media for the isolation and enumeration of proteolytic ruminal bacteria. These media were developed through modification of the media of Hamlin and Hungate (1956) and King and Smith (1955), and of the medium used by Donovan and Vincent (1955) for studying proteolytic organisms from milk. Colonies of proteolytic organisms in these media were characterized by clear zones in opaque skim-milk or plant-protein suspensions in the media. Plant proteins failed to maintain a uniform opacity and were, therefore, of limited value in delineating the proteolytic segment of the flora, even though the plant proteins stimulated total counts by a factor of from three to five. Earlier, Fulghum, King, and Moore (1958) reported that dispersion of whole rumen contents in anaerobic diluting fluid in a blender increased total counts by a factor of four, when compared with total counts obtained from rumen-fluid samples diluted by shaking in anaerobic diluting fluid. Proteolytic counts were the same from both inocula. In later studies (Fulghum, 1958), proteolytic counts were

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also found to be increased by a factor of four when dispersed whole contents were compared with shake dilutions of rumen fluid. Proteolytic and total counts were found to be slightly higher in the dorsal sac of the rumen than in the ventral sac, although this phenomenon was variable with regard to time of sampling after feeding of animals. Similarly, the ratio of proteolytic to total counts varied at different times after feeding. The proteolytic flora remained constant while the total counts varied. The sequence of fluctuation was different in each individual animal.

The present study describes the enumeration of proteolytic bacteria from the bovine rumen, and the isolation and characterization of a representative number of the proteolytic organisms detected.

Materials and Methods

The salts-rumen fluid-protein (SRP) medium of Fulghum (1958) was similar to the rumen fluid-glucose-cellobiose agar medium of Bryant and Burkey (1953*b*). A stock salts solution for the SRP medium was prepared by dissolving 1.0 g of K_2HPO_4, 1.0 g of KH_2PO_4, 2.0 g of NaCl, and 10.0 g of $NaHCO_3$ in 1 liter of boiled, distilled water equilibrated with oxygen-free CO_2. Then, 0.25 g of $CaCl_2 \cdot 2H_2O$, 0.20 g of $MgSO_4 \cdot 7H_2O$, and 2 ml of a 0.1% (w/v) resazurin solution were added separately, complete solution being effected between additions.

The SRP-A medium was made up of 50% stock salts solution, 30% clarified rumen fluid, and 10% CO_2-equilibrated, oxygen-free, distilled water. Cysteine (0.08%, w/v) and, except where noted otherwise, 0.05% (w/v) glucose and 0.05% (w/v) cellobiose were added. The pH was adjusted to 6.8. Portions (9 ml) were dispensed into tubes containing 0.2 g of agar. All additions were made under oxygen-free CO_2 (Hungate, 1950). The stoppered tubes were placed in tightly covered racks and sterilized at 121 C for 20 min. Since protein suspensions were found to coagulate when sterilized in the presence of the salts solution, 1 ml of sterile skim milk was added aseptically to tubes of medium held at 45 C, immediately preceding inoculation. Rehydrated commercial powdered skim milk was prepared with distilled water and sterilized at 116 C for 12 min. The sterile milk was always cooled to and held at 45 C after sterilization, to minimize the re-entry of oxygen into the solution when the milk was rapidly added to the medium.

The SRP-basal medium used for determining cultural and physiological characteristics contained 50% stock salts solution, 20% clarified rumen fluid, and 30% oxygen-free, CO_2-equilibrated, distilled water. Variations of this basal medium are described with the results obtained. These media were designed to duplicate the conditions provided by similar media described by Bryant and Doetsch (1954) and Bryant and Small (1956*a*).

Clarified rumen fluid was prepared from rumen fluid expressed through two layers of cheesecloth. The liquor was heated at 121 C for 20 min under an oxygen-free CO_2 atmosphere in a sealed container. Particulate debris was then removed by centrifugation at 22,000 $\times$ *g* for 20 min.

Rumen samples were obtained from fistulated, mature-grade Hereford and Holstein steers. The animals were maintained on a hay and grain diet. Water was available *ad libitum*. Samples of whole ruminal ingesta were obtained from the center of the hay mat. Dispersed inocula were prepared from 1 g of whole ruminal ingesta and 99 ml of an anaerobic diluting medium containing the salts, resazurin, and cysteine of the SRP-basal medium. The diluted sample was mixed under an oxygen-free CO_2 atmosphere in a Waring Blendor for 1 min. Serial dilutions were then made to 10^{-8} g/ml, using 0.1-ml portions and 9.9-ml anaerobic dilution-medium blanks. Tubes of media inoculated with 10^{-8} g/ml of rumen contents were incubated as roll tubes for 150 hr at 39 C. The time lapse between sampling and the beginning of incubation was less than 1 hr. The anaerobic methods of Hungate (1950) were used throughout this study.

Colony counts were determined with the aid of a 10 $\times$ stereomicroscope. Proteolytic colonies were differentiated by their production of clear zones in the opaque protein suspension in the medium. The Kopeloff-Beerman modification of the Gram stain and the Conklin modification of the Wirtz method of spore staining with malachite green were used. Cell morphology was determined from smears with a nigrosin background stain. Flagella were demonstrated by the Leifson method as described by Skerman (1959).

The purity of each culture was established by diluting isolated colonies in the anaerobic diluting medium and inoculating roll tubes serially through three cultures. Each tube was carefully observed for similarity of colony type. Each colony used in making the series and ten other

colonies in the last tube of the series were examined in Gram-stained smears for the presence of a single-cell morphotype.

Several variations of SRP medium were evaluated to determine their suitability for obtaining total viable counts and for enumerating the proteolytic flora of the rumen. Variations included: SRP-A which contained powdered skim milk as described above; SRP-B was the same as A, with 10% dialyzed skim milk (3.5% solids, w/v) replacing the skim milk; SRP-C was the same as A, with 1% thioglycolate replacing cysteine; SRP-D was the same as A but with 40% rumen fluid.

By use of the above media to determine total and proteolytic colony counts, animal no. 1 was sampled at 9 AM for each of 3 successive days and at 8 and 10 AM on a fourth day; animal no. 2 was sampled on a fifth day at 9 AM; animal no. 3 was sampled on a sixth day at 9 AM. On later dates, this entire sampling regimen was repeated, using SRP-A and SRP-B. Each of the samples was treated as whole-rumen contents, dispersed, and diluted to 10^{-8} g/ml. From each 10^{-8} dilution, duplicate, triplicate, or quadruplicate roll tubes were inoculated. Incubation was at 39 C for 120 hr. Total and proteolytic colony counts were made. One tube of SRP-A medium from each sample in the repeated regimen was selected, and all proteolytic colonies were picked, subcultured, and lyophilized. Of the 65 proteolytic isolates obtained, 38 remained viable for 6-week periods in anaerobic SRP-A medium stabs held at 2 C. These cultures were used for taxonomic studies, and were maintained on SRP-A medium. Isolates were also checked for purity on this medium.

TABLE 1. *Average total and proteolytic colony counts from 10^8 dilutions of whole ruminal ingesta*

Medium	No. of tubes	Avg total colonies/ tube	Avg proteolytic colonies/ tube
SRP-A (skim milk)	49	26.8	9.9
SRP-B (dialyzed skim milk)	43	26.9	10.5
SRP-C (thioglycolate)	23	23.2	7.8
SRP-D (40% rumen fluid)	20	26.1	12.1
All media	135	26	10

RESULTS

From this experiment, 135 roll tubes produced a total of 3,532 colonies, of which 1,358 (38.5%) were proteolytic. Table 1 shows the distribution of average counts obtained on the media described. The data were analyzed statistically to estimate the effect of media, dates, time, media × date, date × time, and experimental error for the total colony-count data and for the proteolytic colony-count data. There were highly significant differences (0.01 level) among dates and among the media when total colony counts were considered. There were highly significant differences among dates, media, and time for proteolytic counts, and highly significant interaction among dates and media with animal no. 1 at the samplings taken at 9 AM. In addition to statistical variations, visual examination of the data revealed variations in total and proteolytic counts among animals. These variations may reflect individual differences that have been observed among other rumen activities in these animals.

Media were inoculated with cells taken from stock stab cultures. Incubation was at 39 C with observations at 24, 48, and 72 hr. Descriptions were made of colony types on SRP-A roll tubes. All isolates were obligate anaerobes which would only grow in the reduced area (colorless to resazurin) in agar shake tubes containing SRP-basal medium plus 1% (w/v) Trypticase (BBL) and 1% agar. These tubes were not gassed during inoculation, so that some oxygen was purposely admitted. Three of the cultures which appeared to

TABLE 2. *Biochemical characteristics of proteolytic bacteria isolated from rumen ingesta*

Organism	No. isolated	Gelatin liquefaction	NO_3 reduction	H_2S production	Indole production	Gas production
Butyrivibrio sp.	30	3	0	2	0	24
Borrelia sp.	1	1	0	1	0	0
Bacteroides sp.	3	1	0	1	0	1
Selenomonadlike B385	2	2	0	0	1	2
Selenomonas ruminantium var. *lactilytica*	1	0	1	1	1	1
Succinivibrio sp.	1	1	0	1	0	0

TABLE 3. *Acid-fermentation products from glucose[a] in mM/100 ml*

Organism	Culture no.	Butyrate	Propionate	Acetate	Formate	Succinate	Lactate	Final pH
Butyrivibrio sp.	Type[a]	0.74			0.28		0.28	
	11	1.47	0.09	0.95	0.76		1.54	5.40
	23[b]	1.15		0.73	2.83		2.00	
	24	1.14	0.30	0.75	0.99		1.43	5.40
	25[b]	1.62			2.79	0.07	1.06	
	26	2.17	0.05		1.57		2.17	5.00
	27	1.51			1.09		2.47	5.30
	28	1.16			1.07		2.19	5.30
	32	1.62			1.08	0.08	2.15	5.35
	33	1.33			1.08		2.09	5.18
	36	0.21	0.09				5.80	4.80
	39	0.36	0.10	0.17			6.45	4.88
	40	1.35	0.23		1.18		1.72	5.30
	41	1.21	0.27	0.25			4.46	5.05
	42[b]	0.17		0.02	0.18		2.87	
	43	1.38	0.06	0.42	0.04		3.10	5.00
	45[b]	0.14		0.27			2.57	
	46	0.99					3.61	5.00
	47	1.51	0.06	0.23	0.07	0.03	3.11	5.10
	49	1.26					3.35	4.90
	50	0.07	0.65			0.33	6.30	4.35
	55	2.11	0.24	0.68	2.08	0.02		5.41
	56	2.05	0.07	0.52	2.34			5.58
	60	1.73		0.30	1.19	0.05	0.32	5.30
	62	1.58		0.44	1.26		0.90	5.18
	63	1.96	0.05	0.56	1.17		2.12	4.98
	69[b]	1.60			2.24		0.52	
	70	2.84	0.11		1.21		2.44	5.10
	71	2.44			0.90		2.06	5.05
	72	1.05	0.15	0.28	1.91		2.41	4.80
	73[b]	1.66		0.19	1.59		0.58	
Succinivibrio sp.	Type[c]			1.24	0.41	1.75	0.16	
	4	0.19	0.18	1.15		2.33		5.46
Selenomonas ruminantium var. *lactilytica*	Type[d]	0.12	1.38	0.89	0.30	0.09	2.05	
	53[b]		0.39		0.54		3.46	
Selenomonadlike B-385	Type[d]	2.38	0.27		0.92		1.88	
	23A[b]	0.77		0.16	2.25	0.12	2.75	
	57A[b]	0.47			1.85	0.49		
Borrelia sp.	12[b]	1.15		0.73	2.83	0.12	2.75	
Bacteroides sp.	9	1.12	0.41	1.27				6.52
	30	1.55	0.46		0.78		1.61	5.22
	35	0.26	0.18	0.78				6.62

[a] Bryant and Small (1956*a*).

[b] Data are from cultures incubated for 1 week at 39 C; chromatogram was developed at room temperature.

[c] Bryant (1956).

[d] Bryant and Small (1956*b*).

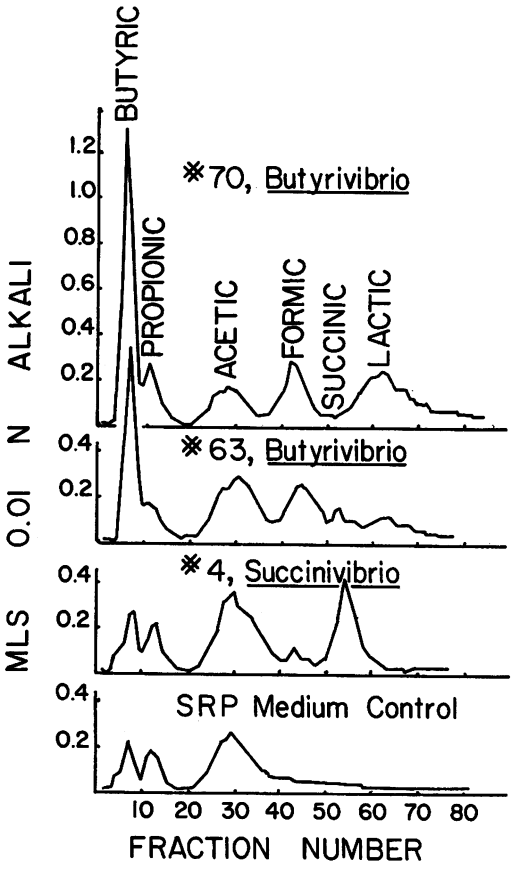

FIG. 1. *Example chromatograms of three cultures and the uninoculated rumen-fluid medium control.*

contain single colonial and cellular morphotypes on SRP-A were shown to be mixed only after transfer to Trypticase medium. On this medium, two distinct colony types were produced. All isolates produced visible growth when Trypticase was used to replace skim milk in SRP-basal-Trypticase broth medium, or when grown as stab cultures in the SRP-basal medium with 0.1% $(NH_4)_2SO_4$ and 1% agar.

All isolates had no requirement for rumen fluid when they were grown as stab cultures in SRP-basal medium with water replacing rumen fluid, cysteine increased to 0.1%, cellobiose and glucose increased to 0.25% each, and the addition 1% each of Trypticase, yeast extract, and agar. A similar medium with 0.5% Phytone (BBL) replacing one-half of the yeast extract also supported growth of all isolates.

To determine gelatin liquefaction, SRP-basal medium with 1% Trypticase and 5% gelatin was used. Incubation was at 39 C, and the tubes were transferred to a refrigerator (2 C) for 30 min before observing for liquefaction. Determinations of indole production and nitrate reduction were made in SRP-basal medium with 2% Trypticase and 0.1% KNO_3. The ability of isolates to produce H_2S was studied by use of SRP-basal medium with 2% Trypticase, 0.05% ferric ammonium citrate, and 1% agar. A blackening along the line of the stab indicated H_2S production. The production of gas was determined by observations of splits or gas bubbles in SRP-A stab cultures or in other agar media used in this study. Final pH was determined with a glass-electrode pH meter in a lightly buffered medium [SRP-basal medium without bicarbonate plus 0.1% $(NH_4)_2SO_4$ and 0.5% glucose]. The atmosphere in these tubes was oxygen-free nitrogen. The chemical tests used were those suggested by the Society of American Bacteriologists Committee on Bacteriological Technic (1957).

Media for the study of substrate fermentation consisted of SRP-basal medium with 0.1% $(NH_4)_2SO_4$ and 0.5% of the substrate to be studied. Glucose-fermentation tubes were incubated for one week at 39 C. Similar preparations without glucose were observed to determine whether growth and fermentation were in response to glucose or to the clarified rumen fluid. No growth or very scant growth occurred in the medium without glucose, whereas moderate to heavy growth occurred in the medium with glucose. The acid-fermentation products of glucose metabolism were determined chromatographically by the method of Neish (1952). In a later study, fermentation tubes were prepared as those above, except that glucose was increased to 1%. These tubes were inoculated with lyophilized cultures of the isolates which had been stored at 2 C for 2 years and were then incubated at 37.5 C for 3 weeks. The acid-fermentation products were determined by the modified chromatographic procedure described by Bruno and Moore (1962).

Biochemical characteristics of the isolates appear in Table 2. Fermentation products appear in Table 3. Fermentation-acid values shown in the tables represent totals of acids produced. Values for acids in the original rumen-fluid medium have been deducted. None of the isolates produced detectable quantities of valeric acid. Figure 1 compares example chromatograms of acid-fermentation products from three of the organisms and the 30% rumen-fluid medium con-

TABLE 4. *Additional characteristics of several proteolytic bacteria isolated from rumen ingesta*

Organism	Strain	Growth on other substrates					Final pH
		Starch	Lactose	Mannitol	Glycerol	Lactate	
Bacteroides sp.	9	+	+	−	−	−	5.8
	30	−	+	−	+	−	4.8
	35	+	+	−	+	−	5.2
Selenomonadlike B-385	Type*	+	+	−	−	−	5.2
	23A	+	+	−	−	−	4.7
	57A	+	+	−	+	+	5.3
Selenomonas ruminantium var. *lactilytica*	Type*	−	+	+	+	+	4.3
	53	+	+	+	−	+	4.3

* Bryant (1956).

trol. Additional characteristics of six of the isolates appear in Table 4.

Lyophilized 12-hr SRP-A broth cultures of the *Selenomonas*, the selenomonadlike B-385, and the *Bacteroides* species remained viable when stored at 2 C for 1 year, whereas most of the *Butyrivibrio* cultures, the *Succinivibrio* culture, and the *Bacteroides* cultures were viable after 2 years of storage.

The morphology and growth characteristics of all of these isolates fit the descriptions of the taxa to which they have been assigned, with the single exception of the *Lachnospira*-like colonial morphology exhibited by one of the selenomonadlike B-385 cultures.

Discussion

The average colony count of organisms from ruminal ingesta on all media was 2.6×10^9, of which 1×10^9 were proteolytic. This represents 13% of the direct microscopic count of 2×10^{10} estimated by Hungate (1957), and agrees with the cultural count obtained by Bryant and Robinson (1961) in an improved nonselective medium with samples from animals on a similar ration.

In the initial determination of acid-fermentation products using the method of Neish (1952), complete separation of succinic and lactic acids was not achieved. A second series of fermentations was carried out in a modified medium with lyophilized cultures as inoculum, and the acid-fermentation products were determined by the method of Bruno and Moore (1962), in which the qualitative identification of each acid component of the fermentation is positive.

Of the 38 cultures, 30 fit the description of the genus *Butyrivibrio* (Bryant and Small, 1956*a*) in that they were obligately anaerobic, gram-negative, small, straight-to-curved rods with rounded ends ranging in size from 0.4 to 0.6 by 1 to 4 μ with polar-to-subterminal, monotrichous flagellation. All produced butyric or lactic acid, or both, often accompanied by acetic and formic acids as well as gas. Two of these organisms showed some production of succinic acid in addition to the fermentation acids characteristic of *Butyrivibrio*.

One of the remaining cultures was classified as *Succinivibrio* species (Bryant and Small, 1956*b*) because it was an obligately anaerobic, gram-negative, small, straight-to-curved rod which had terminal-to-subterminal monotrichous flagellation, measured 0.6 by 1 to 2 μ, and produced large amounts of acetic and succinic acids in the fermentation of glucose.

Three of the seven remaining cultures were larger, obligately anaerobic, gram-variable, curved, rod-shaped organisms with tufted flagellation. One of these fit the description of *Selenomonas ruminantium* var. *lactilytica* (Bryant, 1956) because the tuft of flagella was on the concave side of the cell, because this strain fermented lactate, and because it fermented glucose with the production of lactic, formic, and propionic acids. The cells measured 1 to 1.5 by 2 to 4 μ. The final pH in the lightly buffered medium was quite low (pH 4.3). This organism showed a small amount of growth on glycerol, moderate growth on starch, and abundant growth on lactose and mannitol.

In contrast, the other two cultures of gram-variable rods were smaller, measuring 1 by 2 to 4 μ, had a higher final pH (4.7 to 5.3), and produced butyric and longer-chained fatty acids (but

not propionic acid) from glucose. Formic, lactic, and succinic acids were also produced from glucose. These organisms exhibited little or no growth in lactate- or mannitol-supplemented media, and both utilized starch and lactose. Propionate from the rumen fluid in the medium was utilized by both strains, and one utilized glycerol. The morphology of colonies produced by one strain in roll tubes, slants, and broth cultures was similar to that of *Lachnospira multiparus* (Bryant and Small, 1956*b*). Both of these strains were otherwise similar to the selenomonadlike organisms designated the B-385 group by Bryant (1956), which as yet are unnamed.

Of the remaining four cultures, three were gram-negative, pleomorphic, nonmotile, obligately anaerobic rods measuring 0.5 to 1 by 1 to 3 μ. These strains were classified as members of the genus *Bacteroides*. They grew well in the medium in which yeast extract replaced rumen fluid. They utilized starch and lactose but not mannitol and lactate. Two strains grew poorly on glycerol. The final pH ranged from 4.8 to 5.8 in the lightly buffered medium. These organisms exhibited some characteristics similar to those of *B. ruminicola* subsp. *brevis* (Bryant et al., 1958*a*); however, there were great differences in fermentation products, and, since they produced butyric acid from glucose, they may be more closely related to the lactate-fermenting R-2 group of Bryant et al. (1958*b*) or the proteolytic, butyric acid-producing *Bacteroides* species of Geisecke (1962) or Blackburn and Hobson (1962).

The remaining culture was an obligately anaerobic, gram-negative, motile, helicoidal organism of 0.3 by 5 to 15 μ; it was placed in the genus *Borrelia*. The fermentation products of this spirochete are quite different from those of the rumen strain of Bryant (1952) or the free-living *Treponema zuelzerae* (Veldkamp, 1960).

It is significant that all of the proteolytic ruminal organisms studied in this work represent species or genera already isolated as members of other physiological groups. Unfortunately, not all of the proteolytic organisms isolated in this study remained viable, but there was no morphological evidence that they represented new species.

These observations suggest, as inferred by Bryant (1959), that the proteolytic activity of ruminal ingesta can be largely accounted for by bacterial species which are known to occur in large numbers in the rumen.

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