The Isolation and Classification of Proteolytic Bacteria from the Rumen of the Sheep

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SUMMARY: Proteolytic bacteria were isolated from the rumen of three sheep receiving an adequate protein diet. Using selective anaerobic conditions to suppress the majority of bacteria which were not capable of utilizing casein, proteolytic representatives of several bacterial genera were found. All of these may have been derived from the environment or food of the sheep. The most frequent organism was *Bacillus licheniformis* which existed in the vegetative form in the rumen, although present mostly in the form of spores in the hay fed to the animal.

Since it was reported by Sym (1938) that the bovine rumen contained a highly active proteinase attributable to microbial origin, more recent observations by McDonald (1952), by Annison, Chalmers, Marshall & Synge (1954) and by Chalmers, Cuthbertson & Synge (1954*a, b*) have clearly shown that when protein (in the form of casein, gelatin, ground-nut meal, and to a less extent herring meal) is fed to sheep a rapid and extensive breakdown of the protein may occur. This was found to result in liberation of ammonia in the rumen, with a consequent loss in nutritive value of the protein to the animal. Since the organ itself elaborates no proteolytic enzymes this breakdown must be attributed to the micro-flora or micro-fauna of the rumen, either by the secretion of extracellular proteases, or by intracellular enzymes liberated into the rumen after death and autolysis amongst the microbial population. Liberated intracellular enzymes must be an inevitable component of such a complicated mixed culture as the rumen presents. But the presence of extracellular protease will be determined by the types of actively multiplying micro-organisms present.

Much work has been carried out recently upon the bacterial flora of the rumen, and many rumen bacteria have been isolated and studied in pure culture. Very few of these produced gelatinase. From the bovine rumen an anaerobic Gram-positive rod, designated RO-CL, was isolated by Huhtanen & Gall (1953*b*) which produced acetic and propionic acids from lactic acid, and which was proteolytic; Bryant & Burkey (1953*b*) described a Gram-positive gelatin liquefying rod which they designated +SR -gGXC.

In many cases the rumen bacteria which have been described were among the predominant types present and were isolated from high dilutions (Bryant & Burkey, 1953*a, b*); or they were selected by virtue of their cellulolytic (Hungate, 1950) or saccharolytic properties (MacPherson, 1953; Mann, Masson & Oxford, 1954; Mann & Oxford, 1954). Huhtanen, Rogers & Gall (1952) and Huhtanen & Gall (1953*a, b*) used what was in effect an enrichment method to cultivate bacteria of fastidious nutritional requirements by inoculation of high dilutions into broth. It seems evident that proteolytic bacteria,

if present in the rumen, are not usually amongst the predominant organisms, though they may nevertheless be of great importance in bringing about the first stage of protein degradation, thus providing substrates for attack by many other types of bacteria with subsequent breakdown of amino acids. It was considered therefore that a systematic investigation of the types of bacteria in the rumen of the sheep which produce extracellular protease *in vitro* might provide a useful addition to the existing knowledge of rumen microbiology.

METHODS

The method used for primary isolation of proteolytic bacteria was based upon that of Hungate (1950) but the medium was modified to have a selective effect upon the rumen population and to encourage the growth only of those bacteria capable of utilizing protein. To 45 ml. mineral salt solution 1 (0.3 % K_2HPO_4) and 45 ml. mineral salt solution 2 (0.3 % KH_2PO_4 , 0.6 % NaCl, 0.06 % $MgSO_4$, 0.06 % $CaCl_2$) was added 6 g. agar (Davis), 1 ml. resazurin solution (0.005 %), and either 1.5 g. yeast extract (Difco) or 90 ml. clarified rumen fluid, withdrawn from the rumen of a sheep upon a low protein diet. In some cases 1.5 g. peptone (Evans) was included. The volume was made up to 300 ml. with distilled water. This basal medium was boiled thoroughly and filtered through paper pulp; 200 ml. was then measured and sterilized by autoclaving. The following additions were made aseptically—34 ml. 6 % casein solution (B.D.H., light white soluble casein) previously sterilized by steaming, 13 ml. sterile 6 % Na_2CO_3 , 8.4 ml. sterile 3 % cysteine HCl. CO_2 was bubbled through the hot medium until the resazurin was reduced, and the pH value was varied from 6.8 to 7.4. This medium was transferred aseptically to sterile tubes (18 × 150 mm.) fitted with rubber bungs, under an atmosphere of CO_2 , in 8 ml. quantities, and kept at 48° until required.

Three sheep were used, all with rumen fistulas. Two of these, sheep 969 and sheep 70, were fed 300 g. ground-nut meal, 300 g. flaked maize and 300 g. hay daily. The third, sheep 245, received 100 g. hay with 400 g. stock diet (maize, oats, bran, linseed meal, white fish meal) daily. For each experiment rumen samples of about 30 ml. were withdrawn from deep within the rumen, and as short a time as possible was allowed to lapse between the collection of the sample and inoculation of the medium. Some samples were homogenized for 3 min. in a Waring blender; all were very lightly centrifuged. Serial 1/10 dilutions were prepared either in the anaerobic diluting fluid advocated by Bryant & Burkey (1953*a*) or in normal saline; 1 ml. volumes of appropriate dilutions were used as inocula for the previously prepared tubes of medium, in duplicate or triplicate, from which roll tube cultures were made. These were incubated at 38° for 4 or 5 days.

RESULTS

As this protein-containing medium is initially cloudy because of casein in suspension extracellular 'casease' could be clearly demonstrated in some cases by a zone of clearing around the colonies. This could not however always be

relied upon as a criterion for recognizing a caseolytic organism because in some cases the zone of clearing was so narrow as not to be readily visible in roll tube culture, even when on subculture protease could be demonstrated. On the other hand, with the less dilute inocula, a large area of medium cleared rapidly, and this extensive casein breakdown permitted active growth of colonies not in themselves proteolytic. All isolated colonies available were therefore transferred to slope cultures, purified, and inoculated into nutrient gelatin and litmus milk in the usual manner.

Total viable counts were similar for all three sheep, and, with an accurate technique, were of the order of 10^5 to 10^7 /ml. of rumen fluid, depending upon the composition of the medium. When peptone was included the $1/10^6$ dilution tubes were the most suitable for counting, and the predominant flora consisted of Gram-positive (usually catalase-negative) cocci, with smaller numbers of bacilli. With the addition of fermentable carbohydrate (glucose or cellobiose) the colony count was rather higher and the flora more mixed, but proteolytic anaerobes were not amongst the isolates. In the absence of peptone or carbohydrate, yeast extract as supplement allowed the development of a rather larger number of colonies than did rumen liquor, but with no gross difference in the predominant types developing; in either case bacilli and Gram-negative rods were most frequent, with smaller numbers of micrococci, corynebacteria, and Gram-negative diplococci. Sheep 245 differed from the other two in that many of the Gram-negative rods isolated produced acid and gas in lactose bile-salt broth; with sheep 70 and 969 coliform organisms were infrequent.

Of 176 cultures isolated 77 produced gelatinase and most of these 77 cultures also peptonized casein. All the proteolytic isolates were representative of bacterial genera likely to be found in the environment, food and water of the sheep. With the exception of clostridia, all were facultative anaerobes.

Bacillus species

The majority of proteolytic strains isolated at dilutions of $1/10^4$ to $1/10^7$ of rumen fluid were facultative anaerobic members of the genus *Bacillus*. Of 24 *Bacillus* isolates examined by the methods recommended by Gibson (1944) and by Smith, Gordon & Clark (1946) 19 isolates were clearly identified as *B. licheniformis*. Though these strains exhibited considerable differences in cultural form, from mucoid to rugose or adherent, with or without pigment, their biochemical properties showed them to be closely related. Morphology placed them indisputably in group 1 of Smith *et al.* (1946) with one exception—one strain only exhibited definite equatorial swelling of the mature sporangium, though in all other respects it was typically *B. licheniformis*. All strains liquefied gelatin and peptonized casein in litmus milk rapidly anaerobically at 38° . Fermentation of glucose, xylose and arabinose was, as found by Smith *et al.* (1946), shown more consistently on agar slopes containing an ammoniacal nitrogen source than on peptone agar, and only one strain failed to ferment arabinose, while two others did not ferment xylose. Other properties

agreed with those previously recorded by Knight & Proom (1950). These authors also have shown that *B. licheniformis* requires no growth factors, and all the rumen strains of this species grew well through several subcultures with an ammonium salt as the sole source of nitrogen. The Gibson-Abdel-Malek reaction was positive, though in some cases CO₂ production could be demonstrated convincingly only in 5 % glucose agar milk cultures, and not well in 5 % glucose soft gelatin cultures (Gibson & Abdel-Malek, 1945).

Two cultures appeared related to *Bacillus licheniformis*, but differed in not producing CO₂, and in being able to grow under decreased oxygen tension but not under strict anaerobic conditions. One culture, in which NO₃ reduction and diastase were not demonstrated, appeared to be *Bacillus pumilus*, and the second, by virtue of starch fermentation without NO₃ reduction, was probably a *subtilis-pumilus* intermediate.

Two cultures were identified as *Bacillus cereus*. The average width of these vegetative rods was 1-1.2 μ . Production of phospholipinase was shown by the egg-yolk reaction of McGaughey & Chu (1948). Xylose and arabinose were not fermented; glucose was fermented with formation of acetylmethylcarbinol, without CO₂, but contrary to the findings of Knight & Proom (1950) starch hydrolysis was not demonstrated. Gelatin and casein were attacked rapidly. These strains were not able to utilize ammoniacal nitrogen.

One culture of slender rods, Gram-positive only when very young, and sporulating infrequently within clavate sporangia, was identified as *Bacillus circulans*; both rough and mucoid variants were obtained. The organism liquefied gelatin and, unlike the majority of strains of *B. circulans* examined by Smith *et al.* (1946), peptonized casein in litmus milk. In all other properties observed the rumen strain agreed with the characters described by Smith *et al.* (1946) and by Knight & Proom (1950) for *B. circulans*.

All except two of the rumen bacilli examined grew well under anaerobic conditions at 38° upon an agar medium containing 40 % (v/v) rumen fluid from sheep 245 supplemented with 1 % casein, 0.1 % glucose, inorganic salts and bicarbonate buffer. Under these conditions endospores were very rarely seen, but vegetative growth took the form of pleomorphic Gram-positive rods and filaments, usually very granular. It was only when cultivated aerobically upon a suitable medium, and sometimes at a lower temperature, that the typical *Bacillus* morphology was exhibited. Similar granular pleomorphic rods which were perhaps bacilli were seen in Gram-stained films of rumen fluid.

That *Bacillus* spp. were in fact present in the rumen in the vegetative form rather than as endospores may be deduced from Table 1. After heating rumen fluid for 5 min. in a boiling water bath the numbers of organisms which could be isolated upon the casein roll tube medium were very significantly smaller than the numbers isolated in parallel counts without heating. To facilitate the recognition of bacillus colonies only, these counts were made in Petri dishes incubated under CO₂.

A search for *Bacillus licheniformis* in the hay fed to all three sheep showed the spores of this organism to be present in large numbers. Dry hay (10 g.) was thoroughly ground with 100 ml. broth, and the extract, unheated and after

heating in a boiling water bath for 5 min. plated anaerobically on nutrient agar. The spore count was of the order 10^5 /g. hay, and 15 colonies examined were all found to have the morphology of group 1 bacilli (Smith *et al.* 1946) and to give a positive Gibson–Abdel-Malek reaction, and were therefore presumed to be *B. licheniformis*. On plates of unheated hay extract bacilli were not found at a dilution greater than $1/10^6$, and it thus appears that the majority of facultative anaerobic bacilli in the hay were, in contrast to rumen fluid, in the form of spores.

Table 1. *Colony counts of Bacillus spp. on casein medium in CO₂ with and without preliminary heating*

Exp.	Sheep	Rumen fluid unheated. Dilutions			Rumen fluid heated. Dilutions		
		$1/10^3$	$1/10^4$	$1/10^5$	$1/10^1$	$1/10^2$	$1/10^3$
1	969	91	40	4	—	8 16	1 2
2	969	—	— c. 100	27 40	1 2	0 0	— —

Clostridium species

Four cultures of *Clostridium sporogenes* were isolated, from sheep 70 and 969. Because this actively proteolytic organism produces an alkaline reaction in peptone media, carbohydrate fermentation was not easy to detect by the usual method of inoculation into peptone water sugar broths and the results were variable. Much more consistent results were obtained on peptone agar slopes with the desired carbohydrates and indicator incorporated, as advocated by Smith *et al.* (1946) with reference to those *Bacillus* spp. which were not able to utilize inorganic nitrogen. Under these conditions acid was formed readily from glucose, fructose, galactose, sucrose or maltose, and after 5 days from salicin. Two strains in addition produced acid from xylose, raffinose, dulcitol and inulin after 5 days, while fermentation of starch, lactose and mannitol was consistently negative. Indole was not detected in peptone water cultures, but all other characters observed agreed with those described for *C. sporogenes* (Metchnikoff) in *Bergey's Manual* (1948).

Gram-negative rods

Of 45 isolates of Gram-negative rods only 5 liquefied gelatin, and none of these peptonized casein in litmus milk. Two pigmented strains appeared to be *Flavobacterium arborescens* (*Bergey's Manual*, 1948). Five non-pigmented strains were similar to each other; by virtue of their urease production, fermentation of glucose but not lactose, variable and uncertain production of gas in carbohydrate broth, and motility at 25° but not at 38°, they were considered to belong to the genus *Proteus* (*Bergey's Manual*, 1948); species names could not be assigned.

As Gram-negative rods in general quite frequently elaborate proteases, and as these organisms were evidently quite numerous in the rumen, it seemed

possible that the abundant proliferation of *Bacillus* spp. might mask the presence of proteolytic Gram-negative rods. The bacilli were therefore suppressed by the addition of 10 units penicillin/ml. to the roll tube cultures. The flora then became exclusively Gram-negative, and 19 rods were isolated; none of these liquefied gelatin.

Corynebacteria

Corynebacteria were isolated fairly frequently but only one culture, from sheep 70, was proteolytic. Morphology and method of cell division placed this bacterium clearly in the genus *Corynebacterium* but it was not possible to accord it a species name. Rods were Gram-positive, non-motile, and varied in size from coccoid (liquid media) to rods 3–4 μ . long. Growth on nutrient agar was abundant, aerobically and anaerobically, with formation of yellow pigment; in glucose broth a yellow viscous deposit was formed. When first isolated gelatin liquefaction was rapid; in litmus milk the reaction became alkaline but casein was not digested. Glucose, galactose, sucrose, lactose and maltose were fermented, but not xylose, dulcitol, mannitol, inulin or salicin. Orla-Jensen (1919) described *Microbacterium* spp. possessing properties like this organism in morphology, failure to ferment pentoses or alcohols, production of catalase, reduction of nitrates, and in surviving, in some numbers, a temperature of 80° for 2½ min. when heated in an agar medium.

Micrococci

Of several strains of Gram-positive catalase-positive cocci 6 produced partial liquefaction of glucose nutrient gelatin anaerobically within 7 days, without change of litmus milk. Their proteolytic activity did not in any way compare with that of the bacilli and clostridia. All cultures were non-pigmented; they all fermented glucose. Four strains were VP-positive and therefore belonged to subgroup 2 of Shaw, Stitt & Cowan (1951); 2 strains were VP-negative and therefore fell into their subgroup 3. Though the proteolytic powers were weak a fairly large range of carbohydrates was fermented, and urease was produced by all cultures.

The numbers of each group of organism isolated from individual sheep are recorded in Table 2.

Table 2. *Proteolytic isolates from rumen contents of hay- and concentrate-fed sheep*

Organism	Sheep numbers		
	70	969	245
	Isolates (no.)		
<i>Bacillus</i> spp.	19	24	18
<i>Clostridium</i> spp.	2	2	0
Gram-negative rods	4	0	1
<i>Corynebacteria</i>	1	0	0
Micrococci	4	2	0
% of isolates which were proteolytic	57	42	27

DISCUSSION

Isolations of proteolytic bacteria were made under such conditions of temperature, anaerobiosis, and bicarbonate buffer as would be expected to allow the development of 'true rumen bacteria' in the strict sense, i.e. species having their natural habitat in the rumen and nowhere else. If such organisms exist, there is undoubtedly also a large population of bacteria which are well known in other habitats, which are derived from external sources and are able to multiply within the rumen, and it is to this category that the proteolytic bacteria appear to belong. The most frequently isolated organisms were facultatively anaerobic *Bacillus* spp. These ubiquitous saprophytic bacteria are not commonly associated with an animal host, and although they grew well on a medium containing much rumen fluid, and under such physical conditions as are believed to obtain in the rumen, their metabolic activity there is difficult to assess. The numbers found bear a not insignificant relation to the total population, as the total viable count obtained upon a rich medium under the conditions used in these experiments was of the order 10^9 . The ratio of proteolytic to non-proteolytic bacteria was then, at the most, approximately 1 in 100. All the sheep used in these experiments were ingesting daily considerable numbers of bacilli from their feed in the form of spores, but spores were not detected to any great extent in the rumen, where vegetative forms were present in dilutions of $1/10^4$ to $1/10^7$. If, as appears to be the case, conditions within the rumen are suitable for the prompt germination of *Bacillus* spp. spores then it seems unlikely that multiplication would not follow. A parallel case may perhaps be found in the work of Gutierrez (1953) who reported the isolation of *Corynebacterium acnes* in considerable numbers from hay, and also from the rumen of cattle eating it, and this organism was believed to play an active part in the rumen propionic acid fermentation. The ration fed to the sheep of the present experiment was preponderantly carbohydrate. It would be interesting to determine whether a higher proportion of protein in the ration would result in a greater relative count of proteolytic bacteria.

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REFERENCES

- ANNISON, E. F., CHALMERS, M. I., MARSHALL, S. B. M. & SYNGE, R. L. M. (1954). Ruminal ammonia formation in relation to the protein requirement of sheep. III. Ruminal ammonia formation with various diets. *J. agric. Sci.* **44**, 270.
- Bergey's Manual of Determinative Bacteriology*. 6th ed. (1948). Edited by Breed, R. S., Murray, E. G. D. and Hitchens, A. P. London: Baillière, Tindall and Cox.
- BRYANT, M. P. & BURKEY, L. A. (1953*a*). Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J. Dairy Sci.* **36**, 205.
- BRYANT, M. P. & BURKEY, L. A. (1953*b*). Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. *J. Dairy Sci.* **36**, 218.

- CHALMERS, M. I., CUTHBERTSON, D. P. & SYNGE, R. L. M. (1954*a*). Ruminal ammonia formation in relation to the protein requirement of sheep. I. Duodenal administration and heat processing as factors influencing fate of casein supplements. *J. agric. Sci.* **44**, 254.
- CHALMERS, M. I., CUTHBERTSON, D. P. & SYNGE, R. L. M. (1954*b*). Ruminal ammonia formation in relation to the protein requirement of sheep. II. Comparison of casein and herring-meal supplements. *J. agric. Sci.* **44**, 263.
- GIBSON, T. (1944). A study of *Bacillus subtilis* and related organisms. *J. Dairy Res.* **13**, 248.
- GIBSON, T. & ABDEL-MALEK, Y. (1945). The formation of carbon-dioxide by lactic acid bacteria and *Bacillus licheniformis* and a cultural method of detecting the process. *J. Dairy Res.* **14**, 35.
- GUTIERREZ, J. (1953). Numbers and characteristics of lactate utilizing organisms in the rumen of cattle. *J. Bact.* **53**, 123.
- HUHTANEN, C. N. & GALL, L. S. (1953*a*). Rumen organisms. I. Curved rods and a related rod type. *J. Bact.* **65**, 548.
- HUHTANEN, C. N. & GALL, L. S. (1953*b*). Rumen organisms. II. Two lactate utilizers and six miscellaneous types. *J. Bact.* **65**, 554.
- HUHTANEN, C. N., ROGERS, M. R. & GALL, L. S. (1952). Improved techniques for isolating and purifying rumen organisms. *J. Bact.* **64**, 17.
- HUNGATE, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bact. Rev.* **14**, 1.
- KNIGHT, B. C. J. C. & PROOM, H. (1950). A comparative survey of the nutrition and physiology of mesophylic species in the genus *Bacillus*. *J. gen. Microbiol.* **4**, 508.
- MCDONALD, I. W. (1952). The role of ammonia in ruminal digestion of protein. *Biochem. J.* **51**, 86.
- MCGAUGHEY, C. A. & CHU, H. P. (1948). The egg-yolk reaction of aerobic sporing bacilli. *J. gen. Microbiol.* **2**, 334.
- MACPHERSON, M. J. (1953). Isolation and identification of amylolytic streptococci from the rumen of the sheep. *J. Path. Bact.* **66**, 95.
- MANN, S. O., MASSON, F. M. & OXFORD, A. E. (1954). Facultative anaerobic bacteria from the sheep's rumen. *J. gen. Microbiol.* **10**, 142.
- MANN, S. O. & OXFORD, A. E. (1954). Studies of some presumptive lactobacilli isolated from the rumens of young calves. *J. gen. Microbiol.* **11**, 83.
- ORLA-JENSEN, S. (1919). *The Lactic Acid Bacteria*. Copenhagen: A. F. Høst and Son.
- SHAW, C., STITT, J. M. & COWAN, S. T. (1951). Staphylococci and their classification. *J. gen. Microbiol.* **5**, 1010.
- SMITH, N. R., GORDON, R. E. & CLARK, F. E. (1946). Aerobic mesophilic spore-forming bacteria. *Misc. Publ. U.S. Dep. Agric.* no. 559.
- SYM, E. A. (1938). Hydrolasenwirkung des Blindsackinhaltes des Pferdes und des Panseninhaltes des Rindes. I. Teil Einleitung, allgemeine Methoden und proteolytische Wirkungen. *Acta Biol. exp., Varsovie*, **12**, 192.

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