NUMBERS AND CHARACTERISTICS OF LACTATE UTILIZING ORGANISMS IN THE RUMEN OF CATTLE

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Elsden (1945) has shown that the volatile acids in the rumen fluid of sheep are chiefly acetic, propionic, and butyric. Barcroft, McAnally, and Phillipson (1944) showed that the concentration of volatile acids in the blood draining the rumen was higher than that in the entering blood, indicating absorption from the rumen.

Elsden postulated that propionic acid did not arise directly in cellulose fermentation in the rumen but was derived from lactic acid. In agreement with this view Hungate (1950) has found formic, acetic, and butyric acids, but no propionic acid, produced from cellulose by pure cultures of bacteria isolated from the rumen.

Elsden (1945) cultured an unidentified non-cellulolytic gram positive coccus from the rumen of sheep which was assigned to the genus Propionibacterium and which produced propionic acid, acetic acid, and CO₂ from lactate. The numbers of these organisms were not determined. From New Zealand, Johns (1951) has reported an anaerobic gram negative coccus, Veillonella gasogenes, isolated from the rumen of sheep, which produces propionic and acetic acids, hydrogen, and carbon dioxide. It occurred in numbers of about one million per ml and rapidly attacked succinic acid, forming propionic acid and carbon dioxide.

In the present study an attempt has been made to obtain more information on the lactate utilizing bacteria in the rumen of cattle by determining the kinds and numbers present.

EXPERIMENTAL METHODS

Samples of rumen fluid were obtained with a tube and suction bulb (Hungate, 1950) from a steer with an artificial fistula and from a dairy cow which retained a fistula following recovery from an injury. From normal animals rumen contents were obtained by stomach tube. The samples were inoculated as rapidly as possible (within 30 minutes) into two parallel dilution series, one of which was enriched with 0.5 per cent sodium lactate. Organisms utilizing lactate were identified by the larger colony size in the lactate series as compared with the nonlactate control.

The agar medium used initially for isolation of lactate utilizers contained inorganic salts plus 30 per cent rumen fluid. Cysteine (0.05 per cent) was used as a reducing agent and resazurin as an indicator of oxidation-reduction potential. Anaerobic conditions were maintained by bubbling the tubes with carbon dioxide, and 0.5 per cent sodium bicarbonate was added to buffer the cultures at a pH of approximately 7.0. The culture technique and inorganic salts were similar to those previously used in this laboratory (Hungate, 1950). All cultures were incubated at 37 C.

RESULTS

The lactate utilizers first noted were small gram negative curved rods which appeared after two days of incubation. They formed colonies varying in color from brown to almost black. The black color suggested that hydrogen sulfide might be formed from sulfate, and addition of ferrous sulfate to the medium gave a copious black precipitate of ferrous sulfide, indicating that the organisms belonged to the genus Desulfovibrio. The numbers varied between 50,000 and 100,000 per ml of rumen fluid. Some tested strains attacked malate. The relatively low numbers in which Desulfovibrio is found suggest that it is relatively unimportant as a lactate utilizer in the normal rumen. Their presence in the rumen does suggest that under conditions of high sulfate intake these organisms might produce sufficient quantities of hydrogen sulfide to be toxic (Dougherty, 1942).

1 This investigation was supported in part by funds provided for biological and medical research by the State of Washington, initiative measure number 171.
After 7 to 14 days of incubation another type of colony consisting of gram positive rods appeared in higher dilutions of both lactate and nonlactate series, but with a greater size in the lactate medium. Three strains were isolated using the rumen fluid lactate medium, but they did not show consistently good growth in subcultures. The medium was changed to one per cent yeast extract, one per cent peptone, 0.5 per cent sodium lactate, agar, and inorganic salts. Because this medium gave more rapid and satisfactory growth, it was used for subsequent transfers and for initial isolations. Two more strains were isolated, the lactate utilizers being detectable in the new medium after five days of incubation.

The cells of all strains were 0.5 to 0.8 μ in diameter and 1.0 and 3.0 μ long, with metachromatic granules. Longer rods appeared under acid conditions and showed some branching. On anaerobic yeast extract-glucose agar the colonies were 1 to 3 mm in diameter, butyrous, cream colored, and convex, with old colonies occasionally showing rose pigmentation. Liquid yeast extract cultures developed a ropy, creamy sediment after two days of incubation.

All strains grew best under anaerobic conditions, and oxygen inhibited growth to the extent that a stab culture exposed to air showed only a very slight surface growth. The carbon dioxide requirement was tested by replacing the carbon dioxide used in bubbling the culture tubes with 100 per cent nitrogen and eliminating the sodium bicarbonate buffer. Growth with nitrogen was much slower and colonies were fewer as compared with cultures in which 100 per cent carbon dioxide was used.

An analysis for the fermentation products from glucose and from lactate showed propionic and acetic acids and carbon dioxide. Tests for succinic acid were negative. No neutral products were found. These characteristics indicated that the organisms belonged in the genus Propionibacterium. Other characteristics of the strains are shown in table 1.

These first five isolated strains came from large and characteristic colonies that were recognized easily in the lactate dilution series. The validity of identification by colony appearance was tested in another experiment by picking nineteen additional typical colonies directly into liquid yeast extract plus one per cent sodium lactate. These were picked from nineteen initial dilution series, each inoculated from a different animal. After incubating for five days at 37 C, the acidified cultures were steam distilled and the volatile acids titrated. Inoculated control tubes without lactate were treated similarly and the amount of volatile acid subtracted from that found in the lactate tubes. The difference was assumed to arise from the fermentation of the lactate. The amounts of volatile acid produced from lactate by the nineteen strains ranged from 0.16 to 0.31 meq per 10 ml of medium. Duclaux distillation values for all strains indicated a mixture of acetic and propionic acids. For five of the cultures the volatile acids were separated and identified as acetic and propionic acids using the silica gel partition method of Elsden (1946). In four of these strains the ratio of propionic to acetic acids was about two to one, but less propionic than acetic was found in the fifth.

Using the colony appearance as a means of identifying the propionibacteria, the numbers in the rumen contents of the nineteen different cows were estimated. The numbers varied from 250 thousand to 7 billion (7 × 10⁹) per ml of rumen fluid, with an average of 1.3 billion. Although this high count seemed to indicate an active growth of propionibacteria in the rumen, a check was made to see if these bacteria were present also in hay. A culture count was made on a sample of timothy hay similar to that previously fed the nineteen cows, again using yeast extract-peptone agar with parallel lactate and nonlactate series. Propionibacteria appeared in dilutions which indicated that the number of colonies per gram of hay (air dry wt) was 600 million. A sample of alfalfa hay (Pullman, Washington) gave a count of 300 million per g and one of silage gave 10 million per g. In addition, strains were isolated from cuttings of green alfalfa obtained in Medford, Oregon (200 million per g), and Salinas, California (400 million per g), and were identified as Propionibacterium from the volatile acids produced and from other characteristics. In the Colton, Washington, area the following culture counts were obtained: Red Top hay (Agrostis alba) 2 billion per g, brome hay (Bromus inermis)

* The animals used were pregnant cows which were being fed various diets in an experiment conducted by the Department of Animal Husbandry, the results of which have been reported elsewhere (Galgan and Schneider, 1951).
1 billion per g, and alfalfa hay, 600 million per g. Two dilution series inoculated with soil from a lawn and from an alfalfa field in the Pullman vicinity showed counts of 600 million propionibacteria per g for each soil sample. The strains isolated from these different sources showed cultural and biochemical characteristics similar in most respects to those of the propionibacteria isolated from the rumen fluid of cattle (table 1).

The large numbers of propionibacteria in hay were quite surprising and suggested that they grew readily in this material. To test this the five strains pure cultured from the rumen were inoculated into a liquid medium composed of an extract prepared by boiling 2.0 per cent timothy hay, 0.5 per cent cottonseed meal, and 0.5 per cent ground barley in inorganic salt solution and filtering through cheesecloth lined with cotton.

On agar shake tubes of this medium, colonies were visible in four days. This is slightly faster growth than on yeast-extract lactate and considerably faster than on rumen fluid lactate medium. However, the colonies did not continue to grow to the large size attained with the other media. In a flask culture containing 200 ml of this hay infusion medium, 1.89 meq of a mixture of propionic and acetic acids were produced by strain B38. This indicates that in addition to lactic acid these organisms have the capacity to attack directly some of the materials ingested into the rumen.

In view of the report by Johns (1951) that *V. gazogenes* was an important lactate utilizing bacterium in sheep, it seemed essential to determine whether this species was present also in cattle. Since no rapidly developing colonies an-

### TABLE 1

**Comparison of characteristics of strains of Propionibacterium acnes**

<table>
<thead>
<tr>
<th>RUMEN STRAINS</th>
<th>HAY STRAINS</th>
<th>SOIL STRAINS</th>
<th>HUMAN STRAINS (DATA FROM DOUGLAS AND GUNTER, 1946)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol......</td>
<td>-+ + + + +</td>
<td>+ + + + +</td>
<td>27 strains</td>
</tr>
<tr>
<td>Sucrose.......</td>
<td>+ - + + +</td>
<td>+ + + + +</td>
<td>6 strains</td>
</tr>
<tr>
<td>Glucose.......</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>3 strains</td>
</tr>
<tr>
<td>Glyceral.......</td>
<td>- + + + +</td>
<td>- + + + +</td>
<td>1 strain</td>
</tr>
<tr>
<td>Maltose........</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>Not given</td>
</tr>
<tr>
<td>Mannose.......</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Sorbitol.......</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>All strains positive</td>
</tr>
<tr>
<td>Fructose.......</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>All strains positive</td>
</tr>
<tr>
<td>Galactose.......</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>Rennet curd at 37 C in 14 days followed by peptonisation</td>
</tr>
<tr>
<td>Lactose.......</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>Liquefied in 10 days</td>
</tr>
<tr>
<td>Lactate.......</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>All strains gram positive</td>
</tr>
<tr>
<td>Indole.........</td>
<td>- + + + +</td>
<td>- + + + +</td>
<td>All strains gram positive</td>
</tr>
<tr>
<td>Nitrate reduction...</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Catalase.......</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Litmus milk.....</td>
<td>Curd in from 4 to 10 days, followed by peptonisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin.........</td>
<td>Liquefied in from 5 to 14 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram reaction....</td>
<td>All strains gram positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility.........</td>
<td>All strains nonmotile</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
swering Johns' description had been identified in the various dilution series from cattle rumen, contents from a sheep were used as an inoculum. In this series lactate utilizing colonies were easily detected after 24 hours of incubation, and the pure cultures obtained from them were found to be gram negative cocci using lactate but not glucose, and producing propionic and acetic acids. The small grey, lenticular colonies appeared within 24 hours accompanied by numerous gas bubbles in the agar. Stains showed irregular masses of cocci 2 to 3 μ in diameter. Sugars were not attacked. Indole was not formed and nitrate was not reduced. All characteristics observed agreed with the description of V. gazogenes. Using four sheep, numbers of colonies of V. gazogenes per ml of rumen fluid were found to be 180,000, 280,000, 520,000, and 120,000 per ml, respectively.

Using the information gained from the dilution series inoculated from the sheep, new dilution series inoculated with rumen contents of the fistulated steer were made, and in these it was possible to detect colonies of V. gazogenes in the lower dilutions. The numbers found in three different dilution series inoculated at different times were 2,000, 60, and 200 per ml of rumen contents.

It was interesting to note that when the initial rumen dilution series tubes of both sheep and cattle were held for longer periods of time, colonies of propionibacteria invariably appeared in significantly higher numbers than V. gazogenes. The Veillonella colonies could be seen in 24 to 36 hours whereas the propionibacteria required 6 to 8 days.

**DISCUSSION**

Douglas and Gunter (1946) have studied the metabolism of Corynebacterium acnes and found that it carries on a propionic acid fermentation of various carbohydrates but will not utilize lactate. These workers have proposed a change in the nomenclature of this organism to Propionibacterium acnes with the further provision that the phrase "ferments lactic acid" be deleted from the over-all description of the genus Propionibacterium (Breed et al., 1948).

The strains of Propionibacterium isolated during the present investigation liquefy gelatin, produce indole, and reduce nitrate. These characteristics are not possessed by the species listed in Bergey's Manual (except for nitrate reduction by Propionibacterium pentosaceum) but are described for P. acnes (Douglas and Gunter, 1946). The only significant difference between the characteristics of P. acnes reported by Douglas and Gunter and those found in the present strains is that the latter ferment lactate. Douglas subsequently has found lactate utilization by strains of P. acnes isolated from human sources, which erases this apparent difference. Accordingly, the strains of the present investigation are classified as Propionibacterium acnes. A difference between our strains and those of Douglas is the habitat from which they were obtained, but this does not seem to be a sufficient basis for separating them taxonomically, particularly in view of the great abundance of these bacteria in soil and plant materials and the possibility that human strains may derive from those sources.

It should be reported that in many of the initial dilution series from rumen, hay, and soil, irregularities were noted in the numbers of colonies in the various dilutions. The tubes of lower dilution sometimes showed no propionibacteria colonies, yet the higher dilution tubes would have several large ones. In subcultures from one of these colonies the organisms behaved in the conventional manner with many colonies appearing in the lower dilution tubes and the number diminishing as the dilutions increased. These discrepancies in colony number in initial series were disturbing, and it seemed desirable to find an explanation for them or to validate the reliability of culture counts by some independent method.

Using sterile medium as an inoculum, a test for contamination was performed, using all the materials and methods employed in experimental dilution series. No contaminants appeared after a suitable incubation period. Also, in the experimental dilution series no colonies were observed in the highest dilutions, indicating that contamination could not explain their presence in the lower dilutions.

Another possibility was that the alfalfa hay or other material used as an inoculum contained a heat labile substance which inhibited the organisms in the lower dilutions but was insufficient

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A culture of rumen strain B330B of Propionibacterium acnes has been deposited with the American Type Culture Collection in Washington, D. C., together with a description of the strain.

* Personal communication, 1963.
in the higher ones. This was tested experimentally by inoculating Propionibacterium strain B38 into a dilution series of the usual medium plus a Seitz filtered extract of alfalfa. No suppression of the organisms occurred as compared with a control series without extract.

Finally, as an independent means of substantiating the high numbers of propionibacteria, direct microscopic counts were made in conjunction with tube culture counts on rumen fluid and alfalfa hay. In making the direct counts all organisms morphologically similar to propionibacteria were counted.

Using a rumen fluid sample the culture count was 1,900,000 per ml and the direct count was 6,000,000 per ml. This count was made at a different time of the year than the others reported, which may account for the lower value. With the alfalfa hay a dry, weighed portion was used. Here the tube culture count was 15,600,000 per g and the direct microscopic count was 86,000,000 per g. The magnitude of the culture counts is about that which might be expected if the organisms counted directly were propionibacteria. These observations confirm the results of the culture counts and suggest that the culture count as determined by the higher dilution tubes is approximately correct even though the lower dilutions do not show the expected number of colonies. The partial suppression of propionibacteria in primary dilutions may be due to accompanying organisms.

The finding of large numbers of Propionibacterium in the rumen of both sheep and cattle, and their rapid development on a hay-grain medium suggest that they are important in the formation of propionic acid in the rumen. The significance of the large numbers in the rumen is somewhat difficult to evaluate because of the occurrence of such large numbers in hay. If the propionibacteria are ingested with the hay and do not grow in the rumen, their metabolic products may be relatively unimportant. But if they grow in the rumen, the metabolic activities of such a large number of organisms must be of significance. It is highly probable that they actually do grow in the rumen since it contains the nutrient materials found satisfactory for growth in laboratory cultures. Also, though there was much variation in the number of propionibacteria demonstrated in the rumen and in hay, the average values were somewhat higher for the rumen. This difference is even more marked when it is considered that the counts for the rumen contents were based on the liquid volume whereas those for hay were on a dry weight basis. Since the dry weight of the rumen contents is about 12 to 15 per cent of the wet weight (Carroll, 1952), it is apparent that a very significant growth of propionibacteria must have occurred in the rumen to give the observed counts.

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SUMMARY

Twenty-four strains of propionic acid producing bacteria have been isolated from the rumen of cattle, seven strains have been pure cultured from different types of hay, and four strains have been recovered from soil samples. They resemble Propionibacterium acnes provided that the description of this species be modified to include lactate utilization.

The numbers found in soil suggest that these organisms are widespread in nature, and the relatively large numbers in hay indicate that it may be a particularly favorable habitat. Experiments showed that the isolated propionibacteria can grow rapidly at the expense of constituents in hay and grain and produce considerable amounts of acetic and propionic acids in cultures.

These findings, together with the large numbers and consistent isolation of these bacteria from the rumen fluid of cattle, suggest an active role in the formation of propionic and acetic acids in the rumen, though the occurrence of such large numbers in hay makes it questionable whether the rumen is the site of most active growth.

REFERENCES


