PRODUCTION OF LACTIC ACID AND AN IODINE STAINING SUBSTANCE BY BOVINE RUMEN BACTERIA

R. Q. ROBINSON, R. N. DOETSCH, F. M. SIROTNAK, AND J. C. SHAW
Departments of Bacteriology and Dairy Husbandry, University of Maryland, College Park

Some years ago, Baker (1) divided the microorganisms of the rumen into iodophilic and aniodophilic forms, depending on the color produced within them upon application of an iodine staining solution. Iodophilic forms stained blue-mauve and many were considered to possess cellulolytic abilities. Earlier, Henneberg (10) observed cocci in enzymatically dissolved zones ("frassbette") in plant fragments from rumen contents. These organisms stained wine-red upon application of an iodine staining solution.

Recently, Oxford (16) and Masson and Oxford (15) found that holotrich ciliates from the rumen of sheep were active in depositing intracellular polysaccharide granules. The polysaccharide stained brownish-purple and was considered a reserve substance, starch-like in nature. This substance was synthesized from glucose, fructose, sucrose, inulin, and bacterial levan and to a lesser extent from cellulobiose, but not from maltose. Heald (9) observed rapid increases in stored polysaccharide after feeding, followed by an equally rapid decline. This worker did not agree with Baker's (2) suggestion that polysaccharide synthesis represented a mechanism whereby a source of carbohydrate was available in the intestine, since he believed that most of it was broken down before leaving the rumen. Elsden (7) noted the formation of an iodine staining material in rumen microorganisms when glucose was fed. In vitro observations also were made with whole rumen liquor diluted 1:10 in a buffered salt-substrate mixture incubated anaerobically. Finally, Doetsch et al. (5) reported that an iodine staining substance (ISS) was produced by mixed suspensions of rumen bacteria in vitro from carbohydrates but not from citric acid cycle compounds or fatty acids through C4.

In the paper above (5) it also was stated that lactic acid was not found in rumen liquor of normal animals on a hay and grain diet except in trace amounts. Phillipson and McAnally (18) reported that lactic acid appeared transiently when a cabbage-mangold diet was fed or when glucose was introduced into the rumen. In the work of Elsden (7) cited above it was found that the fermentation of glucose by rumen microorganisms resulted in lactic acid production, but this metabolite was subsequently transformed to lower fatty acids. Further, Elsden demonstrated that the organisms in whole rumen liquor fermented lactate to fatty acids, mainly propionic. Hungate et al. (12) found that considerable non-volatile acid (presumably lactic) accumulated in the rumen of sheep fed glucose or changed abruptly from a diet of hay to grain.

A two-fold effect of carbohydrates on rumen bacteria and protozoa is evident,
namely, lactic acid production and polysaccharide synthesis. Results of further studies on the production of these substances are presented in this paper. If the conditions governing these reactions could be discovered, one might be able to make an estimate of the "normalcy" of the physiological state of the bacterial flora in the rumen of the intact animal. It is suggested that if bacteria taken as mixed suspensions from a given animal are markedly at variance in some physiological way from those of normal animals one might legitimately suspect some malfunction in these bacteria. This condition might further be reflected in some abnormal condition in the animal. It is emphasized that until now this is a speculative view and further work must be done to support or disclaim it. It should be noted that in vivo reactions were verified from in vitro experiments with washed suspensions of rumen bacteria in recent excellent work by Lewis (13).

EXPERIMENTAL PROCEDURE

Collection of sample, preparation of cell suspension, and procedure for the dissimilation test have been described previously (5). The rumen liquor samples were obtained from a 6-year-old Jersey cow fitted with a permanent rumen fistula. The animal's diet consisted of alfalfa hay and a 16% protein grain mixture. Two hundred micromoles of substrate were used in each flask (final volume with cell suspension, buffer, and substrate in each case was 11 ml.). After appropriate incubation time (noted below for each experiment) the fermentation mixture was assayed for lactic acid (3), ISS, and in some cases, volatile fatty acids (8, 14). By "ISS" is meant that substance within the bacteria which imparts a dark blue or brownish-blue coloration to 0.1 ml. of the fermentation mixture when it is treated with one drop of Lugol's iodine solution. The test was carried out on a spot plate. The effect of various factors, possibly attained in vivo under certain conditions, on the production of lactic acid and ISS was studied.

The influence of pH on the two reactions was determined by use of M/15 phosphate buffers at pH 5.0, 6.9, and 8.0. In processing a rumen sample, the cells were finally suspended in buffer at the pH at which they were to be studied. Glucose and maltose were used as substrates, and their complete utilization after 24 hours was noted by testing for residual carbohydrate with Benedict's reagent (11).

The effect of substrate concentration and length of incubation period on the two reactions was studied. Various concentrations of maltose (20 to 2,000 $\mu$M per 11 ml.) were incubated for periods of 1 to 72 hours, and at intervals the fermentation mixture was assayed for lactic acid, ISS, and, occasionally, fatty acids. Compounds postulated to give rise to lactic acid almost directly were tested in high concentrations since they failed to yield this metabolite at the 200$\mu$M level. The sodium salts of pyruvic, malic, and succinic acids were used as substrates at a concentration of 2,000$\mu$M per 11 ml. Lactic acid was determined after 24 hours incubation.
It was observed that ISS produced from a given carbohydrate was utilized within 24 to 48 hours after a qualitative test for reducing sugar was negative. An attempt was made to accelerate this process by adding 0.5 mg each of adenylic acid and magnesium (as MgSO₄ · 7H₂O) in cellobiose dissimilation experiments.

Burroughs et al. (4) reported increased utilization of urea and cellulose in an artificial rumen through use of a mineral salt solution. A study was made to determine the effect of this solution on the utilization of starch and cellobiose. Dissimilation experiments were performed with 200µM of each of these substrates (calculated as starch) and 0.5 ml of salt solution per 11 ml under atmospheres of nitrogen or carbon dioxide. In the latter case 0.5 ml of a 10% solution of NaHCO₃ was added to the buffer before gassing. Appropriate controls were used in all cases.

In order to determine the effect of binding metal ions on the two reactions a chelating agent was employed. In this case sodium versenate (sodium salt of ethylene-diamine tetra acetic acid, EDTA) was added to give a final concentration of 0.0014 M in glucose dissimilation experiments.

Doudoroff et al. (6) reported the direct utilization of maltose by Escherichia coli with the formation of a polysaccharide. These workers found that NaF or sodium iodoacetate inhibited this process. In the present work 0.15 M NaF was employed in cellobiose or maltose dissimilation experiments. The precise amount of reducing sugar present was determined initially and terminally by the Shaffer-Hartman-Somogyi method (19, 20, 21). Tests were conducted under nitrogen or carbon dioxide atmospheres and in the latter case with 0.1 M NaHCO₃ (pH 7.1) as buffer.

## TABLE 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Condition</th>
<th>ISS present after 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>pH 5.0, 8.0</td>
<td>weak +</td>
</tr>
<tr>
<td>Glucose</td>
<td>pH 6.9</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>200 µM instad of 200 µM</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.15 M NaF added</td>
<td>-</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.15 M NaF added</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.0014 M EDTA added</td>
<td>weak +</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>Salt solution added</td>
<td>weak +</td>
</tr>
<tr>
<td>Starch</td>
<td>Salt solution added</td>
<td>-</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>Adenylic acid + Mg added</td>
<td>+</td>
</tr>
</tbody>
</table>

## RESULTS AND DISCUSSION

In Table 1 are summarized the results of experiments on the conditions under which ISS was formed. It can be seen that it was produced at all pH levels used, although at pH 5.0 and 8.0 only in relatively small amounts as compared to that formed at pH 6.9. The substrates were not completely utilized in 24 hours at pH 5.0. A pH of 5.0 or 8.0 represents reactions not normally found in the rumen, and it seems reasonable to assume that a difference in amount of ISS formed might be encountered at sub-optimal pH levels.
ISS produced from $200\mu M$ of substrate is considered a consequence of the presence of a readily available energy source in excess of the immediate capacity of the rumen bacterial suspensions to either ferment or polymerize to another form, viz., capsular substances. When smaller concentrations of carbohydrate ($20\mu M$ per 11 ml.) were incubated for 24 hours, no ISS was detected. However, failure to find this substance was due to the fact that it was completely utilized within this period. When the fermentation mixture was assayed after 1 hour, ISS was found. This means that ISS was formed at a concentration of $1.8\mu M$ of carbohydrate per milliliter and in the presence of a concentration of bacteria much greater per unit volume than found in normal whole rumen liquor. The level of soluble carbohydrate is never very high in the rumen, but from this experiment it is suggested that a concentration of $2\mu M$ of soluble carbohydrate per milliliter of rumen liquor in vivo would be sufficient to give the ISS reaction. ISS was not found after 72 hours incubation of $200\mu M$ of substrate, and this implicates it as a reserve substance. The observations of Oxford (16) and Heald (9) on protozoa and of Elsden (7) on bacteria in this regard are similar to these findings.

Adenylic acid and magnesium ions did not affect the rate of utilization of ISS. It was concluded that these adjuncts must have been present in sufficient amounts in the suspensions or else they were not operative as such in the system.

The utilization of ISS by rumen bacteria in vivo probably occurs much faster because of less available carbohydrate, removal of inhibitory products, etc. Preliminary experiments have shown that acetic, propionic, and butyric acids do not alter the rate at which ISS is formed.

In the presence of the salt solution, little, if any, ISS was found after 24 hours incubation of $200\mu M$ of starch or cellobiose. There was also an increased amount of volatile fatty acids in the fermentation liquor from these experiments. It seems that the inclusion of salts had some accelerating effect on the utilization of ISS. Results were the same with carbon dioxide and nitrogen.

EDTA prevented the utilization of $200\mu M$ of glucose, and only a small amount of ISS was formed. The importance of metallic ions in ISS formation is thus inferred, although the toxicity of EDTA per se has not been completely ruled out. The necessary metallic ions are no doubt present in rumen fluid in excess of their actual need and in any event are not removed absolutely by the method of cell preparation.

Sodium fluoride prevented the formation of ISS and resulted in a 100% increase in reducing sugar when $200\mu M$ of maltose were used. This indicated that the disaccharide fermentation was inhibited after the formation of monosaccharide units. The reaction is thus similar to that described by Doudoroff et al. (6). Greater consistency of results was noted with a carbon dioxide-bicarbonate system than with a nitrogen-phosphate system.

The effect of pH on the production of lactic acid was interesting. No lactic acid was produced at pH 6.9 from $200\mu M$ of glucose, maltose, or cellobiose after 24 hours incubation. On the other hand, lactic acid was produced consistently
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at pH 5.0 and 8.0. This indicated that at pH levels sufficiently different from that normal to the rumen, enzyme systems which dissimilate lactic acid are inoperable and thus it accumulates, or at these levels greater amounts of lactic are formed than can be dissimilated. When substrate concentrations in excess of 200μM per 11 ml. were tested, viz., 2,000μM, rather large amounts of lactic acid accumulated in the fermentation liquor after 24 hours incubation, even at pH 6.9.

The production of lactic acid in experiments involving the use of excess carbohydrate reveals possible applications of this in vitro technique. For example, Phillipson (17) observed that lactic acid was formed in the rumen when flaked maize was included in the diet, and earlier Phillipson and McAnally (18) obtained similar results by feeding glucose. The work of Hungate et al. (12) pointed out similar changes in the nonvolatile acid content of the rumen upon feeding grain or glucose. The results presented here reveal the same thing in a simple, direct manner. It seems that a wide variety of in vitro experimental conditions could be devised and the results used to predict probable in vivo reactions. Expensive, time-consuming in vivo experiments might thereby be eliminated. It is, of course, necessary to verify these reactions in the intact animal, and the technique advocated here is to be construed as a guide and not a final resort.

The production of lactic acid is usually associated with lactic acid bacteria. A good lead is furnished as to the probable physiological type of bacteria to be expected when excess carbohydrate is fed. In the work of Hungate et al. (12) the cultural investigations on the rumen contents revealed the predominance of a lactic acid bacterium. It is our belief that the technique employed by us may be useful not only in predicting probable changes that would occur in the rumen when a particular compound was fed, but that it would furnish information as to the type of bacteria to seek in cultural investigations.

Lactic acid was not produced from xylose or arabinose regardless of concentration employed. This was considered significant, since if true lactic acid bacteria are responsible for the lactic acid production observed one would not expect them to ferment these pentoses.

The speed of centrifuging the final material used in preparing the cell suspension governed to some extent the appearance of lactic acid in the fermentation liquor. No other metabolic difference between cell suspensions prepared at different speeds has been noted. In the work reported on above, the final centrifugation was at 1,952 × G. and obviously these preparations yielded little, if any, lactic acid from 200μM substrate at pH 6.9 after 24 hours incubation. If the final centrifugation was done at 5,782 × G. both maltose and cellobiose were found to yield lactic acid at pH 6.9 after 24 hours incubation. Curiously, lactic acid was not found in glucose dissimilation tests under these conditions. If the fermentation liquor of a glucose dissimilation test was assayed after 2 hours incubation at pH 6.9 some lactic acid was found, and this was interpreted to mean that much more lactic acid results from the dissimilation of the di-
saccharides than from glucose. Approximately 375 to 400 μM of lactic acid accumulated from 200 μM of maltose or cellobiose after 24 hours incubation at pH 6.9 when the new method of cell preparation was employed. Since the new method is now used routinely it will be described as follows: The whole rumen liquor is strained through four layers of cheesecloth and centrifuged for 2 minutes at 771 x G. This is repeated with the supernatant for 10 minutes. Finally, the supernatant is centrifuged for 10 minutes at 5,782 x G. and suspended in buffer and standardized as previously described (5). The supernatant after the last centrifuging is perfectly clear.

Pyruvate, succinate, and malate yielded no lactic acid when supplied at the 200 μM level and from 30 to 60 μM of lactic acid from pyruvate and malate when supplied at the 2,000 μM level. It is unlikely that such high concentrations of these substrates are ever found in the rumen since they are rapidly utilized by rumen bacteria. A possible explanation may be that organisms which rapidly attack these C3 and C4 compounds, viz., Veillonella sp., compete with the lactic acid bacteria for them. Lactic acid is not produced by Veillonella sp. from pyruvate, malate, or succinate; further, they cannot ferment hexose carbohydrates and they would not compete with lactic acid bacteria in this case; hence one might explain the production of lactic acid from hexoses but not C3 and C4 compounds.

SUMMARY

The effect of some conditions on the in vitro formation of an iodine staining substance (ISS) and lactic acid by rumen bacteria has been investigated. Most of the conditions studied could conceivably occur in the rumen at some time; included were alteration of pH, change of substrate concentration, addition of salt mixtures, and inhibitors.

The production of ISS by rumen bacteria is probably a mechanism which enables them to store a reserve energy supply. It is doubtful whether this reaction is confined solely to rumen microorganisms.

Lactic acid was produced from glucose, fructose, maltose, and cellobiose but not from xylose or arabinose. Accumulation of lactic acid occurred when carbohydrate in excess of 200 μM per 11 ml. or cell preparations from high speed centrifugation were used. Lactic acid was detected from glucose (200 μM) after 2 hours incubation but not after 24 hours regardless of the method of cell preparation.

Pyruvate, malate, and succinate yielded scant amounts of lactic acid even when 2,000 μM were used.

Some suggestions are given for use of the washed suspension technique for the solution of practical problems and, more important, for uncovering basic information on the physiological reactions of rumen bacteria when examined as an enzymatically competing population.
REFERENCES