Mitz and Yanari (*8*). The column and collection tubes were cooled with circulating ice water at 3° to 4° C. Fractions of 5 ml. each were collected at a flow rate of 15 ml. per hour. Protein fractions were determined by the absorbances of the fractions at 2760 A. and were plotted against fraction number.

Two major protein peaks were obtained after carbon dioxide elution (Figure 5). Each fraction was assayed for proteolytic activity by the hemoglobin digestion method described. The activity is indicated by the shaded area of the graph. The proteolytic fractions contained activity in an amount equivalent to the total placed on the column with an 18-fold increase in specific activity (Table III) based on the nitrogen content determined by the micro-Kjeldahl method.

While these results seem to indicate that a single proteolytic enzyme has been isolated from meat, more than one enzyme will probably be found upon further fractionation of this material. The kinetic data, such as the relationship of activity to time, temperature, and pH, support the theory that a number of proteolytic enzymes are active in the meat extracts. Thus the partially purified protease preparations should be further purified and separated to characterize more completely the specific enzymes present in the proteolytic system of beef muscle.

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RUMEN BIOCHEMISTRY

Physiological Activities of Rumen Mixed Cell Suspensions

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An attempt was made to determine some of the factors that affect the production of volatile fatty acids and lactic acid by washed cell suspensions of rumen bacteria. Refrigeration or freezing of washed cell suspensions yielded preparations that did not give results comparable to those obtained from untreated washed suspensions. Time of sampling after feeding as well as variations of buffer and/or substrate concentration affects the amounts and ratios of volatile and lactic acids produced. The length of the incubation period influences the quantity of acids present, as does the presence of rumen fluid; the latter seems to have an inhibitory effect on the production of volatile acids. Reactions that require long-term incubations yield results of a variable nature, and hence are much more difficult to interpret in terms of in vivo events.

SEVERAL TECHNIQUES have been employed to study the specialized microbial flora of the rumen with the hope of defining the over-all contribution of the mixed bacterial population (*2, 3*). Among these is the washed cell suspension technique, in which the mixed bacterial (but not protozoan) flora is obtained by differential centrifugation of fresh rumen liquor and then subjected to various physiological experiments. Washed suspensions of rumen bacteria were studied by Johns (*10*), and Elsden and Sijpesteijn (*6*). Subsequently various modifications (*1, 4, 7, 8, 11, 13–15, 19, 20*) were introduced.

Limitations of the washed cell suspension technique have been noted (*3*), but efforts to avoid some of the deficiencies of pure culture experiments in studying one aspect of microbial ecology (*22*) should be encouraged Lewis (*15*) noted that certain treatments of washed cell suspensions would alter the production of various end products. It has been shown also (*18*) that the speed of centrifugation governs to some extent the production of lactic acid.

The purpose of the experiments reported here was to determine the parameters affecting the formation of certain chief end products of carbohydrate metabolism by rumen washed cell suspensions—namely, volatile fatty

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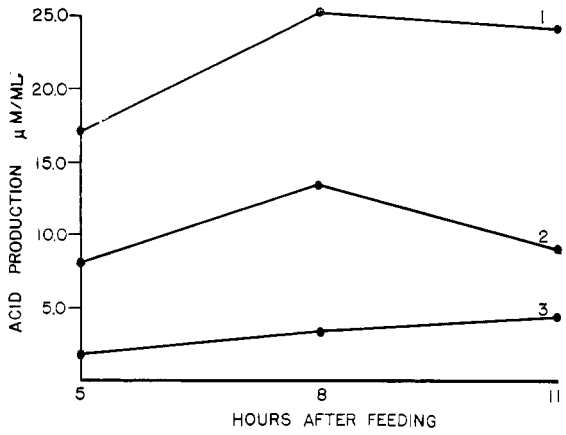

Figure 1. Effect of time of sampling after feeding on the production of volatile fatty acids by a washed cell suspension

1. Propionic acid
2. Acetic acid
3. Butyric acid

Glucose concentration 68 μmoles per ml.

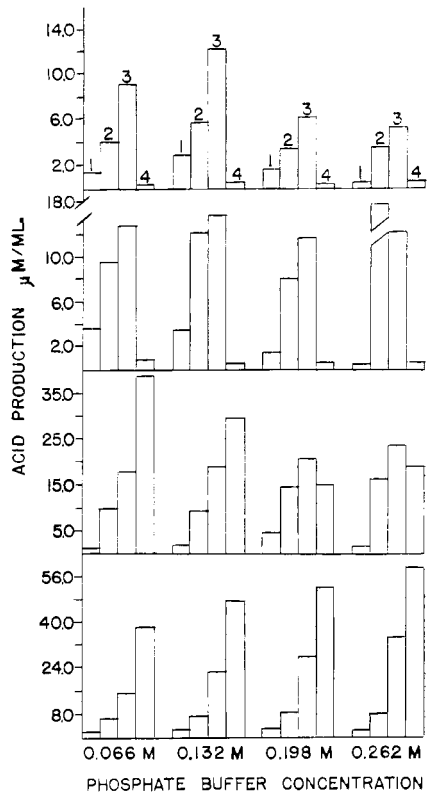

Figure 2. Effect of varying phosphate buffer concentration in conjunction with variations of substrate concentration on the production of volatile fatty acids and lactic acid

1. Butyric acid
2. Propionic acid
3. Acetic acid
4. Lactic acid

Glucose concentrations 11, 22, 55, 111 μmoles per ml.

acids and lactic acid—and concomitantly to observe the influence of a variety of physicochemical conditions on the reactions of such preparations. It is hoped that some critical factors governing the reactions of the total bacterial flora of a unique ecological niche will thereby be revealed.

Materials and Methods

Rumen liquor samples were obtained from a 9-year-old Holstein cow fitted with a permanent bladder-type rumen fistula. The diet consisted of alfalfa hay and a 16% protein grain mixture. Washed cell suspensions were prepared according to Gibbons, Doetsch, and Shaw (*8*). This was used only in the experiment on refrigeration and freezing of washed cell suspensions. In all other experiments, a second preparation obtained by a variation of this technique was employed. The variation was as follows: After "preincubation" (*8*), which was necessary to decrease endogenous respiration, the suspension was centrifuged at 12,500 × G for 10 minutes. The sediment obtained was then resuspended in 0.066*M* phosphate buffer (pH 6.85 ± 0.1) and centrifuged at 1200 × G for 2 minutes. The upper white fraction was separated from a lower green fraction and standardized in the manner employed by Doetsch *et al.* (*4*).

Large scale dissimilation tests were carried out in 50-ml. Erlenmeyer flasks. Each flask contained 5 ml. of washed cell suspensions—some experiments employed 3 ml.—5 ml. of 0.66*M* phosphate buffer, and 1 ml. of a glucose solution. Buffer concentrations were varied from 0.066 to 0.262*M*, and glucose concentrations from 11 to 111 μmoles per ml., and this was dependent upon the experiment involved. Anaerobic conditions were obtained by gassing the flasks for 1 minute with oxygen-free nitrogen and sealing with rubber stoppers. Flasks were incubated at 39 ± 0.5° C. for 24 hours in a constant temperature water bath. Control flasks without substrate were used in each experiment. At the end of the incubation period, flask contents were tested for iodine staining substances (*18*), reducing sugars, and volatile and lactic acids. The latter two were determined by the column chromatographic technique of Wiseman and Irvin (*23*), as modified by McCarthy (*16*).

No multiplication of any component of the washed cell suspension was observed under these conditions—i.e., the preparations are, in fact, composed of "resting cells."

Among the various factors studied as possibly affecting washed cell suspensions were refrigeration, freezing, time of sampling after feeding, effect of buffer and substrate concentration, length of incubation time, periodic neutralization of the reaction flask, and the effect of clarified rumen fluid on the production of volatile fatty acids and lactic acid by a washed cell suspension.

Results

The results of experimenting with the refrigeration of a washed cell suspension in relation to the production of volatile fatty acids are shown in Table I. They reveal that it is not possible to refrigerate washed cell suspensions of rumen bacteria at 4° C. for 24 hours without obtaining variations in the ratio of acetic to propionic to butyric acids. End products of duplicate sets of washed cell suspensions, even after refrigeration for various intervals of time, did not vary more than 3%. Freezing (−20° C.) of washed cell suspensions of rumen bacteria lowered the subsequent production of volatile fatty acids.

The best time to obtain a sample from this particular test animal was 8 hours after feeding (Figure 1). The ratios of the various acids remained virtually the same throughout the sampling period; therefore, time of sampling after feeding may affect the amounts of various acids produced, but should not affect the ratios of acids which may be produced.

Figure 2 depicts results of several experiments on variations in both phosphate buffer and substrate concentrations. Substrate at a level of 111 μmoles per ml. was not completely utilized by the washed cell suspension after 24 hours, as evidenced by a positive Benedict's test. As the substrate concentration per milliliter was increased, there was a proportional increase in the amount of acetic acid produced up to the 111 μmoles per ml. level, regardless of buffer concentration.

However, there was an increase in the amount of propionic acid produced up to either the 22 or 55 μmoles per ml. level, followed by a decrease at 111 μmoles per ml. Lactic acid was not produced at a substrate concentration below 55 μmoles per ml. Figure 2 shows that phosphate buffer concentration had a definite effect on the production of lactic acid. Ratios of acetic to propionic acid varied with changes in both substrate and buffer concentrations.

Results of studies showing the effect of rumen fluid on the production of volatile fatty acids by a washed cell suspension are given in Table I.

Repeated experiments revealed a consistent inhibition of acetic acid production; however, the amount of inhibition varied from experiment to experiment.

The presence of acid end products in rumen fluid might account for the inhibition of the production of fatty acids by a washed cell suspension when suspended in rumen fluid. Results of experiments (Table II) indicate that the fatty acids present in rumen fluid do not inhibit the production of fatty acids, because when these are added to washed cell suspensions, there is comparatively little change in the amounts of fatty acids produced as compared to a control washed cell suspension. A large decrease in the amount of lactic acid was noted.

Preliminary experiments indicated that the time of incubation and subsequent lowering of pH due to the production of volatile and lactic acids was an important factor to be considered. Figure 3 shows results obtained from studies involving these factors. At the end of 11 hours of incubation, a negative Benedict's test was obtained, which indicated complete utilization of the substrate At this point, the total acids began to decrease and the pH began to rise. Between 22 and 26 hours of incubation there was a substantial increase in acetic acid with a simultaneous large decrease in lactic acid. Lactic acid was the main constituent of the total acid content.

Periodic neutralization of the reaction flasks yielded results (Table II) which indicated that there is no appreciable difference in volatile and lactic acid production from a washed cell suspension which has been neutralized periodically and one that has not.

Table I. Effect of Refrigeration and Addition of Rumen Fluid to a Washed Cell Suspension on Production of Volatile Fatty Acids

Treatment	Fatty Acids, μmoles/Ml. Acetic	Propionic	Butyric	ISS[a]	RS[b]
Refrigeration, hours					
0	9.20	16.20	4.70	+	−
24	3.80	11.25	12.80	+	−
90	1.20	1.60	0.00	+	−
Washed cells suspended in					
Phosphate buffer	12.75	16.85	4.85	+	−
Rumen fluid	0.00	13.20	8.45	+	+

[a] Iodine staining substance.
[b] Reducing sugars.

Table II. Effect of Addition of Acid End Products and Periodic Neutralization of a Washed Cell Suspension on Production of Volatile Fatty Acids and Lactic Acid

Treatment	Acids, μmoles/Ml. La	Ac	Pr	Bu	ISS[a]	RS[b]
Addition of acid end products						
Washed cell suspension + acids[c]	0.10	4.15	6.50	5.25	+	−
Control	21.00	3.10	6.90	0.60	+	−
Periodic neutralization						
Experimental flask	21.32	2.97	3.25	0.48	−	−
Control flask	19.83	3.13	3.33	0.66	−	−

[a] Iodine staining substances.
[b] Reducing sugars.
[c] 60 μmoles/ml. acetate, 20 μmoles/ml. propionate.

Discussion

The washed cell suspension is a useful technique for elucidating the biochemical pathways of certain specific reactions known to occur in the rumen (*4*, *6*, *7*, *11*, *13*). If a method of preserving washed cell suspensions could be perfected, it would be possible to use single samples from widely separated areas for a variety of determinations in comparative studies. The results of this phase of the investigation indicated that, during refrigeration or freezing, there is inactivation or destruction of enzyme systems involved in the production of volatile fatty acids. This is in surprising contrast to the behavior of many nonrumen bacteria, which generally withstand exposure to low temperatures without alteration of their physiological characteristics. The remarkable instability reported here could be due to: extreme structural fragility of many of the organisms present; unsatisfactory nature of the suspending medium, or enzymatic inactivation due to processing. The presence of 15% glycerol protects *Escherichia coli*, *Diplococcus pneumoniae*, and *Treponema pallidum* from the damage of freezing and thawing (*9*). The washed cell suspension could possibly be better maintained with a modification of the suspending medium.

Results shown in Figure 1 suggest that there is a significant change in the physiological activity of the bacteria present in the rumen at various intervals after feeding. This change may be due to the accumulation and utilization of end products which may tend to activate or inhibit various enzyme systems, essential to the particular reaction measured.

In the dissimilation of glucose, various

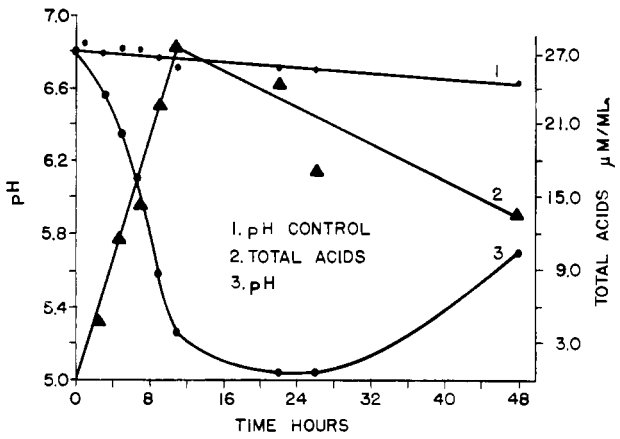

Figure 3. Effect of time of incubation on the production of volatile fatty acids

Glucose concentration 22 μmoles per ml.

ratios of acetic to propionic to butyric acids were produced by washed cell suspensions. In most cases, propionic acid was the main constituent of the total volatile acids. However, rumen fluid from which the washed cell suspension were prepared had a 6:2:1 ratio of the respective acids. Leffel, Brown, and Lakshmanan (*12*) using labeled glucose, found that in in vitro experiments, a predominant percentage of the labeled carbon appeared in the propionic acid fraction. This difference suggests either one of two possibilities: that the amount of volatile fatty acids present in rumen fluid is not representative of the actual production of volatile fatty acids by rumen bacteria, or that the process of preparing washed cell suspensions in some way alters the labile enzyme systems which are responsible for the production of volatile acids. With an in vitro technique, such as used here, there is an accumulation of undefined end products which may affect volatile fatty acid production by washed cell suspensions in unknown ways. A permeable system may offer some advantages over the washed cell suspension in this respect, but evaluation of results is difficult, because of the inherent deficiencies of the artificial rumen.

High concentrations of carbohydrates enhance the production of lactic acid by washed cell suspensions of rumen bacteria (*18*). This has also been shown to occur in sheep fed large quantities of starch (*17*). However, analysis of rumen liquor taken from animals on normal diets generally reveals little or no lactic acid present (*21*). This seems to indicate that lactic acid was utilized as rapidly as it was formed.

The scant production of volatile fatty acids, by a washed cell suspension in rumen fluid, might be caused partially by the inhibitory effect of such acids already present in this fluid. However, the addition of sodium acetate and sodium propionate to a washed cell suspension, in approximately the same concentrations as found in rumen fluid, revealed no inhibition of the production of these acids. Acids beyond propionic may possibly be involved. The postulated substance(s) are obviously not active within the rumen.

The increased production of lactic acid up to 22 hours of incubation, followed by a sudden decrease at 26 hours of incubation cannot be readily explained. There have been numerous reports that lactic acid is utilized in the production of volatile fatty acids. Elsden (*5*) reported that large amounts of volatile fatty acids and lactic acid accumulated during 24 hours of incubation, but disappeared during 48 hours of incubation with a corresponding increase in propionic acid. As absorption is not considered in in vitro experiments, lactic acid is converted to either volatile fatty acids, probably propionic, or to some other undetermined end product. The amount of volatile fatty acids produced from iodine staining substances, after all available carbohydrates have been utilized, is actually a negligible factor.

In view of the variable nature of the results obtained in this study, the washed cell suspension technique should be used with all conditions constant, and should not be employed for reactions which require numerous degradative steps (glycolysis) prior to the final products, unless previous experimentation indicates that the specific reaction under specific conditions is constant. The production of volatile fatty acids and lactic acid, as studied herein, requires long incubation periods, and amounts of substrate in excess of those normally found in the rumen. One- or two-step reactions which require only a short incubation period are to be preferred.

In vitro reactions are not necessarily reactions which occur in vivo. However, in vitro reactions do indicate the potentiality of the organism to perform certain reactions, and may be used as one criterion for actual processes occurring within the rumen. All in vitro reactions, whenever possible, should be substantiated by in vivo experimentation.

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