Continuous Culture as a Method for Studying Rumen Fermentation[1]

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Experiments on continuous culture of rumen contents were undertaken in the hope that a more normal fermentation could be maintained by this procedure than has been possible by batch culture.

In the normal rumen fermentation, end products are removed by flow of ingesta down the digestive tract as well as by absorption through the rumen wall. New substrate is added periodically, and a continuous flow of salivary buffer into the rumen helps to maintain a physiologically normal pH range. Since continuous culture can imitate most of these functions, it would seem that continuous culture would maintain an environment more typical of the rumen than batch culture.

Little work has been done to test the ability of continuous culture to maintain a complex population of microorganisms. Warner (1956) cultured rumen microorganisms semicontinuously by dividing and diluting the cultures five times daily. He reported that this technique gave results more typical of the rumen than a 24- to 48-hr batch culture. Zubrzycki and Spaulding (1957, 1958) continuously cultured normal human fecal flora for 3 weeks, but few of their data were presented. Adler *et al.* (1958) continuously cultured mixed rumen microorganisms for 10 hr on a protein-free diet, but other than observing pH and protozoal motility, they did not determine whether or not a normal rumen fermentation was maintained.

The experiments reported here were conducted as a preliminary test of the reliability of continuous culture for studying the activities of the mixed microbial population of the rumen *in vitro*. The criteria used for comparing the fermentation *in vitro* with the fermentation reported to occur in the rumen were (a) pH, (b) volatile fatty acids—their relative concentrations and rates of production, (c) motility and rates of growth of oligotrich and holotrich protozoa, and (d) concentrations of coliform and amylolytic bacteria.

MATERIALS AND METHODS

Apparatus. A schematic diagram of the pilot apparatus used in these studies is shown in figure 1. The 5.5-L culture was maintained in an 8-L glass vessel within a constant temperature water bath at 39 C. The water bath was covered with black paper to exclude most of the light. The culture was stirred by Lucite (methyl methacrylate) paddles at 33⅓ rpm.

To exclude air, a lid of Lucite with a rubber gasket was held onto the top of the vessel. Various pieces of equipment were fitted into the lid with rubber stoppers. The only opening in the lid which was not air-tight was that through which the stirrer shaft projected. This opening was just large enough to allow the shaft to rotate freely. Since gas was being produced in the vessel, the tendency was for gas to pass out rather than in.

The sampling device consisted of a suction flask connected to ½ in. ID (inside diameter) glass tubing which extended down into the culture to approximately half its depth. When not in use, the rubber tubing connection was clamped off.

A 4-L volume of substrate was stored in a polyethylene bottle, where it was stirred magnetically and refrigerated by an ice water bath. A constant carbon dioxide pressure of ½ psi was maintained in the vessel to minimize the effect of the depth of substrate on total pressure. The amount of substrate in the vessel was not allowed to fall below 2 L so that the maximal drop in hydrostatic pressure was less than 20 per cent of the total pressure. In this way the influence of hydrostatic pressure on the rate of flow from the vessel was diminished.

A ½ in. ID inflow tube led from the substrate vessel to the culture vessel. A vent above the vertical part of the inflow tube was necessary to allow rapid flow of substrate through this part of the tube. Otherwise, sluggish flow of substrate through this vertical segment of the tube caused solid substrate particles to plug the tube by bridging. The vent was connected to the culture vessel so that air was not admitted through it.

A solenoid valve on the inflow tube allowed substrate to flow periodically into the culture vessel. The rate of flow could be varied by changing the frequency with which the solenoid valve opened.

Culture flowed from the culture vessel into a receptacle through a solenoid-controlled outflow tube located near the bottom of the culture vessel. The operation of the solenoid valve was controlled by a float on top of the culture. When the volume of culture

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increased to a certain point, an electrical contact was made on the float, and the solenoid opened, allowing a portion of the culture to flow out. The greatest variation in total culture volume due to this periodic outflow was less than five per cent of the total volume.

All surfaces with which the culture or substrate came in contact were either glass, polethylene, stainless steel, rubber, or Lucite-coated.

Some mechanical difficulties were encountered with this pilot apparatus. The rate of flow, which was supposed to be constant for all experiments, actually ranged from 7.60 to 10.80 per cent of the culture volume per hour, the average being 8.92 ± 0.97 per cent. This variation was caused by unreliable operation of some parts of the flow mechanism and could be eliminated in better constructed equipment.

Treatments. Inocula were obtained from two Holstein steers weighing approximately 900 lb. Each steer was fitted with a 4½-in. ID Lucite cannula into the rumen. One steer received a ration of 20 lb of "U. S. No. 2 Leafy Green Alfalfa Light Grass Mixed Hay." The other steer received a ration of 6 pounds of the above hay plus 10 lb. of a 15 per cent crude-protein concentrate mix. The steers were fed twice daily and received water and salt ad libitum. Each inoculum was obtained 6 or 7 hr after the morning feeding.

Hay substrate was prepared from the same lot of hay as was fed to the steers, by grinding through the 2-mm screen of the Wiley mill and suspending in McDougall's (1948) salt solution at the rate of 5 g of hay (air-dry weight) with 95 ml salt solution.

Hay-concentrate substrate consisted of a 6:10 (w/w) mixture of the hay and concentrate mix, both of which had been ground through the 2-mm screen of the Wiley mill and suspended in McDougall's (1948) salt solution, mixing 5 g of hay-concentrate mixture (air-dry weight) with 95 ml of the salt solution.

Conduct of experiments. Inocula were obtained by squeezing rumen contents through one layer of cheesecloth. The culture vessel was flushed with carbon dioxide, and 3 L of inoculum were added. Substrate flow into the vessel was started at the rate of 900 ml per hr and continued until the culture volume reached 5.5 L. The inflow of substrate was then stopped and the culture was allowed to develop as a batch for approximately 5 hr until a pH of 6 was reached. Then inflow of substrate and outflow of culture were started at the rate of 450 ml per hr, and this was continued

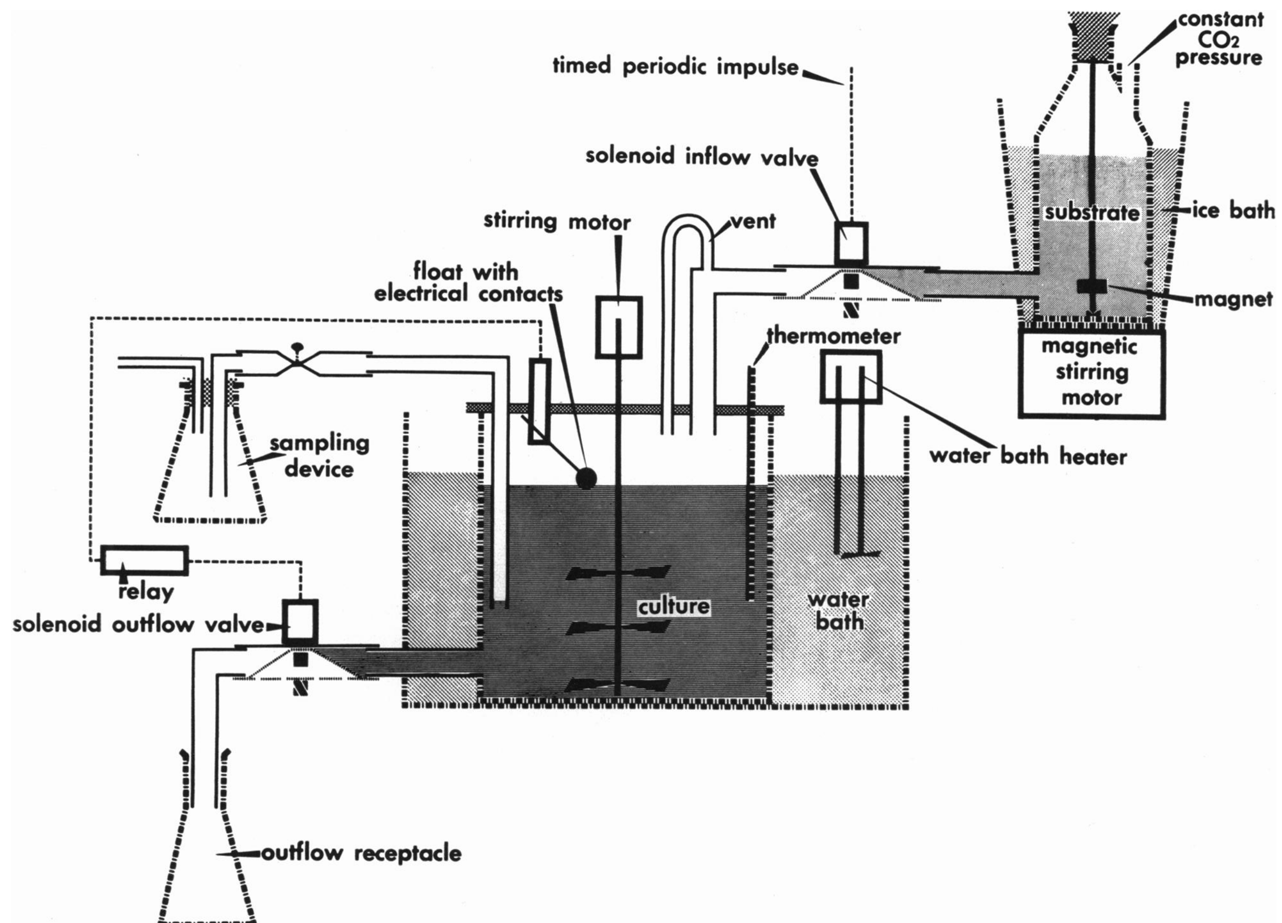

Figure 1. Continuous culture apparatus

for 24 hr or more beyond the time of inoculation. Longer experiments, although desirable, were usually impossible because of mechanical failures.

Sampling. Samples of approximately 100 ml were removed from the culture vessel via the sampling device at (a) the time of inoculation, (b) the start of the 450 ml per hr inflow-outflow, and (c) every 8 hr thereafter. Preliminary experiments had shown that more frequent sampling was unnecessary because only minor fluctuations in the fermentation pattern occurred when the apparatus was working smoothly.

Measurements of pH were made on each 100-ml sample within 1 min after removal from the culture. Then a 10-ml subsample was removed with a large-bore pipette, fixed with 1.1 ml of 40 per cent formaldehyde, and refrigerated until counts of protozoa could be made. From the remaining 90 ml of sample, two 10-ml subsamples for bacterial counts were removed with a large-bore pipette. The remainder of the sample was poisoned with 1 part of a saturated mercuric chloride solution to 50 parts of sample and refrigerated. Later, this sample was centrifuged to remove large debris and some bacteria. The supernatant was refrigerated until analyzed for volatile fatty acids.

Volatile fatty acid analysis. Volatile fatty acids were separated and measured by a modification of the Wiseman and Irvin (1957) procedure.

Counts of protozoa. An equal volume of 66 per cent (w/w) sucrose solution was added to the 11-ml formalinized sample of culture. The resultant suspension was of such a specific gravity and viscosity as to diminish the settling rate of the protozoa. For oligotrich counts, a 1-ml subsample of this suspension was taken with a wide-bore pipette and diluted by a factor of 10, 20, or 40 in 33 per cent sucrose solution. This dilution was stained by adding 1 part of Lugol's iodine to 20 parts of sample. A 0.2-ml portion was then transferred to a counting chamber by means of a measuring pipette having a 1-mm orifice. After allowing the protozoa to settle, the entire 0.2-ml sample was counted under the 100× magnification of a binocular microscope with a mechanical stage. Each slide contained from 100 to 200 organisms.

For holotrich counts a similar procedure was used, but the samples were diluted by a factor of only two or four because of the smaller number of these organisms. The dilutions were stained with methylene blue at the rate of one part of a 0.035 per cent alcohol-water solution to eight parts of sample. There were usually 50 to 100 holotrichs per slide, but in a few samples the number was as small as 20.

Counts of bacteria. Coliform bacteria were counted on poured plates of Bacto violet red bile agar,[4] used as described in the *Difco Manual* (1953). Inoculated plates, with 5 ml of melted medium poured on as a cover, were incubated at 37 C and were counted after 18 to 24 hr of incubation. Only colonies which were purplish red and 1 to 2 mm in diameter were counted. Occasional Gram stains made from these colonies always showed short gram negative rods, sometimes in chains. Usually 25 to 100 colonies were counted per plate, but occasionally plates with as few as 10 coliform colonies were counted in situations where lower dilutions were crowded with other types of colonies, especially spreaders or molds.

Amylolytic cocci, presumably *Streptococcus bovis*, were counted on poured plates of the starch medium described by Higginbottom and Wheater (1954). The procedure used was similar to that used for coliform counts except that the plates were incubated for 3 days. Then they were flooded with iodine solution, and colonies showing starch hydrolysis were counted. Plates with 10 to 100 colonies were counted. However, when more than 15 to 20 colonies were present it could not be ascertained whether they were all amylolytic, because zones of starch hydrolysis overlapped. The majority of colonies did show starch hydrolysis, where this could be determined, and were lenticular, about 1 mm in diameter. Gram stains were made occasionally at random. These almost always showed gram positive cocci, usually in pairs with a flattening on the joined sides characteristic of *S. bovis*.

Experimental design. A 2 × 2 factorial arrangement of treatments with two substrates and two sources of inoculum was replicated twice.

Calculations. The 16- and 24-hr samples were used as the bases for all calculations. It was assumed that the system was then in equilibrium for all criteria except numbers of protozoa, since the values for all measurements except protozoal numbers leveled off during the last half of the incubation period.

The rate of production of volatile fatty acids per volume of culture was calculated by multiplying the rate of dilution times the total volatile fatty acid concentration. This calculation was made assuming that the total volatile fatty acid concentration was constant. This seemed to be a reasonable assumption, since the 16- and 24-hr samples seldom differed by more than 10 per cent, and there was no consistent tendency for an increase or decrease during this period. The differences were probably due to accidental variations in flow rate, which occurred in most experiments, as mentioned earlier.

Since the number of protozoa in the culture was not constant it was necessary to calculate growth rates from the formula of Monod (1950):

$$u = \frac{\log_e x_2 - \log_e x_1}{t_2 - t_1} + D$$

where u = doubling rate, number of divisions per unit time; x = concentration of organism; t = time;

[4] Dehydrated; Difco Laboratories, Inc., Detroit, Michigan.

and D = dilution rate, rate of flow divided by volume of culture.

Generation times were calculated as reciprocals of growth rates.

Results and Discussion

A summary of the results is presented in table 1. Data for individual treatments are given only in cases where there were significant differences ($P < 0.10$). The replicates were not in particularly good agreement because of variability in dilution rates, which were supposed to be constant for all experiments (see Materials and Methods). However, there were no significant differences due to replication, $P < 0.10$.

Hydrogen ion concentration. The average pH values of 6.20 for the experiments using hay substrate and of 5.96 for the hay-concentrate substrate were significantly different at $P < 0.10$. These values *in vitro* are in the range of those reported by other workers (Balch and Rowland, 1957; Briggs, Hogan, and Reid, 1957) for similar substrates *in vivo*, and the depression of pH by the rations containing concentrate is consistent with observations *in vivo*.

Volatile fatty acid concentration. The average volatile fatty acid concentration of 14.8 mEq per 100 ml was somewhat higher than the volatile fatty acid concentrations reported during the period of active fermentation *in vivo* by most workers. However, similar values as well as much higher values have been reported under normal conditions by Annison (1954) and by Reid, Hogan, and Briggs (1957), so this value would not appear to be unphysiological.

Rate of volatile fatty acid production. The average volatile fatty acid production rate of 1.3 mEq per 100 ml per hr for all experiments is comparable to the average value of 1.42 mEq per 100 g per hr, observed by Carroll and Hungate (1954) for hay diets when the total volatile fatty acid concentration averaged 12.77 mEq per 100 g. It is lower than 2.35 mEq per 100 g per hr, which they observed on grain diets where the average volatile fatty acid concentration was 9.15 mEq per 100 g. Stewart (1957) observed a peak production rate of 3.04 mEq per 100 ml per hr, 1 hr after feeding, when the volatile fatty acid concentration was 10.50 mEq per hr. The rate declined rapidly to 1.41 mEq per 100 ml per hr, 6 hr after feeding, when the volatile fatty acid level was 12.61 mEq per 100 ml.

The rate of volatile fatty acid production observed in the present experiments was thus comparable to the lower values reported to occur in the rumen during active fermentation, and it was consistent with values reported where the concentrations of volatile fatty acids were high.

Proportions of volatile fatty acids. The difference in the proportions of acetic acid produced on the two substrates was significant at $P < 0.10$. The values are comparable to proportions of acetic acid which have been reported during active fermentation of similar diets in the rumen and are in agreement with the observation that roughages tend to result in a greater proportion of acetic acid in the rumen than do concentrates (Balch and Rowland, 1957; Reid *et al.*, 1957).

The proportions of propionic acid produced by the two inocula were significantly different at $P < 0.10$. They are similar to observations on the proportions produced in the rumen a few hours after feeding when the fermentation is active (Balch and Rowland, 1957; Reid *et al.*, 1957). The apparent difference due to inoculum source suggests that this may be an important factor in these experiments.

The replicate averages for butyric acid percentage shown in table 1 gave an interaction between inoculum

TABLE 1

Total concentration, rate of production and proportions of VFA, pH, protozoal generation times, and numbers of coliform and amylolytic bacteria observed during continuous culture of rumen contents*

Criteria	Treatments	Averages
Total VFA* conc (mEq/100 ml)	All	14.8
VFA production rate (mEq/100 ml/hr)	All	1.3
Acetic acid (%)	Hay substrate, both inocula	63.0
	Hay-concentrate substrate, both inocula	58.7
Propionic acid (%)	Hay inoculum, both substrates	24.5
	Hay-concentrate inoculum, both substrates	20.0
Butyric acid (%)	Hay substrate, hay inoculum	13.2
	Hay substrate, hay-concentrate inoculum	12.7
	Hay-concentrate substrate, hay inoculum	11.9
	Hay-concentrate, hay-concentrate inoculum	17.2
Valeric acid (%)	All	3.1
pH	Hay-substrate, both inocula	6.20
	Hay-concentrate substrate, both inocula	5.96
Oligotrich generation times (hr)	Hay substrate, hay inoculum	−80
	Hay-concentrate substrate, both inocula	+25
Holotrich generation times (hr)	Hay substrate, hay inoculum	−88
	Hay substrate, hay-concentrate inoculum	+54
	Hay-concentrate substrate, hay inoculum	+39
	Hay-concentrate substrate, hay-concentrate inoculum	+139
Coliform organisms (number/ml)	All	1×10^4
Amylolytic organisms (number/ml)	All	9×10^6

* Volatile fatty acids.

and substrate which was significant at $P < 0.10$. The difference due to inoculum was also significant at this level. In the two cases where the inocula and substrates were comparable, the values of 13.2 and 17.2, for hay and hay-concentrate substrate, respectively, were similar to results which have been reported for comparable substrates in the rumen, the concentrate increasing the proportion of butyric acid present (Balch and Rowland, 1957; Reid *et al.*, 1957).

The valeric acid percentage of 3.1 per cent was comparable to values reported for the rumen (Balch and Rowland, 1957).

Oligotrich generation times. The average generation times for oligotrich protozoa on the two substrates were significantly different at $P < 0.05$. The negative value of -80 hr indicates that the organisms disintegrated faster than they multiplied. The value of 25 hr compares favorably with the best rates of growth reported *in vitro*. Hungate (1942, 1943) observed generation times of from 24 to 48 hr for *Diplodinium*, and Coleman (1958) reported values of approximately 48 hr for *Entodinium*.

It is uncertain whether or not these rates of growth are typical of those which occur in the rumen. It might seem that if each cell divided approximately every 24 hr this would maintain the population of oligotrich protozoa in the rumen if the flow of material through the rumen were equivalent to 100 per cent of the rumen volume every 24 hr. Actually this is an oversimplification, since the rate of flow through the rumen is not uniform, and mixing in of ingested material is not instantaneous. Furthermore, protozoa probably do not grow at a uniform rate in the rumen. A study of the data of Purser and Moir (1959) shows considerable variation with time after feeding in the proportion of dividing forms of oligotrichs in the rumens of sheep. These fluctuations were associated with changes in pH, but they were not shown to be caused by pH, as was concluded by these workers. All these factors make it difficult to estimate the average generation time for the protozoan population in a given rumen at any time.

The fact that concentrate and roughage produced better growth than roughages alone, in these experiments, is consistent with the observation of Oxford and Sugden (1953) that *Oligotricha* are favored by a high starch diet. However, the fact that these organisms showed no growth at all in the experiments with hay substrate cannot be considered comparable to what would occur *in vivo*. Certainly there must have been some growth of these organisms in the rumen of the steer receiving the hay ration, or the inocula from this steer would not have contained them. However, the oligotrich protozoa evidently did not grow as well in the rumen of the hay-fed steer as in the steer receiving concentrates, as evidenced by the fact that their inocula differed 5-fold in content of these organisms, averaging 5.2×10^4 and 2.6×10^5 oligotrichs per ml, respectively.

There was also a difference in the appearance of the oligotrich protozoa on the two substrates. On the hay-concentrate substrate they were dark stained in iodine solution, indicating carbohydrate content. This may have been ingested starch or a storage form of starch. On the hay substrate there were practically no oligotrichs showing iodine staining substance.

Holotrich generation times. The data in table 1 show that holotrich growth rates followed a pattern different from that for the oligotrichs. There was an interaction between substrate and inoculum significant at $P < 0.05$.

It is difficult to explain the fact that the best growth rates were obtained where the substrate and inocula did not correspond. However, it should be noted that there was an apparent positive correlation between growth rates and numbers of holotrichs in the inocula. In the experiments using hay substrate, the hay inocula and hay-concentrate inocula averaged 2.3×10^3 and 6.5×10^3 holotrichs per ml, respectively. In the experiments using hay-concentrate substrate, the hay inocula and the hay-concentrate inocula averaged 9.3×10^3 and 8.0×10^3 holotrichs per ml. It may be that the differences in growth rates were related to the numbers in the inocula rather than to treatments.

As in the case of the oligotrichs, these holotrich growth rates cannot be compared with those obtained *in vivo*, since there is no information on this subject. The only available standard of comparison is the generation time of 48 hr obtained *in vitro* for *Isotricha* by Gutierrez (1955). This is comparable to the generation times observed for two of the four treatment combinations used in these experiments.

It should be borne in mind that these protozoal growth rates represent averages for all the genera in each of the two orders. Also, they were calculated on the assumption that all cells counted were alive, which may not have been true. Therefore they represent a minimum and may have been greater for a given cell or for a given genus.

In all cases both oligotrich and holotrich protozoa showed good motility, as observed in hanging drop preparations.

Coliform bacteria. The average coliform count for all experiments was 1×10^4 per ml. This count is comparable to the counts of 10^4 to 10^5 per ml reported by Mann, Masson, and Oxford (1954), which are among the lowest reported in the literature. One can conclude from this that an abnormal population of these organisms did not develop in these experiments *in vitro*.

Amylolytic bacteria. The average count of amylolytic bacteria was 10^7 for all experiments. The counting procedure was not precise enough to give a more accurate figure. This count is within the range of 10^5 to 10^8 reported *in vivo* for normal diets (Hungate, 1957).

The values observed in these continuous culture experiments for pH, rates of total volatile fatty acid production, proportions of volatile fatty acids, and numbers of coliform and amylolytic organisms appeared to be normal, when compared to reported values for the rumen. Rates of growth of oligotrich protozoa in the experiments using the hay-concentrate substrate were equal to the fastest rates of growth reported *in vitro* to date, there being no data available on their rates of growth *in vivo*. The holotrich protozoa, which seem to lead a precarious existence even in the rumen itself, showed more variable growth rates in these experiments.

These results should encourage further investigation of continuous culture as a method of studying the rumen fermentation. The criteria used for comparison were limited, but the fact that protozoal growth occurred at least in some experiments and that the proportions of volatile fatty acids produced were normal is of significance. To the best of the authors' knowledge, these have never been reported to be normal after more than a few hours in conventional batch cultures of rumen contents.

To some, an attempt to maintain a normal rumen fermentation *in vitro* may seem naive. It is true that *in vitro* there is no normal addition or removal of materials as there is through the rumen wall. Also, there can be no remastication or reinsalivation *in vitro* as occurs *in vivo*. However, it may be possible, by buffering and dialysis, to compensate for the lack of these processes *in vitro* so that, although some gross aspects of rumen activity are lacking, the normal rumen fermentation can still be reproduced on a biochemical and cellular level.

In a stabilized continuous culture the time variable has been eliminated, and the conditions of a given experiment may be typical of only one period in the entire 24-hr cycle *in vivo*. For example, rapid rates of flow would be likely to produce conditions similar to those in the rumen shortly after feeding, whereas slow rates of flow would produce conditions typical of later times. It would be particularly important to keep this fact in mind when attempting to apply the results of experiments *in vitro* to the animal.

Improvements in the apparatus would be essential to further development of this application of continuous culture. Among these improvements should be a more constant flow rate, provision for gas collection and possibly provision for pH control and/or removal of diffusible end products by dialysis when these procedures are desired.

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Summary

On the basis of the limited criteria used in these experiments the continuous cultures appeared comparable to rumen cultures.

Values for pH, rates of total volatile fatty acid production, proportions of volatile fatty acids, and numbers of coliform and amylolytic organisms were similar to values which have been reported in the rumen.

Rates of growth of oligotrich protozoa in the experiments using the hay-concentrate substrate were equal to the fastest rates of growth reported *in vitro* to date. There was apparently no growth of oligotrichs in experiments with hay substrate.

Growth rates of holotrich protozoa ranged from values which were comparable to the best rates of growth which have been reported *in vitro* to values which were negative.

Both holotrichs and oligotrichs showed good motility in all experiments.

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Microbiology of Meat Curing

III. Some Microbiological and Related Technological Aspects in the Manufacture of Fermented Sausages[1]

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In the manufacture of the many varieties of fermented sausage, dependence has been placed traditionally upon chance inoculation with microorganisms contaminating the sausage ingredients or the individual establishments. This method of inoculation may lead to undesirable results including serious economic losses. Two important types of microbial contaminants are required. One type is necessary for the reduction of added nitrate to nitrite for formation of the cured-meat color of the sausage; the second microbial type is needed to effect a fermentation of the added sugar and give rise to the tangy flavor which characterizes these sausages. In years past the acid produced played an integral role in the preservation of the meat. However, today organoleptic contributions are stressed.

Although many sausage manufacturers still employ the traditional method, some no longer depend upon a microbial reduction of nitrate, but rather add nitrite to the sausage mix. However, chance inoculation of fermentative microorganisms is still practiced.

The food industry has successfully circumvented the use of chance inoculation in the preparation of a variety of fermented food products. A large number of cheeses, bread, fermented milk drinks, and alcoholic beverages are examples in which the hazards of chance inoculation have been replaced by deliberate addition of the responsible microorganisms. The development of these "starter cultures" evolved from isolation and identification of the microorganisms responsible for the desired effect, and eventually their addition to the food at the appropriate stage of processing. Thus, it would appear logical that a controlled fermentation could be employed successfully in the manufacture of fermented sausages. To this end, a study of the methods of manufacture and their effect upon the microbial flora, the types and numbers of bacteria in the sausages, and a physiological characterization of the predominant flora was undertaken. A subsequent report will describe the preparation, testing of activity, and utilization of a pure starter culture for sausage manufacture. A preliminary publication regarding some phases of this investigation has been presented (Deibel and Niven, 1957).

Since the completion of this study the results have been applied, and a commercial starter culture employing *Pediococcus cerevisiae* as the fermentative organism has become available.[2] Some aspects of the commercial preparation of the culture have been presented (Harris *et al.*, 1957). Thus, it would be expected that the in-

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