Maintenance of the Rumen Microbial Population in Continuous Culture

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Abstract

Rufener, W. H., Jr. (University of Illinois, Urbana), W. O. Nelson, and M. J. Wolin. Maintenance of the rumen microbial population in continuous culture. Appl. Microbiol. **11**:196–201. 1963.—The design and use of an apparatus for continuous in vitro cultivation of the mixed bovine rumen microbial population are described. Data relative to the concentrations and proportions of volatile fatty acids produced, methanogenesis, carbon dioxide production, and survival of bacteria and protozoa indicated that an essentially normal in vivo fermentation pattern was maintained in vitro for experimental periods of from 3 to 10 days. The continuous cultures were responsive to major changes in type of feed intake. A change from grain to hay resulted in increased acetate and decreased propionate production, whereas decreased acetate and increased propionate resulted when feed intake was changed from hay to grain. Methanogenesis, volatile fatty acid production, and the numbers of microorganisms in the cultures were proportional to the amount of feed materials added up to levels calculated to approximate the in vivo maintenance requirement of the adult bovine.

Investigations of the complex rumen ecosystem have employed a wide variety of techniques, including pure culture studies, in vivo and in vitro perfusion studies, short-term incubation of rumen ingesta, manometric observations on activities of mixed washed-cell suspensions, stabilized enrichment cultures, and the use of various types of fermentation apparatuses. These techniques have permitted the identification and enumeration of the dominant rumen species and have provided data relative to the overall aspects of protein degradation and synthesis, vitamin synthesis, fiber digestion, conversion of feed materials to volatile fatty acids, carbon dioxide, and methane, and other rumen processes.

Continuous culture techniques, which primarily have been developed with pure cultures, have not been used extensively in rumen microbiology. These techniques would appear to be exceptionally useful for further analysis of the rumen ecosystem, especially for studying the interrelationships among the components of the microbial population, the dynamics of population development, the environmental factors influencing population development, and the quantitation of rates of the processes known to occur in the rumen. The continuous culture unit described by Stewart, Warner, and Seeley (1961), and used for short-term experiments (24 hr), demonstrated the feasibility of using this technique in rumen microbiology. Other continuous culture systems for maintaining rumen microbial activity have also been described (Adler et al., 1958). The present report describes the design, operation, and functioning of an apparatus for the continuous cultivation of the mixed rumen microbiota. The design was based on the premise that the maintenance of an in vitro environment as closely as possible to the known in vivo environment would facilitate the achievement of the objective of long-term cultivation in vitro. In addition, it was considered desirable that the apparatus be relatively simple in design, construction, and operation so that several fermentor units could be operated simultaneously.

Materials and Methods

Apparatus. The apparatus consisted of six independent 500-ml fermentation chambers and accessories, and provided for anaerobiosis, constant volume, agitation of the fermentation mixture, collection of effluent liquors and gases, and for pH control by use of mixed ion-exchange resins. A mixture of simulated saliva and water was added continuously to the fermentation chambers at a rate calculated to be proportional to the in vivo intake into the rumen. Samples of rations of hay and grain, or both, were added periodically in a manner calculated to approximate the natural feeding of ruminants.

The components and accessories of the fermentation chamber are illustrated in Fig. 1 in their normal spatial arrangement, with part of the wall of the fermentor (A) cut away to show interior detail.

The fermentation chamber was a modified 500-ml wide-mouth polyethylene bottle. Artificial saliva mixed with water was pumped continuously from the reservoir (B) by an automatic syringe (C) into the fermentor. A screened outlet (D) permitted the escape of gas and effluent liquid from the fermentation chamber. A perforated glass capsule (E), which contained the dialysis bag (F), filled with ion-exchange resins was fitted into the cap of the fermentor. A rubber syringe cap (H) allowed the sampling of gas from the culture.

The plastic fermentation chamber was incubated at 39 C by partial submersion in a thermostatically controlled water bath. Mixing of the culture was accomplished by the

constant oscillation of the vessel around its long axis by means of a reciprocating chain which cradled the bottle at point I, a cog on the undersurface of the bottle. A wooden dowel (J) provided a pivot for rotation.

Effluent liquid and evolved gas passed through tube K into a 750-ml liquid-collection bottle held at 0 C in an ethylene glycol bath. Gas was carried over from the culture via the liquid-collection bottle into a calibrated manometer (M) of 2,700-ml capacity and fitted with a sampling port (N). Entering gas displaced an equal volume of acidified saturated NaCl solution. A float and magnet arrangement in the gas collector permitted the maintenance of a constant pressure in the apparatus equal to that of the external atmosphere.

The integrity of the junctures in the system was assured by sealing the tightly fitted joints with rubber cement. The simultaneous operation of six fermentors in the integrated system is depicted in Fig. 2. The float-magnet device previously mentioned can be seen at point O. Vacuum-operated windshield-wiper motors (P) provided power for constant mixing in the culture vessels through the mediation of connecting rocking arms (Q). Water at 39 C and ethylene glycol at 0 C were circulated to the fermentation bath and fluid collection bath, respectively, by pumping through tubing between the apparatus and thermostatically regulated baths shown at the left in Fig. 2. A third windshield-wiper motor powered six rams which continuously injected saliva-water solution, through the six automatic syringes, into the fermentation vessels.

Operation. The fluid flow rate and substrate intake of the in vitro system were proportional to the in vivo bovine intake of these materials. It was assumed that a bovine weighing 500 kg would have 70 liters of rumen ingesta, would secrete 60 liters of saliva into its rumen, and would drink 40 liters of water per day to yield a ruminal dilution rate of 1.43 volumes per day. Feed materials were added to the cultures on the basis of calculated maintenance requirements for the bovine. According to Morrison (1957), a 500-kg bovine should receive 7.5 lb of total digestible nutrients (TDN) per day to maintain body weight. Thus, a proportional feed intake for a 325-ml culture was:

$$\frac{(7.5\text{ lb of TDN})\ (0.325\text{-liter culture})\ (0.454\text{ kg/lb})}{70\text{ liters of rumen ingesta}} = 15.8\text{g TDN/day}$$

and the proportional saliva-water intake for the 325-ml culture was:

$$(1.43\text{ rumen dilution rate})\ (325\text{-ml culture}) = 465\text{ ml/day}$$

The mineral salt solution simulating the rumen intake of 60 liters of saliva and 40 liters of water was composed of 60 parts of artificial saliva (McDougall, 1948) and 40 parts of tap water. The roughage portion of the rations fed in vivo and added to the in vitro fermentation was alfalfa hay. The concentrate was University of Illinois Herd Mix no. 48, containing 13.3% crude protein and 75% TDN. [Herd Mix no. 48 consisted of the following (in lb): ground, shelled yellow corn (maize), 1,000; ground oats, 200; wheat bran, 600; soy bean oil meal (50% protein), 140; steamed bone meal, 20; ground limestone, 10; and trace mineralized salt (NaCl), 30.] These feed materials were coarsely ground through a 2-mm screen in a Wiley mill to facilitate in vitro mixing and digestion in the absence of normal chewing and rumination. The amounts, proportions, and times of addition of the feeds to the fermentors are described in detail in the Results.

A dialysis sac containing a mixture of 7.1 g of Dowex 2 (×4, 20 to 50 mesh) and 5.2 g of Dowex 50 (×8, 200 to 400 mesh) ion-exchange resins was placed in each fermentation chamber. The mixed resin was prepared by the passage of saturated $NaHCO_3$ through the mixed resins until the eluate gave a negative test for Cl^- ions. The par-

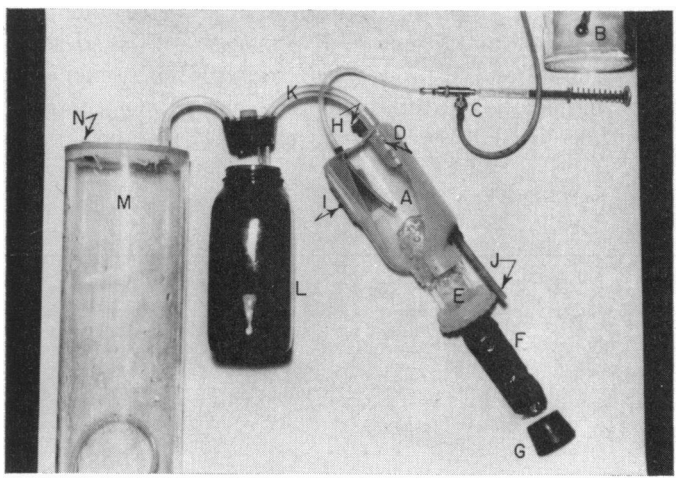

FIG. 1. *Fermentor and accessories. A, fermentor with side wall removed; B, saliva-water reservoir; C, automatic syringe; D, screened effluent outlet; E, perforated glass capsule; F, dialysis sac with ion-exchange resin; G, rubber stopper; H, syringe cap; I, cog for chain cradle; J, wooden dowel pivot; K, effluent tube; L, liquid-effluent collection bottle; M, calibrated gas collector; N, syringe cap.*

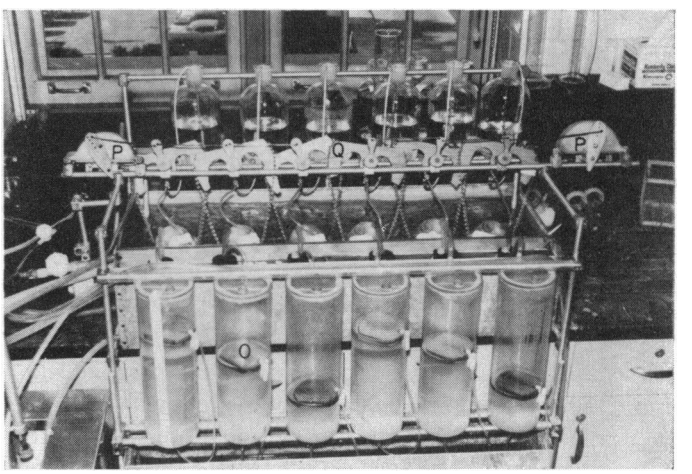

FIG. 2. *Six complete fermentation units. O, float and magnet in gas collector; P, windshield-wiper motors; Q, rocking arm connected to fermentor and windshield-wiper motor.*

tially spent resin was removed and replaced with a fresh resin mixture at the times solid feeds were added to the cultures.

Inocula for the continuous cultures were obtained by aspiration of rumen fluid from fistulated mature bovines maintained on the same roughage and concentrate rations, or both, to be used in the in vitro fermentations. Inocula were transported to the laboratory in preheated, insulated, and stoppered flasks. Each fermentor was initially charged with 300 to 325 ml of fresh undiluted, cheesecloth-filtered rumen fluid. The length of time from removal of rumen ingested to the beginning of in vitro fermentations ranged from 15 to 20 min.

Analytical. The concentrations of the individual fatty acids in fermentation mixtures were measured by chromatography, employing an 18-cm internal indicator column packed with 17 cm of Celite (Johns-Manville, New York, N.Y.; Celite Analytical Filter-Aid) absorbent slurry prepared as described by Wiseman and Irvin (1957). The prepared columns were flushed with 80 ml of BA_2 [acetone in Skelly solve B (Skelly Oil Co., Tulsa, Okla.); subscript of A, per cent of acetone (v/v)] under 5 psi nitrogen before the addition of 4 g of cap material containing the acidified sample. Equal weights of silicic acid and anhydrous sodium sulfate and 1 ml of acidified [one drop of 60% (v/v) H_2SO_4] sample were mixed to form the cap material, which was added to 10 ml of BA_2 on top of the column. The column was then sequentially eluted with 90 ml of BA_2, 50 ml of BA_{10}, and 90 ml of BA_{30}. The acidic bands were collected separately and titrated to a cresol red end point with 0.015 N alcoholic KOH.

The fatty acids absorbed on the mixed resin were removed by flushing with saturated NaCl until the eluate gave a strongly positive test for Cl^- ions. The fatty acids in samples of the resin eluates were measured as described above.

The solvent system used did not permit quantitation of fatty acids of greater chain length than butyric. Higher acids were normally visible as one or more bands preceding the darker butyric band, but were collected and titrated with butyric on the assumption that acids larger than C_4 seldom account for more than 3 to 5% of the volatile acids of the rumen (Annison and Lewis, 1959). Formate and lactate did not normally appear in fermentation mixtures, but were clearly visible on the column when present. Recovery of acids from a standard mixture was 99.8% for total acids, and 99.3, 100.2, and 99.4% for butyric, propionic, and acetic acids, respectively.

Gaseous fermentation products were analyzed by gas-solid chromatography, using a silica gel column in an Aerograph (Wilkens Instrument & Research, Inc., Berkeley, Calif.; model A-100) gas chromatography unit with helium as the carrier gas. Hydrogen was analyzed with nitrogen as the carrier gas. The percentage volume occupied by CO_2, H_2, and air in samples was calculated from standard curves prepared with pure gases.

Total counts of bacteria were made in duplicate on diluted samples using a Petroff-Hausser counting chamber and a phase-contrast microscope. Protozoa were qualitatively observed under phase-contrast microscopy. The pH of fermentation mixtures was determined with a Beckman model G meter.

Results and Discussion

A double-reversal experimental design was used to test the stability of the continuous cultures and to measure the response of those cultures to changes in substrate during a 10-day period of continuous fermentation. Inoculum for one series of three cultures (group H) was obtained from a fistulated bovine maintained solely on a roughage diet of alfalfa hay. Inoculum for the second series of three cultures (group C) was obtained from a fistulated bovine maintained on a high concentrate diet consisting of a daily intake of 10 lb of a 13.3% concentrate mix and from 1 to 2 lb of alfalfa hay.

The substrate additions to the cultures are shown in Table 1. Feeding periods were at approximately 10-hr intervals. The average TDN input per day per culture during the 10-day fermentation period ranged from 8.3 to 9.4 g for the group C cultures and from 8.0 to 8.9 g for the group H cultures. It was calculated that the TDN intake represented 55 to 65% of the maintenance requirement calculated for the cultures, but the input of digestible protein was in excess of the calculated maintenance needs of the cultures.

Also shown in Table 1 are the total milliequivalents of volatile fatty acid produced in the cultures in the respective feeding periods, and the apparent rates of production of the volatile fatty acids. The values shown in Table 1 are averages of the results obtained for the three cultures within each group. The rates of volatile fatty acid production are of the order of magnitude of estimated in vivo values. Carroll and Hungate (1954) have estimated rates of volatile fatty acid production in samples of rumen contents immediately after removal of the rumen contents from the animal. They report rates of 1.42 meq per hr per

TABLE 1. *Stability of continuous cultures and response to substrate changes*

Incubation period	Group C cultures			Group H cultures		
	Substrate	Acid produced*	Production rate	Substrate	Acid produced*	Production rate
hr	*g/feeding*	*meq*	*meq per 100 ml per hr*	*g/feeding*	*meq*	*meq per 100 ml per hr*
0–72	Grain, 5.0 Hay, 0.5–1.0	193	0.87	Grain, 0–0.5 Hay, 7–8	207	0.93
73–165	Grain, 0–0.5 Hay, 7.0	240	0.85	Grain, 5.0 Hay, 0–0.5	232	0.82
166–231	Grain, 5.0 Hay, 0.0	142	0.75	Grain, 0.0 Hay, 7.0	180	0.95

* Total produced in 231 hr.

100 g of rumen contents for hay-fed steers and 2.35 meq per hr per 100 g of rumen contents for grain-fed steers. The rates (Table 1) for the continuous cultures are probably low because substrates became limiting during the measurement periods, as will be shown in a subsequent experiment performed to test this point. There is little doubt, however, that the cultures were able to continue to produce volatile fatty acids at a reasonably normal rate over long time periods. The cultures were also stable to drastic alterations in the substrate fed.

Figure 3 shows the average molar percentage distribution of volatile fatty acids in the two series of continuous cultures during the 10-day incubation period. The values are in the same range usually found in the in vivo rumen. There were some definite trends in the relationship between the type of volatile fatty acid produced and the substrate fed. The use of hay as a substrate consistently yielded a higher acetate-propionate ratio than the concentrate substrate, both at the time of removal of the rumen fluid from the animal and during the in vitro incubation periods.

A switch from hay substrate to high-grain substrate resulted in a decreased acetate-propionate ratio, and a switch from grain to hay substrate led to an increase in the acetate-propionate ratio. The changes in the molar percentage distribution of the volatile fatty acids were independent of the original source of rumen fluid. Butyric acid followed a more variable pattern than acetate or propionate. Lactate and formate were not encountered in the fermentor effluents. The increase in the proportion of propionate in rumen fluid of animals fed increased proportions of concentrate

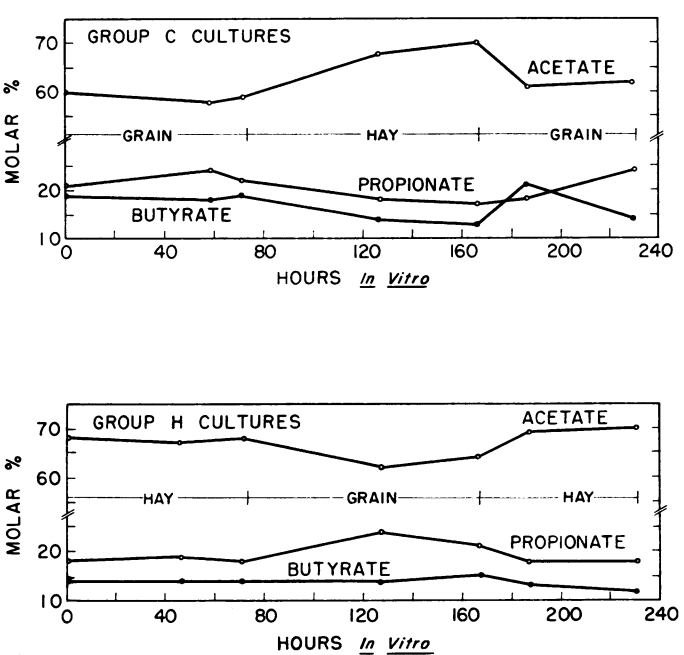

FIG. 3. *Molar per cent distribution of volatile fatty acids in continuous cultures. Group C and group H cultures contained inocula from high concentrate and high rough rations, respectively. The culture feeding regimen is shown in Table 1.*

in their diet has been reported (Elliot and Loosli, 1959). The in vitro continuous cultures, therefore, respond to dietary changes in a manner similar to the in vivo rumen. Interpretation of changes in the proportion of volatile fatty acids in vivo is complicated because changes can be accomplished both by a change in the rate of production of a given volatile fatty acid and by a change in the rate of removal of the volatile fatty acid through the rumen wall. The absorption process is eliminated in the continuous in vitro cultures, and the change in proportion of volatile fatty acids can be interpreted only as a change in microbial fermentation pattern.

Gas production in the continuous cultures proceeded in a relatively normal manner. Methane and carbon dioxide were continuously produced in all cultures. Methane constituted from 22 to 28% and carbon dioxide from 69 to 78% of the evolved gas. Hydrogen was not present in measurable amounts in the fermentation gases.

The inoculum for the group C cultures had good representation of oligotrich and holotrich protozoa and stayed about the same for approximately 2 days. Observations at 59 hr showed considerable thinning of the protozoa, with holotrichs and large oligotrichs being reduced in numbers. Protozoal numbers continued to decline during the experiment until small oligotrichs were the predominant species remaining at 231 hr.

During the course of this experiment, the reaction of the fermentation mixture ranged between pH 5.90 and 6.80 for the group C cultures and between pH 5.85 and 6.80 for the group H cultures. The average daily dilution rates, assuming a 300-ml constant volume in each fermentor, ranged from 1.72 to 1.97 with a mean of 1.88 for group C cultures, and from 2.21 to 2.23 with a mean of 2.21 for the group H cultures.

Data relative to the apparent rates of volatile fatty acid production in the preceding trial suggested that the quantity of substrate added (55 to 65% of the equivalent bovine daily TDN maintenance requirement) may have been completely digested during the intervals between sampling for volatile fatty acid analysis, especially where the maximal apparent rates of production were observed. This possibility was tested in a short-term experiment in which duplicate continuous cultures were fed hay and grain in amounts proportional to 25, 50, and 100% of equivalent bovine TDN maintenance requirements. The rations contained an excess of digestible protein relative to the level of TDN.

Inoculum was obtained from a fistulated bovine maintained on a daily intake of 10 to 15 lb of alfalfa hay and 3 lb of concentrate mix. The inoculum was dispensed in 325-ml amounts in the fermentation chambers. Differing amounts of ion-exchange resins were used in proportion to the level of substrate to be added. The fermentors used for the 25% maintenance level of substrate addition contained 6.3 g of the mixed dried resin, and the medium- and high-level cultures contained 12.6 and 18.9 g, respectively.

The amounts of substrate added are shown in Table 2. Each maintenance level was added to duplicate fermentors at intervals (12 to 13 hr) from zero time up to 60 hr in vitro. Total volatile fatty acid production and the percentage composition of the volatile fatty acids were measured on the pooled effluents and absorbed acids obtained over a 74-hr period in each fermentor. Appropriate corrections were made for the initial amounts of volatile fatty acids in the fermentors. Gas was collected and analyzed twice daily. Excess fibrous material, which tended to accumulate in the cultures receiving the high rate of substrate addition, was removed at 36, 48, and 60 hr prior to the addition of substrate.

TABLE 2. *Feeding regimen for substrate level study*

Per cent of maintenance TDN*	Amount (g) given per feeding	Per cent of maintenance digestible protein
25	Hay, 2.68 Concentrate, 0.77	57
50	Hay, 5.35 Concentrate, 1.54	114
100	Hay, 10.70 Concentrate, 3.07	228

* TDN = total digestible nutrients.

TABLE 3. *Effect of substrate level on total volatile fatty acid production*

Maintenance level fed	Volatile fatty acid production						Total*
	Acetate		Propionate		Butyrate		
	Amt produced	Molar %	Amt produced	Molar %	Amt produced	Molar %	
%	*meq*		*meq*		*meq*		*meq*
25	61.5	70.7	15.0	16.7	11.5	12.6	87.9
50	99.8	66.9	28.8	18.6	22.7	14.5	151.7
100	176.8	63.9	61.3	21.2	42.6	15.0	280.2

* Total produced in 72 hr.

The experimental data demonstrate that an increase in feed intake results in an increase in the total output of microbial products. Table 3 shows the total amount of volatile fatty acids produced in the 72-hr period for each maintenance level fed. Figure 4 summarizes the effect of substrate level on the apparent rates of major product formation. As the level of substrate was increased, CO_2 and volatile fatty acid output were increased to a greater extent than CH_4 production. The molar CO_2-CH_4 ratios in the fermentation gas were 3.35, 3.67, and 4.47 for the low, medium, and high levels of substrate intake, respectively. Similarly the average rates of total volatile fatty acid production were 0.37, 0.65, and 1.20 meq per hr per 100 ml of ingesta. In terms of productive capacity, even the full maintenance-level cultures were evidently not saturated with substrate.

Figure 5 illustrates the proportionality between substrate level and total gas production, and shows that the average apparent rate of gas production was constant with time in vitro. Carbon dioxide and methane were the only

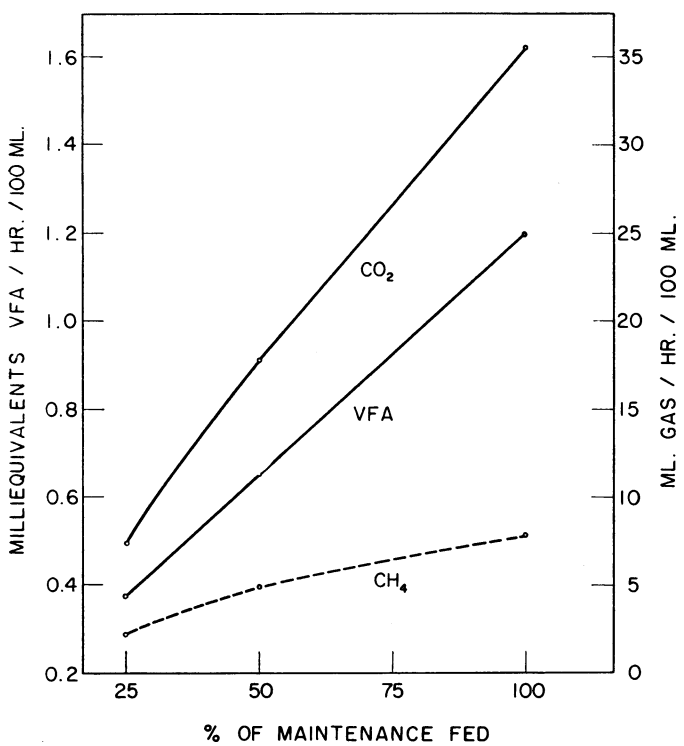

FIG. 4. *Rate of major-product formation with various levels of feed input.*

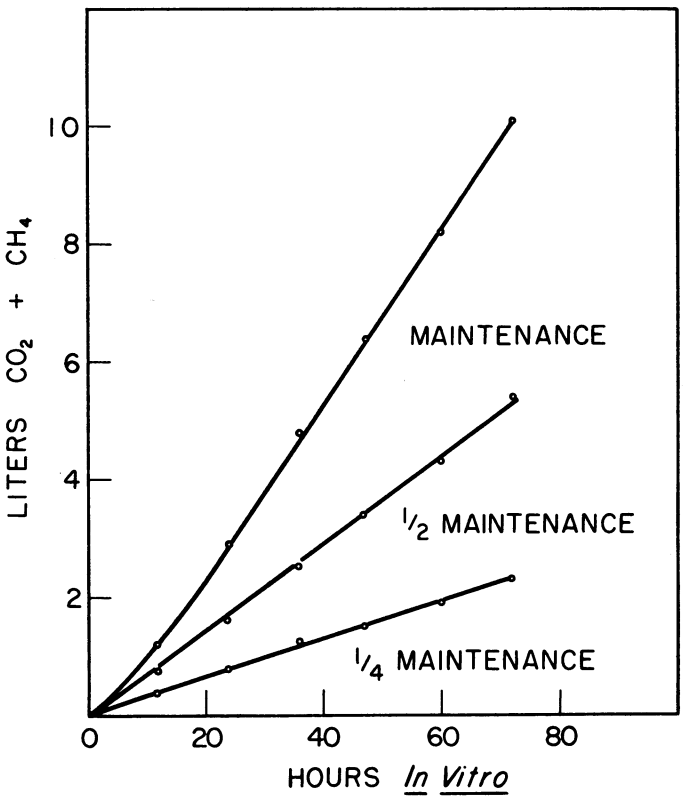

FIG. 5. *Proportionality between feed level used and total gas production in continuous cultures.*

gases produced. Hydrogen was not detected in any of the cultures.

The effect of substrate level on the concentration of bacteria in the fermentation mixture was as follows. At maintenance levels fed of 25, 50, and 100%, the number of bacteria per ml was 2.3×10^9, 3.1×10^9, and 4.4×10^9, respectively. The increase in the number of bacteria per ml with increased feeding level was significant at the 1% level, as tested by analysis of variance. Total counts were made after 72 hr. Those cultures receiving the highest level of substrate showed a decrease in total bacteria from 9.3×10^9 per ml at zero time to 4.5×10^9 per ml at the end of the 72-hr incubation period. As substrate level was decreased, the total bacterial count decreased proportionately in the other fermentors. Protozoal numbers followed the same pattern. Protozoa were sparse in the low-substrate fermentors, moderately abundant in the medium-level substrate cultures, and very numerous in the high-level substrate cultures after 72 hr.

The amount of substrate available had a definite effect on the molar proportions of the total fatty acids that were produced. Cultures receiving low levels of substrates produced lower proportions of propionate and butyrate than did those receiving increased quantities of substrate (Table 3). Conversely, the molar percentage of acetate decreased as substrate levels were increased.

During the 72-hr trial, the reaction of the fermentation mixtures remained relatively constant between pH 6.7 and 6.9 for the low substrate cultures, and between pH 6.6 and 6.9, and between pH 6.2 and 6.7 for the medium and high substrate cultures, respectively.

The results of the experiment show that relatively typical fermentation patterns were observed at all levels of feed input. Feed-input level does, however, limit the total production of fermentation products, at least to the highest level of feed used in this experiment. The bacterial counts and the qualitative observations on the protozoal population suggest that the feed levels used also limit the steady-state level of microorganisms established in the system. It will be of interest to continue these experiments with higher levels of feed than those used in this experiment to determine at what level the system used would become saturated with feed.

The highest rate of acid production obtained in this experiment, 1.20 meq per hr per 100 ml, certainly represents a minimal rate figure because of the probable disappearance of substrate during the experimental measurement period. It should be simple to obtain true maximal production rate values in the system by using shorter time periods between sampling so that substrate does not disappear during the course of the experiment, or by increasing the total amount of substrate used in the fermentors well beyond the maintenance level.

In general, the experimental results suggest that the major rumen population can be cultivated in an in vitro continuous-culture system for extended periods of time. Qualitative and quantitative biological and chemical comparisons of the in vitro continuous system with the in vivo rumen microbial system reveal no major differences. The in vitro system can be maintained with continuous volatile fatty acid, CH_4, and CO_2 production from normal bovine feed materials, with continuous replication of the flora and fauna of the rumen. The system should be extremely useful for a variety of studies concerned with analyzing the rumen microbial system.

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