

Maintenance of a Certain Rumen Protozoal Population in a Continuous In Vitro Fermentation System

FUMINORI NAKAMURA† AND YASUSHI KURIHARA*

Faculty of Science, Biological Institute, Tohoku University, Sendai, Japan

Received for publication 27 October 1977

A continuous culture system suitable for maintaining certain rumen protozoa was developed by modifying the procedure of Weller and Pilgrim (R. A. Weller and A. F. Pilgrim, *Br. J. Nutr.* 32:341-351, 1974) to include a dialyzing system. The concentration of ciliate protozoa, the pH value, and concentrations of volatile fatty acid and ammonia-N could be maintained within normal rumen limits for more than 15 days by appropriate choice of mechanical agitation and of the amount of substrate and physical form of the substrate bags. The average concentration of protozoa in the free fluid around the substrate was about 10% that in the fluid squeezed from solid digesta residues. More than 10^6 protozoa per ml were present in the fresh substrates only 2 h after supplying the substrate. These facts suggest that sequestration of the protozoa among particulate digesta is an important factor in maintaining the concentration of protozoa.

Several devices for continuous in vitro culture of rumen microbial populations have been described previously (1-3, 5-7). These techniques appear to be useful for analysis of the rumen ecosystem, especially for studying the conditions for maintaining a steady state in microbial populations.

In continuous culture techniques analogous to those that primarily have been developed with pure cultures, the concentrations of metabolic products such as volatile fatty acid (VFA) and ammonia-N, and in some cases the concentration of the bacterial population, have been kept similar to those of the natural rumen for extended periods of time. However, success in maintaining the protozoal population has been very limited. The maintenance of protozoa, in numbers comparable to those of the rumen, may be the most reliable means of ascertaining whether the fermentation is kept normal, since it has been suggested that protozoa provide a sensitive indicator of rumen function. Itabashi and Kurihara (3) maintained the protozoal population, consisting mainly of entodinia, at a level of 10^5 per ml for over 3 weeks, using a continuous culture technique similar to that of Slyter et al. (6), and they suggested that some substances originating from the host may be necessary for the growth and maintenance of the protozoal population.

Aafjes and Nijhof (1) demonstrated an artificial rumen comparable to that of a living animal by vertically agitating bags of nylon gauze filled

with hay in a glass vessel covered with a plastic lid. Recently, Weller and Pilgrim (7) reported that an artificial rumen requires physical conditions under which sequestration of particulate digesta is allowed, ensuring a protozoa removal rate less than that of fluid turnover, and they proposed a continuous culture technique similar to that of Aafjes and Nijhof (1).

In this paper we describe and evaluate a continuous in vitro fermentation system developed from those used by Aafjes and Nijhof (1) and by Weller and Pilgrim (7) and present data that demonstrate some of the factors involved in maintaining a protozoal population in a steady state.

MATERIALS AND METHODS

Apparatus. A schematic diagram of the continuous fermentation apparatus is shown in Fig. 1. A 5-liter cylindrical glass vessel (c_1) was divided into two compartments (c_2 and c_3) separated by a polyvinyl chloride membrane (membrane filter was kindly donated by Yuasa Battery Co. Ltd., Osaka, Japan) with a $0.4\text{-}\mu\text{m}$ pore size (Yuasa Battery Co. Ltd.), which is supported on a cylinder of stainless steel mesh (c_4). The substrates, enclosed in nylon bags ($245\text{ }\mu\text{m}$ mesh size), were kept cylindrically shaped by stainless steel wire formers (c_5) and placed in the inner compartment, referred to as the fermentation chamber. A plunger (p), to which the cylindrical wire formers are attached, was driven through a hole (h_1) in the lid by a vertically agitating action, with a stroke length of 80 mm. Buffer solution (7) containing 0.1% Na_2S (s) was pumped continuously into the outer compartment, referred to as the dialyzing chamber, through a hole (h_2) in the glass cylinder, and out to the reservoir (o) at a rate of 4.6 liters per day. The solution was also introduced

† Present address: Medical School, Showa College, Tokyo, Japan.

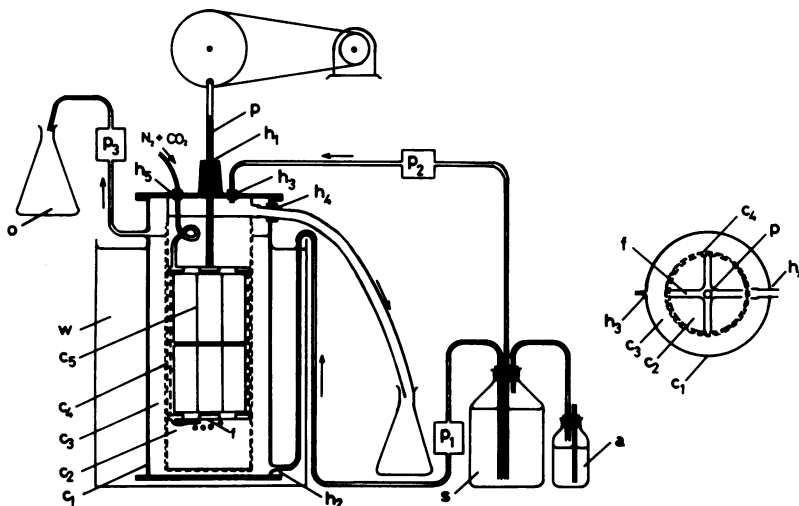


FIG. 1. Artificial rumen apparatus. (a) Pyrogallol solution (oxygen absorbent); (c₁) cylindrical glass vessel; (c₂) fermentation chamber; (c₃) dialyzing chamber; (c₄) stainless steel mesh cylinder; (c₅) stainless wire former; (f) holder for substrate bags; (h₁) hole to guide plunger movement; (h₂) and (h₃) inlet for buffer solution; (h₄) opening for overflow; (h₅) inlet for gas mixture; (o) reservoir; (p) plunger; (p₁-p₂) peristaltic pumps; (s) buffer solution; (w) 39°C water bath. Arrows show flow directions.

into the fermentation chamber through a hole (h₃) in the lid at a rate of 2.3 liters per day, and excess fluid was allowed to overflow through the opening (h₄) in the fermentation chamber. The fermentation chamber was kept anaerobic by a constant flow (two to three bubbles per second), through a hole in the lid (h₅), of gas containing 95% nitrogen and 5% carbon dioxide. The fermentation vessels were immersed in a water bath (w) at 39°C.

Operation of apparatus. The substrate was a mixture of 3 parts crushed alfalfa hay cubes, 2 parts crushed barley, and 1 part uncrushed alfalfa hay pellets. The alfalfa fragments varied in size from powdery particles to strips up to 40 mm long. Initially, 3.4 liters of buffer solution warmed to 39°C was poured into the fermentation and dialyzing chambers. A nylon bag containing about 500 ml of rumen contents from sheep was introduced into the fermentation chamber. The main genera in this inoculum were *Entodinium* (5×10^6 to 5×10^8 per ml) and *Eudiplodinium* (about 10^4 per ml). Another nylon bag, packed with substrate, was placed above the bag containing the inoculum, and the apparatus was set in motion. After running for 24 h, the inoculum bag was removed, the substrate bag was moved down, and a bag of fresh substrate was inserted above it. Thereafter, this procedure was repeated every 24 h. The upper bag of fresh substrate and the lower bag containing digesta residues were designated "new bag" and "old bag," respectively. The fermentation was continued for about 2 to 3 weeks by the daily addition of a new bag and removal of the old bag.

Examination of the samples. Each time an old bag was removed it was squeezed gently until 20 ml of liquid was obtained for experimental purposes. The bag and the small amounts of liquid still contained in

it were then discarded. The pH and the concentrations of protozoa, VFA, and ammonia-N were then measured in the fluid squeezed from the bags and in the effluent from the fermentation chamber. Protozoa were fixed in formalized saline and counted in a 0.2-mm deep Fuchs-Rosenthal counting chamber. The concentration of VFA was determined by gas chromatography using a hydrogen flame ionization detector (Hitachi Electric Co. Ltd.) according to the method of Packett and McCune (4). Ammonia-N was determined on samples previously stored in a freezer at -20°C, by a microdiffusion method. The pH value was determined with an electrode pH meter (TOA Electronic Co. Ltd.) immediately after sampling.

Operating conditions. Cultures were carried out under six separate sets of additions, as shown in Table 1. Experiments 1 through 4 were performed to determine the effects of bag shape and size, the number of bags, and the amount of substrate needed for the maintenance of protozoa concentrations. Experiments 4 through 6 were planned to examine the effects of varying intensities of mechanical agitation.

RESULTS

Experiment 1. In experiment 1, a 90-g portion of substrate was packed in a nylon bag kept cylindrical by wire formers 85 mm in diameter and 100 mm in height. Dilution rates were 1.0 per day for the fermentation chamber and 2.0 per day for the dialyzing chamber. The plunger was driven at a rate of 8 strokes per min with a stroke length of 80 mm.

As seen in Fig. 2, the ciliate protozoa population consisted mainly of *Entodinium* species in-

TABLE 1. Establishing suitable culture conditions^a

Expt no.	Amt of substrate (g supplied/day)	Substrate bag size (mm)		No. of bags		Agitation rate (strokes/min)
		Diam	Ht	New	Old	
1	90	85	100	1	1	8
2	72	85	100	1	1	8
3 ^b	72	50	100	4	4	8
4	72	32	100	3	3	8
5	72	32	100	3	3	12
6	72	32	100	3	3	0

^a Substrate was supplied every 24 h; the given amount of substrate was packed evenly in the bags of the upper group and then placed above the other group, which had undergone fermentation for the previous 24 h in the upper position. The bags were fitted in a container which agitated at a given rate in the fermentation chamber.

^b In experiment 3 only, the bags were without frames; others were performed with bags fitted to stainless wire frames.

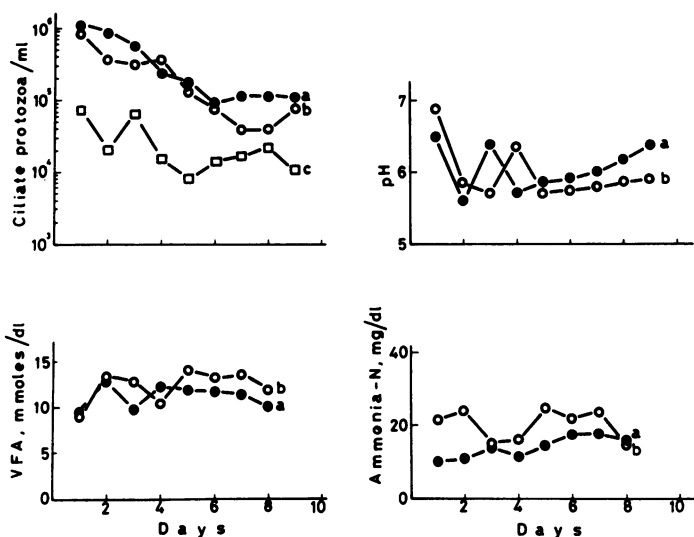


FIG. 2. Concentrations of protozoa, VFA, and ammonia-N and pH values in separate portions of the artificial rumen: receiving 90 g of substrate in one bag per day (experiment 1). Liquid from new bag, ● (a); liquid from old bag, ○ (b); liquid effluent from h₄, □ (c).

cluding *E. simplex*, *E. longinuclatum*, *E. nannellum*, and *E. caudatum* with few other genera present. Concentrations of protozoa decreased sharply with time in both the bags and effluent, and protozoal concentrations in the effluent were consistently much lower than those in the bags. Concentrations of VFA and ammonia-N in both bags fluctuated within a range comparable with in vivo measurements. The pH values fell rapidly to below 6.0 for both bags, fluctuated markedly around 6.0, and then stabilized after day 5.

Experiment 2. The amount of substrate was reduced to 72 g in experiment 2, but other conditions were the same as in experiment 1. Concentrations of protozoa were maintained at a level of 4×10^5 per ml for 6 days in the new bag and 2×10^5 per ml for 4 days in the old bag, and

thereafter both decreased to some extent (Fig. 3). As before, protozoal concentrations in the effluent were much lower than those in the bags throughout the experiment. The pH in the bags fluctuated between 5.5 and 6.5, but VFA and ammonia-N did not show appreciable changes. Their values were similar to those found in vivo, although somewhat higher in the old bag than in the new.

Experiment 3. In experiment 3, an attempt was made to measure the effect of the shape of the bags on the day-to-day variation in the number of protozoa. Substrates enclosed in nylon bags not kept in a cylindrical shape by wire formers were placed in the stainless steel mesh cylinder. The daily supply of 72 g of substrate was packed in four such bags, each containing 18 g.

The concentration of protozoa decreased sharply with time from 1.2×10^6 to 6.3×10^4 per ml in the new bags, and from 3.8×10^5 to 1.8×10^4 per ml in the old bags. In every case protozoa concentrations in effluents were much lower than those in the corresponding fluids from the bags. These results indicate that the physical form of the substrate bag may be an important factor in the maintenance of protozoal populations.

Experiment 4. In experiment 4, three cylindrical bags shaped by wire formers 32 mm in diameter and 100 mm in height were used to contain the daily substrate. Each bag was filled with 24 g of substrate so that the total amount supplied each day remained at 72 g. In all three fluids examined, day-to-day variation in protozoa concentration was reduced (Fig. 4). Concentrations ranged from 2.8×10^5 to 5.4×10^5 per ml in the new bags, and from 1.8×10^5 to 3.4×10^5

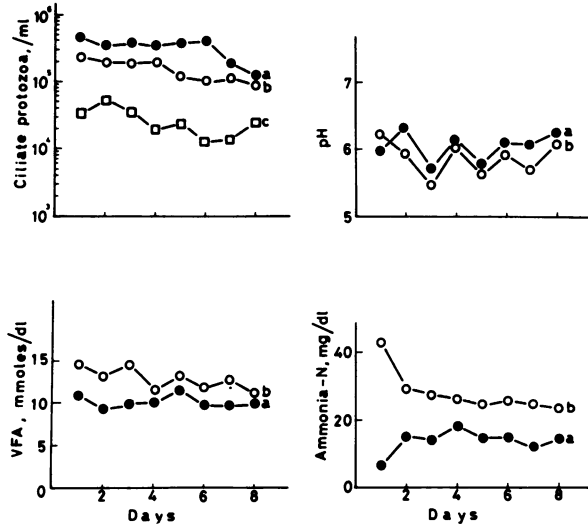


FIG. 3. Concentrations of protozoa, VFA, and ammonia-N and pH values in separate portions of the artificial rumen: receiving 72 g of substrate in one bag per day (experiment 2). Liquid from new bag, ● (a); liquid from old bag, ○ (b); liquid effluent from h₄, □ (c).

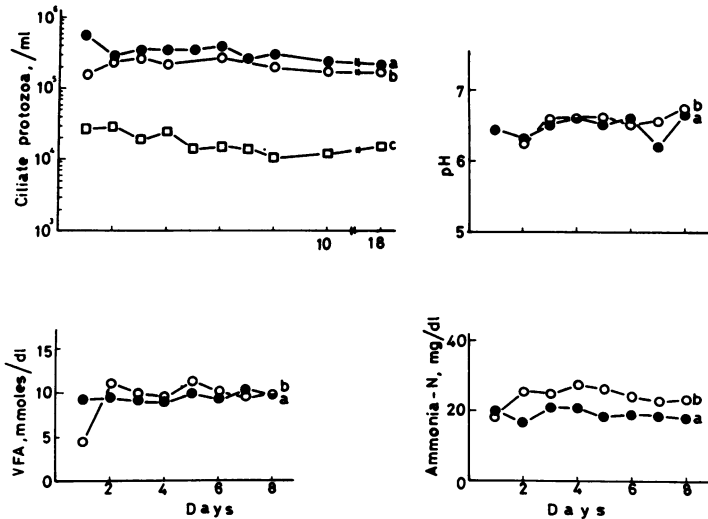


FIG. 4. Concentrations of protozoa, VFA, and ammonia-N and pH values in separate portions of the artificial rumen: receiving 72 g of substrate in three framed bags each day. Liquid from new bag, ● (a); liquid from old bag, ○ (b); liquid effluent from h₄, □ (c).

10^5 per ml in the old bags. The pH values and the VFA and ammonia-N concentrations also remained steady throughout the experimental period. The pH within the bags remained at a level above 6.0, comparable with those from *in vivo* measurements. The concentration of VFA in the bags was about 10 mmol/100 ml, similar to that found *in vivo*. Ammonia-N varied from 18.2 to 27.1 mg/100 ml in the old bags, and from 16.3 to 20.8 mg/100 ml in the new bags, again similar to the range of values measured *in vivo*.

Experiment 5. The effects of the rate of mechanical agitation on day-to-day variations in pH and concentrations of protozoa, VFA, and ammonia-N were investigated in experiment 5. In this experiment, the rate of plunger movement was increased to 12 strokes per min. Other conditions were the same as in experiment 4. Concentrations of protozoa decreased gradually in all three fluids as the trial proceeded, and the levels at day 10 were considerably lower than those in experiment 4, especially in those in the effluent that fell to 6.8×10^3 per ml, approximately half that in experiment 4. The pH values in the old bags fluctuated between 7.5 and 6.3, but variations in the new bags were smaller. Average concentrations of VFA and ammonia-N were 7.7 and 21.6 mg/dl for the old bag, and 9.6 and 19.0 mg/dl for the new bag, respectively; these were somewhat lower than those found *in vivo*, suggesting that increased mechanical agitation accelerates the release of metabolite, as well as protozoa, from the solid residues in the bags.

Experiment 6. In experiment 6 the move-

ment of the plunger was stopped, and other conditions remained as in experiment 5. Concentrations of protozoa decreased gradually with time, just as they did in experiment 5. It should be noted that pH values were very low, and concentrations of VFA were fairly high throughout the experiment. The pH fell to 5.4 in the old bags and 5.2 in the new bags, and VFA concentrations rose to approximately 13 mmol/dl in both bags. The results indicate that the diffusion rate of VFA from solid residues within the bags was reduced in the absence of mechanical agitation, causing the pH to fall almost to 5.0, at which level protozoa become inactive.

Experiment 7. To examine diurnal variations in the concentration of protozoa, samples were taken at 2, 6, 12, 18, and 24 h on day 18 of experiment 6 after supplying the initial new substrate. At each time, the normal daily procedure was followed and fresh substrate was supplied. Other conditions were the same as in experiment 4. pH values and concentrations of protozoa, VFA, and ammonia-N were determined in fluids from the bags and effluent (Fig. 5). At the same time, the diurnal variations in the concentration of dividing protozoa in fluids from within the bags were determined (Fig. 6). It should be noted that more than 10^4 per ml of protozoa were present in the fluid from the fresh substrates after only 2 h, suggesting that protozoa had migrated from the digesta in the old bag and the "free" fluid outside the bags, into the fresh substrates, aided by appropriate mechanical agitation. In the old bags, protozoa were maintained at a level of about 5×10^4 per ml

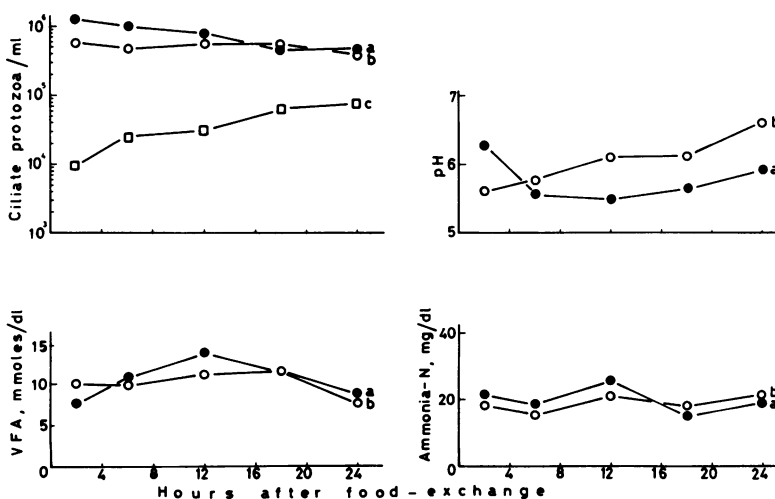


FIG. 5. Diurnal variations in concentrations of protozoa, VFA, and ammonia-N and pH values in separate portions of the artificial rumen under similar conditions as in experiment 4. Liquid from new bag, ● (a); liquid from old bag, ○ (b); liquid effluent from h_4 , □ (c).

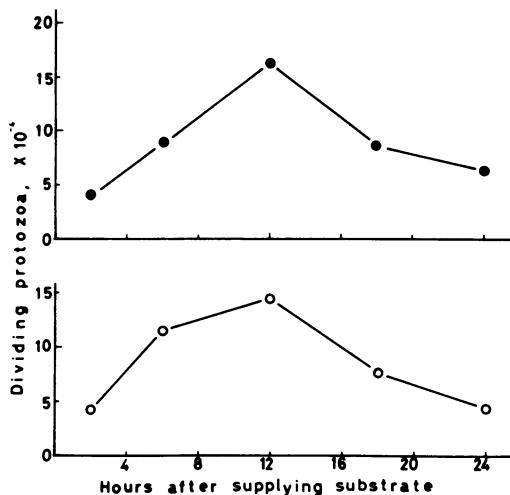


FIG. 6. Diurnal variations in numbers of dividing protozoa from new bags (●) and old bags (○).

during this period. Thus, protozoa concentrations in both bags became almost equal at 24 h. The concentration of protozoa in the effluent was 10^4 per ml at 2 h but steadily increased to nearly 10^5 per ml at 24 h. The initial drop in number may reflect migration to the fresh substrate. In the new bags the pH fell from 6.3 to 5.5 at 12 h, and then increased slightly to 6.0, and the maximum concentration of VFA occurred at 12 h. Thus, the shape of the diurnal curve for VFA contrasted with that of the pH, indicating that microbial decomposition was most active at 12 h. In the old bags, the first of which had been subjected to fermentation for 24 h, the pH value rose with time, and fluctuations in VFA and ammonia-N were smaller. The experiments reported above have been repeated by M. Abe (Ph.D. thesis, Tohoku University, Sendai, Japan, in preparation) with similar results. In all experiments, the inoculum initially contained *Entodinium* and *Eudiplodinium*, as the major genera, but in all cases *Eudiplodinium* died out within a few days, not only in the liquid squeezed from the bags but also in the effluent.

DISCUSSION

A continuous culture system that maintained certain rumen protozoa was developed by including a polyvinyl chloride membrane with 0.45- μ m pore size in the procedure of Weller and Pilgrim (7). The pH value and the concentrations of ciliate protozoa (mainly entodinia), VFA, and ammonia-N could be maintained within normal rumen limits for about 15 days, by appropriate choice of mechanical agitation,

amounts of substrate, and physical form of the substrate bag. Figure 4 indicates that the daily addition of substrate packed in three nylon bags, each kept in a cylindrical shape, led to the maintenance of a steady state in protozoal population, pH, VFA, and ammonia-N. Thus, the volume and physical form of the substrate bag and the amount of substrate supplied need to be considered in establishing a valid artificial rumen system.

The intensity of mechanical agitation is also important for the maintenance of a steady state. A frequency of 8 strokes/min with a strike length of 80 mm gave the best results in these experiments, suggesting that agitation should be sufficient to remove metabolic products from the bags without detriment to the fermentation or the protozoal population.

Studies of the distribution of protozoa within the fermentation chamber under the conditions of experiment 4 (Fig. 4) showed that average concentrations in the free fluid outside the bags were about 10% of those in the fluids squeezed from the residues in the bags. Weller and Pilgrim (7) reported that the passage of protozoa to the omasum was only 6 to 29% of that which would have been expected if protozoa passed from the rumen at the same rate as fluid. Our results may support these findings.

In this connection, the distribution of protozoa in the alimentary tracts of seven slaughtered cattle was studied. Table 2 shows that the concentration of protozoa in omasal fluid was much lower than that in the rumen, which is again consistent with the above results. The reduced concentration of protozoa in the omasum compared with that of the rumen or reticulum supports the suggestion that maintenance of protozoal numbers within the rumen may depend largely on the protozoal removal rate being less than that of fluid turnover. Further evidence of

TABLE 2. Concentrations of ciliate protozoa in segments of bovine alimentary tracts^a

Animal no.	Dorsal rumen	Ventral rumen	Reticulum	Omasum
1	284	226	89	30
2	115	87	91	22
3	99	116	104	33
4	138	147	146	35
5	93	131	89	39
6	72	81	55	21
7	88	77	41	11

^a Samples were taken from the ventral rumen, dorsal rumen, reticulum, and omasum of seven cattle immediately after slaughter. Protozoa were counted with a Fuchs-Rosenthal counting chamber. Individual results are presented ($\times 10^{-4}$ per ml).

a protozoa-conserving mechanism is the appearance within 2 h of high concentrations in bags of fresh substrate (Fig. 5), clearly indicating the sequestration of protozoa among the food solids. Protozoa in free fluid around the bags and in the digesta residues in the old bags apparently migrated to the fresh substrates in the new bags. The excess number formed by division in the new bags, most numerous at 12 h, may be released into the free fluid, with some of them being removed in the effluent according to the rate of fluid turnover. Similarly, metabolic products such as VFA and ammonia-N were removed from the system partly in the effluent and partly in the dialysate.

Whereas the physical form of the substrate bag appears to affect *in vitro* fermentation, it is still unclear why this factor is important. One hypothesis is that the shape and number of bags affect the efficiency of metabolite removal. Another hypothesis can be offered, however. Experiments 1 and 2 show that the quantity of substrate supplied affects fermentation. Since there is no difference between the size and shape of the substrate bag in these two experiments, we can say that the difference in the degree of compaction of the substrate within the bag may be responsible for the difference in the results of the two experiments. The degree of compaction of the substrate within the bag also varied among experiments 2, 3, and 4 because bag size changed while substrate quantity remained fixed, so our results may be attributable to compaction variations rather than to the factors specifically examined. In subsequent research we hope to isolate the effects of compaction from

those of bag shape and size.

Requirements for *in vitro* fermentation in which a population of protozoa is maintained in a steady state may include a suitable physical form of the substrate bag, suitable mechanical agitation for sequestration of protozoa among particulate digesta, and appropriate rates of turnover and dialysis for the removal of protozoa and metabolites.

ACKNOWLEDGMENTS

Our sincere thanks are due to R. A. Weller, Division of Human Nutrition, CSIRO, for reading of the manuscript. H. Komura is also acknowledged for his technical assistance.

LITERATURE CITED

1. Aafjes, J. H., and J. K. Nijhof. 1967. A simple artificial rumen giving good production of volatile fatty acids. *Br. Vet. J.* **123**:436-446.
2. Davey, L. A., G. C. Cheeseman, and C. A. E. Briggs. 1960. Evaluation of an improved artificial rumen designed for continuous control during prolonged operation. *J. Agric. Sci.* **55**:155-163.
3. Itabashi, H., and Y. Kurihara. 1972. Maintenance of rumen microbial populations in an improved artificial rumen. *Jpn. J. Ecol.* **22**:262-265.
4. Packett, L. V., and R. W. McCune. 1965. Determination of steam-volatile organic acids in fermentation media by gas-liquid chromatography. *Appl. Microbiol.* **13**:22-27.
5. Rufener, W. H., Jr., W. O. Nelson, and M. J. Wolin. 1963. Maintenance of the rumen microbial population in continuous culture. *Appl. Microbiol.* **11**:196-201.
6. Slyter, L. L., W. O. Nelson, and M. J. Wolin. 1964. Modifications of a device for maintenance of the rumen microbial population in continuous culture. *Appl. Microbiol.* **12**:374-377.
7. Weller, R. A., and A. F. Pilgrim. 1974. Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous *in vitro* fermentation system. *Br. J. Nutr.* **32**:341-351.