

# Culture of the Rumen Holotrich Ciliate *Dasytricha ruminantium* Schuberg

R. T. J. CLARKE<sup>1</sup> AND R. E. HUNGATE

Department of Bacteriology, University of California, Davis, California

Received for publication 11 October 1965

## ABSTRACT

CLARKE, R. T. J. (University of California, Davis), AND R. E. HUNGATE. Culture of the rumen holotrich ciliate *Dasytricha ruminantium* Schuberg. Appl. Microbiol. 14:340-345. 1966.—The successful cultivation of the anaerobic ciliate *Dasytricha ruminantium* is described. The cultures were established in a salts medium containing 30% clarified rumen fluid. Sucrose and extract of rumen holotrich protozoa were fed once daily for 2 to 4 hr, and *Dasytricha* was then transferred to medium free from these nutrients. Rumen fluid was essential. Omission of protozoal extract resulted in gradual death of the ciliates. Bovine serum satisfactorily substituted for the protozoal extract, but various rumen bacteria, extract of rumen bacteria, and extracts of plant materials could not. There was a positive correlation between formation of methane in the cultures and growth of the ciliates. It is possible that methane bacteria were ingested, but it is not excluded that survival of both dasytrichs and the methanogenic bacteria depended on a low redox potential of the medium.

Evidence increasingly suggests that the rumen ciliate protozoa play an important role in the nutrition of the host (1). The protozoa are of two sorts, entodiniomorphs and holotrichs. The role of the entodiniomorphs has been clarified by observing the foods used by the individual species in the mixed rumen population, by studying the enzymes in protozoa obtained from the rumen (11, 12), and by examining the conditions necessary for artificial cultivation. These protozoa have been grown in vitro for extended periods (2, 5, 9, 15, 16, 19) in the presence of accompanying bacteria which are ingested (6).

Studies of the food and the enzymes of the holotrichs show that these organisms rapidly metabolize certain sugars (13, 14, 20) and synthesize a large fraction of the sugar into starch. Starch storage on occasion can be so extensive that the holotrichs burst.

The rumen holotrichs have not been grown for an extended period outside the host. The most successful culture was probably that of Sugden and Oxford (27) who maintained a flask culture for 30 days without transfer. Division forms were observed, but the total numbers decreased during the latter stages of the culture. Gutierrez (8) maintained very small numbers of *Isotricha* cells,

with division, for 18 days. Quinn et al. (24) maintained holotrichs for 7 days, and Purser and Weiser (23) maintained them for 26 days.

Since the holotrich ciliates may be involved in the etiology of bloat in cattle (3, 4) and, because of their large numbers, must often play a role in nutrition, a further attempt at their cultivation has seemed desirable. The present study was initiated to develop methods for in vitro cultures and to obtain information on factors influencing growth of these protozoa.

## MATERIALS AND METHODS

*Source of rumen ciliates.* The protozoa used as initial inoculum were from a rumen-fistulated cow which was stall-fed on alfalfa hay. They were concentrated and separated from other rumen materials by the method of Oxford (21). After being washed three times by decanting off added salts solution containing cysteine-HCl, the protozoa were inoculated into culture tubes. Both holotrichs and entodiniomorphs were present.

*Culture medium.* The basal medium contained: salt solution, 70 ml; clarified rumen fluid, 30 ml; NaHCO<sub>3</sub>, 0.5 g; cysteine-HCl, 0.02 g; resazurin (0.1%, w/v), 0.1 ml; and extract of rumen holotrich protozoa, 3.0 ml; the medium was equilibrated with oxygen-free carbon dioxide. The salt solution contained (w/v); 0.15% K<sub>2</sub>HPO<sub>4</sub>, 0.60% NaCl, 0.001% MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.001% CaCl<sub>2</sub>. The medium was autoclaved at 15 lb for 15 min to remove O<sub>2</sub>, cooled in ice under a stream of O<sub>2</sub>-free CO<sub>2</sub>, and dispensed under anaerobic

<sup>1</sup> Present address: Plant Chemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand.

conditions in rubber-stoppered culture tubes by use of methods adapted from Hungate (17). The tubes of medium were stored at room temperature.

On occasions, the medium was not autoclaved but was gassed with carbon dioxide for 2 hr to remove O<sub>2</sub>, sterilized by filtration through a membrane filter (Metricel GA-6, pore size 0.45  $\mu$ ; Gelman Instrument Co., Ann Arbor, Mich.), and dispensed aseptically in sterile culture tubes.

The buffer solution used for washing and suspending the ciliates, and for dialysis, was the culture medium without rumen fluid, protozoal extract, or resazurin.

*Clarified rumen fluid.* Rumen fluid was obtained from the same cow which provided the ciliate suspensions. Contents were removed from the rumen through the fistula and strained through gauze. The fluid was then centrifuged at 20,000  $\times$  g, and the clear supernatant liquid was stored at -14 C.

Dialyzed rumen fluid was prepared by dialyzing clarified rumen fluid against several changes of buffer at 4 C.

*Extract of rumen holotrich protozoa.* A thick suspension of rumen ciliates, both holotrich and entodiniomorph, was prepared from several liters of rumen fluid by the method of Oxford (21), and was freed from plant debris and associated bacteria by decanting off added mineral salt solution. The suspension was frozen three successive times by immersing the tube in a mixture of Dry Ice and ethyl alcohol and thawing rapidly, and was then ground in a Tri-R Teflon tissue homogenizer. Holotrich debris and entodiniomorph ectoplasts in the ground suspension were sedimented by centrifugation at 20,000  $\times$  g for 30 min in a refrigerated centrifuge. The viscous, opalescent supernatant liquid, diluted two to three times with buffer, served as the protozoal extract.

Dialyzed protozoal extract was prepared by dialyzing the extract against several changes of buffer at 4 C.

*Technique of culture.* The ciliates were grown in narrow-mouth roll culture tubes 16 by 150 mm (Bellco Glass Inc., Vineland, N.J.), with a small bulge blown on the side about 13 mm from the bottom. Each tube contained 10 ml of medium and was closed with a rubber stopper. Each tube was initially inoculated with about 500 ciliates of which approximately 80% were holotrichs.

The cultures were incubated in an upright position at 38 C in a water bath from which light was excluded. Each day the ciliates were fed by injecting 0.8 ml of an anaerobic buffer solution containing 5 mg of sucrose and 0.3 ml of protozoal extract. After 2 to 4 hr, the tube was placed for 15 min at an angle of about 25°, with the bulge on the lower side. The ciliates settled into the bulge; plant and cell debris remained in the base of the tube. The tube was then transferred without mixing to a similar position in a small Perspex water bath (38 C) on the stage of a dissecting microscope. The stopper was removed, and the debris-free ciliates were transferred with a micro-pipette from the bulge to a tube of fresh basal medium which was then returned to the water bath and incubated in a

vertical position. Less than 1 ml of medium was transferred with the protozoa.

All operations of medium preparation and transfer of protozoa were carried out in a stream of CO<sub>2</sub> freed of O<sub>2</sub> by passage over hot Cu metal which was reduced by H<sub>2</sub>.

*Counting method.* In cultures containing more than 300 cells, 1 ml of culture was removed and mixed with 1 ml of 10% (v/v) formalin. A 0.5-ml sample of an appropriate dilution of the formalin-treated suspension was placed in a flat-bottom circular glass vessel (diameter, 26 mm; volume, 6 ml) with the bottom ruled in 2-mm squares, and the protozoa in alternate squares were counted by use of a dissecting microscope.

Cultures with up to 300 ciliates were counted in the culture tube by use of a dissecting microscope after the water bath had been cooled to 30 C to lessen the movement of the organisms.

*Preparation of test nutrients.* Lyophilized fresh alfalfa and extracts of grass or alfalfa hay were tested as replacements for rumen fluid and protozoal extract.

Grass and alfalfa extracts were prepared by macerating 40 g of fresh orchard grass (*Dactylis glomerata*) or fresh alfalfa in a Waring Blendor with 300 ml of buffer at 4 C, filtering through glass wool, and centrifuging at 20,000  $\times$  g for 30 min. The pale-green solution obtained was gassed thoroughly with CO<sub>2</sub> and frozen overnight. When thawed, some material precipitated and was removed by centrifugation.

Hay extract was prepared by extracting 50 g of chopped alfalfa hay with 1 liter of buffer at 80 C for 30 min. The mixture was then cooled to room temperature, filtered, clarified by centrifugation, and stored at -14 C.

Lyophilized alfalfa was green chop alfalfa, lyophilized and ground to pass through a 200-mesh sieve. A 2-mg amount was added to each culture.

Survival of the ciliates was the index of nutritive value. In some experiments, cultures were started with ciliates freshly removed from the rumen; in others, ciliates which had been grown in culture as long as 21 days were used.

*Preparation of test bacteria.* *Bacteroides succinogens* S85, *Ruminococcus albus*, and *Selenomonas ruminantium* were grown at 39 C in the culture medium with 10% (v/v) clarified rumen fluid and glucose or cellobiose (0.1% w/v) but without protozoal extract. A 2-ml amount of cells and culture fluid from a 24-hr culture was added to each protozoal culture.

Washed mixed rumen bacteria were prepared by centrifuging fresh rumen fluid at 20,000  $\times$  g. The bacteria which were deposited under the plant debris and protozoa were separated and repeatedly washed by resuspension and centrifugation.

The extract of rumen bacteria was prepared by disrupting a thick suspension of washed rumen bacteria in a Hughes press. The resulting mixture was freed from particulate matter by centrifugation.

*Defined nutrients.* The mixture of vitamins and other growth factors contained the following (milligrams per 100 ml): biotin, 0.006; calcium pantothenate, 1.1; choline chloride, 95; folic acid, 0.01; inositol, 0.15; riboflavine, 0.53; thiamine, 2.2; DL-thioctic acid,

0.001; menadione, 2.0; vitamin B<sub>12</sub>, 2.0; adenine-SO<sub>4</sub>, 19; cytosine, 15; guanine-HCl, 4; uracil, 15; xanthine, 15; and thymine, 4. A 1-ml amount of this solution was added to a culture.

The amino acid mixture was that used by Quinn et al. (24).

A 1-ml amount of the following solution of volatile fatty acids or their sodium salts (grams per 100 ml) was added to 100 ml of medium: Na acetate, 21.4; Na propionate, 7.7; Na butyrate, 6.1; *iso*-butyric acid, 0.3; *n*-valeric acid, 1.1; *iso*-valeric acid, 0.1; and  $\alpha$ -methyl-*n*-butyric acid, 0.1.

**Nitrogen analysis.** Analyses of total nitrogen were carried out on the rumen fluid used in the media and on the protozoal extract by the method of Johnson (18). Ammonia in the rumen liquor was also determined.

Analyses of urea and ammonia were made on cultures of holotrichs to determine the nature of the nitrogenous end products. In these experiments, washed mixed holotrichs, both dasytrichs and isotrichs freshly removed from the rumen, were incubated in flasks containing 20 ml of the complete medium. Each experiment was carried out with two flasks which were placed in ice at the beginning of the experiment and two flasks containing potassium penicillin G and chloramphenicol (each 50  $\mu$ g/ml) to control bacterial activity, which were incubated at 39 C for 2 hr. Each flask contained about 2 million organisms.

Ammonia was determined by the method of Conway (7). Urea was measured by the Conway (7) method after treatment with urease.

**Gas analysis.** Gases in the culture tubes were analyzed by use of Perkin-Elmer Vapor Fractometer model 154B, with a silica gel column and thermal conductivity detector.

## RESULTS

*Dasytricha ruminantium* was cultured for periods up to 60 days, when cultures were terminated because of the labor involved in carrying them longer. There was no apparent reason why they could not have been continued much longer if the effort had been made. *Isotricha* did not thrive, although it survived as long as 50 days under the conditions used. The following results apply only to *Dasytricha*.

Routinely, the cultures of *Dasytricha* were not divided regularly, but at least 10% of the cells were lost during transfer each day. Counts showed that in these cultures the maximal population reached was 1,300 per milliliter or a total of 13,000 ciliates in the tube culture. This was from an initial inoculum of 200 cells. Such a culture could be divided every 3 days. In one culture, the number of ciliates doubled every day for 5 days.

In another experiment, 30 cells from a large population already grown in culture for 24 days were inoculated into a tube of fresh medium, and a special effort was made to collect all the cells

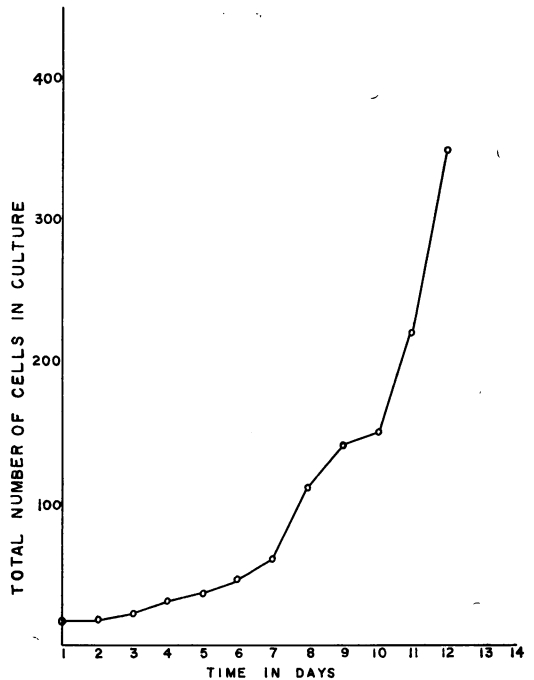


FIG. 1. Growth of a small population of *Dasytricha ruminantium*.

for each transfer. The numbers were counted, with the results shown in Fig. 1. The average rate of increase in this culture was consistent with a division rate of slightly more than once per 48 hr, disregarding lost cells.

The numbers of protozoa during routine transfer after 40 days of culture are shown in Fig. 2. The removal of ciliates for counting caused a 10% drop in the population each day. The growth rate in this culture was much less than that shown in Fig. 1, presumably because nutrients were limiting at this greater concentration of protozoa.

The longevity of cultures receiving various substitutes for rumen fluid and protozoal extract is given in Table 1. Inspection shows that rumen fluid markedly prolonged the life of the cultures as compared with inorganic salts alone. Bovine serum or protozoal extract was essential for continued cultivation. Addition of vitamin B<sub>12</sub> and menadione prolonged the life of the cultures in the absence of serum or protozoal extract.

All of the ciliate cultures contained considerable numbers of bacteria of three morphological types: curved rods, 1.5 by 2.5  $\mu$ , gram-negative; cocci in chains, 1.5 to 2.0  $\mu$  in diameter, gram-variable; cocci, single and in pairs, 0.3 to 0.5  $\mu$ , gram-variable. *Streptococcus bovis* was also present, but was usually removed by treatment with penicillin. Bacterial growth never caused more

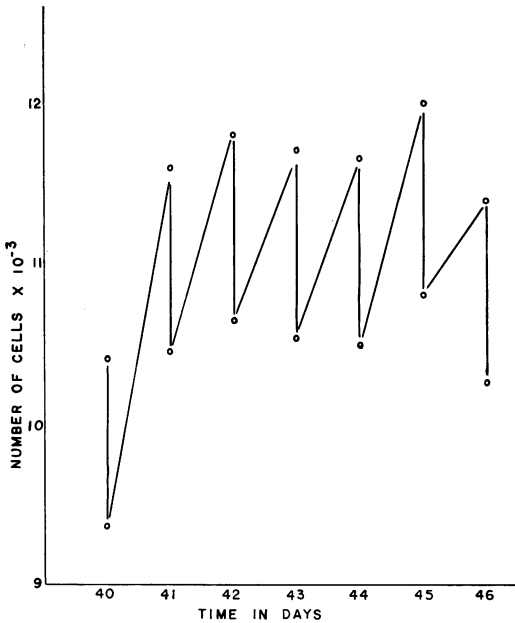


FIG. 2. Growth of a 40-day culture of *Dasytricha ruminantium*. Each day 10% of the cells were removed for counting.

than slight turbidity in the cultures, presumably because sucrose and protozoal extract were available only for 2 to 4 hr each day.

Attempts to prepare rumen ciliates free from associated external bacteria were made by allowing the ciliates to settle in a glass column filled with sterile reduced medium. The column was 125 cm long and had an inside diameter of 7 mm, and was divided into five compartments by glass stopcocks. These divisions prevented mixing of the liquid throughout the tube and allowed retention of the ciliates at will in each compartment. The lower two sections contained potassium penicillin G (75 µg/ml), chloramphenicol (50 µg/ml), and occasionally *p*-fluorophenylalanine (0.005 M). After passage down the column, the ciliates were transferred aseptically to tubes of sterile medium. In subsequent manipulations, care was taken to prevent entry of bacteria.

The washing of the protozoa and the treatment with the antibiotics did not completely remove associated bacteria. On one occasion, only a gram-negative curved rod grew when the culture was inoculated into rumen fluid-glucose-agar. The ciliates in this washed culture survived only 7 days subsequent to the washing process, and did not show division during this period. Other cultures of washed protozoa also survived for only a short period, but, if reinoculated with the mixed bacteria of successful cultures, they sur-

TABLE 1. Survival of *Dasytricha ruminantium* in various media<sup>a</sup>

Additives tested	Survival
	<i>days</i>
No addition, old cells.....	3
No addition, fresh cells.....	6
Rumen fluid, autoclaved.....	15
Rumen fluid, filtered.....	16
Rumen fluid, dialyzed.....	4
Rumen fluid, dialyzed + P. E. <sup>b</sup> + amino acids.....	3
Yeast extract + Casamino Acids (each 0.02%).....	7
Volatile fatty acids + P. E.....	4
RFA <sup>c</sup> + P. E.....	Indefinite
RFA + bovine serum.....	Indefinite
RFA + hay extract.....	15
RFA + alfalfa extract.....	15
RFA + grass extract.....	16
RFA + lyophilized alfalfa.....	13
RFA + PARF <sup>d</sup> (10%).....	14
RFA + <i>Bacteroides succinogenes</i> .....	16
RFA + <i>Selenomonas ruminantium</i> ...	6
RFA + <i>Ruminococcus albus</i> .....	16
RFA + washed rumen bacteria.....	16
RFA + extract of rumen bacteria...	16
RFA + vitamin B <sub>12</sub> + menadione (each 2 µg/ml).....	37
RFA + vitamins.....	37

<sup>a</sup> All cultures were in the inorganic salt solution and received the daily feeding of sucrose plus the additive shown.

<sup>b</sup> Protozoal extract.

<sup>c</sup> Autoclaved rumen fluid.

<sup>d</sup> Autoclaved whole rumen fluid (5).

vived and grew as before washing. Analysis of the gas above the liquid in a number of successful cultures disclosed that they always contained methane. Unsuccessful cultures did not. Growth and division correlated positively with methane production. In cultures with washed protozoa, methane production was negligible. If the culture was inoculated with pure cultures of several non-methanogenic bacteria, methane production returned to normal and the ciliates then flourished. Evidently, the methane bacteria were not removed by washing but were not detected by the rumen fluid-glucose-agar used to test the efficiency of bacterial removal.

All attempts to establish clone cultures of *Dasytricha* were unsuccessful. The smallest number of ciliates which led to a successful culture was 16.

The results of urea and ammonia analyses on cultures containing both dasytrichs and isotrichs are given in Table 2. The results suggest that urea is a normal waste product. The ammonia in the

TABLE 2. Urea and ammonia produced by cultures of mixed rumen holotrichs

Expt	Urea*	Ammonia*
1	3.7	0.9
2	1.2	0

\* Expressed as micromoles per hour.

first experiment could be due to urease activity by residual bacteria in the culture.

#### DISCUSSION

Inability to control storage of polysaccharide may be one of the reasons why previous attempts to culture the holotrichs have failed. Division seems to be impaired in cells distended with storage material. A further difficulty with continuous provision of even low concentrations of sugar is that it favors growth of saccharoclastic bacteria which become so numerous that they consume most of the sugar provided. Feeding sucrose once daily provides a relatively high concentration for a short period, and allows the protozoa to accumulate sufficient storage starch, but prevents bursting. It limits bacterial growth to the period of feeding, and most of the bacteria are eliminated when the protozoa are picked from the substrate-containing medium.

The system of feeding routinely employed (5 mg of sucrose for 2 to 4 hr daily) was not optimal. Cultures which were fed 2.5 mg of sucrose twice daily grew faster. The total population in the cultures was probably limited by both the amount of sucrose and the length of the feeding period. The maximal population of 1,300 ciliates per milliliter is low compared with cultures of some entodiniomorph ciliates (2, 5) and is low compared with the concentrations found in the rumen.

The ciliates had an obligate requirement for rumen fluid and protozoal extract or bovine serum (Table 1). Dialysis of either of these materials diminished growth, so both must provide essential dialyzable factors.

The optimal concentration of rumen fluid (30%, v/v) seems high if provision of growth factors were its only function. Rumen fluid from a hay-fed cow was inadequate for the growth of a culture of sheep ciliates, and rumen fluid from a cow on a 50% grain diet was toxic for the holotrichs from a hay-fed animal. Similar effects of different batches of rumen fluid on the survival of holotrichs in vitro have been shown by Purser and Tompkin (22), who suggested that the effects were indirect, in that the bacterial population was first affected.

Rumen liquor is rich in vitamins and growth factors, and it is hard to see why protozoal extract or serum was required. There was a positive response to a mixture of vitamin B<sub>12</sub> and menadione. One or both of these compounds may not be available to the ciliates in the rumen or may be destroyed during preparation of the rumen fluid. The concentration of vitamin B<sub>12</sub> used here was far in excess of that normally used in microbiological studies and of that in the rumen. In the cultures where menadione and B<sub>12</sub> replaced protozoal extract, the ciliates elongated but did not divide. This resulted in a population of cells two to four times the normal length.

Bacterial ingestion has been demonstrated in *Isotricha* (8) and *Dasytricha* (10), but bacteria have not been shown to be necessary for growth. Bacteria were present in all the present cultures and may have been consumed, but addition of pure cultures or a washed suspension of mixed rumen bacteria did not stimulate growth in the presence or absence of protozoal extract.

Death of cultures after washing could have occurred because bacteria required as food were no longer present. However, failure of added rumen bacteria to promote longevity of the cultures weakens the interpretation that required food bacteria had been eliminated. Some bacteria, including methanogenic cells, remained and could have served as food for at least a few protozoa, provided these particular species were suitable as food.

The bacteria could have played a role in conditioning the medium. Rumen contents have a redox potential at least as low as -350 mv. Cultures of rumen methane bacteria do not grow at potentials above -350 mv (26), and since methanogenesis ceased in washed cultures it is possible that the potential was too high. The optimal pH for *Dasytricha* and the methane bacteria might be of the same order, and the failure to grow after removal of the bacterial contaminants may have been solely the result of a relatively high potential.

The rumen fluid and protozoal extract added daily to each culture contained about 0.5 mg of soluble nitrogen in compounds other than ammonia. On the assumption that half of these compounds were amino acids, the protozoal extract could have supplied the necessary noncarbohydrate nutrients for the relatively low ciliate population.

Neither *Isotricha* nor *Dasytricha* appear to use or excrete ammonia. The ammonia in experiment 1 of Table 2 probably arose from urea via a bacterial urease. Urea is probably the main nitrogenous excretion product of both genera, though

uric acid, which is produced by some protozoa (25), was not determined in the present experiments.

## ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant A1-01266 from the National Institute of Allergy and Infectious Diseases.

## LITERATURE CITED

1. ABOU AKKADA, A. R., AND K. EL SHAZLY. 1964. Effect of absence of ciliate protozoa from the rumen on microbial activity and growth of lambs. *Appl. Microbiol.* **12**:384-390.
2. CLARKE, R. T. J. 1963. The cultivation of some rumen oligotrich protozoa. *J. Gen. Microbiol.* **33**:401-408.
3. CLARKE, R. T. J. 1965. Role of the rumen ciliates in bloat in cattle. *Nature* **205**:95-96.
4. CLARKE, R. T. J. 1965. Diurnal variation in the numbers of rumen ciliate protozoa in cattle. *New Zealand J. Agr. Res.* **8**:1-9.
5. COLEMAN, G. S. 1960. The cultivation of sheep rumen oligotrich protozoa *in vitro*. *J. Gen. Microbiol.* **22**:555-563.
6. COLEMAN, G. S. 1964. The metabolism of *Escherichia coli* and other bacteria by *Entodinium caudatum*. *J. Gen. Microbiol.* **37**:209-223.
7. CONWAY, E. J. 1957. *Microdiffusion analysis and volumetric error*, 4th ed. Crosby Lockwood and Son Ltd., London.
8. GUTIERREZ, J. 1958. Observations on bacterial feeding by the rumen ciliate *Isotricha prostoma*. *J. Protozool.* **5**:122-126.
9. GUTIERREZ, J., AND R. E. DAVIS. 1962. Culture and metabolism of the rumen ciliate *Epidinium ecaudatum* Crawley. *Appl. Microbiol.* **10**:305-308.
10. GUTIERREZ, J., AND R. E. HUNGATE. 1957. Inter-relationship between certain bacteria and the rumen ciliate *Dasytricha ruminantium*. *Science* **126**:511.
11. HOWARD, B. H. 1963. Metabolism of carbohydrate by rumen protozoa. *Biochem. J.* **89**:89.
12. HOWARD, B. H. 1963. Hydrolysis of naturally occurring galactosides by some rumen protozoa. *Biochem. J.* **89**:90-91.
13. HOWARD, B. H. 1959. The biochemistry of rumen protozoa. 1. Carbohydrate fermentation by *Dasytricha* and *Isotricha*. *Biochem. J.* **71**:671-675.
14. HOWARD, B. H. 1959. The biochemistry of rumen protozoa. 2. Some carbohydrases in cell-free extracts of *Dasytricha* and *Isotricha*. *Biochem. J.* **71**:675-680.
15. HUNGATE, R. E. 1942. The culture of *Eudiplodinium neglectum*, with experiments on the digestion of cellulose. *Biol. Bull.* **83**:303-319.
16. HUNGATE, R. E. 1943. Further experiments on cellulose digestion by the protozoa in the rumen of cattle. *Biol. Bull.* **84**:157-163.
17. HUNGATE, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. *Bacteriol. Rev.* **14**:1-49.
18. JOHNSON, M. J. 1941. Isolation and properties of a yeast polypeptidase. *J. Biol. Chem.* **137**:575-586.
19. MAH, R. A. 1964. Factors influencing the *in vitro* culture of the rumen ciliate, *Ophryoscolex purkynei* Stein. *J. Protozool.* **11**:546-552.
20. OXFORD, A. E. 1955. The rumen ciliate protozoa: their chemical composition, metabolism, requirements for maintenance and culture, and physiological significance for the host. *Exptl. Parasitol.* **4**:569-605.
21. OXFORD, A. E. 1958. Bloat in cattle 9. Some observations on the culture of the cattle rumen ciliate *Epidinium ecaudatum* Crawley occurring in quantity in cows fed on red clover (*Trifolium pratense* L.). *New Zealand J. Agr. Res.* **1**:809-824.
22. PURSER, D. B., AND R. B. TOMPKIN. 1965. The influence of rumen fluid source upon viable bacteria counts and the cultivation of oligotrich and holotrich rumen protozoa. *Life Sci.* **4**:1493-1501.
23. PURSER, D. B., AND H. H. WEISER. 1963. Influence of time of addition of antibiotic on the life of rumen holotrich protozoa *in vitro*. *Nature* **200**:290.
24. QUINN, L. Y., W. BURROUGHS, AND W. C. CHRISTIANSEN. 1962. Continuous culture of ruminal microorganisms in chemically defined medium. 2. Culture medium studies. *Appl. Microbiol.* **10**:583-592.
25. SEAMAN, G. R. 1955. Metabolism of free-living ciliates, p. 91-158. *In* S. H. Hutner and A. Lwoff, [ed.], *Biochemistry and physiology of the protozoa*, vol. 2. Academic Press, Inc., New York.
26. SMITH, P. H., AND R. E. HUNGATE. 1958. Isolation and characterization of *Methanobacterium ruminantium* n. sp. *J. Bacteriol.* **75**:713-718.
27. SUGDEN, B., AND A. E. OXFORD. 1952. Some cultural studies with holotrich ciliate protozoa of the sheep's rumen. *J. Gen. Microbiol.* **7**:145-153.