Continuous Culture of Ruminal Microorganisms in Chemically Defined Medium

II. Culture Medium Studies[1]

LOYD Y. QUINN, WISE BURROUGHS, AND WILLIAM C. CHRISTIANSEN

Departments of Bacteriology and Animal Science, Iowa State University, and Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa

Received for publication June 15, 1962

ABSTRACT

QUINN, LOYD Y. (Iowa State University, Ames), WISE BURROUGHS, AND WILLIAM C. CHRISTIANSEN. Continuous culture of ruminal microorganisms in chemically defined medium. II. Culture medium studies. Appl. Microbiol. **10**:583–592. 1962.—Ruminal ciliates have been grown in continuous culture in chemically defined media and in the absence of viable bacteria. Oligotrichic ruminal ciliates seem to require insoluble carbohydrates for growth; the holotrichic ciliates require soluble carbohydrates, but at low concentrations. Both groups of ciliates utilize amino acids as their principal nitrogen source when these are supplied in micromolar concentrations; at millimolar concentrations, amino acids are toxic, possibly from excessive ammonia formation arising from ciliate deaminase activity. Holotrichic ruminal ciliates are destroyed by overdeposition of amylopectin when glucose is present above 0.1% concentration in the medium. Ecological requirements of ruminal ciliates are also described.

Becker and Talbott (1927) reported that only 14 species of ruminal ciliates could be found consistently in the cow and noted wide variations in the incidence of 23 observed species. Of 26 cows periodically examined, 2 had a single species of ciliate in the rumen; most of the cows had 5 to 8 different species of ciliates present, while one cow supported 16 species (exclusive of *Butschlia parva*, which Becker and Talbott never found in cattle). Akkada, Hobson, and Howard (1959) reported that only *Entodinium* spp. survived in the rumen of sheep fed an enriched ration. More recently, Christiansen (*unpublished data*) found that the physical form of the ration of sheep greatly influenced the numbers and types of ciliates found in the rumen. He found that ciliates disappeared from the rumen of sheep fed a pelleted form of finely ground dry ration but that ruminal ciliates thrived when sheep were fed the same pelleted ration prepared initially as a coarse ground feed. Sugden (1953) and Eadie (*private communication*) presented evidence which indicates that a natural antagonism between various species of ruminal ciliates controls the population pattern. Under bloating conditions, many of the larger ciliates disappear, and *Epidinium* (*Diplodinium*) *ecaudatum* increases in the bovine rumen, according to Oxford (1959).

Only in recent years have the ruminal ciliates been cultured outside of their hosts, and even then, defined basal media required supplementation with crude forms of cellulosic materials, such as grass and hay, as well as ground cereal grains (Hungate, 1942). The different types of ruminal ciliates seem to show preferential feeding patterns: *Ophyroscolex caudatus* and *Diplodinium* (*Eudiplodinium*) *magii* have been observed to ingest cellulose, pectin, plant chloroplasts, smaller protozoa, bacteria, and starch grains (Williams, Gutierrez, and Doetsch, 1960); the smaller *Diplodinium* spp. ingest bacteria, starch grains, and cellulose fibriles (Hungate, 1943; Sugden, 1953); *Entodinium* spp. ingest starch but not cellulose (Akkada and Howard, 1960; Annison and Lewis, 1959; Hungate, 1943); Gutierrez and Davis (1959) noted that entodina ingest *Streptococcus bovis* and expressed the opinion that this bacterium is required for the growth of these smaller ciliates.

Holotrichic ruminal ciliates preferentially utilize soluble carbohydrates (Gutierrez, 1955; Heald and Oxford, 1953; Howard, 1959*a*, *b*; Hungate, 1942, 1943; Masson and Oxford, 1951; Oxford, 1955*a*, *b*, 1959; Sugden, 1953; Sugden and Oxford, 1952; Williams et al., 1960), although smaller starch grains may be ingested. Gutierrez (1958) found that *Isotricha prostoma* feeds on small gram-negative rod-shaped bacteria; *Dasytricha ruminantium* ingests cocci (Gutierrez and Hungate, 1957).

The nitrogen requirements of ruminal ciliates have been studied much less than have their carbohydrate requirements. Williams et al. (1961) found that *Ophyroscolex caudatus* utilized plant proteins or free amino acids as nitrogen sources, and that ammonia was formed as an end product of nitrogen metabolism. Akkada and Howard (1962) reported that the *Entodinium* spp. (in normal mixture of species) rapidly hydrolyze casein to peptides, free amino acids, and ammonia; amino acids are not, however, deaminated. They also reported that urea is not utilized by entodina.

[1] Journal Paper No. J-4389 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1357. Supported in part by funds provided by Regional Project NC-25.

TABLE 1. *Composition of culture media*[a]

Component	Quantity (in grams, unless otherwise specified)				
	Medium 1S	Medium 1L	Medium 2	Medium 3	Medium 4
α-Cellulose[b, c]	17.00				
Cellobiose				0.170	0.200
Fructose		0.500	0.500	0.500	0.300
Glucose		0.250	0.250	0.250	0.150
Glycerol				0.190	
Maltose				0.200	0.240
Starch[b, d]	20.00		20.00		
Sucrose		0.750	0.750	0.750	0.480
Xylose				0.120	0.140
Pectin[b, e]	2.20		2.20		
Xylan[b, f]	12.00		12.00		
Sodium acetate[b]	1.04		1.04	1.04	2.14
Sodium butyrate[b]	0.41		0.41	0.410	0.680
Sodium linoleate[b]				0.700	
Sodium oleate[b]	0.017		0.017	0.780	
Sodium palmitate[b]	0.017		0.017	0.130	
Sodium propionate[b]					0.610
Sodium stearate[b]	0.017		0.017	0.059	
Sodium valerate[b]	0.040		0.040		0.310
$(NH_4)_2SO_4$				1.25	
Phytone[g]	10.00				
L-Alanine			1.6 (DL)	0.0050	0.0013
L-Arginine			0.290	0.0070	0.0019
L-Aspartic acid			0.880	0.020 (DL)	0.0029
L-Cysteine·HCl			0.026	0.00070	.140
L-Cystine			0.080	0.0020	0.00053
L-Glutamic acid			0.680	0.017	0.0045
Glycine			0.200	0.0050	0.0013
L-Histidine·HCl			0.150	0.0037	0.00097
L-Isoleucine			0.240	0.012 (DL)	0.0016
L-Leucine			0.400	0.010	0.0026
L-Lysine·HCl			0.420	0.011	0.0029
L-Methionine			0.320 (DL)	0.004 (DL)	0.00053
L-Phenylalanine			0.200	0.010 (DL)	0.0013
L-Proline			0.160	0.004	0.0011
L-Serine			0.240	0.012 (DL)	0.0016
L-Threonine			0.240	0.012 (DL)	0.0016
L-Tryptophan			0.320 (DL)	0.004 (DL)	0.00053
L-Tyrosine			0.160	0.0040	0.0011
L-Valine			0.800 (DL)	0.010	0.0013
Adenine HCl[b]		0.040	0.040	0.020	0.019
Cytosine[b]		0.040	0.040		0.015
Guanine[b]		0.052	0.052	0.020	0.0038
Uracil[b]		0.040	0.040	0.020	0.015
Xanthine[b]		0.040	0.040	0.020	0.015
Ascorbic acid					0.051
Biotin		0.000016	0.000016	0.0000085	0.0000060
α-Tocopherol acetate		0.013	0.013	0.0066	
Calcium pantothenate		0.0028	0.0028	0.0014	0.0011
Choline chloride		0.190	0.190	0.095	0.095
Chlorophyllin A[h]		0.100	0.100	0.025	
Folic acid		0.000034	0.000034	0.000017	0.000013
Inositol		0.0040	0.0040	0.0020	0.00015
Menadione		0.0020	0.0020	0.0010	0.00075
Niacin		0.0045	0.0045	0.0027	0.0020
p-Aminobenzoic acid		0.0010	0.0010	0.00050	0.00037
Pyridoxine·HCl		0.00044	0.00044	0.00022	0.00016
Riboflavine		0.0014	0.0014	0.00070	0.00053
Rutin		0.010	0.010	0.005	
Sodium ascorbate	0.030		0.030	0.030	
NaKMg chlorophyllin A[i]					0.0075
Thiamine		0.00058	0.00058	0.00029	0.0022

TABLE 1.—*Continued*

Component	Quality (in grams, unless otherwise specified)				
	Medium 1S	Medium 1L	Medium 2	Medium 3	Medium 4
DL-Thioctic acid		0.0000010	0.0000010	0.0000010	0.00000075
Vitamin A		0.00010	0.00010	0.000050	0.000034
Vitamin B_{12}		0.00000060	0.00000060	0.00000030	0.000000023
Vitamin D_2		0.0056	0.0056	0.0000017	0.000013
$(NH_4)_6Mo_7O_{24}\cdot H_2O$		0.0060	0.0060	0.0030	0.00045
$CaCl_2$				0.840	0.095
$CoCl_2\cdot 6H_2O$		0.0008	0.0008	0.00004	
$CuSO_4\cdot 5H_2O$		0.0070	0.0070	0.0035	
$FeSO_4\cdot 7H_2O$		0.086	0.086	0.043	
I_2		0.00030	0.00030	0.000015	0.0000023
$MgSO_4\cdot 7H_2O$[b]				0.87	0.095
$MnSO_4\cdot 4H_2O$[b]		0.018	0.018	0.69	
KCl[b]					1.30
K_2HPO_4[b]				3.89	0.047
KH_2PO_4[b]					0.012
$NaHCO_3$					4.44
NaCl[b]	3.00		3.00	5.00	1.19
$Na_2HPO_4\cdot 7H_2O$[b]				1.21	
$ZnSO_4\cdot 7H_2O$				0.0059	
$Na_2EDTACo$[i]					0.000015
$Na_2EDTACu$					0.0014
$Na_2EDTAFe$					0.012
$Na_2EDTAZn$					0.0000020
α-Thioglycerol[k]					0.50
Tween 80	1.50		1.50		0.20
Deionized water (ml)	500	500	1,000	1,000	1,000

[a] All vitamins, amino acids, nucleides, carbohydrates, and fatty acids were from Nutritional Biochemical Corp., Cleveland, Ohio, unless otherwise specified. Inorganic chemicals were of reagent grade, from various sources. These media were alkaline in reaction until saturated with carbon dioxide gas; the pH was then 6.8 to 7.0.

[b] Sterilized by autoclaving. All other components were sterilized by filtration.

[c] Solka-floc BW 40. Brown Co., Boston, Mass.

[d] Potato Starch Powder, Lot No. 6348, General Chemical Division, Allied Chemical and Dye Corp., New York, N.Y.

[e] Pectin, Slow-Set 230. Atlantic Gelatin Division, General Foods Corp., Hollywood, Calif.

[f] Xylan (P) Lot No. B-2016 Mann Res. Labs., Inc., New York, N.Y.

[g] Phytone, Lot No. 903604 Baltimore Biological Laboratory, Baltimore, Md.

[h] Chlorophyll, water-soluble. Aceto Chemical Co., Inc., Flushing, N.Y.

[i] Na K Chlorophyllin, Lot C 1030. Mann Research Laboratories, Inc.

[j] EDTA (Sequestrene) chelated metals. Geigy Industrial Chemicals, Division of Geigy Chemical Corp., Ardsley, N.Y.

[k] α-Thioglycerol 95%. California Corporation for Biochemical Research, Los Angeles, Calif.

The experiments reported in this paper were conducted in an attempt to cultivate the normal mixed population of ruminal ciliates in chemically defined media in the absence of proliferating bacteria. The continuous-culture apparatus employed in this research is described in the accompanying paper (Quinn, 1962).

Materials and Methods

Source of ruminal ciliates. The ciliates used for this work were collected with a suction strainer (Raun and Burroughs, 1962) from the rumen of a fistulated steer maintained on a standard dry ration of the following composition: alfalfa hay, 46 lb; corn cobs, 10 lb; corn (yellow), 30 lb; soybean meal, 6 lb; beet molasses, 7 lb; dicalcium phosphate, 0.5 lb; sodium chloride, 0.5 lb; vitamin A, 30,000 IU; vitamin D, 3,700 IU. The ratio of feed-to-water-intake for the animal was estimated to be 1:5, on a weight basis.

Culture media. In the formulation of chemically defined media, based on the composition of ruminal contents, the following source references were consulted: for chemical analyses of the plant materials in the ration, Committee on Feed Composition (1956, 1958), Balch (1958), Carlson, Forbes, and Hansen (1957), Hansen, Forbes, and Carlson (1958), and Smith (1957); for composition of rumen ingesta, Annison (1956), Annison, Hill, and Lewis (1957), Annison and Lewis (1959), Duncan et al. (1953), Garton (1951), Hubbert, Cheng, and Burroughs (1958*a*, *b*), Kon and Porter (1953, 1954), Phillipson (1952), and Salsbury, Elliott, and Luecke (1958). The composition of media used in this study is given in Table 1.

Medium 1 (obtained in the culture flask when medium 1L and medium 1S were delivered in equal volumes by feeding pumps) is based on the composition of the above-listed all-plant ration, with the components closely approximating the natural form found in plant materials.

Medium 2 differs from medium 1 only in the substitution of equivalent amounts of the constituent amino acids for the phytone of medium 1. Since McDougall's (1958) synthetic saliva was used as the alkaline titrant for cultures grown in media 1 and 2, the mineral salts of the titrant were omitted from the formulation of these media.

Medium 3 was designed to supply, both qualitatively and quantitatively, the soluble nutrilites of ruminal intesta, plus the soluble nutrilites of the plant ration, as reported by numerous workers cited above.

Medium 4 was developed by determining the optimal concentrations of the various groups of nutrilites of medium 3, in terms of ciliate viability and cellular activities. In many cases, the pooled amino acids, pooled carbohydrates, pooled vitamins, and pooled trace minerals were at optimal levels at lower concentration than those found in medium 3. Some toxic components were omitted in the formulation of medium 4.

Titrants. Acid fermentation products were neutralized with McDougall's synthetic saliva in cultures grown in media 1 and 2; with media 3 and 4, filter-sterilized (47-mm HA Millipore Filter, Millipore Filter Corp., Bedford, Mass.) 0.1 N sodium bicarbonate solution was supplied by the automatic titrator.

Gas atmospheres. The complex mixture of gases found in the rumen has been analyzed by various workers (Cole et al., 1945; Erwin, Marco, and Emery, 1961; Flatt and Colovos, 1960; Kingwell et al., 1959; Kleiber, Cole, and Mead, 1943; Pilgrim, 1948; Washburn and Brody, 1937). A synthetic ruminal gas was formulated from the averages of reported analyses of ruminal gases found during active ruminal digestion. The composition of this gas mixture is shown in Table 2.

TABLE 2. *Composition of synthetic ruminal gas*

Gas	Volume
	%
CO_2	65
CH_4	31.7
H_2	2.0
N_2	1.0
O_2	0.2
H_2S	0.17

This gas mixture was prepared and bottled commercially (Matheson Co., Joliet, Ill.) and was used with medium 1. CO_2 gas (The Pure Carbonic Co., Des Moines, Iowa) was used for aeration in conjunction with media 3 and 4.

Preparation of mixed normal bacterial and ciliate inoculum. Procedures described by Coleman (1960) and by Oxford (1955*a*) were used in the preparation of inoculum. The density of the viable ciliate population in the inoculum was checked with a Howard mold-counting cell. Bright contrast objectives (10 and 25 ×) and 10 × periplan eyepieces (Leitz Microscope Laborlux II) were used for phase-contrast visualization. Routinely, the average number of ciliates in 50 low-power fields was used per count; 5% carboxymethylcellulose (Type 70S Extra Low Viscosity Cellulose Gum, Hercules Powder Co., Wilmington, Del.) was used as the suspending medium to slow ciliate motility.

Bacteria-free ruminal ciliate inoculum. Modifications of the procedures of Coleman (1958) and Heald and Oxford (1953) were used in devising the following technique for freeing the ciliate inoculum of viable bacteria.

(i) Freshly collected ruminal ingesta was filtered through six layers of moist surgical gauze to remove the larger feed particles.

(ii) Filtered ruminal ingesta was aerated for a few minutes with CO_2 gas, then held at 37 C in a separatory funnel for 30 min to permit levitation of fine feed particles and the settling of the relatively dense ciliates. The sedimented ciliates formed a distinct layer, which was carefully drawn off from the supernatant ruminal liquor containing numerous bacteria but only a few entodina cells.

(iii) The ciliate layer was packed by centrifugation (Automatic Superspeed Centrifuge, Type SS-3, Ivan Sorvall, Inc., Norwalk, Conn.) at 30 × *g* for 3 min, and the supernatant fluid, which contained plant residues and bacteria, was decanted and discarded. The packed ciliates were gently resuspended in wash solution equivalent to the original volume of ingesta sample. Although various balanced salt solutions were tested, less trauma was produced with complete medium (soluble components only, in the case of medium 1) as the wash solution than with nonnutritive solutions.

(iv) The centrifugal washing operation was repeated until the supernatant solution was almost clear and colorless; usually, three to five washings were sufficient for this purpose.

(v) Washed ciliates were resuspended in culture medium supplemented with 1,000 units/ml each of streptomycin and penicillin, and the ciliates were then incubated at 37 C to eliminate viable bacteria. The presence of living bacteria in the ciliate suspension was checked by inoculating NIH Thioglycollate Broth (Difco) with one drop of the ciliate suspension per tube. Microscopic examinations of wet mounts and gram-stained smears also were made, to check the condition of the ciliates and to check the numbers and types of bacteria present.

RESULTS

Effect of sampling technique on the population pattern of ruminal ciliates in inocula. When collected in a manner designed to reflect the over-all distribution pattern of ciliates in the bovine rumen (Christiansen, Quinn, and Burroughs, J. Animal Sci., *in press*), the ciliate population density was found to vary with time following feed intake, with the maximal population density attained between 2 and 4 hr postprandial. In the fasting ruminant, a high concentration of relatively bacteria-free ciliates may be found in the pool of fluid held in the ruminal cistern.

The larger ciliates tend to concentrate in the ventral section of the filled rumen, while the entodina are more uniformly distributed throughout the mass of ruminal ingesta. The distribution of ciliates in the bovine rumen is not as uniform as has been reported in the ovine rumen (Boyne, Eadie, and Raitt, 1957).

Even with standardization of sampling time and technique, and animal management, variations were encountered in the population pattern among samples of ruminal ingesta collected over a period of several months. With the larger ciliates, one or more species would frequently occur in greatly reduced numbers, or be absent altogether from a sample.

Enumeration of ruminal microorganisms. Difficulties are encountered in direct counting of ruminal microorganisms, because: (i) ciliates, and some of the ruminal bacteria, tend to colonize when permitted to migrate in a thick wet-mount preparation; (ii) ruminal ciliates appear to be gregarious, so that the majority of ciliates of all species present rapidly form a swarm in one or more small areas of the sample-counting chamber; (iii) the density of the larger ciliates is sufficient to carry them out of suspension rapidly so that homogeneous samples are difficult to obtain; and (iv) *Dasytricha ruminantium* attaches rather firmly to the walls of glass containers (Gutierrez and Hungate, 1957).

Total numbers of ruminal ciliates ranged between 70,000 and 100,000 cells/ml of fresh ingesta. Differential counts (average values) for commonly encountered ciliate genera are listed in Table 3.

TABLE 3. *Numbers of viable ruminal ciliates per ml of gauze-strained fresh ingesta*

Genus	Count
Isotricha	6,000
Dasytricha	1,000
Entodinium	60,000
Diplodinium	6,000
Ophryoscolex	3,000

In samples of ruminal bacteria taken over a period of several months, various microscopically recognizable bacterial types ranged in numbers from almost nil to 10^{10} cells/ml of ruminal ingesta. Morphological types noted were: (i) a large encapsulated sarcina, resembling *Sarcina bakeri*; (ii) a long-chain, encapsulated streptococcus, resembling *Streptococcus bovis*; (iii) diplococci with sharply pointed ends, resembling *Peptostreptococcus* spp.; (iv) a selenomonad, resembling *Selenomonas ruminantium*; (v) a large, motile, cigar-shaped multicellular rod, resembling *Oscillospira guilliermondii*; (vi) a short, loosely coiled spirochete, resembling the *Borrelia* sp. of Bryant (1952); and (vii) vibrios, and various forms of small bacilli.

Effects of temperature on ruminal ciliates. Ciliates in ruminal ingesta are adversely affected by chilling. When cooled slowly from 38 to 25 C, the most sensitive forms, the diplodina, become first sluggish in motility and feeding, then contract their membranelles and become nonmotile; on warming to 37 C, normal activity is resumed if the temperature depression has been short in duration. At 25 C, all the *Diplodinium* spp. succumb within 24 hr. The entodina, holotrichs, *Ophryoscolex caudatus*, flagellates, and amoebae all remain active for 24 hr at 25 C, in ingesta. After 48 hr at 25 C, all *Ophryoscolex caudatus* cells usually have autolyzed, while the remainder of the protozoan forms (excepting the diplodina) are still viable, although in somewhat reduced numbers. After 72 hr under these conditions, only *Entodinium minium*, *Isotricha intestinalis*, and the ruminal flagellates are viable; only about 10% of the initial population of ciliates survives this long. At 96 hr, all the protozoa have either lysed or become inactive.

The ruminal ciliates are relatively sluggish in their movements at 30 C, and appear to show maximal motility, feeding activity, and reproductive rates between 37 and 38 C. At 40 C and higher, there is increasing evidence of traumatic effects of heat: ciliates become pleomorphic, develop numerous clear vacuoles, and cease dividing. Similar results were noted by Coleman (1960) with ciliates from the rumen of the sheep.

Oxidation-reduction potential effects. The observation of Hungate (1942) that ruminal ciliates are injured by aerobic conditions was verified and extended in our work. Redox potential measurements and microscopic examination of ruminal ciliates growing in medium 4 provided a number of observations. (i) *Ophryoscolex caudatus* and the *Diplodinium* spp. did not survive above a redox potential of -60 mv, but the isotrichs were not killed by exposure to this relatively aerobic potential for periods of several hours. (ii) *Isotricha* spp. were able to tolerate a potential of $+140$ mv for only a few minutes. (iii) *Entodina* spp. and *Dasytricha ruminantium* fall between *Isotricha* spp. and the *Ophryoscolex-Diplodinium* group in their degree of anaerobiosis. (iv) The optimal redox potential for the entire spectrum of ruminal ciliates seemed to fall between -200 and -260 mv, as measured by Pt electrode. Indigo disulfonate, with $E_m = -125$ mv, was completely reduced in healthy cultures of ruminal ciliates in medium 4. Ascorbic acid, with $E_{m7} = +058$ mv, was very toxic to "resting" ruminal ciliates in the absence of strong reducing agents; cysteine, with an E_h/pH 7/25 C $= -330$ mv (all redox potential values from Clark, 1960), was nontoxic at 0.25 meq/liter concentration. (v) Baldwin and Emery (1960) reported the E_h/pH 7/25 C $= -125$ to -191 mv for normal bovine ruminal ingesta which had a concentration of total reducing agents of 40 to 48 meq/liter. Broberg (1958) found similar redox potentials in the ovine rumen.

Osmotic pressure effects. The various ruminal ciliates differ markedly in their responses to changes in osmotic pressure of the culture medium. *Ophryoscolex caudatus* seems to be the most sensitive of the ruminal ciliates to a nonisotonic environment. Plasmoptysis was complete in

30 min in 0.60% NaCl solution, and plasmolysis occurred within 30 min in 1.4% NaCl solution. The *Isotricha* spp. are most tolerant of unphysiological salt concentrations, with a few individuals in the population surviving for more than 1 hr in 1.5% NaCl solution, and for several hours in 0.5% NaCl solution or other solutions that were osmotically equivalent. The *Diplodinium* spp., *Entodinium* spp., and *Dasytricha ruminantium* were intermediate to the first two groups in their responses to unphysiological salt concentrations. The most favorable osmotic pressure for these ciliates, collectively, seemed to be 260 meq of electrolytes per liter, or approximately 0.80% NaCl equivalent.

Effects of pH. Ruminal ciliates are unable to tolerate conditions below pH 5.5 or above 8.0 for protracted periods of time in chemically defined media. During very active fermentation in poorly buffered media, the pH may drop as low as 3.0 with no deleterious effects, provided the medium is brought to neutrality within a few hours. The optimal pH range for growth of the composite ciliate population seemed to lie between 6.8 and 7.0.

Effects of surface tension. The normal surface tension of ruminal ingesta is in the range of 45 to 59 dynes (Blake, Allen, and Jacobson, 1957), and our findings are that departures from this range lead first to death, then to dissolution of cells of ruminal ciliates. The surface tension of media 3 and 4, as determined by a Cenco-Du Nuoy tensiometer (Central Scientific Co., Chicago, Ill.), was 54 dynes at 37 C.

Oxford (1959) reported that *Epidinium* (*Diplodinium*) *ecaudatum* and *Dasytricha ruminantium* are more sensitive to detergents than are other ruminal ciliates. When the surface tension of medium 3 or 4 was lowered to unphysiological levels by addition of a few ppm of strong surfactants of either the anionic or cationic type, the ruminal ciliates were all quickly immobilized. Nonionic detergents, such as Tween 80, are much less toxic than the ionic types; however, at 0.02% concentration in saline solution and under anaerobic conditions, it caused a rapid decrease in numbers of viable entodina without markedly affecting the behavior of other ruminal ciliates. GE Antifoam 60 (Silicone Products Department, General Electric Co., Waterford, N.Y.) at 5 ppm concentration in medium 4 caused lysis of *Ophryoscolex caudatus* and all *Diplodinium* spp. but did not cause lysis or immobilization of the entodina or isotrichs.

Culture of mixed ruminal protozoa and bacteria. Medium 1 was used, with an initial ciliate population density of 20,000 cells per ml. Incubation temperature was held at 37 C; pH, at 6.8; stirring rate, at 120 rev/min; and the medium turnover time was set at 12 hr for the 200 ml of culture volume. Details of the control apparatus used are given in the first paper (Quinn, 1962).

The morphologically recognizable types of ruminal bacteria, as noted earlier, were all present for the entire 12-hr incubation period, but they gradually increased to abnormally high population densities. As the bacterial population density began to exceed that concentration of bacterial cells normally observed in ruminal ingesta, the larger ciliates became sluggish and tended to undergo autolysis.

Fermentative activity was very high on this medium so that, in 12 hr, 400 ml of McDougall's synthetic saliva (0.1 N base equivalent) were required to maintain a reaction of pH 7.0 in the culture. The salinity controller also required 400 ml of deionized water for control of salinity at 0.65% KCl equivalent.

With this high throughput of liquids, the turnover rate of ciliate culture volume was much greater than the average ciliate generation time. Even though the majority of the entodina cells were observed to be dividing, by the end of the 12-hr run, the total ciliate population had decreased to 10,000 cells/ml.

This experiment was repeated three times, with essentially the same results each time. It was obvious that medium 1 was too readily fermented by mixed populations of ruminal bacteria and protozoa for controlled study of protozoal activities in our culture apparatus. Also, the contributions of bacteria could not be distinguished from protozoal activity in the fermentation process. Therefore, a second series of experiments was conducted, using a ciliate inoculum that had been freed of viable bacteria.

Culture study of bacteria-free ciliates in medium 1. Ruminal ciliates were collected, as in the previous experiment, but were washed and freed of viable bacteria as described in Materials and Methods. Inoculation rate and other experimental conditions were the same as in the first experiment, except that the turnover rate for the culture volume was set at 24 instead of 12 hr.

During the first 4 hr of incubation, a decided lag period was noted, during which the ciliates were sluggish in their movements, and acid production was only 0.05 ml of 0.1 N acetic acid equivalent per ml of culture per hr. None of the species of ciliates (examined microscopically in wet mounts) was observed to be dividing during this lag period.

From the 4th to the 9th hr, the rate of acid production increased sharply to a maximum of 3.0 ml of 0.1 N acetic acid per ml of culture per hr. Between the 9th and 36th hr, there was a steady decline in the rate of production of acid, which paralleled the decreases in density of the ciliate population.

Between the 4th and 9th hr, the ciliates recovered from the lag that was first observed and became more active than the ciliates of freshly collected ruminal ingesta. Of the entodina, 10% were noted to be dividing during this period; this value for the rate of cell division for entodina compares closely with that observed in freshly collected ruminal ingesta. *Isotricha* spp. were filled with amylopectin granules and were near maximal size for the genus (130 by 90 μ). Species observed to be actively feeding included: *Isotricha intestinalis*, *I. prostoma*, *Dasytricha ruminantium*, *Entodinium bursa*, *E. caudatum*, *E. bicari-*

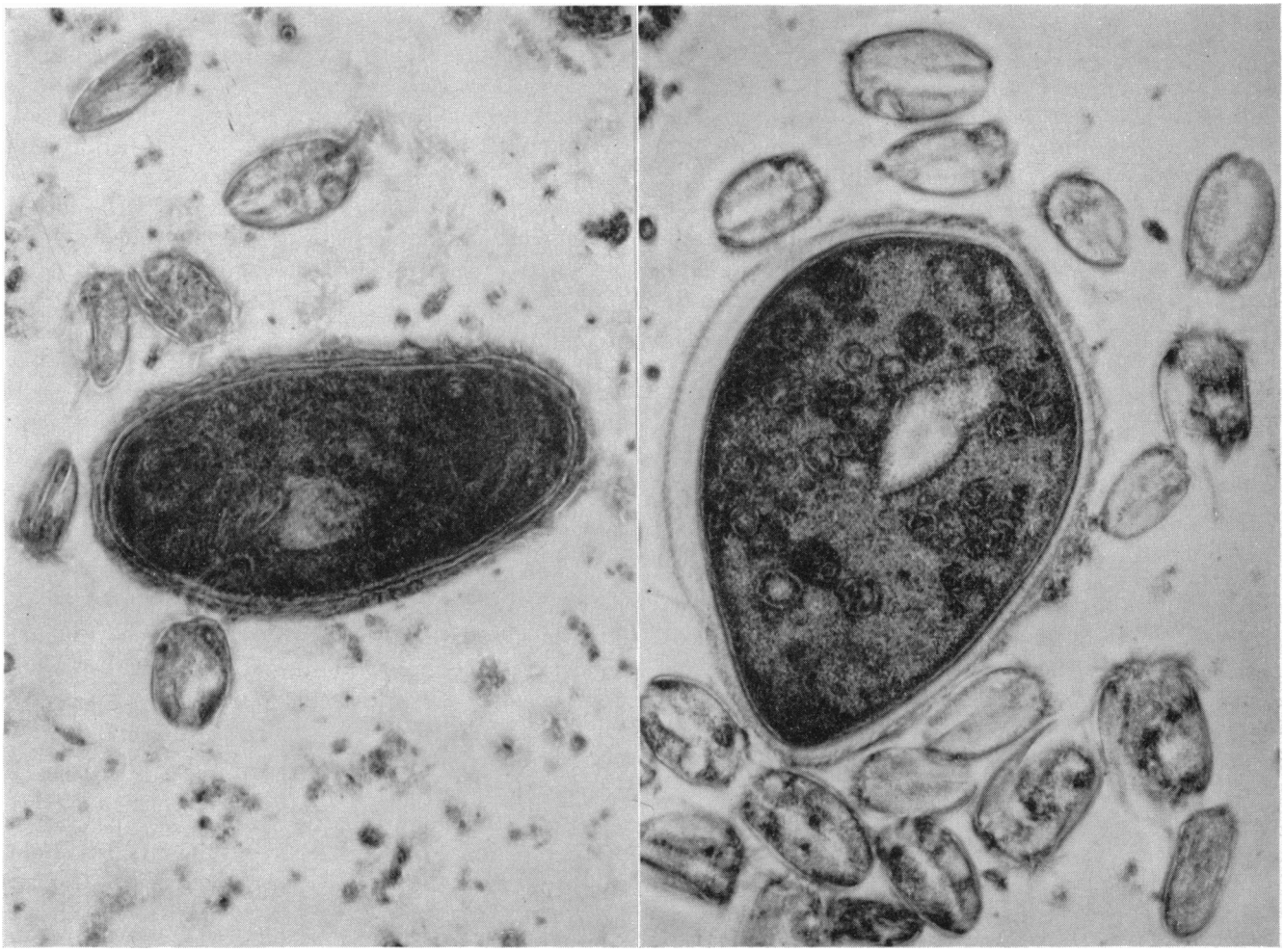

FIG. 1. (left) *Normal cell of Isotricha prostoma and entodina.*
FIG. 2. (right) *Bursting cell of Isotricha prostoma, with characteristic swarm of entodina.*

natum, *E. furca*, *Diplodinium dentatum*, *D. hegneri*, *D. clevelandi*, *D. denticulatum*, *D. medium*, and *Ophryoscolex caudatus*. The flagellates, *Eutrichomastix ruminatum* and *Callimastix frontalis*, also were present in high numbers.

Between the 9th and 10th hr, the *Isotricha* spp. reached nearly twice their normal size, from deposition of intracellular amylopectin granules. A normal cell of *I. intestinalis* is shown in Fig. 1, and a swollen cell of the same species is shown in Fig. 2. The isotrichs soon ruptured from this overdistention, so that, at the end of the 10th hr of incubation, no intact *Isotricha* spp. cells were found in the culture, apparently because of the accumulation of excessive concentrations of soluble carbohydrates. The amylopectin granules thus released were voraciously engulfed by entodina, but were not observed to be ingested by any of the other oligotrichs in the mixed ciliate population. Even before the isotrich cells burst, the entodina swarm around the injured cells, as shown in Fig. 2.

Between the 10th and 30th hr of incubation, *Diplodinium* spp. and *Entodinium* spp. multiplied rapidly in this medium; a higher percentage of these cells was observed in a state of division than is normally noted with cells in freshly collected ruminal ingesta. The addition of chlorophyllin or alfalfa chloroplasts seemed to stimulate further the feeding activity of the oligotrichs.

Phytone, the principal nitrogen source and only undefined component of medium 1, was replaced in medium 2 by an equivalent amount of constituent free amino acids, to give a defined medium, which was used in the next series of experiments.

Culture study of bacteria-free ciliates in medium 2. Conditions of the previous experiment were duplicated in experiments with medium 2.

The ruminal ciliates were immobilized and apparently killed within a few minutes after being inoculated into medium 2. The same result was obtained with ciliate inoculum collected from different cows over a period of several days. When the amino acids were reduced in concentration to 0.01 %, the toxicity of medium 2 disappeared.

It seemed obvious from these results that an unphysiologically high concentration of free amino acids in medium 2 was responsible for the death of ruminal ciliates suspended in it. Medium 3 therefore was formulated, with the average free amino acid composition of ruminal ingesta

(Annison, 1956; Duncan et al., 1953) and with the average concentration of soluble nutrilites present in ruminal ingesta and the plant ration.

Culture study of bacteria-free ruminal ciliates in medium 3. Preparation of inoculum and cultural conditions were the same as with runs involving media 1 and 2.

The bacteria-free ciliates were able to grow well in medium 3 for the first 10 hr of incubation; however, the isotrichs showed the same overdistention from excessive amylopectin storage that was noted with medium 1 in the absence of bacteria. After the 10th hr, all cells of *Isotricha* spp. had burst, and *Dasytricha ruminantium* was markedly reduced in numbers, indicating that the soluble carbohydrate concentration of 0.23% was intolerably high.

Fermentative activity again was similar to that noted with medium 1 in the absence of viable bacteria; an initial lag was followed by vigorous acid production.

At this point, there was clear indication that further nutritional studies on ruminal ciliates required the development of a culture medium which met the narrow tolerance limits of this group of protozoa for various nutrilites. Accordingly, the components of medium 3 were prepared as a series of stock solutions consisting of pooled amino acids, pooled carbohydrates, pooled vitamins, pooled trace minerals, pooled nucleides, pooled lower fatty acids, and glucose. The optimal level of each group of nutrilites was determined by setting up concentration-gradient series of tubes (containing balanced salt solution with increasing concentrations of the specific nutrilite group), inoculating the tubes heavily with bacteria-free (no viable bacteria present) mixed ruminal ciliates, and observing the ciliate responses over a period of several days. The inoculated tubes were gassed with carbon dioxide, sealed with screw caps, and incubated in the dark in a water bath (37 C). In most cases, a definite optimal concentration was noted for each class of nutrilites (above and below which the ciliates were much less active or nonviable). The indicated optimal concentrations for each group of nutrilites were employed in compounding medium 4.

Culture study of bacteria-free ruminal ciliates in medium 4. Preparation of inoculum was the same as for the experiments with medium 3. The continuous-culture apparatus was set to maintain a pH of 7.0, redox potential of −300 mv, stirring rate of 120 rev/min with a Teflon-covered stirring bar of 1-in. length, aeration rate with pure CO_2 of 3 ml gas per ml of medium per min, total electrolyte concentration equivalent to 0.67% KCl, and turnover rate for culture volume of 48 hr. The culture flask was actinic-coated to reduce photodynamic effects on the ciliates.

The entire spectrum of ruminal ciliates was able to maintain themselves in medium 4 for periods up to 168 hr, the longest time that the apparatus could be operated without failure of the control system and consequent development of a lethal environment. The most common cause of failure was fouling of electrodes.

In a few instances, the culture period was abbreviated because of the emergence of penicillin- and streptomycin-resistant strains of bacteria which overgrew the protozoa.

Ophryoscolex caudatus and the *Diplodinium* spp. survived in this medium and were present in the culture flask in appreciable numbers throughout the culture runs; microscopic examination of flask contents only rarely demonstrated a dividing form of these larger oligotrichs, however. Because of their high densities, these large oligotrichs were not often carried over in effluent collected by the constant-level take-off tube. Entodina disappeared quickly from medium 4.

In contrast to *Diplodinium* spp. and *Ophryoscolex caudatus*, isotrichs were observed to multiply in this medium and were able to maintain their initial population density (20,000 cells/ml) for several days before gradually diluting out.

Ciliates were grown for 5 days in medium 4, then were thin-sectioned and examined with an RCA EMU-2A electron microscope; no intracellular microorganisms were seen in the micrographs. Inoculation of culture effluents into thioglycolate medium showed no growth of microorganisms, and gram-stained smears of culture effluent were negative for microorganisms other than ruminal ciliates. These findings were interpreted to indicate that isotrichs are able to grow in the absence of either intra- or extracellular bacteria.

With the low concentrations of fermentable carbohydrates present in medium 4, there was no drop in pH of the culture during the incubation periods. Consequently, with this medium, the addition of alkaline titrant and dilution of the accumulating salts of organic acids by addition of deionized water were obviated.

With a ciliate cell population density of 10,000 cells/ml or higher, the redox potential of the culture remained below −300 mv without addition of reductants by the automatic titrator. Whenever the ciliate census fell much below 10,000 viable cells/ml, addition of a suitable reducing agent, such as cysteine, was required to maintain the redox potential below −300 mv.

Discussion

As judged from sample variability over a period of time, the fauna and flora of the bovine rumen do not conform to a set population pattern. Some of this variation possibly is due to changing flora and fauna of the feedstuffs. Complex inter-relationships (Lewis, 1961; Barnett and Reid, 1961) between the flora and fauna may exist in the rumen: synergism (Hill, 1960), antagonism (Eadie and Hobson, 1962; Eadie, *private communication*; Sugden, 1953), predation (Gutierrez and Hungate, 1957; Gutierrez, 1958; Gutierrez and Davis, 1959), commensalism (Hastings, 1944; Hungate, 1960).

The present research indicates that ruminal bacteria are unnecessary for growth of the isotrichic ruminal ciliates, but the oligotrichs seem to be dependent on a source of cellulose and starch for normal growth. This require-

ment is understandable in view of the strong cellulase and amylase activity of various species of oligotrichs, as reported in papers cited above as well as in reports by Annison and Lewis (1959), Hungate (1943, 1955), and Masson and Oxford (1951).

In the absence of ruminal bacteria, the "synthetic ruminal gas" was toxic to the ruminal ciliates, apparently because of its high content of H_2S. Carbon dioxide seemed to be entirely satisfactory for the gas atmosphere for ruminal ciliates in bacteria-free culture medium.

There are wide differences among the different species of ruminal ciliates with regard to redox potential range for survival, osmotic sensitivity, pH tolerance, response to chilling, and nutritional patterns. In view of these individualistic traits, it is surprising that such a wide variety of protozoal types are able to thrive in such a fixed ecological system as the rumen represents. Apparently, the physiological responses of the ruminant host, and a measure of "self-balancing" exerted by the extremely diverse microbial population, are sufficient to supply the happy medium which permits survival of the complex ruminal microbiota.

Rigorous control of the physical environment is essential to insure growth of ruminal ciliates in the absence of bacteria which normally remove many toxic metabolic products and which set up anaerobic conditions. The bacteria may also serve as a source of growth factors and energy sources ranging in complexity from inorganic molecules to intact bacterial cells.

Acknowledgments

The authors are indebted to Janet Jones, Gordon Miller, and Frank Farrell for technical assistance.

Literature Cited

Akkada, A. R. A., P. N. Hobson, and B. H. Howard. 1959. Carbohydrate fermentation by rumen ologotrich protozoa of the genus *Entodinium*. Biochem. J. **73**:44P–45P.

Akkada, A. R. A., and B. H. Howard. 1960. The biochemistry of rumen protozoa. 3. The carbohydrate metabolism of *Entodinium*. Biochem. J. **76**:445–451.

Akkada, A. R. A., and B. H. Howard. 1962. The biochemistry of rumen protozoa. 5. The nitrogen metabolism of *Entodinium*. Biochem. J. **82**:313–320.

Annison, E. F. 1956. Nitrogen metabolism in the sheep. Protein digestion in the rumen. Biochem. J. **64**:705–714.

Annison, E. F., K. J. Hill, and D. Lewis. 1957. Studies on the portal blood of sheep. 2. Absorption of volatile fatty acids from the rumen of the sheep. Biochem. J. **66**:592–599.

Annison, E. F., and D. Lewis. 1959. Metabolism in the rumen. John Wiley and Sons, Inc., New York.

Balch, D. A. 1958. An estimate of the weights of volatile fatty acids produced in the rumen of lactating cows on a diet of hay and concentrates. Brit. J. Nutr. **1**:18–24.

Baldwin, R. L., and R. S. Emery. 1960. The oxidation-reduction potential of rumen contents. J. Dairy Sci. **43**:506–511.

Barnett, A. K. G., and R. L. Reid. 1961. Reactions in the rumen. Edward Arnold Ltd., London.

Becker, E. R., and M. Talbott. 1927. The protozoan fauna of the rumen and reticulum of American cattle. Iowa State Coll. J. Sci. **1**:345–373.

Blake, J. T., R. S. Allen, and N. L. Jacobson. 1957. The influence of various factors on surface tension and pH of rumen fluid. J. Animal Sci. **16**:190–200.

Boyne, A. W., J. M. Eadie, and K. Raitt. 1957. The development and testing of a method of counting rumen ciliate protozoa. J. Gen. Microbiol. **17**:414–423.

Broberg, G. 1958. Measurements of the redox potential in rumen contents. IV. In vivo measurements. Nord. Vet. Med. **10**:263.

Bryant, M. P. 1952. The isolation and characteristics of a spirochete from the bovine rumen. J. Bacteriol. **64**:325–355.

Carlson, D. M., R. M. Forbes, and R. G. Hansen. 1957. A review of the carbohydrate constituents of roughages. U.S.D.A. NC-25 Regional Publication 88.

Clark, W. M. 1960. Oxidation-reduction potentials of organic systems. The Williams & Wilkins Co., Baltimore.

Cole, H. H., C. F. Huffman, M. Kleiber, T. M. Olson, and A. F. Schalk. 1945. A review of bloat in ruminants. J. Animal Sci. **4**:183–236.

Coleman, G. S. 1958. Maintenance of oligotrich protozoa from the sheep rumen in vitro. Nature **182**:1104–1105.

Coleman, G. S. 1960. The cultivation of sheep rumen oligotrich protozoa in vitro. J. Gen. Microbiol. **22**:555–563.

Committee on Feed Composition, Agricultural Board, National Academy of Sciences. 1956. Composition of concentrate by-product feeding stuffs. Natl. Acad. Sci.—Natl. Res. Council, Publ. 449.

Committee on Feed Composition, Agricultural Board, National Academy of Sciences. 1958. Composition of cereal grains and forages. Natl. Acad. Sci.—Natl. Res. Council, Publ. 585.

Duncan, C. W., I. P. Agrawala, C. F. Huffman, and R. W. Luecke. 1953. A quantitative study of rumen synthesis in the bovine on natural and purified rations. II. Amino acid content of mixed rumen proteins. J. Nutr. **49**:41–49.

Eadie, J. M., and P. N. Hobson. 1962. Effect of the presence or absence of rumen ciliate protozoa on the total rumen bacterial count in lambs. Nature **193**:503–505.

Erwin, E. S., G. J. Marco, and E. Emery. 1961. Volatile fatty analyses of blood and rumen fluid by gas chromatography. J. Dairy Sci. **44**:1768–1771.

Flatt, W. P., and N. F. Colovos. 1960. Quantitative analysis of ruminant respiratory gas by means of gas chromatography. Abstr. 5th Intern. Congr. Nutr., p. 8.

Garton, G. A. 1951. Observations on the distribution of inorganic phosphorus, soluble calcium, and soluble magnesium in the stomach of the sheep. J. Exptl. Biol. **28**:358–368.

Gutierrez, J. 1955. Experiments on the culture and physiology of holotrichs from the bovine rumen. Biochem. J. **60**:516.

Gutierrez, J. 1958. Observations on bacterial feeding by the rumen ciliate *Isotricha prostoma*. J. Protozool. **5**:122–126.

Gutierrez, J., and R. E. Davis. 1959. Bacterial ingestion by the rumen ciliates *Entodinium* and *Diplodinium*. J. Protozool. **6**:222–226.

Gutierrez, J., and R. E. Hungate. 1957. Interrelationship between certain bacteria and the rumen ciliate *Dasytricha ruminantium*. Science **126**:511.

Hansen, R. G., R. M. Forbes, and D. M. Carlson. 1958. A review of carbohydrate constituents of roughages. Bull. 634, Univ. Illinois Agr. Expt. Sta.

Hastings, E. G. 1944. The significance of the bacteria and the protozoa of the rumen of the bovine. Bacteriol. Rev. **8**:235–260.

Heald, P. J., and A. E. Oxford. 1953. Fermentation of soluble sugars by anaerobic holotrich ciliates of the genera *Isotricha* and *Dasytricha*. Biochem. J. **53**:506–512.

Hill, F. D. 1960. In vitro lipolysis of triglycerides by bovine rumen microorganisms. Ph.D. Thesis, Iowa State University, Ames.

HOWARD, B. H. 1959*a*. The biochemistry of rumen protozoa. I. Carbohydrate fermentation by *Dasytricha* and *Isotricha*. Biochem. J. **71**:671–675.

HOWARD, B. H. 1959*b*. The biochemistry of rumen protozoa. 2. Some carbohydrases in cell-free extracts of *Dasytricha* and *Isotricha*. Biochem. J. **71**:675–680.

HUBBERT, F., JR., E. CHENG, AND W. BURROUGHS. 1958*a*. Mineral requirements of rumen microorganisms for cellulose digestion in vitro. J. Animal Sci. **17**:559–568.

HUBBERT, F., JR., E. CHENG, AND W. BURROUGHS. 1958*b*. The influence of potassium, sodium, rubidium, lithium, and cesium on in vitro cellulose digestion by rumen microorganisms with observations upon sodium and potassium influences in lamb fattening rations. J. Animal Sci. **17**:576–585.

HUNGATE, R. E. 1942. The culture of *Eudiplodinium neglectum* with experiments on the digestion of cellulose. Biol. Bull. **83**:303–319.

HUNGATE, R. E. 1943. Further experiments on cellulose digestion by the protozoa in the rumen of cattle. Biol. Bull. **84**:157–163.

HUNGATE, R. E. 1955. Mutualistic intestinal protozoa p. 159–179. *In* S. H. Hutner and A. Lwoff [ed.], Biochemistry and physiology of protozoa, vol. 2. Academic Press, Inc., New York.

HUNGATE, R. E. 1960. Microbial ecology of the rumen. Bacteriol. Rev. **24**:353–364.

KINGWELL, R. G., R. A. OPPERMANN, W. O. NELSON, AND R. E. BROWN. 1959. Factors affecting the composition of rumen gas. J. Dairy Sci. **42**:912–913.

KLEIBER, M., H. H. COLE, AND S. W. MEAD. 1943. Bloat in cattle and composition of rumen gases. J. Dairy Sci. **26**:929–933.

KON, S. K., AND J. W. G. PORTER. 1953. The B-vitamin content of the rumen of steers given various diets. Proc. Nutr. Soc. **12**:xii.

KON, S. K., AND J. W. G. PORTER. 1954. The intestinal synthesis of vitamins in the ruminant. Vitamins Hormones **12**:53–68.

LEWIS, D. 1961. Digestive physiology and nutrition of the ruminant. Butterworths, London.

MCDOUGALL, E. I. 1958. Studies on ruminant saliva. I. The composition and output of sheep's saliva. J. Gen. Microbiol. **43**: 99–108.

MASSON, T. M., AND A. E. OXFORD. 1951. The action of ciliates of the sheep's rumen upon various water-soluble carbohydrates inducing polysaccharides. J. Gen. Microbiol. **5**:664–672.

OXFORD, A. E. 1955*a*. The rumen ciliate protozoa: their chemical composition, metabolism, and requirements for maintenance and culture, and physiological significance for the host. Exptl. Parasitol. **4**:569–605.

OXFORD, A. E. 1955*b*. The bacteriology and protozoology of ruminant digestion. J. Sci. Food Agr. **6**:413–418.

OXFORD, A. E. 1959. Bloat in cattle. XV. Further observations concerning the ciliate *Epidinium ecaudatum*, an inhabitant of the rumens of cattle liable to bloat. New England J. Agr. Res. **2**:365–374.

PHILLIPSON, A. T. 1952. The fatty acids present in the rumen of lambs fed on a flaked maize ration. Brit. J. Nutr. **6**:190.

PILGRIM, A. F. 1948. The production of methane and hydrogen by the sheep. Australian J. Sci. Res. **B1**:130–138.

QUINN, L. Y. 1962. Continuous culture of ruminal microorganisms in chemically defined medium. I. Design of continuous culture apparatus. Appl. Microbiol. **10**:580–582.

RAUN, N. S., AND W. BURROUGHS. 1962. Suction strainer technique in obtaining rumen fluid samples from intact lambs. J. Animal Sci. **21**:454–457.

SALSBURY, R. L., P. ELLIOTT, AND R. W. LUECKE. 1958. Some characteristics of the hemicellulolytic activity of rumen microorganisms. J. Animal Sci. **17**:1190.

SMITH, F. 1957. Investigations of the carbohydrates of forage crops with special emphasis on the hemicelluloses. U.S.D.A. NC-25 Coop. Reg. Res. Project, Annual Report.

SUGDEN, B. 1953. The cultivation and metabolism of oligotrich protozoa from the sheep's rumen. J. Gen. Microbiol. **9**:44.

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.

WASHBURN, L. E., AND S. BRODY. 1937. Growth and development. XLII. Methane, hydrogen, and carbon dioxide production in the digestive tract of ruminants in relation to the respiratory exchange. Mo. Agr. Expt. Sta. Res. Bull. 263.

WILLIAMS, P. P., R. E. DAVIS, R. N. DOETSCH, AND J. GUTIERREZ. 1961. Physiological studies of the rumen protozoan *Ophryoscolex caudatus* Eberlein. Appl. Microbiol. **9**:405–409.

WILLIAMS, P. P., J. GUTIERREZ, AND R. N. DOETSCH. 1960. Protein degradation and other physiological studies on the rumen ciliate *Ophyroscolex caudatus* Eberlein. Bacteriol. Proc., p. 32.