Continuous Culture of Ruminal Microorganisms in Chemically Defined Medium

I. Design of Continuous-Culture Apparatus[1]

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ABSTRACT

QUINN, LOYD Y. (Iowa State University, Ames). Continuous culture of ruminal microorganisms in chemically defined medium. I. Design of continuous-culture apparatus. Appl. Microbiol. **10**:580–582. 1962.—An apparatus is described which has been used for successful continuous culture of the ciliates from the rumen of cattle. Automatic control of feeding rate, pH, oxidation-reduction potential, temperature, stirring rate, aeration rate, salinity, and volume of the culture is provided for, using standard commercial equipment, whenever possible. The operation of this apparatus is described.

Since the pioneering work of Becker and Talbott (1927), many attempts have been made to grow the nutritionally fastidious ciliates of the bovine rumen in vitro. Hungate (1942, 1943) succeeded in culturing *Entodinium* spp. and *Diplodinium* spp. in various natural media supplemented with ruminal fluid. Burroughs et al. (1951), Sugden and Oxford (1952), Sugden (1953), Cheng, Hall, and Burroughs (1955), Oxford (1955*a*, *b*), Gutierrez (1958), and Coleman (1958, 1960) considerably refined and improved techniques for cultivation of ruminal ciliates. These workers, however, were not able to maintain ruminal ciliates in a completely defined medium.

Ruminal ciliates occupy a unique ecological niche, in which pH, salt concentration, temperature, composition of the gas atmosphere, mixing and shearing of ingesta, removal of comminuted and solubilized ingesta, and feeding rate of fresh medium are all closely controlled to maintain a ruminal environment that is nearly optimal for an amazingly complex and dense population, as noted by Hastings (1944). Appreciation of this situation has prompted a number of researchers to construct laboratory culture apparatus modeled on the functioning rumen. Luow, Williams, and Maynard (1949), Warner (1956), Adler et al. (1958), Davey, Cheeseman, and Briggs (1960), El-Shazly, Dehority, and Johnson (1960), and Stewart, Warner, and Seeley (1961) described continuous-culture apparatus in which ruminal microorganisms in normal mixed culture were maintained in natural media for short periods of time without serious alteration in the biological and biochemical patterns associated with *in situ* ruminal microorganisms. Beyond the first 24 hr of incubation, however, these workers noted the appearance of marked deviations from the normal growth and metabolic patterns, particularly when pH control was not provided for.

Heald and Oxford (1953) reported that the ruminal ciliates prefer a salt concentration equivalent to 0.65% KCl. Hungate (1960) found that strict anaerobic conditions are essential for their survival. The pH optimum differs for the different species of ruminal ciliates, but many of the workers cited above found that pH 6.8 to 7.0 is near optimum for ruminal ciliates in mixed population. Incubation temperatures between 35 and 40 C have been used for laboratory cultures of ruminal ciliates, since this range of temperatures has been reported to occur in the rumen (Blake et al., 1957; Annison and Lewis, 1960).

This paper describes a continuous-culture apparatus assembled and used for the study of ruminal ciliates growing under controlled environmental conditions.

Culture flask of the apparatus. The culture flask consisted of a 500-ml Erlenmeyer flask fitted with an umbrella-type closure fabricated from a standard-taper glass joint, size 71/60. Eight glass ports were sealed into the flask closure for feeding, sampling, and similar purposes.

Seven glass standard-taper inner joints (24/20) were sealed into the base of the culture flask to provide connectors for electrodes and probes, which were used to establish electronic control of the culture environment.

Aeration system. The gas atmosphere in the culture flask was controlled by admitting bottled gas of the desired composition through a flowmeter and a sintered-glass sparger into the mechanically stirred culture. A 13-mm, 0.45-μ porosity HA membrane filter (Swinney Hypodermic Adapter, Millipore Filter Corp., Bedford, Mass.) was used in the gas line to maintain sterility of the gas. Foam formation was no problem with normally growing cultures of mixed ciliate populations.

Oxidation-reduction potential and pH control systems. A Beckman automatic titrator (model K) was used to control the pH and redox potential of the culture, using standard glass, calomel, and platinum electrodes, mounted

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in 24/40 standard-taper glass inner joints, as the sensing probes. Electrodes were sterilized either by autoclaving, as described by McKee (1955), or by immersion in Roccal (Winthrop-Stearns, Inc., New York, N.Y.), 1:125, for 10 min, followed by rinsing in sterile deionized water. Alkaline titrants for pH adjustment were either artificial saliva (McDougall, 1958) or 0.1 N sodium bicarbonate. Reducing agents used for oxidation-reduction potential adjustment were: sodium sulfide, ascorbic acid, or cysteine; dilute solutions were filter-sterilized, using a 0.45-μ membrane filter. Both the alkaline titrant and reducing agent solutions were pumped into the culture flask through 47-mm 0.45-μ membrane filters (Model XX 40 047 00 Pressure Filtration Holder and HA Millipore Filters, Millipore Filter Corp.) to protect against contamination of the culture through the titrator.

Salinity control system. The conductivity of the culture medium, measured in a conductivity cell, was adjusted against standard 0.65% KCl solution. The conductivity cell comprised one arm of a resistance bridge circuit which became unbalanced when the salt concentration of the culture became higher than the equivalent of 0.65% KCl. Current flowing in the bridge circuit actuated a relay which turned on a pipetting machine (Brewer Automatic Pipetting Machine, Model 07–40, Baltimore Biological Laboratory, Inc., Baltimore, Md.) that dispensed deionized water until dilution of the culture medium dropped the conductance of the medium to 0.65% KCl equivalent, at which point the conductivity limiter stopped the water pump. The circuitry of this salinity control system will be described elsewhere.

Medium-feeding system. Water-insoluble substrates, in the form of a thin slurry, were metered in by peristaltic pump (Model PA Peristaltic Pump, New Brunswick Scientific Co., New Brunswick, N.J.). Water-soluble medium was metered in by a second pipetting machine (Filametic Vial Filler, Model AB, National Instrument Co., Baltimore, Md.).

Both slurry and soluble media were delivered at a rate which was controlled by a cycle timer (Model CM 7 Cycle Timer, Industrial Timer Corp., Newark, N.J.) that actuated the medium pumps for 10 sec of each 10-min cycle. The volumes delivered by the medium pumps were adjusted to give the desired "turnover rates" of culture-flask contents. The reservoir for slurry medium consisted of a 500-ml Erlenmeyer flask with a sidearm near the base of the flask. Separation of suspended solids was prevented by magnetic stirring with a 1-in. Teflon-covered stirring bar (Model 9235-U11, Octagonal Stirring Bar, A. H. Thomas Co., Philadelphia, Pa.) impelled by a small magnetic stirrer (Model 9235-C Magnetic Stirrer, A. H. Thomas Co.).

The reservoir for water-soluble medium was held in an ice-bath, or when desired was jacketed with an electric heating mantle (Glas-Col Heating Mantle, Model M, Glas-Col Apparatus Co., Inc., Terre Haute, Ind.), whose temperature was held at 37 to 38 C by a rheostat (Model 116 Powerstat, Superior Electric Co., Bristol, Conn.).

Culture-agitation system. Agitation of the culture by rapid aeration was coupled with mechanical stirring to keep the culture homogeneously mixed. The culture flask was supported on an 8-in. diam magnetic stirrer (Magnamix, Precision Scientific Co., Baltimore, Md.), and the flask contents were mixed with a ½-in. long Teflon-covered stirring bar with central spinning pivot at 120 rev/min.

Incubation temperature control system. The culture flask was immersed to the surface level of the medium in a Pyrex glass water bath (7 by 4 in.) heated by a flexible immersion heater (Model S-40825 Heater, 300 w, E. H. Sargent and Co., Chicago, Ill.). The water-bath temperature was controlled by a mercury thermoregulator (Model S-81850, E. H. Sargent and Co.) and a polarized relay (Model 13752A-B Supersensitive Polarized Relay, Central Scientific Co., Chicago, Ill.), which controlled the heater to give temperature regulation within ± 0.5 C of the desired temperature. A thin film of light mineral oil was helpful in controlling evaporation of water from the water bath. A ½-in. thick disc of asbestos board between the water bath and the magnetic stirring motor prevented overheating of the bath by the motor.

Culture constant-level and sampling system. A constant level tube inside the culture flask removed excess volume of culture medium to a sample collection tube, which was in turn connected to a large reservoir flask. Overflow from

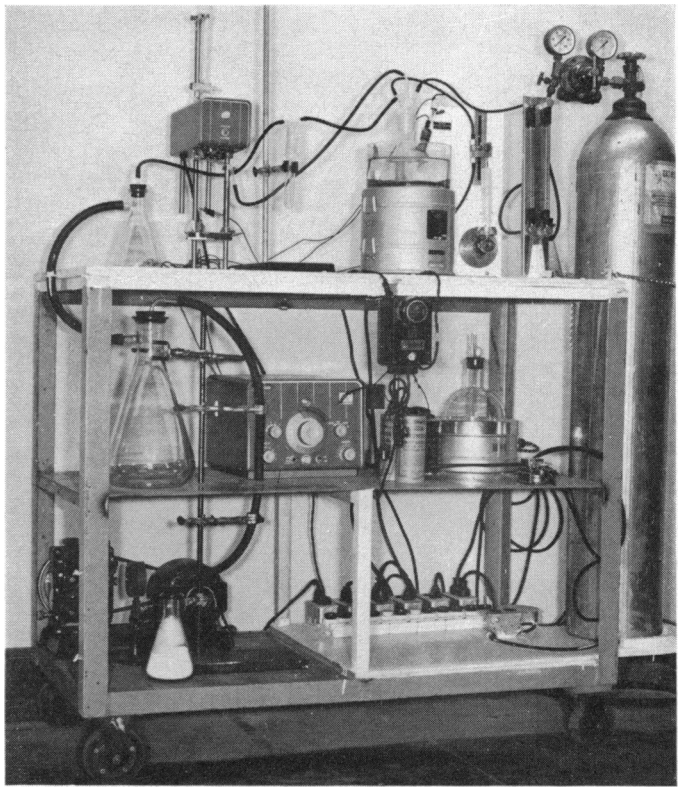

FIG. 1. *Continuous-culture apparatus; prototype model.*

the sample collection tube was collected in the reservoir flask, which was kept at a slight negative pressure by a central vacuum system. The sample collection tube was connected to the vacuum train by a 24/40 standard-taper glass joint so that a filled tube could be replaced readily with an empty and sterile collection tube. The assembled continuous-culture apparatus is shown in Fig. 1.

Discussion

The results obtained with this apparatus are described in the following paper (Quinn, 1962).

Warner (1956), Adler et al. (1958), Davey et al. (1960), and Stewart et al. (1961) were able to maintain essentially normal ruminal microflora and microfauna in vitro for periods of 8 to 24 hr, using continuous-culture methods. Mechanical instability of their apparatus and lack of automatic pH control were cited by these workers as the limiting factors in cultivation of ruminal microorganisms on natural media.

Consequently, long-term stability of all components was the major criterion in selection of "hardware" in designing the continuous-culture apparatus described in this paper. Where possible, commercially engineered and tested apparatus was used; but, in the salinity control system, it was necessary to build the conductivity limiter, because no suitable laboratory-scale apparatus of this type was available.

With this controlled continuous-culture apparatus, the ecological conditions of the bovine rumen have been closely approximated, as indicated by growth of the fastidious ruminal ciliates in the absence of ruminal bacteria.

The principal limitation of the present design of this continuous-culture apparatus is its lack of provision for automatic recording of the biological and chemical changes which occur during growth of cultures.

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