RUMEN STUDIES

by

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RUMEN STUDIES

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INTRODUCTION

Living matter, in order to preserve its inherent integrity, must maintain a dynamic equilibrium between itself and its environment. Thus a study of the mechanisms of living matter in nature would logically require duplication in some manner of this constant interchange to fully replicate this order of integrity. Various methods and techniques have been developed in order to replicate certain biological conditions by holding certain phenomena constant, i.e., nutrition, genetic characteristics, temperature, etc.

In the field of microbiology many techniques of culture have been devised to study microorganisms under different environmental conditions. These methods of culture may be broadly classified into two types: those of the batch culture and those of the continuous-flow types. The development of a technique whereby microorganisms may be kept in a batch type environment with a continuous flow of nutrients through the culture and its application to culture of rumen microorganisms is the major subject of this dissertation.

REVIEW OF LITERATURE

Designs of Continuous Culture Systems

Many reports which describe devices for continuous culturing of microorganisms are found in the literature. All of these systems provide for an inflow of nutrients and an overflow of the entire contents of the culture to maintain constant volume.

Early devices were constructed with external controls such as washout times and nutrient concentration. In recent years, systems operating by internal controls (photometrically and colorimetrically) have been used to control growth rate.

Gerhardt (1946) described a completely continuous system in which the addition of free media and the withdrawal of products was accomplished via vacuum. Similar systems have been devised on the principle of continuous dilution at constant volume to facilitate studies in growth patterns, metabolism, and genetics over extended time intervals at reduced growth rate.

Monod (1950) described a device called the Bactogen that is operated on the principle of external control of nutrient input to maintain a constant flow rate over long periods of time. The growth chamber consists of a round bottomed flask centered on a rotating support which revolves from 200 to 400 turns per minute. The input system has

been devised so as to deliver a certain volume of medium through the pulsating action of a rotating eccentric drum which squeezes the nutrients into the growth chamber. The outflow mechanism consists of a tube attached to an aspirator which keeps the culture volume constant. An aeration tube is also introduced into the system which enters the culture flask through the neck parallel to the axis of rotation as do the inlet and outlet tubes and the sampling tube. The system has been expanded to large scale operation by employing a rotating metal drum of 50 liter capacity to facilitate the growth of nearly one kilogram dry weight of Escherichia coli and Bacillus cereus per day. This author ascribes homogeneity of culture as more critical than the notion of equilibrium conditions as reflected by the design of the apparatus. In addition, the need for equilibrium with the gaseous phase necessitates a large ratio of surface area to volume inside the growth flask.

Novick and Szilard (1950) described a continuous flow system based on the principle of stabilizing the system by a fixed value of flow rate below the maximum growth of the culture. By supplying a large excess of all nutrients but one, limiting growth factors establish growth rates. The mechanisms of operation are similar to that previously described. The nutrient flow is maintained by a fixed pressure across a capillary resistance. A simplified inflow device has been reported by Rotman(1955). The growth chamber consists of a stationary flask or test tube fitted with gassing tubes. The culture volume is maintained

constant by a siphon overflow tube. This system offers the advantages of greater simplicity, fewer moving parts, more accurately controlled culture volume, and greater protection from contamination (Novick, 1955). The Bactogen, on the other hand, provides more effective aeration permitting the use of larger populations.

Modifications of these two systems have been used to study particular organisms (Von Hofsten et al., 1953).

Culture apparatti operating by the principle of internal control by photocells have been reported by various workers. Meyers and Clark (1944) used an elaborate twin photocell arrangement to study algae. One photocell was used to measure transmittance through the culture and the other to control changes in light intensity. The differences in output of these two cells were used as regulators to control nutrient flow into the system by a solenoid valve. These workers reported variations in culture suspensions of one percent. Using a similar system Bryson (1952) studied the effect of toxic agents which were constantly increased in concentration in the nutrient medium via a series of siphons. This device, called a turbidostatic selector, was designed for either continuous or intermittent changes in environment. The apparatus contained two essential components, an electronic circuit to control periodic addition of nutrient to the microorganisms whenever the culture reached a fixed turbidity, and a proportional-feed system for geometrically increasing the concentration of some toxic substance. The proportional feed consisted of four tubes connected by a siphon bridge. Concentrations of material between each adjacent tube were

about tenfold. By using direct feed, concentration gradients of toxic materials were represented as sigmoid curves. With the proportional feed, a geometric increase of the type $y = ab^x$ was obtainable. The latter increase in concentration was more useful in studying toxic substances. Kinetic data were obtained using neutral red placed in the proportional-feed system.

Golle (1953) described a theoretical model in which the outflow of the first culture vessel flowed directly into a second vessel. Growth equations for two culture vessels in series were devised and calculations were made of bacterial growth in the two vessels and their steady state equilibria.

An elaborate stainless steel pilot plant with a 20-liter culture volume was described by Herbert et al. (1956). A theoretical treatment of continuous culture which allows prediction of the steady-state concentrations of bacteria and substrate was given. Mathematical considerations were also given to change in medium concentration and flow rate.

Theory of Continuous Culture Systems

The most extensive expansion of the theory of continuous flow systems was developed by Monod (1950). The author likened the system to that of transfer of a culture to new medium with minimum of dilution. As this frequency reached certain limits, discontinuity of growth and an element of uncertainty would be eliminated.

Rate of growth in a continuous flow system is a function of bacterial mass, growth rate, and loss of the bacterial mass from the growth receptacle with respect to time. The growth in the receptacle becomes $\log_e x_b/x_o = (u-D)t$, where x_b is the bacterial mass in the growth receptacle; x_o, the bacterial mass at time $t = 0$;u, the "rate of Nepherian growth"; D, loss of bacterial mass from the system; and t, time.

The growth rate of the bacterial population at any one time has been described by Spicer (1955) as being a function of its size (n) so that $1/n \cdot dn/dt = f(n)$ where f(n) is some function of n. If medium is being supplied and removes a fraction (b) of the organisms per unit time the equation becomes $1/n \cdot dn/dt = f(n) - b$.

Herbert et al.(1956) used a similar net rate equation of concentration of microorganisms ($dx/dt = ux - Dx$), or increase is equal to growth minus output.

Four different rate processes were described by Moser (1958) to govern population of growth; specific growth rate of viable cells, specific mortality rate, washout rate, and specific assimilation rate involving a limiting nutrient. The combination of the above processes will give the following pattern: $1/n \cdot dn/dt = k_1(c) - V_1(c) - V/W$.

Using continuous cultures of Aerobacter aerogenes, Contois (1959) found that the specific growth rate was a funtion of population density as well as the concentration of the limiting nutrient. The model of bacterial growth was formulated as $R = u_m \cdot S/BP + S$.

The time a particle or microorganism remains in the growth tube has been termed the residence time. Herbert et al. (1956) maintain that residence times are not determined by absolute values of flow rate and culture volume but by their ratio called the dilution rate, D, defined as the number of complete volume-changes per hour. The mean residence time of a particle then becomes 1/D. Equations for denoting particles remaining either longer or shorter than the mean residence time have been given by Moser (1958).

One of the advantages of the continuous culture systems is the ability to study reduced growth by a limiting nutrient. In response to a limiting nutrient, Novick and Szilard (1950b) showed that the entire population was uniformly decreased at reduced growth rate rather than the growth of only a fraction of the population. The relationship between growth rate and limiting nutrient has been conveniently expressed as a hyperbolic function in terms of the form $\log [(p - k)/k] = \log r - x \log c$ (Moser, 1958).

In the stationary state, the concentration of the limiting nutrient in the growth tube was found to be independent of the concentration of this growth factor in the incoming medium with a fixed flow rate (Novick and Szilard, 1950b). Rather, it was found to be a function of growth and flow rates.

The net rate of change of substrate concentration may be obtained from the following equation of Herbert et al. (1956): increase is equal to input minus output minus consumption, where consumption may be expressed as growth/yield constant. Mathematically, the equation

may be stated as $ds/dt = D_{sr} - D_s - ux/Y$.

Synchronization experiments, theory, development, and design have been excellently reviewed by Novick (1955) and Moser (1958). Emphasis has also been placed on the use of continuous culture systems in the study of mutations but will not be reviewed in this dissertation.

Each of these systems involves the passage of medium from reservoir to a growth chamber containing microorganisms and an outlet for the removal of both the medium and the organisms. These systems are not able to retain the microbial population within the system which renders them capable of being used for studies limited to an equilibrium of contents being removed with respect to that in the growth chamber at any one given instant. These systems, then, would be limited in use to that of steady state equilibria among flow rate, generation time, and nutrient concentration. Studies of various stages of the end of log growth phase for variables other than mentioned above, the stationary phase, and the log death phase.

MATERIALS AND METHODS

Description of Growth Chamber. A system for microbial cultures, referred to as a growth chamber, has been devised and successfully operated. Microorganisms are grown in a culture with medium constantly being supplied and removed, thus forming a closed system for bacterial growth and an open system for flow of medium and by-products. The fermentation apparatus allows a liquid solution to pass through a semi-permeable enclosure in which microorganisms are trapped between two bacterial filters (Millipore No. HAWP047A0). The liquid medium supplies the necessary nutrients for bacterial growth and in addition removes the soluble by-products which have been produced by the microorganisms. As shown in Plate I, the vessel consists of two aluminum discs which enclose a glass cylinder. Each end of the cylinder is fitted with an aluminum washer and O-ring to seal and support the bacterial filter which prevents the passage of bacteria from the cylinder. An inoculation port was placed on the side of the cylinder to facilitate inoculation and withdrawal of samples. Two aluminum discs was secured by three brass bolts and the glass cylinder was sealed in the middle (Plate II).

A 7/16 inch magnetic spin bar placed inside the cylinder directly on the outlet filter served to keep the mixture homogenous at all times and prevented clogging of the filter by bacteria and/or other contents.

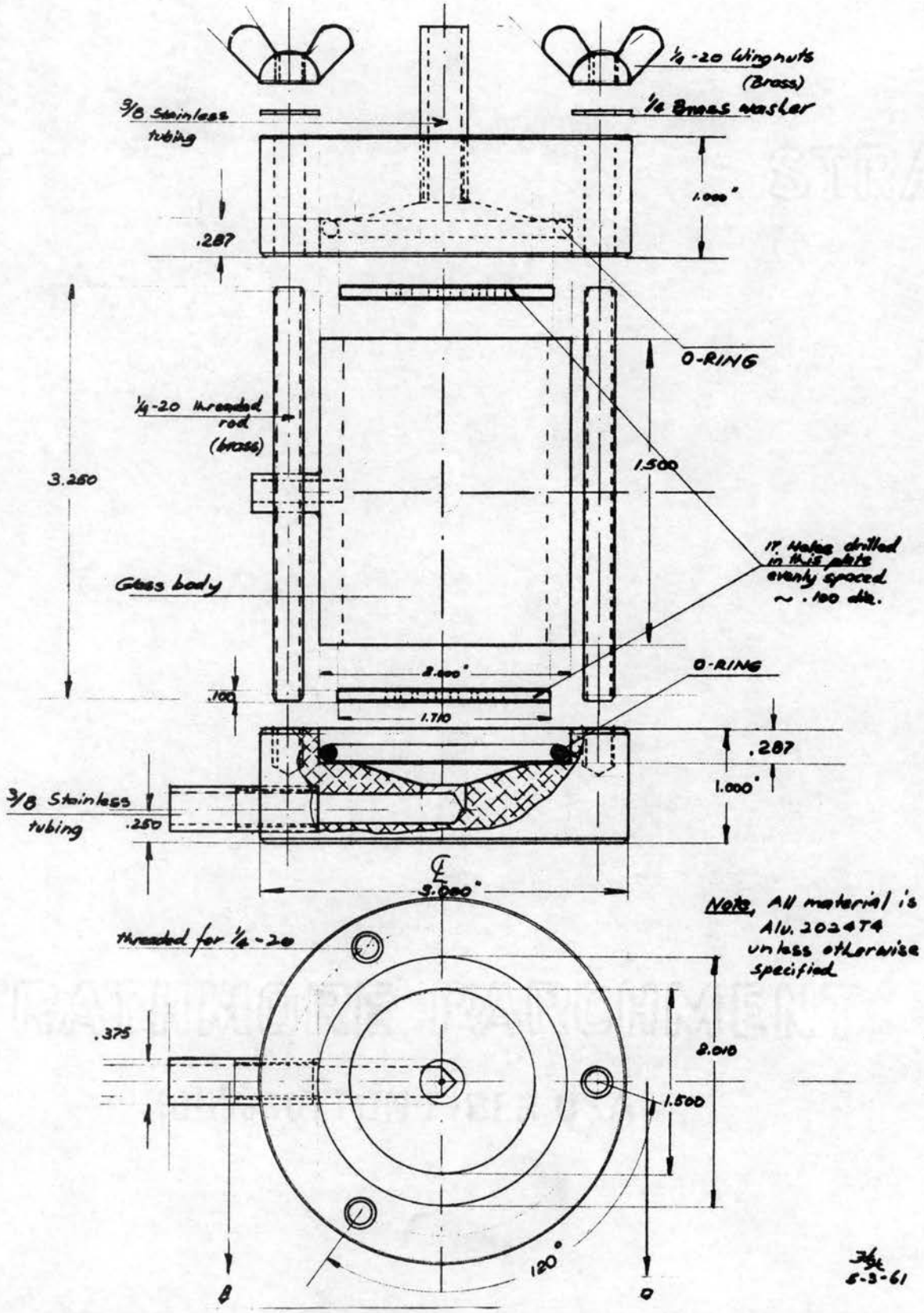

Figure 1. Detailed Drawing of Construction of The Growth Chamber.

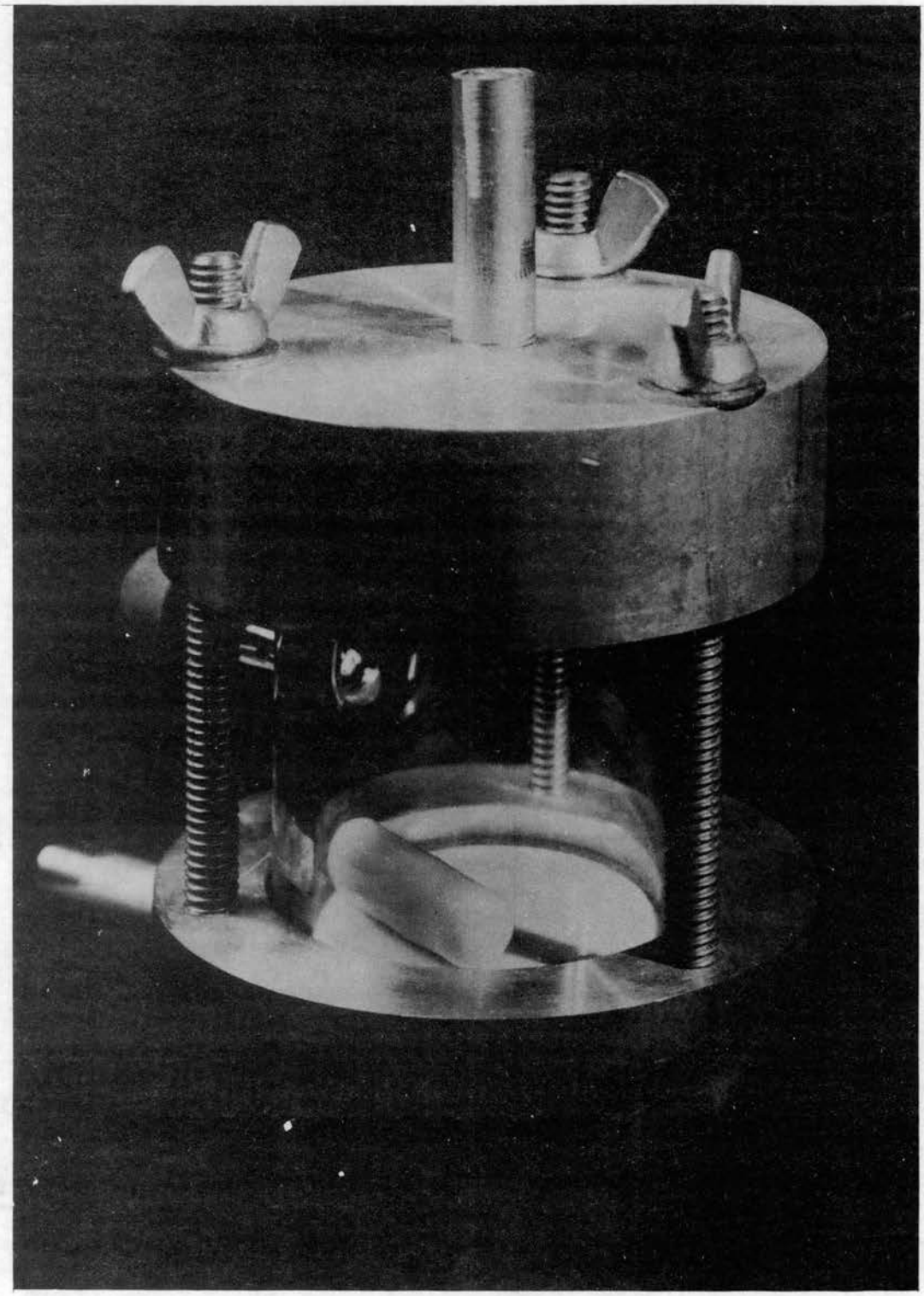

Figure 2. Side View of Assembled Growth Chamber.

The chamber was then placed in vertical position in a water bath with a magnetic stirrer under the water bath directly beneath the growth chamber (Plate III).

Medium was forced through the chamber by gas pressure which also served to maintain either aerobic or anaerobic conditions within the chamber. The medium reservoir was attached to the chamber by surgical tubing with a glass rod containing a sterile cotton plug to prevent bacterial contamination. The medium bottle consisted of a 1-liter flask fitted with a No. 7, 2-hole rubber stopper. Six mm. pyrex tubing was extended through the stopper to the bottom of the flask. To one of the inlets, the gassing tube was placed to force medium out through the second tube which was connected to the inlet of the growth chamber. This inlet connection was fitted with a T-tube (Plate III) to facilitate sampling of medium during the experiment.

Only a true solution or one containing particles smaller than the pore size of the filter can be used for the medium passes through the filter before entering the chamber. Particles of larger size were placed into the chamber through the inoculation port before the bacteria and medium were added or during the experiment.

The outlet apparatus consisted of surgical tubing connected to the outlet of the chamber and glass tubing (I.D. 7 mm.) bent to fit over the edge of the water bath and extending vertically to a large collection tube.

Theory of Operation. The vessel of a closed system for the bacterial population and an open system for the medium flowing into a chamber at a

PLATE III

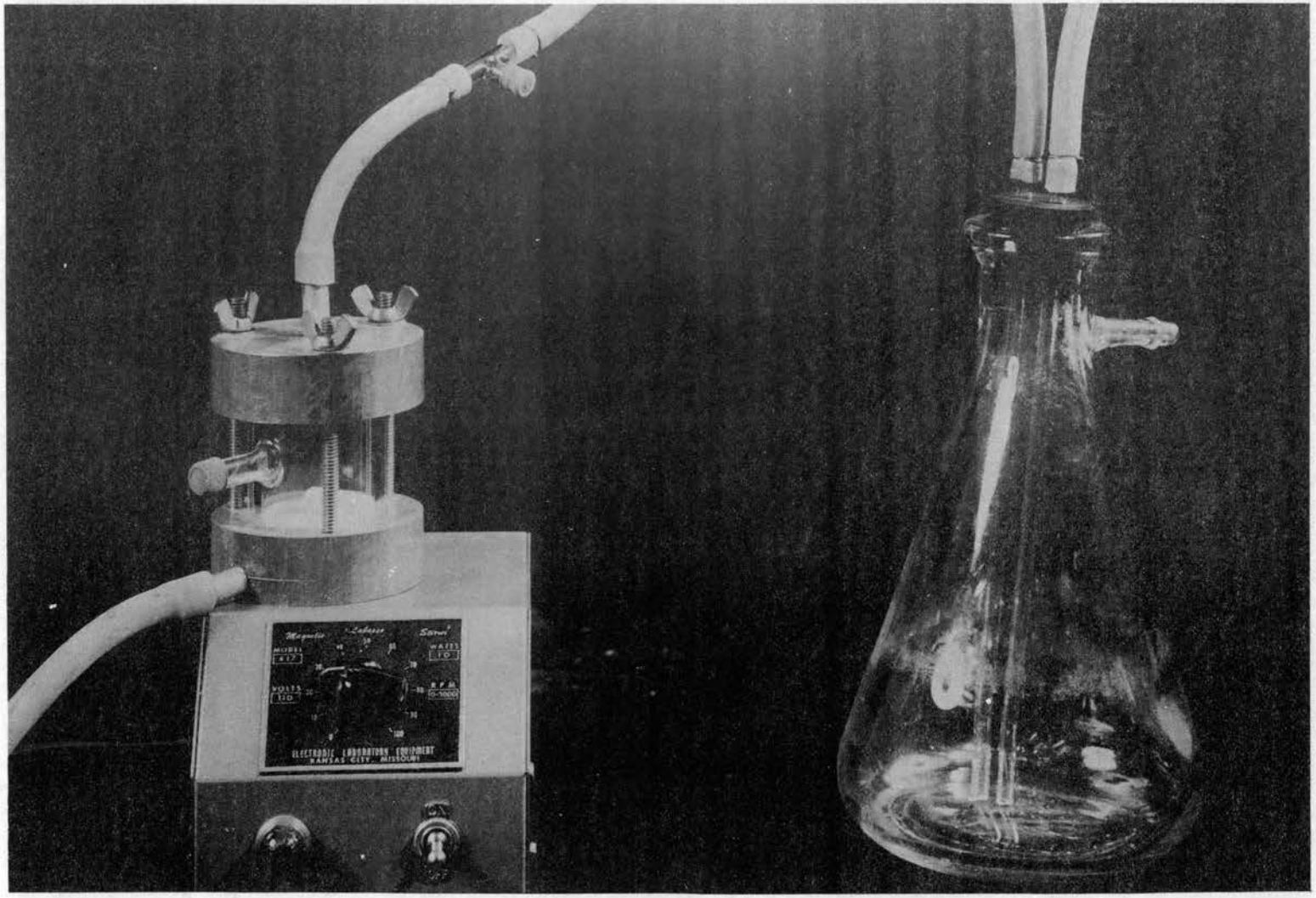

Figure 3. Growth Chamber with Assembled Medium Reservoir and Mixing Assembly.

rate of w/t and flowing out of the chamber at a rate of x/t is considered in developing the following theory. Since the system at any one time is filled, the rate of inflow must equal the rate of outflow, i.e., $w/t = x/t$, and thus, $w = x$.

The flow rate of a given substrate into the chamber per unit time may be given as aw/t. The amount of substrate leaving the chamber per unit time is cw/t, and the amount consumed by the bacterial population inside the chamber may be formulated as kw/t.

The concentration of a given substrate in the growth chamber at any one time is equal to the amount (a) flowing into the chamber, the amount (c) leaving the chamber, and the amount (k) consumed by the bacterial population. This may be expressed as $a = aw/v - cw/v - k$, where (k) is the sum of the microorganisms at any one time (t) multiplied by the consumption coefficient (g_j), $\sum_{j=1}^{j=n} q_j k_j n_j$. The change in concentration with respect to time becomes

$da/dt = aw/v - cw/v - k$, and upon integrating

$$\ln (aw/v - cw/v - k) = wt/v + \ln a_o$$

$$(aw/v - cw/v - k)a_o = e^{wt/v}$$

$$aw/v = cw/v + k + a_o e^{wt/v}$$

$$a = c + kv/w + (a_o v/w)\, e^{wt/v} \qquad (1)$$

If (c) is the amount of substrate leaving the chamber, then the change in this concentration with respect to time becomes

$dc/dt = cw/v - aw/v - k$, and

$dc/dt = (c - a)w/v - k$, then upon integrating,

$$c = a + kv/w + (c_o v/w)\, e^{wt/v} \qquad (2)$$

The amount of product in the chamber at any one time (D) is equal to the amount in the chamber (D), the amount being produced by the microorganisms (k'), and the amount leaving the unit (e). For any instant then,

$$dD/dt = Dw/v - ew/v + k', \text{ and}$$

$$D = e - k'v/w + (D_o v/w)\, e^{wt/v} \tag{3}$$

The amount of the substrate utilized by the microorganisms with respect to time, thus, becomes

$$dk/dt = k - aw/v - cw/v, \text{ and}$$

$$k = aw/v + cw/v + k_o e^{wt/v} \tag{4}$$

The amount of product leaving the chamber (e) at any one time becomes

$$e = D + k'v/w + (e_o v/w)e^{wt/v} \tag{5}$$

and the amount of product formed by the microorganisms (k') at any one time becomes

$$k' = Dw/v + ew/v + k'_o\, e^{wt/v}. \tag{6}$$

From equation (2), the amount of substrate leaving the chamber (c) is a function of microbial action plus dilution factors, but by replacing a certain concentration within the chamber and allowing this concentration to be diluted out without any added substrate (a), the equation becomes $c = a + c_o e^{wt/v}$, thus at any one time the concentration of a substrate within the chamber is equal to the amount in the chamber (a) and the natural log of the flow rate ($e^{wt/v}$). We may predict then, that flow from the chamber is a log function of flow rate.

This may be expressed in any form using the general equation for first order kinetics. This may be rewritten as $c = a + b \cdot \log (wt/v)$ where log (wt/v) may be expressed as any function of volume, i.e., tube number of constant volume, etc., which becomes independent of time (t) as (a) becomes a fixed value in that an original concentration of a_o is not being replaced.

Legend of terms for formulae:

a = concentration of substrate flowing into growth chamber

c = concentration of substrate flowing out of growth chamber

D = concentration of product in the growth chamber

e = concentration of product leaving growth chamber

k = amount of substrate utilized by microorganisms

k' = amount of by-product produced by microorganisms

Testing of Apparatus. Initial experiments were concerned with the physical operation of the growth chamber (establishing and maintaining a constant flow rate and determining dilution factors) to find a satisfactory method whereby the flow of medium through the growth chamber could be maintained at a satisfactory rate.

The chamber was placed in two different positions: horizontally on a shaker and vertically above a magnetic stirrer. In the horizontal position, an old sewing machine cycle system was attached to a wooden box (25 x 35 x 10 cm.) which was oscillated about sixty cycles per minute through an angle of approximately 35°. The assembled chamber was clamped horizontally inside the box perpendicular to the axis of

motion. The medium used was distilled water with or without centrifuged rumen fluid inside the growth chamber. The liquid was forced through the system via constant pressure from a tank of carbon dioxide and/or vacuum. Marbles, glass beads, a dialysis bag, and filter spacers were placed in the unit and flow rate determined. In each case the methods proved unsatisfactory because of low flow rate or complete lack of flow.

Due to the failure to maintain satisfactory flow rate over extended periods of time with the chamber in the horizontal position, it was decided that a magnetic spin bar rotating on the outlet bacterial filter would maintain homogeneity of the contents inside the chamber and could possibly help prolong filter clearance. A 7/16 inch magnetic spin bar was placed inside the chamber to which 20 ml. centrifuged rumen fluid were added. The chamber was placed vertically on a magnetic stirrer which rotated the spin bar at approximately two turns per second. Flow values were determined every hour for 12 hours as shown in Table I. The first hour flow was approximately twice as fast as the other values which were stabilized by the fourth hour. The results indicate that a magnetic spin bar is satisfactory to maintain constant flow for periods up to 12 hours. In addition, homogeneity of the contents inside the growth chamber can be maintained at all times.

Due to fluctuations of flow rate in chambers treated alike with individual gassing tubes, the efficiency of one source of gas for four chambers was studied with constant gas pressure over a period of 24 hours. As may be seen in Table II, this method did not result in constant flow

TABLE I

RATE OF FLOW OF MEDIUM THROUGH GROWTH CHAMBER USING A MAGNETIC SPIN BAR

Time (hr)	Flow (ml)
1	42
2	26
3	26
4	19
5	20
6	20
7	20
8	20
9	20
10	20
11	20
12	19
Avg.	22.7(6.6)

TABLE II

FLOW RATE OF MEDIUM THROUGH FOUR CHAMBERS FROM A CONSTANT PRESSURE SOURCE

Time (hr)	Flow (ml) Chamber Number 1	2	3	4
4	92	15	6	50
9	125	20	9	60
13	32	16	6	53
16	31	17	6	78
20	12	12	4	50
24	6	8	4	14
Avg Flow/hr	12.4 (8.8)	3.7 (0.8)	1.5 (0.5)	12.7 (7.1)

rates for the four chambers. The regulation of flow, thus would be a function of the material on the filter which would be controlled by the speed and/or efficiency of the magnetic spin bar.

Two sets of experiments were conducted in duplicate to establish that the rate of flow out of the chamber followed first order kinetics (page 15). In the first trial, 20 ml. 0.125 N cobalt chloride were placed into the chamber which was then diluted to capacity (50 ml.) with distilled water. Distilled water was forced through the chamber by oxygen pressure to dilute the solution from the chamber. Ten ml. samples were collected and percentage transmittancy taken at 540 um. Concentrations of cobalt chloride were measured by percentage transmittancy and were plotted against log volume (Figure 1). The salt was diluted out at an ever decreasing rate following first order kinetics.

In the second set of trials, a dilute solution of acetic acid was introduced into the chamber and sampled above as in the first dilution trials. Each ten ml. sample from the outlet tube was titrated with 0.01 N sodium hydroxide to a phenolphthalein end point. The milliequivalents of acid were plotted against log volume (Figure 2) which represents a straight line indicating first order kinetics of dilution.

The function of the outlfow of any component through the system having no impairment by the filter, may be expressed as any function of the first order rate equation with (a) equal to zero and (a_o) a constant.

If (a) is being introduced into the chamber, such as acetate by bacteria, the initial equation must be applied, $c = a + kv/w + c_o v/w\, e^{wt/v}$.

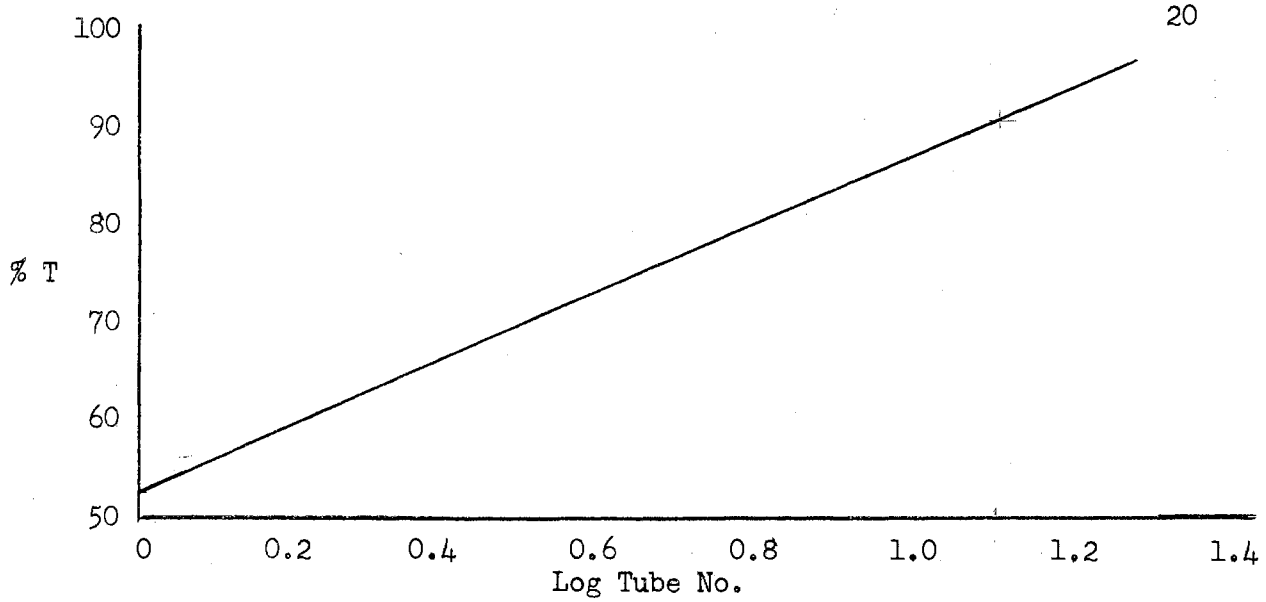

Figure 4. Change in Concentration of Cobalt Chloride With Respect to Log Volume of Outflow

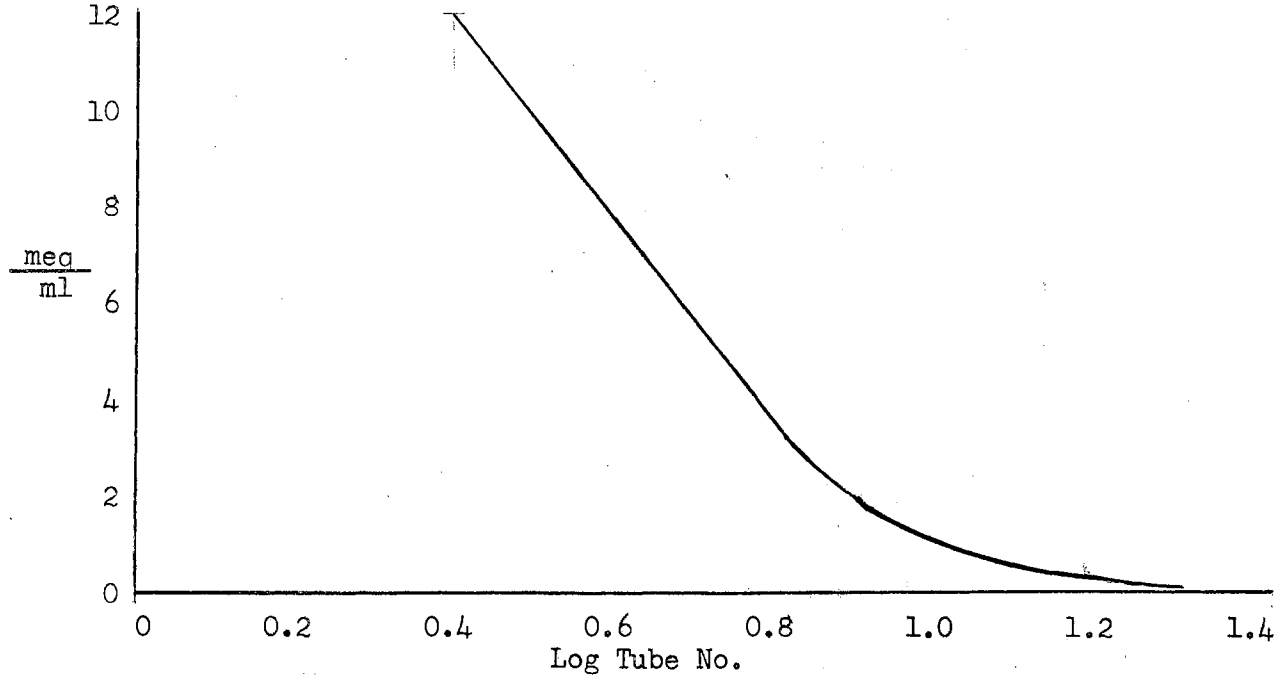

Figure 5. Change in concentration of Acetic Acid With Respect to Log Volume of Outflow

Growth Studies with Escherichia coli. Three experiments using two incubation chambers and 50 ml batch cultures of E. coli were made to determine the growth pattern of this microorganism in the growth chamber.

The growth chambers were sterilized by one of two manners: ultraviolet light sterilization and autoclaving. Initial experiments were made in a growth chamber fitted with a plastic cylinder which could be sterilized only by the use of ultraviolet light. Using this technique of sterilization, the chamber was completely assembled and placed under an ultraviolet lamp for 24 hours. After the chambers were fitted with glass cylinders, the unit was assembled except for the outlet filter and magnetic spin bar which were slipped inside the chamber under sterile conditions after autoclaving. The filter had been previously sterilized (manufacturer) and the spin bar was immersed in 95% ethanol for one minute immediately before placing it inside the chamber. Medium and tubing were autoclaved and assembled under sterile conditions immediately before use.

Nutrient broth was used for all pure culture studies. The solution for each experiment was made in one large container and placed in reservoir flasks for autoclaving. An aliquot was placed in an Evelyn colorimeter tube to serve as a blank for turbidometric determinations of growth. This was autoclaved at the same time as the medium was autoclaved.

Cultures of E.coli were maintained by plating the original culture in nutrient agar and growing specific colonies in nutrient broth at 36.5°C for 18-24 hours. The tubes were placed in the

refrigerator until used. In preparation for chamber studies, the refrigerated cultures were transfered twice. The inoculum was grown 18-24 hours before transfer to the growth chamber.

Each chamber and batch culture had been diluted to a percentage transmittancy of 78 percent at 660 mu with sterilized distilled water. Determinations of growth were made every three hours until the end of the log growth phase after which time samples were taken every six hours. Separate samples of 4.0, 1.0, and 0.5 ml. were aseptically withdrawn from each culture. The percentage transmittances at 660 mu using an Evelyn colorimeter were taken and reported as optical density measurements. The aliquot was further diluted to 10.0 ml. with distilled water and titrated to a phenolphthalein endpoint with 0.01 N sodium hydroxide. These values were recorded as milliequivalents of acid. One 1.0 ml. sample and the 0.5 ml. aliquot were placed in previously tared vials, dried at 100°C for four hours, then further dried at 36°C for eight hours and weighed. The values were recorded as average dry weight per ml. of culture medium. The remaining 1.0 ml. was diluted and plate counts were made using nutrient agar as medium. The values were reported as counts/ ml.

Oxygen was bubbled through the medium to saturate it for maintaining aerobic conditions and to serve as the pressure gas to support flow through the chamber. By previous experimentation it was determined that constant flow could not be maintained because of varied filter permeability, thus no attempt was made to adjust the flow rate.

The following technique was employed in the execution of the experiment with the growth chamber:

Procedure for preparing apparatti:

1. Sterilize gassing and flow apparatti by wrapping in aluminum foil and sterilize for 30 minutes at 15 pounds pressure in autoclave.
2. Attach gassing and flow tubes to chamber and medium reservoir using sterile technique.
3. Open vacuum outlet on medium reservoir and close inlet tube to growth chamber.
4. Allow gas to flush medium for 10 minutes.
5. Open clamp on inlet tube to growth chamber and close flask side outlet.
6. Place sterile needle into T-tube in inlet tube and allow gas to push fluid from reservoir into inlet tubing until partially filled.
7. Attach syringe and remove remaining air.
8. Insert needle (sterile) into growth chamber inoculation port.
9. Apply gas pressure and allow approximately 10 ml. medium to enter the chamber.
10. Add inoculum into chamber by syringe and reapply gas pressure removing air with sterile needle and syringe.
11. Place unit into water bath and adjust speed of magnetic spin bar.

12. Adjust gas pressure by observing bubbling into medium reservoir and place receiving receptacle under outlet tubing.

Procedure for collection of bacterial samples:

1. Remove chamber from water bath and place horizontally with inoculation tube upward.
2. Swab rubber plug with 95% ethanol and insert sterile needle and syringe to remove desired volume (slowly).
3. Turn chamber 180° and slowly remove needle as pressure differences force plug or fluid out.

Growth Study with Bacillus cereus. A growth study was made with this microorganism to determine the growth pattern of a facultative anaerobe in the growth chamber. The preparation of the growth chamber and medium and preparatory culture of the organism was the same as given in the procedure for E. coli. One ml. of a 24 hour culture of B. cereus was inoculated into the growth chamber which was maintained at 36.5°C with oxygen as the gas source. Growth (24 hours) was determined by measuring the percentage transmittancy at 660 mu using an Evelyn colorimeter.

Growth Study with Clostridium sporogenes. Four trials using this microorganism were performed to determine the growth pattern of this strict anaerobe in the growth chamber. The preparation of the experiment was followed as given in the outline for E.coli. The culture was allowed to grow in the absence of flow until visible growth appeared

and observed until the maximum growth was reached as measured turbidometrically. Carbon dioxide was used to propel the medium and to establish and maintain anaerobiosis.

RESULTS AND DISCUSSION

Growth Parameters of Escherichia coli. Optical density, plate counts, dry weight, and acid production were used to determine the growth pattern of E. coli in the growth chamber as compared to that in batch cultures(Tables III and IV).

Optical density measurements indicate the log growth phase began the fifth hour of incubation and reached the stationary phase the 16th hour for the batch culture and first chamber. The growth in the second chamber was slower and reached a maximum (lower) after 20 hours. The log growth phase was apparent in the plate counts after 2 hours and reached the stationary phase between 8 and 10 hours. The dry weight data indicate a general trend in increased growth, but were not sensitive enough to change in numbers as did optical density and plate counts. The samples taken (0.5 and 1.0 ml.) did not contain enough microorganisms to denote any change as the dry weight of the medium may well have been greater than the microbial weight.

Acid production data are in direct opposition to that of the other criteria of response. This discrepancy, particularly in the batch culture is unaccountable since acid production should have followed the growth pattern (Table IV). In the chambers, more acid was

TABLE III

COMPARISON OF BATCH AND CHAMBER GROWTH PATTERNS OF *E. COLI*

Time (hr)	Chamber 1			Chamber 2			Batch Culture		
	OD	Mg/Ml	Plate Count	OD	Mg/Ml	Plate Count	OD	Mg/Ml	Plate Count
0	0.000	7.2(0.2)[1]	$6x10^4(10^2)$	0.000	7.2(0.2)	$1x10^4(10^2)$	0.000	7.2(0.2)	$75x10^4(10^1)$
5	0.029	6.8(0.8)	$24x10^6(10^2)$	0.023	7.6(0.8)	$65x10^5(10^2)$	0.034	7.2(0.9)	$1x10^9(10^2)$
8	0.062	6.6(0.6)	$78x10^7(10^2)$	0.029	6.0(0.5)	$74x10^6(10^2)$	0.120	8.2(0.4)	$48x10^{11}(10^1)$
12	0.085	8.0(4.8)	$2x10^8(10^1)$	0.034	5.5(1.2)	$33x10^7(10^2)$	0.144	5.4(1.2)	$60x10^{11}(10^4)$
20	0.252	8.2(1.3)	$4x10^8(10^2)$	0.138	7.2(0.7)	$32x10^8(10^1)$	0.271	7.0(1.6)	$5x10^{10}(10^3)$
26	0.228	--------	$67x10^7(10^3)$	0.097	--------	$20x10^8(10^2)$	0.197		$12x10^{10}(10^1)$

[1]Standard Deviation.

TABLE IV

COMPARISON OF ACID PRODUCTION OF *E. COLI* IN BATCH AND GROWTH CHAMBER CULTURES

Time (hr)	Chamber 1		Chamber		Batch Culture
	Flow Rate (ml/hr)	Total Meq Acid Prod	Flow Rate (ml/hr)	Total Meq Acid Prod	Total Meq Acid Prod
0	0.0	0.000	0.0	0.000	0.000
5	9.0	0.346	14.4	0.512	0.187
8	12.0	0.292	23.7	0.447	0.175
12	3.0	0.254	11.5	0.325	0.150
20	7.4	0.377	16.0	0.368	0.131
26	14.5	0.517	14.3	0.439	-----

produced with the larger flow rate (Chamber 2) but had the least number of microorganisms as reflected by plate count and optical density measurements.

Four series of experiments were made to determine the effect of various flow rates on maximum growth of E. coli using oxygen as the medium propellant. The results of the individual maximal optical density values at the beginning of the stationary phase and the mean flow rates are given in Table V. The results indicate maximum growth density in independent of flow rate and therefore of nutrient supply as long as the nutreint supply is above that of the needs of the microorganism. A correlation coefficient of - 0.284 was found among all values. It was assumed that under the conditions of the experiments, time was the only variable.

TABLE V

COMPARISON OF FLOW RATE AND MAXIMUM OPTICAL DENSITY OF E. COLI

Maximum Optical Density	Mean Flow Rate (ml/hr)
0.100	14.2
0.138	15.5
0.143	11.3
0.144	21.6
0.150	14.2
0.210	21.6
0.252	9.2

The growth rate of E. coli under carbon dioxide propellant as measured by turbidity may be seen in Table VI. Rate of fermentation by both cultures remained close together during the first six hours of incubation (log phase). The turbidity of the batch culture, after this time and for the remaining fermentation period, remained higher than the turbidity of the growth chamber. This may be explained by the facts that the batch cultures were not under carbon dioxide pressure to the same extent as the chambers or the chambers may have had a leak, although the fluid passing out of the chambers remained visually clear. The drop in O.D. of Chamber 2 could be explained by the presence of a visual leak between the 21st and 27th hour of incubation. The increase in growth in Chamber 1 cannot be explained, however, growth rate may have increased due to the increased flow rate but this cannot be accurately determined since Chamber 2 could not be used for comparisons.

The acid content increased in the chambers (Table VI) after the growth had reached a maximum and was in the stationary phase. This rate continually increased during the remainder of the experiment but may have been due to discrepancies between acidity of the incoming medium and that in the chamber. The decrease in acid in Chamber 2 may be explained by the cracked filter. The continued differences between the chambers with respect to total acid may have been due to total numbers of microorganisms although the turbidity data did not bear this out.

TABLE VI

EFFECT OF CARBON DIOXIDE PROPELLANT ON GROWTH OF *E. COLI* IN THE GROWTH CHAMBER

Chamber No. 1

	Chamber		Outflow		
Time (hr)	Optical Density	Meq Acid per 4 ml	Flow Rate(ml)	Meq Acid per 4 ml	Total Meq Acid Prod
0	0.000	0.032	----	0.032	-----
3	0.060	0.041	13.6	0.043	0.717
6	0.123	0.050	26.0	0.037	0.725
9	0.146	0.041	13.7	0.044	0.451
12	0.126	0.057	37.3	0.048	1.344
21	0.162	0.107	17.8	0.051	2.048
27	0.210	0.123	25.0	0.082	3.075

Chamber No. 2

	Chamber		Outflow		
Time (hr)	Optical Density	Meq Acid per 4 ml	Flow Rate(ml)	Meq Acid per 4 ml	Total Meq Acid Prod
0	0.000	0.033	----	0.032	-----
3	0.059	0.040	5.3	0.031	0.125
6	0.117	0.050	16.7	0.034	0.425
9	0.132	0.047	10.7	0.046	0.368
12	0.126	0.053	16.3	0.052	0.637
21	0.150	0.080	16.8	0.073	2.782
27	0.086	0.073	14.2	0.049	1.045

Batch Cultures

Culture No. 1			Culture No. 2		
Time (hr)	Optical Density	Meq Acid per 4 ml	Time (hr)	Optical Density	Meq Acid per 4 ml
0	0.000	0.032	0	0.000	0.032
3	0.065	0.032	3	0.068	0.033
6	0.144	0.031	6	0.123	0.032
9	0.144	0.032	9	0.138	0.028
12	0.173	0.035	12	0.173	0.035
21	0.185	0.032	21	0.180	0.029
27	0.188	0.031	27	0.195	0.031

Growth of Clostridium sporogenes. Two different cultures of Cl. sporogenes were grown in the chamber to determine the growth pattern of a strict anaerobe in the growth chamber. In the first trial, the log growth phase started after the seventh hour and remained in the log growth phase until 34 hours (Figure 6) when the microorganisms apparently sporulated. The reason for the sporulation may be correlated with the decrease in available nutrients due to the decreased flow rate which became critical at the peak of growth. The decline in flow rate (Figure 6) was believed due to the increased number of microorganisms that clogged the filter. There was no visible evidence of microorganisms in the outflow liquid.

In the second trial, three chambers were used to determine the effect of rate of flow on the growth pattern, particularly, the maximum population density. As may be seen from Table VII, the combinations of all trials indicate growth is proportional to flow rate ($\hat{Y} = 1.38X + 3.41$).

TABLE VII

GROWTH RESPONSE OF CL. SPOROGENES TO FLOW RATES

maximum O.D.	flow rate (ml/hr)
0.071	2.50
0.096	3.54
0.137	5.00
1.456	18.00

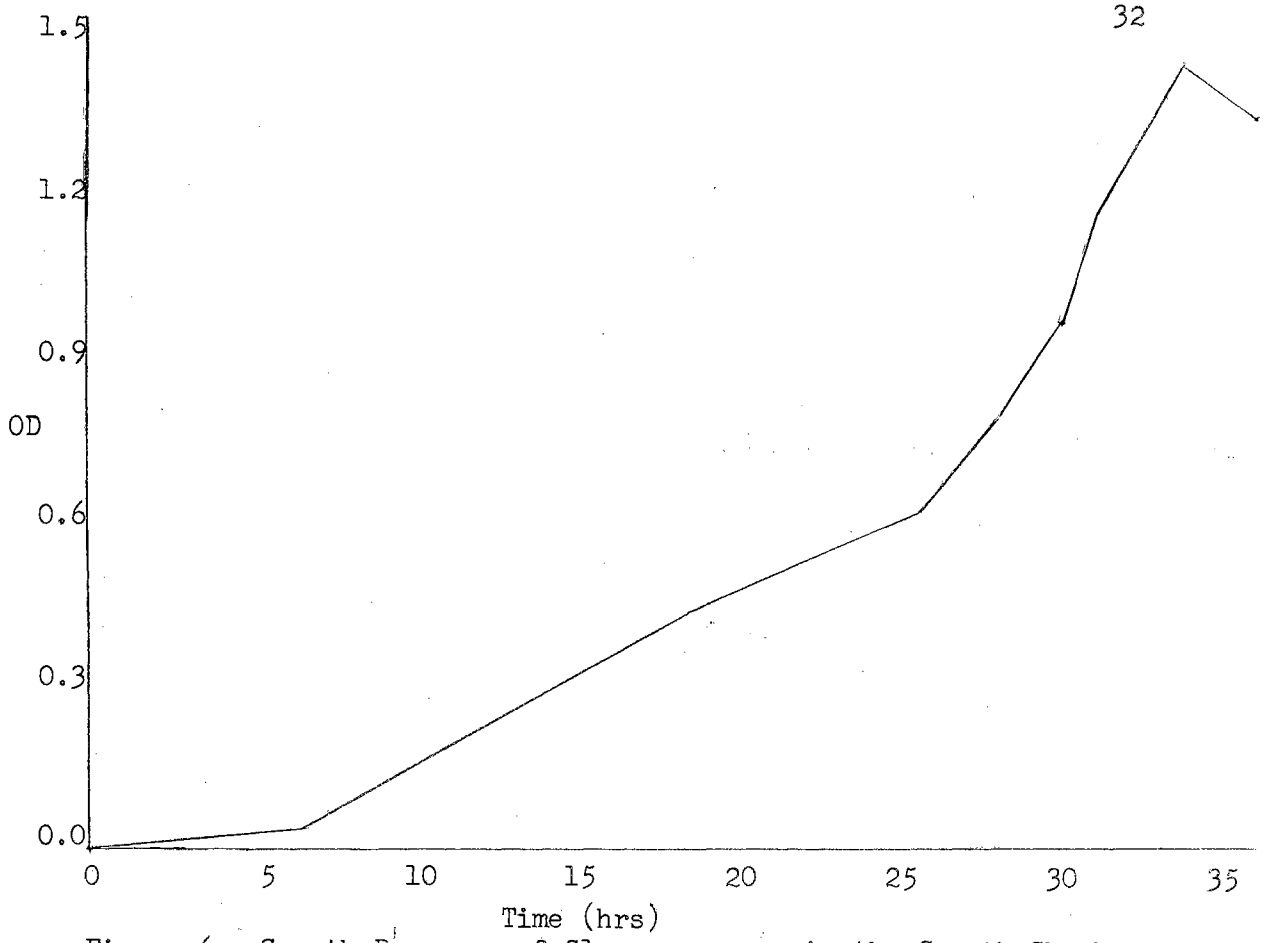

Figure 6. Growth Response of Cl. sporogenes in the Growth Chamber.

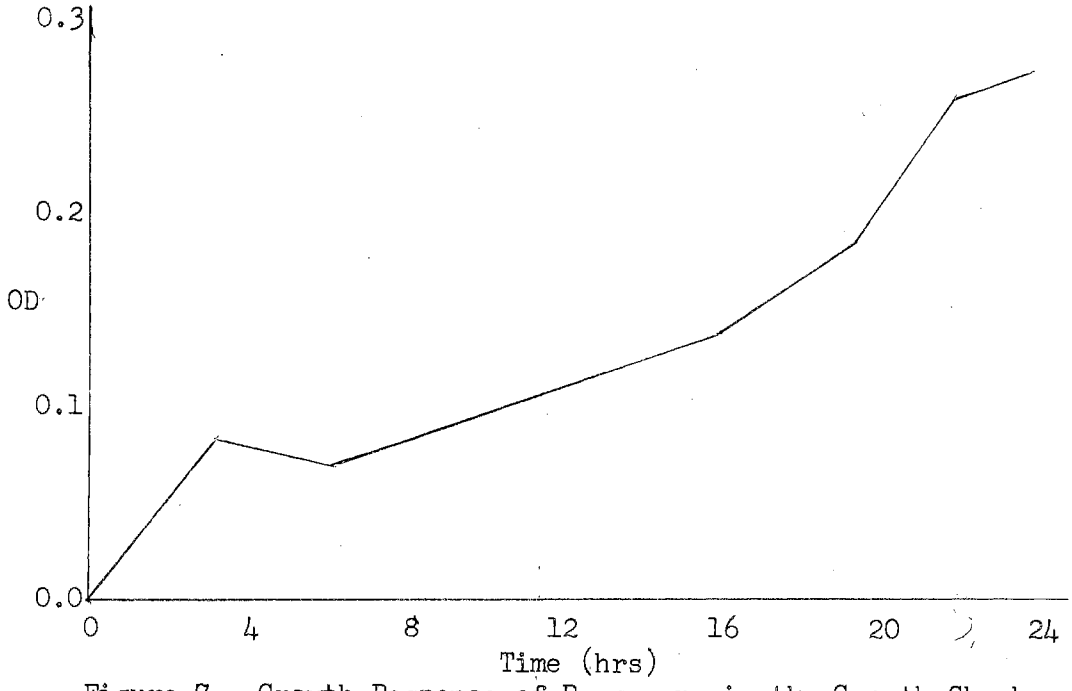

Figure 7. Growth Response of B. cereus in the Growth Chamber.

Growth of Bacillus cereus. The growth of B. cereus as measured by optical density continued in the growth phase 22 hours and began to approach the stationary phase by 24 hours (Figure 7). Even with oxygen as the gas source, this microorganism did not grow as well as expected under the conditions of the experiment.

Pure Cultures in the Growth Chamber. From the results of the E. coli studies it is apparent that this microorganism does not grow well in this chamber due to the limited amount of available oxygen. Other oxygen sources may increase growth but the system was not itself favorable to these microorganisms.

B. cereus showed better growth than E. coli, but this was probably a characteristic of the microorganism rather than the group.

Clostridium sporogenes, a strict anaerobe, has given the best growth of the pure cultures studied. The increase in growth with increased flow rate makes it possible to study growth patterns by varying flow rate as well as limiting nutrient.

From these data it appears that the growth chamber is more adaptable for anaerobic cultures than aerobes, however, growth patterns should be determined for each microorganism.

SUMMARY

The theory and successful application of the growth chamber to three pure cultures have been made. Gas sources are limited to those

which can be dissolved in the medium which limits growth of aerobic cultures. Anaerobes may be successfully maintained in this system.

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CONTINUOUS FLOW ARTIFICIAL RUMEN STUDIES

INTRODUCTION

The anatomy of the ruminant stomach is such that it becomes in a sense a continuous flow system. The esophagus is the inlet tube while the reticulum and rumen wall become the outflow system. Normal fermentation inside the rumen would logically be extended to the capacity of the limiting nutrient in the feed, the length of time the mixture remains in the rumen and the concentration of the by-products. In order to replicate this system, duplication of this flow should be made in some manner. There are few reports in the literature concerned with the use of such systems, therefore, two continuous flow systems were evaluated as possible methods of study of rumen fermentation.

REVIEW OF LITERATURE

The means of studying rumen rumen microorganisms and their activities in isolated systems may be roughly divided into nine methods: direct microscopic observation, pure cultures, fluorescent antisera detection, stabilized enrichment cultures, cell-free enzyme systems, *in vivo* artificial rumen, *in vitro* artificial rumen, washed cell suspensions, and continuous flow systems. In studying the overall reactions the last four have been of greatest value and will be described.

In Vivo Artificial Rumen. Two basic principles are used in this technique: the alteration of either the rumen *per se* or the introduction inside the rumen of an isolated substrate which may be removed at any desired time.

Nichols (1953) isolated a rumen pouch from the dorsal posterior rumen sac without the aid of a distensor. Upon examination (post mortem) it had shrunk to approximately half size after four months. Komarek *et al.* (1960) successfully isolated a pouch of approximately two liters to be representative of the rumen and to include mucosa of both dorsal and ventral sacs without disturbing the innervation and vascular system. The mucosa is likewise expected to approximate that of the normal intact rumen. This type of compartmentalization was preceded by Tsuda'a rumen Pavlov pouch (Komarek *et al.*, 1960).

Substrates with or without selective compartmentalization have been

bathed in the liquor inside the rumen. Hoflund et al. (1948) suspended cotton thread in the rumen with a support to study digestibility in sheep as compared to fluid incubated in a flask with a cotton string. Agreement was very good between in vitro and in vivo digestion, however, the in vitro digestion consistently remained slower than that in vivo. The methods of the determination, however, may well be within experimental error. Quin (1943) used the same technique with fasting animals. In addition surgical gut was used in place of cotton string to study proteolytic activity.

Fina et al.(1958) placed rumen bacteria and cellulose in a semi-permeable porcelain tube and incubated this in the rumen. A protective sleeve was added to prevent contamination and facilitate gassing. In inoculated tubes the digestion of 500 mgm. cellulose was completed in about 48 hours. An initial inoculum continuously digested added increments of cellulose to the extent of ninety percent over a ten-day period. It was found that bacteria were unable to penetrate this porcelain tube. In addition, urease, a small molecular weight enzyme, was not permeable. In vitro studies disclosed, however, that the tubes were permeable to acetic, propionic, valeric, isovaleric, and caproic acids, and substances such as Evan's blue, methyl red, urea, and glucose.

In Vitro Artificial Rumen. The purpose of an artificial rumen is to approximate rumen conditions to study certain metabolic patterns for overall reaction(s) using a complete rumen bacterial population outside the animal body.

For an artificial rumen to be a valid method of studying rumen microorganisms, it should, to some extent, resemble *in vivo* conditions. An excellent treatise on technique validity has been reported by Warner (1956). The criteria have been based on bacterial populations, fermentation of usual substrates, and interactions between substrates. These criteria will be briefly discussed here.

Numbers of microorganisms. The numbers of microorganisms before and after incubation cannot be used to estimate true mean generation times owing to the complexity of the system because protozoa consume bacteria in large numbers, protozoa consume other protozoa and bacteria, and selemonads possible consume protozoa.

Motility of microorganisms. This includes the maintenance and numbers of normal appearance of bacteria, selemonads, and protozoa. These microorganisms should be present after fermentation. By adding substrate it appears that only the dividing protozoa could be used as criterion for multiplication.

Maintenance of normal rates of digestion of cellulose, starch, and protein and of normal interactions between these components. The use of cellulose digestion as a function of ruminal activity may not be valid in all cases as the actual rate of cellulolysis in the rumen as distinct from the overall rate for the whole animal is of doubt. Next, the rate limiting step in cellulose digestion is unknown; if it occurs at an early stage, 24-hour incubation *in vivo* may be different from that of a new inoculum *in vitro.* Because of essentiality in straining

rumen fluid, some decrease in rate may be expected. Using a dialysis bag Warner (1956) found a rate of digestion of ten gms. cellulose/liter rumen liquor/ day. Assuming the sheep's rumen contains approximately a five-liter capacity, it was found that in vivo digestion was approximately 50 gm./ day. This then is in very good agreement.

The rate of starch digestion has been found to vary with the diet of the animal. Starch in the diet enhanced digestion in vitro. A similar situation was noted with casein which reached a peak in 6-8 hours. In the rumen, 4-5 hours; it had passed through the abomasum in 8-10 hours. The in vitro seems to be intermediate. Here again a lag phase may be involved although no data are available to substantiate this point.

The end products of digestion, ammonia and volatile fatty acids, agree fairly well in vivo and in vitro. It would logically follow that if a lag phase is observed in rate of digestion, rate of end-product would follow accordingly.

Ability to predict quantitative results in vivo. Biological criteria are simplier to apply and are of more value in long term experiments. Criteria such as rates of digestion of substrate or of production of metabolite(s) can be quite sensitive over short periods of time. In this connection, the expression of results in units such as gms./ liter/ hour or day rather than as digestion or other relative values would make comparisons easier between different techniques.

Not only must the physical environment in an artificial rumen

approximate normal (maintenance of suitable temperature, pH, gas phase, provision for removal of metabolites) but also the substrates tested must approximate in nature and quantity to the diet of the animal from which rumen liquor was taken, if in vitro results serve as results in vivo. In vitro techniques may show or predict what new products will do, but in this adaptation the behavior of the first few days following the diet change is unpredictable. This adaptation period does, however, seem prolonged as far as induction by microorganisms is concerned. The problem then becomes one certainly of both rumen population and the host animal. Conjecture would advocate an adjustment by the microflora and microfauna followed by a gradual change in the ruminant animal itself. This phenomenon has not yet been explored.

Chemical criteria have been attributed mostly to proper cellulose digestion although other relative values as protein synthesis, ammonia and volatile fatty acid production have been used. Little attention has been paid to rate of digestion which seems to be a very logical criterion for measurement of activity.

It is Warner's contention that in no case where incubation has been continued for more than a few hours has complete success been reached in meeting even the few criteria most workers tend to adopt. In addition, not much has been done to estimate the validity of the results in terms of normal rumen function, and some of what has been done is open to criticism.

Advantages and disadvantages of the in vitro artificial rumen techniques. The artificial rumen has been found to be an effective screening device for various products. The precision is usually good, the cost low, and time in obtaining results short.

On the other hand the results may not be representative. End products resulting cannot be controlled and different types of organisms may develop in different trials on the same substrate. There is activity in the absence of substrate indicating a proliferating population coupled with ever-present end products due to insufficient separation in processing of samples. To do so, however, may deplete unknown essential co-factors. Population increases are ever present which may have been altered because of substrate and/or environmental changes.

The in vitro artificial rumen systems may be divided into four main types: 1) undiluted or slightly diluted rumen liquor incubated with substrate in an all-glass impermeable system, 2) whole rumen liquor diluted to one-half strength with a mineral mixture resembling saliva and incubated with a substrate in an impermeable system, 3) various fractions of rumen liquor used in an impermeable system, and rumen liquor, whole or diluted, with a substrate in a semi-permeable system with contents dialyzed against a mineral solution.

Rumen liquor in an all-glass impermeable system. One of the first successful attempts to use this system was made by Pearson and Smith (1943). With undiluted samples of 2-3 liters rumen contents were transferred to a flask packed in a bucket of sawdust. This was

strained through muslin and incubated at 39°C. These workers reported that their system remained normal for 2-4 hours and that numbers and types of, mainly iodophilic bacteria, showed no significant changes during that time. Using the same technique McNaught and Owen (1949) showed the concentration of o-phenanthroline which suppressed urea utilization *in vitro* corresponded to a concentration of ferrous ion of the same order as found *in vivo*. Gray *et al.* (1951) inoculated fodder samples with rumen fluid in an Erlymeyer flask fitted with inlet and outlet tubes to flush the system with carbon dioxide or nitrogen either before or after introduction of inocula. Anaerobic state was preserved by closing the outlet tube with a water seal and incubated at 38-40°C. From these studies a number of criteria were successfully applied to their system: 1) microscopic observation of the activity of the protozoa, 2) the ratio of methane produced to fodder supplied, 3) digestion of cellulose and pentosans. All these were found to be similar *in vivo* and *in vitro* but the rates of digestion of the substrates and methane production were one-half that of *in vivo*. McNaught (1951), using a similar system free of protozoa, found the carbon dioxide to methane ratio of evolved gasses similar to that found *in vivo*, although larger. In very short experiments, Quin (1943) and McAnally (1943) found the rate of gas output following glucose addition was similar *in vivo* and *in vitro*.

Whole rumen liquor diluted to one-half strength with artificial saliva in an all-glass impermeable system. Burroughs *et al.*(1950a,

1950b) used a 50% (v/v) inoculum of material from the preceding fermentation and claim that there were no marked changes in numbers, size, or predominant types of bacteria throughout 5-6 successive transfers of 36-hour incubation periods, but that some of the types of protozoa failed to survive. The main criterion used by the workers was, however, cellulose digestion. Although this criterion has been used extensively, Warner (1956) notes two points. First, the absolute account of cellulose was small, approximately 3 gm./ liter rumen contents in early experiments and later 10-20 gm./ liter. Contents were taken from an animal on a high roughage ration. These animals would perhaps receive 50 gm cellulose/ liter rumen contents/ day as well as hemicelluloses, etc., which might well involve the same digestive enzymes so that the actual amount, in gm. cellulose/ liter/ day, is much less in vitro than in vivo. Secondly, the criterion relied on, cellulose digestion, over several fermentation periods does not seem very reproducible. Arias et al. (1951) and Burroughs et al.(1951) used the same system but one fermentation for 40 hours. Brooks et al.(1954) claimed that the numbers of bacteria before and after incubation were approximately equal and the effects of added fat on cellulose digestion in vivo and in vitro were very similar. Examination of the data according to Warner (1956) shows, however, that a dose of 32 gm. corn oil to sheep decreased cellulose digestion from 41.9% to 20.0% while a dose of 160 mgm. corn oil/ 25 ml. diluted rumen liquor in vitro (same concentration assuming a rumen volume of 5 liters) decreased digestibility from 36.2% to 2.2%, much more marked. Brooks et al.(1954) also tested the effect of various

steroids on cellulose digestion *in vivo* and *in vitro* anad again more marked differences were found *in vitro*.

Various fractions of rumen licour in an impermeable system. Rumen liquor was freed from protozoa by McNaught (1951). Marston (1948) suspended all the rumen microorganisms in a mineral solution and incubated these contents in an elaborate mechanism designed to simultaneously measure various physical constants. A 3.5-liter glass pot, hermatically sealed with a metal lid and rubber gasket, contained six stoppered ports for various measuring devices. An attached stirrer rotated very slowly. All metal parts within the pot were coated with paraffin wax. Ports were used for inlet and outlet of gas, inlet for standard alkali solution, glass electrode for pH, platinum electrode for oxidation-reduction potential, calomel half-cell as reference potential, a closed pipette to draw samples to check the accuracy of the glass electrode, and a toluene mercury thermoregulator to keep the temperature 40°C $\pm$ 1.5°C.

Rumen liquor, whole or undiluted with substrate in a semipermeable system. Contents in the permeable bag were dialyzed against a mineral mixture as described by Louw (1949) who showed that the semipermeable artificial rumen permitted better digestion than the impermeable system with the same inoculum. Applying the technique of Marston (1948) with different concentrations of substrate it was found that the semipermeable system digested approximately 45% more cellulose than the impermeable system. No evidence was found that the bacteria attacked the cellulose membrane (dialysis bag). This setup is very similar to Warner's (1956)

apparatus which consisted of a 20 x 5 cm. glass tubing enclosed with rubber stoppers and containing a cellophane dialysis bag (12 x 3 cm.). This sack usually contained 50 ml. rumen liquor and substrate. The outer chamber was filled with a mineral solution to the level of the contents inside the dialysis bag. A slow stream of nitrogen with five per cent carbon dioxide was bubbled through both solutions. At the end of the trial, volumes of both compartments were measured and interval times measured. Incubation periods up to eight hours were thought to approximate in vivo conditions. Louw (1949) used a stirring rod instead of gas to aggitate the contents.

Washed Cell Suspensions. A suspension of rumen bacteria is obtained by differential centrifugation, i.e. successive centrifugations at increasing speeds to remove endogenous materials. The advantages of this system are: a controlled non-proliferating population; little activity in the absence of substrate; short experimental period; small amounts of substrate. There are certain disadvantages that may well be noted: possible loss of co-factors; selection of certain organisms; extensive exposure to oxygen; and insoluble substrates do not work well (Thornton, 1952).

Microscopic examination by Doetsch and Robinson (1953) showed that all the chief morphological types of bacteria are present when compared with those of a collection of pure cultures isolated from the rumen. However, because of a mixed population, side reactions may complicate the principle reaction or make it difficult to find immediately.

It is assumed in this system (Doetsch and Robinson, 1953) that the enzymes operating *in vitro* are also operating *in vivo* and that the quantitative aspect, i.e., the same density of cells at each determination is controlled. The qualitative aspect is assumed to remain constant although there is considerable doubt on the basis of density measurements alone. It is well to remember that one is working with an essentially concentrated, mixed, enzymatically competing population. Nevertheless, better control over the fermentation can be had for studying in detail the properties of the mixed rumen population because of the elimination of such complications as absorption and the presence of a range of potential substrates.

Continuous Flow Systems. The criteria for a continuous flow artificial rumen, based on the biochemical activities of the micro-organisms (Adler *et al.*, 1958), are for short periods and the authors make no attempt to evaluate growth rates. The semipermeable systems do remove end-products but no account is made of population growth. The use of a continuous flow system could possibly overcome some of the obstacles that are present in impermeable systems. The requirements for continuous growth (Adler *et al.*, 1958) must include the removal of the "excess" population as well as end products. These workers used a five-necked round bottom flask based on the principle of the chemostat with additional inlets and soutlets for pH readings, temperature control, buffering, and sampling. The incubation chamber was kept at a constant volume with a device to deliver nutrients at will.

Davey et al.(1960) modified the semipermeable system of Warner (1956) by dialyzing the rumen contents against a continuous flow of saline solution over prolonged periods of time. Physiological saline, previously dialyzed against rumen contents to obtain volatile fatty acid equilibria, was run through the apparatus at a rate of 500 ml./day. The saline volume in the container was 2550 ml. and 850 ml. rumen contents were used in the dialysis bag. Criteria of validity were quantitative and qualitative bacteriological data, volatile fatty acid production, pH levels and digestion rates. All of these measurements agree closely with in vivo observations. The butyrate values were, however, slightly higher due to rapid breakdown by rumen epithelium in vivo. The advantage of such a system is the close control over prolonged periods of time by adjustment of saline flow. The life of the dialysis bag (seven days) becomes the limiting factor, however, this may easily be replaced.

MATERIALS AND METHODS

Two types of continuous flow systems, the growth chamber and a modified chemostat, were employed to evaluate their use in *in vitro* artificial rumen systems.

Growth Chamber. Nine series of experiments were conducted with this system to determine its value as an *in vitro* artificial rumen. The chamber was assembled as given in the pure culture procedure (page 21), except that cellulose was placed into the system before complete assembly. The system was not autoclaved due to the large inoculum. The chamber was filled with 50 ml. centrifuged rumen fluid and incubated at 39.5°C for 24 hours. The centrifuged rumen fluid was obtained from steers fitted with permanent rumen fistulae. The ingesta was strained through four layers of cheesecloth into previously warmed thermos bottles and transported to the laboratory where the contents were immediately centrifuged at 450 x g for one minute. The supernatant was decanted and placed into the chamber. The residual cellulose was removed after incubation and determined by the method of Crampton and Maynard (1938). The values were expressed as mgm. cellulose hydrolyzed per 24 hours. At various time intervals rate of flow was measured and visual observation made for the condition of the bacterial filters.

Modified Chemostat. A modified system of that described by Novick and Szilard (1950) and Monod (1950) was used to test the utility of such a system as an *in vitro* artificial rumen. This system could possibly duplicate *in vivo* conditions due to the outflow of microorganisms and by products similar to that of rumen ingesta passing from the rumen to the reticulum.

A 500 ml. round bottom flask was fitted with a four-hole rubber stopper. The medium inlet, 6 mm capillary tubing fitted with a ground glass stopper, extended to approximately 5 cm. above the reaction mixture as did a gas outlet tube (6 mm, ID). A gassing tube (6mm capillary tubing) extended to the bottom of the flask to maintain homogeneity of the mixture and to maintain anaerobiosis. A 6 mm capillary tube extended 2 cm. from the bottom of the flask and was bent at right angles over the water bath to form a siphon for the overflow. The medium reservoir was similar to that described by Rotman (1955). A 500 ml. ground glass reagent bottle and glass extension were inserted into a syringe which served to maintain a constant pressure head.

Centrifuged rumen fluid (150 ml.) plus 2.0 gms. purified cellulose were placed into the flask with 150 ml. medium. The total volume of fluid in the flask was maintained at 39.5°C for 24 hours. At various intervals of time, the amount of fluid was measured and the cellulose in the outflow separated from the fluid by centrifugation. At the end of the incubation period, the cellulose remaining in the flask and that collected from the outflow were measured (Crampton and Maynard, 1938). Criterion of response was cellulose hydrolysis as

related to flow.

Three types of media (inorganic, organic, and cell-free rumen fluid) were utilized to determine the value of each as a nutrient source with respect to cellulose hydrolysis at various flow rates.

The inorganic medium was that used by Cheng et al. (1955) for washed cell suspensions. Urea was added to make 0.1% solution which served as the sole nitrogen source. The medium was used without autoclaving. Nutrient broth was employed to determine its use in the artificial rumen. The procedure of preparation is the same as that for pure culture studies (page 21). Cell-free rumen fluid as defined in this thesis is the filtered solution obtained directly from the rumen via suction through a semi-permeable porcelain tube. The porcelain tube and connection apparatus has previously been described in detail by Fina et al.(1958). The operation was modified by connecting the outlet tube to a 1-liter suction flask and applying a vacuum (aspirator) to withdraw fluid from the rumen. The entire apparatus was sterilized before use by autoclaving at 15 lbs. pressure for 30 minutes. The preparation of the porcelain tube for use has been described (Fina et al., 1958). After the fluid had been withdrawn from the rumen, the tubing was clamped and the apparatus taken to the laboratory where the tubing was aseptically removed and the fluid either used immediately or stored at 4°C until used. The fluid was clear and the color resembled that of nutrient broth. The brownish color was assumed to be material from the ration. A precipitate was noticed that was dissolved by carbon dioxide which lowered the

pH from 7.2 - 7.5 to 6.0-6.3. The vacuum was thought to have removed some of the volatile fatty acids while the pH was below neutral and carbon dioxide which resulted in a higher pH than that of the contents inside the rumen.

RESULTS AND DISCUSSION

Growth Chamber. The data from the three types of media have been summarized in Tables VIII, IX, and X. Correlation coefficients were made to determine the significance of flow rate on cellulose hydrolysis.

Cellulose digestion has been expressed as gm. cellulose hydrolyzed/ liter rumen fluid/day to standardize inocula volume. The work of Warner (1956) gave supporting evidence that this velocity expression quantitizes in vivo hydrolysis with in vitro data more accurately than fractional units. A second expression of cellulose hydrolysis (gm. cellulose hydrolyzed/liter rumen fluid/ liter medium) was used to standardize both inocula volume and digestion on the basis of flow rate. This expression appears the most logical in correcting for flow rate variation since exact replication of flow rate has been found virtually impossible.

The effect of varied flow rate on cellulose hydrolysis using inorganic medium has been summarized in Table VIII. A decrease was noted in rate of hydrolysis as flow rate increased from 1.4 to 8.6 ml/hr and was greatly decreased when expressed on a medium basis. Non-significant correlation coefficients of -0.337 and -0.167, however, were found between mean flow rate and velocity of hydrolysis and velocity of hydrolysis/liter medium respectively. Variation may

TABLE VIII

EFFECT OF INORGANIC MEDIUM ON CELLULOSE HYDROLYSIS BY RUMEN MICROFLORA IN THE GROWTH CHAMBER

Mean Flow Rate(ml/hr)	Turnover No. (hr)	Velocity[1] (gm/l)	% of Standard	Velocity/Liter[2] (gm/l^2)
1.4	35.71	4.916	101.9	146.300
8.6	5.81	1.590	33.0	7.696
9.0	5.56	0.932	19.3	4.306
9.6	5.21	0.462	9.6	2.005
9.7	5.15	0.333	6.9	1.419
11.5	4.34	1.642	34.0	5.944
19.5	2.56	1.028	21.3	2.200

[1] Gms. Cellulose Hydrolyzed/ Liter Rumen Fluid/ Day
[2] Gms. Cellulose Hydrolyzed/ Liter Rumen Fluid/ Day

may well be due to differences in digestibility among samples as found in all in vitro artificial rumen systems. The data indicate, that for all practical purposes, flow of inorganic medium was detrimental to cellulose hydrolysis. Theoretically, the absence of flow (extrapolation to zero flow) should give greater hydrolysis. The increase in velocity at higher flow rates can be explained, partially, by the effect of medium as reflected in the velocity corrected for a constant medium fraction. These differences may be due to three errors. A nutrient could be limiting in the medium or perhaps a cofactor or nutrient produced by other microflora in the chamber which was washed out before utilization. On the other hand, cellulase may have been washed out before hydrolysis was completed.

Gill and King (1956) suggested that cellulase is an extracellular enzyme and could thus have been removed from the chamber with the added action of the magnetic spin bar.

It appears thus, that inorganic medium is not sufficient to promote cellulose hydrolysis except at very low flow rates.

From the reults of the use of inorganic medium, nutrient broth was used as a source of medium for the growth chamber at various flow rate levels. The effect of varied flow rate on cellulose hydrolysis is given in Table IX. Flow rate had little effect on velocity of hydrolysis ($r = -0.220$) but decreased hydrolysis with respect to velocity/liter of medium ($r = -0.815$, $P < 0.01$). The optimum flow rate appears to be between one and four ml./hr. corresponding to a minimum turnover of 12 hours. This flow may be correlated to a theoretical turnover in the rumen of a 12-hour interval between feeding periods. Only in the case of the 26.3 hour turnover was hydrolysis greater than that obtained in the impermeable system (4.823 gm. cellulose hydrolyzed/liter rumen fluid). This amount is still below the suggested amount of 50 gm. in vivo (Warner, 1956).

These data indicate that nutrient broth did not increase cellulose hydrolysis above that of inorganic medium, attesting that a limiting nutrient(s) is not a factor or that the limiting nutrient(s) is not present in the nutrient broth.

In order to test the hypothesis of a limiting growth factor or nutrient, cell-free rumen fluid was used as the medium source. As

TABLE IX

EFFECT OF NUTRIENT BROTH ON CELLULOSE HYDROLYSIS BY RUMEN MICROFLORA IN THE GROWTH CHAMBER

Mean Flow Rate (ml/hr)	Turnover No.(hr)	Velocity (gm/l)	% of tandard	Velocity/Liter (gm/l^2)
0.5	100.0	0.596	12.4	49.665
1.0	50.0	3.854	79.9	160.596
1.5	33.3	3.548	79.8	98.528
1.9	26.3	5.278	109.4	115.694
3.7	13.5	2.330	48.3	26.236
11.3	4.4	0.426	8.8	1.572
12.0	4.2	1.314	27.2	4.560
12.4	4.0	0.292	6.1	0.981
12.4	4.0	3.056	63.4	10.268
12.7	3.9	0.524	10.9	1.719
13.8	3.6	0.276	5.7	0.834
14.4	3.5	2.528	52.4	7.306
15.0	3.3	2.056	42.6	5.716
18.8	2.7	0.142	2.9	0.315

may be seen from Table X, the velocity of hydrolysis was independent of flow rate ($r_{=}$ - 0.122). The hydrolysis at 2.5 ml./hr. appears to be low,however, in correcting for flow (hydrolysis/liter medium) this value is adjusted somewhat and this set of values approaches significance ($r_{=}$ - 0.909). The rate of hydrolysis with 2.6 hours turnover appears near that of <u>in vivo</u> hydrolysis. This value would substantiate the hypothesis of limiting nutrient for the inorganic and nutrient broth media. Trials were limited due to the extreme difficulty in obtaining the cell-free rumen extract.

TABLE X

EFFECT OF CELL-FREE RUMEN FLUID ON CELLULOSE HYDROLYSIS BY RUMEN MICROFLORA IN THE GROWTH CHAMBER

Mean Flow Rate (ml/hr)	Turnover No. (hr)	Velocity (gm/l)	% of Standard	Velocity/Liter (gm/l^2)
2.5	20.00	2.725	56.5	45.400
19.1	2.62	15.210	315.4	33.160
25.0	2.00	0.415	8.6	0.690

Assuming all cofactors and nutrients are available in the cell-free rumen fluid, the limiting hydrolysis appears to be due to the excessive stirring of the microflora by the magnetic spin bar which released cellulase from the microorganisms and resulted in loss of activity by the passage of the enzyme from the chamber and/or denaturation.

These data indicate that under the specified experimental conditions, cell-free rumen fluid is the best source of medium for use in the growth chamber as in in vitro artificial rumen system. Identification of these factors could alleviate the necessity of the fluid. Factors that influence cellulose hydrolysis have been found by Bentley et al. (1954; 1955) and include the short-chained fatty acids, valeric, caproic, iso-butyric, and iso-valeric; the amino acids, valine, proline, leucine, and isoleucine (Dehority et al, 1957). These factors were assumed available in the cell-free rumen fluid.

Modified Chemostat. The results of the use of a modified chemostat as an artificial rumen have been summarized in Table XI. The use of inorganic medium as the nutrient source did not increase cellulose

TABLE XI

THE EFFECT OF THREE TYPES OF MEDIA AT VARIED FLOW RATES UPON CELLULOSE HYDROLYSIS IN A MODIFIED CHEMOSTAT

Medium	Mean Flow Rate(ml/hr)	Turnover No.(hr)	Velocity (gm/l)	% of Standard	Velocity/Liter (gm/l^2)
1	12.7	23.6	1.321	27.4	4.33
1	18.1	16.6	2.767	57.4	6.36
2	5.6	53.6	1.610	33.4	11.98
2	14.6	20.5	2.189	45.4	6.24
3	5.2	57.7	4.942	102.5	39.78
3	28.3	28.3	4.252	88.2	6.26
3	60.0	5.0	1.721	35.7	1.20

hydrolysis over that of the standard glass impermeable system indicating that microorganisms were either washed out of the system or environmental conditions were not satisfactory or both. The increase in hydrolysis with increased flow rate, however, would tend to eliminate the former explanation in favor of the hypothesis of a "diluting out" of essential nutrients in the extracellular fluid. The use of nutrient broth as compared to inorganic medium did not increase cellulase activity over that of inorganic medium with respect to velocity. Correcting for flow rate showed an increase in hydrolysis with decreased flow rate indicating that low flow rate may influence activity. The

use of cell-free rumen fluid at the low flow level indicates the inorganic medium and nutrient broth were deficient in growth factors as evidenced by the hydrolysis of 102.5 percent of that of the normal standard. With increased flow rate and therefore increased washout rates, decreased hydrolysis was also apparent with this medium. It thus appears that this system is capable of reproducing cellulolytic activity similar to that in the impermeable glass system, but does not approach the rate of 10-20 gm./liter in Burrough's data (1950) as calculated by Warner (1956) nor to the theoretical value of 50 gm./day as reported by Warner(1956).

The values reported may well be below absolute rate as the cellulose washed out of the unit was added to that remaining in the flask at the end of incubation. With complete mixing, the cellulose was washed out at decreasing rates according to the amount in the flask at any one given time, thus supplying less amount of substrate with time. The importance of this aspect with the washout rate of microorganisms is unknown.

SUMMARY

Two continuous-flow systems, the growth chamber and a modified chemostat, have been evaluated as possible culture vessels for in vitro artificial rumen investigations.

Twenty four trials involving three different media and various flow rate indicate that the growth chamber is suitable as an artificial rumen at low flow rates using cell-free rumen fluid. Cellulose

hydrolysis is not significantly different from that of an all-glass impermeable system.

A modified chemostat has been evaluated as a possible artificial rumen culture vessel. The results of seven trials indicate the system is comparable to the all-glass system with the use of cell-free rumen fluid at low flow rates. Cellulose hydrolysis was the only criterion used in this evaluation.

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IN VITRO FRACTIONATION STUDIES OF CELLULOSE DIGESTION

INTRODUCTION

Animal variation influences results of *in vitro* artificial rumen trials as in any other type of measurement. With respect to cellulose hydrolysis, variations are assumed, due chiefly to the nutritional environment and genetic makeup of the host animal. Consistent differences in cellulose digestion among animals treated alike would indicate direct differences in the activities of the microflora or indirect differences due to inherent environmental conditions of the host animal which alter optimum conditions for microbial action.

The role of microorganisms and their nutrients in two animals of different cellulose digestibilities is the subject of this chapter.

REVIEW OF LITERATURE

A search of the literature has failed to reveal any work with respect to the addition of various fractions of rumen fluid from more than one ruminant mixed together in the same fermentation flask. Various fractionation studies of rumen fluid and microflora have, however, revealed quantitative and qualitiative differences that warrant discussion in this chapter.

Doetsch et al. (1952) and McNeill et al. (1954) found that rumen fluid contains essential factors for optimal growth of rumen microflora. The cellulolytic factor activity has been demonstrated in hot water extracts of alfalfa leaf meal, timothy hay, ladino clover, autolyzed yeast, molasses, cow manure, and in partial hydrolysates of casein, soybean, hair, chicken feathers and bone meal(Burroughs et al., 1950a, 1950b; Ruf et al., 1953; Bentley et al., 1954; Hall et al., 1954). Further fractionation studies of rumen fluid by Bentley et al. (1954; 1955) showed that valeric caproic, iso-butyric, and iso-valeric acids stimulated cellulose digestion whereas acetic, propionic, and butyric acids did not. Bryant and Doetsch (1955) found that Bacteriodes succinogenes needed iso-valeric, iso-butyric, and valeric acids for growth in pure medium. Other studies indicate that iso-valerate is converted to leucine by Ruminococcus flavefaciens (Allison et al., 1959). Leucine per se cannot be utilized

by this microorganism.

Hershberger (1955) and Dehority et al.(1957) found that valine, proline, and the leucines were active in hydrolysis of cellulose. These are probably the amino acids which gave the stimulation in the partial protein hydrolysates although the action of peptides is only conjecture at present (Hall et al., 1954). Organic nitrogen sources such as peptone and amino acids were found necessary for R. flavefaciens (Ayers et al., 1958).

These data indicate that various nutrients are essential for cellulose hydrolysis. The absence or decrease in numbers of the organism(s) responsible for the production of the nutrients as end-products or of hydrolysis products may well have an effect on the overall rate of cellulose hydrolysis.

MATERIALS AND METHODS

A series of studies using the *in vitro* artificial rumen technique was made to study the variation in cellulose digestibility of two fistulated animals maintained under the same conditions with respect to feed, water, and shelter.

Experiments were designed to study cellulose digestibility of separate and combined fractions of fluid from two steers. The artificial rumen technique employed was similar to that described by Burroughs *et al.* (1950) with several modifications. Rumen samples were collected from two Hereford steers fitted with permanent fistulae and maintained on a high roughage ration. The fluid was strained through four layers of cheese cloth into thermos bottles previously warmed to 39°C with tap water. The bottles were stoppered, transported to the laboratory, and the ruminal fluid was immediately centrifuged at 450 x g for one minute. The supernatant was transferred to 50 ml. centrifuge tubes containing 120 mgm. purified cellulose (Solka Floc) plus 5 ml. double strength inorganic medium (Cheng *et al.*, 1955). A total of 15 ml. rumen fluid was placed into each fermentation tubes fitted with gassing tubes (CO_2) to maintain homogeneity and anaerobiosis. The microorganisms were allowed to incubate at 39°C in a water bath for 24 hours. Residual cellulose was determined by the method of Crampton and Maynard (1938) and expressed as gm. cellulose hydrolyzed/ liter rumen fluid/ day.

In the first trial percentages of 100, 75, 50, 25, and zero of the fluid from Steer I were placed in the tubes and run in triplicates. In the same tubes, corresponding percentages of fluid of zero, 25, 50, 75, and 100 were added from Steer II. From the results of trial 1, it was decided to test the hypothesis that bacterial density was the cause of digestibility differences in addition to the percentage studies. The optical densities of the fresh rumen samples were taken at 540 mu and the denser fluid (Steer I) was diluted to the same density as that of the less dense fluid (Steer II) with distilled water. The percentages of the undiluted fluid were 39 and 50 for Steers I and II, respectively. This dilution should correspond to 100 percent of Steer II. In the third trial, the denser fluid (Steer I) was diluted to 90 percent to match the 100 percent fraction of Steer II. This ratio, again, should correspond to the digestibility of Steer II. Trials 4 and 5 were repeat experiments of trial 3.

The conditions of all trials remained the same throughout the experimental period with time being the only known variable.

RESULTS AND DISCUSSION

The criterion of response of the percentage of fluid from two steers was cellulose hydrolysis. The results are summarized in Table XII and it is apparent that the fluid from Steer I consistently hydrolyzed cellulose at a greater velocity than that from Steer II ($P < 0.05$). The 75:25 ratio likewise was signficantly greater than that of the 0:100 ratio. The

TABLE XIII

CELLULOSE HYDROLYSIS OF FRACTIONS OF TWO FISTULATED STEERS USING THE IN VITRO ARTIFICIAL RUMEN TECHNIQUE

fraction of fluid from each steer		gm cellulose hydrolyzed/ liter rumen fluid/day					
		trial					
I	II	1	2	3	4	5	avg
100	0	3.960	2.760	1.464	1.312	2.904	2.480(1.1)
75	25	3.888	3.192	0.712	1.224	2.640	2.328(1.3)
50	50	3.912	1.832	0.456	1.336	2.200	1.944(1.3)
25	75	3.152	1.896	0.568	1.544	2.776	1.984(1.0)
0	100	2.912	0.816	1.056	1.504	1.272	1.512(0.8)

decrease in the fraction of fluid from Steer I with subsequent decrease in hydrolysis appears to approach linearity ($P < 0.05$) indicating that the ruminal fluid per se was responsible for the consistantly different digestibility. The variation among trials was likewise highly significant which would tend to indicate a consistant error in the trial affecting all tubes alike. Assuming this to be true, time has been

confounded with technique of assay and we cannot be sure that the variation is all due to technique. The variation in the replications does not appear to be due, in a large percentage, to steer variation since the slope of the means of each trials followed the same general trend. Because these trials were run over a period of several months, it appears that Steer I will constantly digest a greater percentage of the cellulose than Steer II even though Steer II had been previously inoculated with the flora of Steer I. These differences could be due to a greater population of microorganisms that digest cellulose or microflora that enhance, in some manner, rate of hydrolysis of cellulose as evidenced by the above results, or a decreased water content in the rumen as observed by the larger optical density measurements in the fluid from Steer I. It is also possible that the host (Steer II) may have certain antibodies acting across the rumen wall to influence microbial action. Although this is strictly conjecture, antiurease activity has been shown to act across the intestinal wall in rats and chicks.

The results of diluting the fluid from Steer I to that of Steer II as measured by optical density measurements may be found in Table XIII. The data indicate that mixing on a 50:50 ratio after dilution of fluids to the same optical densities,decreased hydrolysis slightly over that of fluid from Steer I indicating, as expected, the combined fractions were inferior. The fact that in two of the cases hydrolysis was greater than that of Steer II would indicate the presence of microorganisms in some way is stimulating cellulose hydrolysis. The isolation studies of Bryant and Doetsch (1955) indicate the necessity of branched chain fatty

TABLE XIII

CELLULOSE HYDROLYSIS OF RUMEN FLUID FROM TWO STEERS STANDARDIZED TO EQUIVALENT ABSORBANCIES

Steer I	Steer II	Combination[1] of fluid	Steer I[2] (diluted)
2.760(0.05)[3]	0.816(0.19)	2.240(0.60)	-----
1.464(0.08)	1.056(0.56)	0.616(0.07)	0.704(0.05)
1.312(0.55)	1.504(0.16)	1.216(0.05)	1.000(0.65)
2.904(0.30)	1.272(0.12)	2.062(0.05)	0.168(0.07)

[1] Fluid was added on 50:50 ratio with respect to optical density.
[2] Fluid from Steer I diluted to same optical density as Steer II.
[3] Standard deviation.

acids are present in enough quantity due to the apparent health of the animal and are therefore present in such quantity to sufficiently provide enough substrate for microbial action. Dilution of the fluid from Steer I to that of Steer II and using this as the sole microbial source was less than that from either of the steers and appeared to be even too variable for speculation.

The question of the cause of these differences still remains unanswered as does the apparent factor (s) involved in increased hydrolysis in one of the animals. The answer may be a simple limiting nutrient produced by a single species of microflora or may involve the enviornment provided by the host animal in complex with entire bacterial population in the rumen.

SUMMARY

In five trials involving two steers treated alike, it was found that one steer consistently hydrolysed cellulose at a greater velocity than the other. Various ratios of each steer's fluid indicate rate of hydrolysis is a function of fluid concentration. Standardization of fluid on the basis of optical density measurements is inconclusive.

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