DEVELOPMENT OF AN IMPROVED *IN VIVO* ARTIFICIAL RUMEN (VIVAR) TECHNIQUE AND SOME OF ITS APPLICATIONS

by

CHESTER LEROY KEITH

B. S., Kansas State University, 1958

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1963

Approved by:

Louis R Fina

Major Professor

TABLE OF CONTENTS

INTRODUCTION

In recent years there has been considerable interest in the role played by the bacterial population of the rumen in the digestive processes of the host animal. It is now well known that to feed the animal one must first feed the rumen microorganisms. These organisms are responsible for the breakdown of complex foodstuffs into soluble products which are then assimilated by the host animal. During this process cellulose is degraded to form volatile fatty acids. The amino acid composition of the protein is altered and certain B vitamins are synthesized.

The rumen may be regarded as a large fermentation vat which provides a suitable environment for the continuous culture of the microbial population. Reasonable constancy of conditions in the rumen is provided by the following: The frequent intake of food by the animal provides a regular supply of substrate for the microorganisms; soluble products of microbial metabolism are absorbed through the rumen wall thus preventing accumulation with resultant inhibition of enzyme activity; a constant temperature of 38-42 C is maintained; the volume of rumen contents is regulated by frequent passage of liquid to the omasum; the animal secretes large volumes of saliva which acts as a buffer.

The rumen maintains very complex and interdependent flora and fauna, which are undoubtably disorganized when removed from their natural environment. However, the approaches that have been made toward gaining an understanding of the types and functions of the organisms involved in rumen metabolism have been through <u>in vitro</u> techniques. The microorganisms are usually studied by one or more of the following: (1) microscopic examinations, (2) cultural techniques, (3) biochemical studies of washed cell suspensions, and (4) use of rumen fluid as inocula in an artificial rumen.

These techniques are valuable in identification, classification, and enumeration of rumen organisms, but the use of *in vitro* artificial rumen methods, using rumen fluid as inocula, as means of studying the symbiotic activity of rumen organisms leaves much to be desired. Nevertheless, it is the artificial rumen technique that has been most useful in giving insight into rumen function.

The term "artificial rumen" is used to designate an apparatus designed to duplicate as nearly as possible the conditions that exist in the rumen. The rapid development of abnormal conditions has appeared to be the limiting factor in the *in vitro* fermentations. *In vivo* conditions are met for only a few hours. This has been sufficient time for gaining much information, but has meant that the exact symbiotic relationships have not been maintained.

To more nearly duplicate rumen conditions the artificial rumen must meet certain criteria. The criteria that have been previously suggested are: the maintenance of numbers and normal appearance of the bacteria and protozoa of the rumen; the maintenance of normal interaction between these; and the ability to predict quantitative results *in vivo*.

Recently a new approach was made toward the elucidation of the complexities of rumen function. This was by the use of an *in vivo* artificial rumen (VIVAR) apparatus consisting of porcelain test tubes which were suspended in the rumen. Technical difficulties limited its use although the method appeared to more nearly meet the aforementioned criteria. The study reported here was undertaken to improve the *in vivo* technique and demonstrate its potential as a tool for studies in microbial ecology of the rumen.

REVIEW OF LITERATURE

Artificial rumen techniques are attempts to impose upon the withdrawn rumen samples conditions which duplicate those within the rumen. Changes may be made in these conditions and their effects noted. In the method of Pearson and Smith (1943), samples of rumen ingesta were strained through muslin and incubated in flasks placed in a 39 C water bath. These investigators studied the effect of pH, temperature, and gaseous environment. Later work on the same project by McNaught (1951) was carried out on strained rumen contents. The material was centrifuged at 2,000 rev/min for 5 min, to remove protozoa. Incubation (4 hr) was carried out in flasks under CO_2. Since these were short term experiments, no elaborate attempts were made to exactly duplicate the rumen conditions. Marston (1948) used a similar system, but devised methods to control the pH, temperature, and anaerobiosis. The fermentation vessel consisted of a 3.5 liter closed jar with stirrer, electrodes for pH, Eh, and a thermoregulator. During fermentation the Eh was in the area of -0.380 v.

A more elaborate device for *in vitro* study of rumen digestion was suggested by Louw, Williams, and Maynard (1949). It consisted of a glass jar suspended in a water bath at a controlled temperature. Fermentation products could be dialyzed through a Visking casing suspended in the growth medium. Various inlets and outlets were available for gassing, sampling, and the addition of substrate or buffer. These investigators compared their method with that of Marston (1948), and showed much more digestion using the "semipermeable" membrane. The criticism of the all glass system of Marston (1948) was that no provision was made for removal of nongaseous fermentation products. Wasserman *et al.* (1952) used a modification of the Louw *et al.* (1949) and Marston (1948) methods in studies on the effect of antibiotics on *in vitro* cellulose digestion.

Huhtanen and Gall (1952) developed a miniature artificial rumen similar to Louw's apparatus. The smaller dialysis sack was suspended in an outer bath of synthetic saliva and cysteine-bicarbonate solution gassed with CO_2 to maintain the proper Eh and pH. They claimed their apparatus was easier to handle and was excellent for pure culture studies because it required a smaller inoculum and substrate.

Burroughs et al. (1950a, b, c) also have made use of an artificial rumen in studying cellulose digestion, digestion of good and poor quality roughages, and digestion as influenced by grain and protein-rich foods. The artificial rumen devised by this group consisted of 500 ml flasks incubated in a water bath at 40 C. Three-holed rubber stoppers were inserted in the flasks for CO_2 inlet and outlet and for regulating the pH of the contents. The fermentations were carried out continuously in 36 hr periods. The original material came directly from the rumen, but the inoculum for the second 36 hr period consisted of one half the residue of the preceding fermentation. Burroughs et al. (1950b) considered the advantages of the artificial rumen to be speed in obtaining results, precision, and low cost. They admitted that the following limitations attend the use of the device: Results may not be truly representative, the end products resulting can not be controlled, and different types of organisms may develop in different runs on the same substrates.

The permeable type of artificial rumen has been found to give better results than the impermeable type (Louw et al., 1949). The permeable type has a semipermeable sack immersed in the medium, through which fermentation products may diffuse. The use of such a system obviates some criticism directed against the artificial rumen. However, Warner (1956) claimed that in vivo conditions were not met when fermentations proceeded beyond 8 hr. This

investigator observed numbers of bacteria and protozoa in a system similar to that of Louw et al. (1949). After 8 hr, there was a decrease in motility as well as a decrease in numbers of bacteria and protozoa.

El-Shazly, Dehority, and Johnson (1960) made comparisons of the all glass, semipermeable, and continuous flow types of apparatuses for in vitro rumen fermentations. The continuous flow apparatus was similar to that of Warner (1956) (a modification of Louw's apparatus), with the exception that it was constructed to allow the dialysis fluid to flow through the outer jacket enclosing the semipermeable reaction sack. A comparison of values for per cent cellulose digestion after 30 hr for each type, using second stage alfalfa as substrate, were: all glass, 48%; continuous flow, 51.6%; and semipermeable, 51.2%. Microscopic examination of the flora in all three systems showed little difference after 30 hr of fermentation.

Since the advent of the artificial rumen techniques, much information has been gained from studies of rumen microbiology and forage utilization. Kamstra, Moxon, and Bentley (1958) used an all glass system in studies on the effect of stage of maturity and lignification on the digestion of cellulose in forage plants. Quicke et al. (1959a) used a similar technique to evaluate digestibility of forage cellulose. Quicke and Bentley (1959b) also determined in vitro that differences in lignin content of hays cut at different stages of maturity were too small to account for the observed differences in the digestibility of the cellulose. LaFevre and Kamstra (1960) compared cellulose digestion of 22 rations by in vitro and in vivo methods and found the 48 hr in vitro fermentations to yield cellulose digestion coefficients similar to the values obtained in vivo. Dehority and Johnson (1961) studied the effect of particle size upon the in vitro cellulose digestibility of forages.

Digestion leveled off after 30 hr of fermentation with no marked increases occurring up to 48 hr. They also reported that ball-milling the substrate increased the rate and extent of digestion *in vitro*. These studies point out the value of the artificial rumen techniques. However, only in a few instances have these results been verified *in vivo* (LeFevre and Kamstra, 1960).

In vivo studies generally have been made using fistulated animals. Usually these experiments take the form of feeding or withholding some substance from the test animal and withdrawing rumen samples periodically. The same animal or group of animals is used throughout the experiment.

For examples of some of the earliest *in vivo* work, the experiments of Monroe and Perkins (1939) and Smith (1941) may be cited. Studies were done on pH values of rumen ingesta as influenced by different rations. Other techniques have consisted of suspending cotton threads or silk bags containing ground hay in the rumen (Balch and Johnson, 1950; Miles, 1951). Hueter *et al.* (1958) introduced carbohydrates and other materials into the rumen. Their conversion into volatile fatty acids and their effect upon pH values was followed, both *in vitro* and *in vivo*. Feeding experiments including digestibility trials (Meyer *et al.*, 1960) and effects of antibiotics (Bartley, Fountaine, and Atkeson, 1950) have been run, but it was difficult to relate the results to the exact role played by the rumen. Experiments have also been performed in which C^{14} labeled alfalfa was introduced into the rumen of fistulated animals (Yadava, 1962). Samples of blood, urine, feces, and rumen contents were analyzed for C^{14} activity.

Fina, Teresa, and Bartley (1958) developed a new *in vivo* technique in an effort to more closely simulate rumen conditions. The apparatus, which was suspended in the rumen, consisted of porcelain test tubes equipped with gas

escape rubber tubes. Experiments using this apparatus showed filter paper to be digested continuously over a period of 10 days using the same inoculum. When a diluted inoculum was used initially, packed cell volumes increased through 48 hr. This demonstrated that any growth factors required by the cellulolytic organisms were gaining entrance to the interior of the tubes. The method appeared to more nearly meet the criteria suggested by Warner (1956), but the apparatus itself had major limitations. It was cumbersome to assemble, pore size was not uniform, selection of pore size was limited to several grades, and the porcelain tubes were difficult to clean between experiments (Fina et al., 1962).

MATERIALS AND METHODS

Description of Apparatus

The apparatus used in this work consisted of a specially designed stainless steel drum with a side arm[1] (Plate I). The drum could be fitted with a wide variety of membranes. Inner and outer stainless steel wire mesh screens, rubber gaskets, and membranes were held in place on the drum by outer nuts (Plate II). Screens of 40 x 40 mesh were placed on each side of the membranes to prevent punctures caused by ingested plant stems and to support against hydrostatic pressure in the rumen. The side arm of the drum served as a sampling port as well as a gas escape outlet. A piece of stainless steel screen was inserted in the drum to prevent obstruction of the side arm by particulate substrate. For gas escape, rubber tubing of inside diameter 9 mm was attached to the side arm and was passed through a no. 15 rubber plug

---

[1]Mott Metallurgical Corp., 272 Huyshope Ave., Hartford 14, Conn.

secured by the plastic cap of the fistula. The length of this tubing regulated the depth of the apparatus in the rumen. The rubber tubing was joined by a polyethylene connector[1] to a rubber tube extended from a manifold. The manifold consisted of one or more aluminum "T" joints connected with short pieces of rubber tubing, and was positioned parallel and slightly above the left side of the animal. The tubings extending from the manifold were kept free from kinking and tangling by a weight and pulley arrangement. A constant stream of nitrogen was passed through the manifold at a rate of approximately 60 bubbles/min. This flow of nitrogen served to prevent entrance of atmospheric oxygen, kept the system at essentially atmospheric pressure, and maintained anaerobic conditions while allowing free escape of fermentation gases.

The respective manifold section and "T" were removed when an apparatus was taken from the rumen. The hole in the rubber plug of the fistula was then stoppered. As many as seven of these apparatuses could be placed simultaneously in the fistulated animal.

Method Used in Obtaining Inocula

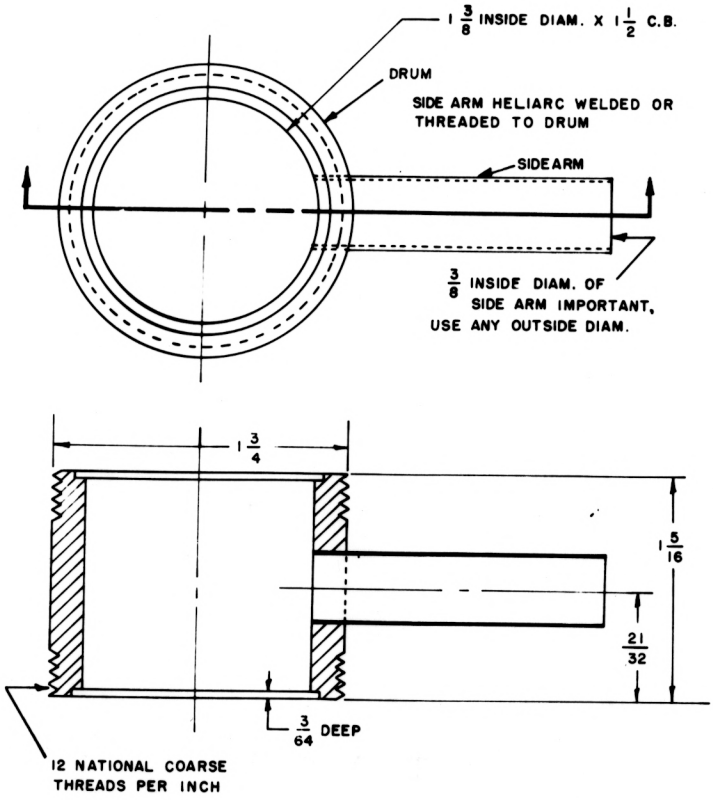
Samples of rumen fluid for use as inocula were taken 4-6 hr after feeding the animal. Before sampling, the rumen contents of a fistulated animal were manually mixed. Rumen fluid was obtained by inserting into the rumen, through the fistula, a rubber tube with an attached metal strainer. Two liters of fluid then were collected in a vacuum flask under partial vacuum. The flask, no longer under vacuum but sealed from air, was immediately placed in a 40 C water bath. After 10-15 min the fluid stratified into three layers. Heavier

---

[1]Laboratory supply houses.

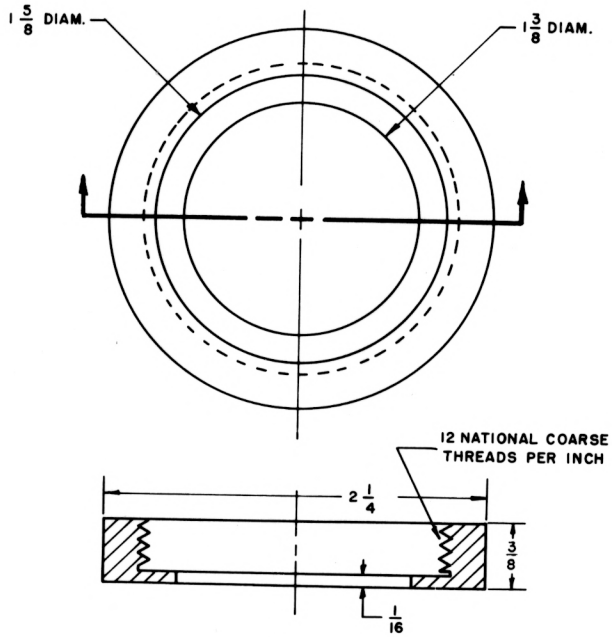
EXPLANATION OF PLATE I

A diagram of the stainless steel drum with side arm and nut.



DRUM & SIDE ARM

MAT. STAINLESS STEEL
FINISH SMOOTH



NUT

MAT. STAINLESS STEEL
FINISH SMOOTH

EXPLANATION OF PLATE II

A photograph of the stainless steel VIVAR with nut, inner and outer screens, rubber gaskets, and membrane.

PLATE II

particles settled to the bottom, the lighter, more coarse particles floated to the top. This left a center layer of yellowish fluid with very small particles in suspension. A portion of this fluid was collected by the following method. A section of glass tubing was inserted through a hole in the stopper of the flask and into the center layer. Fluid was then drawn into a 250 ml flask under partial vacuum. This flask was equipped with a cannula which extended to the bottom. A section of rubber tubing connected the cannula to a two-way valve for use in continuous pipetting while inoculating the apparatuses. This technique allowed rapid inoculation with a minimum of temperature change and exposure to atmospheric oxygen. A 25 ml sample, delivered by a 30 ml syringe with a long, 18 gauge needle, was used for each inoculation.

General Procedure for Setting Up *In Vivo* Experiments

Prior to each experiment, one side of the VIVAR was outfitted with screens, gaskets, and membranes. The substrate was added through the open end of the drum, after which the remaining membrane, gaskets, and screens were secured by the nut. The rubber tubing from the side arm was extended through the rubber fistula plug and the assembled apparatus was taken to the metabolism room in which the animal was kept. The rumen fluid, obtained as previously described, was then thoroughly mixed and a 25 ml inoculum was injected into the VIVAR through the side arm tubing. During the inoculation process the VIVAR was submerged in a 40 C water bath.

The manifold and gas escape line were swept free of atmospheric oxygen before connection to the side arm tubing. The inoculated VIVAR was immediately placed in an upright position against the left wall of the rumen directly under the fistula. The plastic fistula cap was then secured. The flow of

nitrogen through the manifold was adjusted to the desired rate (60 bubbles/min), as indicated by a bubbler.

Experimental Animal

The animal used in these studies was a three year old Jersey cow obtained from the Kansas State University herd. This animal was fistulated at the age of one year and fitted with a plastic cannula-type fistula. The animal was fed a ration of seven pounds of chopped alfalfa hay twice daily. Water was offered ad lib. during all experimental periods.

Procedure for Determining In Vitro Diffusibility of Volatile Fatty Acids

To test diffusion properties, the stainless steel VIVARs were assembled with membranes in place, filled with distilled water, and submerged in test solutions containing one of the C_2-C_6 fatty acids. Samples were taken periodically both from the test solution outside and from VIVARs. The concentration of acid was determined by titration with standard NaOH, using phenolphthalein as the indicator. Four samples were withdrawn from each VIVAR over a 48 hr period. The per cent equilibrium of the solution inside the drum as compared to the test solution at each sampling time was recorded. Membranes tested were of the following types: 0.1 μ and 0.3 μ Millipore,[1] Vycor glass,[2] sintered stainless steel,[3] ceramic,[4] and Visking.[5]

---

[1]Millipore Filter Corp., Bedford, Mass.

[2]Corning Glass Works, Corning, N. Y.

[3]Mott Metallurgical Corp., 272 Huyshope Ave., Hartford 14, Conn.

[4]Coors T35 fired to cone 04, 1.5 mm thickness with pore size less than 0.4 μ; Coors Porcelain Co., Golden, Colo.

[5]Laboratory supply houses.

Analysis for Total Volatile Fatty Acids

Total volatile fatty acids were determined by a method devised by Markham (1942) and modified by Fina and Sincher (1959). The method was as follows. One milliliter of 85% phosphoric acid and 1 ml of the sample to be analyzed were added to the sample tube. It was steam distilled until 40 ml of distillate was collected. The amount of volatile fatty acids was then determined by titration against standard NaOH using phenolphthalein as the indicator. Carbon dioxide-free nitrogen was bubbled through the solution during titration to prevent absorption of atmospheric CO_2. Results were expressed as micromoles of total volatile fatty acids per milliliter of rumen fluid.

Procedure for Determining *In Vivo* Diffusibility of Total Volatile Fatty Acids

To test the diffusion properties of the membranes under conditions within the rumen, three VIVARs were assembled as previously described and placed in the rumen. The VIVARs were equipped with 0.1 μ, 0.2 μ Millipore, and Visking membranes then filled with distilled water and placed in the rumen. Samples of rumen fluid as well as samples from each VIVAR were withdrawn at various time intervals for 48 hr. Concentrations of total volatile fatty acids in both the rumen and VIVAR samples were determined by the previously described method.

Procedure for *In Vitro* Testing of VIVARs for Sterility

Tests were conducted *in vitro* to determine whether *Serratia marcescens*, selected because of its small size and ease in detection, could penetrate into broth surrounding VIVARs equipped with various types of membranes. Six VIVARs

were assembled, each with one of the following types of membranes: sintered stainless steel, Vycor glass, ceramic, Visking, and Millipore of 0.1 μ and 0.2 μ pore sizes. The VIVARs were filled with 30 ml of nutrient broth and each was placed in a separate beaker containing 300 ml of the same broth. Each beaker was covered by a double layer of cotton which was taped to the side arm of the VIVAR to prevent contamination of the broth. The beakers containing the broth and VIVARs, covered by cotton, were autoclaved for 20 min at 120 C. After cooling, the broth inside the drum of each VIVAR was inoculated with a suspension of S. marcescens. Incubation was at room temperature for 72 hr. The broth surrounding the VIVARs would become cloudy and reddish if the membranes were permeable to S. marcescens.

Procedure for In Vivo Testing of VIVARs for Sterility

Tests were conducted in vivo to determine whether bacteria would penetrate into sealed, sterile VIVARs. Three VIVARs were equipped with screens, gaskets, and membranes of the following types: 0.1 μ, 0.2 μ Millipore, and Visking dialyzing membrane. Each VIVAR was filled with 30 ml of distilled water and placed in a beaker of water. A length of rubber tubing was connected to each side arm and was passed through the rubber fistula plug, removed from the animal. The protruding ends of tubing were wrapped in paper. The complete apparatus, with VIVARs in water, was autoclaved for 20 min at 120 C. After the tubing ends had cooled and dried, they were unwrapped and cotton from sterile applicators was forced a distance of 2 in. down each tube. Following the cotton, a 1 in. length of 1/4 in. wooden doweling was inserted into each tube. The ends of the tubes were coated with paraffin to prevent entrance of moisture. The sealed, sterilized VIVARs were then placed in the

rumen and left for 120 hr.

Upon removal from the rumen, the fistula plug was slipped off the side arm tubes which were then wrapped in cotton soaked in $HgCl_2$ (1:1000). Scissors, sterilized by flaming alcohol, were used to cut through the tubing and cotton. Samples of the fluid were removed for sterility testing by inserting a long sterile needle into the drum through the side arm. Sterile syringes were used to collect 5 ml of the fluid which were added to 50 ml of sterile tryptose broth. The inoculated broth was incubated at 39 C for 10 days. Fluid from each VIVAR was also examined microscopically, using the Gram's stain.

Procedure for Qualitative Testing of Membrane Permeability to Urease, Glucose, and Soluble Starch

VIVARs filled with distilled water and equipped with Millipore (pore size, 0.1 μ and 0.2 μ) and Visking membranes were placed in a solution of urease (1 mg/ml). At various time intervals 1/2 ml samples were withdrawn from the VIVAR drums and added to tubes containing 20 mg of urea, with phenol red to indicate alkalinity resulting from hydrolysis. The tubes were placed in a 37 C water bath and observed at the end of 30 min.

Similar tests were conducted to determine membrane permeability to glucose and soluble starch.[1] The Molisch test and iodine solution were used to detect diffusion of glucose and soluble starch, respectively.

---

[1]Baltimore Biological Laboratories, Baltimore 18, Md.

Procedure for Determining Susceptibility of Membranes to Digestion *In Vivo*

The ability of the Millipore membranes to resist digestion in the rumen was determined. Six membranes were weighed and placed in a wire cage which was suspended in the rumen for 5 days (120 hr). Upon removal from the rumen the membranes were thoroughly washed in distilled water and dried. Weights of the membranes before and after exposure in the rumen were compared. Similar experiments were done using Visking membranes.

Procedure for Studying the Utilization of Plant Cellulose

The dried material was ground to a particle size of 2 mm in a Wiley mill. A known amount of the cellulose or whole plant material was added to each VIVAR. The VIVARs were inoculated with 25 ml of rumen fluid, then incubated in the rumen for various time intervals. After removal from the rumen, the amount of cellulose digested was determined on the entire contents of each VIVAR. A cellulose analysis was also done on 25 ml of the inoculum. When studying the utilization of C^{14} labeled alfalfa, only total volatile fatty acids were analyzed.

Analysis for Cellulose

The amount of cellulose digested was determined from an analysis made upon the entire contents of each VIVAR at the end of the designated time periods. The contents were washed from the VIVAR and sedimented by centrifugation at 2,000 rev/min for 10 min. The cellulose was extracted following the method of Crampton and Maynard (1938) with modifications such that the major part of the extraction process could be carried out in 50 ml centrifuge tubes

equipped with ground glass joints. (See page 71 of the appendix for procedure.)

Procedure for Collection of Volatile Fatty Acid Samples

Samples of rumen fluid to be analyzed for volatile fatty acids were withdrawn from a VIVAR while in its original position in the rumen. The VIVAR was equipped with a 20 gauge needle, 50 cm in length. The needle pierced the wall of the side arm tubing outside the rubber fistula plug, and extended down into the drum. A strainer was constructed from the bottom end of a 1 ml plastic centrifuge tube and was closed by a small rubber stopper. The strainer was placed in the drum and held in position by the end of the needle. Perforations in the strainer were made using a 25 gauge needle. The small perforations prevented clogging of the needle as fluid samples were withdrawn by a syringe. The needle and attached strainer remained in the VIVAR during the sampling period (3-5 days).

Analysis of Carbon-14 Labeled Total Volatile Fatty Acids

Rumen fluid, from the VIVAR, was distilled as previously described. Forty milliliters of distillate was collected and divided into two equal parts. Phenolphthalein, indicator, was added to one part which was titrated with standard NaOH, with nitrogen bubbling into the distillate to remove CO_2. The amount of alkali used was recorded and the same amount of alkali, but no indicator, was added to the second half of the distillate. The first half was discarded. The second half was evaporated to about 1 ml over a steam bath. The material was transferred to a 2 ml, weighed, glass boat and dried to

constant weight under a heat lamp. After drying and weighing the boat, 1 ml of distilled water was added to dissolve the residue (sodium salts of volatile fatty acids). The glass boat was then placed in a glass counting vial and 10 ml of phosphors solution[1] was added. The radioactivity of the residue in the vial was determined using a liquid scintillation spectrometer. The observed counts per minute were corrected for background and efficiency of the counting method (50%). Corrected counts were expressed as disintegrations per minute per milligram of the sodium salts of total volatile fatty acids.

Description of Biosynthesis Chamber and Accessories Used for Growing Carbon-14 Alfalfa

The chamber (Plate III) was constructed of 3/8 in. clear Plexiglas[2] held together by brass machine screws (10 x 24 x 3/4 in.) at 2 in. intervals. The Plexiglas edges were softened with ethylene dichloride before they were joined. This formed airtight joints; however, all seams were calked on the inside with a nonhardening calking compound as a precaution against leakage.

An 18 in. hole in the top of the chamber was closed by a Plexiglas lid 22 in. square by 1/4 in. thick. The lid was held in place by eight, 3/4 in. screws which passed through the lid and screwed into the chamber top. An airtight seal was formed using nonhardening calking compound.

A dehumidifier[3] was placed in the chamber through the hole and mounted on a base of plywood and polystyrene blocks to absorb vibration. The relative

---

[1]Dioxane 250 ml, 2,5-diphenyloxazol (PPO) 1.75 g, 1,4 bis-2 (5-phenyloxazolyl)-benzene (POPOP) 12.5 mg, and naphthalene 25 g.

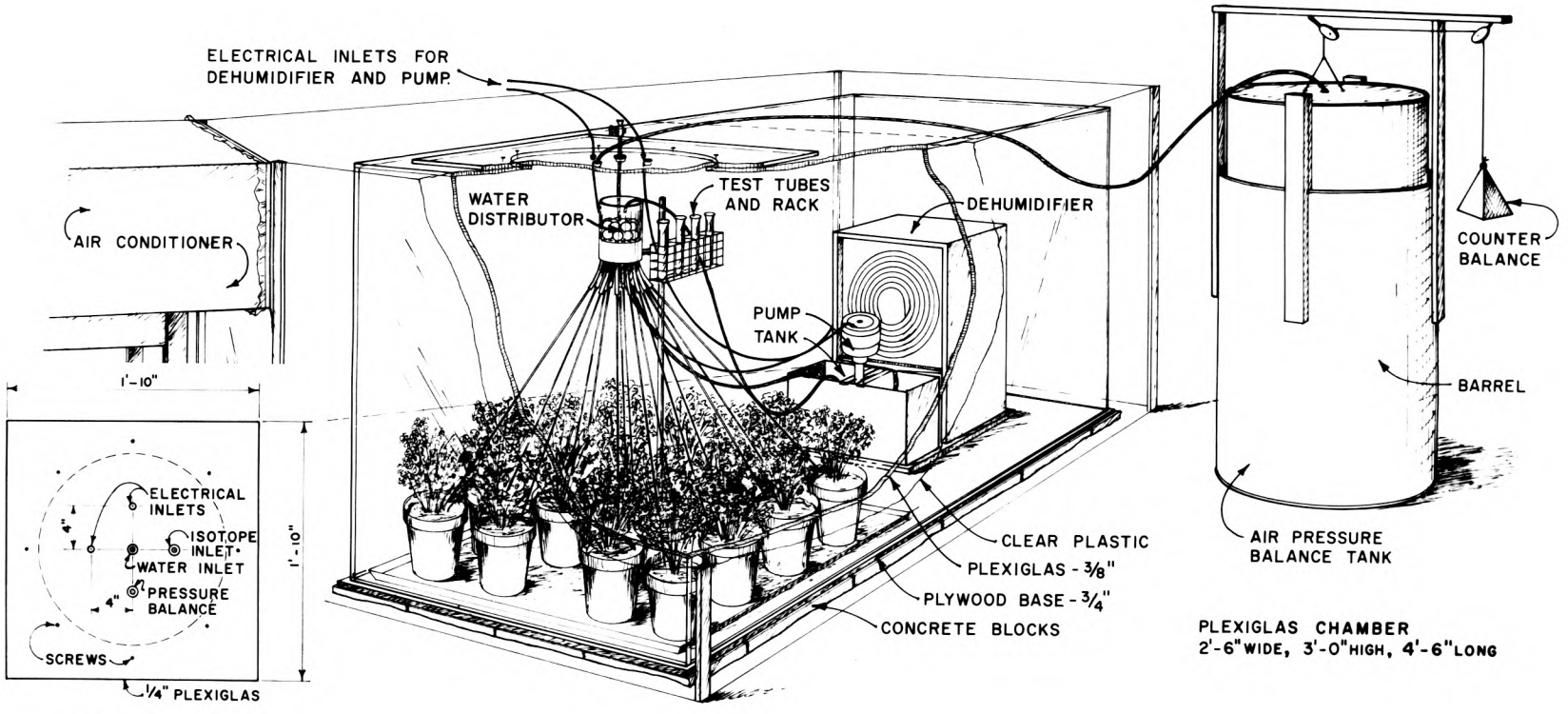
[2]Rohm & Haas Inc., Washington Square, Philadelphia 5, Pa.

[3]Westinghouse, Model No. ESC 70002.

EXPLANATION OF PLATE III

The biosynthesis chamber and related accessories used in labeling plants.

PLATE III


ELECTRICAL INLETS FOR DEHUMIDIFIER AND PUMP
AIR CONDITIONER
WATER DISTRIBUTOR
TEST TUBES AND RACK
DEHUMIDIFIER
PUMP TANK
COUNTER BALANCE
BARREL
AIR PRESSURE BALANCE TANK
CLEAR PLASTIC
PLEXIGLAS - 3/8"
PLYWOOD BASE - 3/4"
CONCRETE BLOCKS
PLEXIGLAS CHAMBER
2'-6" WIDE, 3'-0" HIGH, 4'-6" LONG
1'-10"
1'-10"
ELECTRICAL INLETS
ISOTOPE INLET
WATER INLET
PRESSURE BALANCE
4"
4"
SCREWS
1/4" PLEXIGLAS
LID FOR CHAMBER

humidity of the chamber was maintained at 55-60% to prevent fungal growth which appeared at higher humidities.

The water condensed by the dehumidifier was recirculated to the plants by an electric vertical immersion pump placed on the collection tank. The pump was activated, when necessary, by a switch placed outside the chamber. Water was pumped through a 5/8 in. plastic tube into a one liter container supported on a ring stand and mounted above the plants. A no. 15 rubber stopper served as the base of the container. Sixteen 4 1/2 in. sections of 1/4 in. copper tubing extended through holes in this stopper. They were connected by rubber tubing to 3/16 in. diameter glass tubes positioned over each plant. Distribution of water was controlled by pre-set screw cocks. When additional water was required it was passed into the dehumidifier tank through a glass stopcock in the lid and then pumped to the plants. When excess water accumulated it was siphoned out through the same stopcock. Electrical wires to the dehumidifier and pump entered the chamber through rubber stoppers inserted in the lid.

Pressure changes within the chamber due to temperature and barometric fluctuations were absorbed by a spirometer consisting of a 45 gal airtight drum inverted over 50 gal of water layered with 1 1/2 in. of mineral oil. Air passed from the chamber to the spirometer through a polyethylene tube extending through a rubber stopper in the lid. The inverted drum was held in place during ascent and descent by four wooden guides placed around the circumference of the outer barrel. Pressure created in raising the drum was counterbalanced by a weight and pulley arrangement. The drum seldom moved more than 8-10 in. during labeling runs.

The growth chamber was maintained in a conventional greenhouse. To

prevent excessive temperature variations a canopy of clear Flex-O-Glass[1] was built around the chamber. It extended about 6 in. from all sides and the top, excluding the access portion. An air conditioner was positioned to blow into the canopy. On extremely hot days a cheesecloth shade was pulled over the chamber top. In this way the interior of the chamber remained below 80 F even when the greenhouse temperature rose to 100 F.

Procedure for Labeling Alfalfa Plants with Carbon-14

The alfalfa plants used were obtained from a spaced-plant nursery and transferred while dormant to 6 in. pots. Soon after growth initiation, 16 plants treated with an insecticide were placed in the chamber on a grill supported by pans to allow for good drainage (Plate IV). A complete nutrient solution was provided prior to placing the plants in the chamber. Natural day length was supplemented by artificial light to provide an 18 hr day.

A measured amount of $BaC^{14}O_3$ was placed in test tubes and mixed with distilled water. The tubes were held in a rack fastened to the ring stand, and placed directly under a hole in the lid. The hole was plugged by a rubber serum stopper. To release the $C^{14}O_2$ from the $BaC^{14}O_3$, 5 ml of 85% H_3PO_4 was injected through the serum stopper by a long needle attached to a syringe. Levels of C^{14} activity were monitored by withdrawing 10 ml of the chamber atmosphere with a gastight syringe. The CO_2 was absorbed in hydroxide of hyamine[2] and counted in a liquid scintillation spectrometer.

Plants were removed at the early bloom stage and placed in the dark

---

[1]Warp Bros., 1100 N. Cicero Ave., Chicago 51, Ill.

[2]p-(diisobutylcresoxyethyl)-dimethyl benzyl-ammonium hydroxide.

EXPLANATION OF PLATE IV

A photograph of the biosynthesis chamber and accessories during a labeling run.

PLATE IV

(usually for 48 hr) to metabolize reserve sugars. They were then harvested, placed in paper bags, and dried at 100-120 F in a forced draft oven for 48 hr.

Procedure for Determining Carbon-14 Activity of Alfalfa Plant Material

The alfalfa plant material was assayed for total C^{14} by a combination and modification of the Van Slyke and Folch (1940) and Claycomb, Hutchens, and Van Bruggen (1950) methods of wet oxidation. The C^{14} activity of the extracted cellulose was determined by the same procedure. Duplicate 3-4 mg samples of ground plant material were oxidized to CO_2 in Van Slyke and Folch oxidation apparatuses. Five milliliters of the Van Slyke, Plazin, and Weisiger (1951) oxidation reagent,[1] prepared according to Calvin et al. (1949), were used for combustion of the samples. The liberated CO_2 was trapped in 3 ml of hydroxide of hyamine (Passmann, Radin, and Cooper, 1956). Two milliliters of this hydroxide of hyamine were transferred to a glass counting vial containing 10 ml of phosphors solution, according to a modification of the method used by Werbin, Chaikoff, and Imada (1959). The radioactivity of the material in the vial was determined using a liquid scintillation spectrometer. The observed counts per minute were corrected for background, efficiency of the counting method (30%), and for the quantity of the sample counted. Corrected counts were expressed as disintegrations per minute per milligram of plant material. The average disintegration rate per minute per milligram of the two samples was used to calculate the C^{14} content of the dried plant material in millicuries.

---

[1]Chromic trioxide 25 g, phosphoric acid (density 1.7) 167 ml, fuming sulfuric acid containing 20% free sulfur trioxide 333 ml.

EXPERIMENTAL RESULTS

The *In Vitro* Diffusion Characteristics of VIVAR Membranes

The diffusion properties of the membranes were investigated *in vitro* to determine their relative permeabilities to the C_2-C_6 volatile fatty acids. The data presented in Table 1 represent the per cent equilibriums attained using different test solutions and different membranes. The form of the acid used seemed to affect the rate of diffusion, as shown by the more rapid rate of diffusion of n-hexanoic acid (potassium salt) than the valeric acids (free acids). The fatty acids examined (Table 1) passed more rapidly through the Millipore membrane possessing the smaller pore size of the two tested. The most rapid rate of diffusion through all the membranes took place during the first 4 hr. Diffusion then continued at a slower rate until equilibrium was reached or the tests were terminated. All membranes were found to be permeable to the 8 and 10 carbon fatty acids (potassium salts). Autoclaving at 120 C for 15 min had no effect on the diffusion properties of the Millipore and Visking membranes. (Only compounds checked were acetic, propionic, and n-valeric acids.)

The *In Vivo* Diffusion Characteristics of Millipore and Visking Membranes

The permeability characteristics of the Millipore and Visking membranes under *in vivo* conditions were examined to determine the diffusibility of total volatile fatty acids. The data presented in Table 2 represent the amounts of total volatile fatty acids present in the VIVARs at designated time intervals compared with the amount in the rumen at the same time. The per cents of

Table 1. *In vitro* diffusion characteristics[1] of VIVAR membranes

Time (hr)	Per cent equilibrium attained using different membranes[2] A	B	C	D	E	F
	Acetic acid, 342 μmole solution outside					
0	0	0	0	0	0	0
1	65	31	22	8	2	2
8	92	75	62	38	21	10
18	98	84	82	41	38	20
24	99	85	87	45	46	26
48	100	86	92	48	60	41
	Propionic acid, 186 μmole solution outside					
0	0	0	0	0	0	0
4	69	58	40	23	16	5
8	78	75	60	27	35	9
24	92	91	86	33	46	23
48	99	99	97	38	69	41
	n-Butyric acid, 186 μmole solution outside					
0	0	0	0	0	0	0
3	55	53	24	33	39	5
8	74	71	48	32	43	7
24	84	91	83	36	51	20
48	91	97	94	45	61	37
	iso-Butyric acid, 189 μmole solution outside					
0	0	0	0	0	0	0
4	56	54	12	29	10	5
8	83	80	34	36	18	8
24	90	91	71	43	42	24
48	100	99	87	45	58	41
	n-Valeric acid, 183 μmole solution outside					
0	0	0	0	0	0	0
4	54	51	22	3	16	5
8	61	60	35	41	17	6
24	84	82	66	40	37	15
48	96	92	85	44	48	29
	iso-Valeric acid, 188 μmole solution outside					
0	0	0	0	0	0	0
4	54	51	28	34	18	5
8	63	59	38	37	24	6
24	88	86	77	40	45	17
48	97	96	92	44	54	30

Table 1. *In vitro* diffusion characteristics[1] of VIVAR membranes (concl.)

Time (hr)	Per cent equilibrium attained using different membranes[2]					
	A	B	C	D	E	F
	n-Hexanoic acid, potassium salt, 183 μmole solution outside					
0	0	0	0	0	0	0
4	91	82	49	38	11	5
8	100	100	73	45	22	8
24	105	97	85	44	43	16
48	100	111	100	53	75	42

[1]Determined using free acids, unless otherwise stated.

[2]A-Millipore membrane, 0.1 μ pores; B-Millipore membrane, 0.3 μ pores; C-Visking; D-sintered stainless steel; E-ceramic; F-Vycor porous glass.

equilibrium attained with the rumen were also expressed in Table 2.

As was noted previously (Table 1), the membrane possessing the smaller pore size demonstrated the most rapid rate of diffusion. However, when comparisons are made between per cent equilibriums attained *in vitro* (Table 1) and *in vivo* (Table 2) it is seen that *in vivo* percentages are consistently lower at each sampling time. Reasons for this apparent decrease in diffusibility will be explained in the discussion. Nevertheless, it was shown that in 24 hr the per cent equilibrium attained with the 0.1 μ Millipore membrane was within 18% of the acid concentration in the rumen. The per cent equilibrium reached using this membrane was still increasing at the time the experiment was terminated.

In Vitro Sterility Tests Using *Serratia marcescens*

In vitro incubation was done to test the ability of six different membranes to hold back *Serratia marcescens*. Sintered stainless steel discs proved to be no barrier. Other membranes tested (page 14) were found to be impermeable to *S. marcescens*. The test organism passed through three different sets of stainless steel discs within 6 hr after inoculation. The organisms growing in the broth surrounding the VIVARs were transferred to nutrient agar slants and incubated at room temperature. The typical red pigment of *S. marcescens* was produced. The organisms were found to be small Gram-negative rods when examined microscopically.

In Vivo Sterility of the VIVAR

It was found that VIVARs would remain sealed and sterile during 120 hr of exposure to conditions in the rumen. Clear amber fluid was collected

Table 2. *In vivo* diffusion characteristics of Millipore and Visking membranes

Time in hours	Milliliters of 0.11 N NaOH used in titration				Micromoles of total volatile fatty acids per ml				Per cent equilibrium attained		
		Membranes[1]				Membranes			Membranes		
	Rumen	A	B	C	Rumen	A	B	C	A	B	C
0	1.00				110.0						
4	1.35	0.45	0.38	0.35	148.5	49.5	41.8	38.5	33.3	28.1	25.9
8	1.17	0.62	0.59	0.58	128.7	68.2	64.9	63.8	52.9	50.4	49.5
12	1.20	0.77	0.75	0.63	132.0	84.7	82.5	69.3	64.1	62.4	52.5
16	1.22	0.89	0.77	0.72	134.2	97.9	84.7	79.2	72.9	63.1	59.0
24	0.96	0.79	0.73	0.71	105.6	86.9	80.3	78.1	82.2	76.0	73.9
32	0.96	0.79	0.72	0.68	105.6	86.9	79.2	74.8	82.2	75.0	70.8
40	1.09	0.93	0.82	0.76	119.9	102.3	90.2	83.6	85.3	75.2	69.7
48	1.17	1.03	0.90	0.80	128.7	113.3	99.0	88.0	88.0	76.8	68.3

[1]A-Millipore membrane, 0.1 μ pores; B-Millipore membrane, 0.2 μ pores; C-Visking membrane.

aseptically from VIVARs equipped with Millipore and Visking membranes. No microbial contamination was found when the fluid was cultured in tryptose broth. Microorganisms were not evident in stained smears prepared from the VIVAR contents or from the broth after incubation.

The Qualitative Permeability of Membranes to Urease, Glucose, and Soluble Starch

Qualitative tests for urease were positive within 1 hr after the VIVARs fitted with Millipore membranes were placed in a solution of urease (molecular weight approximately 480,000). However, the VIVARs equipped with Visking membranes contained no detectable urease after 48 hr in the test solution. Both Millipore and Visking membranes were permeable to glucose and soluble starch. Permeability of both types of membranes was not affected by sterilization in the autoclave. Identical results were obtained when the three tests were repeated.

The Ability of Millipore Membranes to Resist Digestion <u>In Vivo</u>

The results in Table 3 show that the Millipore membranes were not digested by cellulolytic organisms of the rumen. The weights of the membranes were essentially the same before and after exposure in the rumen for 120 hr. Similar results were obtained when testing Visking membranes.

Table 3. Comparisons of Millipore membrane weights before and after exposure in the rumen

Membrane No.	Hours in vivo	Weight before placed in rumen	Weight after removed from rumen	Net change in weight
		(g)	(g)	(g)
1	120	0.0950	0.0951	+0.0001
2	120	0.0945	0.0947	+0.0002
3	120	0.0943	0.0944	+0.0001
4	120	0.0917	0.0916	-0.0001
5	120	0.0914	0.0911	-0.0003
6	120	0.0916	0.0918	+0.0002

Reproducibility of Digestion of Alfalfa Hay Cellulose Using the VIVAR

Table 4 and Fig. 1 present the results of three digestion trials conducted at 16 day intervals. One animal, maintained on chopped alfalfa hay, was used. The alfalfa hay used in the digestion trials was of the same cutting (second) and of the same particle size (2 mm). Table 4 demonstrates that reproducible results can be obtained when using the VIVAR, even though experiments are performed several days apart and with different inocula. Very similar values for per cent cellulose digestion were recorded for all trials at the 24, 36, 48, 60, and 72 hr periods. It should be noted that all three trials demonstrated a 12 hr period, between 36 and 48 hr, during which less than 1% of cellulose was digested. Following this period, the three trials exhibited a slight increase in cellulose digestion, extending from 48 to 72 hr. The values recorded for cellulose digestion in this 24 hr period were 1.8, 5.3, and 10.7%. Trial No. 1 showed a lag period in digestion during the first 12 hr; however, at the end of 24 hr, per cent cellulose digestion in Trial No. 1 was approaching that reported for trials No. 2 and 3. A possible reason for the lag period in Trial No. 1 will be explained in the discussion. It was interesting to note the slight amount of cellulose that disappeared from the

Table 4. Reproducibility of digestion of alfalfa hay cellulose using the VIVAR[1]

Trial No.	Time in hours	Alfalfa added	Cellulose in alfalfa	Cellulose in 25 ml of inoculum	Total cellulose added	Cellulose recovered	Cellulose digested	Per cent cellulose digested
		(mg)	(mg)	(mg)	(mg)	(mg)	(mg)	(%)
1	12	1,000	329	1.0	330.0	274.8	55.2	16.7
	24	1,000	329	1.0	330.0	171.8	158.2	47.9
	36	1,000	329	1.0	330.0	129.6	200.4	60.7
	48	1,000	329	1.0	330.0	129.5	200.5	60.7
	60	1,000	329	1.0	330.0	125.0	205.0	62.1
	72	1,000	329	1.0	330.0	123.7	206.3	62.5
	72	1,000	329	no inoculum[2]	329.0	327.0	0*	0*
2	12	1,000	329	36.0	365.0	178.3	186.7	51.1
	24	1,000	329	36.0	365.0	159.2	205.8	56.3
	36	1,000	329	36.0	365.0	139.4	225.6	61.7
	48	1,000	329	36.0	365.0	139.3	225.7	61.8
	60	1,000	329	36.0	365.0	124.3	240.7	65.9
	72	1,000	329	36.0	365.0	119.8	245.2	67.1
	72	1,000	329	no inoculum	329.0	324.0	0	0
3	12	1,000	329	16.9	345.9	173.8	172.1	49.7
	24	1,000	329	16.9	345.9	154.3	191.6	55.3
	36	1,000	329	16.9	345.9	147.5	198.4	57.3
	48	1,000	329	16.9	345.9	145.1	200.8	58.0
	60	1,000	329	16.9	345.9	130.6	215.3	62.2
	72	1,000	329	16.9	345.9	108.1	237.8	68.7
	72	1,000	329	no inoculum	329.0	326.5	0	0

[1]Also see Table No. 9 in appendix.

[2]Control-distilled water added.

*Within experimental error of method used.

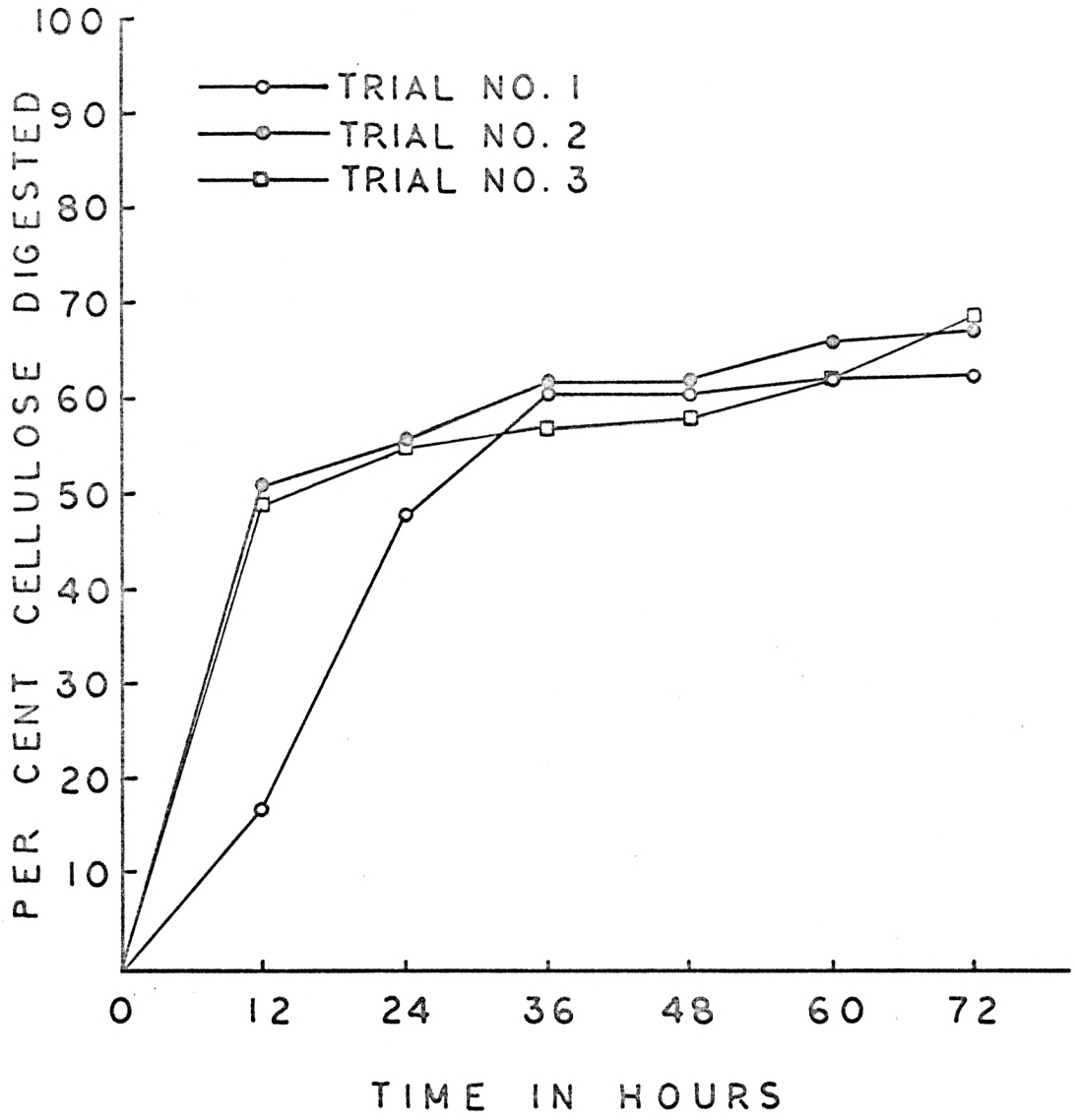


Fig. 1. Reproducibility of digestion of alfalfa hay cellulose using the VIVAR on three different occasions.

control VIVARs in these three trials as well as in subsequent trials.

Digestion of Extracted Alfalfa Cellulose Using the VIVAR

The results again demonstrate that good reproducibility can be obtained when experiments are performed several days apart and with different inocula (Table 5 and Fig. 2). Alfalfa cellulose, extracted according to Crampton and Maynard (1938) and ground to a particle size of 2 mm in a Wiley mill, was the sole energy source in both trials. The results showed extracted cellulose to be digested at a more rapid rate and to a greater extent than cellulose in whole plant material (Table 4). The rate of digestion of extracted cellulose was rapid for the first 36 hr, 78.8 and 79.8% of the cellulose being digested in this period. During the 36 to 72 hr period, the values for cellulose digestion were 7.1 and 9.8%. Digestion of the extracted cellulose proceeded to within 10.4 and 14.9% of completion in 72 hr as compared with the range of 31.3 and 37.5% reported for digestion of whole plant cellulose (Table 4).

Recovery of Carbon-14 in Dried Alfalfa Plant Material

The quantities of C^{14} recovered in the dry weight of the total plant material and cellulose from several labeling runs are shown in Table 6. On the average, 33.8% of the isotope released was recovered in the plant tissue. The per cent of C^{14} recovered in the plant material was similar whether all the $C^{14}O_2$ was released at periodic intervals during growth or only once (Table 6). Losses in the oil or the water used in the spirometer were insignificant. After 60 mc of C^{14} had been released in the chamber the counts on the water and mineral oil were 5 to 10/ml above background or approximately 1 μc in all the water and oil in the barrel.

Table 5. Digestion of extracted alfalfa cellulose using the VIVAR[1]

Trial No.	Time in hours	Extracted cellulose added	Cellulose in 25 ml of inoculum	Total cellulose added	Cellulose recovered	Cellulose digested	Per cent cellulose digested
		(mg)	(mg)	(mg)	(mg)	(mg)	(%)
1	12	300	17	317	126.5	190.5	60.0
	24	300	17	317	95.9	221.1	69.7
	36	300	17	317	67.1	249.9	78.8
	48	300	17	317	54.5	262.5	82.8
	60	300	17	317	47.1	269.9	85.1
	72	300	17	317	44.4	272.6	85.9
	72	300	no inoculum[2]	300	296.0	0*	0*
2	12	329	4	333	147.4	185.6	55.7
	24	329	4	333	95.1	237.9	71.4
	36	329	4	333	67.0	266.0	79.8
	48	329	4	333	55.3	277.7	83.3
	60	329	4	333	40.5	292.5	87.8
	72	329	4	333	34.5	298.5	89.6
	72	329	no inoculum	329	327.1	0	0

[1]See also Table No. 10 in appendix.

[2]Control-distilled water added.

*Within experimental error of method used.

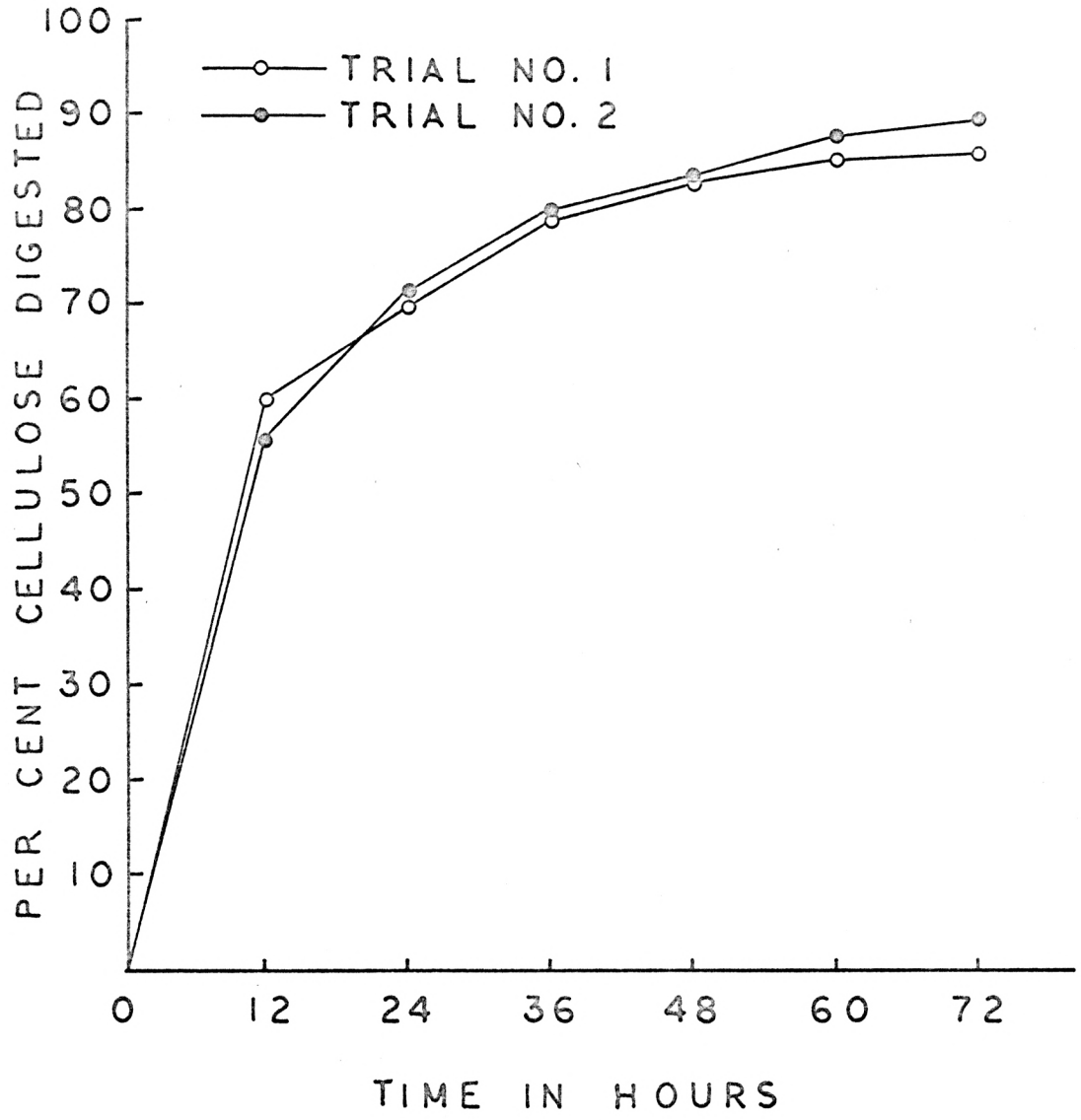


Fig. 2. Digestion of extracted alfalfa cellulose using the VIVAR on two different occasions.

Table 6. Carbon-14 recovery in harvested alfalfa (dry matter basis) from five labeling runs

Weight of material	Per cent cellulose	Isotope released	Amount recovered: Cellulose C^{14}	Amount recovered: Total C^{14}	Isotope recovered
(g)		(mc)	(mc)	(mc)	(%)
84.0	40.9	5.0	0.919	1.682	33.6[a]
32.6	32.2	10.0	1.000	2.838	28.3[a]
71.5	39.2	10.0	1.640	4.090	40.9[b]
49.5	36.4	10.0	1.459	3.342	33.4[b]
52.5	42.4	7.9	1.002	2.601	32.8[b]

[a]One release.

[b]Four releases.

Volatile Fatty Acid Production from Carbon-14 Labeled Alfalfa Using the VIVAR

Trial No. 1. The results plotted in Fig. 3 and 4 and presented in Table 11 of the appendix are the average of two trials, A and B, each using 1 g (40 μc) of whole C^{14} alfalfa and two trials, C and D, each using 319 mg (7 μc) of C^{14} alfalfa extracted with hot water to remove soluble carbohydrates (Hansen, Forbes, and Carlson, 1958). Samples were taken from each VIVAR at the designated times. Results were expressed as disintegrations per minute per milligram of the sodium salts of total volatile fatty acids (which means specific activity). The levels of C^{14} activity in volatile acids from both whole and extracted C^{14} alfalfa are shown to be similar, differing only in magnitude. Labeled volatile acids were produced within 15 min of fermentation from both types of material. A peak in C^{14} activity occurred in the acids in each case after 4 hr of fermentation. The peaks in activity then declined until 46 hr. During the 46 to 66 hr periods, it is shown that second peaks in C^{14} activity occurred. Carbon-14 activity from both types of material slowly fell after 66 hr, but remained significant at the end of 107 hr.

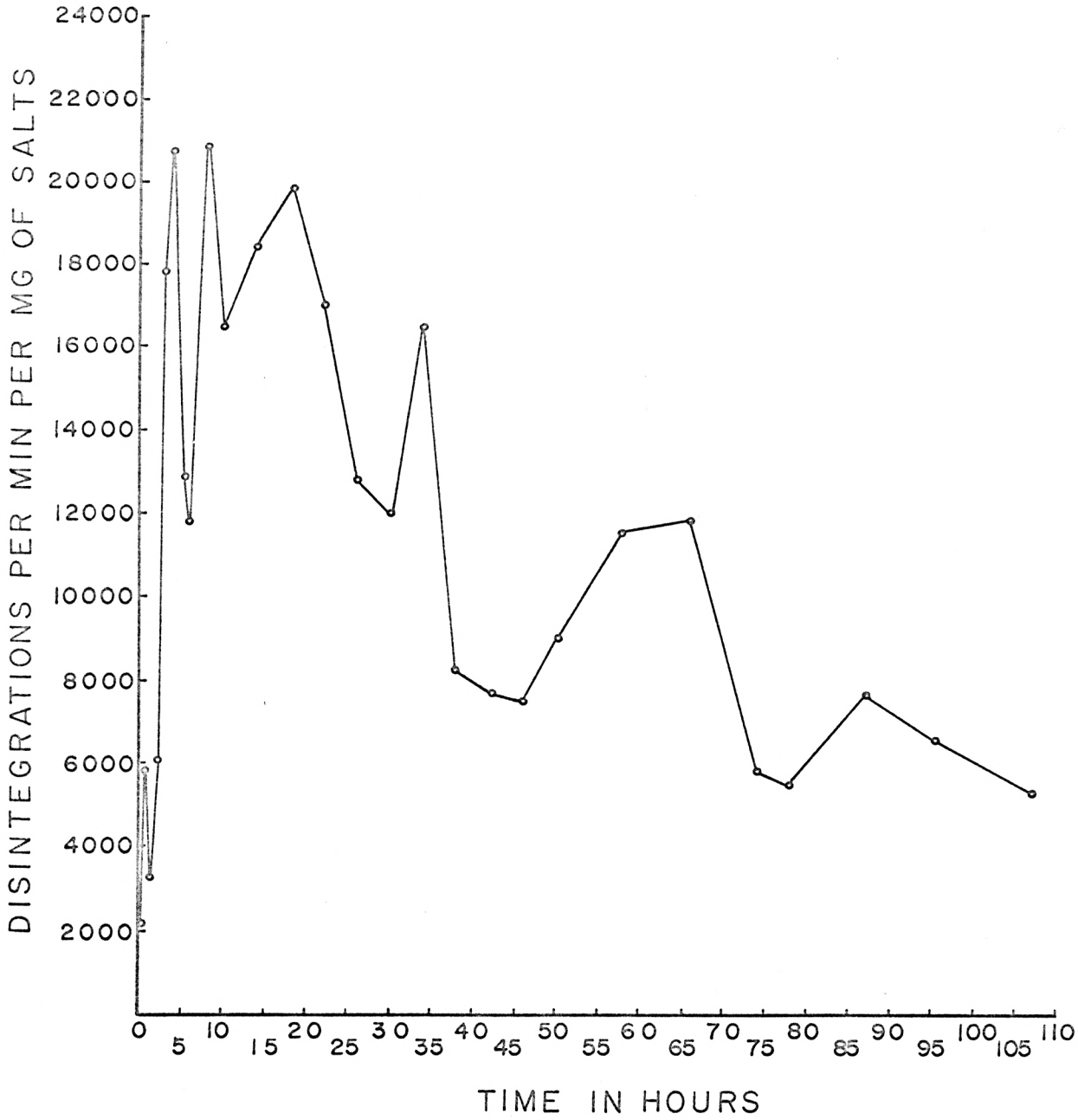


Fig. 3. Average specific activities of total volatile fatty acids (Na salts) produced from whole C^{14} alfalfa in Trial No. 1.

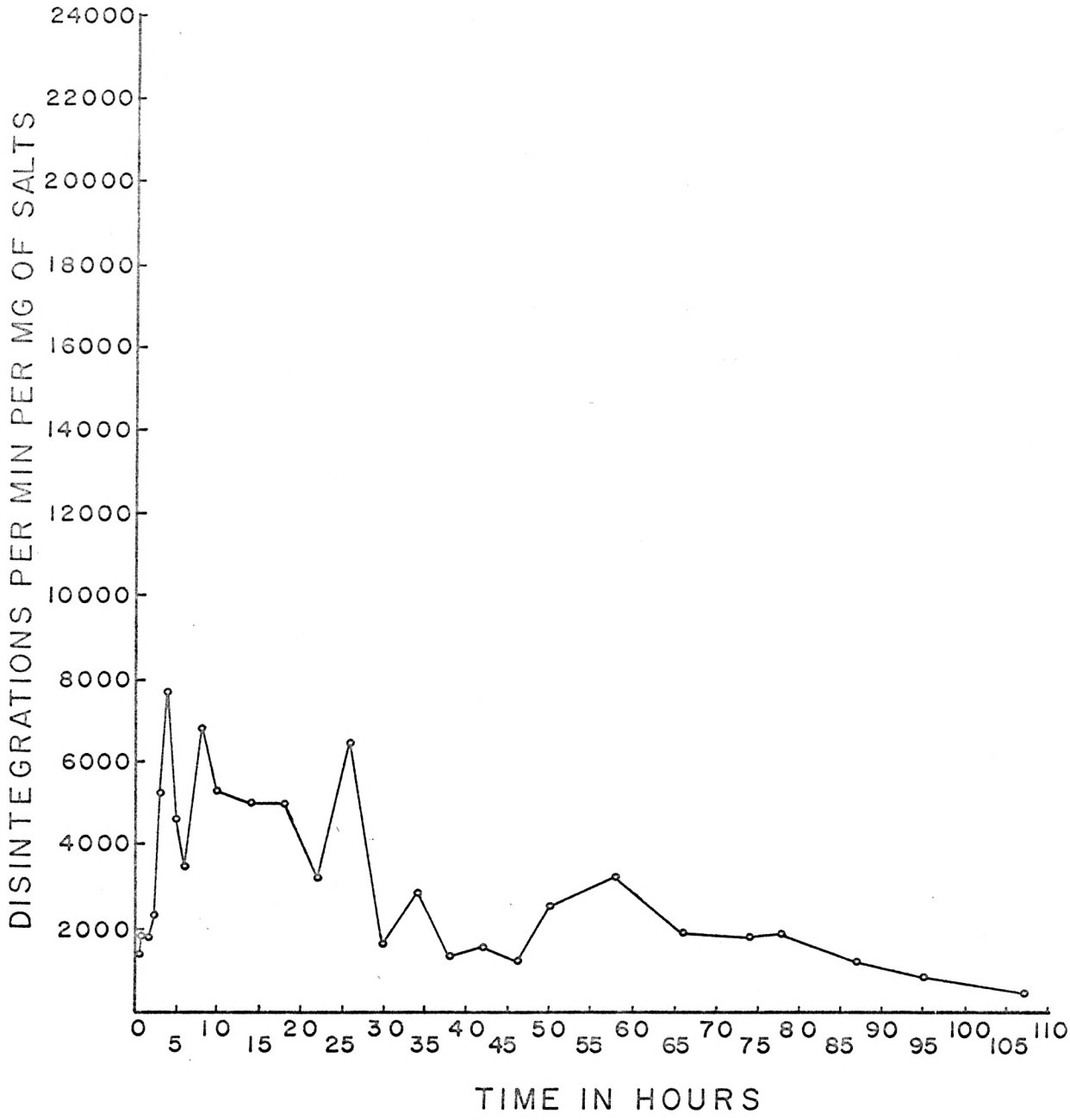


Fig. 4. Average specific activities of total volatile fatty acids (Na salts) produced from extracted C^{14} alfalfa in Trial No. 1.

Trial No. 2. The results plotted in Fig. 5 and presented in Table 12 of the appendix are the average of two trials, A and B, each using 1 g (10 μc) of whole C^{14} alfalfa and two trials, C and D, each using the extractive-free residue (7 μc) from 1 g of the same whole alfalfa. Results were expressed as disintegrations per minute per milligram of the sodium salts of total volatile fatty acids. The levels of C^{14} activity in volatile acids from both types of substrate were shown to be very similar except that C^{14} activity from the extracted (sugar free) alfalfa was consistently lower than that shown for whole plant. This difference became less pronounced as length of incubation increased. The results were similar to those of Trial No. 1 (Fig. 3 and 4) in that C^{14} activity was detected as early as 15 min and had reached a peak between 4 and 30 hr. Results of Trial No. 2 (Fig. 5) differed from those of Trial No. 1 by showing a more gradual decline in C^{14} activity with no sharp second peaks in activity appearing. Again, a significant amount of C^{14} activity remained in the volatile fatty acids at the end of the trial (100 hr).

Trial No. 3. Experimental procedure differed from that of Trials No. 1 and 2. Four VIVARs, each containing 1 g (34 μc) of whole C^{14} alfalfa, were inoculated and placed in the rumen of the fistulated animal. The animal was then fasted for 72 hr. Samples of rumen fluid for C^{14} volatile fatty acid analyses were taken alternately from each VIVAR. The results in Fig. 6 and Table 13 of the appendix show that the specific activity of the sodium salts of the volatile acids rose rapidly and remained at about the same level of C^{14} activity throughout the experiment. There was no gradual decline in C^{14} activity of the volatile acids as seen in the previous trials. The factors that might have caused the specific activity of the volatile acids to decrease in Trials No. 1 and 2 will be explained in the discussion.

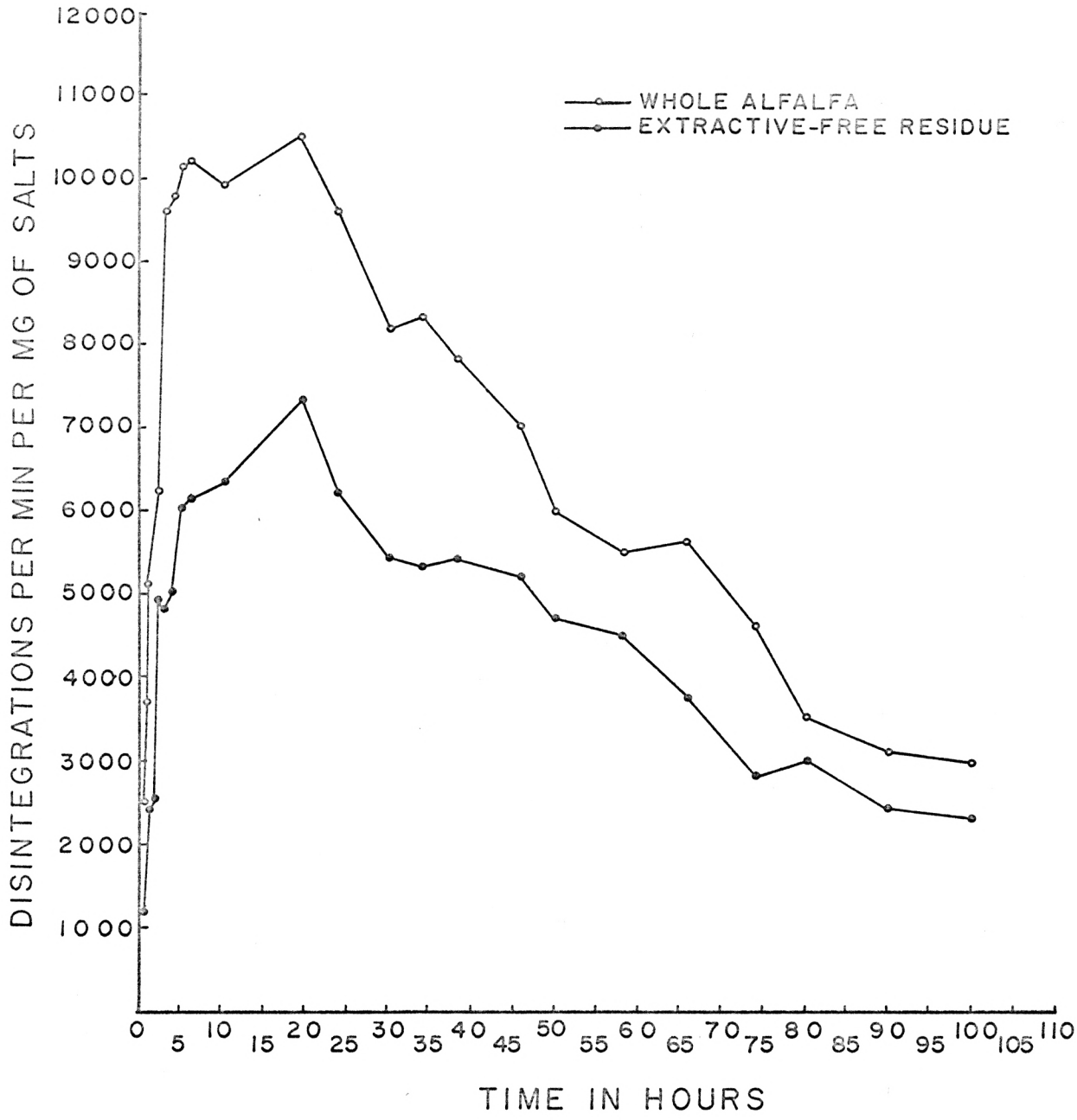


Fig. 5. Average specific activities of total volatile fatty acids (Na salts) produced in Trial No. 2.

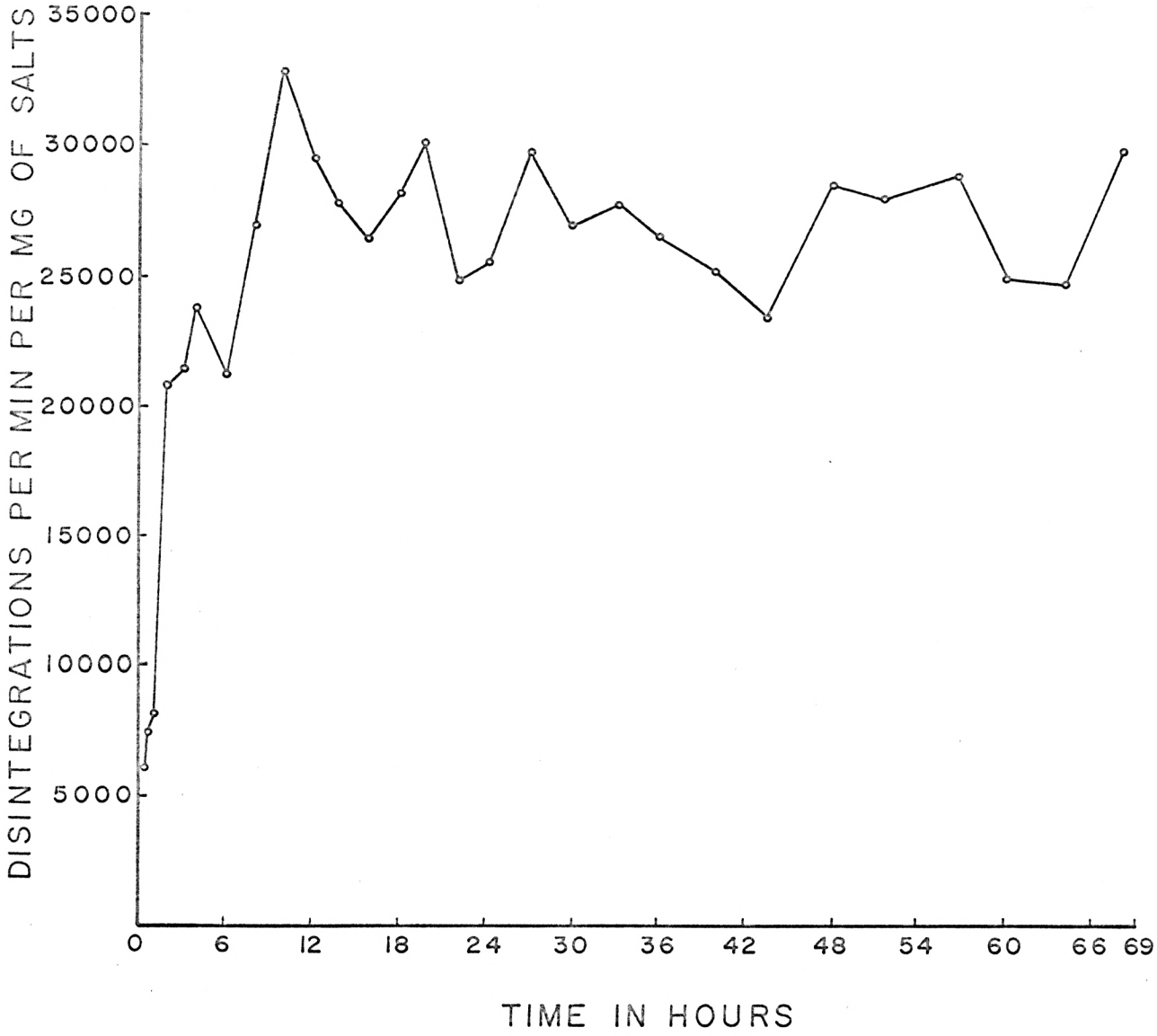


Fig. 6. Specific activities of total volatile fatty acids (Na salts) produced in Trial No. 3.

The Digestion of Prairie Hay Cellulose Using the VIVAR

The data presented in Table 7 and plotted in Fig. 7 were the results of two digestion trials conducted 7 days apart. The prairie hay used in each trial was of the identical source and particle size (2 mm). Values for per cent cellulose digestion at all time intervals in both trials compared favorably. The results showed cellulose digestion to proceed at a nearly constant rate during the first 48 hr of each trial. Digestion then continued at a much reduced rate until the trials were terminated at 78 hr. It is important to note the differences between digestion of prairie hay cellulose and alfalfa hay cellulose as seen in Fig. 1. Possible reasons for these differences will be discussed.

The Effect of Added Lignin on Digestion of Extracted Alfalfa Cellulose

This experiment was designed to determine whether or not added lignin would inhibit digestion of extracted alfalfa cellulose. A 250 mg sample of extracted cellulose was placed in each of four VIVARs. Two VIVARs each received 170 mg of 60% lignin[1] in addition to the cellulose. The VIVARs were inoculated with 25 ml of rumen fluid and placed in the rumen of the animal. They were removed at the end of 48 hr and the per cent cellulose digestion determined. From this preliminary study it is seen (Table 8) that cellulose digestion was depressed approximately 13% when the lignin was present.

---

[1]Dried lignin liquor obtained from Consolidated Water Power & Paper Co., 1130 E. John St., Appleton, Wis.

Table 7. Digestion of prairie hay cellulose on two different occasions using the VIVAR[1]

Trial No.	Time in hours	Prairie hay added (mg)	Cellulose in prairie hay (mg)	Cellulose in 25 ml of inoculum (mg)	Total cellulose added (mg)	Cellulose recovered (mg)	Cellulose digested (mg)	Per cent cellulose digested (%)
1	24	1,000	354	5	359	210.0	149.0	41.5
	36	1,000	354	5	359	161.1	197.9	55.1
	48	1,000	354	5	359	115.6	243.4	67.7
	54	1,000	354	5	359	108.1	250.9	69.8
	66	1,000	354	5	359	100.4	258.6	72.0
	78	1,000	354	5	359	97.3	261.7	72.8
	78	1,000	354	no inoculum[2]	354	352.0	0	0
2	24	1,000	354	8	362	218.3	143.7	39.6
	36	1,000	354	8	362	170.5	191.5	52.9
	48	1,000	354	8	362	137.9	224.1	61.9
	54	1,000	354	8	362	121.7	240.3	66.3
	66	1,000	354	8	362	116.5	245.5	67.7
	78	1,000	354	8	362	105.8	256.2	70.7
	78	1,000	354	no inoculum	354	351.2	0	0

[1]Also see Table No. 14 in appendix. (Complete data)

[2]Control-distilled water added.

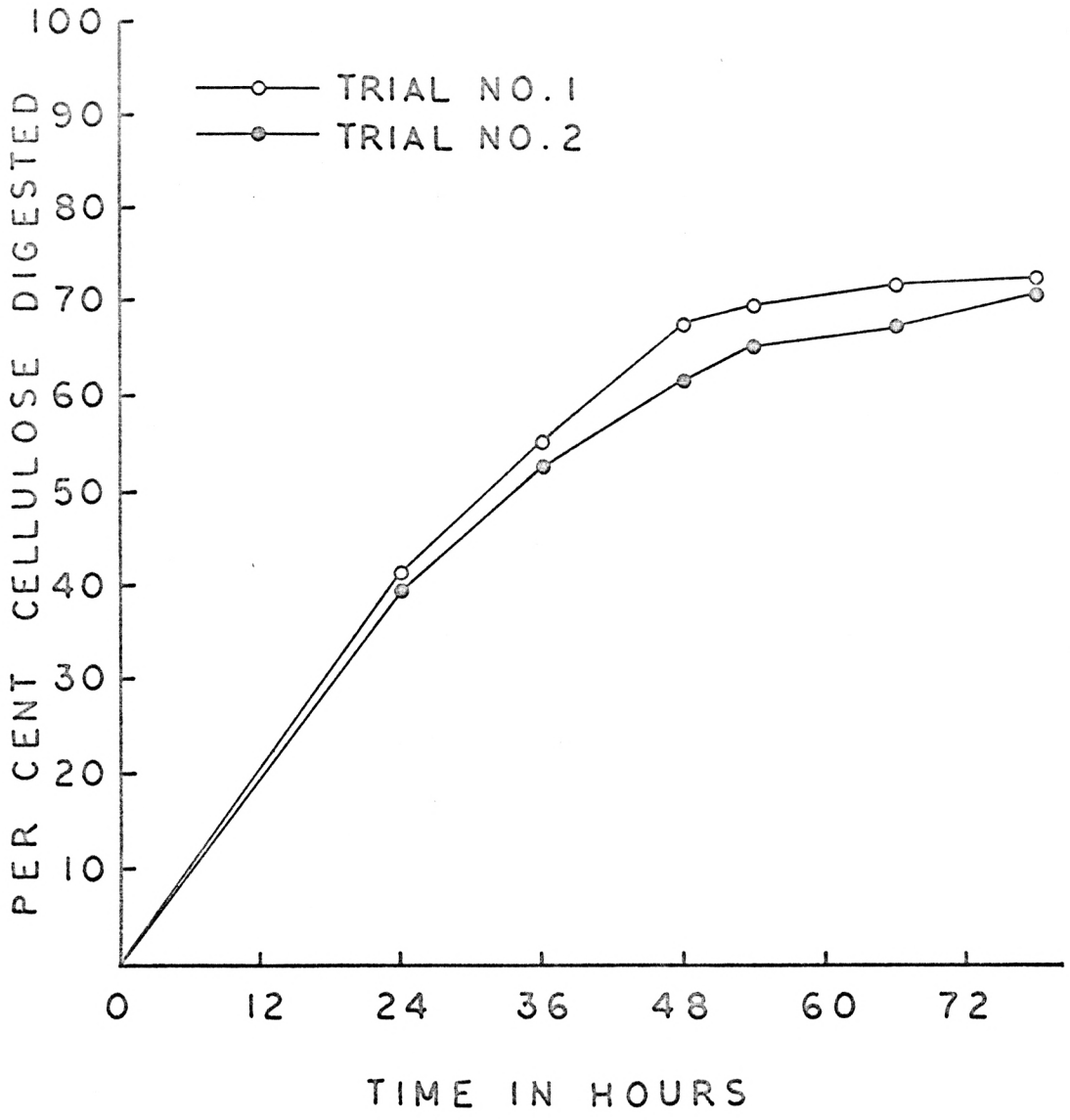


Fig. 7. Digestion of prairie hay cellulose using the VIVAR on two different occasions.

Table 8. The effect of added lignin on digestion of extracted alfalfa cellulose[1]

VIVAR No.	Extracted cellulose added (mg)	Cellulose in 25 ml of inoculum (mg)	Total cellulose added (mg)	Lignin added (mg of 60%) (mg)	Time in hours	Cellulose recovered (mg)	Cellulose digested (mg)	Per cent cellulose digested (%)
1	250	4	254	0	48	45.4	208.6	82.1
2	250	4	254	0	48	35.5	218.5	86.0
3	250	4	254	170	48	72.5	181.3	71.3
4	250	4	254	170	48	73.5	180.5	71.0
5	250	no inoculum[2]	250	0	48	249.0	0	0

[1]Also see Table No. 15 in appendix. (Complete data)

[2]Control-distilled water added.

DISCUSSION

It has been the purpose of this thesis to report on an improved *in vivo* artificial rumen technique and show some of its applications in the study of rumen microbiology.

In vitro observations (Table 1) revealed that all membranes tested were permeable to the C_2-C_6 volatile fatty acids, with the membrane possessing the smallest pore size (0.1 μ) exhibiting the most rapid rate of diffusion. Experimental conditions *in vivo* would be quite different from the conditions of these experiments, thus the data shown in Table 1 are illustrative only of the relative diffusion barriers presented by the different membranes. Evidence of these differences was pointed out in Table 2 in which the results of *in vivo* diffusion tests, using Millipore and Visking membranes, were shown. From the results in Table 2 it is seen that the total volatile acids diffused through the membranes, but never reached, during the experimental period, equilibrium with the ruminal volatile acids, nor were the per cents of equilibrium attained *in vivo* as great as those attained *in vitro* (Table 1). There is the possibility that this apparent decrease in diffusibility may have been due to clogging of the membranes *in vivo* by finely divided forage particles. Nevertheless, it was shown that within 24 hr the concentration of volatile acids in the VIVAR equipped with 0.1 μ Millipore membranes was within 18% of the volatile acid concentration in the rumen. The concentration of volatile acids in this VIVAR was still rising at the end of 48 hr. The above mentioned *in vitro* and *in vivo* diffusion studies were undertaken for the purpose of selecting membranes that would allow a rapid rate of exchange between soluble VIVAR contents and those of the rumen. These soluble products would include volatile acids, vitamins, and any known or unknown growth

factors. The diffusibility of selected membranes would also be important from the standpoint of elimination of microbial waste products that might otherwise build up to inhibitory levels in the VIVAR. Vycor glass, ceramic, and sintered stainless steel discs were not tested *in vivo* because of their slow diffusion characteristics as seen *in vitro* (Table 1).

Results of *in vitro* sterility tests showed the Millipore, Visking, Vycor, and ceramic membranes to be suitable for use in pure culture studies. Sintered stainless steel discs were easily penetrated by the test organism, *Serratia marcescens*. *In vivo* sterility tests using Millipore and Visking membranes showed that no organisms penetrated into the sealed, sterile VIVARs even after 120 hr in the rumen. Fluid from these VIVARs was examined microscopically and cultured in tryptose broth. Special media were not used and strict anaerobic techniques were not followed when culturing the fluid. It was felt that the membranes would not be selectively permeable to only the fastidious anaerobic organisms. In view of this and the numbers of aerobes reported present in rumen contents (Gall and Huhtanen, 1951) culturing only for aerobes seemed reasonable.

Qualitative tests conducted *in vitro* showed Millipore and Visking membranes to be permeable to glucose and soluble starch. Similar tests for urease showed detectable amounts of the enzyme had diffused through the Millipore membranes (0.1 μ and 0.2 μ pore sizes) within 1 hr, but no urease was detected in a 48 hr test on contents of the VIVAR equipped with the Visking membranes. The impermeability of Visking to urease was not altered by sterilization in the autoclave. It was not determined whether urease found in rumen fluid (Pearson and Smith, 1943) would penetrate through Millipore membranes. The *in vitro* diffusion of glucose and soluble starch was used only as another

indicator of membrane permeability to various compounds. The importance of glucose and soluble starch diffusion under in vivo conditions would be questionable because of the very low concentration of these substrates in the rumen. They are fermented quite rapidly (Annison and Lewis, 1959; Sutherland, personal communication to Fina).

The ability of the Millipore and Visking membranes to resist digestion in the rumen was examined and no significant loss in weights was detected. There were no apparent signs of weakening or digestion after exposure in the rumen for 120 hr. That these membranes were not digested was rather unexpected, since both types consist of cellulosic material.

Digestion trials, with alfalfa hay as substrate, were undertaken to determine if reproducible results could be obtained using the VIVAR technique on different days with different inocula. The results (Table 4 and Fig. 1) clearly show this to be possible. There was close agreement in values of per cent cellulose digestion for all trials at each time interval, with the exception of the 12 and 24 hr samples in Trial No. 1. This trial differed from trials 2 and 3 in that the rumen fluid used for the inoculum contained only 1 mg of cellulose compared to 36 and 16.9 mg in the inocula of the other trials (Table 4). There is the possibility that the inoculum used in Trial No. 1 contained fewer numbers of cellulolytic organisms, thus a lag period might be demonstrated. Possibly this lag period persisted until a sufficient population of cellulolytic organisms was built up, as seen by digestion proceeding in agreement with the other two trials from 36 to 72 hr. The microscopic work by Baker and Harriss (1947) has indicated that cellulolytic organisms are found in close association with their cellulose substrates. If this is the case, then an inoculum low in cellulose particles could well be

low in numbers of cellulolytic organisms, thus causing a lag period in cellulose digestion. It would appear from these results (Table 4) that a more constant amount of cellulose in the inoculum might result in even better reproducibility of digestion in the first 12-24 hr of an experiment. It was of interest to note the slight amount of cellulose that disappeared (2-5 mg) from the control VIVARs in this and subsequent trials. This was within experimental error of the method of cellulose extraction (Crampton and Maynard, 1938). The failure to digest cellulose in uninoculated VIVARs helps substantiate the absence of extra-cellular cellulase in rumen fluid (Hungate, 1950; Kitts and Underkofler, 1954) which otherwise might have diffused into the VIVARs. The fact that digestion of cellulose ceased between 36 and 48 hr and then resumed between 48 and 72 hr, in all three trials, raises some interesting questions. A similar leveling off period has been reported for alfalfa by Dehority and Johnson (1961), using in vitro digestion techniques. Their fermentations ceased at 30 hr and showed no more digestion by 48 hr, the time at which the experiments were terminated. They believed that all of the available cellulose was digested in 30 hr and that lignin encrustation prevented digestion of the remaining cellulose. The digestion of cellulose in 48 hr by their in vitro method was given as 58.1%. The average per cent of cellulose digested in three trials at 48 hr using the VIVAR was 60.1%, with an average value of 66.1% digested at the end of 72 hr. If Dehority and Johnson (1961) are correct in assuming all available cellulose to be digested by 30 hr, would this also hold true using the VIVAR technique? Apparently so, as seen by the leveling off period between 36 and 48 hr. The question now arises as to why digestion resumes after 48 hr in the VIVAR method. As to the answer, one can only speculate. It is possible that lignin which covers or encrusts the plant

cellulose (Hansen et al., 1958; Dehority and Johnson, 1961) has two effects on cellulose digestion. One well known effect is that lignin acts as a physical barrier between the cellulose and the cellulolytic organisms (Baker and Harriss, 1947). The second effect may be that lignin itself, because of its aromatic composition (Hansen et al., 1958), might exert bacteriostatic action against cellulolytic organisms if it undergoes degradation in the rumen. Lignin has been reported as indigestible in ruminants (Crampton and Maynard, 1938), but Ely, Jacobson, and Kane (1951) claimed as much as 14% of ingested lignin was utilized. Crampton and Maynard (1938) state that lignin is not attacked, "perhaps because of a certain degree of antiseptic action resulting from its phenolic nucleus". With this evidence in mind, one could speculate that the reason cellulose digestion levels off at 36 hr in the VIVARs might be a result of bacteriostasis caused by degradation products of lignin. As the inhibiting products were utilized by other bacteria, cellulolytic activity might resume at a rate dependent upon the decreasing concentrations of the inhibitory substances. When digestion of extracted alfalfa cellulose was studied it was seen (Table 5 and Fig. 2) that digestion was more rapid and more complete than digestion of whole plant cellulose (Table 4 and Fig. 1). The cellulose used in this experiment was extracted from the same plant material used in the trials previously discussed. When comparisons were made between the percentages of cellulose digestion of whole plant (Table 4) and extracted cellulose (Table 5) it became evident that cellulose unprotected by lignin was readily attacked by the organisms (Baker et al., 1959). The amount of extracted cellulose digested in 12 hr (Table 5) was nearly equal to the amount digested in 24 hr (Table 4) using whole plant material. Again, good reproducibility was demonstrated between trials performed on different days

and with different inocula. It was not determined whether a lag in digestion occurred between 0 and 12 hr as reported by Dehority (1961), who showed a lag of 12 hr when using 100 mesh cellulose *in vitro*. If a lag period occurred in these trials (Table 5) it would surely have been short considering the amount of cellulose digested (60.0 and 55.7%) in 12 hr.

Preparation of labeled alfalfa used in this work required a special growth chamber in which $C^{14}O_2$ of high specific activity was utilized in the photosynthetic process. Several such apparatuses have been constructed (Brown, Tiffin, and Holmes, 1958; Fisher, 1958; and Scully *et al.*, 1955), but their use was impractical for this work because of high construction costs or small capacity. A Plexiglas growth chamber (page 20) was used to label alfalfa plants for use in these studies. The quantities of C^{14} recovered in the dry weight of the total plant material and cellulose from several labeling runs may be seen in Table 6. On the average 33.8% of the isotope released was recovered in the plant tissue with the greatest recovery of C^{14} occurring in the healthiest and most vigorously growing plants. Carbon-14 per cent recovery was similar whether all the $C^{14}O_2$ was released at periodic intervals during growth or only once (Table 6).

While the purpose for which alfalfa was prepared did not require a high degree of uniform labeling, it should be possible, using stricter control measures (Scully *et al.*, 1955), to obtain uniformly labeled plant material. Nonuniform distribution of radioactivity in plants may be due to a number of factors. The $C^{14}O_2$ may be diluted by soil CO_2, and unlabeled carbon present in the roots may be translocated to the first few inches of new growth (Jenkinson, 1960). Material of greater C^{14} activity was obtained when plants were placed in the chamber early and when the same plants were used repeatedly

to benefit from root stored C^{14}.

The digestion trials using C^{14} alfalfa as substrate in the VIVARs were intended to give information on the breakdown of plant components such as soluble carbohydrates and cellulose. The specific activity per milligram of Na salts of the volatile fatty acids was followed as an indication of fermentation of the substrate. The results plotted in Fig. 3 show that the C^{14} alfalfa (Trial No. 1) was rapidly attacked, C^{14} activity in the volatile acids was detected as early as 15 min and quickly rose to a peak which was maintained between 4 and 30 hr. The C^{14} activity then demonstrated a gradual decline until about 38 hr. From 38 to 46 hr C^{14} activity dropped very little, but immediately following, between 46 and 66 hr, a second peak occurred in C^{14} volatile acids. The C^{14} activity then fell off, but was still present at 107 hr. The hot water extracted C^{14} alfalfa[1] demonstrated essentially the same pattern of radioactivity. Using both types of labeled material two peaks of C^{14} activity were seen. The first occurred between 4 and 30 hr and the second peak between 46 and 66 hr. Since an early peak was observed in volatile acids produced from supposedly sugar free C^{14} alfalfa (Fig. 4), one must assume that other plant materials were undergoing fermentation during this period. The results of alfalfa hay cellulose digestion trials (Table 4) indicated that most of the available cellulose was digested between 0 and 36 hr, with a slight increase in digestion occurring after 48 hr. If cellulose digestion occurs in two steps then it seems that any volatile fatty acids produced should, if labeled, appear as two C^{14} peaks to reflect the increases in concentration. Before going further, the effect of unlabeled ruminal volatile acids upon the

---

[1]Extracted to remove soluble carbohydrates.

C^{14} activity of the labeled volatile acids in the VIVARs should be explained. The experimental animal was fed at 12 hr intervals; after each feeding large quantities of unlabeled volatile acids were produced. These acids peak in concentration at 3-4 hr after feeding (Knox, 1961). Absorption by the rumen gradually lowers the concentration to that observed prior to feeding (Knox, 1961). This means that after each feeding the labeled volatile acids were diluted by unlabeled ruminal volatile acids diffusing into the VIVARs. Thus the specific activity of the acids in the VIVARs would show a gradual but constant decrease in C^{14} activity. Sampling the VIVARs would speed this dilution process as fluid passed in to take the place of that removed. Nevertheless, since the dilution of specific activity should be fairly constant, any increase in C^{14} volatile acid formation in the VIVARs would be reflected by an increase in the specific activity of the volatile acids sampled. Consequently it appears, from Fig. 3 and 4 and information based on Table 4, that digestion of alfalfa hay, using the VIVAR, takes place over an extended period of time with cellulose utilization occurring in a two step fashion.

Trial No. 2 was an attempt to duplicate the results described above obtained from Trial No. 1 (Fig. 3 and Fig. 4), and to actually demonstrate a relationship exists between the patterns of C^{14} volatile acids produced from whole and hot water extracted C^{14} alfalfa. Here again (Fig. 5) both types of material showed detectable C^{14} activity in the volatile acids at 15 min. Major peaks in activity occurred between 4 and 30 hr, followed by a gradual decline in C^{14} activity. Results of this trial differed from Trial No. 1 in that no pronounced peaks occurred between 46 and 66 hr, only very slight increases in activity appeared. This difference may have been because fewer samples were withdrawn from the VIVARs, thus decreasing the dilution of labeled

acids by unlabeled ruminal volatile acids. The specific activity would not reflect any minor increase in volatile acid production in the VIVAR because only the concentration of labeled acids would change, not the ratio of labeled to unlabeled acids. It is significant that C^{14} activity from the extractive-free residue of whole C^{14} alfalfa was much lower than C^{14} activity from whole labeled alfalfa during the 4-30 hr period. This probably was due to the loss during the extraction process of soluble labeled substances. This difference in C^{14} activity between the two materials became progressively less as length of fermentation time increased.

Trial No. 3 was designed to show that the specific activity of volatile fatty acids produced in VIVARs would remain constant when unlabeled volatile acids were not diffusing inward. Four VIVARs were used for alternate sampling so that less inward diffusion of unlabeled ruminal acids would occur, hence less dilution of the specific activity of the volatile acids. The fistulated animal was fed when the VIVARs were placed in the rumen, then fasted for the following 72 hr. From the results in Fig. 6 it was seen that the specific activity of the volatile acids remained fairly constant up to the time the experiment was terminated. As each point on the curve (Fig. 6) represents the specific activity of the acids in a different VIVAR, there was some unevenness. The concentrations of total volatile fatty acids in the VIVARs at 44 and 68 hr were 109 and 104 μmoles/ml, respectively. At these same times, the concentrations of volatile acids in the rumen were only 20-30 μmoles/ml. Therefore, diffusion of labeled volatile acids would tend to be toward the lower concentration found in the rumen. As a result, the specific activity of the acids in the VIVARs would not be decreased, but would remain more constant as seen in Fig. 6.

Because of the results obtained with the alfalfa hay (Table 4 and Fig. 1), it was believed that studies with the VIVAR using prairie hay might be of interest. Dehority and Johnson (1961) had suggested that some basic difference appeared to exist with regard to cellulose digestion of grass and legume hays in relation to lignin. They had shown that at about the same lignin content, the legume (alfalfa) cellulose was digested to a lesser extent than the cellulose of grass hays. Results using the VIVAR technique tended to contradict their digestion studies, but they had not used prairie hay. During the first 36 hr of fermentation in the VIVAR, more alfalfa hay cellulose than prairie hay cellulose was digested (Table 7 and Fig. 7). However, at 48 hr, the per cent prairie hay cellulose digested was greater than that reported for alfalfa hay cellulose (Table 4 and Fig. 1). The results indicated cellulose digestion of prairie hay proceeded at a slower but more constant rate than digestion of alfalfa hay cellulose. It was also shown that prairie hay digestion exhibited no leveling off period between 36 and 48 hr, instead, the digestion proceeded at an average rate of about 3 mg of cellulose per hour. From these results it appears that there is a difference in cellulose digestion of prairie hay and alfalfa hay, but how this difference may have related to lignin content is not known. Lignin determinations were not performed on the hays.

Lawton et al. (1951) suggested that the theory of encrustation of cellulose by lignin might not be the true reason for depression of cellulose digestion. They theorized that lignin might contain bacteriostatic compounds or groups. In an attempt to gain further information on the effect of lignin on cellulose digestion, samples of dried lignin (wood) were added to VIVARs containing extracted alfalfa cellulose. The amount added was 170 mg of 60% lignin, which would be nearly equal to the amount of pure lignin found in

green, mature alfalfa (Hansen et al., 1958). Cellulose digestion of these samples was then compared with the digestion of extracted cellulose alone. From the results (Table 8) it appeared that the added lignin had depressed cellulose digestion by an average of 12.9%. It was thought that residual H_2SO_4 from the lignin extraction process might be the reason for the apparent inhibition of digestion. Therefore, the effect of the lignin on pH values of distilled water and of rumen fluid was examined. The pH value of distilled water containing 170 mg of lignin was 4.3. However, the lignin had no effect on the pH value of rumen fluid; the value remained at 6.8. It is realized that degradation products vary with the source of the lignin (Hansen et al., 1958). Consequently, the results of this experiment might not hold true with alfalfa lignin. Kamstra et al. (1958) reported that lignin isolated from full bloom alfalfa had no effect on digestion when added to in vitro fermentations of cellulose. However, there is the possibility that the in vitro technique may alter a particular organism that might degrade lignin.

The experimental work discussed touches only a minimum of the potential areas for study through use of the VIVAR technique. Its application in the areas of pure culture studies, symbiotic relationships, enzyme isolation, and metabolic pathways should be possible.

As with any system which attempts to duplicate natural conditions, especially largely undefined conditions, there are both advantages and disadvantages. The apparent advantages of the VIVAR technique consist of the following: The rumen itself serves as the incubator, growth factors required by the organisms may diffuse into the VIVARs, metabolic end products are eliminated more rapidly, the apparatus consists of few parts and can be easily assembled, and a great variety of membranes are available to facilitate different experiments.

The disadvantages of the VIVAR technique are the following: a fistulated animal must be available; and experiments are difficult to replicate, because of the limited number of VIVARs which can be placed in the rumen.

SUMMARY

An improved *in vivo* artificial rumen (VIVAR) technique was developed in an attempt to more closely duplicate actual rumen conditions. The apparatus consisted of a stainless steel drum with a side arm and could be fitted with a variety of membranes. The membranes were held in place by screens, gaskets, and outer nuts. Rubber tubing was attached to the side arm which served as a sampling port and a gas outlet. The apparatus was placed in the rumen in an upright position directly under the fistula of the animal and against the left wall of the rumen. The rubber tubing extending from the side arm was passed through the fistula plug and connected to a rubber tube suspended from a manifold. A constant stream of nitrogen was passed through the manifold to maintain anaerobic conditions while allowing free escape of fermentation gases. The tubing from the manifold was kept free of tangling by a weight and pulley.

In vitro diffusion studies revealed that all membranes tested were permeable to the C_2-C_6 volatile fatty acids, with the membrane possessing the smallest pore size (0.1 μ) exhibiting the most rapid rate of diffusion. Similar diffusion tests *in vivo* showed VIVARs equipped with 0.1 μ Millipore membranes to contain, at 24 hr, a volatile acid concentration within 18% of that found in the rumen. Further tests of the various membranes showed that sealed, sterilized VIVARs would remain sterile, both *in vitro* and *in vivo*, except when they were equipped with sintered stainless steel discs. Also, Millipore membranes were found to be permeable to glucose, soluble starch, and

urease. Visking membranes were permeable to all but urease. In addition, it was found that Millipore and Visking membranes were not digested during 120 hr in the rumen.

Digestion trials using substrates of whole alfalfa hay and extracted alfalfa cellulose demonstrated that reproducible results could be obtained when experiments were performed on different days and with different inocula. Cellulose digestion of whole plant material appeared to take place in two steps, one between 0 and 36 hr and another between 48 and 72 hr. Digestion of extracted alfalfa cellulose was more rapid and more complete than that of whole plant cellulose. Average per cents of cellulose digested at 72 hr were 87.7% for extracted cellulose and 66.4% for whole plant cellulose. The possible effect of lignin was also discussed in relation to cellulose digestion.

Interpretation of results of digestion trials using prairie hay, in the VIVARs, indicated that grasses and legumes may be digested differently. Cellulose digestion did not level off between 36 and 48 hr as it did in the digestion trials with alfalfa. Also, per cents of cellulose digested were greater for whole alfalfa between 0 and 36 hr than for prairie hay.

Preliminary studies showed that dried lignin (from wood) depressed digestion of extracted alfalfa cellulose an average of 12.9%, thus indicating possible bacteriostatic action of lignin rather than simple encrustation.

Alfalfa labeled with C^{14} was grown in a specially constructed chamber. On the average, 33.8% of the isotope released was recovered in the plants. Digestion trials using labeled alfalfa demonstrated C^{14} activity in the volatile fatty acids after 15 min of fermentation in the VIVARs. The C^{14} activity of the acids remained high between 4 and 30 hr, then declined until 48 hr at which time it increased again, followed by a drop at about 66-70 hr. Hot

water extracted alfalfa (soluble carbohydrate free) showed essentially the same picture except that the first peak in C^{14} activity was of lesser magnitude. An experiment in which the animal was fasted demonstrated that the specific activity of the C^{14} volatile fatty acids produced in the VIVARs would remain relatively constant when there was little inward diffusion of unlabeled acids.

The advantages and disadvantages of the VIVAR technique were enumerated, with the conclusion that it is believed the VIVAR can be used to contribute much valuable information to the knowledge of rumen microbiology and function.

ACKNOWLEDGMENT

The author wishes to express his sincere gratitude to Dr. L. R Fina for his guidance, encouragement, and material assistance in the undertaking and completing of this work, and in the preparation of this manuscript.

Gratitude is also expressed to Dr. E. E. Bartley for his suggestions and assistance and for providing the fistulated animals for these studies.

The author is likewise indebted to Dr. E. L. Sorensen for his advice and for providing greenhouse facilities. Appreciation is expressed to Dr. H. C. Moser for suggestions in relation to tracer techniques and for the use of the counting equipment.

The author wishes to express thanks to Mr. D. W. Stanton and Mr. Carl Barnes for their many suggestions and for their assistance in this investigation.

The author gratefully acknowledges the support of the United States Department of Agriculture, North Central Regional Research Project 25 and 63, which provided research funds that made this study possible.

LITERATURE CITED

Annison, E. F., and D. Lewis. 1959.
Metabolism in the rumen. Methuen and Co., Ltd., London. 184 p.

Baker, F., and S. T. Harriss. 1947.
The role of the microflora of the alimentary tract of herbivora with special reference to ruminants. 2. Microbial digestion in the rumen (and caecum) with special reference to the decomposition of structural cellulose. Nutr. Abstr. Rev. 17: 3-12.

Baker, T. I., G. V. Quicke, O. G. Bentley, R. R. Johnson, and A. L. Moxon. 1959.
The influence of certain physical properties of purified celluloses and forage celluloses on their digestibility by rumen microorganisms in vitro. J. Animal Sci. 18: 655-662.

Balch, C. C., and V. W. Johnson. 1950.
Factors affecting the utilization of food by dairy cows. 2. Factors influencing the rate of breakdown of cellulose (cotton thread) in the rumen of the cow. Brit. J. Nutrition 4: 389-394.

Bartley, E. E., F. C. Fountaine, and F. W. Atkeson. 1950.
The effects of an APF concentrate containing aureomycin on the growth and well being of young dairy calves. J. Animal Sci. 9: 646-647.

Brown, J. C., L. O. Tiffin, and R. S. Holmes. 1958.
Carbohydrate and organic acid metabolism with C-14 distribution affected by copper in thatcher wheat. Plant Physiol. 33: 38-42.

Burroughs, W., N. A. Frank, P. Gerlaugh, and R. M. Bethke. 1950a.
Preliminary observations upon factors influencing cellulose digestion by rumen microorganisms. J. Nutr. 40: 9-24.

Burroughs, W., H. G. Headley, R. M. Bethke, and P. Gerlaugh. 1950b.
Cellulose digestion in good and poor quality roughages using an artificial rumen. J. Animal Sci. 9: 513-522.

Burroughs, W., J. Long, P. Gerlaugh, and R. M. Bethke. 1950c.
Cellulose digestion by rumen microorganisms as influenced by cereal grains and protein-rich feeds commonly fed to cattle using artificial rumen. J. Animal Sci. 9: 523-530.

Calvin, M., C. Heidelberger, J. C. Reid, B. M. Tolbert, and P. E. Yankwich. 1949.
Van Slyke-Folch wet oxidation, p. 92-94. In Isotopic carbon. John Wiley & Sons, Inc., New York.

Claycomb, C. K., T. T. Hutchens, and J. T. Van Bruggen. 1950.
Techniques in the use of C^{14} as a tracer. 1. Apparatus and technique for wet combustion of non-volatile samples. Nucleonics 7: 38-41.

Crampton, E. W., and L. A. Maynard. 1938.
The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutr. 15: 383-395.

Dehority, B. A. 1961.
Effect of particle size on the digestion rate of purified cellulose by rumen cellulolytic bacteria in vitro. J. Dairy Sci. 44: 687-692.

Dehority, B. A., and R. R. Johnson. 1961.
Effect of particle size upon the in vitro cellulose digestibility of forages by rumen bacteria. J. Dairy Sci. 44: 2242-2249.

El-Shazly, K., B. A. Dehority, and R. R. Johnson. 1960.
A comparison of the all-glass, semipermeable membrane, and continuous flow types of apparatus for in vitro rumen fermentations. J. Dairy Sci. 43: 1445-1451.

Ely, R. E., W. C. Jacobson, and E. A. Kane. 1951.
Metabolism of lignin in orchard grass hay cut at various stages of maturity. (Abstr.) J. Dairy Sci. 34: 492.

Fina, L. R, C. L. Keith, and E. E. Bartley, P. A. Hartman, and N. L. Jacobson. 1962.
Modified in vivo artificial rumen (VIVAR) techniques. J. Animal Sci. 21: 930-934.

Fina, L. R, and H. J. Sincher. 1959.
Improved micro steam distillation apparatus. Chemist-Analyst 48: 83.

Fina, L. R, G. W. Teresa, and E. E. Bartley. 1958.
An artificial rumen technique for studying rumen digestion in vivo. J. Animal Sci. 17: 667-674.

Fisher, A. C., Jr. 1958.
You and the obedient atom. Natl. Geog. Mag. 114: 303-353.

Gall, L. S., and C. N. Huhtanen. 1951.
Criteria for judging a true rumen organism and a description of five rumen bacteria. J. Dairy Sci. 34: 353-362.

Hansen, R. G., R. M. Forbes, and D. M. Carlson. 1958.
A review of the carbohydrate constituents of roughages. Univ. Illinois Agr. Expt. Sta. Bull. 634, 47 p.

Hueter, F. G., R. J. Gibbons, J. C. Shaw, and R. N. Doetsch. 1958.
Comparison of in vivo and in vitro techniques in ruminology studies. J. Dairy Sci. 41: 651-661.

Huhtanen, C. N., and L. S. Gall. 1952.
The miniature artificial rumen and its uses. (Abstr.) J. Animal Sci. 11: 766.

Hungate, R. E. 1950.
The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. 14: 1-49.

Jenkinson, D. S. 1960.
The production of ryegrass labelled with carbon-14. Plant and Soil 13: 279-290.

Kamstra, L. D., A. L. Moxon, and O. G. Bentley. 1958.
The effect of stage of maturity and lignification on the digestion of cellulose in forage plants by rumen microorganisms in vitro. J. Animal Sci. 17: 199-208.

Kitts, W. D., and L. A. Underkofler. 1954.
Digestion by rumen microorganisms. Hydrolytic products of cellulose and the cellulolytic enzymes. J. Agr. Food Chem. 2: 639.

Knox, K. L. 1961.
Rumen concentrations of volatile fatty acids as affected by feeding frequency. J. Dairy Sci. 44: 1550.

LeFevre, C. F., and L. D. Kamstra. 1960.
A comparison of cellulose digestion in vitro and in vivo. J. Animal Sci. 19: 867-872.

Lawton, E. J., W. D. Bellamy, R. E. Hungate, M. P. Bryant, and E. Hall. 1951.
Some effects of high velocity electrons on wood. Science 113: 380-382.

Louw, J. G., H. H. Williams, and L. A. Maynard. 1949.
A new method for the study in vitro of rumen digestion. Science 110: 478-480.

Markham, R. 1942.
A steam distillation apparatus suitable for micro-Kjeldahl analysis. Biochem. J. 36: 790-791.

Marston, H. R. 1948.
The fermentation of cellulose in vitro by organisms from the rumen of sheep. Biochem. J. 42: 564-574.

McNaught, M. L. 1951.
The utilization of non-protein nitrogen in the bovine rumen. 7. A qualitative and quantitative study of the breakdown of carbohydrate which accompanies protein formation in bovine contents during in vitro incubation. Biochem. J. 49: 325-332.

Meyer, J. H., W. C. Weir, L. G. Jones, and J. L. Hull. 1960.
Effect of stage of maturity, dehydrating versus field-curing and pelleting on alfalfa hay quality as measured by lamb gains. J. Animal Sci. 19: 283-294.

Miles, J. T. 1951.
Rumen digestion of some crude fiber constituents. (Abstr.) J. Dairy Sci. 34: 492.

Monroe, C. F., and A. E. Perkins. 1939.
A study of the pH values of the ingesta of the bovine rumen. J. Dairy Sci. 22: 983-991.

Passmann, J. M., N. S. Radin, and J. A. D. Cooper. 1956.
Liquid scintillation technique for measuring carbon-14-dioxide activity. Anal. Chem. 28: 484-486.

Pearson, R. M., and J. A. B. Smith. 1943.
The utilization of urea in the bovine rumen. 2. The conversion of urea to ammonia. Biochem. J. 37: 148-153.

Quicke, G. V., O. G. Bentley, H. W. Scott, and A. L. Moxon. 1959a.
Cellulose digestion in vitro as a measure of the digestibility of forage cellulose in ruminants. J. Animal Sci. 18: 275-287.

Quicke, G. V., and O. G. Bentley. 1959b.
Lignin and methoxyl groups as related to the decreased digestibility of mature forages. J. Animal Sci. 18: 365-373.

Scully, N. J., W. Chorney, G. Kostal, R. Watanabe, J. Skok, and J. W. Glattfeld. 1955.
Biosynthesis in C^{14}-labeled plants; their use in agricultural and biological research. Proc. Int. Conf. Peaceful Uses Atomic Energy 12: 377-385.

Smith, V. R. 1941.
In vivo studies of hydrogen ion concentrations in the rumen of the dairy cow. J. Dairy Sci. 24: 659-665.

Van Slyke, D. D., and J. Folch. 1940.
Manometric carbon determination. J. Biol. Chem. 136: 509-541.

Van Slyke, D. D., J. Plazin, and J. R. Weisiger. 1951.
Reagents for the Van Slyke-Folch wet carbon combustion. J. Biol. Chem. 191: 299-304.

Warner, A. C. I. 1956.
Criteria for establishing the validity of in vitro studies with rumen micro-organisms in so-called artificial rumen systems. J. Gen. Microbiol. 14: 733-748.

Wasserman, R. H., C. W. Duncan, E. S. Churchill, and C. F. Huffman. 1952. The effect of antibiotics on in vitro cellulose digestion by rumen microorganisms. J. Dairy Sci. 35: 571-580.

Werbin, H., I. L. Chaikoff, and M. R. Imada. 1959. Rapid sensitive method for determining H^3-water in body fluids by liquid scintillation spectrometery. Soc. Exptl. Biol. Med. 102: 8-12.

Yadava, I. S. 1962. Studies on the rate of passage of nutrients through the bovine rumen and the rumen metabolism of C-14 labeled roughage. Ph.D. Thesis, Kansas State University (L. C. Card No. Mic 62-4493) 189 p. Univ. Microfilms. Ann Arbor, Mich. (Dissertation Abstr. 23: 775)

APPENDIX

Procedure for Cellulose Extraction

1. Place a 1 g air dried sample in a 50 ml centrifuge tube.
2. Add 15 ml of 80% acetic acid and 1.5 ml of concentrated nitric acid.
3. Place the tube, fitted with a reflux condenser, in a beaker of boiling water. Boil for 40 min.
4. Transfer the residue (cellulose), with alcohol from a wash bottle, into a porous-bottomed crucible.
5. Wash the residue with hot benzene, hot alcohol, and ether. Use suction.
6. Dry the crucible and residue, weigh.
7. Calculate the cellulose as weight lost after ignition.

Table 9. Reproducibility of digestion of alfalfa hay cellulose using the VIVAR on three different occasions

Trial No.	Time in hours	Alfalfa added (mg)	Cellulose in alfalfa (mg)	Cellulose in 25 ml of inoculum (mg)	Total cellulose added (mg)	Empty wt of crucible (g)	Wt of crucible and cellulose (g)	Difference in wt (mg)	Wt of crucible and ash (g)	Wt of cellulose minus ash (mg)	Cellulose digested (mg)	Per cent cellulose digested (%)
1	12	1,000	329	1.0	330.0	18.6962	18.9728	276.6	18.6980	274.8	55.2	16.7
	24	1,000	329	1.0	330.0	18.8404	19.0412	200.8	18.8694	171.8	158.2	47.9
	36	1,000	329	1.0	330.0	16.8388	16.9703	131.5	16.8407	129.6	200.4	60.7
	48	1,000	329	1.0	330.0	18.2770	18.4077	130.7	18.2782	129.5	200.5	60.7
	60	1,000	329	1.0	330.0	17.8475	17.9738	126.3	17.8488	125.0	205.0	62.1
	72	1,000	329	1.0	330.0	18.2764	18.4026	126.2	18.2789	123.7	206.3	62.5
	72	1,000	329	no inoculum[1]	329.0	16.4320	16.7594	327.4	16.4328	326.6	0	0
2	12	1,000	329	36.0	365.0	18.6958	18.8766	180.8	18.6983	178.3	186.7	51.1
	24	1,000	329	36.0	365.0	18.8407	19.0030	162.3	18.8438	159.2	205.8	56.3
	36	1,000	329	36.0	365.0	18.7133	18.8558	142.5	18.7164	139.4	225.6	61.7
	48	1,000	329	36.0	365.0	18.2768	18.4213	144.5	18.2820	139.3	225.7	61.8
	60	1,000	329	36.0	365.0	18.2762	18.4030	126.8	18.2787	124.3	240.7	65.9
	72	1,000	329	36.0	365.0	17.8468	17.9692	122.4	17.8494	119.8	245.2	67.1
	72	1,000	329	no inoculum	329.0	20.4384	20.7662	327.8	20.4391	327.1	0	0
3	12	1,000	329	16.9	345.9	18.6951	18.8774	182.3	18.7036	173.8	172.1	49.7
	24	1,000	329	16.9	345.9	18.2512	18.4108	159.6	18.2565	154.3	191.6	55.3
	36	1,000	329	16.9	345.9	18.8399	18.9927	152.8	18.9452	147.5	198.4	57.3
	48	1,000	329	16.9	345.9	18.0684	18.2188	150.4	18.0737	145.1	200.8	58.0
	60	1,000	329	16.9	345.9	18.0199	18.1550	135.1	18.0244	130.6	215.3	62.2
	72	1,000	329	16.9	345.9	18.7128	18.8261	113.3	18.7180	108.1	237.8	68.7
	72	1,000	329	no inoculum	329.0	23.6519	23.9787	326.8	23.6529	325.8	0	0

[1] Control-distilled water added.

Table 10. Digestion of extracted alfalfa cellulose using the VIVAR on two different occasions

Trial No.	Time in hours	Extracted cellulose added (mg)	Cellulose in 25 ml of inoculum (mg)	Total cellulose added (mg)	Empty wt of crucible (g)	Wt of crucible and cellulose (g)	Difference in wt (mg)	Wt of crucible and ash (g)	Wt of cellulose minus ash (mg)	Cellulose digested (mg)	Per cent cellulose digested (%)
1	12	300	17	317	18.6954	18.8322	136.8	18.7057	126.5	190.5	60.0
	24	300	17	317	18.0685	18.1739	105.4	18.0780	95.9	221.1	69.7
	36	300	17	317	18.2562	18.3317	75.5	18.2646	67.1	249.9	78.8
	48	300	17	317	18.8400	18.9035	63.5	18.8490	54.5	262.5	82.8
	60	300	17	317	18.0200	18.0761	56.1	18.0290	47.1	269.9	85.1
	72	300	17	317	18.7130	18.7648	51.8	18.7204	44.4	272.6	85.9
	72	300	no inoculum[1]	300	17.0688	17.3664	297.6	17.0697	296.7	0	0
2	12	329	4	333	16.2907	16.4394	148.7	16.2920	147.4	185.6	55.7
	24	329	4	333	16.4319	16.5435	111.6	16.4484	95.1	237.9	71.4
	36	329	4	333	17.0632	17.1313	68.1	17.0643	67.0	266.0	79.8
	48	329	4	333	18.2756	18.3328	57.2	18.2775	55.3	277.7	83.3
	60	329	4	333	17.8472	17.8902	43.0	17.8497	40.5	292.5	87.8
	72	329	4	333	18.2762	18.3130	36.8	18.2785	34.5	298.5	89.6
	72	329	no inoculum	329	18.2227	18.5503	327.6	18.2235	326.8	0	0

[1] Control-distilled water added.

Table 11. The assay data for total volatile fatty acids produced from whole and extracted C^{14} alfalfa in Trial No. 1

Sampling time	Wt of Na salts of volatile acids (mg)				Observed counts per min per 2/3 sample wt				Disintegrations/min mg of Na salts of volatile acids[1]				Average disintegrations/min mg of Na salts of volatile acids	
	Trials				Trials				Trials				Trials	
	A	B	C	D	A	B	C	D	A	B	C	D	A & B	C & D
(hr)														
.25	4.4	4.7	1.9	6.9	471	3,772	1,006	202	534	4,012	2,646	146	2,273	1,396
.5	5.2	5.1	4.3	5.5	7,092	4,872	775	3,343	6,819	4,776	900	3,033	5,797	1,969
1	5.9	4.7	4.6	5.7	1,581	4,988	2,532	1,198	1,339	5,306	2,752	1,050	3,322	1,901
2	5.9	2.3	4.7	5.0	5,028	3,674	283	4,328	4,261	7,986	300	4,328	6,123	2,314
3	6.6	6.9	5.6	4.9	25,983	21,998	9,436	2,051	19,683	15,940	8,425	2,091	17,811	5,258
4	6.7	7.0	5.0	4.5	27,739	1,426	7,725	6,913	20,700		7,724	7,680	20,700	7,702
5	7.0	6.4	6.3	5.0	19,331	15,156	8,635	2,406	13,807	11,846	6,852	2,406	12,828	4,629
6	6.1	6.3	5.2	4.9	11,466	16,826	3,824	3,255	9,555	14,021	3,824	3,254	11,788	3,539
8	7.0	7.2	5.7	7.2	21,473	47,036	8,842	8,442	15,337	26,405	7,756	5,862	20,871	6,809
10	9.1	8.1	7.4	6.4	28,968	27,412	5,205	9,032	15,916	16,921	3,516	7,055	16,418	5,286
14	4.4	7.4	5.7	5.3	18,593	23,125	5,442	5,556	21,127	15,624	4,773	5,241	18,375	5,007
18	6.8	5.9	5.2	4.3	34,793	16,672	6,394	3,312	25,532	14,145	6,148	3,851	19,863	4,999
22	6.8	7.2	5.2	6.0	20,159	27,650	3,884	3,239	14,822	19,201	3,734	2,698	17,011	3,216
26	8.5	6.6	5.9	5.8	17,745	20,293	3,994	11,055	10,438	15,373	3,384	9,529	12,905	6,456
30	6.4	7.0	5.6	5.6	10,832	21,784	912	2,546	8,462	15,560	814	2,273	12,011	1,543
34	6.0	8.4	5.5	5.3	13,819	36,085	2,034	4,134	11,515	21,478	1,849	3,900	16,496	2,874
38	7.0	7.1	5.2	5.5	8,362	14,648	674	2,144	5,972	10,315	648	1,949	8,143	1,298
42	7.4	6.5	6.3	4.1	8,142	12,963	958	1,895	5,501	9,171	760	2,310	7,736	1,535
46	6.0	6.5	5.1	5.1	5,938	13,218	505	2,021	4,948	10,167	494	1,981	7,557	1,237
50	6.1	7.1	4.5	4.8	11,935	11,903	2,074	2,534	9,782	8,382	2,304	2,639	9,082	2,471
58	5.6	5.7	4.4	4.9	13,208	12,818	2,462	3,496	11,793	11,244	2,797	3,567	11,518	3,182
66	5.1	5.7	5.8	5.1	12,626	12,949	1,822	2,359	12,378	11,358	1,570	2,312	11,868	1,941
74	5.2	4.1	4.9	4.1	7,686	3,612	1,708	1,592	7,390	4,404	1,742	1,940	5,897	1,841
78	5.9	5.7	4.8	3.7	6,019	7,000	1,470	1,611	5,100	6,034	1,531	2,176	5,567	1,853
87	5.6	5.7	5.2	4.5	10,174	7,002	880	1,370	9,084	6,140	846	1,522	7,612	1,184
95	5.0	5.1	3.5	4.1	8,927	4,129	882	336	8,926	4,047	1,260	409	6,486	834
107	5.7	1.0	3.6	3.5	7,803	747	270	326	6,849	3,733	375	465	5,291	420

[1] Observed counts corrected for background (35 count/min), efficiency of counting method (30%), and quantity of sample counted.

Table 12. The assay data for total volatile fatty acids produced from whole and extracted[1] C^{14} alfalfa in Trial No. 2

Sampling time	Wt of Na salts of volatile acids (mg)				Observed counts per min per total sample wt				Disintegrations/min mg of Na salts of volatile acids[2]				Average disintegrations/min mg of Na salts of volatile acids	
	Trials				Trials				Trials				Trials	
	A	B	C	D	A	B	C	D	A	B	C	D	A & B	C & D
(hr)														
.25	4.6	4.0	3.7	4.0	8,004	3,588	2,406	3,196	3,480	1,794	1,700	799	2,637	1,249
.5	3.9	3.4	3.9	4.0	8,552	5,228	5,442	4,478	4,384	3,074	2,790	2,238	3,729	2,514
1	4.2	4.4	2.6	3.6	11,706	10,082	10,694	3,977	5,574	4,582	3,096	2,208	5,078	2,652
2	5.6	9.2	4.6	4.3	15,088	32,784	9,436	11,198	5,388	7,126	4,630	5,208	6,257	4,919
3	8.8	5.7	3.8	4.5	44,256	26,098	16,246	10,566	10,058	9,156	4,966	4,696	9,607	4,831
4	5.9	3.5	4.7	3.0	32,826	14,689	10,888	9,294	11,130	8,392	6,912	3,098	9,761	5,005
5	7.3	8.0	3.5	1.4	38,924	38,576	15,647	4,044	10,664	9,644	6,220	5,776	10,154	5,998
6	5.8	7.2	5.0	5.7	33,217	32,227	16,194	17,101	11,454	8,952	6,258	6,000	10,203	6,129
10	6.6	7.1	5.1	5.7	33,599	34,196	20,218	17,832	10,180	9,632	6,350	6,256	9,906	6,303
19.5	6.4	5.1	5.6	3.9	33,810	26,732	19,700	14,618	10,564	10,482	7,220	7,496	10,523	7,358
24	5.3	5.8	6.1	4.7	27,921	24,998	17,398	14,014	10,536	8,602	6,458	5,962	9,578	6,210
30	5.4	6.7	6.2	4.7	23,898	25,092	15,838	12,340	8,850	7,490	5,612	5,250	8,170	5,431
34	5.2	5.4	5.2	5.0	22,562	21,674	12,364	11,253	8,676	8,026	6,090	4,500	8,351	5,295
38	5.8	4.9	5.3		23,407	18,902	14,394		8,070	7,700	5,430		7,885	5,430
46	3.7	3.7	3.7	4.0	12,908	13,012	9,376	10,408	6,976	7,032	5,064	5,204	7,004	5,134
50	4.5	5.0	4.5	3.4	14,564	13,700	10,148	8,408	6,472	5,480	4,510	4,944	5,976	4,727
58	4.3	2.8	3.5	2.5	13,067	6,739	8,291	5,268	6,076	4,812	4,736	4,214	5,444	4,475
66	3.8	2.8		2.8	10,202	8,134		5,229	5,368	5,810		3,734	5,589	3,734
74	3.9	2.8	3.5	4.0	8,677	6,500	5,714	4,873	4,480	4,642	3,264	2,436	4,545	2,850
80	3.8	3.4	3.3	3.7	8,811	4,138	5,202	5,256	4,636	2,438	3,152	2,840	3,535	2,996
90	3.9	3.5	0.8	3.0	7,873	3,881	801	4,070	4,036	2,216	2,000	2,712	3,126	2,356
100	3.7	3.1	3.2	3.2	6,771	3,558	4,356	3,616	3,660	2,296	2,354	2,260	2,978	2,307

[1] Extractive-free residue from 1 g of whole C^{14} alfalfa.

[2] Observed counts corrected for background (35 count/min) and efficiency of counting method (50%).

Table 13. The assay data for total volatile fatty acids produced from whole C^{14} alfalfa in Trial No. 3[a]

Sample No.[1]	Sampling time (hr)	Wt of Na salts of volatile acids (mg)	Observed counts per min per sample wt	Disintegrations per min per mg[2] of Na salts of volatile acids
A1	.25	7.8	23,236	5,958
B2	.5	6.7	27,046	8,072
C3	1	9.3	39,216	8,432
D4	2	6.8	66,293	19,496
A5	3	12.9	136,098	21,100
B6	4	11.6	139,089	23,980
C7	6	13.2	137,611	20,850
D8	8	13.0	158,187	26,336
A9	10	10.9	176,316	32,350
B10	12	10.5	148,897	28,360
C11	14	12.6	170,942	27,132
D12	16	12.3	158,814	25,822
A13	18	12.4	169,244	27,296
B14	20	11.2	169,151	30,204
C15	22	11.4	141,840	24,884
D16	24	13.4	170,025	25,376
A17	27	10.9	162,298	29,778
B18	30	9.8	129,435	26,424
C19	33	11.6	157,046	27,076
D20	36	9.3	121,504	26,130
A21	40	7.6	94,988	24,996
B22	44	2.5	28,471	22,776
C23	48	8.5	117,350	27,610
D24	52	11.8	161,068	27,298
A25	56	6.9	95,364	27,640
B26	60	6.5	75,178	23,130
C27	64	6.1	75,434	24,732
D28	68	8.1	121,130	29,908

a Experimental animal was fasted for 72 hr.

1 Samples were taken alternately from VIVARs A, B, C, and D.

2 Observed counts were corrected for background (35 count/min) and efficiency of counting method (50%).

Table 14. Digestion of prairie hay cellulose using the VIVAR on two different occasions

Trial No.	Time in hours	Prairie hay added (mg)	Cellulose in prairie hay (mg)	Cellulose in 25 ml of inoculum (mg)	Total cellulose added (mg)	Empty wt of crucible (g)	Wt of crucible and cellulose (g)	Difference in wt (mg)	Wt of crucible and ash (g)	Wt of cellulose minus ash (mg)	Cellulose digested (mg)	Per cent cellulose digested (%)
1	24	1,000	354	5	359	18.6961	18.9438	247.7	18.7338	210.0	149.0	41.5
	36	1,000	354	5	359	18.0686	18.2448	196.2	18.1037	161.1	197.9	55.1
	48	1,000	354	5	359	18.8403	18.9894	149.1	18.8738	115.6	243.4	67.7
	54	1,000	354	5	359	18.2575	18.4024	144.9	18.2943	108.1	250.9	69.8
	66	1,000	354	5	359	18.7102	18.8453	135.1	18.7449	100.4	258.6	72.0
	78	1,000	354	5	359	18.0204	18.1527	132.3	18.0554	97.3	261.7	72.8
	78	1,000	354	no inoculum[1]	354	16.4328	16.8163	383.5	16.4635	352.8	0	0
2	24	1,000	354	8	362	17.0642	17.3320	267.8	17.1137	218.3	143.7	39.6
	36	1,000	354	8	362	16.2896	16.5078	218.2	16.3373	170.5	191.5	52.9
	48	1,000	354	8	362	16.8366	17.0205	183.9	16.8826	137.9	224.1	61.9
	54	1,000	354	8	362	18.2780	18.4468	168.8	18.3251	121.7	240.3	66.3
	66	1,000	354	8	362	17.8494	18.0070	158.3	17.8912	116.5	245.5	67.7
	78	1,000	354	8	362	18.2794	18.4246	145.2	18.3188	105.8	256.2	70.7
	78	1,000	354	no inoculum	354	18.7135	19.0958	381.9	18.7423	353.1	0	0

[1] Control-distilled water added.

Table 15. The effect of added lignin on digestion of extracted alfalfa cellulose

VIVAR No.	Time in hours	Extracted cellulose added (mg)	Cellulose in 25 ml of inoculum (mg)	Total cellulose added (mg)	Lignin added (60% pure) (mg)	Empty wt of crucible (g)	Wt of crucible and cellulose (g)	Difference in wt (mg)	Wt of crucible and ash (g)	Wt of cellulose minus ash (mg)	Cellulose digested (mg)	Per cent cellulose digested (%)
1	48	250	4	254	0	18.0687	18.1152	46.5	18.0698	45.4	208.6	82.1
2	48	250	4	254	0	18.2580	18.2945	36.5	18.2590	35.5	218.5	86.0
3	48	250	4	254	170	18.0207	18.0952	74.5	18.0225	72.7	181.3	71.3
4	48	250	4	254	170	18.7105	18.7850	74.5	18.7115	73.5	180.5	71.0
5	48	250	no inoculum[1]	250	0	18.2763	18.5246	248.3	18.2768	247.8	0	0

[1] Control-distilled water added.

DEVELOPMENT OF AN IMPROVED IN VIVO ARTIFICIAL RUMEN (VIVAR) TECHNIQUE AND SOME OF ITS APPLICATIONS

by

CHESTER LEROY KEITH

B. S., Kansas State University, 1958

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1963

An improved *in vivo* artificial rumen (VIVAR) technique was developed in an attempt to more closely duplicate conditions in the rumen. The *in vivo* artificial rumen consisted of a stainless steel drum with a side arm. The substrates and inocula were placed inside the drum which was sealed by membranes held in place by screens, gaskets, and outer nuts. Rubber tubing was attached to the side arm which served as a sampling port and gas outlet. The apparatus was placed in the rumen of a fistulated animal.

In vitro studies revealed that all membranes tested were permeable to the C_2-C_6 volatile fatty acids. *In vivo* tests showed that after 24 hr the concentration of total volatile fatty acids in VIVARs equipped with Millipore membranes was within 18% of the concentration found in the rumen at the same time. Further tests showed that VIVARs could be kept sterile both *in vitro* and *in vivo*, except when fitted with sintered stainless steel discs. Millipore membranes were found to be permeable to urease, glucose, and soluble starch; Visking was permeable to all but urease. Millipore and Visking membranes were not digested during 120 hr in the rumen.

Digestion trials using substrates of whole alfalfa hay and extracted alfalfa cellulose showed that reproducible results could be obtained when trials were performed on different days with different inocula. Cellulose digestion of whole plant material appeared to take place in two steps, one between 0-36 hr and another between 48-72 hr. Very little digestion took place between 36 and 48 hr. Extracted cellulose was digested more rapidly and to a greater extent than was whole plant cellulose. It was found that the addition of dried wood lignin depressed digestion of extracted alfalfa cellulose approximately 13%.

It was found that prairie hay was digested at a more constant rate than

alfalfa hay, and that cellulose digestion of prairie hay did not level off between 36 and 48 hr as it did in digestion trials with alfalfa hay.

Alfalfa labeled with C^{14} was grown in a specially constructed chamber. Approximately 34% of the isotope released was recovered in the plants. When this plant material was fermented in the VIVAR C^{14} activity was detected in volatile fatty acids after 15 min. The C^{14} activity of the acids remained high between 4 and 30 hr, then it declined until the 48-66 hr period when radioactivity increased. Hot water extracted C^{14} alfalfa showed essentially the same picture except the first peak in C^{14} activity was of lesser magnitude. When experiments were conducted in a fasted animal, the specific activity of the C^{14} volatile fatty acids produced in the VIVARs remained more constant.

This study has described the improved VIVAR technique and demonstrated its applications in some areas of rumen microbiology and function.