FURTHER OBSERVATIONS ON THE TECHNIQUE OF STUDYING PROTEIN SYNTHESIS IN THE RUMEN

by

RAMBHAU KAHANDERAO RAIKAR

G.B.V.C., Bombay Veterinary College, Bombay, (India), 1945
B.Sc. (Vet.), University of Bombay, Bombay, (India), 1955

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Animal Husbandry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1963

Approved by:

D. Richardson

Major Professor

TABLE OF CONTENTS

INTRODUCTION

In most of the classical physiology books, emphasis is mainly based on the study of monogastric animals and it is only comparatively recently that the physiology of the ruminant has been intensively studied. Schalk and Amadon, are considered as pioneer workers on ruminant digestion. Their work provided a firm foundation for much subsequent work. In later years an impetus to further research was provided by the work of Barcroft, Phillipson and Elsden during World War II. Today many aspects of digestion and metabolism with an emphasis on what is happening in the rumen has been unfolded due to the brilliant work by the research workers from United States of America, United Kingdom, Australia and other parts of the World.

In herbivorous animals a large quantity of coarse, fibrous food, having low nutritive and caloric value, must be ingested, to derive nutrients and energy for maintenance of life and production. This has resulted in a specialized development of the alimentary canal. In the ruminants this has occurred in the expansion of the part represented by the rumen. The rumen is a complex fore-stomach of considerable size and is a unique organ not comparable to organs of other species of animals as regards its functions. This peculiar organ is called by some a "Lunch basket" and by others a 'Fermentation Vat'. Fermentation of the ingesta takes place in this roomy compartment as a result of microbial attack and many nutrients are formed, supplying nutrients to the host. Besides, the phenomenon of rumination is developed as a further adjunct to the digestion of this coarse food, since with remastication a second period of maceration of vegetable fibers provides an increased surface for microbial attack.

The microorganisms consisting of microflora and microfauna, play a

definite role in the functions attributed to this organ. One of the most important functions carried out by the microbial population in the rumen is the synthesis of protein from non-protein nitrogenous material and thus improving the protein quality. There is as yet a big gap in understanding of this process as little work is done so far on this aspect. The symbiotic relationship between the host and the microbial population, their relationship with each other, the rate of protein synthesis and catabolism, and what quantity and proportion of amino acids are absorbed by the animal as a result of these actions needs further exploration in the field of research.

Certain techniques are used by various workers to study the microbial population and protein synthesized by them. These include (1) microscopic, (2) pure culture, (3) washed suspensions of the microorganisms from rumen and (4) the artificial rumen technique studies.

These techniques do not give us the exact picture as to what interactions are taking place amongst the microbes in the rumen. The difficulties in technique must be overcome so that in vivo work can be undertaken.

It was demonstrated by Regier (1961) that dialising sacs can be used to study rumenology with reference to protein synthesis by microorganisms quite successfully. It was also shown by him that pure cultures of bacteria that are normally found in the rumen, can be used for the study of protein synthesis by these organisms when favorable conditions as found in the rumen are provided as far as possible, in the dialising sacs. Since his studies were not conclusive, further observations were made to study the technique developed by him to study protein synthesis by the microbial population in the rumen. The work is divided in two parts. The first part deals with the work of Regier (1961), in which a combination and improvement of the pure

culture, diet, or media, artificial rumen, fistulated animal, method is dealt with. The dializing sacs made of material, selectively permeable due to pore size, placed in a protective cover of polythelene bottles with experimental diet or media, and microorganisms inoculated in the sacs, were incubated in the rumen of fistulated steers. The media consisted of rumen liquor strained and sterilized or sterilized normal saline. Ability to remain sterile and synthesis of protein by the microbes in the sac was studied.

As the technique could not be worked out satisfactorily, it was decided to work out a technique of studying rumenology by taking representative samples of rumen contents by thoroughly mixing the rumen contents for five minutes to ten minutes. Samples were collected from anterior, middle and posterior side of the rumen and weighed. The entire rumen contents were removed and placed in a tub, representative samples were taken of the contents after thoroughly mixing each time. The tub with rumen contents and the samples were weighed. The rumen contents from the tub with some warmwater were reintroduced in the rumen. The samples were dried, ground in a mill and analysed for nitrogen content.

REVIEW OF LITERATURE

Microflora and Microfauna of the Rumen

The mystery of the rumen has been steadily unfolding in the past decade through fundamental research. The study of the microorganisms of the rumen received a great impetus as a result of an interesting hypothesis by Zuntz (1891), cited by Loosli (1949), that these microorganisms might utilize dietary non-protein nitrogen (N.P.N.) for their growth with resultant synthesis of protein which would become available to the host. Intensive

work has been done since 1940 on microbiology of the rumen.

According to Oxford (1955), for the microbiologist the interesting difference between ruminants and other herbivores is that, ruminants preferment their food before digestion proper in that part of the paunch known as the rumen. Hence the almost inevitable variety of microorganisms in that special kind of fermentation vat in which the typical fermentation processes get established under highly reducing conditions when the animal is quite young and the rumen is relatively small.

Annison and Lewis (1959) state that the rumen is an essentially anaerobic, highly reducing system at a slightly acid, but buffered ph with a temperature of 39° C and under a gas phase composed mainly of carbon dioxide, methane and nitrogen. This environment favors development of a specialized population.

Doetsch and Robinson (1953) stated that the importance of studies on the rumen bacterial flora to problems of general interest such as ecology, synergism, antibiotics and symbiosis is fully realized now. Such practical problems as meat and milk production, utilization of feedstuffs, cellulose decomposition, vitamin and protein synthesis need no further comment to justify exploration. The indigenous bacterial flora of the rumen develops under conditions imposed by this unique and intricately constructed organ.

Baker (1942) states that the elucidation of the factors concerned in digestive assimilation by cattle of starch, cellulose, proteins and protein substitutes, necessitates an accurate knowledge of the functional activities of the rumen microorganisms.

Considering the importance of this microbial population in the rumen to host, Elsden et al. (1948) stated, it is essential that the population

of the rumen as a whole be given at least as much attention as the study of individual organisms.

The microbial population of the rumen is enormous. Continuous growth of this population is ensured by a periodic intake of food; a continuous flow of saliva, passage of the contents along the digestive tract and absorption of the end products of metabolism through the rumen wall. The growth and division of the microorganisms is accompanied by death and autolysis of others.

According to Oxford (1955), the rumen mixed culture is so complex an association that relatively little is known with certainty about important microbial inter-relationships within it. Rumen microbiology is undoubtedly a field in which the great unifying discoveries have yet to be made.

Studies of rumen bacteria of cattle and sheep by means of direct counts, gram staining and anaerobic cultural techniques, were made by Gall et al. (1949). According to them the counts exceeded 50×10^8 per gram of the rumen contents.

Bryant et al. (1953) using safranin staining techniques found the count of bacteria to be 10^{10} per gram of rumen contents. Hungate (1957) obtained a count of 10^8 on hay and concentrate ration.

A consideration of the overall values suggest that the mean number of bacteria in rumen contents is around $10^8 - 10^{10}$/ml. and that the figure is higher when the animal is fed a ration rich in concentrates than when fed on hay or grass (Annison and Lewis, 1959).

Barnett and Reid (1961) stated the problem of microbial population, the study of which should be given certain considerations; (1) a preliminary classification of ruminal microorganisms into two groups, -- Protozoa and

bacteria. (2) Rumen reactions in the main, are the result of symbiotic relationships, and it would be unprofitable, except in few cases, to consider the in vitro behavior of microorganisms isolated in pure culture. (3) It is precedent to accept limitations set by Gall and Huhtanen (1951) that before an organism can be looked upon as a typical component of the microflora population the following main criteria should be satisfied:

(a) The organism must be able to live anaerobically.

(b) It should be able to produce the type of end products found in the rumen.

(c) Rumen ingesta should contain not less than one million of the particular organism per gram of rumen ingesta.

Barnett and Reid (1961) are of the opinion that the rumen bacteria for an anaerobic environment are not as stringent as was formerly supposed. Doetsch and Robinson (1953) and Heald et al. (1953) obtained high viable counts of microorganisms without imposing strictly anaerobic conditions.

Barnett and Reid (1961) stated that there are at least thirty species of protozoa to be found in a population of rumen microorganisms. Numbers of protozoa, though smaller as compared to bacteria, may reach 10^6 per gram of rumen contents (Alstad, 1958).

According to Annison and Lewis (1959) the number of protozoa may be 1,000,000 per gram of rumen contents and the bulk may be equivalent to the bacteria from the rumen.

Johnson et al. (1944) found that protozoa of the rumen may be separated in a fairly high state of purity by filtration and centrifugation. The deposition of storage polysaccharide increases the cell density and this property forms the basis of a technique for separation of the holotrich

ciliates (Heald and Oxford, 1953; Oxford, 1955). Gutierrez (1955) separated several individual species of protozoa by decantation following the deposition of the storage material. Further separation was effected by washing and differential sedimentation in a special fluid with appropriate density properties.

Johnson et al. (1944) determined the bacterial and protozoa counts of the rumen at intervals for 24 hours after feeding and observed that the greatest number of bacteria and fewest number of protozoa were found one hour after feeding. Later the number of bacteria decreased while the protozoa increased for 16 hours.

Barnett and Reid (1961) pointed out that the rumen protozoa may supply one-fifth of the food utilized by the host. The possible significance of protozoa, until recently has been overlooked. This is largely due to the fact that the protozoa ingested large quantities of bacteria and of claims that fermentation was effected by the bacterial enzyme systems rather than protozoal cells.

The modern view is so divorced from the traditional one in this matter that the rumen protozoa are now considered to have considerable biochemical activity in their own right and much work is being done in examining the role of individual groups of protozoa in relation to metabolism of the bacteria and to the nutrition of the host animal.

Annison and Lewis (1959) stated that the exact role of the protozoa in the rumen has not been established. It has been suggested that the storage of polysaccharide is of nutritional importance for the host animal, that the structural protein is of considerable nutritional value and that they actively contribute to the breakdown of cellulose, starch and protein.

The general problems of bacterial-protozoan interrelationships are very complex and it is possible that some of the properties assigned to protozoa are due to contaminating bacteria. Some of the problems involved have been discussed in excellent reviews by Oxford (1955) and Hungate (1955).

A symbiotic relationship exists between the ruminant and microorganisms, especially the bacteria found in the rumen, in that the ruminant animal supplies food material to be utilized by the bacteria, and the bacteria in turn break down these substances to products which may be absorbed into the blood stream of the host animal (Thornton et al., 1952). It is possible that this type of cooperation may also be existing between various members of the microbial population.

Various aspects of "rumenology" have been excellently reviewed in articles by Marston (1939), Goss (1943), Baker (1943), Elsden and Phillipson (1948), Doetsch and Robinson (1953), and Hobson (1959).

Doetsch and Robinson (1953) reviewed papers concerned principally with the bacteriology of the bovine rumen. Baker (1943) has excellently discussed the species of bacteria that are normally found in the rumen. Huhtanen et al. (1952) have used improved techniques in isolating the microorganisms from the rumen. Hobson (1959) has described organisms concerned with nitrogen metabolism in the rumen.

Experimental Methods and Approaches for the Study of Rumenology

Alstad (1958) has pointed out that there is an unfortunate gap in systematic and methodological appraoches to the rumen microflora studies. This is due to the fact that none of the techniques, used nowadays satisfactorily cover the interaction involved between various representatives

of rumen population.

The various approaches that have been made to the problem of elucidating both the types and functions of microorganisms involved in rumen metabolism have been in two main directions (Davey et al., 1961).

The functional approach has entailed the use of whole or fractionated rumen contents to provide information on the metabolic activity and end-products of a natural mixture of rumen organisms on specified substrates during incubation in vitro. Therefore, in this case the approach to study rests on the substrates and not on organisms involved in the reactions. Such in vitro incubation of rumen contents has been adopted and elaborated in many ways. One of the advantages is that the experimental apparatus and procedures may be varied to suit different types of investigations, (Davey et al., 1961).

The other main method of approach has been to isolate and identify as many microorganisms as possible from the rumen and then armed with qualitative and quantitative information an attempt is made to fit them into an overall metabolic pattern of the rumen. In this case the specificity lies with the microorganisms and not so much with the substrate. Direct microscopic observations have also been made of rumen contents and some estimates of microbial numbers have been made by this means, Gall et al.,(1949)and Gall and Huhtanen (1951).

Doetsch and Robinson (1953) state that the technical problems involved in bacteriological studies of the bovine rumen are many and varied. Difficulty is also encountered immediately when the problem of sampling is considered. They mention two methods of approach in sampling technique: (1) A sample is taken by means of a stomach tube; (2) A sample is taken via a permanent

rumen fistula.

The first method is simple and does not require any alteration in the animal but the difficulty is that we are at a loss to know whether we are taking a representative sample of the entire rumen ingesta. The second difficulty lies in handling of the animal if no facilities are available and if the animal is "off-feed".

The second method is useful when the same animals are to be studied over a period of time. Samples can be obtained conveniently from various locations of the rumen. Greater quantities of material can be obtained within a short time. The disadvantages of this method are that it requires a surgical operation, the alteration is permanent, and represents an artificiality which may influence the rumen in ways not as yet perceived.

Elsden et al. (1950), Doetsch and Robinson, (1953), Hobson (1959) have pointed out that there is still a wide gap and lack of knowledge on our part to understand the interrelations between various types of bacteria to vitamin synthesis, minerals, protein synthesis and antibiotics. The synergistic reactions and antagonism is also not understood. To understand this intricate machinery, it is essential to know the physiological functions of these rumen microorganisms and to discover how these physiological types altered with age, changes in diet, disease, effect of antibiotics and other conditions. New techniques are developing which will help to unfold these difficult problems.

According to Doetsch and Robinson (1953), after a rumen sample is taken, it is usually studied by one or more of the following methods:

(1) Microscopic studies;

(2) Pure culture studies;

(3) By differential centrifugation to obtain mixed bacterial suspensions, and

(4) As inoculum for an artificial rumen.

Annison and Lewis (1959) stated that the character of the rumen population can usually be defined by measuring the total number of organisms present and the number of certain individual species.

The method consists of diluting a rumen sample, shaking or agitating by some means, placing on a measured area of a glass slide or in a counting chamber, and examining microscopically with one or several objectives. The material on slide may be dried, stained and then examined. Organisms concerned with starch and cellulose digestion can be stained with iodine (Doetsch and Robinson, 1953).

Kohler (1940) cited by Doetsch and Robinson 1953, diluted rumen samples and centrifuged lightly to remove protozoa, food particles, etc. The supernatant was then passed through filter membranes to further remove the bacterial clumps and residual food particles. One one-hundredth ml. amount was spread on a known area of a glass slide, dried and stained with carbol fuschsin. Gall et al. (1949) made gram stains with 1:10 and 1:100 dilutions and also with 1:1000 by nigrosine. Pounden and Hibbs (1950) examined undiluted rumen samples in order to observe the bacteria attached directly to food particles. Moir and Williams (1950) working with sheep, made 1:500 dilutions in a thoma pipette andused 0.1 percent aniline blue solution. The samples were studied in Petroff-Hauser counting chambers.

The advantage of the microscopic examination is that the investigator can study directly the morphological types of bacteria in a given sample. Organisms not cultivatable also may be seen and quantitative estimates of

the population be made. Variations in certain species may be detected and thus a useful guide in population changes (Doetsch and Robinson, 1953).

Annison and Lewis (1959), pointed out that there are certain disadvantages inherent in the methods of bacterial counts and each method be regarded as complimentary to each other. A reliable total count is difficult to obtain because of the problems of distinguishing living bacteria from other particles present. The significance of a viable count is not easy to asses. Differential counts are complicated by difficulties in accurate recognition of organisms, the large number of species present and the correlation of results to the conditions in vivo.

In cultural methods samples are processed in a variety of ways, depending upon the interest of the investigator. According to Annison and Lewis (1959) much of the published work on pure cultures of rumen organisms is of limited value from the standpoint of rumen metabolism, since the two criteria mentioned by Elsden and Phillipson (1948) have not been established. These criteria are (1) the organism must be capable of carrying out a reaction known to occur in the rumen and (2) be present in sufficient numbers to account for the extent of the reaction.

Alstad (1958) has severly criticised this method of studying microorganisms. According to him it is difficult to equate numbers of a given species of bacteria found on a particular medium, with its natural habitat in the rumen. Moreover, species isolated as pure cultures on an artificial medium may show characteristics not consistent with those found within the natural environment of the rumen.

In spite of these criticisms and objections, the pure culture method in the hands of several workers has yielded much valuable information. Until

the nutritional requirements of the rumen bacteria are better understood, this method of approach may be considered sound. Bryant (1959) has extensively reviewed the pure culture studies with a reference to the species of bacteria cultured. He observed 'The species will probably have to be defined to include many variable characteristics, or the number of species will become unmanageable.'

According to Annison and Lewis (1959), washed suspension method is a microbiological tool in the study of rumenology. The suspension is obtained by a process of 'differential centrifugation', i.e., successive centrifuging at various speeds to remove the heavier fractions of rumen sample. The supernatant is then centrifuged at high speed for 30 minutes. The residue is suspended in a phosphate buffer kept under anerobic conditions. The suspension is washed a second time in buffer before use and incubated with test substrate and fermentation products estimated. Biochemical techniques give information as regards enzymatic capabilities of the mixed culture of organisms. Alstad (1958) states that it is assumed that enzymes observed in vitro are also operative in the rumen, and that one is working with an essentially concentrated, mixed, enzymatically competing population.

Doetsch and Robinson (1953) stated that the chief advantages of washed suspensions are:

(1) A controlled non-proliferating population;

(2) Little activity in the absence of substrate;

(3) Short experimental period, and

(4) The necessity for only small amounts of substrate, (Thornton et al. 1952).

Annison and Lewis (1959), pointed out that there are several advantages

in this procedure over *in vivo* studies or the use of untreated rumen contents. Washing the organisms reduces the carry-over of the end products of rumen fermentation and so minimizes the blank values obtained in the analysis. It is considered that the organisms are in a 'resting state', i.e., metabolically active but with little growth or reproduction. Thus changes in the population during experimental periods of incubation are not marked.

Huster et al. (1958), compared *in vivo* and *in vitro* rumen bacterial dissimilations of some carbohydrates, amino acids and organic acids of metabolic importance by washed cell suspension method. In general, they reported qualitative agreement between *in vivo* and *in vitro* washed cell suspension. They concluded that this technique appears most useful for studying short one or two step reactions presumed to occur in the rumen.

Barnett and Reid (1961) stated there are two types of approach to the study of the problems of ruminal activity and function--namely the *in vivo* and the *in vitro*. There must be two schools of thought on these two different approaches. Some are of the opinion that the reactions which take place in any form of artificial rumen are not the same as those which take place in the natural rumen of the living animal. In support it is stated that, under artificial conditions, products of microbial metabolism are either not removed, although their action may be suppressed or that their removal is not directly related to the mode of removal appertaining to the rumen, which may be looked upon as a large, but not ideal, semi-permeable sac. It is often stated that under *in vitro* conditions a microbial population may develop which may not bear any relation to the one found in the rumen. The population that develops *in vitro* is adapted to perform functions

which would not necessarily take place in vivo.

Lewis (1961) has pointed out that the in vitro study of rumen microbial fermentations also has marked disadvantages, chiefly consequent upon the removal of the test material from the animal. The rapid development of abnormal conditions has proved to be the main limiting factor in such in vitro fermentations. Though a majority of them maintain in vivo conditions for only a few hours. This has proved to be sufficient time for many valuable observations to be made. But this means that the interdependent and sequential relationships of microbial reactions have not been maintained.

Annison and Lewis (1959) describe this method as at first merely involving incubation of rumen contents in a vessel to which a substrate was added and the disappearance of this or appearance of end products was followed.

In the earlier artificial rumen experiments Wegner et al. (1940) added urea to rumen contents and after incubation at 37° C. determined inorganic nitrogen, but obtained negative results. With modifications, ammonia nitrogen disappearance was measured as a criterian of bacterial growth. They criticized the then existing artificial rumen techniques as lacking movement and diffusion. Since then further developments have been aimed at reproducing more closely the in vivo conditions particularly with regard to salt concentration, buffering power, anaerobiosis and nature of substrate. Pearson and Smith (1943), strained rumen ingesta through muslin cloth and incubated at 39° C. in a water bath for two to four hours. They studied the effect of pH, type of carbohydrate present, temperature, gaseous environment and urea concentration on the activity of rumen microorganisms. The conversion of urea to ammonia, synthesis and breakdown of

protein and correlation between protein synthesis and microbial activity were involved. McNaught et al. (1950) and McNaught (1951) using this technique studied the effect of various treatments on the synthesis of protein by rumen bacteria. Among these studies were the effect of metals and the breakdown of carbohydrates which accompanies protein formation in the rumen.

Marston (1948) described an artificial rumen consisting of a 3.5 litre glass pot for fermentation equipped with stirrers and an outlet for gas. He felt that this technique appeared to simulate more closely the natural environment found in the rumen. A year later, a rather elaborate device for in vitro study of rumen digestion was suggested by Louw et al. (1949). It consisted of a glass jar suspended in a water bath at controlled temperature. Fermentation products dialyzed through a visking casing suspended in growth medium. Inlets and outlets were available for gassing, sampling and the addition of buffers. The material was kept in constant motion by use of a stirrer.

Burroughs et al. (1950, 1951) also made use of an artificial rumen technique in studying cellulose digestion and the effect of energy on urea utilization. They devised an artificial rumen consisting of 500 ml. glass fermentation flasks incubated in a water bath at 40° C. Three-holed stoppers were inserted in the flasks for carbon dioxide inlet and outlet and for regulating the pH of the contents. The fermentations were carried out continuously in 36-hour period.

Gall and Glaws (1951) stated that the permeable type of artificial rumen gives better results than the impermeable. The permeable type has a cellophane sac immersed in the medium. Fermentation products diffuse

through it. The reactions take place within the sac. The use of such a system obviates some criticism directed against artificial rumen.

Arias et al. (1951) have developed an apparatus which consisted of two water baths maintained at 39° C. They were used simultaneously, each containing 6 immersed 1000 ml. Erlenmeyer fermentation flasks that in turn were connected together with appropriate tubing for purposes of continuously bubbling carbon dioxide through the fermentation contents. The carbon dioxide stream helped in maintaining anaerobiosis and stirring of the sacs. The flasks were inoculated with microorganisms taken from live animals. Feed ingredients were added every 24 hours over a 4-5 day period and materials were withdrawn at periodic intervals to maintain a constant volume of fermenting material.

Recently Adler et al. (1958) have developed an artificial rumen with an incubation chamber with approximately constant volume of liquid and temperatures. It had a device to deliver nutrients into the chamber at a constant rate. A constant flow of nitrogen gas over the contents of the incubation flask maintained anaerobiosis. The magnetic stirrer maintained the homogenicity of the contents. As nutrients were added the liquid overflowed keeping the contents changing to remove metabolic end products and excessive population. Growth was measured by using a protein free solution in the incubation flask and then measuring the increase in protein in aliquots removed every two hours over a period of time as a measure of the increase in microorganisms.

Fina et al. (1958) developed a technique which they believed would more closely resemble actual rumen conditions than other methods. Their artificial rumen consisted of a porcelain test tube attached to a glass

frothing tube and a gas escape mechanism. Substrates and inocula were placed in the tube and the apparatus suspended in the rumen of a fistulated animal. The porcelain tubes were found to be permeable to two-carbon and six-carbon fatty acids and glucose but not to bacteria.

Regier (1961) used semi-permeable dializing sacs in the study of protein synthesis by microorganisms.

In vivo studies have been made, generally, by using fistulated animals. The essence of this method is that the experiment is performed within the animal and afterwards the results are interpreted. The same animal or group of animals is used throughout the experiment.

Wegner et al. (1941) used the in vivo method to determine how the level of protein in the ration influences the rate of conversion of urea nitrogen to protein. Many workers have contributed to the use of this technique. This method according to Doetsch and Robinson (1953) requires no further verification since no artificialities are introduced.

Warner (1956a) maintained that the criteria of normal rumen function which can be applied to in vitro work are as follows: (1) maintenance of numbers and normal appearance of bacteria, selemomonads and protozoa of the rumen; (2) maintenance of normal rates of digestion of cellulose, starch and protein; (3) of normal interrelation between these; and (4) the ability to predict quantitative results in vivo. To meet these criteria up to 8 hours at a time, a cellophane sac containing rumen liquor and substrate is dialyzed against a complex mineral mixture solution like that of the rumen and incubated at 39° C. in an atmosphere of nitrogen and carbon dioxide. He further stated that to be normal, an artificial rumen must have suitable temperatures, pH value, gas phase and provision for the removal of metabolites.

The substrate must approximate in nature and quantity the diet of the animal from which the rumen liquor inoculum was taken if *in vitro* results are to serve as indications of results *in vivo*.

Belasco (1954) regards the artificial rumen technique as an *in vitro* approach capable of rapid screening of nitrogen compounds as candidate nitrogen feeds.

Burroughs et al. (1950a) stated that the artificial rumen can best be used as a screening device in studying influential factors of feeds in rumen physiology, from which most promising must be checked in animal experimentation. They consider the advantages of artificial rumen to be (1) speed with which determinations can be made in a series of laboratory flasks as compared to similar determinations carried out in the rumen of live animals. (2) Precision which can be exercised over various conditions in the laboratory which are left to chance in experimental studies using live animals; (3) less expenses for animals.

They also list certain disadvantages in use of this technique--(1) the results may not be truly representative; (2) the end products formed cannot be controlled; (3) different types of organisms may develop in different runs on the same substrate.

Elsden et al. (1952) have added to this list of disadvantages by saying that the end products are already present. There is already an activity without substrate. There are population increases and experiments are relatively long term as compared to washed suspensions.

Barnett and Reid (1961) stated that all modes of experimentation are necessary and complimentary to each other and that the *in vitro* exponent plays a valuable part in promotion of knowledge.

Nutritional Requirements of Rumen Microorganisms

During recent years attention has been given to qualitative and quantitative requirements of rumen microorganisms for organic nutrients. But only limited attention has been given to the inorganic nutrient requirements of the rumen microflora.

Warner (1956) suggested a need for an investigation of the mineral constituents of media used in artificial rumen studies. This would evaluate the various *in vitro* techniques being used. He emphasizes that this is necessary because:

(1) The basal medium used for testing feed materials for stimulation of cellulose digestion by microorganisms must contain an optimal level of inorganic nutrients to rule out the possibility of an apparent stimulation being a result of correcting a deficiency of some inorganic substance.

(2) A measure of the toxic level of various inorganic elements would be of value both for *in vitro* studies and for possible relation to conditions that could exist in the rumen of the animal.

Barnett and Reid (1961) stated that the lack of knowledge as to the nutrient requirements of the rumen bacteria, and the inability to culture more than a comparatively small number of the organisms actually present in the rumen has been a real barrier to progress in the field of rumenology. Further studies on the nutrient requirements of individual organisms are needed.

The evidence concerning nutritional requirements of the rumen microorganisms, especially rumen bacteria, has been derived almost completely from experiments made on pure cultures and from observations made from

artificial rumen studies. Thus the methods for studying the problems have been indirect. McNaught et al. (1950) have written:

> Since the growth of rumen bacteria in pure culture is beset with many difficulties, it is not possible to study the mineral requirements by growing bacteria on a synthetic medium made purposely deficient in a given element. Moreover there are grave doubts as to whether results obtained with pure cultures would help much in elucidating the process occurring in the normal rumen where mixed populations of bacteria and protozoa are always found and where symbiotic relationships of one species of microorganisms to another almost certainly obtain.

Tosic and Mitchell (1948) in studies with sheep, found that rumen microorganisms concentrate cobalt from their external environment. They considered that the cobalt requirement may be more necessary for the rumen microorganisms than for the ruminant itself. Burroughs et al. (1951a) lists three general nutrient requirements of rumen microorganisms namely (1) energy, (2) protein or its elements like nitrogen, and (3) inorganic constituents involved in enzyme or enzyme systems of the microorganisms.

Lewis (1954) has shown that sulphate is reduced to sulphite in the rumen. Feeding 150 grams of $Na_2SO_4 \cdot 10H_2O$ to sheep resulted in 14.7 micromoles of sulphite/ml. rumen liquor. No ill effects were observed. Thus the ruminant is quite tolerant of the high levels of sulphur.

Hobson (1959) reports that sulphur is a constituent of some amino acids and seems to be needed in the processes of nitrogen utilization in the rumen. A source of sulphur is necessary for protein synthesis from added or endogenous ammonia in the rumen and can be supplied in the form of sulphate. Burroughs (1951a) stated that a conventional protein and in some cases a sulphur containing amino acid could exert an influence upon the conversion of urea into rumen bacterial protein by contributing a mineral element like phosphorus or sulphur needed by the microflora in the

synthetic process.

Arias et al. (1951) report that rumen microorganisms have energy requirements and the degree to which these requirements are fulfilled has considerable influence upon their utilization of urea or other ammonia supplying compounds.

Adequate balance of nutrients for the microorganisms appears to be of importance. Arias et al. (1951) stated that dextrose or other readily available carbohydrates in too large a quantity is not needed, in an attempt to make maximum utilization of urea as a protein supplement. They explain that unfavorable fermentation conditions for cellulose digestion may occur and utilization of urea interfered with.

Burroughs et al. (1951) using the artificial rumen technique, made observations on the mineral requirements of rumen bacteria. They found that cellulose digestion could be favored by addition of a mineral mixture to the artificial rumen. They reported that there are varying needs for sodium, potassium, calcium, magnesium, phosphorus, sulphur and chlorine by rumen microorganisms. An artificial saliva containing these elements was devised by them, and added to the artificial rumen. Phosphorus and iron were found effective in stimulating urea utilization and cellulose digestion.

McNaught et al. (1950) reported that 10 ppm. of copper was the maximum level that could be present in rumen liquid when the criterian of rumen microorganisms activity was the conversion of non-protein nitrogen to protein. They found that 10 ppm. of cobalt would reduce in vitro conversion of non-protein to protein by rumen microorganisms. They further observed that a concentration of from 10 to 100 ppm. of iron had no influence on

the conversion of non-protein to protein, but 1000 ppm. sharply reduced the synthesis when centrifuged rumen liquor was used.

Bryant and Burkey (1953) stated that many of the groups of bacteria isolated required carbonate in the culture medium before growth was obtained and several groups did not grow well unless rumen fluid was included in the medium.

Chance et al. (1953) studied the effect of aureomycin on rumen digestion and the rate of passage of certain nutrients from the rumen. Aureomycin was fed at 0.5 g per day for 15 days and then 1.0 g per day for the next 15 days. The rumen contents were completely evacuated, weighed, sampled, and replaced in the rumen before feeding (0 hour) and again at 6 and 12 hours after feeding. The rate of removal of dry matter, crude fiber, crude protein and nitrogen free extract was the highest when 0.5 g of aureomycin was fed. There was accumulation of these products when 1.0 g of aureomycin was fed in the ration suggesting depression of digestion.

Proper nutrition of the bacteria, for the well-being of the host, is also necessary to function well in protein synthesis. Baker (1946) stated that for synthesis of protein to exceed breakdown, ample readily available source of energy in form of carbohydrate, other than cellulose must be present and the content of soluble protein must be low in the ration.

Smith and Baker (1944) in an attempt to find a correlation between protein synthesis and microbial activity found that maltose promoted protein synthesis. Glycerol did promote synthesis, but tended to cause hydrolysis of protein and an increase in non-protein nitrogen. In a study of amino acid metabolism of bovine rumen bacteria, Sirotnak, et al. (1953) found fermentable carbohydrates to enhance dissimilation of amino acids and reported

that maltose, cellulose and glucose caused a significant increase in the production of ammonia and carbon dioxide from aspartic acid.

Agrawala (1951) found a much higher level of amino acids in rumen samples from cattle on normal rations than from animals on purified diet. Burroughs et al. (1951a) stated that nitrogenous requirements of rumen microorganisms are relatively simple, essentially involving only ammonia and not the more complex forms of nitrogen, such as amino acids.

McDonald (1948, 1952) showed that there was a fall in ammonia concentration in rumen contents when starch was added and an increase in volatile fatty acids from starch when small amounts of casein were also added. These observations were extended by Lewis and McDonald (1958), and the effect of carbohydrate supplements on casein fermentation was examined in detail. They concluded that the type of carbohydrate material present regulated ammonia concentration in the rumen and presumably the extent of microbial protein synthesis.

The importance of ammonia in the nutrition of rumen microorganisms was further emphasized by Bryant, et al. (1959) in their work with a ruminal celluloytic bacterium. They found that ammonia is essential for the growth of Bacteroides succinogenes even with the presence of 19 amino acids, a mixture of purines and pyrimidines, and all the B-complex vitamins. They found delayed growth when cystein or glutathione was the only source of amino acids available.

Information in the literature on the trace mineral requirement for optimum microbial activity is limited. According to Alstad (1958) a great deal remains to be done regarding nutritional requirements of the microorganisms of the rumen and the effect of deficiencies on overall rumen

function. Certain observations on requirements for trace minerals, fatty acids, vitamins, etc. for the rumen microorganisms have been made. But generally they have been made with some sort of artificial rumen techniques. How far these results can be applied to the tremendous mixed population of microorganisms of the rumen is still to be studied. Therefore major work in this direction lies still ahead.

The Fate of Nitrogenous Compounds in the Rumen

During the last decade considerable progress has been made in the study of the fate of protein and other nitrogenous materials consumed by ruminants. In the light of these results it is desirable to consider means of assessing the efficiency of various proteins in satisfying the nitrogen requirements of the ruminants (Lewis and McDonald, 1958).

It is usually considered that the nitrogenous constituents which leave the rumen are digested in a manner similar to the nitrogenous constituents of the diet of non-ruminants. But in assessing the value of proteins to the ruminants, two additional factors must be taken into account: the way in which nitrogenous compounds are modified within the rumen and the extent to which the ingested nitrogen is absorbed from the rumen. Attention must be given to the extent of ammonia absorption from the rumen, the degree of microbial protein synthesis in the rumen and the value of this protein to the animal (Lewis and McDonald, 1958).

Annison and Lewis (1959) stated that the nitrogen metabolism in the rumen affords another striking example of the influence of rumen micro-organisms on the nutrition of the host animal. Ingested proteins, like other foodstuffs, are subjected to the attack of the rumen microbial

population and undergo extensive degradation before passing on to the abomasum and small intestine. The main contribution of the rumen to nitrogen metabolism is that it can modify or supplement the amino acids of the ingested protein and alter the amount of nitrogen that is made available to the animal.

Barnett and Reid (1961) stated that the value of food protein in the nutrition of the ruminant is dependent upon three things: (1) the ability of the animal to break it down to compounds which are capable of absorption or utilization, (2) the inherent value of some of the individual breakdown components and the potential value of some of the individual breakdown components and (3) the potential value of some of the others which may be utilized by the rumen microflora.

McDonald (1954) stated that the interest in the role of microorganisms in protein metabolism stems from the hypothesis of Zuntz (1891), that these organisms might utilize dietary non-protein nitrogen for their growth, with resultant synthesis of protein which would become available to the host. In spite of the activity in this field, comparatively little attention has been given to the possibility of microbial digestion of food protein in the rumen. In normal ruminant diets, most of the nitrogen occurs in the form of protein. The facts that secretory glands do not occur in the rumen, that the saliva contains no amylolytic or proteolytic enzymes (Wegner et al., 1940) and that the rumen contents are strong proteolytic suggest that ruminant organisms play an active part in the digestion of protein.

Pearson and Smith (1943) observed that the breakdown of protein occurs in vitro when rumen liquor is used as media. McDonald (1948, 1952) showed that soluble protein was extensively degraded in the rumen with the formation

of ammonia.

The extent to which ruminal microbes convert fodder protein into the protein of their own structures has been the subject of several speculations, though little experimental effort has been directed to the problem (McDonald, 1954).

Schwarz (1925) concluded that the greatest part of the protein requirement of cattle was met by the digestion of microbial protein derived from fodder protein. His data was based on the analysis of rumen contents alone and therefore such a conclusion was not desired. He found on an average 60 percent of the total nitrogen of rumen contents in the fodder residues and 32 percent in the microorganisms.

Ferber et al. (as cited by McDonald, 1954) concluded that rumen protozoa provided about 2 percent of the protein requirement of the sheep. Hungate (1942) considered this value to be too low. He found that in culture, the protozoa, Eudiplodinum neglectum, has a division rate of about once a day and hence calculated that protozoa provided about 20 percent of the hosts daily protein requirements.

Kohler (1940) as cited by McDonald (1954), attempted to assess the daily output of microbial nitrogen from the rumen of cattle by comparisons of bacterial numbers or weight in the contents of the rumen and the duodenum, but found his method inadequate to yield acceptable results.

Thaysen (1945) estimated that a minimum of 180 g. per day of microbial protein passed from the rumen of cattle. He based his calculations on analysis of centrifuged rumen liquor that flowed from the rumen to abomasum each day. McNaught and Smith (1947), using data of Pearson and Smith, found 100-150 g. of bacterial protein per day in the rumen of cattle. Using the

data of Schwarz (1925) and an assumed rate of flow of digesta from the rumen of the ox, 40 kg. per day, they calculated that 75 g. of bacterial protein would be formed per day.

Moir and Williams (1950) observed in sheep, a constant increase in the numbers of ruminal microorganisms with increasing protein intakes. They suggested that a constant proportion of the food protein was converted into bacterial protein. They calculated that approximately 50 percent of the dietary protein was converted into bacterial protein. They subsequently recognized that such a calculation is not possible with their data.

Moore and King (1958) in the determination of intraruminal distribution of soluble nitrogen used an analytical fractionation procedure for nitrogen balance studies of the rumen fermentations. The procedure was evaluated in an *in vivo* experiment. They concluded that regardless of dietary treatment, most of the nitrogen (65-78 percent) was observed to be found in feed residues and microbial cells. Only small amounts of nitrogen (less than 2 percent) were encountered in the form of dissolved protein.

El Shazley (1952), studying the degradation of protein in the rumen of sheep found that an increased ammonia concentration was correlated with increasing concentration of iso-butyric acid and of five carbon chain acids. He considered these acids as a result of attack by microbes on protein in the rumen.

McDonald (1954) calculated that when zein was fed as the main nitrogenous constituent of the ration some 60 percent of the protein might pass unchanged from the rumen. If soluble casein was included in the ration as a source of nitrogen more than 90 percent was degraded (McDonald and Hall, 1957). Annison (1956) reported proteolytic activity of rumen contents with washed

cell suspensions of rumen bacteria and demonstrated extensive degradation of casein, arachin and soybean proteins. After feeding a ration containing protein but devoid of free amino acids or peptides to sheep, he was able to demonstrate the presence of peptides and amino acids in the rumen. This is evidence that proteolysis is the first step in protein digestion and peptides and amino acids were described as intermediates in the breakdown by microbial proteolytic enzymes.

McDonald (1948, 1954) stated that the protein entering the rumen is partly broken down to ammonia under the action of microorganisms present and partly converted to microbial protein. There is simultaneous synthesis of microbial protein from the non-protein nitrogen available in the rumen. Chalmers and Synge (1954), pointed out that the nitrogen utilization of the animal largely depends upon the relative proportions of these three processes. Lewis (1954) observed that the complexity of the problem is appreciated when it is realized that this balance is largely controlled by the nature of the microbial flora which in turn is dependent on the feeding regime.

It was shown by McDonald (1948) that some part of ammonia that is formed in the rumen is absorbed into the ruminal veins and carried along the portal vein to the liver (Annison et al., 1955). Since ammonia is not found in peripheral circulation, it has been presumed that all the ammonia absorbed from the rumen is converted to urea in the liver. It is probable that the urea so formed is partly returned to the rumen in the saliva (McDonald 1948) and partly lost in the urine.

Lewis (1954) stated that the efficiency of utilization of protein consumed by the ruminant must therefore in part depend upon the quantity of ammonia produced in the rumen, which in turn is reflected by the ammonia

concentration in portal blood.

Lewis (1961) stated that protein that enters the rumen is fermented by the microorganisms, giving rise to peptides, amino acids and ammonia. The NPN consumed can also produce amino acids and ammonia. There is a synthesis of microbial protein using the NPN compounds in the rumen. It is not only amino acids and peptides that can be used in these synthetic reactions but ammonia nitrogen can also be readily incorporated into microbial protein, Phillipson, Dobson and Blackburn, (1959). Two major factors that regulate this balance are the solubility of the ingested protein and the amount and type of carbohydrate present. Some unchanged protein, microbial protein and NPN is continually passed along the alimentary tract. The digestive process, from this point onwards is probably similar to that in non-ruminants. The significance of the rumen action, therefore, lies partly in the extent to which food protein is converted into microbial protein. This effect is dependent upon the relative nutritive values of the ingested protein and that synthesized in the rumen, or alternately it depends upon the degree of synthesis of necessary amino acids.

As summarized by Alstad (1958), protein, urea, amino acids, ammonia, nitrates, etc. enter the rumen. Hence the proteins are hydrolyzed to amino acids which are in turn absorbed by the rumen microorganisms or deaminated and decarboxylated with liberation of fatty acids. He suggests the possibility of a mutual oxidation-reduction between certain pairs of amino acids, yielding keto acids, fatty acids, ammonia and carbon dioxide. Ammonia may then be absorbed through the rumen or utilized by the microorganisms to synthesize their amino acids and proteins. Nitrates may be reduced to ammonia and any material which is not attacked passes on to the

abomasum and intestine.

Dissimilation of Non-Protein Nitrogen Compounds by Rumen Microorganisms

The belief that certain simple nitrogenous compounds such as urea are capable of supplying the ruminant a significant proportion of its nitrogen requirement is many years old. The theory most commonly advanced to account for the utilization of these simple compounds is that they are built up into protein by certain microorganisms of the rumen which use them as a source of nitrogen for their own multiplication. The protein so formed is subsequently broken down in the small intestine to amino acids which are absorbed into the blood stream of the host along with those resulting from the digestion of the diet.

Loosli et al. (1949) stated that in 1891 Zuntz presented the view that bacteria in the rumen of animals utilize non-protein nitrogenous compounds to protein, which in turn was used by the animals. They further stated that in recent years it has been conclusively shown that protein is formed in the rumen from dietary urea and ammonium salts. The protein thus formed appears to be of relatively low biological value. But to their knowledge, no attempt was made to measure the amino acid composition of protein synthesized in the rumen. In the course of their studies with sheep on a purified diet containing urea, they observed that ten essential amino acids are synthesized in large amounts in ruminants fed urea as the only dietary source of nitrogen.

Reid (1953) observed that, as recently as 1937, it was not commonly accepted that urea is converted to proteins in amounts of any great significance to ruminants. It was suggested that the protein-sparing action

of urea may have been the result of such mechanisms as neutralization by ammonia of organic acids formed in the digestive tract of the ruminants.

Wegner et al. (1940, 1941a and 1941b) revealed that urea nitrogen utilization must occur within 4-6 hours after feeding since urea and ammonia nitrogen are in negligible amounts after that time.

In 1941, Benesch reviewed the work done on urea noting the necessity of finding substitutes for dietary proteins in the form of simple nitrogenous compounds. The work was continued further by Mills et al. (1942). They observed that when the protein level in the concentrate mixture was above 18 percent, any added urea was poorly converted into protein. They further observed that urea is well utilized when added to corn or oat ration, due to the abundance of starch in the grains. Addition of corn molasses to a grain mixture did not increase the efficiency of urea utilization. When timothy hay alone was fed both the ammonia nitrogen and total protein were at low levels and remained constant throughout the trial. When urea was fed with hay, hydrolysis of urea to ammonia was delayed, being incomplete at one hour after feeding and disappearance of the ammonia was very slow, about half remaining as such in the paunch at 6 hours after feeding. The protein level was slightly lower than timothy hay alone. Therefore they concluded that no abundant and active flora was operating in the rumen.

In contrast, when starch was fed along with hay and urea, microbial activity was great. The urea was completely hydrolyzed in less than one hour and ammonia thus formed had practically disppeared in six hours. As the ammonia nitrogen level fell, there was a concurrent rise in protein indicating that the ammonia was built into protein. The total rise in protein was approximately equivalent to the amount of ammonia disappearing.

Pearson and Smith (1943a, 1943b) were perhaps the first to suggest the significance of ammonia in the rumen in relation to their studies on the utilization of urea in the bovine rumen. They tested the theory that certain microorganisms which multiply in the rumen build up their own protein from this simple form of nitrogen. They noted that the optimum pH for this conversion to be between 7 and 9, with little occurring below 3 or above 9.5. A slight increase in the conversion of urea was brought about with increased substrate concentration. The influence of time of sampling from the rumen appeared to have little effect as to the ability of the rumen liquor to convert urea to ammonia. They reported the absence of urease activity in feed, and that none is separated into the rumen. Consequently this enzyme must be produced by microorganisms in large amounts. Quinone, cysteine, sodium fluroide, boric acid and borax, all appeared to have an inhibitory effect upon urease. Smith and Baker (1944) confirmed the assumption of Pearson and Smith (1943c) that as non-protein nitrogen content decreases in vitro total nitrogen remained constant, protein was synthesized. This synthesis was accompanied by a great increase in the number of microorganisms, with iodophilic counts greatly increased when synthesis predominated. Accompanying the synthesis of protein, was the increase in polysaccharide which was starch-like. By centrifuging the rumen liquor before incubation, protein synthesis was shown to occur in the absence of protozoa. These workers believed that there can be little doubt that the bulk of the synthesized polysaccharides and protein is incorporated in the small rods, cocci and vibriophiles of the microiodophilic population.

Bartlett and Blaxter, (1947) showed that urea has a depressant effect upon milk production when fed at levels of ingestion that would be necessary

to justify its use economically.

Annison and Lewis (1959) stated the most of the experimental work has been devoted to finding the optimum conditions for feeding urea and only indirect information on actual protein synthesis has resulted. Such evidence for synthesis has been obtained in several ways, including measurement of the changes in the proportions of ammonia and protein in rumen contents on feeding urea (Mills et al., 1942); by analysis of the total nitrogen of the microbial fraction after feeding (Gray et al., 1953); or by analysis of rumen contents before and after some time, feeding a diet containing urea (Agrawala et al., 1953).

Lewis (1951) has established that nitrate as found in the fodders, may be reduced to ammonia by rumen microflora. He further states that the toxic effect of nitrates in bovines may be due to the accumulation of ammonia (Lewis, 1957).

Barnett and Reid (1961) stated that while it is generally accepted that ammonia is a main end product in the hydrolysis of urea, the biochemical pathway for its formation is still ill-defined.

Hobson (1959) calls attention to the fact that urea can replace part of the protein in the diet if carbohydrates, supplying readily available energy, are available and that urea is always present in saliva, which is broken down to ammonia and utilized for bacterial growth.

Alstad (1958) notes that the validity of the claim that non-protein nitrogen compounds can replace part of protein of ruminant rations has been questioned but cites the annual use of approximately 75 thousand tons of urea as feed supplements for ruminant rations, as evidence concerning acceptibility of the idea.

Hart et al. (1939) showed that utilization of ammonium bicarbonate and ammonium sulphate as substitutes showed the efficiency of ammonium bicarbonate to be equal to that of urea and greater than of ammonium sulphate. The optimum concentration of urea was found to be between 45 and 100 mgms. per 100 g. of rumen contents. Concentrations above and below resulted in decreased synthesis.

Urea can be utilized as a nitrogen source for ruminants but the extent of its use is limited because of possible toxic effects. Biuret, a condensation product of urea, has been shown to be less toxic to animals than urea. Meiske et al. (1955) showed that urea produced toxic symptoms and death while the same and higher levels of biuret were not toxic. Campbell et al. (1960) studied utilization of biuret in vivo. They stated that biuret can be used as a nitrogen source for ruminants.

Fate of Rumen Microorganisms in the Digestive Tract of Ruminants

Doetsch and Robinson (1953) stated that a symbiotic relationship exists between the ruminant and the bacteria found in the rumen. The host supplies the bacteria with materials to be utilized and the bacteria in turn breakdown these substances to products which may be absorbed into the blood stream. The degradation of bacteria take place as a result of action of enzymes, effect of metabolic end products, etc.

McNaught et al. (1947) stated that Mullar in 1906 was probably the earliest who attempted to investigate the nutritive value of rumen bacteria. He approached the problem by inoculating a cultural medium with rumen bacteria and allowed it to grow for three days. The mixture was then precipitated with

ammonium sulphate and the precipitate washed with ethanol. The dried product was then fed to a bitch. The nitrogen retention in the bitch was found to be similar to that on a ration combining albumin and casien.

McDonald (1952) stated that the formation of ammonia in the rumen leads to two opposing tendencies. First, since substances such as urea, which are nutritionally valueless to the host, can be converted to ammonia and utilized for growth of bacteria, that is for synthesis of body protein by the bacteria which can be subsequently digested and used by the host. Thus a gain of nitrogen access to the host animal. By contrast the degradation of protein to ammonia, which can be directly absorbed from the rumen, implies a source of loss of nitrogen to the host animals. They further state that the interaction of these opposing tendencies is probably a major factor leading to the relative constancy of the biological value of food nitrogen (crude protein) for ruminants.

McDonald (1952) stated that under ordinary conditions of feeding, the nitrogen entering the rumen will comprise chiefly protein together with varying amounts of non-protein nitrogenous substances as peptides, amino acids, amides, purines, pyrolls, simple bases such as choline and the betaines, inorganic nitrogen as ammonia, nitrates and nitrites and traces of other substances. The nitrogenous bases and amino compounds may be deaminated while nitrates are reduced to ammonia (Lewis, 1951). Ammonia is also produced by the degradation of proteins. In addition, small but significant amounts of nitrogen are added to the rumen contents by the saliva, in which the most important component is urea, which is readily converted into ammonia. Ammonia is utilized by the microorganisms for growth together with amino acids produced by the activity of bacterial

proteases. Protein leaving the rumen, by passage in the ingesta to the more distal parts of the gastro-intestinal tract consists of a mixture of undigested food protein and the protein of the microorganisms. Some of the nitrogen utilized by the ruminal microorganisms for growth would appear as nucleic acids (and other non-protein substances) which are probably of very limited, if any, value to the host when the bacteria are disintegrated in lower part of the gastro-intestinal tract.

Schwarz (1925) concluded that the greatest part of the protein requirement of cattle was met by the digestion of microbial protein, derived from the fodder protein.

McDonald (1954) pointed out that the literature reveals that it is likely that the growth of microorganisms (bacteria and protozoa) in the rumen result in a significant degree of conversion of animal's food nitrogen into microbial protein, but that the extent of this conversion and the factors affecting it are not evident.

The nitrogenous substances absorbed by the host are not merely those of the diet, but a mixture of dietary constituents, products of microbial metabolism in the rumen and the constituents of microorganisms themselves.

Hart et al. (1939) stated that the products of bacterial synthesis in the rumen is made available to the host for subsequent digestion in the lower gastro-intestinal tract, and the products of this breakdown are utilized by the host animal. Benesch (1941) observed that it seems possible that not only does the bacterial synthesis in the rumen depend on many factors like the nature of the diet, the type of microbial population, ph etc., but that the bacterial protein also thus formed can be digested to variable extent by digestive enzymes.

Pounden et al. (1950) stated that complete utilization of the nutrients synthesized in the rumen by the microorganisms consisting of the microflora and the microfauna, presupposes later disintegration of the microorganisms, in order that the products incorporated in their cells may be absorbed by the host animal. The concept that the rumen microorganisms are digested by the host animals after passage from the forestomachs is quite generally accepted.

Hastings (1944), stated that ruminants live to a large extent on protozoa and bacteria which are constantly carried to the true stomach, killed and digested.

McNaught et al. (1947) consider that it would be almost impossible to explain this ability that ruminants possess for utilizing non-protein nitrogen as compared to other animals, like the pigs, rats, poultry, unless the conversion is accomplished by rumen microorganisms which later release their products to the host. Johnson et al. (1944), mentioned the ability that the bacteria utilize products like urea and that the bacteria are later digested by protozoa and these in turn are digested by the host animal. Ciliates apparently are destroyed during their passage through the digestive tract as they disappear in the abomasum, Bartlett et al., (1947). Baker (1943) has also shown that they are digested by peptic and tryptic enzymes.

Pounden et al. (1950) further stated that according to Baker (1943), iodophilic bacteria of the rumen, although not affected by gastric secretions, are inconspicuous and present only in limited numbers in coecum and feces of cattle and sheep. Partially digested microorganisms are seen frequently in caecal contents. The breakdown and disappearance by the time the material reached the coecum, strongly iodophile, oscillospira organisms which were

prevalent in the contents of sheep rumen were reported by Baker and Harris (1947). Pounden et al. (1950) confirmed the findings of others that the destruction of protozoa by abomasal fluid does occur. They concluded that the ultimate fate of rumen microorganisms varies between the extremes of complete destruction in the abomasum to passage entirely through the digestive tract of the host.

McCarthy (1962) stated that the main value of the rumen to nitrogen metabolism of the bovine is that the microorganisms present can modify or supplement the amino acids of the diet, and can alter the amount of nitrogen, available to the host. Much of the protein that enters the rumen is converted to organic acids and ammonia. New protein, with all essential amino acids, of bacterial origin is synthesized and when these microbes pass to the lower digestive tract, the protein becomes available to the host animal.

PART I

General Methods and Procedure

The Dializing Sac. Seamless regenerated cellulose dializing tubing was made into sacs and incubated in the rumen of fistulated steers housed in the metabolism room of the Animal Husbandry Department, the object being to study further its effective use in an attempt to provide an *in vivo* artificial rumen combination for the study of protein synthesis in the rumen. The permeability of this tubing was described by the manufacturers as having an average pore size of 24A°, which was determined by the rate of flow of water through the film. The tubing permits passage of water and low molecular

weight substances in aqueous solution to diffuse while the high molecular weight substances such as protein and bacteria are not permitted to diffuse in and out of the sac, the sacs permitted 80-90 ml. of fluid to be accomodated.

Preparation and Incubation. The dialysis tubing was cut into 7-8 inch length pieces, which were soaked in a tray of distilled water for a few minutes. Soaking moistened the sacs and the two folds could be separated easily. To one end of the sac, a serum rubber stopper was inserted and tied firmly with a rubber band. Care was taken to see that the fore end of the tubing protruded out of the rubber stopper about 0.2 inch, so that this end of the tubing could be sealed with plaster of paris.

Before tieing the other end, distilled water was poured in the sac and gentle pressure was applied from the sides to detect any leakage from the rubber stopper end. The water was removed and then the end tied with a nylon string about 3/4-inch from the end. Plaster of paris was applied to both ends of the sac and was allowed to dry and harden for twelve hours during which time the sacs were prevented from drying and cracking, by putting wet paper towels on them. After the sacs were ready for use, 30 ml. sterilized rumen liquor or 30 ml. sterilized saline was injected into the sacs through the stoppered end. The sacs were then sterilized in the autoclave for 3 to 5 minutes. After cooling, one gram of cornstarch dissolved in ten ml. distilled water and 1/2 ml. bacterial suspension or 1 ml. fresh rumen liquor, according to treatments, were injected into each sac through the stopper. A further safety measure was adopted to prevent leakage by dipping both ends of the sac alternately in liquid wax several times. The sacs were then introduced into one litre polythelene bottles having holes on sides. The bottles were stoppered and introduced to the ventral part

of the rumen, for incubation as per scheduled time. Rumen contents entered the bottles through the holes but coarser particles of hay were prevented from entering the bottles due to the size of the holes, thus the sacs were protected from damage.

Collection of Rumen Liquor. Rumen liquor from the ventral sac of the rumen was collected in 250 ml. beakers. It was filtered through a strainer into the collecting bottle. This bottle along with normal saline, syringes and other glassware were sterilized in the autoclave at 15 pounds pressure for half an hour and allowed to cool in the autoclave. The rumen liquor was filtered again before use if there were still coarse particles present.

For sac inoculations, fresh rumen liquor was collected and strained through the strainer and cheese cloth into a clean sterilized test tube. One ml. of this fresh liquor was used for each sac inoculation according to the treatments.

Nitrogen Determination. After the period of incubation of the sacs in vivo, in the rumen of two sets of identical twin, fistulated steers, the bottles were removed and cleaned with water. The sacs were removed gently from the bottles, washed with water and then flushed with distilled water. The water was removed from the sides with a paper towel. The end of the sac to which string or nylon was tied to seal, was snipped with scissors and contents of the sac were poured into an Erlenmyer flask containing 60 ml. of 10 percent Trichloroacetic acid. The sac was rinsed 3-4 times and the contents poured into the flask. The protein was allowed to precipitate for 24 hours. Then it was filtered through a funnel, in which a filter paper was placed, by gentle suction. The filter paper with precipitate was introduced into a Kjeldahl digestion flask. The funnel

was cleaned with filter paper and this also added to the Kjeldahl flask.

Protein nitrogen was determined by the use of a Kjeldahl apparatus. True protein was estimated by multiplying the protein nitrogen values by 6.25.

Trial No. 1

The object of this trial was to study the difference, if any, in protein synthesis by the microorganisms, when sterilized rumen liquor or sterilized normal saline was used as a source of media and when the sacs were incubated in the rumen of fistulated steers for a period of 48 hours.

Experimental Procedure. The experimental design, treatments to the dializing sacs, period of incubation are shown in Table 1. The sacs were divided into two groups and were incubated in the rumen of identical twins.

Table 1. Protein synthesis by microorganisms, incubated for 48 hours with rumen liquor and normal saline as media.

Sample Sac No.	Steer No.	Period of incubation hrs.	Treatments	Protein Nitrogen %	True Protein %
1		48	Rumen liquor + 1 gm. starch + 1 ml. fresh R.L.	0.368	2.300
2	3	48	R.L. + starch + 1 ml. fresh R.L.	0.351	2.200
3		48	R.L. + starch (Blank)	0.225	1.406
4		48	Normal saline + starch + 1 ml. fresh R.L.	0.099	0.619
5	4	48	Normal saline + starch + 1 ml. fresh R.L.	0.095	0.594
6		48	Normal saline (Blank)	0.037	0.231

Results and Discussion. The results of the trial as shown in Table 1 indicate that the difference between rumen liquor and normal saline as a medium for growth and synthesis of protein by the microorganisms is marked. This might be due to readily available source of nutrients and energy for the microorganisms from rumen liquor.

Trial No. 2

The object of this trial was to determine the difference between streptococcus faecalis and Ln. mesenteroides, as regards their ability to synthesize protein. It was also the intention to find out the possibilities of the use of dializing sacs for pure culture studies. Since sterilized rumen liquor and normal saline was used as a source of media, the difference between the two could also be determined.

Experimental Procedure. The experimental design with various treatments, period of incubation of the sacs and results are shown in Table 2.

Results and Discussion. The results of the test indicate that those sacs incubated after inoculation with microorganisms showed turbidity as contrasted with the sacs which were not inoculated. The contents of sacs which were not inoculated showed clear brownish fluid. This reveals growth of the microorganisms in sacs in which they were inoculated.

It was further revealed that sterilized rumen liquor as compared to sterilized normal saline was a better media for growth and nourishment for the microorganisms.

As shown in Fig. 2, no difference in protein synthesis by the two species of bacteria used in the experiment was observed.

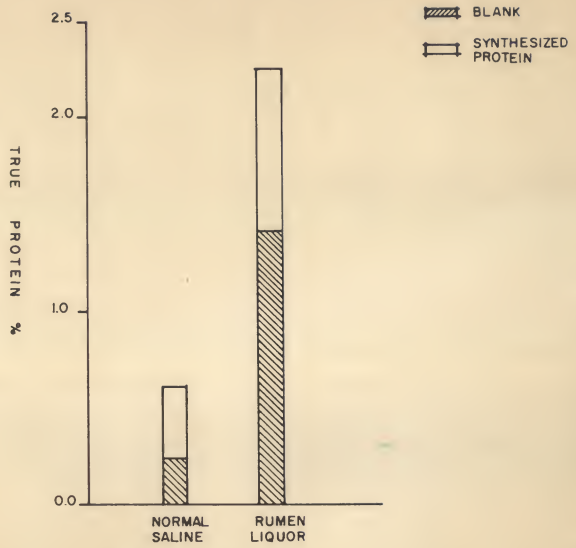

FIG. I COMPARISON OF RUMEN LIQUOR AND NORMAL SALINE AS MEDIA FOR MICROBIAL SYNTHESIS OF PROTEIN.

FIG. 2 COMPARISON OF PROTEIN SYNTHESIZED BY LN. MESENTEROIDES AND S. FAECALIS INCUBATED FOR 24 HOURS WITH RUMEN LIQUOR OR NORMAL SALINE AS MEDIA.

Table 2. Comparison between Ln. mesenteroides and S. faecalis for protein synthesis when incubated for 24 hours with rumen liquor or normal saline as media.

Sample Sac No.	Steer No.	Period of Incubation hrs.	Microorganism Used	Treatments	Protein Nitrogen %	True Protein %
1		24	Ln. mesenteroides	R.L.[1] + starch (Blank)	0.098	0.613
2		24	" "	R.L. + starch + 1/2 ml. fresh culture	0.215	1.344
3	4	24	" "	" "	0.226	1.413
4		24	" "	Saline + starch (Blank)	0.037	0.231
5		24	" "	Saline + starch + 1/2 ml. fresh culture	0.068	0.425
6		24	" "	" "	0.085	0.536
7		24	S. faecalis	R.L. + starch (Blank)	0.099	0.624
8		24	" "	R.L. + starch + 1/2 ml. fresh culture	0.260	1.413
9		24	" "	" "	0.262	1.640
10	3	24	" "	Saline + starch (Blank)	0.037	0.231
11		24	" "	Saline + starch + 1/2 ml. fresh culture	0.088	0.536
12		24	" "	" "	0.068	0.425

[1]R.L.—sterilized rumen liquor

Trial No. 3

The objective of this trial was to find out (1) the difference, if any, in protein synthesis in the sacs by microorganisms when the polythelene bottles were introduced and incubated in the rumen with or without weights added to them, the object being to push the bottles in the liquid portion of the rumen contents; (2) the effect of incubation of the sacs for 36 and 48 hours on protein synthesis by the microorganisms; (3) the difference between sterilized normal saline and rumen liquor as media for the growth of the microorganisms.

Experimental Procedure. This trial consisted of four groups. The experimental design for these four groups with treatments and results are shown in Table 3. One gram of starch in five ml. distilled water was injected in each sac. The microorganism used was Ln. mesenteroides.

Results and Discussion. The results reveal that: (1) there was no difference in protein synthesis in bottles containing the sacs with weights and those without weights; (2) there was a marked difference in protein synthesis by the microorganisms between sterilized rumen liquor and sterilized normal saline used as media. It shows that rumen liquor was a better medium than normal saline; (3) there was no difference observed in the synthesis of protein by the microorganisms, when the bottles were incubated in the rumen of fistulated identical twins.

Trial No. 4

The objective of the trial was to determine (1) the difference, if any, in protein synthesis by the microorganisms when the sacs were incubated for 24 and 48 hours; (2) the difference between rumen liquor and normal

Table 3. Synthesis of protein nitrogen by Ln. mesenteroides, incubated for 36 and 48 hours with or without addition of weights to bottles.

Sample Sac No.	Steer No.	Weight	Period of Incubation hrs.	Microorganisms Used	Treatments	Protein Nitrogen %	True Protein %
1		Without weight	36	Leuconostoc mesenteroides	Saline + starch (Blank)	0.037	0.231
2	3	" "	36	" "	Saline + starch + 1/2 ml. bacteria	0.069	0.431
3		" "	36	" "	R.L.[1] + starch (Blank)	0.387	2.419
4		" "	36	" "	R.L. + starch + 1/2 ml. bacteria	0.429	2.662
5		" "	48	" "	Saline + starch (Blank)	0.037	0.231
6	4	" "	48	" "	Saline + starch + 1/2 ml. bacteria	0.037	0.231
7		" "	48	" "	R.L. + starch (Blank)	0.408	2.550
8		" "	48	" "	R.L. + starch + 1/2 ml. bacteria	0.430	2.812
9		With weight	36	" "	Saline + starch (Blank)	0.032	0.197
10	3	" "	36	" "	Saline + starch + 1/2 ml. bacteria	0.032	0.197
11		" "	36	" "	R.L. + starch (Blank)	0.367	2.294
12		" "	36	" "	R.L. + starch + 1/2 ml. bacteria	0.375	2.344
13		" "	48	" "	Saline + starch (Blank)	0.032	0.197
14		" "	48	" "	Saline + starch + 1/2 ml. bacteria	0.032	0.197
15	4	" "	48	" "	R.L. + starch (Blank)	0.375	2.344
16		" "	48	" "	R.L. + starch + 1/2 ml. bacteria	0.391	2.444

saline as a source of medium for the synthesis of protein by the microorganisms.

Experimental Procedure. The experimental design with various treatments and the period of incubation of the sacs in the rumen are shown in Table 4.

Results and Discussion. There was a definite indication of greater protein synthesis by microorganisms when sterilized rumen liquor was used as media and the sacs incubated for 24 hours in the rumen. The protein synthesis by microorganisms was at a lower level when normal saline was used as media as compared to rumen liquor.

At 48 hours of incubation the synthesized protein was found to be less than at 24 hours. It was probable that the synthesized protein might have been decomposed. It might be possible that the metabolites that were formed due to the activity of the microbial population interferred with protein synthesis by the microorganisms. Or sufficient nutrients were not provided for the growth of the microorganisms.

Trial No. 5

The objective of this trial was to determine the synthesis of protein by the microorganisms when the sacs were incubated for 12, 24, 36 and 48 hours in the rumen of fistulated steers. Also to confirm the findings of the previous trials that sterilized rumen liquor was a better medium than normal saline for nourishment and growth of the microflora and microfauna of the rumen when in vivo artificial rumen technique was used.

Experimental Procedure. The experimental design, treatments to the dializing sacs, period of incubation and the results of the trial are shown

Table 4. Synthesis of protein by rumen microorganisms using rumen liquor or normal saline as media, incubated for 24 and 48 hours.

Sample Sac No.	Steer No.	Period of Incubation hrs.	Microorganisms Used	Treatments	Protein Nitrogen %	True Protein %
1	3	24	Fresh rumen liquor	R.L.[1] + starch (Blank)	0.205	1.274
2	3	24	" "	R.L. + starch + fresh R.L. one ml.	0.305	1.906
3	3	24	" "	R.L. + starch + fresh R.L. one ml.	0.305	1.906
4	3	24	" "	Saline + starch (Blank)	0.079	0.494
5	3	24	" "	Saline + starch + fresh R.L. one ml.	0.147	0.919
6	3	24	" "	Saline + starch + fresh R.L. one ml.	0.144	0.900
7	4	48	" "	R.L. + starch (Blank)	0.157	0.982
8	4	48	" "	R.L. + starch + fresh R.L. one ml.	0.210	1.313
9	4	48	" "	R.L. + starch + fresh R.L. one ml.	0.206	1.297
10	4	48	" "	Saline + starch	0.070	0.437
11	4	48	" "	Saline + starch + fresh R.L. one ml.	0.112	0.700
12	4	48	" "	Saline + starch + fresh R.L. one ml.	0.110	0.688

[1]R.L.— sterilized rumen liquor

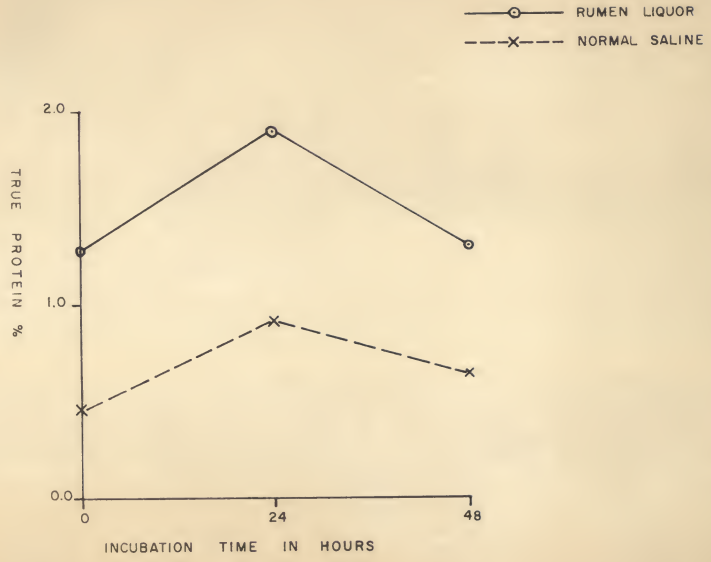

FIG. 3 PROTEIN SYNTHESIZED BY MICROORGANISMS WITH RUMEN LIQUOR OR NORMAL SALINE AS MEDIA, INCUBATED FOR 24 AND 48 HOURS.

in Table 5.

Results and Discussion. The results reveal protein synthesis by the microorganism was greatest when the sacs were incubated for twelve hours in the rumen of a fistulated steer. Protein was present at a lower level when sacs were incubated for 24 hours. After this period the protein nitrogen decreased and was minimum at 48 hours incubation of the sacs. It might be possible that the protein gradually decreased from 12 to 48 hours of incubation, due to degradation. Probably also because of lack of sufficient nutrients available to the microorganisms as a result of accumulation of metabolites in the sacs. It might be possible that the rate of diffusion inward and outward might not be sufficient and therefore these waste products were not removed at a sufficient rate to permit microbial activity.

Similarly a low level of synthesis of protein was observed in normal saline media, which decreased at 24 hours of incubation and remained stationary up to 48 hours of incubation.

The results confirmed the findings of previous trials that sterilized rumen liquor was a better medium than sterilized normal saline for growth and nutrition of the rumen microorganisms.

The results also show that in vivo artificial rumen technique, using semipermeable membrane, cannot be used with success when protein synthesis by microorganisms is to be studied for more than 12 hours at a time.

PART II

As the technique of the first part of the experiment could not be worked out satisfactorily, it was decided to work out a technique of studying rumenology by obtaining representative samples of the rumen

Table 5. Synthesis of protein by rumen microorganisms using rumen liquor or normal saline as media, incubated for 12, 24, 36 and 48 hours.

Sample Sac No.	Steer No.	Period of Incubation hrs.	Microorganism Used	Treatments	Protein Nitrogen %	True Protein %
1	4	12	Fresh rumen liquor	Saline + starch + 1 ml. fresh R.L.[1]	0.252	1.590
2	4	12	" "	Saline + starch (Blank)	0.057	0.356
3	4	12	" "	R.L. + starch + 1 ml. fresh R.L.	0.556	3.475
4	4	12	" "	R.L. + starch (Blank)	0.320	2.000
5	3	24	" "	Saline + starch + 1 ml. fresh R.L.	0.131	0.822
6	3	24	" "	Saline + starch (Blank)	0.073	0.456
7	3	24	" "	R.L. + starch + 1 ml. fresh R.L.	0.488	3.150
8	3	24	" "	R.L. + starch (Blank)	0.315	1.969
9	2	36	" "	Saline + starch + 1 ml. fresh R.L.	0.152	0.950
10	2	36	" "	Saline + starch (Blank)	0.068	0.425
11	2	36	" "	R.L. + starch + 1 ml. fresh R.L.	0.341	2.131
12	2	36	" "	R.L. + starch (Blank)	0.278	1.738
13	1	48	" "	Saline + starch + 1 ml. fresh R.L.	0.152	0.950
14	1	48	" "	Saline + starch (Blank)	0.070	0.437
15	1	48	" "	R.L. + starch + 1 ml. fresh R.L.	0.210	1.313
16	1	48	" "	R.L. + starch (Blank)	0.206	1.297

[1] R.L.--sterilized rumen liquor

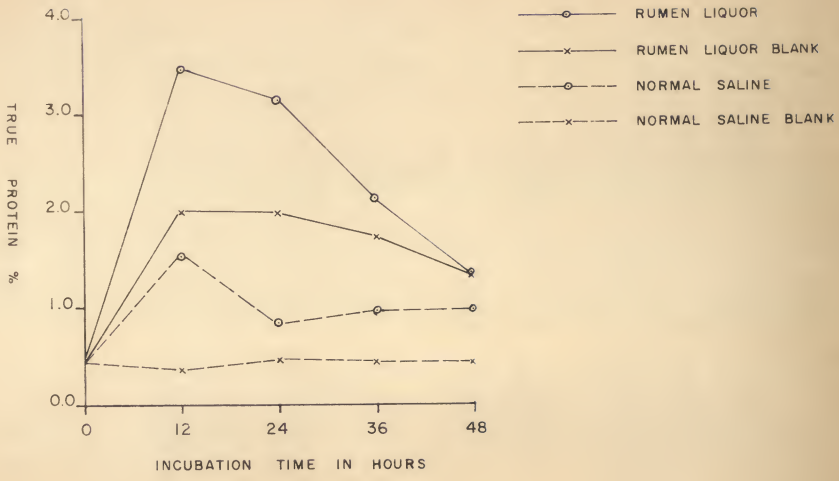

FIG. 4 SYNTHESIS OF PROTEIN BY MICROORGANISMS WITH RUMEN LIQUOR OR NORMAL SALINE AS MEDIA, INCUBATED FOR 12, 24, 36, AND 48 HOURS

contents from fistulated steers.

Review of Literature

The sampling of material from a fistulated animal is a matter of some complexity owing to the fact that even when food has been in the rumen for some time the rumen contents are by no means homogenous (Barnett and Reid, 1961). This difficulty is especially encountered when food has been given a short time before a sample is taken.

Smith et al. (1956) made an examination of the ingesta of fistulated steers at different levels in the rumen. Analysis showed that the content of fibre, total nitrogen, sugars and volatile fatty acids was higher at the top of the rumen, while ether extract, pH and capacity to digest cellulose were higher in the lower levels.

Fistulation appears to have little effect on the normal digestion processes of the ruminant. In comparative trials with fistulated and unfistulated lambs maintained on different forages over an extended period of time it has been found that no significant differences are apparent between animals with respect to digestibility coefficients for dry matter, crude fibre, cellulose, energy and crude protein (Reid et al., 1959).

In clear recognition of sampling difficulties, some workers remove the total rumen ingesta through the fistula and after taking a representative sample, replace the main bulk of the material in the rumen.

Nicols (1955) has described a sampling apparatus for removing material from the rumen. This consists essentially of two tubes, one of which slides into another. The tubes are rotated so that slots at the base of each tube lie opposite each other, thereby closing the opening. The apparatus is then

introduced to any desired area of the rumen, the inner tube turned until the slots coincide and, when sufficient rumen fluid has been collected, the tube is again rotated and the sample withdrawn.

Using the "complete removal" method, Hale et al. (1947a) demonstrated its reproducibility in two experiments with the same animals. Employing ordinary digestibility data based on total nutrients fed and total material excreted, these workers were able to obtain values for the percentage of digestion taking place in the rumen.

Agrawala et al. (1953) have used fistulated animals and the technique of complete removal of rumen contents to demonstrate the in vivo synthesis of protein from non-protein nitrogen.

Due to sampling difficulties, in an attempt to overcome these difficulties, different workers have used certain devices to study biochemical change in the rumen of fistulated animals by suspending samples in the ruminal mass itself. Quin (1943) described a means of investigating the rate of disintegration of test substances by enclosing them in a cloth bag and withdrawing the bag for examination at given intervals of time after immersion. A very similar procedure was used by Balch and Johnson (1950) to determine the site of optimum cellulolytic activity in the rumen. Belasco et al. (1958) in evaluating the digestibility of roughages following nitrogen application and Fina et al. (1958) have used a porcelain tube, connected to a gas escape device and immersed in the rumen to study different aspects of cellulose digestion.

Yadava (1962) in his studies on the rate of passage through the bovine rumen and the rumen metabolism of C-14 labelled roughage used two methods of study, i.e., in vivo artificial rumen technique and taking samples from

the fistulated steers. The rumen was completely emptied of all solid and liquid ingesta immediately before the morning feeding. The solid portion was removed with hands and the remaining liquid portion of the rumen ingesta was removed with a plastic beaker. The rumen contents were weighed, thoroughly mixed, and a 400 g. sample was taken for analysis. The remaining ingesta were replaced in the rumen as soon as possible. It took approximately 20 minutes for the whole operation.

Hale et al. (1947a) using fistulated animals and removing the entire rumen contents for obtaining samples, have shown that during the first six hours after feeding predominant activity in the rumen is centered on the breakdown of protein and easily utilizable carbohydrates. Over the next 6-hour period cellulose breakdown is the major operation. After 12 hours, unless the animal has a further intake of food, ruminal digestion practically ceases.

General Methods and Procedure

Sampling Technique. Five hundred ml. beakers, pans, a fifteen gallon capacity tin tub and plastic buckets were weighed and labelled accordingly. The entire rumen contents of the fistulated steer were thoroughly mixed by inserting the hand in the rumen and stirring for 5 to 10 minutes. After ascertaining the homogenicity of the mixture of rumen contents, a 250 ml. beaker was introduced into the rumen to collect the sample. Care was taken to place the palm of the hand over the beaker so that no material entered. The rumen contents were stirred again and the palm of the hand released from the top of the beaker. The rumen contents entered the beaker, which was then removed from the rumen and emptied into a 500 ml. beaker. Three

such samples were obtained from anterior, middle and posterior portion of the rumen, stirring the rumen contents each time. The 500 ml. beakers with rumen contents were weighed. Then the entire rumen contents were emptied into a tub and buckets, stirred thoroughly for 3-5 minutes and samples were taken of the ingesta, stirring each time a sample was taken. All the samples with their containers were weighed.

After the samples were dried to a constant weight, the samples with the containers were weighed. The difference between the initial and the final weight gave the moisture content from which moisture percentage in the rumen contents was calculated. Moisture content of the samples obtained from inside the rumen and outside the rumen were compared.

Each dried sample obtained from inside and outside the rumen was ground in a Wiley mill and the material collected in respective bottles which were labelled previously. About two grams of representative sample was taken in duplicate of each sample and total nitrogen determined.

In later trials it was decided not to remove the entire rumen contents but only to obtain samples from anterior, central and posterior part of the rumen after the necessary preliminaries were completed.

Trial No. 1

The objective of this trial was to devise a sampling technique (1) obtain representative samples of rumen ingesta for analysis from inside and outside the rumen, (2) determine percentage of total nitrogen.

Experimental Procedure. The procedure that was followed to collect rumen ingesta samples is described in the general methods and procedure.

Results and Discussion. The results of the trial are shown in Table 6.

Table 6. Analysis of rumen ingesta obtained from inside and outside the rumen for comparison.

Sample No.	Steer No.	Total Nitrogen mgms.	Percent Nitrogen	Average Nitrogen %	Total Protein %	Moisture Content %
I	3	0.0376	2.099	2.101	13.13	87.21
I	3	0.0361	2.103			
1	3	0.0424	2.141	2.143		87.09
1	3	0.0386	2.145			
2	3	0.0363	2.091	2.093	13.35	86.23
2	3	0.0384	2.096			
3	3	0.0332	2.172	2.161		88.74
3	3	0.0431	2.151			

I - Sample from evacuated rumen contents.
1 - Sample from anterior portion of rumen.
2 - Sample from central portion of rumen.
3 - Sample from posterior portion of rumen.

There is no marked difference between the values of the samples. There is practically no difference between total protein values in the samples obtained from inside the rumen and outside the rumen. The results indicate that a representative sample can be obtained from the rumen ingesta of fistulated steers without emptying the entire rumen contents.

Trial No. 2

The objective of this trial was to (1) try the sampling technique with the other twin steer of the one used in the first trial, (2) obtain representative samples from inside and outside the rumen, of rumen ingesta for total nitrogen analysis and comparison.

Experimental Procedure. The exact procedure adopted to collect rumen samples is described in the general methods and procedure, Part II. As the rumen contents in the particular steer from which samples were to be taken appeared more solid than the rumen contents of the steer used in Trial No. 1, it was decided to stir for ten instead of five minutes as in Trial No. 1.

Results and Discussion. The results of the experiment are shown in Table 7. The results indicate that there is no marked difference between the total protein content of the rumen ingesta samples collected from anterior, central and posterior portion of the rumen.

There is a slight difference in the values of total protein of the samples collected from inside and outside the rumen. No marked difference was observed in the total protein content of the rumen ingesta from steer No. 3 as compared to the protein content of the rumen ingesta of steer No. 4, which are identical twins.

Trial No. 3

The object of the trial was to (1) verify the sampling technique using a steer from another identical twin pair, (2) determine the difference, if any, in nitrogen content of the samples collected from inside and outside the rumen, (3) determine the difference, if any, in nitrogen content of the samples collected from different locations in the rumen.

Experimental Procedure. The experimental procedure that was followed in collecting samples from the rumen was the same as stated in the general methods and procedure.

Results and Discussion. The results of the experiment are shown in Table 8. The results reveal that there is practically no difference in

Table 7. Analysis of samples obtained from inside and outside the rumen of steer No. 4 for total nitrogen.

Sample No.	Total Nitrogen mgms.	Percent Nitrogen	Average Nitrogen %	Total Protein %	Moisture Content %
IIa[1]	0.036	2.08			88.25
IIa	0.041	2.06			
IIc[2]	0.034	1.97	2.00	12.50	88.63
IIc[2]	0.032	2.00			
IIP[3]	0.033	1.96			88.79
IIP	0.028	1.93			
IIE[4]	0.042	1.95			86.79
IIE	0.040	1.90	1.90	11.88	
IIE	0.036	1.87			
IIE	0.022	1.88			86.53

[1]a = Sample from the anterior portion of the rumen.

[2]c = Sample from the central part of the rumen.

[3]P = Sample from the posterior portion of the rumen.

[4]E = Exterior samples from evacuated rumen contents.

percentage nitrogen in respective duplicate samples and in samples from different locations from the rumen. There is a slight difference in average total nitrogen and total protein in samples from inside and outside the rumen. It may be possible that there might have been some error in collecting samples.

Table 8. Percentage nitrogen and moisture of samples of rumen ingesta obtained from inside and outside the rumen of steer No. 2.

Sample No.	Total Nitrogen mgms.	Percent Nitrogen	Average Nitrogen %	Total Protein %	Moisture Content %
III[1]	0.040	2.02			92.00
III	0.045	2.05			
III[2]	0.046	2.08	2.05	12.82	92.33
III	0.029	2.08			
III[3]	0.022	2.03			92.33
III	0.027	2.03			
III[4]	0.0357	1.58	1.98	12.38	92.32
III	0.034	1.97			

[1]Anterior portion of rumen.

[2]Central portion of rumen.

[3]Posterior portion of rumen.

[4]From emptied rumen contents.

Trial No. 4

The objective of this trial was to reconfirm the sampling technique with a steer which was identical twin to steer used in Trial No. 3.

Experimental Procedure. The experimental procedure was same as given in general methods and procedure, Part II.

Results and Discussion. The results of the trial are shown in Table 9. It is clear from the table uniform results were obtained from the samples collected from various locations from inside the rumen. The total protein content of the rumen ingesta samples in case of steer No. 1 was higher than the total protein content of the rumen ingesta of steer No. 2 in Trial No. 3. Individuality factors may be responsible for this.

Table 9. Analysis of samples of rumen ingesta obtained from inside and outside the rumen of steer No. 1 for nitrogen and moisture percent.

Sample No.	Total Nitrogen mgms.	Percent Nitrogen	Average Nitrogen %	Total Protein %	Moisture Content %
IV[1]	0.040	2.41			91.97
IV	0.044	2.42			
IV[2]	0.043	2.38	2.41	15.06	91.24
IV	0.044	2.38			
IV[3]	0.066	2.44			92.05
IV	0.044	2.41			
IV[4]	0.046	2.28	2.27	14.19	91.97
IV	0.040	2.26			

[1]Samples from the anterior portion of the rumen.

[2]Samples from the posterior portion of the rumen.

[3]Samples from the central portion of the rumen.

[4]Samples from the exterior of evacuated rumen contents.

Trial No. 5

The objective of this trial was to (1) find out whether grinding the dried rumen ingesta to a coarse or a fine grind had any effect on determination of protein nitrogen, (2) find out the difference in precipitation of the protein by 15 percent and 57 percent trichloroacetic acid.

Experimental Procedure. The experimental procedure followed in this trial was similar to Trials 3 and 4 except that no outside samples were collected as the rumen contents were not evacuated.

The samples were dried in the oven and ground in a Wiley mill. Half of each sample was ground to a coarse form, 2 mm mesh, and the other half to a fine grind, 1 mm mesh. Approximately two grams of each sample, in

duplicate, was treated with 15 percent and 57 percent trichloroacetic acid. The samples were allowed to precipitate for 24 hours on an electric shaker. The precipitate was collected in Kjeldahl digestion flasks after centrifugation and filtration.

Results and Discussion. The results of the experiment are presented in Table 10. They indicate that coarse grinding and fine grinding of the rumen ingesta does not make any difference in protein nitrogen values.

There was a difference in average percent nitrogen values when the protein of the rumen ingesta was precipitated with 15 percent and 57 percent trichloroacetic acid. The values were lower with 57 percent trichloroacetic acid precipitation. It may be possible that the protein nitrogen deteriorated when precipitated with a higher concentration of Trichloroacetic acid.

Trial No. 6

The objective of this trial was to analyze wet samples of rumen ingesta, and to determine the difference, if any, in average total nitrogen between the wet samples and the dried and ground samples of the previous trials, also to determine the uniformity of sampling technique.

Experimental Procedure. The rumen ingesta samples were collected in beakers as per usual procedure. The samples collected from anterior, central and posterior portion of the rumen ingesta in the rumen were then kept in a deep freeze until the next morning so that no moisture from the samples was lost. Each of these samples were thoroughly mixed with a glass rod, until it became homogenous. About 15 to 20 grams of each sample in duplicate was taken for nitrogen determination.

Results and Discussion. The results of the experiment are presented

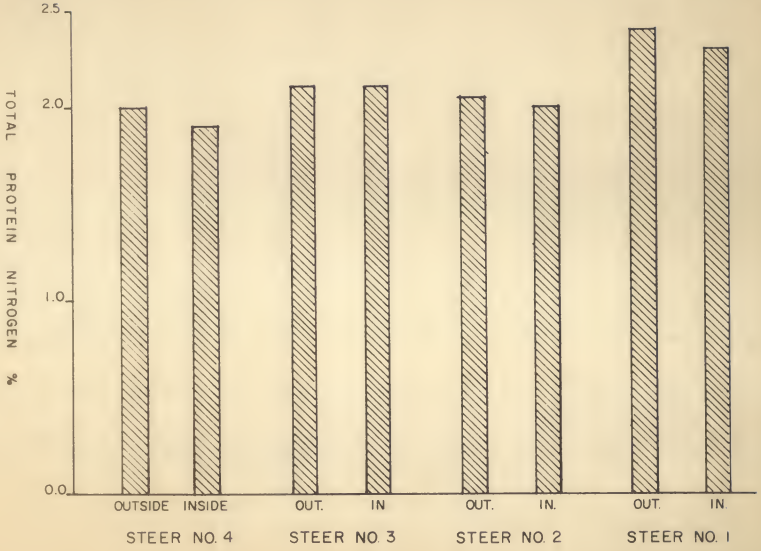

FIG. 5 COMPARISON OF SAMPLES FROM INSIDE AND OUTSIDE THE RUMEN OF FOUR FISTULATED, IDENTICAL TWIN PAIR STEERS FOR PROTEIN NITROGEN.

Table 10. Difference between coarse grind and fine grind rumen ingesta precipitated by 15 percent and 57 percent trichloroacetic acid in nitrogen content.

Sample No.	Type of grind	Steer No.	Percent Trichloroacetic acid used	Sample size gms.	Protein Nitrogen mgms.	Protein Nitrogen %	Average Protein Nitrogen %	True Protein %
Ia	Coarse	3	15	1.0590	0.017	1.61	1.635	10.25
Ia	"	3		1.2080	0.020	1.66		
Ia	"	3	57	0.8251	0.013	1.58	1.565	9.81
Ia	"	3		1.1621	0.018	1.55		
Ia	Fine	3	15	1.7313	0.028	1.62	1.625	10.19
Ia	"	3		1.7210	0.028	1.63		
Ia	"	3	57	1.5403	0.024	1.56	1.560	9.75
Ia	"	3		1.4138	0.022	1.56		

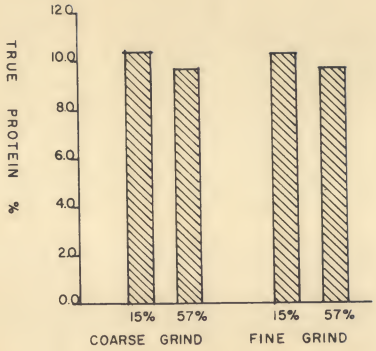

FIG. 6 COMPARISON OF TRUE PROTEIN VALUES OF COARSE AND FINE GRINDING OF THE RUMEN INGESTA FROM STEER NO. 4 PRECIPITATION WITH 15% AND 57% TRICHLORACETIC ACID.

in Table 11. These results reveal that the total protein values obtained with wet samples are slightly lower than those obtained from dried and ground samples in Trial No. 2. Comparison is shown in Fig. 7.

The values of total nitrogen obtained from anterior and posterior portion of the rumen ingesta from inside the rumen are similar but the value of total nitrogen from the central sample is slightly higher.

Table 11. Analysis of wet samples of rumen ingesta collected from anterior, posterior, and central portion of the rumen of fistulated steer.

Sample No.	Steer No.	Sample size gms.	Total Nitrogen mgms.	Total Nitrogen %	Average Nitrogen %	Mean Nitrogen %	Total Protein %	Total Protein % from Trial 2
S4A	4	19.6	0.265	1.656	1.643			
S4A	4	20.3	0.261	1.631				
S4C	4	17.4	0.276	1.725		1.67	10.44	12.50
S4C	4	20.2	0.272	1.700	1.712			
S4P	4	14.4	0.264	1.650				
S4P	4	15.5	0.264	1.650	1.650			

Trial No. 7

The object of this trial was to determine the true protein values when the wet samples were precipitated with 15 percent trichloroacetic acid.

Experimental Procedure. The rumen ingesta samples were collected in beakers as per usual procedure. The samples were kept in deep freeze until the next morning. Then these samples were thoroughly mixed with a glass rod for some time till each of the samples became homogeneous. Separate glass rods were used for each sample. Approximately 10 to 15 grams of each sample

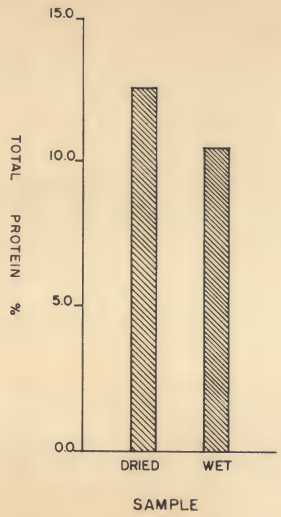

FIG.7 COMPARISON BETWEEN DRY AND WET SAMPLES FROM STEER NO. 4, FOR TOTAL PROTEIN.

were taken for protein nitrogen determination.

Results and Discussion. The results of the trial are presented in Table 12. They indicate that the values of true protein nitrogen of the wet samples from different locations of the rumen are practically uniform.

Table 12. Rumen ingesta wet samples precipitated with 15 percent trichloroacetic acid. Protein nitrogen values.

Sample No.	Steer No.	Sample size gms.	Total Protein Nitrogen:	Protein Nitrogen %	Average Protein Nitrogen: %	Mean Protein Nitrogen: %	True Protein %
ShA	4	9.3	0.226	1.415	1.426		
ShA	4	8.7	0.230	1.438			
ShC	4	11.1	0.239	1.494	1.500	1.46	9.13
ShC	4	11.8	0.241	1.506			
ShP	4	11.0	0.236	1.475	1.440		
ShP	4	12.1	0.225	1.406			

GENERAL DISCUSSION

Part I

The purpose of these studies was to learn the technique of studying protein synthesis by the microbial population of the rumen. Dializing sacs were used to study an in vivo artificial rumen technique devised by Regier (1961). It was observed that with 48 hours incubation of the sacs in the rumen of fistulated steers the sacs remained intact but they were ballooned and enlarged due to the accumulation of gases. This finding of the sacs remaining intact is in close agreement with the findings of Louw et al. (1949)

who devised the in vitro artificial rumen technique making use of dializing tubing. They claimed after examining the tubing under microscope that the microorganisms did not attack the visking casing. They further stated that the failure of microorganisms to attack the casing may be explained on the basis that attack on the cellulosic materials usually begins at broken or torn ends and no such surfaces are exposed when the tubing is made into a closed sac.

The dializing sac which was used as an artificial rumen, when incubated in the rumen, remained as a separate entity deriving the nutrients from the rumen and diffusing out the metabolites which were formed due to activity of the microorganisms in the sac. The objects of the study were to find out how long the dializing sac could be used as an artificial rumen successfully when incubated in the rumen for long periods and whether protein synthesis by the microorganisms would continue for such periods under the conditions found in the sacs.

Warner (1956a) lists a suitable temperature as one of the criteria for normal artificial rumen function. Since the sacs were incubated in the rumen itself, it is obvious that the temperature of the sac should be the same as that of the rumen.

Warner (1956a) also lists a suitable pH value as one of the necessary criteria for normal functioning of an artificial rumen. In the studies undertaken, it is probable that the favorable pH was maintained in the sac for microbial synthesis of protein when incubated for periods up to 12 hours. It is doubtful whether such favorable conditions were maintained when the sacs were incubated for 48 hours in the rumen. The accumulation of gases and metabolites in the sacs would change the pH of the medium. The results

reveal so, because synthesis of protein by the microbial population in the sac decreased with longer incubation period.

With the incubation of the sacs for 36 and 48 hours, it was doubtful whether the gas phase and the provision for removal of metabolites, the criteria stated by Warner (1956a), were met. The results indicate so. The protein nitrogen values should have been the same as those obtained with 12 and 24 hours incubation of the sacs. It is, therefore, doubtful whether the technique that was devised by Regier (1961) can be used for the study of protein synthesis by the microbial population in sacs for more than 12 hours.

From the results of Trials 2 and 3, it appears that the technique could be used to study behavior of pure cultures as to synthesis of protein when inoculated in the sacs and incubated in the rumen. It was presumed that the nutrients would diffuse from the rumen contents into the sacs in sufficient quantities so as to promote protein synthesis and microbial growth. The two cultures of bacteria used in Trial 2 were Leuconostoc mesenteroides and Streptococcus faecalis. In Trial 3, Leuconostoc mesenteroides was used. There was practically no difference found in protein synthesis by these two species of bacteria when incubated for 24 hours in the rumen of identical twin fistulated steers.

There is no doubt that the rumen microorganisms can thrive and function actively in the sac when incubated in the rumen up to 24 hours but after that period the activity decreases considerably. As Regier (1961) pointed out, it is still not known whether the nutrients diffused into the sacs support the growth of all rumen populations or a part of the microbial population. Whether the nutrients promote all the activities of all the populations or

some activities of some populations, is yet to be known. Further work in this direction will probably clear up the obstacles in the understanding of this problem. It might also be possible that the nutrients from the sacs might have diffused out into the surrounding medium of the rumen ingesta or the products of metabolism due to the activity of the microorganisms accumulated in the sacs which brought about a static condition of the microorganisms when incubated for 36 and 48 hours. It can also be reasoned that the decrease in protein nitrogen might be due to breakdown of the protein and its deterioration, when the sacs were incubated for longer periods, under which conditions sufficient nutrients were not available for microbial growth.

Of interest is the fact that there is appreciably more protein nitrogen in the sacs containing sterilized rumen liquor as media than those sacs containing normal saline. This finding is according to the expectations. It is possible that the microorganisms get a readily available source of nutrients and energy for growth and activity. It appears that some time lapsed before the nutrients entered the sacs containing normal saline or sufficient nutrients did not diffuse in the sacs from the surrounding rumen ingesta so as to bring about growth and activity of the organisms.

In the early trials it was noticed that the bottles containing sacs, which were put in the rumen of fistulated steers, remained on top of the ingesta in the rumen. It was thought that the microorganisms in the sacs were not getting sufficient nutrients from the solid rumen ingesta. In order that the bottles remain in liquid portion of the ingesta, heavy iron pieces were tied to the bottles before they were introduced in the rumen for incubation. A comparison of protein nitrogen values was made between sacs

contained in bottles which had weights attached and those without weights. Results in Table 3 reveal that no practical difference was observed between the two groups.

The possible inconsistency in the results as seen from Tables 1 to 5 might be due to inadequate removal of the metabolites formed and accumulated in the sacs as a result of microbial activity. Unsuitable gas phase and imbalance between the minerals and other nutrients may also be contributory factors. It might also be possible that the number of microorganisms in 1 ml. rumen liquor or 1/2 ml. culture were not sufficient to utilize nutrients from the sacs for maximum growth and activity.

Part II

As the combination of artificial rumen, in vivo technique did not work satisfactorily, it was decided to switch to the study of rumenology in the rumen of fistulated steers. In the first instance, it was decided to establish a sampling technique to obtain representative samples of the rumen ingesta for analysis.

The rumen may contain up to 200 lbs. of contents and, the material is much drier near the esophageal end than at the omasal end. Balch (1950) has also stated that the contents of the ventral sac are always more moist than those of the dorsal sac. On the basis of dry matter composition, the contents vary at different depths in the rumen. It is clear therefore, that sampling of rumen contents may mean anything and in the realization of potential errors in the procedure some workers have resorted to removal of the total rumen contents mixing them, taking an aliquot and replacing the rest.

In view of the difficulties encountered by the previous workers, an attempt was made to establish a sampling technique which could be used in in vivo studies.

Samples of rumen ingesta were obtained from four fistulated identical twin pairs for determination of total nitrogen or protein nitrogen as per general methods and procedure Part II.

In Trial 1 and 2, comparison was made between the total nitrogen content of samples obtained from inside and outside the rumen. The results were nearly uniform. The average total nitrogen content of samples from steer No. 3, was 2.14 mgms. per cent which in steer No. 4, was 1.95 mgms. per cent.

The total protein content of the rumen ingesta from steer No. 3 was slightly higher as compared to the total protein content of the rumen ingesta from steer No. 4, which is his identical twin. Similarly the total protein content of the rumen ingesta samples from steer No. 1 were higher than that from steer No. 2 which is his identical twin.

All four steers were getting the same ration and the same quantity. In all cases samples were taken from the rumen ingesta six hours after the morning feeding. Even with these identical conditions, differences in total protein content of the samples was observed. This might be due to difference in utilization of protein and non-protein nitrogen by these four steers. But in all the steers total protein values of samples of rumen ingesta obtained from different locations of the rumen and samples obtained after evacuating the rumen contents were uniform when considered on an individual steer basis. From the first four trials the results reveal that if proper mixing of the rumen ingesta is done, representative samples of the rumen

ingesta could be obtained from the rumen for the study of rumenology.

In Trial No. 5, an attempt was made to study the effect of coarse and fine grinding of the rumen ingesta on the protein nitrogen values when protein was precipitated with 15 per cent and 57 per cent trichloroacetic acid. It was observed that grinding of the samples to coarse or fine grind had no effect on the protein nitrogen values. But a slight difference was observed in protein nitrogen values when protein of the rumen ingesta samples was precipitated with 15 per cent and 57 per cent trichloroacetic acid. It might be possible that the higher concentration of trichloroacetic acid decomposed protein of the samples of rumen ingesta.

In Trials 6 and 7, instead of using dried and ground samples, wet samples were used for analysis. Difficulty was encountered in using wet samples to get representative samples from beakers for weighing. Even with all the necessary precautions and thorough mixing, analysis of samples obtained from different locations of the rumen as seen in Table 11, reveal slight differences in average total nitrogen content. The sample from the central portion gave slightly higher values.

In Trial No. 7, samples collected from different locations were precipitated with 15 percent trichloroacetic acid. The precipitate was analyzed for protein nitrogen. Results from Table 12 reveal that the average protein nitrogen content of the wet samples from different locations gave nearly uniform results. Moisture content of the samples obtained from inside the rumen and after evacuating the rumen and those obtained from identical twins did not show any difference. The moisture content of the samples from the rumen of steer No. 1 and 2 was higher than that of steer No. 3 and 4.

Thus from these trials on "sampling technique" it was observed that

when carefully used, the method will be of immense use to the research worker to study rumenology in vivo.

SUMMARY AND OBSERVATIONS

Part I

Further observations were made on the technique of studying protein synthesis by microorganisms in the rumen, devised by Regier (1961), to determine whether the technique can be used for longer duration experiments. This technique consists of the use of dializing tubing made into 7-8 inch length sacs which were incubated in the rumen of fistulated steers. The sacs were filled with sterilized normal saline or strained and sterilized rumen liquor. After being sterilized in the autoclave for 3-5 minutes and cooling, one gram of corn starch dissolved in 10 ml. distilled water was added to each sac. The sacs were then inoculated with fresh strained rumen liquor or bacterial suspension. After sealing the ends the sacs were incubated in the rumen. The selective permeability of the sacs allows diffusion in and out of nutrients and removal of metabolites from the sacs. At the end of incubation the contents of the sacs were removed and nitrogen determinations made.

The sacs remained intact and sterile up to 48 hours incubation in spite of agitation and stirring in the rumen. The possibility of pure culture studies is thus advanced.

Addition of weights to bottles in which sacs were introduced and incubated did not reveal any marked difference in protein nitrogen as compared to those without weights.

Trials 1 to 5, were conducted to study protein synthesis by bacteria or ruminal microorganisms inoculated into the sacs incubated in vivo and to determine whether conditions were favorable for microbial growth within this environment. As regards protein synthesis, the results of these five trials are not consistent and uniform. Therefore no definite conclusions can be drawn. However it was observed that the protein nitrogen values of the contents of the sacs decreased as the incubation time was increased from 12 hours to 48 hours. There was ballooning of the sacs due to accumulation of gases when incubated for 48 hours.

The results of all five trials gave an indication that rumen liquor was a better source of media than normal saline. Rumen liquor appears to supply nutrients for immediate use by the microorganisms.

It appears that the artificial rumen in vivo technique cannot be used for longer duration experiments because the protein nitrogen values decreased and the metabolic products formed due to microbial activity were not removed at a sufficient rate so as to promote microbial growth.

Part II

Due to inconsistent results obtained in Part I of the experiment, a sampling technique to obtain representative samples of rumen ingesta was studied.

The rumen ingesta of the fistulated steers was stirred by hand thoroughly for 5-10 minutes. Samples were taken from anterior central and posterior part of the rumen. The entire rumen contents were evacuated in a tub and representative samples were obtained. The samples were dried in oven and ground in Wiley mill. Total nitrogen determinations were made

using Kjeldahl apparatus.

Analysis of rumen samples obtained from inside and outside the rumen for total nitrogen in the first four trials revealed that there were differences in the total nitrogen values in the samples from the four fistulated steers. Even in identical twin steers the sample values for total nitrogen differed slightly. But in the same steer total nitrogen of the samples from inside and outside the rumen as well as in duplicate samples was uniform and did not show marked difference.

Coarse grinding and fine grinding did not affect the true protein values of samples in Trial 5, when precipitated with 15 per cent and 57 per cent trichloroacetic acid. Fifty-seven per cent trichloroacetic acid appeared to decompose protein as it gave slightly lower values than 15 per cent trichloroacetic acid precipitation.

In Trials 6 and 7, instead of ground dry samples, wet samples from different locations of the rumen were analyzed for total protein in Trial 6 and for true protein in Trial 7. Difficulty was encountered in obtaining representative samples for weighing.

A method of sampling technique has been devised. It is felt that this method would be of immense use in the study of rumenology in vivo.

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude and indebtedness to his Major Professor, Dr. Draytford Richardson, for his encouragement, patient supervision and guidance throughout the course of study, experimental work, and preparation of this manuscript.

Gratitude is likewise expressed to Dr. W. S. Tsien for his guidance and assistance in carrying out the laboratory work.

The author is indebted to Dr. Rufus F. Cox, Head of the Department for providing facilities in carrying out this investigation.

The author wishes to express his deep sense of appreciation to his colleague Mr. Muhammad Akram of Pakistan, for his timely help in carrying out this investigation. Appreciation is likewise expressed to Mr. Daniel Peters for his help.

The author is also indebted to Dr. Indrajit Yadava for his valuable suggestions and to Dr. J. M. Bowen for demonstration of fistulation technique in sheep and goats.

LITERATURE CITED

Adler, J., J. A. Dye, D. E. Boggs, and R. H. Williams. 1958.
Growth of rumen microorganisms in an in vitro continuous-flow system on a protein-free diet. The Cornell Vet., 48:53.

Agrawala, I. P. 1951.
Comparative study of the synthesis of the ten essential amino acids and riboflavin, niacin and pantothanic acid in the rumen of cattle on normal and purified diets. Mich. Uni. Microfilm Abstr., 11:481.

Agrawala, I. P., C. W. Duncan, and C. F. Huffman. 1953.
A quantitative study of rumen synthesis in the bovine on natural and purified rations. I. Protein, dry matter and non-protein nitrogen. J. Nutri., 49:29.

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

Alstad, George W. 1958.
Rumen microbiology. Agradata, 2:1. Chas. Pfizer and Co., Inc.

Annison, E. F., M. I. Chalmers, and R. L. M. Synge. 1954.
Ruminal ammonia formation in relation to the protein requirement of sheep. III. Ruminal ammonia formation with various diets. J. Agri. Sc., 44:270.

Annison, E. F., K. J. Hill, and D. Lewis. 1955.
Portal blood analysis in the study of the absorption of ruminal fermentation products. Biochem. J., 60:XIX.

Annison, E. F. 1956.
Nitrogen metabolism in the sheep. Protein digestion in the rumen. Biochem. J., 64:703.

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

Archibald, J. G. 1943.
Feeding urea to dairy cows. Massachusetts Agr. Expt. Sta. Bull. 406.

Arias, C., W. Burroughs, P. Gerlaughh, R. M. Bethke. 1951.
The influence of different amounts and sources of energy upon in vitro urea utilization by rumen microorganisms. J. An. Sc., 10:683.

Baker, F. 1942.
Normal rumen microflora and microfauna of cattle. Nature, 149:220.

Baker, F. 1942.
Microbial synthesis and autolysis in the digestive tract of Herbivora. Nature, 149:582.

Baker, F. 1943.
Direct microscopical observations upon the rumen population of the ox. I. Qualitative characteristics of the rumen population. Annals. Appt. Biol., 30:230.

Baker, F. 1946.
The rumen process as a fundamental field. An attempt at synthesis. Nature, 158:609.

Balch, C. C., and B. C. Johnson. 1950.
Factors affecting the utilization of food by dairy cows. I. The rate of passage of food through the digestive tract. Brit. J. Nutr., 4:361.

Barnett, A. J. G., and R. L. Reid. 1961.
Reactions in the rumen. London: Edward Arnold (Publishers) Ltd.

Bartlett, S., and K. L. Blaxter. 1947.
The value of urea as a substitute for protein in the rations of dairy cattle. I. Field trials with dairy cows. J. Agr. Sc., 37:32.

Belasco, I. J. 1954.
New nitrogen feed compounds for ruminants--a laboratory evaluation. J. Ani. Sci., 13:601.

Belasco, I. J., M. F. Gribbius, and D. W. Kolterman. 1958.
The response of rumen microorganisms to pasture grasses and prickly pear cattus following foliar application of urea. J. Ani. Sc., 17:209.

Benesch, R. 1941.
Chemical substitutes for dietary protein. Nature, 147:531.

Bentley, O. G., R. R. Johnson, S. Vaneko, and C. H. Hunt. 1954.
Studies on factors needed by rumen microorganisms for cellulose digestion in vitro. J. Ani. Sc., 13:581.

Bryant, M. P. 1959.
Bacterial species of the rumen. Bact. Reviews., 23:125.

Bryant, M. P., and L. A. Burkey. 1953.
Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. J. Dairy Sc., 36:205.

Bryant, M. P., Nola Small, Cecilia Bauna and Isadore Robinson. 1958.
Studies on the composition of the ruminal flora and fauna of young calves. J. Dairy Sc., 41:1747.

Burroughs, W., and Paul Gerlaugh. 1949a.
The influence of SBOM. upon roughage digestion in cattle. J. Ani. Sc., 8:1.

Burroughs, W., Paul Gerlaugh, B. H. Edgington, and R. M. Bethke. 1949b.
Further observations on the effects of protein upon roughage digestion in cattle. J. Ani. Sc., 8:9.

Burroughs, W., Paul Gerlaugh, B. H. Edgington, and R. M. Bethke. 1949c.
The influence of corn starch upon roughage digestion in cattle. J. Ani. Sci., 8:433.

Burroughs, W., C. Arias, P. Gerlaugh, and R. M. Bethke. 1950.
The use of artificial rumen in studying urea utilization by micro-organisms taken from the paunch of cattle (Abs.). J. Ani. Sc., 9:650.

Burroughs, W., L. S. Gall, P. Gerlaugh, and R. M. Bethke. 1950.
The influence of casein upon roughage digestion in cattle with rumen bacterial studies. J. Animal Sc., 9:214.

Burroughs, W., A. Latona, P. DePaul, P. Gerlaugh, and R. M. Bethke. 1951.
Mineral influence upon urea utilization and cellulose digestion by rumen microorganisms using the artificial rumen technique. J. Ani. Sc., 10:693.

Campbell, T. C., R. C. Warner, and J. K. Loosli. 1960.
Urea and biuret for ruminants. Proc. Cornell Nutr. Conf. 1960.

Chalmers, M. I., D. P. Cuthertson, and R. L. M. Synge. 1954.
Ruminal ammonia formation in relation to the protein requirement of sheep. J. Agri. Sc., 44:254.

Chalmers, M. I., and R. L. M. Synge. 1954.
Protein digestion in ruminants. Advances in protein chemistry. 9:98.

Chance, C. M., C. K. Smith, C. F. Huffman, and C. W. Duncan. 1953.
Antibiotics in rumen digestion and synthesis. III. Effect of aureomycin on rumen microorganisms with special reference to the streptococci and coliform groups. J. Dairy Sc., 6:743.

Davey, L. A., and C. A. E. Briggs. 1961.
Descriptive microbiology of the rumen. Digestive Physiology and Nutrition of the Ruminant. London:Butterworths. 1961.

Doetsch, R. N., R. O. Robinson, R. E. Brown, and J. C. Shaw. 1953.
Catabolic reactions of mixed suspensions of bovine rumen bacteria. J. Dairy Sc., 36:825.

Doetsch, R. N., and R. Q. Robinson. 1953.
The bacteriology of the bovine rumen. A review. J. Dairy Sc., 36:115.

Elsden, S. R. 1952.
A discussion on symbiosis involving microorganisms. Proc. Royal Soc., 13:139-193.

Elsden, S. R., and A. T. Phillipson. 1948.
Ruminant digestion. Ann. Rev. Biochem. 17:705.

ElShazly, K. 1952.
Degradation of protein in the rumen of the sheep. 1. Some volatile fatty acids including branched-chain isomers, found in vivo. Biochem. J., 51:640.

Fina, Lewis R., G. W. Teresa, and E. E. Bartley. 1958.
An artificial rumen technique for studying rumen digestion in vivo. J. Ani. Sc., 17:667.

Gall, L. S., W. Burroughs, P. Gerlaugh, and B. H. Edgington. 1949.
Rumen bacteria in cattle and sheep on practical farm rations. J. Ani. Sc., 8:441.

Gall, L. S., and W. L. Glaws. 1951.
The bacteriology of the artificial rumen. Bact. Proc., 20.

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

Goss, H. 1943.
Some peculiarities of ruminant nutrition. Nutr. Abst. and Rev., 12:531.

Gray, F. V., A. F. Pilgrim, and R. A. Weller. 1953.
Conversion of plant nitrogen to microbial nitrogen in the rumen of the sheep. Nature, 172:347.

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

Hale, E. B., C. W. Duncan, and C. F. Huffman. 1947a.
Rumen digestion studies. I. A method of investigating chemical changes in the rumen. J. Nutri., 34:733.

Hamilton, T. S., W. B. Robinson, and B. Connor Johnson. 1948.
Further comparisons of utilization of nitrogen of urea with that of some feed proteins by sheep. J. Ani. Sc., 7-8:26.

Hart, E. B., G. Bohstedt, H. G. Deobald, and M. I. Wegner. 1939.
The utilization of simple nitrogenous compounds such as urea and ammonium bicarbonate by growing calves. J. Dairy Sc., 22:785.

Hastings, E. G. 1944.
The significance of bacteria and protozoa of the rumen of the bovine. Bact. Rev., 8:235.

Heald, P. J., N. Krogh, S. O. Mann, J. C. Appleby. 1953.
Fermentation of soluble sugars by anaerobic holotrich ciliate protozoa of the general Isotricha and Dasytricha. J. Gen. Microbiol., 9:207.

Hershberger, T. V., T. A. Long, E. W. Harsook, and R. W. Swift. 1958.
Use of the artificial rumen technique to estimate the nutritive value of forages. J. Ani. Sc., 17:1189.

Hobson, P. N. 1959.
Nitrogen metabolism in the rumen with information regarding the organisms concerned. Oklahoma Conf. Radioisotopes in Agr. U. S. Atomic Energy Commission. April 1959, 203.

Hoflund, S., J. F. Quin, and R. Clark. 1948.
Studied on the alimentary tract of Marino sheep in South Africa. XV. The influence of different factors on the rate of cellulose digestion, (a) in the rumen (b) in the ruminal ingesta studied in vitro. Onderstepoort J. Vet. Sc. Ani. Ind., 23:367.

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

Huhtanen, C. N., and L. S. Gall. 1952.
The miniature artificial rumen and its uses. J. Agri. Sc., 11:766.

Huhtanen, C. N., M. R. Rogers, and L. S. Gall. 1952.
Improved technique for isolating and purifying rumen organisms. J. Bact., 65:548.

Hungate, R. E. 1957.
Microorganisms in the rumen of cattle fed a constant ration. Canadian J. Microbiol., 3:289.

Hungate, R. E., D. W. Flecher, R. W. Dougherty, and B. F. Borrentine. 1955.
Microbial activity in the bovine rumen; its measurement and relation to bloat, Appl. Microbiol., 3:161.

Johnson, B. C., T. S. Hamilton, H. H. Mitchell, and W. B. Robinson. 1942.
Efficiency of urea as a protein substitute in the ration of ruminants. J. Ani. Sci., 1:256.

Johnson, B. C., T. S. Hamilton, W. B. Robinson, and J. C. Carey. 1944.
On the mechanism of non-protein nitrogen utilization by ruminants. J. Ani. Sc., 3:287.

Jurtshuk, P., Jr., and F. G. Hueter. 1955.
In vitro studies on the dissimilation of purines and pyrimidines by bovine rumen bacteria. Maryland Agr. Expt. Sta., 238:4.

Lewis, D. 1951.
The reduction of nitrate by rumen bacteria. (Abs.) J. Gen. Micro., 4:XI.

Lewis, D. 1951.
The metabolism of nitrate and nitrate in the sheep. 2. Hydrogen donators in nitrate reduction by rumen microorganisms in vitro. Biochem. J., 49:149.

Lewis, D. 1955.
Amino acid metabolism in the rumen of the sheep. 1955. Brit. J. Nutr., 9:215.

Lewis, D. 1957.
Blood urea concentration in relation to protein utilization in the ruminant. J. Agr. Sc., 48:438.

Lewis, D. 1961.
Digestive physiology and nutrition of the ruminant. Proc. of the Uni. of Nothingham Seventh Easter School in Agri. Sc., 1960.

Lewis, D., and S. R. Elsden. 1955.
The fermentation of L. thuconine, L. serine, L. cystein and acrylic acid by a grain negative coccus. Biochem. J., 60:683.

Lewis, D., I. W. McDonald. 1958.
The inter-relationship of individual proteins and carbohydrates during fermentation in the rumen of the sheep. I. The fermentation of casein in the presence of starch or other carbohydrate material.

Loosli, J. K., 1952.
Meeting the sulphur requirements of ruminants. Feed Age 2:44.

Loosli, J. K., H. H. Williams, W. E. Thomas, F. H. Ferris, and L. A. Maynard. 1949.
Synthesis of amino acids in the rumen. Science, 110:144.

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.

Marston, H. R. 1939.
Ruminant Nutrition. Ann. Rev. Biochem., 8:557.

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

McCarthy, R. D. 1962.
The importance of rumen metabolism in relation to milk composition. J. Agri. and Food Chemistry. April:126.

McDonald, I. W. 1948.
The absorption of ammonia from the rumen of the sheep. Biochem. J., 42:584.

McDonald, I. W. 1952.
The role of ammonia in ruminal digestion of protein. Biochem. J., 51:86.

McDonald, I. W. 1954.
The extent of conversion of food protein to microbial protein in the rumen of the sheep. Biochem. J., 56:120.

McDonald, I. W., and R. J. Hall. 1957.
The conversion of casein into microbial protein in the rumen. Biochem. J., 67:400.

McNaught, M. L. 1951.
The synthesis of lysine by bacteria during incubation of rumen contents in vivo (Abs.) Biochem. J., 50:i-ii.

McNaught, M. L., and J. A. B. Smith. 1947.
4. Nitrogen metabolism in the rumen. Nutr. Abs. and Rev., 17:18.

McNaught, M. L., K. M. Henry, S. K. Kon, and J. A. B. Smith. 1950a.
The utilization of non-protein nitrogen by the bovine rumen. 5. The isolation and nutritive value of a preparation of dried rumen bacteria. Biochem. J., 46:32.

McNaught, M. L., E. E. Owen, and J. A. B. Smith. 1950b.
The utilization of non-protein nitrogen in the bovine rumen. 6. The effect of metals on the activity of the rumen bacteria. Biochem. J., 46:36.

McNeil, P. P., R. N. Doetsch, and J. C. Shaw. 1954.
Some nutritional requirements of bovine rumen bacteria. J. Dairy Sc., 37:81.

Meiske, J. C., W. J. Vanarsdell, R. W. Luecke, and J. A. Hoefer. 1955.
The utilization of urea and biuret as sources of nitrogen for growing-fattening lambs. J. Ani. Sc., 14:941.

Mills, R. C., A. N. Booth, G. Bohstedt, and E. B. Hart. 1942.
The utilization of urea by ruminants as influenced by the presence of starch in the ration. J. Dairy Sc., 25:925.

Moir, R. J., and V. J. Williams. 1950.
Ruminal flora studies in sheep. II. The effect of level of nitrogen intake upon the total number of free microorganisms in the rumen. Australian J. Scientific Res., 3:381.

Moore, W. E. C., and K. W. King. 1958.
Determination of the intraruminal distribution of soluble nitrogen. J. Dairy Sc., 41:1451.

Nicols, R. E. 1955.
A sampling tube for rumen fluid. Am. J. Vet. Res., 16:410.

Owen, E. C., J. A. B. Smith, and N. C. Wright. 1943.
Urea as a partial protein substitute in the feeding of dairy cattle. Biochem. J., 37:44.

Oxford, A. E. 1955.
The bacteriology and protozoology of ruminant digestion. J. of Sci. Food and Agri., 8:413.

Pearson, R. M., and J. A. B. Smith. 1943a.
The utilization in the bovine rumen. 1. Methods of analysis of the rumen ingesta and preliminary experiments in vivo. Biochem. J., 37:142.

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

Pearson, R. M., and J. A. B. Smith. 1943c.
The utilization of urea in the bovine rumen. 3. The synthesis and breakdown of protein in rumen ingesta. Biochem. J., 37:153.

Phillipson, A. T., M. J. Dobson, T. M. Blackburn. 1959.
Assimilation of ammonia nitrogen by rumen bacteria. Nature, 183:402.

Pounden, W. D., L. C. Ferguson, and J. W. Hibbs. 1950.
The digestion of rumen microorganisms by the host animal. J. Dairy Sc., 33:565.

Quin, J. I. 1943.
Studies on the alimentary tract of Marino sheep in South Africa. VII. Fermentation in the forestomach of sheep. Onderstepoort J. Vet. Sc., 18:91.

Quin, J. I., W. Oyasent, and R. Clark. 1951.
Studies on the alimentary tract of Marino sheep in South Africa. XVIII. The effect of fasting on the activity of the ruminal flora of sheep and cattle. Onderstepoort J. Vet. Res., 25:51.

Regier, Fremont A. 1961.
A new technique for the study of protein synthesis in the rumen. A Master's Thesis. Kansas State University.

Reid, J. T. 1953.
Urea as a protein replacement for ruminants. A review. J. Dairy Sc., 36:955.

Reid, R. L. 1959.
Unpublished data.

Schwarz, C. 1925.
The physiological use, as food, of microorganisms in the stomachs of ruminants. Biochem. Z., 156:134.

Sirotnak, F. M., R. N. Doetsch, R. Q. Robinson, and J. C. Shaw. 1953.
Amino acid metabolism of bovine rumen bacteria. J. Dairy Sc., 36:1117.

Sirotnak, F. M., R. N. Doetsch, R. Q. Robinson, and J. C. Shaw. 1954.
Aspartate dissimilation reactions of rumen bacteria. J. Dairy Sc., 37:531.

Smith, J. A. B., and F. Baker. 1944.
The utilization of urea in the bovine rumen. IV. The isolation of the synthesized material and the correlation between protein synthesis and microbial activity. Biochem. J., 38:496.

Smith, P. H., H. G. Sweeny, J. R. Rooney, K. W. King, and W. E. C. Moore. 1956.
Stratification and kinetic changes in the ingesta of the bovine rumen. J. Dairy Sc., 39:598.

Thaysen, A. C. 1945.
The microbiological aspects of rumen digestion. Proc. Nutri. Soc., 3:195.

Thornton, H. G., J. W. Howie, F. Baker, A. T. Phillipson, R. L. M. Synge, and S. R. Elsden. 1952.
A discussion on symbiosis involving microorganisms. Proc. Royal Soc. B., 139:113.

Tosic, and R. L. Mitchell. 1948.
Concentration of cobalt by microorganisms and its relation to cobalt deficiency in sheep. Nature, 162:502.

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

Warner, A. C. I. 1956b.
Proteolysis by rumen microorganisms. J. Gen. Microbiol., 14:749.

Wegner, M. I., A. N. Booth, G. Bohstedt, and E. B. Hart. 1940.
The in vivo conversion of inorganic nitrogen to protein by microorganism from cow rumen. J. Dairy Sc., 23:1123.

Wegner, M. I., A. N. Booth, G. Bohstedt, and E. B. Hart. 1941a.
Preliminary observations on chemical changes of rumen ingesta with and without urea. J. Dairy Sc., 24:51.

Wegner, M. I., A. N. Booth, G. Bohstedt, and E. B. Hart. 1941b.
The utilization of urea by ruminants as influenced by the level of protein in the ration. J. Dairy Sc., 24:835.

Yadava, Indrajit Singh. 1962.
Studies on the rate of passage of nutrients through the bovine rumen and the rumen metabolism of C-14 labelled roughage. Ph. D. Dissertation, Kansas State University, 1962.

FURTHER OBSERVATIONS ON THE TECHNIQUE OF STUDYING PROTEIN SYNTHESIS IN THE RUMEN

by

RAMBHAU KHANDERAO RAIKAR

G.B.V.C., Bombay Veterinary College, Bombay, (India), 1945
B.Sc. (Vet.), University of Bombay, Bombay, (India), 1955

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Animal Husbandry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1963

Seamless regenerated cellulose dializing tubing having thickness between 0.0018 to 0.0023 inches with a width of about 3 to 4 inches was made into 7 to 8 inch long sacs to accomodate 80-90 ml. fluid. One end of the sac was tied with nylon thread and the other end with rubber stopper and rubber band. After filling the sacs with sterilized normal saline or sterilized rumen liquor were sterilized again for 3 to 5 minutes and after cooling were inoculated with fresh rumen liquor or bacterial suspension. One gram of starch in 10 ml. distilled water was added to each sac. Both ends of the sacs were further sealed with plaster of paris and wax and incubated in the rumen of fistulated steers for varying lengths of time. This provided an in vivo artificial rumen combination for protein synthesis by the microorganisms. At the end of incubation the contents of the sacs were emptied into flasks containing 10 percent trichloroacetic acid. The precipitate was analyzed for protein nitrogen.

It was demonstrated that the sacs remained sterile up to 48 hours. Therefore possibilities of pure culture study are suggested. At the end of 48 hours of incubation, the sacs were ballooned and tense due to accumulation of gases which must have prevented the growth of microorganisms.

Trials were conducted to study protein synthesis by microorganisms inoculated into the sacs filled with normal saline or sterile rumen liquor. The amount of protein nitrogen in the blanks and those inoculated with microorganisms was determined. The results of all the trials show that there was no appreciable increase in protein synthesis during incubation. The results were also not consistent. Maximum amount of protein nitrogen was found after 12 hours incubation and minimum at 48 hours incubation. It was demonstrated that sterilized rumen liquor was a better medium than normal saline

for growth and protein synthesis by the microorganisms.

It was concluded that the technique devised by Regier (1961) cannot be used for studies of longer than 12 hours duration.

A sampling technique to obtain representative samples of rumen ingesta was studied. The rumen ingesta of fistulated steers were thoroughly stirred by hand for 5-10 minutes. Samples were collected from anterior, central and posterior parts of the rumen. Then the entire rumen contents were removed and samples were collected. The samples were weighed, dried in oven and reweighed. The dried samples were ground and representative samples from the ground material were analyzed for total nitrogen.

Total nitrogen values differed slightly in the identical twins and with those from the other identical twin pair. But the results of the samples from inside and outside the rumen of individual steers revealed no marked difference. No difference in the moisture content of the samples in identical twins was observed.

No difference was observed in protein nitrogen content of the samples obtained from inside the rumen when ground with a find and coarse mesh and precipitated with 15 per cent and 57 per cent trichloroacetic acid. Fifty-seven per cent trichloroacetic acid appeared to have a slight deteriorating effect on protein.

Weighing wet samples appeared to be rather difficult because representative samples from these wet samples was difficult to obtain.

A sampling technique to obtain representative samples of rumen ingesta is established. It is believed that this technique will have advantage over other techniques because there are no artificialities and results would be more reliable.