A NEW TECHNIQUE FOR THE STUDY OF PROTEIN SYNTHESIS IN THE RUMEN

by

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INTRODUCTION

The rumen is a unique organ, performing many functions not known to be duplicated elsewhere in nature. The microorganisms in the rumen are known to play a very definite role in these functions. Their activity in the field of protein synthesis from non-protein material and for improving protein quality is of special interest. Much more work remains to be done on protein synthesis by bacteria; for example, so far few have been able to adequately grow bacteria in pure culture and demonstrate protein synthesis by any of the specific organisms that appear in the rumen. The relationship among specific bacteria in this process needs to be worked out thoroughly. Investigators have seldom touched upon the synergistic, antagonistic, and symbiotic interaction phenomena on the synthesis of protein in the rumen.

The techniques in use at present, namely, (1) microscopic, (2) pure culture, (3) washed suspensions, and (4) artifical rumen do not give satisfactory indications of the interactions involved among various representatives of the rumen population. Before valid in vivo work can be done, further developments are necessary to overcome these deficiencies in technique.

The studies in this experiment were designed to investigate the possibilities of the development of a new method for the study of the synthesis of protein in the rumen. This method includes a combination and improvement of the current pure culture, diet or media, artifical rumen, and fistulated animals. Dialyser sacs made of material selectively permeable because of pore size placed in a protective cover with experimental diet or media and microorganisms were incubated in the fistulated rumen. The media consisted of rumen contents sterilized by filtration and the sacs were sterilized when desired by boiling. A study was made of the ability of the sac to remain intact and

sterile while maintaining selective diffusibility during incubation. Work was also done in an effort to demonstrate protein synthesis in the sac.

REVIEW OF LITERATURE

Microbiology of the Rumen

Intensive research on rumen microorganisms and their functions began in the early 1940's, although these studies by no means constituted the initial attack launched in this direction. As early as 1891 Zuntz as cited by Loosli (1949) suspected that microorganisms of the rumen split the proteins fed to the host animal.

Annison and Lewis (1959), p. 22, state that the rumen is essentially an anaerobic highly reducing system at a slightly acid, but buffered pH, at a temperature of 39° C. and under a gas phase composed mainly of carbon dioxide, methane, and nitrogen. A very specialized microbial population develops in this environment. In addition to the well known cellulose digestion, rumen microorganisms contribute protein synthesis, B vitamin synthesis, fat alteration and soluble carbohydrate digestion to the nutrition of the host animal (Alstad, 1958). Baker (1943) states that the microflora and microfauna of the rumen make an important contribution to the process of digestion and assimilation in the ruminant herbivora.

The total population of the microorganisms of the rumen reach quite high numbers. Studying rumen bacteria of cattle and sheep by means of direct slide counts, gram stains, and anaerobic cultural techniques Gall, et al. (1949) found bacterial counts to exceed 50×10^9 per gram of rumen contents. Alstad (1958) reports that numbers of bacteria may reach 10^{10} per gram of rumen

contents. Claypool, et al. (1961) recorded counts of total viable anaerobic bacteria ranging through 10^9 per milliliter of rumen content while Annison and Lewis (1959), p. 22, state that bacteria may number 10^{10} per gram of rumen contents. According to these workers the growth of one organism may be peculiarly dependent on the others present, and the total numbers or the proportions of each species can fluctuate under different conditions. A number of physiological types may be present in each of the morphological groupings of bacteria but they list small rods and cocci as the most prominent groups. Due to the heterogeneity of rumen contents it is quite difficult to obtain representative samples for microbiological examination. However Gall, et al. (1949) found that bacteria from animals on pasture appeared morphologically similar to those on a winter ration, with a few different types in addition. They also reported a noticeable increase in fast-growing organisms when the amount of grain in the ration was increased.

Numbers of protozoa in the rumen are of a smaller degree, but may reach 10^6 per gram of rumen contents according to Alstad (1958). Annison and Lewis (1959), p. 23, regard protozoa to number roughly 1,000,000 per gram of rumen content, but believe that their bulk may be equivalent to that of the bacteria in the rumen. Johnson, et al. (1944) found that protozoa may be separated from paunch contents in a fairly high state of purity by filtration and centrifugation. They determined the bacteria and protozoa counts in paunch contents at intervals for 24 hours after feeding and discovered the greatest number of bacteria and fewest protozoa one hour after feeding. Later the number of bacteria decreased while the protozoa increased for 16 hours.

A symbiotic relationship exists between the ruminant and the bacteria found in the rumen in that the host supplies materials 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 (Thornton et al. as cited by Doetsch and Robinson, 1953). Similar cooperation may also exist between various members of the microbial population. Dohner and Cardon (1954) reported an interesting case of symbiosis between two rumen strains of E. Coli. In their studies pure cultures failed to ferment lysine. However when cultured together, the two strains were able to ferment the amino acid giving ammonia and butyric and acetic acids as products of the fermentation.

Annison and Lewis (1959), p. 23, note 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 of protozoa is of considerable nutritional value and that they actively contribute to the breakdown of cellulose, starch, and protein.

Various aspects of rumen microbiology have been excellently reviewed. Doetsch and Robinson (1953) reviewed papers concerned principally with the bacteriology of the bovine rumen which have been published since 1946. Baker (1943) in a discussion of rumen microbiology gives a systematic description of the species normally present and an elucidation of functional activities of the microorganisms described. Further information regarding the specific organisms concerned with nitrogen metabolism in the rumen is given by Hobson (1959).

Nutrient Requirements of Rumen Microorganisms

The evidence concerning nutritional requirements of rumen bacteria has been derived almost completely from experiments made on pure cultures and from observations made on various artifical rumen systems. Burroughs, et al. (1951b) states that utilization in the ruminant, like cellulose digestion, is

dependent, to a large extent, upon considerations involving the nutrient requirements of the rumen microorganisms. Their work suggests that a roughage could be utilized most efficiently when each of the nutrient requirements were adequately supplied. Burroughs, et al. (1951a) lists three general nutrient requirements of rumen microorganisms: energy, protein or its elements such as nitrogen, and inorganic constituents involved in enzyme or enzyme systems of the rumen microorganisms. Bleden, et al. (1961) found certain branched-chained volatile fatty acids such as isovaleric acid to be essential for the growth of several species of cellulolytic rumen bacteria. Aries, et al. (1951) reports 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 NH_3 supplying compounds. Hart, et al. (1939) studying the utilization of urea and ammonium bicarbonate nitrogen by growing calves came to the conclusion that the most efficient utilization occurs when some soluble sugar such as corn molasses is fed in the ration. In 1940 Wegner, et al. showed that rumen bacteria used energy in the form of sugar from corn molasses. An addition of this sugar to rumen fluid incubated in vitro increased the disappearance of ammonia nitrogen, which was used as a measure of bacterial activity.

In studying nitrogen metabolism in sheep, Annison (1956) found that the rate of disappearance of ammonia and amino acids from the rumen of sheep after feeding casein or casein hydrolysate was increased in the presence of carbohydrates. Hueter, et al. (1958) reported similar findings with in vivo trials, showing enhanced ammonia utilization by rumen microorganisms with glucose as a source of readily fermentable carbohydrates. Work done by Phillipson et al. (1959) supports the hypothesis that a depression in the concentration of ammonia nitrogen in the rumen that occurs when a starch rich food is added to

e high protein ration may be partly due to an increase in the concentration of bacteria that can assimilate ammonia nitrogen. Mills, et al. stated in 1942 that the function of the starch is to serve as a readily available energy source for microorganisms enabling them to build new protoplasm in which the nitrogen, from urea in this case, is incorporated. When timothy hay was the sole ingredient of the basal ration, utilization of added urea took place only partially, if at all. In the presence of starch a suitable substrate was provided for the development of an active flora and urea was efficiently utilized.

The correct balance of nutrients for the microorganisms appears to be of importance. Aries, et al. (1951) state that dextrose or other readily available carbohydrate in too large a quantity is not warranted in an attempt to make maximum utilization of urea as a protein supplement. They explain that unfavorable fermentation conditions for cellulose digestion will occur, and then urea utilization fails to occur past a short initial fermentation period.

Proper nutrition of the bacteria is also necessary for these organisms to function well in synthesizing protein. Baker, et al. (1947) found protein synthesis to occur readily, but stated that for synthesis to exceed breakdown, ample carbohydrate other than cellulose must be present, and the content of protein, particularly soluble protein, must be low. Similarily Smith and Baker (1944) in an attempt to find a correlation between protein synthesis and microbial activity found that maltose promoted protein synthesis. In these tests glycerol did not promote the synthesis, but tended to cause a 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, cellobiose, and glucose caused a significant increase in the production of ammonia and carbon dioxide from aspartic acid.

Burroughs, et al. (1951a) notes that nitrogenous requirements of rumen microorganisms are relatively simple in nature, essentially involving only ammonia and not involving the more complex forms of nitrogen, such as amino acids. In a test conducted to learn the nature of the influences exerted by conventional proteins already present in rations upon the utilization of added simple nitrogenous compounds these workers found that the major interest of the rumen microorganism in protein appeared to be for energy rather than for ammonia. In fermentations conducted in vitro where adequate ammonia of urea origin was available, the presence of added gelatin or casein resulted in large amounts of additional ammonia formed. It appears that this ammonia comes from proteins being utilized first and foremost as energy sources in which the accompanying ammonia liberated may or may not be utilized later depending upon the quantitative needs of the rumen microorganisms.

The importance of ammonia in the nutrition of rumen microorganisms was further emphasized by Bryant, et al. (1959) in their work with a ruminal cellulolytic bacterium. They found ammonia to be essential for the growth of this Bacteroides succinogenes even in the presence of 19 amino acids, a mixture of purines and pyrimidines, and all the B vitamins. They found none of the amino acids to be essential for growth but somewhat delayed growth was had when cysteine or glutathione was the only source of amino acids available.

When casein was added to a timothy hay-starch-urea ration, Mills, et al. (1942) found the utilization of urea to be reduced, and only a slow hydrolysis of urea to ammonia to occur. Though such results are difficult to explain they suggested that it could be because casein was a "preferred" source of protein and it also maybe drastically changed the rumen flora.

Mineral requirements of ruminants may be higher than those of monogastric animals due to the trace mineral requirements of rumen microflora (Alstad,

1958). Burroughs, et al. (1951b) found stimulating effects upon microbial activity in urea utilization and cellulose digestion from the complex mineral assortment found in the ashes of plants or plant products. They studied water extracts from immature clover hay, rumen ingesta, manure, sugar and ash of blackstrap cane molasses, and mature timothy hay, as well as specific mineral elements alone and in presence of molasses-ash. Ashes of molasses, the clover hay, and the timothy hay were found approximately comparable on a weight basis in stimulating fermentations. Phosphorous and iron were found to be effective in stimulating urea utilization and cellulose digestion by rumen microorganisms. They also presented evidence that other elements besides iron and phosphorous were included in rumen bacterial physiology in addition to the sodium, potassium, calcium, magnesium, chlorine, and sulphur routinely used in the artifical saliva in the fermentation flask.

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. 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 phosphorous or sulphur needed by the microflora in this synthesis (Burroughs, 1951a). Minerals could also help in urea utilization by supplying those needed in cellulose digestion thereby releasing additional energy for rumen bacterial development.

McNaught, et al. (1950b) used the decrease of non-protein nitrogen in bovine rumen liquid when incubated in vitro as an index of bacterial growth to investigate the effects of metals on bacterial activity. In muslin strained, centrifuged rumen liquid, bacteria were able to tolerate 100 ppm iron,

10 ppm copper, less than 10 ppm cobalt and between 100 and 1000 ppm molybdenum. The inhibition of bacterial growth was demonstrated with 1000 ppm iron, 25 ppm copper, 100 ppm cobalt, and 2000 ppm molybdenum. They noted that the amount of iron associated with microorganisms increased on incubation in vitro, and that 1-2 ppm of iron in rumen liquid produced good bacterial growth. In in vitro incubated rumen filtrate rich in protozoa and bacteria Pearson and Smith (1943b) found phosphate to have a retarding effect upon the conversion of urea to ammonia.

Alstad (1958) feels that a great deal remains to be done concerning nutritional requirements of authentic rumen bacteria and the effect of deficiencies on overall rumen function. It seems likely that some vitamin requirements of rumen bacteria are met by others; but little is known concerning this. Observations on requirements for trace minerals, fatty acids, vitamins, etc. have been made usually with some sort of artificial rumen system; and only empirical results are obtained with this technique. This worker further asserts that the bulk of definite work concerning nutritional requirements of rumen bacteria still lies ahead.

Breakdown and Utilization of Protein by Rumen Microorganisms

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 (Annison and Lewis, 1959, p. 92). Studies on protein digestion in connection with ruminal microflora have not been as numerous as those dealing

with various carbohydrates. It is only comparatively recently that experimental evidence has accumulated demonstrating extensive degradation of ingested protein in the rumen. As proteolytic activity is absent in bovine saliva and no proteolytic enzyme or secretory glands occur in the rumen (Wegner, et al. 1940) protein breakdown by microorganisms takes on great importance.

El Shazly (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 acids. He considered these acids to arise from microbial attack on protein. McDonald (1952) discovered that the addition of 25 grams of casein to the rumen through a fistula was followed by a pronounced rise in ammonia concentration. The addition of zein, on the other hand, produced no change in concentration of ammonia in the rumen. He concluded that for all practical purposes, the whole of the digestion of protein in the rumen is effected by microorganisms. Annison (1956) reported proteolytic activity of rumen contents with washed suspensions of rumen bacteria, demonstrating extensive degradation of casein, arachin, and soyabean 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 was given as evidence that proteolysis is the first step in protein digestion and the peptides and amino acids were described as intermediates in the process of protein breakdown by microbial proteolytic enzymes. Lewis (1955) reported results that strengthened the probability that amino acids are intermediate steps in the breakdown of protein in the rumen. He detected free amino acids to a greater extent in rumen fluid three hours after feeding than before, using successive desications over concentrated sulfuric acid to concentrate the rumen contents.

Amino acids are not absorbed from the rumen (Annison 1956, Hobson 1959)

and the next step in the breakdown of protein in the rumen appears to be degradation of amino acids to ammonia. Sirotnak (1953) tested 22 amino acids and found aspartic acid, glutamic acid, serine, arginine, cysteine, and cystine to be attacked by suspensions of mixed rumen microorganisms. The principle degradation products were carbon dioxide, ammonia, acetic acid, propionic acid, and butyric acid. Warner (1956b) found both rumen bacteria and protozoa to be proteolytic suggesting that proteolysis in the rumen takes place in the usual way, through peptides of decreasing chain length to free amino acids which are then deaminated to ammonia. El Shazy (1952) found amino acids to give rise to ammonia and volatile fatty acids in the presence of rumen bacteria both in vivo and in vitro in sheep. He determined that the principal source of ammonia is deaminative attack by microorganisms on amino acids of the protein fed. These results are in accord with the findings of Lewis who found individual amino acids placed in the rumen of sheep to give rise to ammonia. Also in accordance are the results of Looper, et al. (1959) who demonstrated significant deamination of beta-alanine, D L aspartic acid, and L glutamic acid as these amino acids were broken down to ammonia and other products. Chalmers, et al. (1954) and Dohner and Cardon (1954) further emphasize the importance of ammonia in the process of protein metabolism.

Bacteria appear to utilize a portion of the ammonia formed by amino acid deamination as a source of nitrogen for their own body proteins. Gray, et al. (1953) fed wheaten hay to sheep and found that at least half of the protein is degraded, and later resynthesized to microbial protein by the bacteria and other microorganisms in the rumen. On a partially purified diet for adult sheep, when casein provided 87 per cent of the nitrogen, McDonald and Hall (1957) found at least 90 per cent of the casein to be degraded in the rumen and utilized for synthesis of microbial proteins. This conclusion was based

on the estimation of casein in the mixed ingesta leaving the stomach, after the development of a chemical procedure for determining small amounts of casein in the presence of other proteins.

Weller, et al. (1958) analyzed rumen contents of sheep slaughtered at different times after feeding a ration of wheaten hay and found that between 2 and 24 hours after feeding, microbial protein accounted for 63 per cent to 82 per cent of the total. At the same time soluble nitrogen comprised 5 per cent to 10 per cent, the rest being plant nitrogen. This agrees with the observation of Hobson (1959) that ammonia nitrogen in the rumen decreases due to utilization for bacterial growth and absorption from the rumen. In a study of the intraruminal distribution of soluble nitrogen Moore (1958) found that most of the nitrogen was in feed residues and microbial cells regardless of dietary treatment. The synthesis of amino acids and protein by the microorganisms of the rumen is further confirmed by various workers (McDonald, 1954; Smith and Baker, 1944; McNaught, 1951; and Loosli, et al., 1959; and Wegner, et al., 1940 and 1941b).

As summarized by Alstad (1958), protein, urea, amino acids, ammonia, nitrate, etc., enter the rumen. Here the proteins are hydrolyzed to amino acids which are in turn absorbed by the rumen microorganisms or deaminated and decarboxylated with liberation of the corresponding fatty acid. 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. Nitrate may be reduced to ammonia; and any material which is not attacked passes on to the abomasum and intestine.

Utilization of Non-Protein Nitrogen by Rumen Microorganisms

An important facet of the subject of rumen microbiology was revealed in 1891 by Zuntz as cited by Loosli (1949). At this time Zuntz presented the view that bacteria in the rumen of animals might utilize non-protein nitrogenous compounds to form protein which in turn could be used by the host animal. It was this hypothesis that initiated the interest in the role of ruminal microorganisms in protein metabolism, and literature on this phase of the work is very extensive. Muller in 1907 (a, b) experimenting with dogs found little difference between a ration with amide products extracted from hay and one with blood albumin as the protein source. He concluded that such non-protein nitrogenous constituents of hay could be used for tissue building in the body and so could replace protein, stating further that these amides could even possibly become proteins. Voltz in 1919 advanced the view that urea could assume the role of food protein in the metabolism of the ruminant whereby urea is first built up into bacterial protein in the digestive tract, 80-90 per cent of which is afterwards absorbed from the intestine. In 1937 Fingerling, et al. advocated the introduction of urea as a protein substitute in agricultural practice. Hart, et al. in 1939 with two separate experiments involving utilization of urea and ammonium bicarbonate nitrogen showed that such nitrogen could be used for at least part of the supply of protein nitrogen. Wegner, et al. (1940, 1941a, 1941b) continued this work finding that urea nitrogen utilization in the rumen must occur within 4-6 hours after feeding since urea nitrogen and ammonia nitrogen are negligible in the rumen after that time (1941a). In 1941 Benesch reviewed the work on urea done up to that time noting the necessity of finding substitutes for dietary proteins in the form of simple nitrogenous compounds of a non-protein nature. The work was continued in 1942 by Mills, et al. and

Axelsson. Axelsson (1942) described certain thermal and iodophilic acid-forming cocci and others which synthesize protein from urea nitrogen for use in their bodies, which later die and are digested and the protein utilized by the host animal. He also revealed the possibility of urea poisoning if fed in a pure form in too great amounts by rapid formation of ammonia and carbonic acid through the bacterial actions in the rumen.

Pearson and Smith (1943a, 1943b, 1943c) 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 proceded to test the theory that certain microorganisms which multiply in the rumen build up their own protein from this simple form of nitrogen. Those microorganisms then pass further along the alimentary tract where their protein is digestad with the ordinary protein of the diet. Incubated samples of rumen ingesta with and without urea were used to test the effect of various factors on the conversion of urea. They found the conversion to be insignificant at 4° C., while increases in temperatures brought about an increase in the conversion rate to a maximum at 49° C. As the temperature increased, conversion decreased to 79° where it became null. They noted the optimum pH for this conversion to be between 7 and 9, with little occuring below 3 or above 9.5. A slight increase in the conversion of urea was brought about with increased substrate concentration. The influence of the time of sampling from the rumen appeared to have little effect as to the ability of the rumen liquor to convert urea to ammonia. These workers reported the absence of urease activity in feed, and that none is secreted into the rumen. Consequently this enzyme must be generated by microorganisms in large amounts relative to the small organisms. Quinone, cysteine, sodium flouride, boric acid, and borax all appeared to have an inhibitory effect upon urease. Included in their publication is a detailed description of the methods

of analysis used by these workers.

Smith and Baker (1944) confirmed the assumption of Pearson and Smith (1943c) that as the non-protein nitrogen content decrease in vitro while total nitrogen remained constant, protein was synthesized. This synthesis was accompanied by a great increase in the number of microorganisms, with the iodophile counts greatly increased when synthesis predominated. These counts decreased or showed little change when hydrolysis predominated. Also accompanying the synthesis of protein was the synthesis of a starch-like polysaccharide. By centrifuging the rumen liquor before incubation, protein synthesis was shown to occur in the absence of protozoa, causing these workers to believe that these organisms do not contribute to the synthesis. From dried microscopial examinations and other considerations it was concluded that there can be little doubt that the bulk of the synthesized polysaccharide and protein is incorporated in the small rods, cocci, and vibriones of the microiodophile population.

In 1947 urea was found to have a depressant effect upon milk production when fed at levels of ingestion that would be necessary to justify its use economically (Bartlett and Blaxter, 1947). Two years later Loosli, et al. (1949) demonstrated the synthesis of the ten essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) in ruminants fed urea as the only dietary source of nitrogen. These workers were prompted to conclude that the fact that the animals continued to gain weight on the urea diet, containing no protein, for over three months is further evidence of the formation of amino acids. Wasserman, et al. (1953) stated that the relative simplicity of the nutritive requirements of the anaerobic bacteria isolated from rumen contents and their occurance in the rumen of animals receiving non-protein nitrogen and soluble carbohydrate may indicate that they are important converters of non-protein nitrogen to

protein nitrogen in ruminants.

A laboratory evaluation of various nitrogen food compounds for ruminants was made by Balasco (1954). Using in vitro techniques he showed, in addition to urea, the high availability of the nitrogen of numerous organic and inorganic ammonium salts. Ammonium formate, alpha-ketoglutarate, malate, and especially ammonium succinate and lactate showed higher rates of nitrogen utilization and lower free ammonia levels than urea. They postulated that the organic fragments of these salts enter into some biosynthetic process stimulatory to nitrogen fixation by rumen microflora. Salts of guanidine as the sole nitrogen source in the artifical rumen gave low levels of free ammonia and provided an excellent nitrogen source for the growth of rumen microorganisms. Included in this category were amidines such as creatinine and creatine. Amides of monocarboxylic acid also produced good bacterial growth, but gave less response than urea. Diamides of dicarboxylic acids were not found to provide available nitrogen for bacteria utilization. He further states that purines also provide nitrogen for microorganisms. Annison and Lewis (1959), p. 96, also mention the large volume of saliva that enters the rumen containing a significant quantity of urea. Campbell, et al. (1960) compared soybean oil meal, urea, and biuret as nitrogen supplements to ruminant rations. From the growth and feeding trials conducted and nitrogen balances, digestion coefficients, and net protein values studied, they concluded that all these supplements are useful sources of nitrogen, probably in the descending order as listed above. Hobson (1959) calls attention to the fact that urea can replace part of the protein in the diet if the carbohydrates are available, and that urea is always present in saliva, which is broken down to ammonia and utilized for bacterial growth.

Further work with various non-protein nitrogen sources was reported by Phillipson, et al. (1959) and Hershberger, et al. (1959). Alstad (1958) notes

that the validity of the claim that non-protein nitrogen can replace part of the 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 acceptability of the idea.

Eventual Fate of Rumen Microorganisms

As far back as 1943 the view was expressed that infusoria might have a special nutritional significance (Gruby and Delafond as cited by Benesch 1941). Probably the earliest attempt to investigate the nutritive value of rumen bacteria was made by Muller in 1906 (cited by McNaught, et al. (1947). He approached the problem by inoculating a cultural medium with rumen bacteria and allowing it to grow three days. The mixture was then precipitated with ammonium sulfate and the precipitate washed with ethanol. The dried product was then fed to a bitch. The nitrogen retention on this ration was found to be similar to that on a ration combining albumin and casein.

Zuntz in 1891 as cited by Loosli (1949) suggested that bacteria in the rumen of animals utilize non-protein nitrogenous compounds to form protein, which in turn could be used by the animals. Voltz in 1919 said that 80 per cent to 90 per cent of the bacterial protein in the digestive tract is afterwards absorbed from the intestine. An examination of the nitrogen partition of the contents of the first stomach of ruminants during digestion indicated to Schwarz (1925) that bacteria furnish an important source of nitrogenous food.

It is clear that the nitrogenous substances absorbed by the host animals are not merely those found in the diet, as in the monogastric animal. Rather, they comprise a mixture of dietary constituents, products of microbial metabolism in the rumen and the constituents of the microorganisms themselves

(McDonald 1954). Johnson, et al. (1944) made a dried preparation of sheep rumen bacteria, of protozoa and also of an organism which they isolated in pure culture from mixed rumen flora in an effort to determine the nutritive value of the bacterial protein of the rumen. The bacterial fraction which was isolated less satisfactorily contained 44.50 per cent protein when dried, while the bacterial fraction from cultures on synthetic media had 58.81 per cent protein. Digestibility of the purer preparation was 82.4 per cent with a biological value of 66 per cent. These results agree with the idea that in ruminants much of the food nitrogen, either protein or non-protein in nature, is first synthesized by the bacteria into their own cellular proteins, and then, at least to a considerable extent, the protozoa utilize bacterial protein in their own growth, and finally the host digests the protozoal protein, and remaining bacterial protein. Thus up to the maximum amount of nitrogen capable of utilization by the microorganisms in the paunch, all food nitrogen would exhibit a biological value characteristic of the mixed microorganisms which reach the duodenum. These investigators believe this biological value to be approximately 60. Nitrogen consumed above that required by the microorganisms should exhibit a biological value approximately (probably somewhat less) the same as a non-ruminant of similar requirements.

McNaught (1950a) also determined the nutritive value of an isolated preparation of dried rumen bacteria. Bacterial sludge separated by centrifugation and dried after incubation with maltose and urea contained on a dry weight basis 44.4 per cent protein and 7.1 per cent ash. They calculated that 58 per cent of the bacterial protein was present in liquid as it came from the rumen. The 42 per cent remaining was synthesized during incubation. Using young rats the biological value was found to be 88.3 and the true digestibility 73.2.

Baker (1947), Benesch, et al. (1941), and Hart, et al. (1939) mention the

products of bacterial synthesis in the rumen made available for subsequent digestion. Benesch observes that it seems possible that not only does the bacterial synthesis in the rumen depend on variable conditions, but that also the bacteria protein thus formed can be digested to variable degrees by digestive enzymes.

Observations conducted on samples from various parts of the digestive tract of cattle for the types of bacteria characteristically present in rumen samples of cattle were made by Founden, et al. (1950). This interesting study was conducted by investigating the presence of morphologically identifiable types in various sections or areas of the tract. The large coccoids were observed in all parts of the tract with a lower concentration in the posterior part some of the time. Cigar-shaped organisms disappeared in the abomasum and were missing in the rest of the tract. Small rods and bacteria in the form of square ended rods appeared to disintegrate gradually as they reached the posterior part of the tract, while some were observed in samples from the caecum or colon. It was concluded that the ultimate fate of rumen microorganisms varies between extremes of complete destruction in the abomasum to passage entirely through the digestive tract of the host.

Techniques of Study

According to Alstad (1958) there is an unfortunate gap in methodological approaches to rumen microflora studies. This deficiency lies in the fact that none of the techniques in current general use satisfactorily covers the interaction involved between various representatives of rumen population. This deficiency in technique has to be overcome before valid in vitro work can be done. Doetsch and Robinson (1953) describe the lack in knowledge in the field

of the relationships of specific bacteria to vitamin synthesis, minerals, protein synthesis, and antibiotics. The synergistic reactions and antagonisms are also not known. They state that it has become necessary to know the physiological functions of the rumen organisms, and to discover how these physiological types are altered with age, changes in diet, disease, and other conditions. They predict that new techniques will be introduced to facilitate solutions to the difficult problems accompanying the study of rumen bacteria. Hobson (1959) also calls attention to the lack of knowledge concerning the microorganisms responsible for certain processes in the rumen.

The principal methods used by most investigators are roughly classified by Alstad (1958) into: (a) pure culture studies; (b) washed suspension studies; and (c) artifical rumen studies. In the first method samples are processed in a variety of ways, depending upon the interests of the investigator. Here one is working with living organisms and can usually isolate many of them in pure culture. This method allows one to study metabolic activities of the predominant bacterial species which were presumably physiologically active in the rumen, under controlled physical and chemical conditions. Alstad (1958) criticizes this method noting that it is difficult to equate numbers of a given kind of bacterium found on a given medium, with its importance in the rumen. Further, species isolated as pure cultures on an artificial medium may show characteristics not consistent with those found within the natural environment of the rumen. These objections not withstanding, the pure culture method in the hands of several workers has yielded much valuable information. Bryant (1959) has written an extensive review limited to pure culture studies with emphasis on the species of bacteria cultured.

In the method using washed suspensions of mixed population, whole rumen liquor is "differentially centrifuged" to obtain the bacterial fraction. Usual

techniques of biochemistry are then used to study enzymatic capabilities of the mixed cells. 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 (Alsted 1958). There are several advantages in this procedure over *in vitro* studies or the use of untreated contents of the rumen (Annison and Lewis 1959, p. 52). Washing the organisms reduces the carry over of the end-products or rumen fermentation and thus minimizes the blank values obtained in the analyses. It is considered that the organisms are in a "resting" state, i.e. metabolically active, but with little growth or reproduction since the removal of potential substrates by washing reduces fermentation. Hueter, *et al.* (1958) compared *in vivo* and *in vitro* rumen bacterial dissimilations of some carbohydrates, amino acids, and organic acids. In general they reported qualitative agreement between *in vivo* and *in vitro* washed cell suspension experiments. They concluded that the washed cell suspension technique appears to be most useful for studying short one- or two-step reactions presumed to occur in the rumen.

There have been numerous attempts to examine the activities of the rumen population *in vitro* and many of these attempts used are referred to as "artificial rumen" techniques. Annison and Lewis (1959), p. 54, 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 the appearances of end-products was followed. Wegner, *et al.* (1940) criticized the existing artificial rumen techniques as lacking movement and diffusion. Since that time these artificial rumen techniques have taken on varied and complicated aspects, using various preparations of the rumen liquor to be incubated.

In 1948 Marston described an artificial rumen consisting of a 3.5 liter glass pot for fermentation equipped with a stirrer and an outlet for gas. He

felt that this technique appeared to simulate more closely the natural environment, but pointed out one significant omission -- no provision was made for the removal of non-gaseous fermentation products as they accumulate, which might be expected to slow the rate of activity of the bacteria and eventually inhibit digestion. A year later Louw (1949) reported a rather elaborate device consisting of a glass jar suspended in a water bath at controlled temperature. Fermentation products dialyzed through a Visking casing suspended in the growth medium. Inlets and outlets were available for gassing, sampling, and addition of buffers. The system was kept in constant motion by a stirrer.

Burroughs, et al. (1950b) used an artificial rumen technique in studying roughage digestion of cattle feeds and urea utilization. They attempted to produce conditions which resembled as closely as practicable those found in the rumen of cattle. Arias, et al. (1951) further described their apparatus. Two water baths maintained at 39° C. were used simultaneously, each of which contained 6 immersed 1000 milliliter Erlenmeyer fermentation flasks that in turn were connected together with appropriate tubing for purposes of continuously bubbling carbon dioxide through the respective fermentation contents. This carbon dioxide stream maintained anaerobiosis and stirred the fermentation mass. These flasks were inoculated with microorganisms taken from live animals. Feed ingredients were added every 24 hours over a 4-5 day period similar to the feeding of the animals and materials were withdrawn at periodic intervals to maintain a constant volume of fermentating material at all times.

More recently Adler, et al. (1958) developed an apparatus with an incubation chamber with approximately constant volume of liquid and constant temperatures. It also 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 while a magnetic stirrer kept the

contents homogenous. 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 eliquots 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 approximate actual rumen conditions than other methods. It consisted of a porcelain test tube attached to a glass frothing tube and 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.

Warner (1956a) maintained that the criteria of normal rumen function which can be applied to in vitro work are as follows: maintainance of numbers and normal appearance of bacteria, selenomonads and protozoa of the rumen; maintainance of normal rates of digestion of cellulose, starch and protein; of normal interrelations between these; and the ability to predict quantitative results in vivo. To meet these criteria up to 8 hours at a time he used a cellophane sac containing rumen liquor and substrate dialysing against a complex mineral solution like that of the rumen, all incubated at 39° C. in an atmosphere of nitrogen and carbon dioxide. He stated further 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 techniques as an in vitro approach capable of rapid screening of nitrogen compounds as candidate nitrogen

feeds. Burroughs, et al. (1950a) states that the artificial rumen can best be used as a screening device in studying influential factors of feeds in rumen physiology, from which the most promising must be ultimately checked in animal experimentation. As the chief advantages of the artificial rumen these workers list (1) speed with which determinations can be carried out in a series of lab 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 expense for animals and the small amount of feed used in tests. These workers also see some limitations of this technique, expressing doubt that in vitro conditions are truly representative of those which occur in natural conditions in live animals. The saliva composition must be approximated, the end-products resulting can not be controlled and different types of organisms may develop in different runs of substrates. They expressed the idea that these limitations may be minor, however.

GENERAL METHODS AND PROCEDURE

Material of the Sac

The use of seamless regenerated cellulose dialysis tubing made into sacs and incubated in the rumen of fistulated steers was investigated in an attempt to provide an in vivo-artificial rumen combination for the study of protein synthesis in the rumen. The permeability of this dialysis tubing was described by the manufacturer[1] as having an average pore radius of 24 Å as determined by

[1]Visking Company, Chicago.

the rate of flow of water through the film. The tubing is permeable to water and will allow low molecular weight compounds in an aqueous solution to diffuse while refusing passage to higher molecular weight materials, such as proteins and bacteria. Tubing labeled by the manufacturer as C-85 and C-75 with a thickness between .0018 inches and .0023 inches and a width of three to four inches was used for making the sacs prior to incubation. The sacs were made of a length to allow for approximately 50-75 ml. capacity.

Formation and Incubation of the Sac

To completely seal the end of the tubing, it was tied with nylon cord at two locations approximately three-fourths inches apart at each end. Then to each end was applied a proper amount of plaster of paris which was allowed to dry into a hard lump effectively sealing the ends. As further precaution against leakage into or out of the sac, the ends covered with hardened plaster were dipped several times in melted wax to give them a tightly fitting wax cover. When it was necessary to inoculate the sacs after sterilization or to withdraw the contents of the sac aseptically, through a hypodermic needle, one end was fitted with a rubber serum stopper tied in with the nylon cord and sealed with plaster and wax. In all but the first of the trials requiring sterilization the sacs were sterilized by boiling for three to five minutes prior to incubation. In such cases the wax was applied immediately after boiling, as boiling temperatures would soften the wax.

In a preliminary trial a sac made by tying the wetted ends of the tubing into a knot was incubated directly in the rumen connected to a nylon cord tied to the cap of the rumen fistula. This proved unsatisfactory as sharp pieces of hay, etc., in the rumen ingesta pierced the sac and it was torn from the

string by movement of the ingesta. A copper wire basket constructed to hold the sac and protect it from tearing and piercing in the rumen during incubation proved satisfactory. An easier and more practical method was then developed using 250 ml. and 500 ml. polyethelene bottles to hold and protect the sac during incubation. Using a heated cork borer, holes approximately three-eighth inch in diameter were punched at regular intervals into the bottles. This in effect, served as a protective cover while allowing free movement of the liquid and smaller sized portions of the rumen ingesta through the sides of the bottle, enabling contact with the sac which was sealed with the plaster and wax as described above.

The sacs were incubated in vivo in the rumen of two sets of identical twin, fistulated steers. The steers were fed a ration consisting of five lb. of ground yellow corn, one lb. soybean oil meal, one lb. alfalfa hay and four lb. of prairie hay per head daily. In Trial VIII one steer from each pair was fed urea as a nitrogen source in place of the soybean oil meal.

Nitrogen Determinations

Determinations of urea nitrogen were made using a modified micro-Kjeldahl distillation apparatus developed in this laboratory. Samples were incubated in distillation flasks with urease at 39° C. for one to one and one-half hours to produce a breakdown of the urea. Sodium hydroxide was then added in the typical Kjeldahl distillation procedure and steam introduced into the sample to drive off the ammonia which was collected after condensation in boric acid containing a bromocresol indicator. This was then titrated with sulphuric acid.

Protein nitrogen was determined by the use of a micro-Kjeldahl apparatus

EXPLANATION OF PLATE I

Picture portraying the developmental history of the sacs in the experiment:

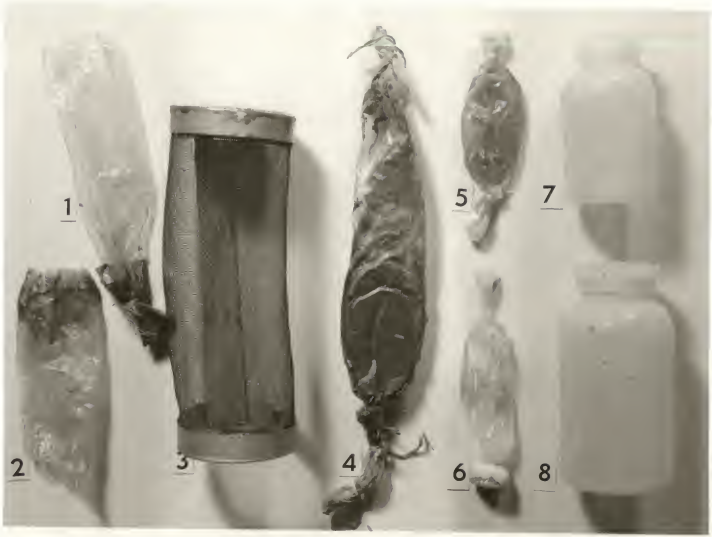
1 and 2. Original sacs put in the rumen which were torn from the nylon cord by rumen ingesta.

3 and 4. Protective wire basket and sac which it successfully held preventing damage to it.

5. Sac as finally prepared sealed with plaster of paris and wax containing rumen liquor sterilized by filtration.

6. Sac as finally prepared, fitted with serum stopper in lower end and sealed with plaster of paris and wax containing distilled water.

7 and 8. Polyethelene bottles as prepared and used to provide a protective cover for sacs during incubation.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after precipitation with 10 per cent trichloroacetic acid and subsequent separation by suction filtering. Protein precipitation appeared to be more consistent and complete with trichloroacetic acid than with tungstic acid from sodium tungstate and sulfuric acid.

In most cases the medium placed in the sac was rumen liquor as described below in the particular cases. Because of the heterogeneous nature of rumen fluid collected by squeezing through muslin, dialyzer tubing was also used in most cases to collect rumen liquor in a more homogeneous state. Using tubing measuring five inches wide and .0035 inches in wall thickness, large sacs were made and filled with distilled water and sealed as described earlier and put into 1 liter polyethelene bottles, prepared similarily to those previously mentioned. After three to four days of incubation in the rumen the sacs were found to be filled with a clear, homogeneous, amber colored liquid containing the various nutrients and compounds of small molecular weights which diffused into the sac.

TRIAL I

Experimental Procedure

The objective of Trial I was to discover if the dialyzing sac would still be intact after four days of incubation in the rumen. This trial consisted of four groups having three sacs in each group. In Group 1, three lengths of dialyzing tubing were soaked in 95 per cent alcohol for one hour, washed, and incubated in a polyethelene bottle for four days in the rumen. Group 2 had three pieces of tubing soaked in toluene for one hour, washed, and incubated similarily to those of Group 1. Group 3 consisted of threeepieces of tubing

soaked in distilled water for one hour before the four days incubation in vivo. In Group 4 were three lengths of tubing receiving no treatment or incubation. After the tubing of Groups 1, 2, and 3 were incubated four days, they, together with the tubing pieces from Group 4, were made into sacs. Into each sac was then added the albumin from one egg and a solution of sugar dissolved in water. Each sac was then placed in a water bath for 18 hours to give ample time for diffusion to take place.

Following the dialyzation period, the water of this water bath was tested for the presence of sugar by the Molish Test for carbohydrates, and the presence of protein by using the Biuret test.

Results and Discussion

In all four groups the water in which the sacs had been dialyzed gave a positive Molish test and a negative Biuret test. Evidence was thereby given that the dialysis tubing remained intact in the rumen for four days while retaining the properties of selective diffusion. The negative Biuret tests demonstrated the inability of large molecular weight substances, such as protein, to pass through the membrane while the positive Molish tests showed that the tubing remained permeable to smaller molecular weight substances such as simple sugars. Since the larger molecular weight substances as protein cannot pass through the tubing the inference was made that the tubing would also remain impermeable to the microorganisms of the rumen for periods up to 96 hours.

The alcohol and toluene methods of sterilization did not appear to have any differential effect upon the dialyzing properties of the tubing, when compared to each other and to the control Groups 3 and 4. However, sterilization by soaking in alcohol or toluene proved more tedious and more difficult to

keep the sacs sterile while filling with the desired medium and subsequent sealing of the end of the sac. The further possibility exists of some alcohol or toluene remaining in the sac and becoming toxic to bacteria with which the sac would be inoculated later. The membrane also seemed to harden somewhat and showed a tendency to easier cracking after soaking in these solutions. The tubing is manufactured to withstand the high temperatures of steam sterilization. Consequently, sterilization, when desired, was accomplished by boiling in subsequent trials.

TRIAL II

Experimental Procedure

Trial II was conducted to determine the possibilities of the use of the incubated dialysis sac for pure culture study. In this test there were three treatment groups of seven sacs each. The sacs in the first group were partially filled with 50 ml. of redistilled water and sterilized by boiling three to five minutes. These sacs were then inoculated with a hypodermic needle dipped in fresh rumen liquor, sealed and incubated in the rumen for periods of 30, 60, 90, and 120 minutes, 24, 48, and 72 hours. In the second group seven sacs were also sterilized by boiling and incubated without inoculation for the same periods of time in the rumen. The sacs in Group 3 were sterilized by boiling, inoculated similarily to Group 1 and incubated in distilled water at 40° C. in an anaerobic incubator[2] for similar periods of time.

At the end of the incubation periods the contents of each sac were

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[2]National Appliance Company, Portland, Oregon. Model 3640.

released asceptically into a small, sterilized vial and incubated in the anaerobic incubator at 40° C. for an additional 48 hours.

Results and Discussion

All of the sacs which were inoculated and incubated *in vivo* (Group 1) showed positive bacterial growth at the end of the 48 hours additional incubation *in vitro*. This growth was evidenced by a cloudy appearance and precipitate in the water in the vial with a white viscus membrane-like substance on the surface and walls of the vial.

On the contrary, those sacs incubated *in vivo* but not inoculated after sterilization (Group 2) showed no growth at the end of the subsequent 48 hours *in vitro* incubation. The liquid in the vials was transparent and clear but those incubated for increasing lengths of time showed a deeper yellowish color from the greater concentration of diffused substances from the rumen.

The sacs incubated *in vitro* in the incubator, even though inoculated after sterilization as in Group 1, showed no growth at the end of 48 hours subsequent incubation.

This trial was partially repeated using two uninoculated, sterilized sacs incubated *in vivo* for each of the various periods of time. The contents were then transferred asceptically into sterile vials and observed to be clear and transparent, lacking microbial growth. One vial of each time interval was then inoculated and all were incubated in the anaerobic incubator. Similar results were observed when growth appeared in the inoculated vials but failed to do so in those uninoculated.

As a further test of the ability of the sterilized sac to remain sterile during 96 hours of incubation *in vivo* contents of uninoculated sacs thus

incubated were used to inoculate sterile petri dishes containing nutrient agar and sterilized rumen liquor. After subsequent incubation in the anaerobic incubator no colonies of growth were observed on the agar, demonstrating the sterile properties of the sac.

From these trials it was concluded that the sac could be used in vivo to study pure cultures of specific species of bacteria without contamination by other organisms of the rumen. Further, it was concluded from the sacs incubated only 30 minutes in the rumen that periods as short as this are adequate to allow sufficient nutrients to diffuse into the sac from the rumen fluid to support growth of the microorganisms inoculated into the sac.

TRIAL III

Experimental Procedure

In order to compare the method in question with a conventional artificial rumen technique Trial III was conducted using pH changes in the sac as a basis of comparison.

A total of 42 sacs in four treatment groups comprised Trial III. Each sac contained 50 ml. of fresh rumen liquor strained through four layers of muslin. To each sac was added 60 mg. of urea as a substrate for microbial activity.

In Group 1, sacs were incubated in vivo for seven different periods of time, namely 20, 60, 120, 180 minutes, and 24, 48, and 72 hours. In Group 2 sacs were incubated in vivo for those periods of time after a potassium phosphate buffer was added to equal approximately 0.6 per cent of the solution in each sac. Group 3 consisted of sacs incubated for each of the same periods in

distilled water in the anaerobic incubator at 40° C. with no buffer added to the sacs. Group 4 consisted of sacs incubated with the potassium phosphate buffer for each of the similar periods of time in water in the anaerobic incubator also at 40° C. At the end of the incubation periods all sacs were opened and emptied into beakers and the pH of the contents tested and recorded. The pH of fresh rumen liquor samples taken directly from the rumen during this test was 6.60. The results of this trial are given in Table 1 and Figure 1.

Table 1. Average pH value of sac contents after incubation.

Incubation Period	In Vivo		In Vitro	
	Without Buffer	With Buffer	Without Buffer	With Buffer
20 minutes	6.95	7.40	7.20	7.80
60 minutes	6.98	7.03	7.15	7.50
120 minutes	6.80	6.90	7.15	7.40
180 minutes	6.60	6.88	7.13	7.10
24 hours	6.50	6.55	7.37	----
48 hours	6.05	6.35	8.10	7.85
72 hours	6.10	6.10	8.42	8.20

Nearly 50 readings other than those avereged in Table 1 of pH values of the contents of sacs incubated in vivo for periods up to 72 hours have been recorded in other trials. Similar results were observed where the pH value remained very near to that of normal rumen contents as long as 48 hours with a slight drop in value after 72 hours incubation.

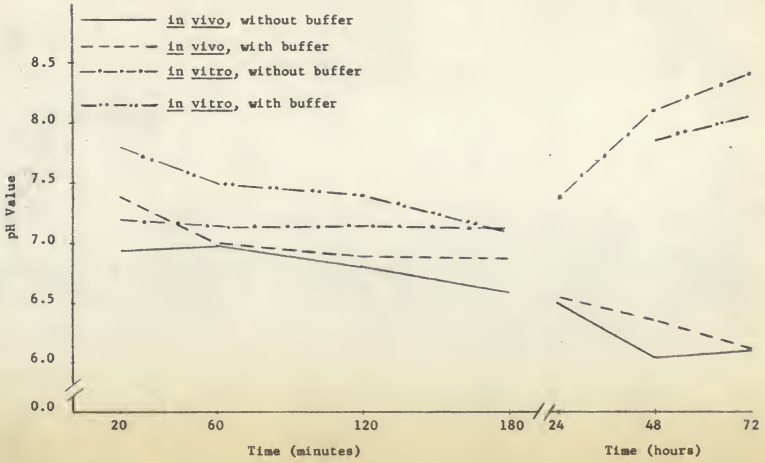

Figure 1. Average pH value of sac contents after incubation.

Results and Discussion

Very little change in pH value of the contents of the dialyzing sacs was observed in either of the groups incubated *in vivo* (Groups 1 and 2). At 24 hours the pH value was yet very near to that of the fresh liquor of the rumen. At the end of the longest period, 72 hours, the pH was becoming slightly more acidic. There was very little difference between Group 1 without the added buffer and Group 2 with the buffer added to the sacs, but the pH value of those with the buffer tended to be a little higher at the various levels, being slightly above neutrality after 20 and 60 minutes.

The sacs incubated *in vitro*, however, showed a marked increase in pH value. All values read above pH 7 and by the end of 72 hours read as high as pH 8.42. The difference between those with and without buffer in Groups 3 and 4 was marked, with a tendency for the pH to again be higher for those with buffer added at all time levels except after 180 minutes and 72 hours where the sacs without buffer were slightly higher.

It was concluded that in identical sacs incubated *in vivo* and *in vitro* the pH will remain more constant *in vivo* where the dialysation ection of the sac in the rumen helps maintain this constant pH level. Though the pH did become slightly more acidic than that of normal rumen contents at 72 hours, it was still not far outside the range of pH value needed for normal microbial growth given by Pearson and Smith (1943c). At the end of 24 hours of incubation the values were almost exactly those of the normal rumen. At the same time those incubated *in vitro* showed a rise in pH that would be too alkaline to remain within the normal range for rumen microbial growth. While little difference between sacs with buffer and those without buffer was observed *in vivo*, there was a more marked difference in this respect in those tested *in vitro*.

TRIAL IV

Experimental Procedure

In Trial IV and V an attempt was made to study the diffusion of small molecular weight substances through the dialyzing tubing. Urea was used in this case as a nitrogen source, and when put directly into the sac as a substrate for the microorganisms inoculated into the sac, it rapidly diffused out of the sac in about 2½ hours, thereby becoming unavailable to the microorganisms. Consequently, a method was developed to keep a source of nitrogen available to the microorganisms growing in the sac over a longer period of time.

As a preliminary effort in this direction 60 mg. of urea was placed in small packages made of dialyzing tubing several layers thick folded over and held shut by stapling. These small packages were then placed in the regular sacs with the idea that the urea would diffuse first out of the package and then become available to microorganisms in the sac. This method, though simple and fast, proved unsatisfactory as the holes made in the tubing by the staples allowed the dissolved urea to leak out into the sac very rapidly, showing little advantage over putting the urea directly into the sacs.

A more satisfactory procedure was then developed. Glass tubing one-fourth inch in inside diameter was cut into three inch lengths and each end was covered with several thicknesses of dialyzing tubing and sealed to the glass after the desired amount of urea and water was placed in the glass cylinder. The dialyzing tubing was cut into small squares and stretched tightly over the end of the glass cylinder. A small band made by cutting one-fourth inch rubber tubing into three-eights inch lengths was then stretched over the membrane around the end of the glass tube holding it stretched tightly over the end of

the glass. The membrane over the ends of the glass tubing was then covered to prevent its becoming waxed as the cylinder was dipped in melted wax to effect a seal where the membrane was held against the glass by the rubber band. The protective cover was then removed, exposing the membrane. In this way the urea was held in the glass cylinder and could escape only by diffusing through the membrane stretched over the open ends of the glass cylinder. This sealed glass cylinder was then placed in the sacs along with the inoculated medium where the urea could slowly diffuse out into the sac, thus becoming available to the microorganisms over a longer period of time.

Trial IV consisted of two groups differing only in the number of thicknesses of membrane covering the ends of the glass tubing in which 300 mg. of urea were placed. Group 1 had two layers of membrane over each end of the glass cylinders while Group 2 had four layers of the membrane over each end. These glass cylinders were each put into a sac with 120 mg. of potassium phosphate buffer and 25 ml. of rumen fluid collected by the use of a large dialyzing sac incubated in the rumen 72 hours as described earlier. This fluid was inoculated with one ml. of fresh rumen liquor strained through four layers of muslin cheesecloth. One sac from both groups was incubated <u>in vivo</u> for each of the following periods of time: 15, 30, 45, 105, 165, 225 minutes and 24, 48, and 72 hours. At the end of the incubation period the sac was opened. The glass tube was removed, opened, and carefully washed out into a micro-Kjeldahl distillation flask and the urea nitrogen was determined as described previously. The results of Trial IV are given in Table 2.

Results and Discussion

As can be seen from the data presented in Table 2 the method described

using the capped glass cylinders to make the nitrogen source available over a long period of time was successful. At the end of each successively longer period of time there was less urea nitrogen left in the cylinder demonstrating the prolonged diffusion through the membrane. Though the difference between the groups having two and four thicknesses of membrane over the glass tubing ends is slight, it is generally evident that the urea was retained longer and diffused slower in Group 2.

Table 2. Mg. urea nitrogen in glass cylinder after incubation.

	15 min.	30 min.	45 min.	105 min.	165 min.	225 min.	24 hrs.	48 hrs.	72 hrs.
Group 1 Two layers membrane over end of glass tube	130.4	128.2	126.1	114.9	106.7	104.8	21.0	4.9	2.0
Group 2 Four layers membrane over end of glass tube	136.4	136.2	128.6	113.7	116.8	98.7	37.2	9.9	2.6

TRIAL V

Experimental Procedure

Trial V was conducted largely as a repetition of Trial IV having two identical groups to give two replications in the determinations. Several time levels were deleted from Trial IV and others added to make Trial V.

A glass cylinder again containing 300 mg. of urea was put in each sac along with 50 ml. of rumen liquor collected and inoculated as in Trial IV. The in vivo incubation periods used in Trial V were 15, 30, 45, 60, 90, 120, 150 minutes and 24, 48, and 72 hours and the urea nitrogen remaining in the glass tube was again determined as in Trial IV. The results of Trial V are

presented in Table 3.

Table 3. Mg. urea nitrogen in glass cylinder after incubation.

	15 min.	30 min.	45 min.	60 min.	90 min.	120 min.	150 min.	24 hrs.	48 hrs.	72 hrs.
Group 1[1]	136.5	132.3	122.9	120.1	98.1	115.1	98.3	35.7	7.9	3.9
Group 2[1]	120.5	124.3	124.5	129.6	105.2	114.2	114.9	14.3	10.0	2.8

[1]Identical treatment in each group - four layers of membrane over ends of glass tube.

Results and Discussion

The results of Trial V presented in Table 3 are similar to those of Trial IV. With the exception of one low figure from the sac incubated for 90 minutes, the results of Group 1 show a very regular decrease in the amount of urea nitrogen left in the glass cylinder. Group 2 shows somewhat low figures for 15 minutes and 90 minutes incubation, but in general exhibits a general decreasing trend in urea nitrogen left in the cylinder. No buffer was used in the sacs in Trial V and the pH value of the sac contents after various periods of incubation were similar to those of Trial IV.

TRIAL VI

Experimental Procedure

Trial VI was conducted to study protein synthesis by bacteria inoculated into sacs incubated *in vivo* and to determine if conditions were favorable for

the microorganisms to grow in this environment. The trial consistad of three groups of sacs incubated in the rumen of the fistulated steers for various periods of time. The sacs in Group 1 and 2 contained 25 ml. of rumen liquor collected by the use of a large dialyzing tubing sac incubated 72 hours as described earlier. Each sac was inoculated with 1 ml. of fresh rumen liquor strained through four thicknesses of muslin. To each sac was added 120 mg. of potassium phosphate buffer and a glass cylinder containing 300 mg. of urea sealed as described earlier. The glass cylinders in the sacs of Group 1 were covered with two layers of dialyzing membrane and those in Group 2 with four thicknesses. The sacs in these groups were incubated in vivo for the following lengths of time: 15, 30, 45, 105, 165, 225 minutes and 24 and 48 hours.

Group 3 contained sacs filled with 50 ml. of rumen liquor similar to that of Groups 1 and 2 and inoculated in the same manner. Three hundred mg. of urea in glass cylinders sealed with 4 thicknesses of membrane at each end were enclosed in the sacs in each group. Two sacs were incubated in vivo for each of the following periods of time: 15, 30, 45, 60, 90, 120, and 150 minutes and 24, 48, and 72 hours.

At the end of the incubation periods each sac was opened and the contents carefully emptied and washed out of the sac. The pH of the sac contents was measured and the protein was precipitated and filtered with subsequent protein nitrogen determination being made as described earlier. Table 4 records the results of this test. The blank spaces in this table indicate that no incubation period of the particular level lacking was used in that group. The results are presented in this way to allow all of the results of Trial VI to be recorded in one table. Figure 2 gives the results of Groups 1 and 2; Figure 3 gives Group 3, parts 1 and 2.

Table 4. Mg. protein nitrogen in the sac at the end of incubation.

	15 min.	30 min.	45 min.	60 min.	90 min.	105 min.	120 min.	150 min.	165 min.	225 min.	24 hrs.	48 hrs.	72 hrs.
Group 1[1]	2.10	1.68	1.05	----	----	0.11	----	----	0.00	0.42	5.46	0.35	----
Group 2[2]	3.36	2.52	0.84	----	----	-0.21	----	----	-0.11	0.12	2.52	0.58	----
Group 3[3] Part 1	-0.11	0.32	0.11	0.53	0.11	----	-0.17	-0.06	----	----	0.11	0.17	0.04
Group 3 Part 2	0.00	0.11	0.11	0.11	0.11	----	-0.06	-0.17	----	----	-0.17	-0.11	-0.49

[1]Urea in glass tube with two layers membrane over ends of glass tube, 25 ml. rumen liquor in sac.

[2]Urea in glass tube with four layers membrane over ends of glass tube, 25 ml. rumen liquor in sac.

[3]Urea in glass tube with four layers membrane over ends of glass tube, 50 ml. rumen liquor in sac.

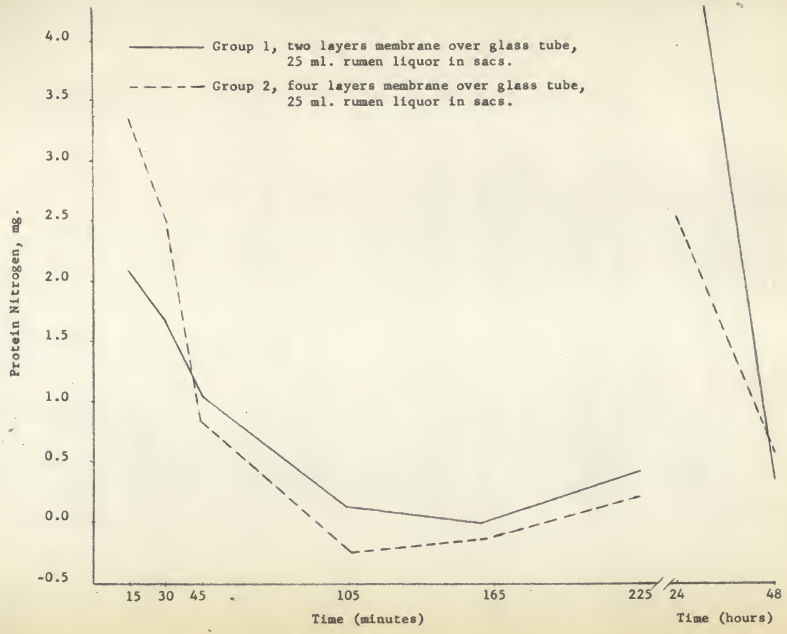

Figure 2. Protein nitrogen in sac at end of incubation.

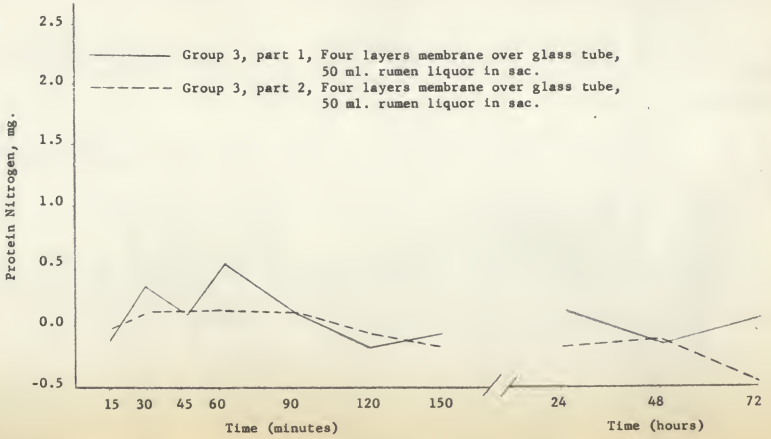

Figure 3. Protein nitrogen in sac at end of incubation.

Results and Discussion

The results of Trial VI are rather inconclusive regarding protein synthesis from the urea by the microorganisms inoculated into the sac. In some cases the values determined for the protein nitrogen in the sac at the end of incubation are below that in the blank determinations made from samples prepared identically to the incubation sac contents. The blanks were used as a measure of the protein contributed to the sac by the one ml. inoculation of rumen fluid at the beginning of incubation. Thus, the negative figures in the table and graph are explained as a result of substracting this initial blank value from the final value of the protein nitrogen. It is difficult to establish any definite trend in protein synthesis from these results. From Groups 1 and 2 it appears that there occurs an initial drop in protein content from the beginning of incubation until 105 minutes. Here a state of equilibrium is reached which was maintained through 48 hours of incubation, except for the high values at the end of 24 hours for which no explanation is available. The two parts of Group 3 show an initial rise in protein nitrogen and a subsequent decrease and low state of equilibrium similar to that of Groups 1 and 2. Though no buffer was used in Group 3 pH values remained similar to those of Groups 1 and 2. Further discussion on these results is given later.

TRIAL VII

Experimental Procedure

The objective of Trial VII was to again study the synthesis of protein in the dialyzing sac and to compare the two members of each of the two sets of

fistulated identical twin steers used in the test in this respect. Steers 1 and 2 were one set of twins and steers 3 and 4 were the other set of twins. All steers were set up identical in treatment to compare the twins and sets of twins against each other. Six sacs were used in each steer. Two sacs were incubated in the rumen of each steer for 12 hours and two for 24 hours. One sac was incubated for 36 hours and one for 48 hours. One of the two sacs incubated for 12 hours, and one of the two incubated for 24 hours contained 50 ml. of rumen fluid collected by the use of a large dialyzing sac incubated for 96 hours by the method described earlier. The other sac of each of these two periods of incubation and the sac from each of the two longer periods contained 50 ml. of distilled water. All sacs were inoculated with one ml. of fresh, strained rumen fluid from the rumen of the steer in which they were incubated. At the end of the incubation periods the contents of the sacs were emptied and the sac washed out. The protein was then precipitated, filtered, and determined as described previously. The results of this test are given in Tables 5 and 6 and Figure 4. In Tables 5 and 6 the blank values were not subtracted from the final figures but were recorded as the zero time value.

Table 5. Mg. protein nitrogen in the sac incubated with distilled water.

	0 hrs.	12 hrs.	24 hrs.	36 hrs.	48 hrs.
Steer 1	0.47	0.42	0.42	0.42	0.42
Steer 2	0.47	1.05	0.53	0.42	0.47
Steer 3	0.47	0.84	0.95	0.63	0.53
Steer 4	0.47	1.26	0.42	0.84	0.53

Table 6. Mg. protein nitrogen in sacs incubated with rumen liquor.

	0 hrs.	12 hrs.	24 hrs.
Steer 1	1.37	0.63	0.84
Steer 2	1.37	0.96	0.84
Steer 3	1.37	0.84	0.84
Steer 4	1.37	1.16	0.74

When each polyethelene bottle contsining a sac initially filled with the distilled water was withdrawn from the rumen at the end of the incubation periods a 50 ml. sample of rumen juice was taken from the location of the bottle in the rumen. This juice was strained through four layers of muslin and precipitated, filtered, and the protein nitrogen determination made by the Kjeldahl method. Table 7 gives the amount of protein in terms of mg. of protein nitrogen per ml. of the rumen fluid.

Table 7. Mg. protein nitrogen/ml. rumen fluid from rumen samples taken at time and location of withdrawal of sacs from rumen.

	9:00 p.m. End of 12 hr. period	9:00 a.m. End of 24 hr. period	9:00 p.m. End of 36 hr. period	9:00 a.m. End of 48 hr. period
Steer 1	0.65	1.22	1.16	1.41
Steer 2	1.21	1.39	0.64	1.02
Steer 3	0.90	1.30	0.46	1.06
Steer 4	1.01	1.47	0.91	1.37
Averages	0.94	1.35	0.79	1.22

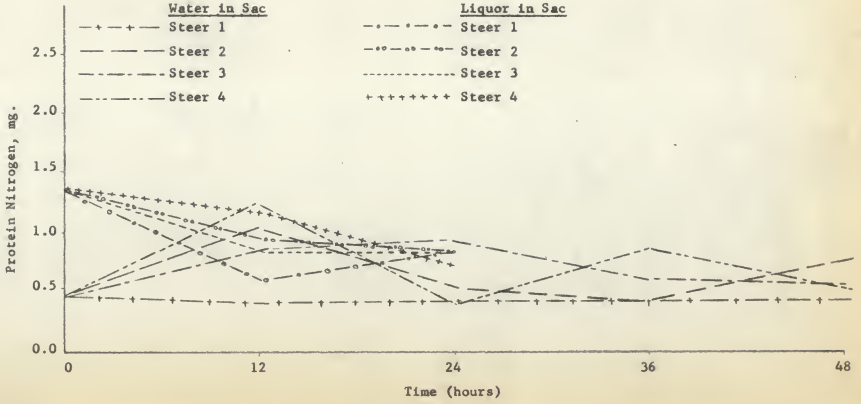

Figure 4. Protein nitrogen in sac at end of incubation.

Results and Discussion

As in Trial VI it is rather difficult to distinguish any definite trend in protein synthesis in Trial VII. There also does not appear to be any consistent appreciable difference between the steers in the two sets of twins nor any comparison that can be made between the steers within each set of twins. However, steer 2 did appear to show values somewhat higher than his twin, in this respect. The sacs incubated with rumen liquor showed no higher values for protein nitrogen than those incubated with distilled water. As indicated in Tables 5 and 6, all the values recorded for the sacs with the rumen liquor were below those having distilled water when compared to the respective blanks at zero level. No satisfactory explanation for the higher blanks recorded in Table 6 is available. Several possible explanations for the lack of microbial synthesis of protein in the sacs in Trials VI and VII are discussed later.

As evidenced in Table 7 no apparent correlation existed between the amount of protein nitrogen synthesized in the sac and the amount of protein nitrogen in the rumen ingesta itself.

TRIAL VIII

Experimental Procedure

Trial VIII was conducted in an effort to determine if the apparent lack of bacterial protein synthesis in the sac in previous trials was due to a lack of available nitrogen or available energy or both.

For this test the steers were numbered A through D with steers A and D comprising one set of twins and steers B and C comprising the other set. One

steer from each set of twins, numbers C and D, were fed a urea supplement for approximately two and one-half weeks prior to and during the incubation test period. For these two steers one-half pound of corn and 60 grams of urea were used to replace the soybean oil meal of the previously described ration with the rest of the ration remaining unchanged. The purpose of feeding the urea was to increase the concentration of non-protein-nitrogen in the rumen available to diffuse into the sac to provide the nitrogen needed for bacterial synthesis of protein. To act as a control, the other steers of each pair of twins, numbers A and B were continued on the original ration as set forth earlier.

This trial consisted of six treatment groups of four sacs each. One sac was incubated for 12 hours and one for 24 hours in the rumen of each of the two steers comprising each group. All of the sacs were inoculated with one ml. of fresh rumen fluid strained through muslin. A sample of rumen liquor from each of the two steers receiving no urea was mixed to provide the inoculation material for the sacs incubated in the rumen of these steers. In a similar manner inoculation material was prepared from rumen liquor of the two steers receiving urea in the ration. This was done in order that the bacteria inoculated into the sacs would be accustomed to the environment of the rumen ingesta in which the respective sacs were incubated.

In Group 1 the contents of the sacs consisted of 50 ml. of distilled water with ten mg. of corn starch added as an increased source of energy for bacterial growth. The sacs in Group 2 were filled with 50 ml. of rumen liquor collected over a 72 hour period as described and used earlier. Ten mg. of starch was also added to the sacs in this group. Group 3 was a replication of Group 1 with the exception that the sacs in this group were incubated in the rumen of steers receiving urea in the ration. Group 4 was designed as a control

for Group 3 as the sacs contained 50 ml. distilled water with no starch added. Group 5 was a replication of Group 2, again with the exception that the sacs were incubated in the rumen of steers fed urea. Finally Group 6 acted as a control of Group 5 with the sacs containing 50 ml. of rumen liquor as in Group 5, but with no starch added. The sacs in Groups 1 and 2 were incubated in the rumen of steers receiving no urea and those of Groups 3, 4, 5, and 6 in the rumen of steers receiving urea in the ration.

At the end of the incubation periods the sacs were removed from the rumen and the contents were washed into flasks for the precipitation of the protein which was subsequently determined as described earlier.

The results of Trial VIII are presented in Table 8 and Figure 5. The figures in the seventh and ninth columns of this table are the average values in terms of mg. of protein nitrogen for each of the treatment groups. The fifth column of this table gives the blank values for each of the treatment groups. These blanks are a measure of the mg. of protein nitrogen in the sac at the initiation, or zero time, of the incubation periods. The origin of this protein is the one ml. of rumen fluid used to inoculate each of the sacs, and was determined from samples prepared similar to the contents of the sacs in each treatment group. These blank values are used in Figure 5 as the initial base level of protein nitrogen at zero time from which to chart the values after 12 and 24 hours incubation.

Results and Discussion

From the data presented in Table 8 the difficulty of determining any definite trends in bacterial protein synthesis is again seen. However, the results are somewhat more encouraging than those of previous trials.

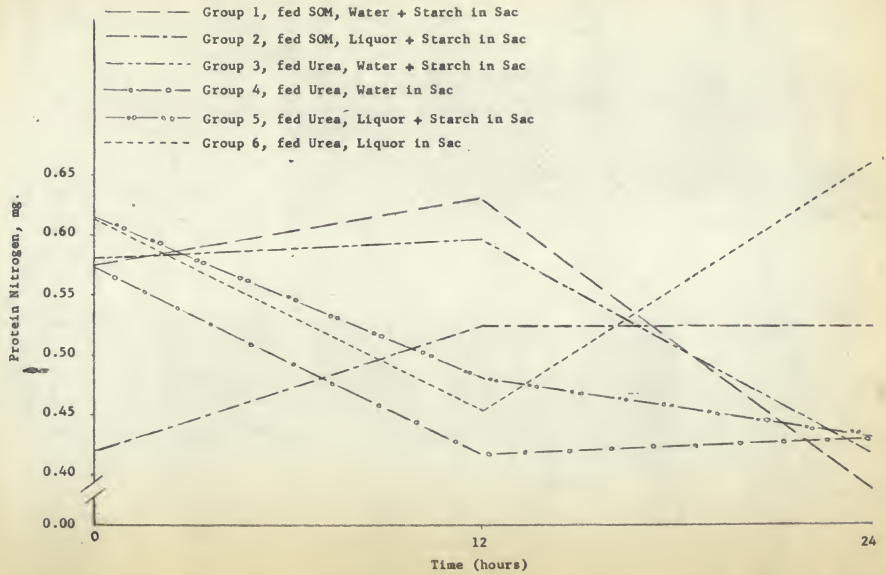

Figure 5. Average protein nitrogen in sac at end of incubation.

Table 8. Mg. protein nitrogen in sac at end of incubation.

Steer	Group	Added Nitrogen Source	Sac Treatment	Blank Value	12 Hours	Group Average	24 Hours	Group Average
A	1	Soybean oil meal	Water + starch	0.575	0.609	0.630	0.420	0.389
B	1	"	"	0.575	0.651		0.357	
A	2	"	Liquor + starch	0.420	0.420	0.525	0.525	0.525
B	2	"	"	0.420	0.630		0.525	
C	3	Urea	Water + starch	0.578	0.696	0.596	-----	0.420
D	3	"	"	0.578	0.442		0.420	
C	4	"	Water	0.578	0.273	0.420	0.483	0.431
D	4	"	"	0.578	0.567		0.378	
C	5	"	Liquor + starch	0.619	0.546	0.483	0.441	0.431
D	5	"	"	0.619	0.420		0.420	
C	6	"	Liquor	0.619	0.525	0.456	0.420	0.651
D	6	"	"	0.619	0.378		0.882	

In comparing Groups 1 with 3 and 2 with 5 there appears to be no increase in bacterial growth in the sac due to feeding urea to the steers, as the results are inconsistent, in this respect.

By comparing Group 1 with 2, 3 with 5, and 4 with 6, it can be seen that rumen liquor in the sacs does not consistently effect a higher rate of protein synthesis than distilled water. However, there does appear to be some effect upon growth of the microorganisms in the sac from added starch. Although no direct control for Groups 1 and 2 was included in this respect there is an

appreciable initial increase in both of these groups in protein nitrogen after 12 hours incubation. Similar results are observed when Groups 3 and 4 are compared in this respect. After 12 hours incubation, the average value for the sacs incubated with added starch was decidedly higher in protein nitrogen. Results are much the same for Group 5 compared with 6, again showing an advantage after 12 hours incubation for the sacs with added starch.

The drop or leveling off in protein nitrogen level in the sacs from 12 to 24 hours incubation which occurred in four of the groups is not completely understood. It is probable that, with the amount of energy available in this test, synthesis in the sac occurs at or before 12 hours. Further indication of an earlier peak is given in previous trials.

TRIAL IX

Experimental Procedure

The object of Trial IX was to test further the addition to the sacs of energy in the form of corn starch in an effort to stimulate bacterial growth. Various levels of starch were used in an attempt to determine the optimum level of energy.

This trial consisted of four groups of six sacs containing 50 ml. of distilled water each. Two of the four steers used in this trial were fed the ration containing soybean oil meal and two the ration using urea for the nitrogen supplementation as described in Trial VIII. One ml. of a mixture of strained rumen fluid from the two steers on each of the rations was used to inoculate the sacs incubated in the rumen of the steers, as done in Trial VIII. The sacs of Group 1 were incubated 12 hours in the rumen of one of the steers receiving

soybean oil meal and the sacs of Group 2 were incubated 24 hours in the rumen of the other steer on this ration. Similarly the sacs of Group 3 were incubated 12 hours in the rumen of one of the steers receiving urea in the ration, and those of Group 4 were incubated 24 hours in the rumen of the other steer on the urea-containing ration.

The treatment of the sacs, other than the length of incubation, was the same in each group. The first sac of each group contained only the inoculated distilled water. To the second was added 100 mg. of starch; to the third 200 mg.; to the fourth 500 mg.; to the fifth 1000 mg.; and to the sixth was added 2000 mg. of starch. The sacs were then sealed as described earlier. At the end of the incubation period the usual protein nitrogen determination was made.

The results of Trial IX are given in Table 9 and Figure 6. The blank values given in this table are again a measure of the initial protein nitrogen in the sacs at the beginning of incubation and were not substracted from the subsequent figures. The starch used in this trial was found by the Kjeldahl method to contain 0.06 per cent protein nitrogen, and this nitrogen value was substracted from the final figures for the sacs containing added starch. Thus the values given are corrected to actual protein nitrogen in the sac other than that from the starch which was added.

Results and Discussion

From the results of the trial it is obvious that the lack of protein synthesis in previous trials was due, at least in part, to an energy deficiency of the microorganisms in the sac. As evidenced in Table 9, all of the sacs containing starch showed values higher than that of the blanks, both after 12

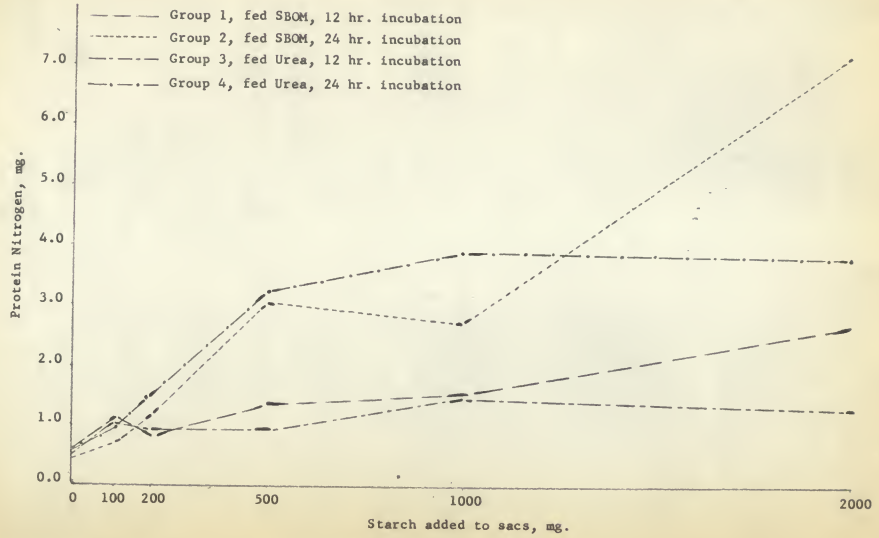

Figure 6. Protein nitrogen in sacs at end of incubation.

and 24 hours incubation. In all cases the sacs with no starch added remained at or fell below the blank value indicating a breakdown of protein in the absence of sufficient energy.

Table 9. Mg. protein nitrogen in sac at the end of incubation.

Group	Added Nitrogen Source	Incubation Time	Blank Value	No Starch	100 mg. Starch	200 mg. Starch	500 mg. Starch	1000 mg. Starch	2000 mg. Starch
1	SBOM	12 hrs.	0.651	0.567	1.116	0.846	1.359	1.542	2.622
2	SBOM	24 hrs.	0.651	0.420	0.675	1.182	3.060	2.697	7.137
3	Urea	12 hrs.	0.504	0.462	1.011	0.867	0.897	1.437	1.257
4	Urea	24 hrs.	0.504	0.504	0.927	1.497	3.207	3.852	3.756

There is a direct relationship between the amount of protein nitrogen in the sacs after incubation and the amount of starch added. Though there are several low values, the general trend is for an increased protein synthesis with increasing amounts of starch added to the sacs. The groups incubated in the rumen of steers fed soybean oil meal (1 and 2) showed a continued rise in protein synthesis up to the 2000 mg. level of starch. The peak for the sacs incubated in the rumen of steers fed urea (3 and 4) appears to be at the 1000 mg. level of added starch.

In comparing the groups incubated for 24 hours (2 and 4) with those incubated 12 hours (1 and 3) a decided increase in protein nitrogen is observed, indicating a continued synthesis of protein over the 24 hour period. The feeding of urea again appears to have no beneficial effect on the growth of the microorganisms in the sac (comparing Groups 1 and 2 with 3 and 4).

GENERAL DISCUSSION

In this experiment it was shown that incubation sacs made of dialyzing tubing remained intact as long as four days while incubated in the rumen of fistulated steers. This is in agreement with the findings of Louw, et al. (1949) who found no microscopic evidence of attack by ruminal organisms on the Visking casing used in an artificial rumen. Though the manufacturers state that bacteria may attack the cellulose from which the tubing is constructed, Louw, et al. (1949) stated that the observed lack of microbial attack may be explained on the basis that attack on cellulosic materials usually begins at broken or torn ends and no such surfaces are exposed when the tubing is made into a closed sac. Evidence that the ruminal microorganisms may eventually attack the material of the sac is given by a sac left for eight days in the rumen. Upon opening the polyethelene bottle containing it, the sac was observed to be almost completely digested except at the ends near the plaster and wax seal.

When urea was added to the sacs some deterioration was observed in some cases at the end of four days of incubation. A chemical problem was suspected in this case rather than microbial attack, as the sacs were generally in good condition after 96 hours in the rumen when no urea was added.

Thus it appears that this method could be used for the studies using pure cultures as the sacs can be kept sterile and nutrients will diffuse into them. The lack of microbial growth in sterilized sacs inoculated and incubated in vitro in distilled water is to be expected as there were no nutrients available to support bacterial growth in the sac. On the contrary, the diffusion of nutrients into the sacs incubated in the rumen materially increases its resemblence to actual conditions. However, it is still not known whether the nutrients

diffused into the sac support the growth of all rumen populations or a part of the populations; all the activities of all the populations or some activities of some populations.

Warner (1956a) lists a suitable pH value as one of the necessary criteria for normal function of an in vitro artificial rumen. The same is true for sacs incubated invivo and this further resemblence to actual rumen conditions is provided by the normal pH value maintained within the sac for incubation periods of 24 hours or longer. Thus no detrimental effects upon protein synthesis in the sac should be experienced due to pH changes.

Pearson and Smith (1943c) list the normal range in pH of the rumen as 6.3 - 7.4 which includes all of the values recorded in this experiment up to 24 hours of incubation.

It was concluded that on the basis of pH changes in the sac studied, the technique of incubating these sacs in vivo has considerable advantages over the conventional in vitro method. This is attributed to the ability of the buffering action of the rumen to keep the inside of the sac nearly normal by dialyzation.

Warner (1956a) also lists a suitable temperature as a requirement for normal artificial rumen function. It is quite obvious that the temperature of the sacs incubated in the rumen would be held at the exact temperature of the rumen itself.

Belasco (1954) found urea to be particularly suited as a feed ingredient having a high nitrogen content and biological availability. He stated that the amount that could be used in a feed is limited by the rapid rate at which it can be converted to ammonia in the rumen. Of a similar problem was the rapid diffusion out of the sac of the urea placed there as a substrate material for bacteria in the sac. This problem was overcome to a large extent by

placing the urea in glass tubes fitted with the diffusing membranes over the ends, thus retaining the urea and making it available over a longer period of time.

The disappointing lack of consistent protein synthesis in the sacs in Trial VI may be due to a number of individual factors or combination of factors. In this trial urea diffused out of the glass tube into the sac as an added nitrogen source for the bacteria. The steers involved, however, were not fed any urea prior to, or during the incubation test periods. Werner (1956a) states that the substrate must approximate in nature 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. This obviously would not be the case in this trial as no preliminary period of time was given for the microbial population of the rumen to become accustomed and adjust to urea as a substrate in the rumen ingesta. Thus it is probable that the bacteria in the rumen fluid used to inoculate the sacs found the urea in the sac unsatisfactory as a substrate, and possibly some toxic effect was present from the urea.

That rumen organisms do thrive and function actively in the sac while incubated in the rumen is evidenced by a preliminary test run at the initiation of the experimental work herein presented. About 100 grams of rumen ingesta, including coarse pieces of hay, etc., were put in a sac and the sac inserted into the rumen in the protective wire basket mentioned earlier. At the end of 72 hours incubation the contents of the sac was observed to be reduced into a fine pulp with no coarse pieces of hay remaining. The possibility exists that this digestion was due to the large number of ruminal organisms included in the 100 grams of ingesta put into the sac, and that the one ml. inoculations of strained rumen liquor used in later trials did not contain a sufficient population to effect an amount of protein synthesis detectable by the laboratory

methods employed. It is also likely that the 100 grams of rumen ingesta put into the sac mentioned above would have a large amount of carbohydrate to support microbial growth. The results of Trial IX where added starch produced a substantial increase in growth and protein synthesis indicated that an energy deficiency was responsible for the lack of growth in Trials VI, VII, and VIII. Warner (1956a) pointed out that the maintainance of numbers and normal appearance of bacteria, salenomonads and protozoa of the rumen is another criterion of normal rumen function which can be applied to in vitro studies.

In Trial VI and the subsequent trials some of the values for protein nitrogen are lower at the end of incubation than that given for the initial blank value of protein nitrogen in the sac from inoculation. This could be explained by an initial proteolysis of microbial and other protein in and subsequent diffusion out of the sac.

No urea was fed to the steers in Trial VII and in further an effort to maintain a compatable relationship between inoculum and substrate inside the sac, the sacs were inoculated with rumen liquor from the specific rumen in which they were to be incubated.

It was expected that the sacs in this trial containing rumen liquor would support a greater microbial population and greater protein synthesis at the initiation of the incubation period than those containing distilled water. The reason for this thinking was the more immediate availability of nutrients already in the sac at the beginning of the incubation period before diffusion began into the sacs of water. This, however, failed to be the case.

A probable explanation is that any nutrients contained in the rumen fluid would after a short time also diffuse into the sac containing water. As demonstrated in Trial II, periods of incubation as short as 30 minutes are adequate to allow sufficient nutrients to diffuse into the sac from the rumen fluid to

support some growth of certain of the microorganisms inoculated into the sac.

Whether or not there should be any correlation between the amount of protein nitrogen synthesized in the sac and the amount of protein nitrogen in the rumen ingesta itself is questionable. The possibility existed, however, that a particularly high concentration of protein nitrogen in the rumen ingesta surrounding the polyethelene bottle containing the sac might have an indirect connection with similar levels in the sac. If conditions in the rumen stimulated a high level of bacterial growth and synthesis, thus a high concentration of protein nitrogen, it was thought possible that to some extent the same conditions would exist inside the sac. One factor producing such a condition could be a high amount of nutrients available for bacterial growth in the rumen which could then diffuse into the sac to produce a similar condition within the sac. As mentioned earlier, however, such a correlation did not appear to exist.

Of interest is the fact that there is appreciably more protein nitrogen in the samples withdrawn from the rumen at 9:00 a.m. before feeding than at 9:00 p.m. several hours after feeding. This is explainable in the fact that soon after feeding much of the protein nitrogen is in the form of feed ingested, and being in coarser particles would not be strained through the cheese cloth into the sample upon which the determination was made. On the contrary, when a longer time lapse after feeding occurred a greater percentage of the protein nitrogen of food matter would have been broken down into fine particles and also incorporated into microbial protein. These minute particles and microorganisms could then pass through the cheese cloth increasing the protein content of the sample thus obtained.

The values recorded for the protein nitrogen in the rumen fluid are in fairly close agreement with the figures of 50 - 110 mg./100 ml. given by McDonald (1952).

It was suspected that one factor contributing to the absence of protein synthesis in Trials VI and VII was the lack of sufficient nutrients available for the bacteria in the sac. The supplement of urea fed as one consequence of this question did not appear to affect a higher rate of bacterial growth in Trial VIII. Pearson and Smith (1943b) state that the first step in urea utilization in the rumen of dairy cattle is the conversion of urea to ammonia. They believe that any amount of urea fed is likely to be converted into ammonia in one hour. If this is the case the greater concentration of non-protein nitrogen in the rumen desired in Trial VIII from the supplemented urea would largely be in the form of ammonia. It is possible that this ammonia then would not diffuse into the sac. As a consequence, the non-protein nitrogen concentration would not be increased within the sac, and no increase in bacterial growth would be effected. However, whether the ammonia diffused into the sac or not, the results of Trial IX indicate that nitrogen was not the limiting factor. Rather, as discussed later, an energy deficiency accounted for the lack of protein synthesis in these sacs.

Pearson and Smith (1943c) found protein synthesis to be increased in rumen fluid incubated in vitro by the addition of non-protein nitrogen. The maximum amount of synthesis was obtained by these workers when the added urea nitrogen amounted to 75 or 100 mg. per 100 grams of rumen liquor.

Pearson and Smith (1943c) also report that with in vitro incubation of rumen fluid they were able to cause synthesis of protein to dominate over hydrolysis by the addition of three to ten grams of starch per liter of rumen liquor. However, during the in vitro incubation used by these investigators no nutrients other than those present in the liquor at the initiation of the incubation could become available for bacterial growth without outside addition. This is not wholly the case with sacs incubated in vivo as nutrients may pass

directly into the sac by diffusion from the rumen ingesta. It is possible, however, that this diffusion of nutrients into the sac may not be sufficient to maintain adequate growth, as indicated by the slight stimulus given to protein synthesis by the addition of ten mg. of starch to the sacs in Trial VIII. It was suggested that this stimulus could be increased materially by the addition of greater amounts of starch to the sacs. This suggestion led to Trial IX which gives conclusive evidence that the previous lack of protein synthesis was due to a deficiency of energy.

The results of Trial IX indicate that the ten mg. of starch added to the sacs in the previous trial provided a very inadequate amount of energy. The optimum level of added starch in Trial IX appeared to be somewhere between 1000 and 2000 mg. for 50 ml. of distilled water inoculated with one ml. of strained rumen fluid. The ten mg. of starch used in Trial VIII was sufficient to cause a slight increase in protein synthesis during 12 hours of incubation, but was not adequate to cause synthesis to predominate over hydrolysis for longer periods of time. As mentioned earlier Pearson and Smith (1943c) found that the addition of non-protein nitrogen and a carbohydrate source were effective in causing synthesis to predominate over hydrolysis in in vitro incubation of rumen fluid. In the light of their work and the results of Trial IX, it seems likely that in all the sacs without added starch hydrolysis dominated at certain times causing a low final protein nitrogen value and resulting low synthesis value.

Another difference between the incubation of strained rumen fluid as done by Pearson and Smith (1943c) and rumen liquor collected as described in this work with dialysing tubing needs to be pointed out. A vastly greater number of microorganisms exist in the strained fluid than in the liquor collected by dialysation inoculated with only one ml. of strained rumen fluid. However, as

indicated in the last trial, with adequate energy, one ml. of inoculation material appears sufficient to produce an appreciable increase in protein nitrogen in the sac.

A further possible explanation for the lack of protein synthesis in the sacs in Trials VI, VII, and VII is the lack of proper removal of metabolites and a suitable gas phase which are also listed by Warner (1956a) as requirements for proper functioning of an artificial rumen. Upon removal from the rumen after incubation the sacs were usually turgid and the blank space filled with gas. This production of gas is in itself evidence of microbial growth, but an over accumulation in the sac could hinder further growth of the organisms concerned. It is thought, however, that the selectively permeable sac allows the elimination by diffusion of the fermentation products, thereby approaching the situation in the rumen where the end products of fermentation are absorbed or pass out. It is possible that a vent for accumulated gas could be attached to the sac by means of rubber tubing arranged to prevent the entrance of oxygen into the sac. The artificial rumen technique developed by Fina, et al. (1958) includes such a feature. This problem could also be alleviated to some extent by making the sac larger to allow more potential space to hold the gas. However, the effect of such an accumulation of gas upon bacterial action may be slight, especially since synthesis was appreciably increased by the addition of starch in Trial IX.

The technique described for the study of protein synthesis appears to have certain advantages over some of the more commonly used artificial rumen techniques. Marston (1948) noted that a glass fermentation pot made no provision for the removal of non-gaseous fermentation products which might upon accumulation be expected to slow the rate and eventually inhibit the activity of rumen organisms. Wegner, et al. (1940) had similar criticism of an

artificial rumen technique lacking diffusion. In contrast the selectively permeable sac allows the elimination by diffusion of fermentation products, thereby more nearly duplicating the situation in the rumen where the end-products of fermentation are absorbed or pass out. Though the apparatus described by Marston (1948) had a stirrer, Wegner, et al. (1940) criticized their artificial rumen for having no movement. Stirring is obviously adequately accomplished as the polythelene bottle containing the sac is carried about in the rumen by the movements of the rumen ingesta.

Burroughs, et al. (1950a) mentions as one of the chief advantages of the artificial rumen technique, the precision which can be exercised over various conditions in the laboratory which are left to chance in experimental studies using live animals. With the technique described herein it appears possible to carry some of this precision control which they mention into in vivo work. The amount and type of substrate and bacteria involved can be carefully controlled by the use of the sac.

There is an unfortunate gap in methodological approaches to rumen microflora studies, and according to Alstad (1958) it lies in the fact that none of the techniques in current general use satisfactorily cover the interaction involved between various representatives of the rumen population. Doetsch and Robinson (1953) also mention the lack in knowledge in the field of relationships of specific bacteria to vitamin synthesis, minerals, protein synthesis and antibiotics. They state further that the synergistic reaction and antagonisms are also not known in this connection. There is also little data on microorganisms responsible for urea breakdown in the rumen (Hobson, 1958). Once basic mechanisms are found to occur, organisms specifically capable of carrying them out may be studied (Sirotnak, et al. 1953). However, Annison and Lewis (1959), p. 38, state that it is often difficult to decide whether or not

an individual rumen organism has a significant role in rumen metabolism. They explain that certain in vivo studies can throw light on the over all fermentation pattern but not on the activities of the individual microorganism.

That the sac herein described can be kept sterile during in vivo incubation is significant in the light of the foregoing discussion concerning the gap in methodological techniques. The study of pure cultures by inoculating sterilized sacs with specific organisms prior to incubation could apparently be used to give additional information regarding the problems posed here.

SUMMARY AND OBSERVATIONS

A new method for the study of protein synthesis in the rumen has been developed. The technique consists of the use of sacs made of dialyzing tubing incubated in the rumen of fistulated steers. The sacs were filled with distilled water or rumen liquor sterilized by filtration and inoculated with strained rumen fluid. Various microbial substrates can be added to the sacs prior to incubation. The selective permeability of the sac allows the diffusion out of the sac of certain metabolism end products. At the end of incubation the contents of the sacs were removed and the several nitrogen determinations made.

The ability of the sacs to withstand sterilization by alcohol, toluene and boiling and to remain intact in the rumen as long as 96 hours was demonstrated. Sealed with plaster of paris and wax, the sacs were shown to remain sterile during in vivo incubation while maintaining normal pH values, temperature and stirring. The possibility of pure culture studies is hereby advanced.

A technique was developed to make urea or other nitrogen source material available to the bacteria inoculated into the sac over an extended period

of time. The urea was placed in three inch lengths of one-fourth inch diameter glass tubing. Over the open end of these cylinders varying thicknesses of dialyzing membrane were stretched, held in place by rubber bands made by cutting three-eighth inch lengths of one-fourth inch rubber tubing, and sealed to the sides of the cylinders with wax. These glass cylinders were then placed within the sacs before sealing and incubation. The urea was then able to diffuse out over a period of time into the sac to become available to microorganisms in the sac. That the urea was held within the cylinder longer and the diffusion rate slower when using four thickness of membrane as compared to two thicknesses was shown.

Four trials (VI, VII, VIII, IX) were conducted to study protein synthesis by bacteria inoculated into sacs incubated in vivo and to determine if conditions were favorable for microbial growth within this environment. The results of the first three of these trials are rather inconclusive regarding protein synthesis. In the first of these trials (VI) there appeared to be an initial drop in protein nitrogen in the sacs for approximately two hours or more. After this a slight increase in protein nitrogen was generally noted followed by an apparent leveling off into a state of equilibrium which was maintained during 48 hours incubation.

The results of the second of these three trials (VII) failed to give any conclusive evidence regarding difference in protein synthesis in the sac between pairs of twin steers or between individuals within a set of twins.

There appeared to be no correlation between the amount of protein nitrogen synthesized in the sac and the amount of protein nitrogen in the rumen ingesta at the time and location of the removal of the sac from the rumen. Appreciably more protein nitrogen was noted in the samples withdrawn from the rumen at 9:00 a.m. before feeding than at 9:00 p.m. several hours after feeding.

In the third of these trials (VIII) no evidence is given of increased synthesis in the sac from feeding urea in an attempt to increase the concentration of non-protein nitrogen in the rumen. However, an increase in protein synthesis during the first 12 hours of *in vivo* incubation was effected by adding corn starch to the sac prior to incubation.

In the last of these trials (IX) the addition to the sacs of greater amounts of starch was found to substantially increase protein synthesis. In this trial appreciable protein synthesis was maintained through 24 hours of incubation.

In several trials rumen liquor placed in the sac at the initation of incubation did not consistently effect a higher rate of protein synthesis than did distilled water.

It was concluded that the method described may yield significant information regarding protein synthesis in the rumen and specific organisms involved. The sac described meets many of the criteria for a successful artificial rumen (Warner, 1956a) such as temperature, pH and removal of at least some of the metabolites while adding the decided advantage of *in vivo* incubation.

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A NEW TECHNIQUE FOR THE STUDY OF PROTEIN SYNTHESIS IN THE RUMEN

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The use of seamless regenerated cellulose dialysis tubing made into sacs and incubated in the rumen of fistulated steers was investigated in an attempt to provide an *in vivo*- artificial rumen combination for the study of protein synthesis in the rumen. Tubing with a thickness of between 0.0018 and 0.0023 inches with a width of three to four inches was tied off into lengths to allow for 50-75 ml. capacity. After being filled with the desired substance, they were tied with nylon cord and sealed with plaster of paris and wax. The sacs were sterilized when desired by boiling and inoculated with strained rumen liquor. The sacs were filled with distilled water or sterile rumen liquor collected by filtration through similar dialyzing membrane. At the end of incubation periods of varying lengths, the contents of the sac were removed and analyzed for urea or protein nitrogen.

It was demonstrated that the sterilized, sealed sacs remained sterile during *in vivo* incubation up to 96 hours, while maintaining the properties of selective diffusion. The possibility of pure culture studies is therefore suggested. It was also shown that the pH value within the sac remained very close to that of normal rumen liquor up to 24 or 48 hours incubation, increasing its similarities to actual rumen conditions.

A method was developed to provide a source of nitrogen to the bacteria in the sac over an extended period of time. One-fourth inch diameter glass tubing was cut into three inch sections. The cylinder thus formed was filled with urea and fitted and sealed with varying thicknesses of dialyzing membrane over the ends which allowed the urea to diffuse into the sac in which the cylinder was placed.

Trials were conducted to study protein synthesis by microorganisms inoculated into the sac filled with water or rumen liquor. The amount of protein nitrogen in the sacs before and after incubation was determined. The results

of several of these trials failed to show any great increase in the amount of protein nitrogen during incubation and were rather erratic. An initial drop in protein nitrogen during two hours incubation was observed with a slight increase thereafter and subsequent state of equilibrium being reached at the end of 12 hours of incubation. However, in the final trial the addition of starch to the sacs was shown to substantially increase protein synthesis. This synthesis was maintained and continued to increase over 24 hours of incubation.

The addition of urea to the ration of the steers in an attempt to increase the concentration of non-protein nitrogen in the rumen failed to effect an increase in protein nitrogen in the sac.

The use of rumen liquor sterilized by filtration appeared to have no advantage in encouraging bacterial growth over distilled water when placed in the sacs and inoculated with strained rumen fluid. It was shown that nutrients diffuse into the sac in 30 minutes or less.

No appreciable difference was shown to exist in protein synthesis between the sacs incubated in the rumen of pairs of identical twin steers, or between the individuals within pairs of twins. No correlation was seen to exist between the amount of protein synthesized in the sac and the amount of protein nitrogen in rumen samples withdrawn at the end of various incubation periods.

It was concluded that the method described may have certain advantages over artificial rumen techniques currently in use.