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NUTRIENT AND OTHER ENVIRONMENTAL INFLUENCES UPON RUMEN PROTOZOAL GROWTH AND METABOLISM

by

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TABLE OF CONTENTS

	Page
INTRODUCTION.	1
PART I. IN VITRO TECHNIQUES, THEIR USE AND RESULTS FOR STUDYING RUMEN PROTOZOA	4a
REVIEW OF LITERATURE.	4b
EXPERIMENTAL PROCEDURE AND RESULTS.	20
A Multiple-Tube Laboratory Technique for Studying Volatile Fatty Acid Production by Rumen Protozoa .	20
Methods and procedures.	20
Results and discussion.	26
In Vitro Volatile Fatty Acid Production by Rumen Protozoa	29
Responses of rumen protozoa to pH changes and time of in vitro fermentations.	31
Methods and procedures	31
Results and discussion	37
Responses of rumen protozoa to substrate and protozoal concentration changes of in vitro fermentations	41
Methods and procedures	42
Results and discussion	44
Responses of rumen protozoa to diethylstilbestrol and antibiotics of in vitro fermentations	48
Methods and procedures	49
Results and discussion	51
Responses of rumen protozoa to sodium, potassium and phosphorus of in vitro fermentations	57
Methods and procedures	58
Results and discussion	63
CONCLUSIONS AND SUMMARY	78

Page

PART II. PRODUCTION OF VOLATILE FATTY ACIDS AND AMMONIA IN THE RUMEN OF FAUNATED AND PROTOZOA-FREE LAMBS 82a

REVIEW OF LITERATURE. 82b

Techniques for Obtaining Protozoal-Free Lambs. . . . 82b
In Vivo Production of Rumen Volatile Fatty Acids and Ammonia in Faunated and Protozoal-Free Lambs . 89

EXPERIMENTAL PROCEDURE AND RESULTS. 98

Rearing Isolated New-Born Orphan Lambs 98

Methods and procedures. 98
Results and discussion. 105

Defaunating the Rumen of Lambs Having Protozoal Populations. 113

Methods and results 113
Discussion. 117

Volatile Fatty Acid Production in the Rumen of Faunated, Partially Faunated and Protozoal-Free Lambs. 119

Methods and procedures. 120
Results and discussion. 125

Ruminal Ammonia Production in the Rumen of Faunated, Partially Faunated and Protozoal-Free Lambs. . . . 138

Methods and procedures. 139
Results and discussion. 140

CONCLUSIONS AND SUMMARY 149

Page

PART III. RATION CHARACTERISTICS INFLUENCING RUMEN PROTOZOA AND THE EFFECT OF PROTOZOA UPON GROWTH PERFORMANCE OF LAMBS 152

REVIEW OF LITERATURE. 153

EXPERIMENTAL PROCEDURE AND RESULTS. 164

Influence of Level of Feed Intake, Physical Form of Ration and Rate of Passage of Ingesta Upon Rumen Protozoal Populations. 164

Methods and procedures. 165
Results and discussion. 169

Influence of Diethylstilbestrol Upon Rumen Protozoal Numbers and the Effect of Protozoa Upon Growth Performance of Lambs 180

Methods and procedures. 181
Results and discussion. 186

CONCLUSIONS AND SUMMARY 192

GENERAL DISCUSSION. 195

LITERATURE CITED. 200

ACKNOWLEDGEMENTS. 211

APPENDIX. 212

INTRODUCTION

Ruminants such as cattle and sheep are known to harbor large numbers of protozoa (fauna) as well as bacteria (flora) in the rumen or fore part of their digestive tract. The simultaneous growth (fermentation) of these two populations of microorganisms is known to benefit the nutrition of ruminants by digesting starch and cellulosic or fibrous feed constituents and by synthesizing needed vitamins, particularly B vitamins and useful microbial proteins. It is obviously evident that the study of nutrient requirements of these microorganisms is vital if progress is to be made in furthering our knowledge of ruminant nutritional inadequacies.

The protoplasmic mass of rumen protozoa has been estimated as being approximately equal to the protoplasmic mass of rumen bacteria under average or normal feeding conditions. Therefore, it has been assumed that each of these populations of rumen microorganisms is roughly equally capable of contributing to the nutrition of the ruminant host. The only commonly known exception to this is the evidence that protozoal proteins are somewhat superior nutritionally as compared to bacterial proteins. This assumption was strengthened by the research evidence which demonstrated that defaunated ruminants digest fibrous feeds about as well as faunated ruminants.

The physiological and biochemical relationships between

rumen protozoa and bacteria and their influences upon the host are poorly elucidated. The development of *in vitro* techniques for studying fermentative cultures of rumen protozoa free of bacteria has been obviously lacking.

Despite all the evidence which tended to relegate rumen protozoa to a rather minor roll in the nutrition of the ruminant host, nevertheless, the subject appeared worthy of re-investigation from several standpoints. One of these was the belief that nature did not universally place protozoa within the rumen of animals without having some biological symbiotic relationship involved. It also seemed reasonable that rumen protozoa might exert some beneficial influence upon their host other than that concerned with digestion and synthesis within the digestive tract of ruminants. For example, it seemed reasonable that the end products of protozoal metabolism, such as volatile fatty acids or related rumen acids, might have specific significance to the nutrition of cattle and sheep. Since the utilizable energy is different for the various rumen acids, it appears important to understand the causal agents of levels and ratios of these acids.

This work was initiated to study and appraise the influences of nutrient, environmental and dietary factors upon rumen protozoal populations. In order to test the proposed hypotheses, it was also necessary to develop certain techniques for studying rumen protozoa. Therefore, much of the research lead-

ing to the completion of this thesis was concerned with developing suitable techniques for studying the contributions of rumen protozoa to the nutrition of their host. Also, information was needed concerning influential dietary factors in respect to rumen protozoal disappearance and propagation.

In view of these known evidences as well as factors yet unidentified concerning the overall function and importance of rumen protozoa, more intensive research studies were instigated in hopes of furthering the knowledge and understanding of ruminant nutrition.

PART I. *IN VITRO* TECHNIQUES, THEIR USE AND RESULTS FOR STUDYING RUMEN PROTOZOA

REVIEW OF LITERATURE

The importance of rumen protozoa in the over-all nutrition of cattle and sheep has been considered by some investigators to be insignificant. This attitude may be incorrect and may have discouraged nutritional studies concerning rumen protozoal metabolic activities and their effects on the host animal. The *in vitro* incubation of rumen microorganisms with specified substrates under various treatments and types of incubation techniques have been studied for several years but because of the inadequacies of these techniques in culturing bacterial-free rumen protozoa in chemically and physically defined conditions, their importance has been essentially ignored. The following technique was developed to study the nutritional requirements as an aid to understanding the over-all function or functions of rumen protozoa.

The significance of volatile fatty acid production arising from fermentations within the rumen of cattle and sheep has become an active area of research in ruminant nutrition and physiology over the past few years. It is well established that rumen volatile fatty acids are formed during fermentations involving mixed populations of bacteria and protozoa.

In searching for a technique for studying rumen protozoal fermentations, several different techniques were encountered in

the literature for employing rumen bacteria or mixed rumen microorganisms for *in vitro* fermentations. Marston (1948) reported a technique which closely simulated rumen conditions in the live animal. Since the publication of this technique, investigators have modified this procedure. Louw *et al.* (1949) described a method for *in vitro* fermentations which involves the use of semi-permeable bags which are suspended in an aqueous growth medium. Huhtanen *et al.* (1954) simplified an *in vitro* fermentation technique to a miniature form which consisted of a small cellophane sac into which the substrate was placed. The sac was suspended in a jar containing bacterial-nutrient medium. The method of Burroughs *et al.* (1950) involved microbial fermentation over a period of days; the culture was 'halved' at 36-hour intervals and volume reconstituted with fresh medium. Cheng *et al.* (1955) developed a method which utilized washed suspensions of rumen microorganisms and a relatively short fermentation period. These methods apparently served well where the objectives permitted use of mixed populations of rumen organisms or rumen bacteria. However, for studying rumen protozoal fermentations it was apparent that a method, different in certain respects from those already described, must be developed. Data taken from the following cited literature greatly aided the development of such a technique.

Separation of rumen protozoa from other materials present

in samples taken from the rumen was essential. Heald and Oxford (1953) found that protozoa could be separated from other rumen materials by incubating for one to two hours in the presence of 0.4 to 0.5 percent glucose which caused protozoa to settle as a distinct layer on the bottom of the container. The supernatant liquid was then decanted and discarded. Gutierrez (1955) reported that 0.4 percent additions of glucose aided settling of rumen protozoa to the bottom of a fermentation container. In further studies, Gutierrez (1958) separated rumen protozoa from other rumen materials by treating with 0.5 percent glucose and incubation in a separatory funnel at 39 degrees centigrade for about one hour. The white bottom layer (protozoa) was drawn off in a buffered medium. This investigator reported further that Isotrichs were separated from other rumen protozoa by using a tube 1.5 centimeters in diameter and 60 centimeters long which was fitted with a stopcock. The heavier Isotrichs settled faster and were drawn off free of other protozoa. Oxford (1955) further reported that when galactose was added to fermentation media, bacterial fermentation was enhanced and debris and scum floated to the top thereby allowing faster and more complete settling of rumen protozoa.

With the aid of these references, a method for separating protozoa from other rumen constituents of rumen ingesta was developed, however, further separation was necessary, namely, protozoa separation from bacteria. Heald et al. (1952) and

Heald and Oxford (1953) showed that rumen protozoa tolerated higher levels of antibiotics than did rumen bacteria. About 0.05 percent streptomycin or 0.025 percent each of streptomycin and penicillin inhibited bacteria while rumen protozoa were relatively unharmed. Oxford (1955) pointed out that 0.05 percent streptomycin along with two to three days starvation of the microorganisms gave complete eradication of rumen bacteria. Oxford (1955) stated that rumen protozoa do indeed possess their own digestive enzymes since fermentation continued at a rapid rate in the presence of antibiotics which kill the bacteria present. Even though rumen protozoa were processed in a way that enabled other rumen materials and bacteria to be removed or killed, intracellular energy stores were present in large enough quantities to prevent maximum response from added nutrients. Quinn (1962) developed a medium for rumen microorganism fermentations which was based upon the analysis of a balanced ration for ruminants. This medium enabled the culture of all rumen protozoa for two to three days and some specific types for seven to eight days. Modifications of this medium has enabled the depletion of any given nutrient or nutrients after which requirements of such nutrients can be studied.

In determining suitable substrates for rumen protozoa, it was found, in general, that rumen bacteria and protozoa attack or utilize the same compounds. Hungate (1942) demonstrated that cellulose and grass were utilized in supporting continuous cultures for extended periods. Cellulolytic enzymes were demon-

strated with glucose as an end product of cellulose breakdown. Hungate (1943) showed that rumen protozoal breakdown of cellulose led to a rapid synthesis of food reserves, such as amylopectin or other starch granules. Further studies by Hungate revealed a rapid storage and utilization of starch by rumen protozoa. On the other hand, Heald et al. (1952) indicated that maltose and lactose were not utilized by rumen protozoa. Oxford (1955) postulated that many of the carbohydrates, such as sucrose, glucose, cellobiose, insulin and levans, were used by protozoa to synthesize amylopectin. Sugden (1953) observed that excess soluble carbohydrates, such as glucose and sucrose, caused protozoal cells to burst. Since four-fifths of the cell weight may be amylopectin, additional soluble carbohydrates may cause additional reactions leading to excess carbon dioxide gas which probably is the cause of cell bursting, especially in Diplodinium (Sugden, 1953). Studies by Heald and Oxford (1953) indicated that Isotrichs and Dasytricha produce high levels of rumen glucose, fructose, sucrose, raffinose, inulin, rye grass levan and cellobiose. The formed acids were primarily acetic, butyric and lactic, with traces of propionic.

Utilization of many of the soluble sugars by rumen protozoa has been demonstrated by several investigators (Sugden, 1953; Oxford, 1951; Hungate, 1942 and 1943; Gutierrez, 1953; Heald et al., 1952). Hungate (1942) showed that holotrichs readily utilized glucose, fructose, sucrose, galactose, cellobiose, inulin and levan. Sugden (1953) and Hungate (1943)

showed that the oligotrichs have similar capabilities for utilizing these soluble sugars. It is of interest to note that maltose was poorly, if at all, utilized by rumen protozoa, while sucrose with a similar chemical structure was readily utilized. In general, rumen protozoa rapidly ingest and ferment large amounts of most carbohydrates giving rise to evolution of hydrogen, carbon dioxide and fatty acids.

In considering appropriate criteria for estimating treatment responses by rumen protozoa, volatile fatty acid production was logically selected as the best single criterion. Oxford (1955) stated that holotrich (Isotrichs and Dasytricha) protozoa store amylopectin synthesized from starch materials which enabled the continuous synthesis of volatile fatty acids. Gutierrez (1955) concluded that Isotrichs are capable of producing acids at the rate of 2.5 to 3.2 millimicromoles per cell per hour when conditions were suitable. Gutierrez (1958) postulated that rumen protozoa supply the host with a continuous source of fatty acids, probably 20 to 25 percent of their energy requirement. Further observations indicated that holotrichs are capable of producing fatty acids at the rate of 230 to 250 grams per 100 kilograms of rumen contents per day. These acids consisted of acetic, butyric, lactic and traces of propionic. Hungate (1942) has shown that some rumen protozoa produce fatty acids at the rate of 70 percent of their weight each day and may supply 20 to 25 percent of the requirements of the host. Calculations by Hungate

(1955) indicate that rumen contents of the mature animal may contain 2.2 kilograms of volatile acids, of which holotrichs supplied 10 percent and oligotrichs 10 percent. It is believed that rumen protozoa readily utilize both carbohydrates and proteins, synthesizing acetic, butyric, propionic and formic, as well as lactic and succinic acids as the important end products (Hungate, 1960). Williams et al. (1960) suggested that rumen protozoa utilize proteinaceous material very readily. Hungate (1960) concluded that 60 to 70 percent of the end products of protozoal fermentation were fatty acids. Considering both, the importance of total fatty acids to ruminants and the apparent ability of rumen protozoa to produce them, total titratable acidity appeared to be the most appropriate method for estimating protozoal metabolic activities.

Most in vitro techniques involving microbial fermentations have utilized incubation periods of about 24 hours in duration. For our purposes, it was of interest to know whether strictly rumen protozoal fermentations would be different from either mixed or rumen bacterial fermentations. Cheng et al. (1955) found that the rate of cellulose digestion was greatest 16 to 20 hours after incubation began and after 24 hours decreased rapdily. Gutierrez (1955) found that Isotrichs cell division (generation time), in vitro, was 48 hours but in vivo was only about 24 hours. In laboratory studies where the medium was changed daily, Hungate (1942) found that the rate of cell division for oligotrichs was about one new cell per 24 hour inter-

val. In later studies, Hungate (1960) reported that rate of cell division for rumen protozoa, as a whole, was from 18 to 24 hours. Under less desirable conditions, where media were not properly balanced and growth factors were not restored regularly, generation time may be much greater. Oxford (1955) indicated that, in general, rumen protozoal generation time may be about 12 hours, but much longer under less favorable conditions. These studies indicated that the length of fermentation for best response may be 16 to 24 hours. This was subsequently substantiated.

As a guide in determining the optimum pH for protozoal activity, values were taken from various investigations where rumen pH values were obtained while lambs were fed various types of rations (Rhodes, 1961; Raun, 1961). These observations indicate that rumen pH values commonly fell in the range of pH 5.4 to 6.0 when lambs were fed high concentrate rations; pH values of 6.0 to 6.6 were found on high roughage rations. Hungate (1942) has shown that a pH of about 5.8 was optimal for cellulose digestion by culturing rumen protozoa while bacteria were inhibited with toluene. He also showed that the endoplasm of Diplodinium was acidic with a pH of about 5.0. Hungate (1942) also found that a pH of 8.0 or higher was lethal to protozoa while a pH of 5.5 stopped rumen protozoal activity. An interesting symbiotic relationship is exhibited between termites and their normal intestinal protozoa. Cleveland (1924) found the optimum pH for termite protozoa to be near neutrality

with a tolerable limit near pH 5.0 for short periods of time. The reaction of the internal contents of rumen protozoa, in general, is in the range of pH 4.5 to 5.5.

While it appears from the above observations that optimum pH for growth of rumen protozoa is from pH 6.0 to 7.0, according to Cheng et al. (1955) cellulose digestion by rumen bacteria was greatest when the pH ranged from 6.5 to 7.5. This indicated that rumen protozoa can tolerate more acidic conditions than can rumen bacteria.

When considering the size of inoculum or the concentration of rumen protozoa used during incubation, it was realized that different animals fed different rations would yield cultures with different fermentative patterns. Cheng et al. (1955) stated that the amount of rumen fluid required to give optimum digestion, even though variable under different conditions, was about 40 milliliters for making up 20 milliliters of fermentation medium. That is, microorganisms from 40 milliliters of rumen fluid were used in 20 milliliters of fermentation medium. When working directly with rumen protozoa, Sugden and Oxford (1952) found that rumen protozoa obtainable from 100 milliliters of rumen fluid placed in 20 milliliters of fermentation medium gave suitable protozoal concentrations for their in vitro studies. Aided by these findings, along with further observations by the author, procedures were worked out which gave satisfactory protozoal concentrations for in vitro investigations herein reported.

Because of the reported influences of hormones upon protozoa harbored by termites and wood roaches, it was of interest to speculate their possible effects upon rumen protozoa. Observations made by Cleveland (1924) during and after molting of termites (loss of the tissue lining the gut) have shown that protozoal numbers increased greatly during and following this molting period. Further studies by Cleveland (1950) have shown that protozoa of the termite and wood roach do indeed possess a sexual reproductive process which is correlated with the molting of the host. Hungate (1955) postulates that both sexes, male and female, are exhibited by protozoans and thus it appears that the sexual reproduction supplements the asexual process thereby enhancing the total number of protozoa. Cleveland (1950) theorized that this sexual reproductive process developed through a series of evolutionary events and is dependent upon not only hormones but also certain physiological conditions. In theorizing about the hormonal effects upon ruminants and their protozoa, the following literature was helpful. Ferber (1928) noted a correlation between the number of rumen protozoa and an increased assimilation of nitrogenous compounds within the rumen. It was also noted that pregnant and lactating heifers contained twice as many rumen protozoa as heifers not pregnant or lactating. Undocumented research with goats during the early 1900's in France indicated that protozoa increased in number in the rumens of estrogen treated as compared to non-estrogen treated animals. These findings

may suggest that protozoal numbers are enhanced by the level of nitrogenous compounds within the rumen as a result of higher and more select food material being ingested. But more likely, they suggest a possible estrogenic enhancement of protozoal numbers due to an increase in rate of cell division.

In studying fatty acid production by rumen protozoa with in vitro techniques, it was realized that extracellular as well as intracellular bacteria must be inhibited in order to assure quantitative estimates of acids formed by protozoa. Heald et al.(1952) found that streptomycin killed rumen bacteria without altering the metabolic activities of rumen protozoa. Gram stain preparations of rumen contents made after incubating ingesta at 40 degrees centigrade for 48 hours in the presence of streptomycin failed to reveal any living rumen bacteria. Using in vitro fermentations of rumen protozoa and bacteria with soluble sugars as substrates, Heald and Oxford (1953) found that relatively high levels of streptomycin did indeed kill bacteria with little or no harm to the protozoa. Oxford (1955) demonstrated that starvation of rumen microorganisms for 24 to 48 hours in the presence of 0.05 percent streptomycin caused the death of bacteria. When using protozoa treated in this manner, these workers clearly believed the resulting fermentation products were entirely due to protozoan activity. Sugden (1953) found that 0.04 to 0.06 percent streptomycin killed most, if not all, bacteria without affecting rumen protozoa in laboratory fermentations. With recent availability of anti-

biotics exhibiting lethal effects upon rumen bacteria but without harmful effects upon rumen protozoa, the problem of developing techniques for studying in vitro fermentations of protozoa has been greatly enhanced.

When studying the metabolism of organisms, their requirement for nutrients are of primary interest. With the development of artificial rumen techniques, the requirements for various nutrients by rumen bacteria have been given a great deal of investigation. Nutrient requirements of rumen protozoa have been given a great deal less investigation. In studying rumen protozoal metabolism, their nutrient requirements are of immediate concern. Hungate (1942) observed that 0.5 to 0.7 percent sodium chloride greatly enhanced the viability of in vitro cultured rumen protozoa. Sugden and Oxford (1952) reported that medium consisting of 0.5 percent sodium chloride, 0.15 percent sodium acetate, 0.1 percent sodium bicarbonate, 0.1 percent potassium phosphate, 0.01 percent magnesium sulfate and 0.01 percent calcium chloride was suitable for in vitro protozoal cultures. Other studies by these researchers showed increased protozoal activity when media were supplemented with grass juice. In addition to the nitrogenous aspects, the feeding of many major and minor minerals was undoubtedly responsible for stimulated responses. Hungate (1950) demonstrated an unusually low requirement for nitrogen by termite protozoa. Termites grew satisfactorily on diets consisting of wood that contained only 0.046 percent nitrogen. Calculations made on

data from these studies indicate that for each gram of nitrogen consumed, including that assimilated and voided in the feces, 350 grams of wood were decomposed. Protozoal protein has been shown to be of relatively high biological value (McNaught et al., 1954). It is believed that rumen protozoa compete with bacteria for fermentable carbohydrates and that they may even use bacteria as energy or protein sources. Appleby et al. (1956) postulated that rumen protozoa may be destroyers of various vitamins, especially the B-vitamins. They further stated that rumen protozoa may have no requirement for dietary B-vitamins. The lack of a more thorough understanding of nutrient requirements by rumen protozoa is apparent from the scanty literature above.

It is generally agreed that rumen protozoa are obligate anaerobic organisms (Hungate, 1942; Sugden and Oxford, 1952; Heald et al., 1952; Sugden, 1953). Termite and roach protozoa are also considered to be obligate anaerobes (Cleveland, 1925d). However, when oxygen was introduced into their presence, protozoa reduced it so rapidly that oxygen tensions remained nearly unchanged except immediately after oxygen administration. Research by Cleveland (1925d) indicated that in order to kill termite protozoa with oxygen, at least 24 hours of treatment with oxygen tensions equal to 2 or 3 atmospheres were required. It appears that these protozoa were able to tolerate relatively high oxygen tensions for a considerable length of time. Rumen protozoa are not believed to be able to

tolerate oxygen tensions as high as these, however, rapid fermentation ties up nearly all oxygen in the form of carbon dioxide (Hungate, 1955). The holotrichs are less sensitive to oxygen than the oligotrichs but were killed when tensions were high for extended periods (Oxford, 1955).

With the average temperature of the rumen being about 39 degrees centigrade, it seems reasonable to expect that protozoa would tolerate temperatures from 35 to 45 degrees centigrade. In laboratory experiments this was found to be not the case. Temperatures 20 to 30 degrees below body temperature are tolerated for several hours, while 5 to 6 degrees above body temperature for short periods results in death of rumen protozoa. In the case of termite and roach protozoa, Cleveland (1925d) has shown that when the host was incubated at 6 to 8 degrees centigrade above normal host body temperature, within 24 hours all intestinal protozoa were dead. When incubated at 15 degrees above host body temperature, they were all dead within 10 minutes. On the other hand, when they were incubated 20 to 25 degrees below body temperature, neither host or protozoa was harmed. This indicated a low tolerance to high temperatures and a high tolerance to low temperatures.

It soon becomes apparent, when studying literature concerned with rumen protozoa, that a symbiotic relationship exists between bacteria, protozoa and the ruminant host. Whether or not this symbiosis is an association of mutualism, commensolism or parasitism is not so apparent and is difficult

to show direct evidence to prove any of these relationships. With termites, Cleveland (1924, 1925a, 1925b, 1925c and 1925d), by defaunation, was able to show an association of the protozoa which was essential for the life of its termite host. This vital role of protozoa does not, however, exist in the maintenance of ruminant life. Perhaps a more vital and interesting symbiosis exists between ruminant protozoa and bacteria. It has been shown by many investigators that most types of rumen protozoa ingest bacteria (Gutierrez, 1957, 1958 and 1959; Appleby *et al.*, 1956; Oxford, 1955; Hungate, 1950; Eadie *et al.*, 1959). Appleby *et al.* (1956) used staining techniques and the culture of crushed and intact protozoa to detect ingested bacteria. They concluded that *Entodinium* and *Diplodinium* did contain rumen bacteria, and believed that *Isotrichs* and *Dasytrichs* did not. Gutierrez (1958), however, showed that *Isotricha prostoma* exhibited a selective preference for ingesting certain rod-shaped bacteria of the rumen. In further research, Gutierrez and Hungate (1957), in order to demonstrate the ingestion of large numbers of bacteria, found it necessary to starve the protozoa so they would ingest nutrients rapidly. Gutierrez (1958) suggested that ingested bacteria traveled to the anterior end of the protozoal cell, gradually disintegrating and releasing its contents to the protozoa.

The nitrogen requirements of rumen protozoa, in general, are not understood but are believed to be relatively low. Some investigators believe that ingested bacteria may supply the

nitrogen needs of some protozoa (Appleby et al., 1956; Hungate, 1955; Oxford, 1955). Hungate (1950) demonstrated an unusual nitrogen sparing effect of termites containing intracellular protozoa. Being aware of the studies by McNaught et al. (1954) which showed that protozoal protein was superior in nutritive value to that of bacteria, it appears that bacteria synthesize proteins from raw materials, such as urea, nitrogen and ammonia. Protozoa then synthesized proteins from bacteria or other nitrogenous compounds giving rise to proteins with a better balance of essential amino acids which enhanced its nutritional value for the ruminant host. Weller (1957) found that rumen protozoal protein contained higher amounts of leusine, isoleusine, phenylalanine and especially lysine than contained by rumen bacterial protein. The importance of protozoa in utilizing simple nitrogenous compounds, such as urea and ammonia, have been tested and found a biological value index of 49 for urea fed to protozoal-free sheep and 62 when fed to normal faunated sheep (Hungate, 1950). With the possibility that bacteria may serve as a source of nitrogen for protozoa, it must be kept in mind that the value of protozoal proteins for the host depends upon the ability of protozoa to synthesize proteins of superior quality from bacteria or despite bacterial-protozoal competition for other nitrogenous materials within the rumen.

EXPERIMENTAL PROCEDURE AND RESULTS

A Multiple-Tube Laboratory Technique for Studying Volatile Fatty Acid Production by Rumen Protozoa

Little is known quantitatively concerning the formation of volatile fatty acids by rumen protozoa in the absence of bacteria even though it has been demonstrated that rumen protozoa possess relatively large amounts of amylolytic, cellulolytic and proteolytic enzymes (Hungate, 1942; Williams et al., 1960). According to Ferber (1928), protozoa may constitute 6 to 10 percent of the weight of rumen contents and supply 20 to 25 percent of the total energy requirements of sheep and cattle. With these and other symbiotic relationships between rumen protozoa and the host animal in mind and with awareness of the inadequacies of presently used artificial rumens, it appeared desirable to develop a technique for studying bacteria-free protozoal cultures under chemically and physically defined conditions. To meet this requirement an impermeable artificial rumen system adapted primarily for bacteria-free protozoal populations was developed. A description of this technique is presented in the following section of this thesis and by Christiansen et al. (1962).

Methods and procedures

The technique developed consists of four principal steps. The first step involved harvesting a relatively large mass of mixed types of viable protozoa from rumen fistulated cattle or

sheep for subsequent laboratory usage. The second step consisted of separating the protozoa from food particles and freeing them of viable bacteria. The third step involved multiple-tube protozoal fermentations for several hours, with experimental variations imposed between different tubes. The fourth and final step consisted of titration of total volatile fatty acids which were produced, with comparative analysis being made between tubes. Rumen fluid was obtained from a mature, fistulated steer which was receiving a ration composed of 30 percent alfalfa-grass hay, 30 percent ground corn cobs, 30 percent ground shelled corn, 6 percent soybean oil meal and 4 percent molasses. These four steps are described in detail below.

Step 1. Collection of rumen protozoa About five liters of rumen liquid, relatively free of solids, were collected into insulated containers from a rumen fistulated steer using a syringe and suction apparatus described by Raun and Burroughs (1961). Approximately 50 percent of the liquid was withdrawn from the floor of the rumen, 25 percent from the mid-area and the remainder from the upper part of the rumen ingesta in an effort to get a representative protozoal sample for usage in the laboratory. Collection studies showed that the larger protozoa (Isotricha, Diplodinium and Ophryoscolex) were more concentrated in the lower part of the rumen, while the smaller protozoa (Dasytricha and Entodinium) were nearly homogenously distributed.

Step 2. Separation of protozoa Immediately upon

reaching the laboratory the rumen liquid was strained through cheesecloth into large flasks and incubated at 39 degrees centigrade with 0.1 percent maltose and 0.5 percent sucrose for 1 to 2 hours (as modified from Gutierrez, 1955; Oxford, 1955). These soluble sugars caused rapid fermentation releasing carbon dioxide which aided in carrying feed particles and bacteria to the top. This 1 to 2 hours of incubation was followed by decantation and discarding of approximately two-thirds of the upper liquid containing most of the solid food particles. The remaining liquid which contained most of the protozoa was transferred to a separatory funnel. The incubation was allowed to proceed for an additional 1 to 2 hours until the protozoa were visibly layered in a small volume (80 to 100 milliliters) at the bottom of the separatory funnel. During these short incubation periods, the active protozoal fermentations were relied upon to maintain anaerobiosis. The protozoa were carefully withdrawn by quickly opening and closing the separatory funnel petcock several times, allowing a short time to elapse between each withdrawal.

The protozoa thus separated were next suspended in 1 liter of a special energy-deficient antibiotic-containing medium (table 1). Bacteria were killed by adding 0.05 percent streptomycin or 0.025 percent each of streptomycin and penicillin (Heald et al., 1952; Heald and Oxford, 1953). Protozoa were then incubated anaerobically for 16 hours at 39 degrees centigrade for purposes of destroying viable bacteria and reducing

Table 1. Depletion medium[a]

Compound	mg./l.	Compound	mg./l.
L-alanine	1.31	Folic acid	0.01
L-arginine	1.90	$CaCl_2$[b]	94.50
L-aspartic acid	2.89	KCl[b]	1298.00
L-cysteine HCl	40.17	KH_2PO_4[b]	12.00
L-cystine	0.53	K_2HPO_4[b]	46.50
L-glutamic acid	4.46	$MgSO_4 \cdot 7H_2O$[b]	94.50
Glycine	1.31	$NaAc \cdot 3H_2O$[b]	3542.00
L-histidine HCl	9.97	NaCl[b]	1185.00
L-isoleucine	1.57	$NaHCO_3$[b]	4444.00
L-leucine	2.63	Na_2Cu[d]	1.36
L-lysine HCl	2.89	H_2Fe[d]	11.92
L-methionine	0.53	Na_2CO[d]	0.01
L-phenylalanine	1.31	Iodine	0.00225
L-proline	1.05	Na_2ZN[d]	0.002
L-serine	1.57	Ascorbic acid	50.60
L-threonine	1.57	Biotine	0.006
L-tryptophane	0.53	Choline	95.0
L-tyrosine	1.05	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.45
L-valine	1.32	Indigo disulfonate	10.00
Adenine HCl[b]	19.12	iso-propyl alcohol[e]	6.83
Cytosine[b]	15.00	Ca pantothenate	1.12
Guanine[b]	4.69	Pyridoxine HCl	0.16
Uracil[b]	15.00	Riboflavin	0.53
Xanthine[b]	15.00	Na chlorophyllin	7.50
Na butyrate[b]	682.00	p-aminobenzoic acid	0.37
Na propionate[b]	608.00	Inositol	0.1495
Na valerate	310.00	Thiotic acid	0.00075
Menadione	0.75	Vitamin A	0.0341
a-thioglycerol	700.00	Vitamin D_2	0.01308
Tween 80	400.00	Vitamin B_{12}	0.000023
Thiamine	2.19	Streptomycin	500.00
EtOH[c]	93.40	Deionized H_2O	1000 ml.
Niacine	2.05		

[a]Developed by Quinn (1962).

[b]Sterilized by autoclaving - all other compounds sterilized by filtration.

[c]EtOH (ethanol, 95%) was introduced into medium through stock solutions of vitamin D_2, menadione (vitamin K), and thiotic acid (lipoic acid protogen).

[d]Chelated (EDTA) minerals.

[e]i-propyl alcohol introduced through stock solution of vitamin A.

protozoal energy stores and at the same time maintaining the protozoa in a viable condition. At the termination of this incubation period the protozoa were again separated from the bulk of the medium with the use of a separatory funnel as previously described.

Step 3. Multiple-tube fermentation The protozoa were next suspended in 1 liter of an energy-containing medium (table 2) fortified with antibiotic, maintained under anaerobic condi-

Table 2. Composition of incubation medium

Constituent	Amount
	(mg./liter)
NaCl	3000
KCl	2000
$NaC_2H_3O_2$	3000
$KC_2H_3O_2$	2000
$NaHCO_3$	2000
K_2SO_4	1000
$Na_2HPO_4 \cdot 7H_2O$	3000
KH_2PO_4	2500
$MgSO_4 \cdot 7H_2O$	100
Starch (powder)	8000
Starch (soluble)	2000
Cellulose	2000
Sucrose	1000
Streptomycin	300

tions, and stirred by bubbling with carbon dioxide gas. The pH of this medium was adjusted to 6.8 to 7.0 with a saturated solution of sodium carbonate. Energy substrate was supplied from starch, sucrose and cellulose as indicated in table 2. Twenty milliliter aliquots were then pipetted into (for example) fifty 75 milliliter tubes, 30 of which were fitted with

carbon dioxide inlet and outlet tubes according to the method of Cheng et al. (1955). The 20 fermentation tubes without carbon dioxide inlet and outlet tubes served as titration controls with fermentation terminated immediately without incubation, with 1 milliliter of saturated mercuric chloride. These titration controls were titrated with 0.1 N sodium hydroxide to a pH of 9.0 using an automatic titrating apparatus. The 30 experimental tubes with carbon dioxide inlet and outlet tubes were incubated anaerobically at 39 degrees centigrade for 16 to 20 hours, after which the fermentation was terminated with mercuric chloride and each of the tubes was also titrated to a pH of 9.0. All respective control and fermentation tubes were treated alike in that the volume remained constant.

Step 4. Titrating total acids produced The protozoal fermentation activity of a given tube during the 16 to 20 hour incubation period was determined by subtracting the appropriate control-tube-titration-value from the experimental-tube-titration-value obtained. All values thus obtained were then transposed and expressed as milliequivalent volatile fatty acid produced per tube. In expressing these values it was realized that a negligible amount of these acids was not volatile. Data from experiments using gas chromatographic analysis have shown that the majority of end products of fermentation are indeed volatile fatty acids.

Microscopic observations were made from time to time during the 16 to 20 hour incubation period to check for the gener-

al viability of the major types of protozoa present as well as the absence of bacteria.

Results and discussion

A typical experiment in which information concerning the influence of hydrogen ion concentration upon rumen protozoal volatile fatty acid production will be used to illustrate the results of an experiment using this technique. It should be pointed out that this experiment is presented as an example rather than the specific effects of pH upon rumen protozoa. The pH in each five tubes was varied (prior to incubation) from 4.0 to 8.5 in 0.5 graduations by additions of a saturated solution of sodium carbonate. The results of the volatile fatty acid values for the titration-control-tubes and the incubated-tubes are presented in table 3 and other experiments are plotted graphically in figure 1. These values are expressed as milliliters of 0.1 N sodium hydroxide required to titrate tube contents to a pH of 9.0. Protozoal total acid production during the incubation period is represented by the incubation-tube values minus the titration-control-tube values expressed as milliequivalents of total volatile fatty acids. This technique provides a good method for studying the requirements of nutrient factors for rumen protozoa. The titration-control-tubes were usually duplicated while the incubation-tubes were run in triplicate or quadruplicate. Values taken from a typical experiment, where the nutrient solution was as listed in table 2

Table 3. Influence of pH on VFA production by rumen protozoa[a]

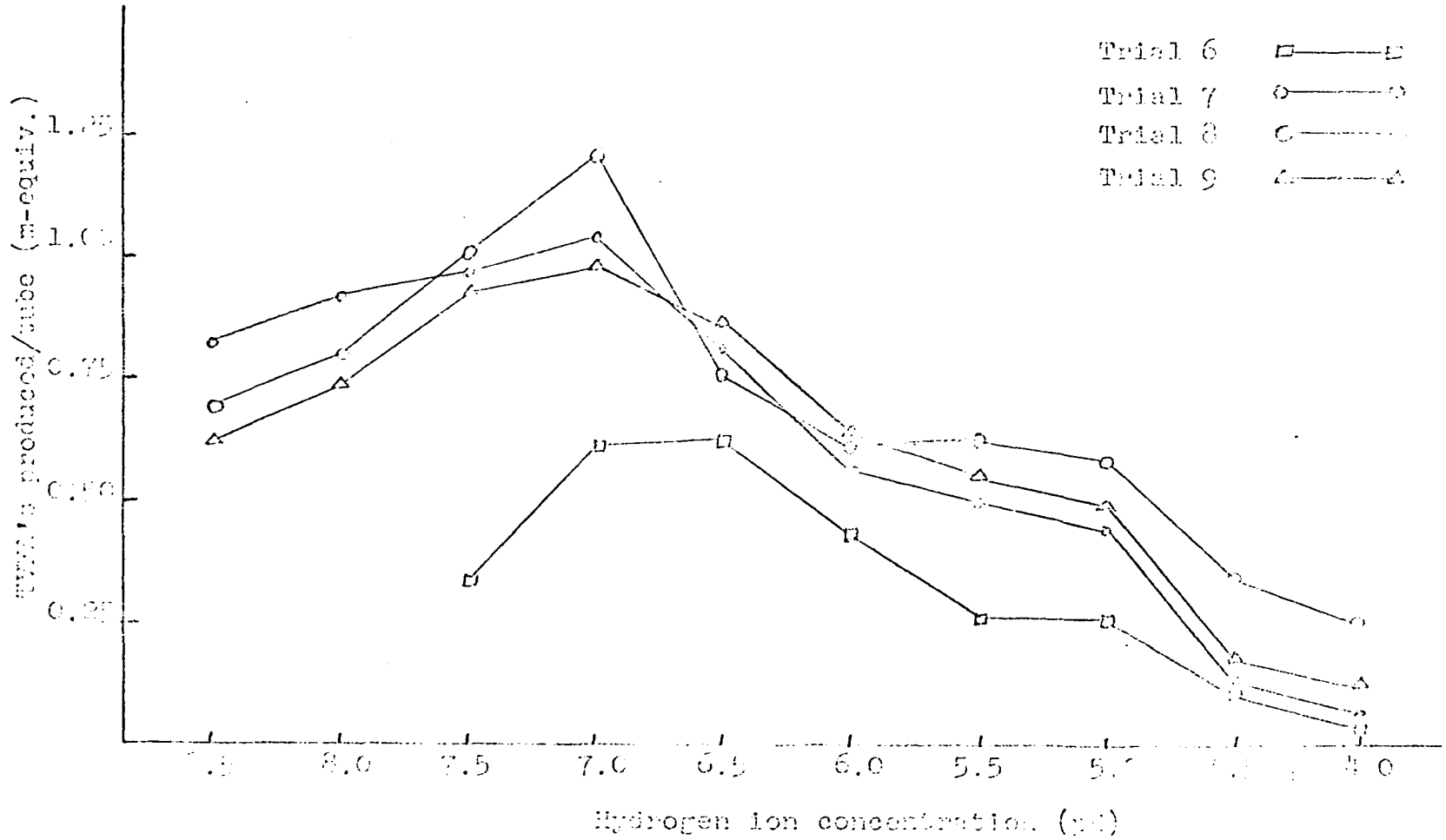
Initial pH	Expressed values per tube	Control tubes[b]			Incubated tubes[c]			
		First	Second	Av.	First	Second	Third	Av.
8.5	NaOH(0.1N),ml.	4.2	4.5	4.4	13.4	11.8	12.2	12.5
	VFA,m-equiv.	-	-	-	0.9	0.8	0.8	0.8
8.0	NaOH(0.1N),ml.	5.5	5.6	5.6	14.8	15.0	14.2	14.7
	VFA,m-equiv.	-	-	-	0.9	1.0	0.9	0.9
7.5	NaOH(0.1N),ml.	6.5	6.7	6.6	16.0	16.6	16.5	16.4
	VFA,m-equiv.	-	-	-	0.9	1.0	1.0	1.0
7.0	NaOH(0.1N),ml.	7.2	7.0	7.1	17.9	17.8	17.5	17.7
	VFA,m-equiv.	-	-	-	1.1	1.1	1.0	1.1
6.5	NaOH(0.1N),ml.	8.0	7.9	8.0	15.9	15.9	16.3	16.0
	VFA,m-equiv.	-	-	-	0.8	0.8	0.8	0.8
6.0	NaOH(0.1N),ml.	12.5	12.9	12.7	17.9	18.9	18.8	18.5
	VFA,m-equiv.	-	-	-	0.5	0.6	0.6	0.6
5.5	NaOH(0.1N),ml.	13.0	13.3	13.2	18.9	18.5	18.2	18.5
	VFA,m-equiv.	-	-	-	0.6	0.5	0.5	0.5
5.0	NaOH(o.1N),ml.	18.0	18.5	18.3	22.0	21.9	22.5	22.1
	VFA,m-equiv.	-	-	-	0.4	0.4	0.4	0.4
4.5	NaOH(0.1N),ml.	22.4	22.0	22.2	23.0	23.5	23.5	23.3
	VFA,m-equiv.	-	-	-	0.1	0.1	0.1	0.1
4.0	NaOH(0.1N),ml.	25.0	25.8	25.4	25.5	26.0	25.8	25.8
	VFA,m-equiv.	-	-	-	0.0	0.1	0.0	0.0

[a]VFA's represents titratable acidity (non-volatile acids were negligible).

[b]Fermentation terminated before incubation and served as correction factors for converting values from incubated tubes to true acid production during the experimental incubation period.

[c]Fermentation terminated after the experimental incubation period.

Figure 1. Influence of pH on VFA production by rumen protozoa.


Trial 6
Trial 7
Trial 8
Trial 9
1.0
0.5
8.0
7.5
7.0
6.5
6.0
5.5
Hydrogen ion concentration. (pH)

and the incubation time 18 hours, are as follows: the protozoal acids formed were about 5 grams expressed as acetic acid equivalent per 100 grams of organic matter per hour.

Visual examination by microscope following the incubation period did not reveal any bacterial growth in conjunction with the protozoa. Also, electron micrographs of similar cultures maintained for 3 to 4 days did not reveal bacterial growth. It thus appeared that this technique provides quantitative analysis of total acids arising from the action of rumen protozoa. These results indicate that protozoa *per se* are capable of producing large quantities of volatile fatty acids, since at the height of activity as much as 8 grams of protozoal acids as acetic acid equivalents per hour per 100 grams of organic matter were produced.

The present technique offers a means whereby factors influencing protozoal volatile fatty acid production can be systematically and quantitatively studied in furthering knowledge bearing upon ruminant nutrition and physiology.

In Vitro Volatile Fatty Acid Production by Rumen Protozoa

Rumen protozoa appear to have a tremendous potential for ingesting carbohydrate materials, storing them as amylopectin and then through fermentative synthesis, supplying the host with a continuous source of fatty acids. Gutierrez (1955) estimated that weight of protozoal acids formed from 100 kilograms of rumen content was about 24 grams per day and comprised

20 to 30 percent of the total rumen acids formed. Heald and Oxford (1953) indicated the significance of rumen protozoa to the host may be two-fold: first, the rumen bacteria are prevented from fermenting all soluble carbohydrates; second, when soluble carbohydrates remain free in the rumen, endogenous fermentation of the protozoan glucosan releases into the rumen a steady flow of lactic, acetic and butyric acids. Lactic acid is readily converted to propionic acid by rumen bacteria (Johns, 1951a, 1951b, 1952).

Hungate (1942) showed that rumen protozoa supply about 30 to 35 percent of the fatty acids required by the host. It is believed by some investigators that fatty acids comprise 55 to 65 percent of the total end products of protozoal fermentations.

This section contains data pertaining to some nutrient, physical and chemical requirements of rumen protozoa for maximum production of fatty acids. Since the determination of the amount of fatty acids produced by the in vitro fermentations of rumen protozoa was the primary objective, the technique previously described in this thesis and by Christiansen et al. (1962) was used to estimate protozoal activity by the amount of fatty acids produced.

The Tukey test as described by Snedecor (1959) was used to determine a difference required for significance at the .05 probability level for comparison of treatment means in each trial.

Responses of rumen protozoa to pH changes and time of in vitro fermentations

Methods and procedures

The influence of pH changes upon in vitro fatty acid production by rumen protozoa The normal pH range, within the rumen, for optimal microbial activity is believed to be from 6.0 to 7.0. A diet consisting almost entirely of concentrates can give a rumen pH of 5.0 or less. To test the effects of pH upon in vitro protozoal fermentations, the procedure previously described was used. Rumen protozoa used for these trials were processed according to the procedure previously described in this thesis and by Christiansen et al. (1962).

Trial 1 The pH in each set of five tubes (three fermentation and two titration control tubes) was varied from 7.0 to 4.0 in 0.5 graduations by additions of a dilute solution of hydrochloric acid. Because of changes in pH due to protozoal fermentations, every two hours during the incubation period the pH was restored to the original level. When more basic than original, a dilute solution of hydrochloric acid was used and when more acid they were adjusted by adding saturated sodium carbonate. The amount used in adjusting the pH in each set of three fermentation tubes was added to the respective titration control tubes. Protozoal activity in the titration control tubes was stopped immediately, without incubation, with 1 milliliter of saturated mercuric chloride per tube. The fermentation tubes (three per treatment) were incubated anaero-

bically, using carbon dioxide gas, at 39 degrees centigrade for about 18 hours. After this incubation period, protozoal fermentations were terminated by adding 1 milliliter of saturated mercuric chloride per tube. All tubes, fermentation and titration controls, were then titrated with 0.1 N sodium hydroxide to a pH of 9.0 using an automatic titrating apparatus. All respective control and fermentation tubes were treated alike in that the volume remained constant and treatments other than fermentation were the same.

The protozoal activity of a given tube during the 18-hour incubation period was determined by subtracting the appropriate control-tube-titration-value from the fermentation-tube-titration-value obtained. All values thus obtained were then transposed and expressed as milliequivalents of acid produced per 20 milliliters of fermentation medium. A typical experimental design and an example of the calculations, but not from this trial, are presented in table 3. The results of this trial are summarized and presented in table 4. In expressing these values it was realized that a negligible amount of these acids was not volatile. Data from experiments, using gas chromatographic analysis, have shown that the majority of the end products of fermentation were indeed volatile fatty acids.

Microscopic observations were made from time to time during the 18-hour incubation period in observing the general viability of the major types of protozoa present as well as the absence of bacteria.

Trial 2 Methods and procedures of this trial which were common to trial 1 are not repeated.

The pH in each set of 5 tubes was varied from 7.5 to 4.0 in 0.5 graduations by additions of dilute hydrochloric acid. At hourly intervals, for 8 hours after incubation began, the pH was adjusted to the original level using dilute hydrochloric acid or saturated sodium carbonate. At the end of 8 hours of incubation, pH adjustments were discontinued. At the end of 16 hours of incubation the protozoal fermentations were terminated and the tube contents were analyzed as described in trial 1. Results of this trial are presented in table 4.

Trial 3 Methods and procedures of this trial which were common to trial 1 are not repeated. The pH in each set of five tubes was varied from 8.5 to 4.0 in 0.5 graduations by additions of dilute hydrochloric acid. At two hour intervals, for 10 hours after incubation began, the pH was adjusted to the original level using dilute hydrochloric acid or saturated sodium carbonate. At the end of a 22-hour incubation period, protozoal fermentations were terminated and tube contents were analyzed as described in trial 1. Results of this trial are presented in table 4.

Trial 4 Methods and procedures of this trial which were common to trial 1 are not repeated. The pH in each set of five tubes was varied from 4.0 to 8.5 in 0.5 graduations by additions of saturated sodium carbonate. At two hour intervals, for 10 hours after incubation began, the pH was adjusted

to the original level using dilute hydrochloric acid and sodium carbonate. At the end of 22 hours of incubation the tube contents were analyzed as described in trial 1. Results are presented in table 4.

Trial 5 Methods and procedures of this trial which were common to trial 1 are not repeated. The pH in each set of five tubes was varied initially from 4.0 to 8.5 in 0.5 graduations by additions of a saturated solution of sodium carbonate. No pH changes were made during the incubation period. At the end of a 16-hour incubation period the tube contents were analyzed as described in trial 1. Results of this trial are summarized and presented in table 3.

Trials 6, 7, 8 and 9 Methods and procedures of these trials which were common to trial 1 are not repeated. The pH of each set of five tubes was varied initially from pH 4.0 to 7.5 in trial 6 and from 4.0 to 8.5 in trials 7, 8 and 9 in 0.5 graduations by additions of a saturated solution of sodium carbonate. No pH changes were made during the incubation period. At the end of a 16-hour incubation period for trial 6 and 22 to 24 hours for trials 7, 8 and 9 the tube contents were analyzed as described in trial 1. Results of these trials are plotted graphically and presented in figure 1.

The influence of time upon in vitro fatty acid production by rumen protozoa Using an artificial rumen technique with rumen bacteria, Cheng et al. (1955) found that the rate of cellulose digestion was greatest 16 to 20 hours after

beginning the incubation period. Hungate (1960) and Oxford (1955) indicated that the generation time of some types of rumen protozoa was 18 to 24 hours in length. The following trials were conducted to determine the effect of time upon *in vitro* acid production by rumen protozoa.

The methods and procedures used in these trials which were common to trial 1 in the previous section concerned with pH influences are not repeated in this section.

Trials 1 and 2 The fermentation medium used for these trials was the same as that described in table 2 except that the substrate was supplied by 1.8 percent starch and 0.2 percent sucrose. Medium and intracellular energy stores were depleted for a 24-hour incubation period using the depletion medium described in table 1. The four tubes representing the zero hour served as the titration-control-tubes in that fermentation was terminated prior to incubation by adding 1.0 milliliter of saturated mercuric chloride. Protozoal fermentation in the experimental tubes was stopped with mercuric chloride at 2, 4, 8, 16, 24, 48 and 72 hours after the beginning of incubation. Each time listed was replicated by protozoal fermentations from contents of four tubes within each trial. At the end of these fermentation periods, the tube contents were analyzed as previously described in the *in vitro* technique section. The results are summarized and presented in table 5.

Trials 3 and 4 The fermentation periods in

these trials were 0, 1, 2, 4, 8, 16, 24 and 48 hours, respectively. In trials 1 and 2 the fermentation periods started with 2 and ended at 72 hours, while in these experiments they started at 1 and ended at 48 hours with the intermediate intervals being the same in all four trials. All other methods and procedures used in trials 3 and 4 which were common to trials 1 and 2 are not repeated.

Trials 5, 6 and 7 The fermentation medium used in these trials was the same as used for trials 1, 2, 3 and 4 except that the substrate was supplied by 0.8 percent starch and 0.2 percent sucrose. Rumen protozoa used in these trials were not depleted of energy stores. They were placed into the fermentation medium as described above and incubation began immediately. Protozoal fermentations were terminated at 0, 2, 4, 8, 16, 24, 48 and 72 hours and were replicated in four tubes. Tube contents were analyzed at the end of these periods as previously described. The data from the results of these trials are presented in table 5.

Trial 8 The fermentation medium used for these trials was the same as used for trials 1 and 2. The only difference between trials 1 and 2 and 3 and 4 is that the latter trials were not depleted of energy stores. Fermentations were terminated at 0, 2, 4, 8, 16, 24, 48 and 72 hours after the beginning of fermentation. Tubes contents were analyzed as previously described. Data from the results of these trials are presented in table 5.

Results and discussion

The influence of pH upon in vitro fatty acid production by rumen protozoa The results of the trials of this experiment are presented in tables 3 and 4. It is apparent

Table 4. Influence of pH upon in vitro fatty acid production by rumen protozoa[a]

pH[b]	Acid produced (m-equiv./20 ml.)			
	Trial 1[c]	Trial 2[c]	Trial 3[d]	Trial 4[e]
8.5			0.58	0.64
8.0			0.75	0.88
7.5	0.54	0.64	0.96	1.03
7.0	0.65	0.98	1.06	1.20
6.5	0.50	0.84	0.79	1.01
6.0	0.33	0.62	0.65	0.95
5.5	0.27	0.40	0.63	0.74
5.0	0.28	0.30	0.54	0.88
4.5	0.12	0.22	0.18	0.56
4.0	0.00	0.10	0.21	0.24

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for trials 1, 2, 3 and 4 are 0.31, 0.27, 0.35 and 0.33, respectively.

[b]All treatment values are based on the mean of three determinations within a trial.

[c]The pH was adjusted with a dilute solution of HCl.

[d]The pH was adjusted from 8.5 with a dilute solution of HCl.

[e]The pH was adjusted from 4.0 with a saturated solution of sodium carbonate.

from these data that pH exerted an influence upon protozoal acid production. The optimum pH for maximum acid production was approximately 7.0. Below or above this pH the amount of acids produced was progressively less. This same conclusion

was reached when this experiment was repeated on different days. The average results of 4 such trials are plotted graphically in figure 1. Even though the actual values in one trial were somewhat less than those in the other 3 trials, nevertheless, the same influence of pH upon fatty acid production prevailed. These lower values may be the result of a small harvest of protozoa from the steer at the beginning of the experiment or due to a shorter incubation period.

The results obtained in trials 4, 5, 6, 7, 8 and 9 may appear to be due to the influence of sodium carbonate additions *per se* upon protozoal activity rather than the specific influence of pH. However, data from trials 1, 2 and 3 using hydrochloric acid to adjust pH gave similar pH influences.

Microscopic observation of the contents of the incubation tubes showed that the small protozoa (*Entodinium* and *Dasytricha*) became non-viable during low pH conditions (approximately 5.5), while the larger protozoa (*Diplodinium*, *Isotricha*, *Epidinium* and *Ophryoscolex*) remained viable and fermentatively active. From personal observations, as well as those by Eadie and Hobson (1962), on rumen fluid from intact sheep where the pH was approximately 5.5 or less, the opposite condition appears to be true. During low pH conditions in the intact sheep the larger protozoa disappear first and the smaller protozoa remain. These observations suggest factors other than low pH are exerting inhibitory effects on rumen protozoa. The rate of

passage of ingesta is discussed in part 3 of this thesis in connection with this subject. It is postulated that as the diet particle size decreases and rate of consumption increases, rate of passage subsequently increases. Since the larger protozoa require a longer period for cell division (18 to 24 hours) than the smaller protozoa (12 to 18 hours), it appears that a washing out or a depletion of protozoa from the rumen may take place. When ruminants consume this type of ration it is realized that the pH is also lower than normal and may also contribute to the disappearance of rumen protozoa.

The influence of time upon *in vitro* fatty acid production by rumen protozoa The results of 8 trials on this experiment are summarized and presented in table 5. In microbial fermentations the length of incubation influenced the amount of end products. In trials 1 and 2, acid production (milliequivalents per 20 milliliters) was determined at 0, 2, 4, 8, 16, 24, 48 and 72 hours after the beginning of incubation. Substrate, in the form of starch and sucrose, was supplied at the level of 2 percent. Acid production increased progressively up to and including 72 hours; however, the major portion was produced by 16 to 24 hours of incubation. In trials 3 and 4, with substrate at 2.0 percent and acid determination made at 0, 1, 2, 4, 8, 16, 24 and 48 hours after incubation began, similar results were obtained.

In trials 5, 6 and 7, where substrate was supplied at 1.0 percent, it is of interest to note that maximum acid production

Table 5. Influence of time upon *in vitro* fatty acid production by rumen protozoa[a]

Time (hrs.)[b]	Acid produced (m-equiv./20 ml.)			
	Trials 1&2[c]	Trials 3&4[c]	Trials 5,6&7[d]	Trial 8[e]
0	None	None	None	None
1	--	0.31	--	--
2	0.39	0.76	0.35	0.37
4	0.44	0.93	0.48	0.56
8	0.68	1.09	0.65	0.63
16	0.96	1.40	0.78	0.79
24	1.10	1.65	0.94	0.85
48	1.24	1.80	0.81	0.88
72	1.46	--	0.53	0.98

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for trials (1 & 2), (3 & 4), (5, 6 & 7) and 8 are 0.22, 0.28, 0.18 and 0.30, respectively.

[b]All treatment values are based on the mean of four determinations within a trial.

[c]Values are based on the mean of two separate trials where substrate was 2.0 percent and 24 hour nutrient depletion had been performed.

[d]Values are based on the mean of three separate trials where substrate was 1.0 percent and depletion of nutrients had not been performed.

[e]Values are based upon one trial where substrate was 2.0 percent and depletion of nutrients had not been performed.

was obtained at about the 24 hour period. At the 48 and 72 hour periods acid production was progressively less. With only 1.0 percent substrate and after 20 to 30 hours of fermentation, it is theorized that starch and sucrose were depleted leaving the acids previously produced as the only energy substrate available to the organisms. This is supported by the fact that

pH levels were lowest at the 24 hour period and then increased thereafter indicating a decrease in acidity. Nevertheless, during the first 24 hour period the same pattern of the previous trials is evident.

In trial 8, substrate was supplied at 2.0 percent and acids determined at 0, 2, 4, 8, 16, 24, 48 and 72 hour periods. As in the other trials, the major portion of protozoal acids were produced at the end of the 16 to 20 hour periods. The relatively lower acid production in trials 5, 6, 7 and 8 as compared to the other trials is likely due to lack of a 24 hour nutrient depletion period. Protozoa from trials 1, 2, 3 and 4 were depleted for 24 hours suspended in the depletion medium.

From these data it can be concluded that an incubation period of 16 to 24 hours, when studying acid production by rumen protozoa, was most satisfactory.

Responses of rumen protozoa to substrate and protozoal concentration changes of in vitro fermentations

Artificial rumen techniques involving rumen bacterial fermentations have used substrate levels of 0.5 to 2.0 percent of the media. Cheng et al. (1955) found that when cellulose was supplied at 0.25, 0.50, 0.75, 1.00 and 1.25 percent digestion was 75.7, 57.3, 64.6, 53.6 and 38.1 percent, respectively. Studying oligotrich protozoa of in vitro fermentations, Sugden (1953) supplied substrate at 0.1 percent in the form of powdered cotton-wool and powdered hay. Oxford (1955) used 1 percent glucose as the substrate for culturing holotrich protozoa.

When considering the concentration of rumen microorganisms for *in vitro* fermentations, Cheng *et al.* (1955) obtained the following results. When using 10, 20, 40, 60 and 80 milliliters of rumen fluid to prepare 20 milliliters of washed suspension, cellulose digestion was 27.7, 65.9, 92.8, 93.2 and 95.3 percent, respectively. Sugden (1953) prepared 100 milliliters of fermentation medium which contained protozoa from 200 milliliters of rumen fluid. It is important to indicate the objectives of the above studies were either to obtain optimum cellulose digestion or to maintain viable cultures of microorganisms. Since the primary objective of the *in vitro* experiments of this study was to obtain maximum fatty acid production, optimum substrate levels and protozoal concentration were determined from this point of interest.

Methods and procedures The rumen protozoa used for this experiment were collected and processed the same as described in the first phase of this work and by Christiansen *et al.* (1962). Methods and procedures used in this phase which were common to the first phase are not described here.

The fermentation medium shown in table 2, minus carbohydrates, was used for the studies in this phase. Treatments were assigned using a completely randomized system and were replicated with 3 tubes per treatment in 2 trials and 2 tubes per treatment in the third trial. A factorial arrangement of treatments was used in this experiment to study the effects of levels of substrate and protozoal concentrations as well as

their interrelationships. Protozoal concentrations are designated as "N" which represents the number of milliliters of rumen fluid used to obtain protozoa to prepare a milliliter of fermentation medium.

Trial 1 Rumen protozoa were suspended in the incubation medium at a dilution giving a protozoal concentration of 16 N. After the respective tubes of this treatment were inoculated with 15 milliliters of this suspension, the volume was doubled giving a protozoal concentration of 8 N and the respective tubes inoculated. This was repeated in order to give 4 N and N protozoal concentrations. Carbohydrates were added to the incubation medium at a concentration so that 5 milliliters pipetted into the respective tubes gave 3.20 percent carbohydrate. By doubling the remaining volume of this medium with carbohydrate-free incubation medium, 1.60 percent carbohydrate containing medium was obtained, of which the respective tubes were inoculated. This was repeated in order to give 0.80 and 0.40 percent carbohydrate in the incubation medium. With this technique all tubes contained 20 milliliters of medium. Homogeneous mixtures of medium containing protozoa and carbohydrates, prior to pipetting, were maintained by agitating with a magnetic stirrer. After incubating the experimental-tubes of this trial for 18 hours, protozoal activity was terminated and the fatty acids of the tube contents were analyzed as described previously. The results of this trial are presented in table 6.

Trials 2 and 3 These trials were conducted using essentially the same methods and procedures as trial 1. Trial 2 included a 2 N protozoal concentration level and carbohydrate percentages of 0.15, 0.30, 0.60, 1.20 and 2.40 which differed from trial 1. In trial 3, protozoal concentrations tested, which were different from the other two trials, were 0.5 N, 6 N and 12 N. Carbohydrate levels tested were the same as trial 1 except for the 0.20 percent level.

Following an 18 to 20 hour incubation period, fermentations were terminated and tube contents were analyzed for acids produced as described in the first phase of this work. Treatments were replicated with 3 tubes in trial 2 and 2 tubes in trial 3. Results from trials 2 and 3 are presented in tables 7 and 8, respectively.

Results and discussion The concentration of energy substrate and rumen protozoa used in the medium also affected the amount of fatty acid production per 20 milliliters during a given incubation period. The results of 3 trials are presented in tables 6, 7 and 8.

It is evident from the data of table 6 that fatty acid production increased progressively as both substrate and protozoal concentrations were increased. With carbohydrate substrate levels of 0.40, 0.80, 1.60 and 3.20 percent, fatty acid production (milliequivalents per 20 milliliters) increased from 0.40, 0.49, 0.51, to 0.67, respectively. When protozoal concentrations were increased from N to 4 N, 8 N and 16 N ("N" re-

Table 6. Trial 1 - influence of substrate and protozoal concentrations upon *in vitro* fatty acid production[a]

Substrate level[b]	Protozoal concentrations[c]				
	N	4N	8N	16N	Av.
% CHO					
0.40	0.40	0.48	0.59	0.90	0.59
0.80	0.49	0.56	0.74	1.15	0.74
1.60	0.51	0.67	1.01	1.36	0.89
3.20	0.67	0.99	1.20	1.60	1.12
Av.	0.52	0.68	0.89	1.25	

[a]A difference (D) of 0.21 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of three determinations per treatment.

[c]"N" represents the number of milliliters of rumen fluid used to obtain protozoa to prepare a milliliter of fermentation medium.

presents the number of milliliters of rumen fluid used to obtain protozoa to prepare 1 milliliter of fermentation medium) fatty acids produced increased from 0.40 to 0.48, 0.59 and 0.90, respectively. An additive effect from substrate and protozoal concentrations was noted when observing the factorially arranged data. When substrate level was 3.20 percent and protozoal concentration was 16 N, milliequivalents of fatty acids were 1.60.

In trial 2, with data given in table 7, it is seen that the results were similar to trial 1. Substrate levels were 0.15,

Table 7. Trial 2 - influence of substrate and protozoal concentrations upon in vitro fatty acid production[a]

Substrate level[b]	Protozoal concentration[c]					
	N	2N	4N	8N	16N	Av.
% CHO						
0.15	0.34	0.39	0.43	0.56	0.72	0.48
0.30	0.39	0.42	0.48	0.60	0.85	0.54
0.60	0.43	0.49	0.53	0.71	1.01	0.63
1.20	0.51	0.54	0.64	0.95	1.26	0.78
2.40	0.65	0.68	0.88	1.23	1.69	1.03
Av.	0.46	0.50	0.59	0.81	1.10	

[a]A difference (D) of 0.24 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of three determinations per treatment.

[c]"N" represents the number of milliliters of rumen fluid used to obtain protozoa to prepare a milliliter of fermentation medium.

0.30, 0.60, 1.20 and 2.40 percent and protozoal concentrations were N, 2 N, 4 N, 8 N and 16 N. Fatty acid production was essentially doubled at the highest concentration of substrate and protozoa when compared to the control. An additive effect was also apparent, with the substrate level of 2.40 percent and protozoal concentration of 16 N, milliequivalents of fatty acid was 1.69

The results of trial 3 are presented in table 8. The pro-

Table 8. Trial 3 - influence of substrate and protozoal concentrations upon *in vitro* fatty acid production[a]

Substrate level[b]	Protozoal concentrations[c]							
	½N	N	2N	4N	6N	8N	12N	Av.
% CHO								
0.20	0.30	0.33	0.34	0.39	0.39	0.51	0.70	0.42
0.40	0.35	0.44	0.33	0.39	0.49	0.59	0.79	0.48
0.80	0.39	0.48	0.45	0.45	0.58	0.66	0.84	0.55
1.60	0.44	0.52	0.59	0.62	0.71	0.88	1.16	0.68
3.20	0.52	0.55	0.62	0.70	0.91	1.26	1.78	0.91
Av.	0.40	0.46	0.47	0.51	0.61	0.78	1.05	

[a]A difference (D) of 0.28 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of two determinations per treatment.

[c]"N" represents the number of milliliters of rumen fluid used to obtain protozoa to prepare a milliliter of fermentation medium.

gressive increase in acid production due to increases in substrate and protozoal concentrations is also apparent in this trial. At the low levels (0.5 N protozoal concentrations and 0.20 percent substrate) milliequivalents fatty acid were 0.30 and at the high levels separately (12 N protozoal concentrations and 3.20 percent substrate) milliequivalents fatty acids were 0.70 and 0.52, respectively. When these high levels were combined, an additive response was obtained giving 1.78 milli-

equivalents of fatty acid per 20 milliliters of medium.

When considering the results of these trials and the amount of rumen fluid which can be collected normally, the 4 N or 6 N concentration of rumen protozoa and 1.5 to 2.5 percent substrate appeared to be most desirable for studying *in vitro* fatty acid production by rumen protozoa. Most of the previously listed and all of the following experiments were conducted with about 2.0 percent substrate and 4 N protozoal concentrations.

Responses of rumen protozoa to diethylstilbestrol and antibiotics of *in vitro* fermentations

Methods and procedures The rumen protozoa used for this phase of study were collected and processed the same as described in the first phase of this work and by Christiansen *et al.* (1962). Methods and procedures used in this phase which were common to the first phase are not described here.

The fermentation medium used for these experiments is shown in table 2. Treatments were assigned completely at random.

Influence of diethylstilbestrol (DES) upon rumen protozoa The experimental design for testing level of DES was completely random with each treatment replicated with four tubes within a trial. The DES was suspended in alcohol and dispersed in respective tubes to give levels of none, 0.195,

0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25.0, 50.0 and 100.0 micrograms per 20 milliliters of medium. Protozoal counts are based on log number per milliliter and were performed according to the method described by Moir and Somers (1957).

Protozoal counts were made after the incubation period in trial 1, and both before and after incubation in trial 2. Following a 20-hour incubation, protozoal counts were made on each tube and total fatty acids produced were analyzed as previously described. The results of these trials are presented in table 9.

Influence of streptomycin and penicillin upon rumen protozoa and bacteria To test the effects of level of streptomycin a completely randomized design was used. To test the effects of level of streptomycin and penicillin as well as their relationships, a completely randomized design, with a 4 x 4 factorial arrangement of treatments, was used. In six trials where levels of streptomycin were tested, each treatment was replicated with four tubes within each trial. In the other trial, involving the factorial arrangement, each treatment was replicated with three tubes.

Streptomycin was suspended in distilled water and dispersed into respective tubes to give levels of none, 0.25, 0.05 and 0.10 percent (w/v) in three trials and levels of none, 0.25 and 0.05 percent in three other trials. In the other trial, streptomycin and penicillin suspended in distilled water were dispersed in respective tubes with a factorial arrangement

to give levels of none, 0.0125, 0.025 and 0.05 percent of both antibiotics. Following a 20 to 22-hour incubation total fatty acids produced were analyzed as previously described. The results of these trials are presented in tables 10 and 11.

In two other trials, a completely random design with a 2 x 4 factorial arrangement of treatments was used to test the level and relationship of sodium and streptomycin in regard to their influence upon rumen protozoa. Treatments were replicated with four tubes within each trial. In both trials streptomycin was tested at 0.05 percent as well as a control with none. The effects of sodium as sodium acetate were tested at 0, 50, 100 and 200 milligrams (Na) per 20 milliliters of fermentation medium. Total protozoal fatty acids produced were analyzed at the end of the incubation period as previously described. The results of these trials are presented in table 12. Microscopic observations were made from time to time during and following the incubation period observing for the presence or absence of bacteria.

Results and discussion

Diethylstilbestrol (DES) The summarized results of two trials are given in table 9. In trial 1, the addition of 0.195 to 12.5 micrograms of DES per 20 milliliters of fermentation media stimulated protozoal fatty acid production. Maximum fatty acid production was obtained at the 1.56 to 12.5 microgram levels while the 50.0 and 100.0 microgram levels gave depression in protozoal fatty acid production. Protozoal num-

Table 9. Influence of diethylstilbestrol (DES) upon *in vitro* fatty acid production by rumen protozoa[a]

DES levels[b]	Acid produced		Protozoal count		
			Trial 1	Trial 2	
	Trial 1	Trial 2	After[c]	Before[c]	After[c]
(mcg./20 ml.)	(m-equiv./20 ml.)		(x1000)	(x1000)	(x1000)
None	0.42	0.38	11.4	9.6	7.2
0.195	0.52	0.42	10.4	11.5	11.8
0.390	0.56	0.44	9.6	10.6	12.8
0.780	0.53	0.48	11.7	11.5	12.7
1.560	0.62	0.50	13.4	9.5	13.9
3.120	0.69	0.53	14.3	10.4	14.7
6.250	0.59	0.45	14.1	9.7	13.3
12.500	0.68	0.48	12.4	11.6	12.9
25.000	0.41	0.40	11.8	10.5	8.4
50.000	0.33	0.39	10.9	9.4	9.1
100.000	0.33	0.36	9.4	9.8	9.3
Av.				10.4	12.4

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for trials 1 and 2 are 0.16 and 0.13, respectively.

[b]All treatment values were based on the mean of four determinations per treatment within a trial.

[c]Before and after refer to the fermentation periods.

bers appear to be enhanced at the 1.56 to 12.5 microgram levels with a slight depression noted at the 100.0 microgram level. In trial 2, the same trend is evident. Protozoal fatty acid production was stimulated from the 0.195 to 25.0 microgram levels. Maximum fatty acid production resulted at DES levels of 0.78, 1.56, 3.12, 6.25 and 12.5 micrograms per 20 milliliters of fermentation medium. Depression was slight at the 100.0 microgram level.

With protozoal counts made both before and after incubation in this trial, it appeared that all levels of DES except 25.0, 50.0 and 100.0 micrograms enhanced protozoal numbers. Maximum numbers were obtained at the 3.12 microgram level. In trial 2, the average number of protozoa per milliliter before incubation was 10.4×10^3 for all treatment levels while the average number after incubation was 12.4×10^3. It should be noted that the control, with no DES, decreased in number from 9.6×10^3 before to 7.2×10^3 after incubation.

When calculating the DES concentration within the rumen of 1000 pound steers fed 20 milligrams per head per day, it was found that the level would be about 2 to 4 micrograms per 20 milliliters of rumen fluid. It can be noted that in both trials maximum protozoal numbers and fatty acids produced were obtained at levels of 1.56 to 12.5 micrograms per milliliters of liquid. The increases noted are believed to be due to an estrogenic enhancement of protozoal cell division. This is supported by studies of Cleveland (1949) that showed protozoa of the termite and roach possess a sexual reproduction process which can be enhanced or depressed by the presence or absence of insect hormones. It was also shown by Ferber (1928) that pregnant and lactating heifers contained about double the rumen protozoa as compared to heifers not pregnant or lactating. It is well known that the level of circulating estrogenic hormones are higher in pregnant and lactating mammals than in non-pregnant and non-lactating mammals.

Streptomycin The results of six trials summarizing this experiment are presented in table 10. When compared

Table 10. Influence of streptomycin upon *in vitro* fatty acid production by rumen protozoa[a]

Trial no.[b]	Streptomycin levels			
	None	0.025%	0.05%	0.10%
1	0.55	0.48	0.42	0.44
2	0.88	0.65	0.64	0.65
3	0.48	0.36	0.37	0.38
4	0.74	0.58	0.53	
5	0.65	0.49	0.51	
6	0.93	0.72	0.70	
Av.	0.71	0.55	0.54	0.49

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for trials 1, 2 and 3 is 0.18 and for trials 4, 5 and 6 is 0.22.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based upon the mean of five determinations per treatment within a trial.

to none, levels of 0.025, 0.05 and 0.10 percent streptomycin decreased fatty acid production (milliequivalents per 20 milliliters) from 0.71 to 0.55, 0.54 and 0.49, respectively. In all six trials a marked depression was obtained when streptomycin was added as compared to the control which contained no streptomycin. It can also be noted that levels of 0.05 and 0.10 percent streptomycin did not further depress acid production as

compared to the 0.025 percent level.

Microscopic observations indicated that bacteria were abundantly present in tube contents not treated with streptomycin. These observations also failed to reveal viable bacteria from streptomycin treated tube contents.

Streptomycin and penicillin The results of the factorial experiment conducted to study the level and interrelationships of streptomycin and penicillin are presented in table 11. Additions of streptomycin and penicillin at levels

Table 11. Influence of streptomycin and penicillin upon *in vitro* fatty acid production by rumen protozoa[a]

Penicillin levels[b]	Streptomycin levels				
	None	0.0125%	0.025%	0.05%	Av.
None	1.42	1.09	0.92	0.89	1.08
0.0125%	1.20	0.91	0.84	0.90	0.96
0.025%	1.15	0.95	0.88	0.80	0.95
0.05%	1.25	0.72	0.85	0.78	0.90
Av.	1.26	0.92	0.87	0.84	

[a]A difference (D) of 0.26 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based upon the mean of three determinations per treatment.

of 0.0125, 0.025 and 0.05 percent, when compared to none, depressed fatty acid production considerably. The control level of fatty acid (milliequivalents per 20 milliliters) was 1.42,

while the respective levels of streptomycin showed a decrease to 1.09, 0.92 and 0.89. With penicillin the decrease was from 1.42 to 1.20, 1.15 and 1.25, respectively. Maximum depression of acid production was obtained at the combined levels of 0.0125 percent of streptomycin and 0.05 percent penicillin. It is of interest to note that higher levels of these antibiotics failed to further depress fatty acid production. It is apparent that streptomycin had a further depressing effect, at comparable levels, than penicillin. Streptomycin and penicillin levels of 0.025 each or 0.05 percent streptomycin appear to be the optimum for inhibiting bacterial growth in these short time fermentations.

Sodium and streptomycin The results of 2 trials involving factorial arrangement of treatments of sodium and streptomycin are summarized and presented in table 12. Additions of 0.05 percent streptomycin gave similar depression of fatty acid production as the previous trials. This depression was relatively constant when 50, 100 and 200 milligrams of sodium per 20 milliliters of liquid were added. The average depression in fatty acid production for the two trials due to streptomycin was 0.24 milliequivalents per 20 milliliters of medium.

Evidence that the depression in fatty acid production is due to inhibition of bacteria is, first, microscopic observation failed to reveal motile bacteria on antibiotic treated cultures; secondly, the substantial depression obtained at the

Table 12. Influence of sodium and streptomycin upon in vitro fatty acid production by rumen protozoa[a]

Streptomycin level[b]	Na acetate level (mgm./20 ml.) 0	50	100	200	Av.	Av. of 2 trials
Trial 1						
None	0.70	0.81	0.85	0.83	0.80	
0.05%	0.53	0.58	0.61	0.69	0.60	
Av.	0.61	0.69	0.73	0.76		
Trial 2						
None	0.76	0.85	0.83	0.87	0.83	0.815
0.05%	0.48	0.57	0.56	0.60	0.55	0.575
Av.	0.62	0.71	0.70	0.73		
Av. of 2 trials	0.615	0.700	0.715	0.745		

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for trials 1 and 2 are 0.22 and 0.19, respectively.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of four determinations per treatment within a trial.

low levels of antibiotic was not progressive when levels were increased. It thus appears that bacteria are inhibited with little or no detrimental effects upon rumen protozoa.

Responses of rumen protozoa to sodium, potassium and phosphorus in vitro fermentations

Methods and procedures Rumen protozoa used for this phase of study were collected and processed as described in the first phase of this work and by Christiansen et al. (1962).

Methods and procedures used in this phase which were common to the first phase are not described in this section.

The fermentation medium used for these experiments is shown in table 2. When studying rumen protozoal requirements for a given mineral, sodium for example, the medium was left free of that particular mineral. Treatments were assigned completely at random.

Influence of sodium upon in vitro fatty acid production by rumen protozoa To study the effects and requirements of sodium on rumen protozoa, sodium chloride and sodium acetate were used most extensively. To study possible differences of source of sodium, several sodium containing compounds were used. Treatment levels listed always indicate the amount of sodium per se, not the complete compound.

The experimental design for testing sodium effects upon rumen protozoa was completely random with each treatment replicated with three or four tubes within a trial.

All sodium containing compounds used were suspended in distilled water and dispersed in the respective tubes to give the specified levels. The volume of all tubes was equalized with distilled water.

Trials 1, 2 and 3 To test the effects of level of sodium and the interrelationships of sodium chloride and sodium acetate, a completely randomized design with treatments arranged in a 4 x 4 factorial plan was used.

In trial 1, with results presented in table 13, sodium chloride and sodium acetate were each tested at levels of 0, 25, 50 and 100 milligrams of sodium per 20 milliliters of fermentation liquid. In trial 2, with results presented in table 14, sodium chloride was tested at levels of 0, 50, 100 and 200 while sodium acetate was at 0, 25, 100 and 400 milligrams per 20 milliliters of fermentation liquid. In trial 3, with results given in table 15, sodium chloride and sodium acetate were each added at the levels of 0, 50, 100 and 200 milligrams of sodium per 20 milliliters of liquid.

Trial 4 To study the effect of levels of sodium, a completely randomized design was used. With three different compounds (sodium chloride, sodium acetate and sodium sulfate) level effects were determined at 0, 12.5, 25, 50, 100, 200 and 400 milligrams of sodium per 20 milliliters of fermentation liquid. The results of this trial are presented in table 16.

Trial 5 In determining the effect of several

different sodium containing compounds upon fatty acid production by rumen protozoa, a completely randomized design was used. In the first trial (5a) ten different compounds were used. They were as follows: sodium chloride, sodium acetate, sodium carbonate, sodium bicarbonate, sodium sulfate, sodium sulfite, sodium phosphate, sodium borate, sodium citrate and sodium butyrate. In the second trial (5b) all of these compounds except sodium chloride, sodium acetate, sodium citrate and sodium butyrate were tested. For comparison, two sodium-free controls with four replications each were included.

In all trials pertaining to sodium effects upon rumen protozoa, the incubations were terminated 18 to 20 hours from the beginning. The fatty acids produced per tube were analyzed as previously described.

Influence of sodium and potassium upon in vitro fatty acid production by rumen protozoa To study the interrelationships of sodium and potassium upon acid production by rumen protozoa, sodium chloride and potassium chloride were used in a completely random design with a 5 x 5 factorial arrangement of treatments. Treatments were replicated with three fermentation tubes.

Sodium and potassium chlorides were suspended in distilled water and dispersed in the respective tubes to give levels of 0, 50, 100, 200 and 400 milligrams of each compound per 20 milliliters of liquid. The interrelationships were determined with the combination levels of each compound as stated. Total

protozoal fatty acids produced were analyzed as previously described at the end of an 18 to 20 hour incubation period. Results of this trial are shown in table 18.

Influence of potassium upon in vitro fatty acid production by rumen protozoa To determine the effects and requirements of potassium on rumen protozoa, potassium chloride, potassium acetate, potassium phosphate and potassium sulfate were used. Treatment levels listed indicate the amount of potassium per se.

The experimental design used to determine the effect of potassium upon rumen protozoa was completely random with each treatment replicated with three or four tubes within a trial.

All potassium containing compounds used were suspended in distilled water and dispersed in the respective tubes to give the specified levels. The volume of all tubes were equalized with distilled water.

Trial 1 To determine the effect of level of sodium and the interrelationships of potassium chloride and potassium acetate, a completely randomized design with treatments arranged in a 3 x 3 factorial method was used. As seen in table 19 (results of this trial) potassium chloride and potassium acetate were each studied at levels of 0, 50 and 100 milligrams of potassium per 20 milliliters of fermentation liquid.

Trial 2 The same experimental design and levels were used in this trial as in trial 1. The effects of

sodium supplied by sodium phosphate and sodium sulfate were studied. Results of this trial are given in table 20.

Trial 3 To determine the requirement for potassium, 3 trials were conducted using 3 different potassium containing compounds. These compounds, potassium chloride, potassium acetate and potassium sulfate, were studied at levels of 0, 12.5, 25, 50, 100, 200 and 400 milligrams of potassium per 20 milliliters of fermentation liquid. A completely random design with treatments replicated with four tubes was used to determine the requirement level of potassium for maximum fatty acid production by rumen protozoa. The results of these trials are presented in table 21.

Influence of phosphorus upon in vitro fatty acid production by rumen protozoa To study the effects and requirements of phosphorus upon rumen protozoa, sodium phosphate and potassium phosphate were used in a completely random design with a 4 x 4 factorial arrangement of treatments. Treatment levels listed represent the amount of phosphorus per se. All phosphorus containing compounds used were suspended in distilled water and dispersed into the respective tubes to give the specified levels.

Trials 1 and 2 Sodium phosphate and potassium phosphate were used in a factorial experiment at levels of 0, 37.5, 75.0 and 150.0 milligrams of phosphorus per 20 milliliters of liquid in studying protozoal fatty acid production. The results of trial 1 are shown in table 22.

In trial 2 these same phosphorus containing compounds were used at levels of 0, 50, 100 and 200 milligrams per 20 milliliters of liquid. The results of this trial are presented in table 23.

Trial 3 To determine the effect of levels of phosphorus upon rumen protozoa, a completely randomized design was used. With two compounds (sodium phosphate and potassium phosphate) level effects were determined at 0, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 milligrams of phosphorus per 20 milliliters of liquid. Two controls per trial with three replications each, free of phosphorus, were conducted for the purpose of treatment comparisons. The results of this experiment are given in table 24.

Results and discussion

Influence of sodium upon in vitro fatty acid production by rumen protozoa The results of the additions of various levels of sodium chloride and sodium acetate, alone and in combination, to protozoal fermentation media are given in table 13. When 25, 50 and 100 milligrams of sodium chloride and sodium acetate were added per 20 milliliters of medium, a progressive increase was obtained as compared to the sodium-free control. The increase was of even greater magnitude when the combination of these compounds was added. Maximum protozoal fatty acid production was obtained when combination levels of 100 milligrams of sodium chloride and sodium acetate were added.

Table 13. Trial 1 - influence of sodium upon *in vitro* fatty acid production by rumen protozoa[a]

Na acetate level[b]	NaCl level (mgm./20 ml.)				
	0	25	50	100	Av.
(mgm./20 ml.)					
0	0.28	0.31	0.36	0.42	0.34
25	0.35	0.38	0.41	0.46	0.40
50	0.39	0.41	0.46	0.51	0.44
100	0.44	0.45	0.53	0.58	0.50
Av.	0.36	0.39	0.44	0.49	

[a]A difference (D) of 0.18 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of three determinations per treatment.

In trial 2 (results shown in table 14), levels of 50, 100 and 200 milligrams of sodium chloride stimulated a progressive increase while levels of 100 and 400 milligrams of sodium acetate failed to increase fatty acid production as compared to the sodium-free control. Maximum production of protozoal fatty acids was obtained at the 200 milligram level of sodium chloride and 100 milligrams of sodium acetate. The failure of the 400 milligram level of sodium acetate to stimulate fatty acid production may have been due to toxicity effects at that level. The low level of fatty acid production at the lowest level of each compound is thought to be an abnormal result since other trials showed stimulation at these levels.

Table 14. Trial 2 - influence of sodium upon *in vitro* fatty acid production by rumen protozoa[a]

Na acetate level[b]	NaCl level (mgm./20 ml.)				
	0	50	100	200	Av.
(mgm./20 ml.)					
0	0.30	0.27	0.38	0.48	0.36
25	0.27	0.33	0.34	0.44	0.35
100	0.33	0.35	0.41	0.53	0.41
400	0.33	0.44	0.47	0.48	0.43
Av.	0.31	0.35	0.40	0.48	

[a]A difference (D) of 0.15 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of three determinations per treatment.

The results of trial 3 are shown in table 15. When compared to the sodium-free control, levels of 50, 100 and 200 milligrams stimulated a progressive increase in fatty acid production. This was nearly true with these levels of sodium acetate as well. An additive influence of both compounds is evident, although maximum fatty acid production was obtained at the 200 milligram sodium chloride and 100 milligram sodium acetate levels. At the 100 and 200 milligram levels of sodium chloride, where 200 milligrams of sodium acetate were present, a depression in fatty acid production is noted.

The results of the additions of various levels of sodium

Table 15. Trial 3 - influence of sodium upon *in vitro* fatty acid production by rumen protozoa[a]

Na acetate level[b]	NaCl level (mgm./20 ml.)				
	0	50	100	200	Av.
(mgm./20 ml.)					
0	0.50	0.49	0.56	0.66	0.55
50	0.61	0.65	0.64	0.67	0.64
100	0.59	0.65	0.54	0.75	0.63
200	0.62	0.82	0.59	0.71	0.69
Av.	0.58	0.65	0.58	0.72	

[a]A difference (D) of 0.13 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of three determinations per treatment.

as sodium chloride, sodium acetate and sodium sulfate are shown in table 16. From these data it is indicated that maximum fatty acid production results at the 100 to 200 milligram level and in every case the 400 milligram level depressed protozoal fatty acid production. In trial 4c, the results indicate that the 200 milligram level was toxic, but since higher levels produced greater amounts of fatty acids, it may have been an atypical response. From these results and those from trials 1, 2 and 3, it appears the optimum level of sodium for maximum protozoal fatty acid production is about the 200 milligram level (1 percent, w/v).

Table 16. Trial 4 - influence of sodium levels upon *in vitro* fatty acid production by rumen protozoa[a]

Sodium level[b]	Acid produced (m-equiv./20 ml.) Trial 4a	Trial 4b	Trial 4c	Av.
(mgm./20 ml.)	(NaCl)	(Na acetate)	($NaSO_4$)	
0	0.36	0.30	0.45	0.37
12.5	0.44	0.39	0.57	0.47
25	0.52	0.49	0.78	0.60
50	0.54	0.56	0.98	0.69
100	0.60	0.66	1.30	0.85
200	0.57	0.64	0.60	0.60
400	0.40	0.44	0.88	0.57
Av.	0.49	0.50	0.65	

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for trials 4a, 4b and 4c are 0.23, 0.16 and 0.29, respectively.

[b]All treatment values are based on the mean of four determinations per treatment within a trial.

The results of the effects of the additions of various sources of sodium upon *in vitro* fatty acid production by rumen protozoa are shown in table 17. In these trials (5a and 5b) 100 milligrams of sodium per 20 milliliters of liquid as sodium chloride, sodium acetate, sodium sulfite, sodium phosphate, sodium borate, sodium citrate and sodium butyrate were studied. When compared to the sodium-free controls, in trial 5a, all sodium containing compounds, except sodium citrate and sodium butyrate, stimulated fatty acid production by about 100 percent. In this trial sodium citrate and sodium butyrate depressed fatty acid production nearly 100 percent.

In trial 5b, the sodium containing compounds tested (so-

Table 17. Trial 5 - influence of sodium from various sources upon *in vitro* fatty acid production by rumen protozoa[a]

Sodium source[b]	Na level	Acid produced (m-equiv./20 ml.) Trial 5a	Trial 5b
	(mgm./20 ml.)		
None	0	0.73	0.63
None	0	0.74	0.58
NaCl	100	1.43	
Na acetate	100	1.42	
$NaCO_3$	100	1.54	1.30
NaH_2CO_3	100	1.30	1.38
Na_2SO_4	100	1.30	0.98
Na_2SO_3	100	1.50	1.09
Na_2HPO_4	100	1.28	1.10
Na borate	100	1.90	1.59
Na citrate	100	0.39	
Na butyrate	100	0.36	

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for trials 5a and 5b are 0.32 and 0.38, respectively.

[b]All treatment values are based on the mean of four determinations per treatment within a trial.

dium complexed with carbonate, bicarbonate, sulfate, sulfite, phosphate and borate) stimulated acid production similar to the stimulation in trial 5a.

It is of interest to note that in both trials, sodium borate yielded a greater increase of acid production than any other compound. It is possible that rumen protozoa have a requirement for boron. This, of course, has never been demonstrated for animal life as it has for plants.

From these data it is concluded that the optimal sodium requirement for maximum acid production by rumen protozoa is

0.75 percent plus or minus 0.25 percent (w/v). It also appears that sodium can be supplied in several different forms.

Influence of sodium and potassium, alone and in combination, upon in vitro fatty acid production by rumen protozoa The results of the factorial experiment on the effects of various levels of sodium (sodium chloride) and/or potassium (potassium chloride) on acid production by rumen protozoa are presented in table 18. The 50, 100 and 200 milligram levels of

Table 18. Influence of sodium and potassium upon in vitro fatty acid production by rumen protozoa[a]

KCl level[b]	NaCl level (mgm./20 ml.)					
	0	50	100	200	400	Av.
(mgm./20 ml.)						
0	0.50	0.55	0.57	0.64	0.49	0.53
50	0.55	0.56	0.59	0.58	0.47	0.55
100	0.58	0.61	0.65	0.59	0.54	0.59
200	0.56	0.54	0.51	0.51	0.48	0.52
400	0.35	0.15	0.10	0.30	0.35	0.25
Av.	0.51	0.48	0.48	0.50	0.47	

[a]A difference (D) of 0.21 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of three determinations per treatment.

sodium chloride progressively stimulated acid production, while the 400 milligram level was similar to the sodium-free control.

Levels of 50, 100 and 200 milligrams of potassium chloride stimulated while 400 milligrams depressed fatty acid production. Maximum fatty acid production resulted when sodium chloride and potassium chloride were in combination each at 100 milligrams per 20 milliliters of fermentation medium.

The highest level of sodium chloride was not detrimental to fatty acid production by rumen protozoa but the highest level of potassium chloride depressed fatty acid production. It is of interest to note that fatty acid production was almost completely inhibited when potassium chloride was added at 400 milligrams and sodium chloride at 100 milligrams. Fatty acid production increased at the high level of potassium chloride when sodium chloride was present at 200 and 400 milligrams per 20 milliliters of fermentation medium.

Influences of potassium upon *in vitro* acid production by rumen protozoa The results of the factorial experiment used to study the effects of level and interrelationships of potassium chloride and/or potassium acetate upon acid production by rumen protozoa are shown in table 19. Additions of potassium (potassium chloride) at 50 and 100 milligrams per 20 milliliters increased acid production by about 30 percent compared to the potassium-free control. Similar increases in fatty acid production were obtained when 50 and 100 milligrams of potassium acetate were added.

Maximum fatty acid production was obtained when 50 milligrams of potassium chloride and potassium acetate were added in

Table 19. Trial 1 - influence of potassium upon *in vitro* fatty acid production by rumen protozoa[a]

K acetate level[b]	KCl level (mgm./20 ml.) 0	50	100	Av.
(mgm./20 ml.)				
0	0.59	0.77	0.78	0.71
50	0.70	0.87	0.76	0.78
100	0.75	0.54	0.53	0.61
Av.	0.68	0.73	0.69	

[a]A difference (D) of 0.25 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of three determinations per treatment.

combination. It can be seen from table 19 that slight depressions in fatty acid production resulted when 100 milligrams of potassium acetate and either 50 or 100 milligrams of potassium chloride were added.

In another trial, the effect of potassium, in the form of potassium phosphate and potassium sulfate, upon fatty acid production by rumen protozoa was studied and the results are shown in table 20. When potassium phosphate was added at the 50 milligram level, an increase of fatty acid production of about 300 percent was obtained as compared to the potassium-free control. However, when 100 milligrams were added per 20 milliliters of liquid, the response was only about 200 percent of the potas-

Table 20. Trial 2 - influence of potassium upon *in vitro* fatty acid production by rumen protozoa[a]

K_2SO_4 level[b]	KH_2PO_4 level (mgm./20 ml.) 0	50	100	Av.
(mgm./20 ml.)				
0	0.58	1.75	1.17	1.17
50	0.73	1.95	1.00	1.23
100	0.67	2.58	0.87	1.37
Av.	0.66	2.14	1.01	

[a]A difference (D) of 0.28 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of three determinations per treatment.

sium-free control.

Additions of 50 and 100 milligrams of potassium sulfate gave increases in fatty acid production of a much lesser magnitude, about 20 percent. It is of interest to note the tremendous increase in fatty acid production at the 100 milligram level of potassium sulfate and the 50 milligram level of potassium phosphate. There was almost a 5-fold increase over the potassium-free control. This, of course, indicates a synergistic effect of potassium phosphate and potassium sulfate.

From the data of these trials, it appears that the optimum level of potassium for maximum fatty acid production is from 0.50 to 0.75 percent of the fermentation medium. These data

also indicate a different response magnitude with different sources and combinations of potassium containing compounds. In trial 2, the abnormally high responses may have been due to the sulfate and phosphate effect upon rumen protozoal acid production.

The results of adding various levels of potassium in three forms, potassium chloride, potassium acetate and potassium sulfate, upon the fatty acid production of rumen protozoa are given in table 21. Each of these compounds was added at 12.5,

Table 21. Trial 3 - influence of level of potassium upon *in vitro* fatty acid production by rumen protozoa[a]

Potassium level[b]	Acid produced (m-equiv./20 ml.)			
	Trial 3a	Trial 3b	Trial 3c	Av.
(mgm./20 ml.)	(KCl)	(K acetate)	(K_2SO_4)	
0	0.51	0.58	0.45	0.51
12.5	0.60	0.65	0.52	0.59
25	0.73	0.70	0.68	0.70
50	0.84	0.88	0.65	0.79
100	0.73	0.82	0.66	0.74
200	0.31	0.42	0.30	0.41
400	0.22	0.33	0.15	0.23
Av.	0.56	0.60	0.54	

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for trials 3a, 3b and 3c are 0.21, 0.19 and 0.24, respectively.

[b]All treatment values are based on the mean of four determinations per treatment within a trial.

25, 50, 100, 200 and 400 milligrams per 20 milliliters of fermentation liquid. Potassium chloride stimulated fatty acid production progressively up to the 50 milligram level and the

100 milligram level response was nearly 50 percent above the potassium-free control. A response pattern very similar to trial 3a was obtained in trial 3b when testing sodium acetate. In trial 3c, when potassium sulfate was studied, the response was progressive to the 25 milligram level and a plateau resulted at the 100 milligram level. In all three trials the 200 and 400 milligram level depressed fatty acid production drastically. When adding 400 milligrams of potassium sulfate per 20 milliliters of medium, fatty acid production was almost totally suppressed.

Considering the results of all trials studying potassium containing compounds and their effect upon rumen protozoa, the optimum level for maximum fatty acid production appears to be 100 plus or minus 50 milligrams of potassium. There appears to be a response difference from different sources of potassium.

Influence of phosphorus upon *in vitro* fatty acid production by rumen protozoa The results of additions of phosphorus in two forms (sodium phosphate and potassium phosphate) upon fatty acid production of rumen protozoa are given in tables 22 and 23. Using a factorial experiment, this study involved sodium phosphate and/or potassium phosphate at levels of 37.5, 75.0 and 150.0 milligrams of phosphorus per 20 milliliters of medium. These additions resulted in tremendous progressive increases over the phosphorus-free control. In the sodium form more than a 4-fold increase was obtained and with potassium form more than a 5-fold increase resulted. Maximum

Table 22. Trial 1 - influence of phosphorus upon *in vitro* fatty acid production by rumen protozoa[a]

KH_2PO_4 level[b]	Na_2HPO_4 level (mgm./20 ml.)				
	0	37.5	75.0	150.0	Av.
(mgm./20 ml.)					
0	0.64	0.77	1.10	2.95	1.72
37.5	0.97	1.86	2.60	3.63	2.27
75.0	2.22	1.97	2.84	2.08	2.28
150.0	3.35	2.59	1.88	0.95	2.19
Av.	1.79	1.78	2.11	2.40	

[a]A difference (D) of 0.20 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of three determinations per treatment.

response resulted at the combined high level of sodium phosphate and the 37.5 milligram level of potassium phosphate.

In trial 2, where sodium phosphate and potassium phosphate at levels of 50, 100 and 200 milligrams were tested, progressive responses in acid production were obtained similar to that of trial 1. The overall responses were of less magnitude, however. In both cases the response was about a 3-fold increase over the phosphorus-free control. Maximum response was obtained at the combined level of 100 milligrams of each compound.

It can be concluded from these studies that the optimum level of phosphorus for maximum protozoal fatty acid production

Table 23. Trial 2 - influence of phosphorus upon *in vitro* fatty acid production by rumen protozoa[a]

KH_2PO_4 level[b]	Na_2HPO_4 level (mgm./20 ml.)				
	0	50	100	200	Av.
(mgm./20 ml.)					
0	0.38	0.50	0.85	0.98	0.68
50	0.62	0.78	0.95	1.42	0.94
100	0.92	1.38	1.64	0.85	1.20
200	1.13	1.34	0.72	0.43	0.91
Av.	0.76	1.00	1.04	0.92	

[a]A difference (D) of 0.26 required for significance at P = .05 level for comparison of treatment means.

[b]All treatment values are expressed as milliequivalents of acid per 20 milliliters and are based on the mean of three determinations per treatment.

is about 200 milligrams per 20 milliliters of fermentation liquid.

In trials 3a and 3b, studying the influence of phosphorus levels upon fatty acid production of rumen protozoa, various levels of sodium and potassium phosphates were used and the results are summarized and presented in table 24. With levels of phosphorus beginning at 0.39 milligrams per 20 milliliters of medium and doubled for each of 10 treatment levels to give a maximum 400 milligrams, the fatty acid production stimulation was progressive up to and including the 200 milligram level. The acid production at the 400 milligram level was much greater

Table 24. Trial 3 - influence of phosphorus levels upon *in vitro* fatty acid production by rumen protozoa[a]

Phosphorus level[b]	Acid produced (m-equiv./20 ml.) Trial 3a	Trial 3b	Av.
(mgm./20 ml.)	(KH_2PO_4)	(Na_2HPO_4)	
0	0.63	0.47	0.55
0	0.61	0.43	0.52
0.39	0.67	0.56	0.61
0.78	0.76	0.50	0.63
1.56	0.82	0.68	0.75
3.125	0.87	0.74	0.81
6.25	0.95	0.85	0.90
12.5	1.10	0.89	0.99
25	1.31	0.99	1.15
50	1.93	1.24	1.59
100	2.32	1.38	1.85
200	2.44	1.60	2.02
400	1.48	0.98	1.23
Av.	1.29	0.89	

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for trials 3a and 3b are 0.26 and 0.28, respectively.

[b]All treatment values are based on the mean of three determinations per treatment within a trial.

than the phosphorus-free control but greatly less than the three other highest levels of phosphorus.

In both trials maximum fatty acid production resulted at the 200 milligram level. From the data of all trials on phosphorus studies, it is indicated that the optimum level for maximum fatty acid production by rumen protozoa is about 200 milligrams of phosphorus per 20 milliliters of fermentation medium. It is further indicated that the phosphorus needed can be supplied by either sodium phosphate or potassium phosphate.

CONCLUSIONS AND SUMMARY

A series of laboratory investigations were conducted in an attempt to develop an *in vitro* technique by which fatty acid production by rumen protozoa could be measured. As a result of these investigations, a multiple-tube laboratory technique for studying factors influencing fatty acid production by rumen protozoa in the absence of bacteria was developed and is described and illustrated by example. The technique involved harvesting a large mass of protozoa from a mature rumen fistulated steer, separation of the protozoa from bacteria and food particles and multiple-tube fermentation tests with imposed variables with results assayed by titration of total fatty acids following incubation. The technique is illustrated by results with a high degree of repeatability from the experiments testing the effects of various factors upon fatty acid production by rumen protozoa.

It is apparent from the data of this work that pH exerted an influence upon *in vitro* fatty acid production of rumen protozoal fermentations. The optimum pH for maximum fatty acid production was approximately 7.0. Below or above this pH, the amount of fatty acids produced was progressively less. The presence of a pH of about 7.5 or more was very lethal to rumen protozoa while pH levels of 5.5 or less were very inhibitory to protozoal fatty acid production but many protozoa remained motile during low pH conditions.

The amount and rate of fatty acid production was affected by the length of incubation. Fatty acid production increased progressively up to 72 hours of incubation; however, the major portion was produced at 16 to 24 hours of incubation. It was concluded that an incubation period of 16 to 24 hours when studying fatty acid production by rumen protozoa was most satisfactory.

The concentration of energy substrate and size of inoculum of rumen protozoa also affected the amount of protozoal fatty acid production. Progressive increases of fatty acid production resulted as energy substrate was increased from 0.15 to 3.20 percent (with various intermediate levels). Similar progressive increases were obtained as protozoal concentrations (N equals the number of milliliters of rumen fluid used to prepare a milliliter of fermentation medium) were increased from 0.5 N to 16 N (with intermediate levels). In factorial experiments with various levels of substrate and/or protozoal concentrations, additive responses were obtained. It is concluded that 1.5 to 2.5 percent substrate and 4 to 6 N protozoal concentrations produce satisfactory results.

Rumen protozoal numbers and fatty acid production were affected by various additions of diethylstilbestrol (DES) to the in vitro fermentation medium. This study indicates that the optimum level of DES for maximum protozoal numbers and fatty acid production is from about 2 to 6 micrograms per 20 milliliters of fermentation medium. It was of interest to note that

the amount of DES in rumen liquid of 1000 pound steers fed 10 milligrams per day is in the range of 2 to 4 micrograms per 20 milliliters. Levels of 25, 50 and 100 micrograms appeared to be toxic to rumen protozoa since protozoal numbers and fatty acid production were depressed.

Antibiotics (streptomycin and penicillin) were used to inhibit bacterial activities. All levels of streptomycin and penicillin depressed fatty acid production, but the higher levels (0.05 and 0.10 percent) failed to increase the depression. The higher level of fatty acid produced by the antibiotic-free control was apparently due to bacterial contamination, which was prevented by streptomycin and penicillin. Streptomycin and penicillin levels of 0.025 or 0.05 percent streptomycin appear to be optimum for inhibiting bacterial growth during short time fermentations.

The addition of various amounts of sodium, potassium and phosphorus in various forms and levels affected the fatty acid production by rumen protozoa.

These studies indicate that about 0.75 percent of sodium was optimal for maximum production of fatty acids by rumen protozoa. Sizable protozoal fatty acid stimulations were obtained when sodium was added in several different forms (sodium as the chloride, acetate, carbonate, bicarbonate, sulfate, sulfite, phosphate and borate). With three different compounds, sodium chloride, sodium acetate and sodium sulfate, tested at various levels, maximum protozoal fatty acid production was obtained at

the 0.50 percent level. Depression in fatty acid production resulted when sodium was added at 1.0 percent or higher levels.

When sodium chloride and/or potassium chloride were studied, maximum protozoal fatty acid production resulted when 0.5 percent of each compound was present.

From the data of experiments studying the potassium needs of protozoa, it appears that the optimum level for maximum fatty acid production is from 0.50 to 0.75 percent. Depression in protozoal fatty acid production resulted when 1.0 and 2.0 percent potassium was added. The data also indicate a different response magnitude with different sources and combinations of potassium containing compounds.

The additions of various amounts and forms of phosphorus resulted in stimulation of protozoal fatty acid production. It was concluded from data of these studies that the optimum level of phosphorus for maximum protozoal fatty acid production is about 1.0 percent of the volume of the fermentation liquid. When studying various levels, a progressive increase in fatty acid production resulted when phosphorus was added up to 1.0 percent while a depression was obtained at the 2.0 percent level. These studies further indicate that phosphorus can be supplied by either sodium phosphate or potassium phosphate.

PART II. PRODUCTION OF VOLATILE FATTY ACIDS AND AMMONIA IN THE RUMEN OF FAUNATED AND PROTOZOA-FREE LAMBS

REVIEW OF LITERATURE

Techniques for Obtaining Protozoal-Free Lambs

Interest was stimulated when becoming aware of the unusual and peculiar association represented by the teeming mass of protozoa harbored by wood-eating termites and roaches. Calling to mind the ability of termites to live on a diet of wood and being aware of the similarity of the termite and ruminant protozoa, the question arises as to the role of the protozoa within the rumen of cattle and sheep. It is not uncommon to find the mass of protozoa in the rumen equal to or greater than that of rumen bacteria (Oxford, 1955; Hungate, 1955). Katzin and Kirby (1939) estimated that one-third of the weight of termites may be due to their protozoa content. It is well established that anaerobic protozoa of the rumen, wood roach and termite have great potentials for storage of carbohydrates in the form of amylopectin and that they continually synthesize fatty acids from this material (Gutierrez, 1954; Eadie, 1959; Hungate, 1959; Oxford, 1955). Protozoal protein supplied to the host may be about 20 percent of its requirement (Hungate, 1955). Experimental studies by McNaught et al. (1954) and Weller (1957) have shown that protozoal protein was of higher biological value than bacteria or yeast protein which was probably due to a better balance of the essential amino acids, particularly lysine. To further study the value of protozoa to their ruminant host, the study of rumen metabolism, with and

without protozoa, appeared to be an appropriate approach to the problem. Techniques for obtaining protozoal-free lambs were developed in attempting to investigate this approach.

When attempting to obtain protozoal-free lambs, determining the normal source of their rumen protozoa is of primary importance. In the late 1800's some investigators believed that grasses, grains and water harbored the protozoa which subsequently established themselves in the rumen of animals that consumed these materials. Some workers believed hay contained protozoal cysts and when entering the rumen became vegetative and gave rise to the protozoal populations. Since investigators proposed these sources of rumen protozoa, it has been shown that neither hay, grain or water give rise to rumen protozoa (Becker and Hsiung, 1929). The early proposals are explainable on the basis that the test animals were not completely free of protozoa and thus protozoa reappeared when lambs resumed normal eating. Becker and Hsiung (1929) attempted to faunate protozoa-free lambs by feeding them dried or fresh feces taken from faunated ruminants or horses. Their attempts were not successful. After other studies using different grasses and hays fed to defaunated ruminants, these investigators have concluded that young ruminants obtain their protozoa from other faunated ruminants. Protozoa are transmitted through animal to animal contact or through the feed, water or pen facilities or by animals or man that may carry them from faunated to defaunated ruminants. Ingestion of saliva or mouth contents loaded with

rumen protozoa, regurgitated from the rumen, enables the young ruminant to become faunated. Becker and Hsiung (1929) found that defaunated goats obtained rumen protozoa from faunated goats in pens which permitted mouth to mouth contact of the defaunated and faunated goats.

For obtaining protozoal-free lambs, one of the most positive methods, although not easily accomplished, was the prevention of protozoa from becoming established in the very young ruminant. Pounden and Hibbs (1948a and 1948b) have shown that calves normally establish protozoal populations at 4 to 8 weeks of age. Young lambs and goats may become faunated at 2 to 4 weeks of age. The age at which faunation occurs is dependent upon rumen development which, in turn, is dependent upon the ingestion of a roughage or bulky-type diets by the young ruminant. Pounden and Hibbs (1948a and 1948b) found that protozoa failed to become established in milk-fed calves but were easily established in milk and alfalfa hay-fed calves. They also found that inoculation with ingesta from faunated ruminants assisted in establishing rumen protozoal populations. If calves or lambs are removed from the presence of their mothers at 2 to 3 days of age and placed in isolation from faunated animals, faunation should not take place, providing other modes of transmission are adequately controlled. Eadie and Hobson (1962) have successfully prevented faunation of lambs by removing them from their mothers at 2 to 3 days of age and maintaining them isolated from any possible source of ruminant proto-

zoa. Lambs were weaned on a starter liquid and then given grass ad libitum at 13 weeks of age. Jacobson (1959) found that dairy calves weaned on milk-replacers accepted roughages and mixed grains as early as 4 days of age and within 2 weeks their ration could be predominantly dry feeds. After rumen development has occurred, care must be exercised to prevent faunation. Protozoa are easily transmitted by way of feed, water and pen facilities, by faunated ruminants or by animal caretakers having contact with faunated ruminants (Becker and Hsiung, 1929).

Becker (1929), treating starved goats with copper sulfate, was able to establish and maintain defaunation. The animals were starved, except for water, for 3 days afterwhich 50 milliliters of a freshly prepared 2 percent solution of copper sulfate, mixed with about 1 pint of distilled water, was poured into the rumen. Twenty-four hours later the same treatment was repeated with starvation continued. About 4 hours after the second treatment, the animal was offered a small amount of alfalfa hay. Usually the appetite was lost, probably due to the lack of microbial fermentation, but returned gradually within a few days. If protozoa do not return within 2 weeks, the animal should remain defaunated unless reinoculation takes place (Becker, 1929).

The acidity of the rumen has been thought to influence the activity of the microbial population. Under low pH conditions (4.0 to 5.0) rumen protozoa usually disappear (Eadie et al.,

1956; Oxford, 1955; Hungate, 1960). Undocumented research by Liebetanz in the early 1900's found that hydrochloric and acetic acid administration via capsule and stomach tube resulted in death of the animals. In later studies, administration of acetic acid through a rumen cannula into starved animals resulted, eventually, in the loss of protozoa. It was interesting to note that defaunation success took place with young animals while most of the failures occurred with adult animals. Cleveland (1925b), using sulfuric and hydrochloric acids, was able to kill most protozoa but injury to the termite host also resulted. This was probably true with the ruminant also since it requires relatively high acid concentrations to kill 100 percent of the protozoa. Another disadvantage of this method is that it cannot overcome the tremendous biological buffering system of the ruminant. Over a period of a few days, it requires large amounts of acids to change the overall rumen pH appreciably.

When proteins are metabolized a number of putrefactive compounds are formed. For example, when tryptophane is catabolyzed, indole and skatole are formed. These compounds, when in solution at near saturated levels, cause injury or death to rumen protozoa. A study of organic compounds exhibiting harmful effects on rumen protozoa has been made by Eadie *et al.* (1956). From their results the following compounds, when administered at saturated levels, were lethal to rumen protozoa. They are: 7-methoxy-1-methyl-indole, 5-methoxyindole, 1-meth-

oxyindole, menthol-β-glucoside, menthol, geraniol, eugenol, skatole, indole, 1-phenylethanol, 2-phenylethanol, toluene and tryphtophol. Most of these compounds are very toxic to rumen protozoa, especially when used in a closed system (such as a test tube). On the other hand, in the rumen, where absorption, passage and other changes take place, it was very difficult to obtain a complete and lasting defaunation with these compounds.

Other compounds studied by Eadie et al. (1956) that exhibit harmful effects for rumen protozoa were borneol, isoborneol, borneol-α-glucoside, cineole and naphthalene. Terpene alcohols, tween 80, mannose, α and β-glucosides have shown injury or death to rumen protozoa. Cleveland (1925a) killed termite protozoa by soaking their wood diet with solutions of calcium chloride, potassium chloride, sodium chloride, quinine sulfate, copper sulfate and mercuric chloride. Although protozoa were killed, in many cases the hosts also were either killed or injured. With high enough concentration over extended periods, several compounds proved lethal to protozoa; however, there is a question whether such treated animals can be considered worthy of experimental research.

When termites were starved, their protozoa began to disappear at the end of 3 days and were nearly all dead at the end of 20 days (Cleveland, 1925c). Certain types of protozoa disappeared readily upon starvation, while others were difficult to kill by this method. Protozoa probably supply food for the host, thereby enabling the host to remain alive. Severe starva-

tion may injure the host and thus the animal may be of little value for experimentation. Cleveland (1925d) defaunated termites by incubating them at 10 to 15 degrees above the body temperature of the host. He found that incubation at 36 degrees centigrade for 24 hours or at 45 degrees centigrade for 10 minutes caused death of the protozoa without apparent injury to the host.

Rumen as well as termite and roach protozoa have been shown to be obligately anaerobic (Hungate, 1942; Sugden and Oxford, 1952; Cleveland, 1925). It has further been shown that certain types of protozoa are more resistant to oxygen than others (Heald and Oxford, 1952; Cleveland, 1925d). Oxygen in rather excessive amounts is toxic to many, if not all, forms of animal life. Realizing this, Cleveland (1925d) attempted to kill termite protozoa by increasing oxygen tensions. Upon placing termites in an atmosphere of nearly pure oxygen for 24 hours, he found most protozoa were killed. In a series of experiments, this investigator found that oxygenation of the host at one atmosphere of pure oxygen killed one type of protozoa, while starvation for 6 days plus this oxygen treatment killed a second and different type. Starvation for 8 days plus oxygenation caused the disappearance of a third type of protozoa. Starvation plus 72 hours of oxygenation completely removed all intestinal protozoa. This method appeared particularly useful since it resulted in a differential defaunation which enabled the study of the different types of protozoa. It was also

shown that by altering the length of starvation and oxygenation, various combinations of four types of protozoa were obtained within the termite gut. When termites were treated so they contained at least one type of protozoa, it was found that certain single types were adequate to sustain the life of the termite while other types were not.

In Vivo Production of Rumen Volatile Fatty Acids and Ammonia in Faunated and Protozoal-Free Lambs

Rumen fatty acids are the primary products of carbohydrate fermentation, the majority of which are volatile fatty acids comprised largely of acetic, propionic, butyric, valeric and formic acids. With various types of ration and feeding regimes, total concentrations and ratios of volatile fatty acids are widely variable. These acids constitute the major source of energy to ruminants since a large portion of ingested carbohydrates are degraded in the rumen.

Microbial degradation of proteinaceous feedstuffs results in the production of ammonia, some of which in the ruminant is absorbed through the rumen wall. It is then transported by the blood to the liver where it is transformed into urea. This urea may be lost by way of the urine or recycled through the saliva to the rumen. Recycled urea may be of benefit to the ruminant; however, with its lower efficiency coupled with urine losses, ammonia production may be, under certain conditions, a loss of otherwise more efficient utilizable nutrients.

It is proposed that rumen protozoa supply the host with a continuous source of fatty acids and nitrogenous compounds, probably 20 to 30 percent of its requirements (Hungate, 1942; Heald and Oxford, 1953; Oxford, 1955; Hungate, 1955; Gutierrez and Hungate, 1957; Gutierrez, 1958; Hungate, 1960). Microbial produced rumen acids are the primary sources of energy for ruminants (Annison and Lewis, 1959; Barnett and Reid, 1961; Hungate, 1960). In rumen fluid, the following (w/v) percentages of acids are most commonly found: (as their salts) acetate, 50 or more; propionate, 20 to 35; butyrate, 10 to 20; and higher acids combined, 1 to 8 (Phillipson, 1952; el-Shazly, 1952; Annison, 1954; Balch and Rowland, 1957; Oxford, 1955; Hungate, 1960). Rumen acids beyond valeric are normally present only in minute amounts (Annison, 1954). Lactate may be produced at considerable levels in the rumen but, due to a rapid fermentation that involves fixation of carbon dioxide and decarboxylation, lactate is converted to propionate (Johns, 1951a and 1951b). Oxford (1958) concluded that acetate was a primary product of cellulose digestion while propionate and lactate, as well as acetate were products of starch and soluble sugar degradation. There is little evidence for the occurrence, under normal conditions, of more than traces of rumen acids of chain length greater than C_5, and although formic acid has been found under certain conditions, it is not usually present (Annison and Lewis, 1959).

It is generally agreed that concentrations and ratios of

rumen acids are dependent upon ration composition and feeding regimes. There is lack of agreement as well as understanding, however, as to what the specific causal mechanisms are which are responsible for concentration and ratio changes. Rhodes (1961) and Raun (1961) showed that increasing the amount of concentrates consumed by lambs caused a narrowing of the acetate-propionate ratio. Balch (1958) showed that higher propionate levels were produced when lactating cows were fed finely ground hay than when they were fed long hay. It was demonstrated by Annison (1954) and Balch and Rowland (1957) that raising the protein level of a flaked maize diet increased production of butyric and branched chain acids. Phillipson (1952) observed that additions of cobalt to a diet containing flaked maize produced a narrowing of the acetate-propionate ratio.

Experimental studies by Phillipson (1952), Ensor et al. (1959), Balch and Rowland (1957) and Shaw (1959) showed that substitution of flaked corn for non-heated corn narrowed the acetate-propionate ratio and lowered butyrate levels. Jamieson (1959) observed that nitrate administration narrowed acetate-propionate ratios. It is of interest to note that he associated high propionate levels with periods of poor growth. Annison et al. (1959a and 1959b) found elevated total volatile fatty acids, ruminal ammonia, amino nitrogen and lactic acid levels, as well as narrowed acetate-propionate and acetate-butyrate ratios, when sheep were shifted from dry roughage to lush green pasture. Stewart and Schultz (1958) observed that

urea consistently increased volatile fatty acid production and narrowed the acetate-propionate ratio *in vitro*. Barnett and Reid (1957) noted that whereas the addition of a water extract of grass to an *in vitro* medium caused a widening of the acetate-propionate ratio, addition of the extracted grass markedly narrowed this ratio.

Since it is generally accepted that antibacterial agents exhibit harmful effects upon rumen bacteria while rumen protozoa are apparently unharmed by them, it is of interest to note their general effects upon rumen volatile fatty acids. In bloat studies, Mangan *et al.* (1959) observed that penicillin decreased volatile fatty acid and ruminal ammonia production as well as protein breakdown. Hungate *et al.* (1955) concluded that, although chlortetracycline feeding altered the rumen microbial population, the potential for microbial activity was not greatly affected. Visek *et al.* (1959) suggested that certain antibiotics suppressed bacterial urease activity.

It is evident in the literature cited that rumen acid concentrations and especially ratios are easily altered by feeding different types and physical forms of rations to ruminants. Lacking, however, is an explanation of the effect of these diet changes upon the rumen microbial populations which, of course, are responsible for the production of rumen acids. While reviewing studies concerned with microbial fermentations, it was evident that different types of organisms not only exhibit specificity for certain substrates but may be sole producers

of one or more specific fatty acids.

The following citations from Bryant (1959), in a review paper, point out the differeing abilities of different types of bacteria in producing fatty acids. Elsden (1945) isolated lactate-fermenting strains of Propionibacterium from rumen contents of sheep and cattle using lactate enrichment cultures. Gutierrez (1953) described proteolytic, lactate-fermenting, propionic acid producing bacterium from the rumen of cattle. Mann and Oxford (1954) found a strain of Lactobacillus in calves which produced lactic acid. Bauman and Foster (1956) isolated a different strain of Lactobacillus from the rumen of cattle on a diet supplemented with urea and alfalfa hay which produced acetic and lactic acid in 2:1 ratio. Ramibacterium isolated from young calves produced acetic, lactic and formic acids (Bryant et al., 1958). Maki and Foster (1957) isolated an anaerobic succinic acid producing bacterium from the rumen of cattle. Johns (1951a and 1951b) concluded that Veillonella gazogenes was the primary organism involved in converting pyruvate to propionate via carbon dioxide fixation and decarboxylation. Johns (1952) demonstrated that Clostridium propionicum forms propionate from lactate.

In vitro protozoal studies by Gutierrez (1955) have shown that the holotrichs, Isotricha and Dasytricha, ferment carbohydrates giving rise to acetic and butyric acids. Isotricha when cultured alone produced large quantities of lactic acid. This suggests that Isotricha protozoa are able to synthesize

lactate while other types of protozoa are not able to ferment it. It has been shown that Entodinium ingests amylolytic Streptococcus bovis which are producers of acetic acid (Gutierrez and Davis, 1959). It was also concluded that other types of protozoa may be enhanced in their ability to digest cellulose by the presence of certain bacterial strains.

Highest total volatile acid concentrations generally occur 3 to 6 hours after feeding (Gray and Pilgrim, 1951; Stewart et al., 1958). In silage fed sheep, Williams and Christian (1959) observed peak ammonia and volatile acid levels 1 hour after the start of feeding. Stewart et al. (1958) and Shaw et al. (1959) found volatile fatty acid ratios to be fairly constant throughout the day. Gray and Pilgrim (1951) found the acetate-propionate ratio to be narrow shortly after feeding and widened thereafter. In an in vitro study, Bladen and Doetsch (1959) observed fairly constant volatile fatty acid ratios throughout the day when animals were fed all-hay rations. On the other hand, when more concentrated rations were fed, fermentation peaks 2 to 6 hours after feeding were noted (Balch and Rowland, 1957). In addition to these investigators, Reid et al. (1957) observed narrowed acetate-propionate ratios and increased butyrate levels when high concentrate rations were fed. They also noted that propionate levels increased for a short time after feeding.

The utilization of protein and non-protein nitrogen by rumen microorganisms is an extremely interesting reactive sys-

tem of bacteria and protozoa interposed between diet and host in ruminant animals. Underwood and Moir (1956) proposed the following pathways of protein utilization. The first is that part of the diet protein passes unchanged to the abomasum and to the small intestine where hydrolytic change to amino acids presumably occurs. Another path is the microbial attack in which proteins are broken to peptides and amino acids within the rumen and then yield ammonia and fatty acids. The ammonia, totally or partially, is utilized by bacteria and protozoa to yield their respective proteins which pass to the small intestine and are utilized by the host. It is well known that amino acids and ammonia produced by microbial fermentations are formed more rapidly than the rumen flora and fauna can utilize so that they, especially ammonia, accumulate in the rumen. McDonald (1948) has shown that ammonia is absorbed from the rumen which may account for a considerable loss of ingested nitrogen, especially if the level of free ammonia in the rumen is in excess. Ammonia absorbed from the rumen is usually transferred to the liver and kidneys and converted to urea. With urine losses and a relatively low utilization efficiency of the urea recycled by way of the saliva, this path is clearly a path of nutrient losses.

Even though dietary protein may be relatively less efficiently utilized by ruminants than non-ruminants, apparent differences in nutritive value of protozoal and bacterial protein stimulate interest for continued study of microbial metabolism

of nitrogenous feedstuffs. McNaught et al. (1954), using rat assay techniques, found that the nutritive values of yeast, bacterial and protozoal proteins were 74, 84 and 91, respectively. Weller (1957) indicated that the superior value of protozoal protein was probably due to a better balance of amino acids, particularly lysine. Gutierrez and Davis (1959) concluded that at least some types of rumen protozoa are dependent upon bacteria as a protein source. It was shown by Hungate (1950) that termite protozoa live very well when the termite wood diet contained as little as 0.046 percent nitrogen. For each gram of nitrogen consumed, 350 grams of wood were decomposed. On the other hand, Williams et al. (1960) suggested that rumen protozoa utilize proteinacious materials very readily. This may indicate a large tolerance range for nitrogen required or the ability of protozoa to utilize these materials.

Various studies have shown that the rate of ammonia production from different proteins differs greatly in rumen microbial fermentations. McDonald (1952), comparing the rate of ammonia production from casein, gelatin and zein when administered directly to the rumen, found that production of excess ammonia was linked with the solubility of the proteins. Ellis et al. (1956) reported a marked increase in ammonia production when urea was fed as the nitrogen source. In laboratory studies, Arias et al. (1951) found that urea was utilized to a greater extent upon the addition of readily available carbohydrate energy sources. Lewis and McDonald (1958) showed that

ruminal ammonia production was decreased when carbohydrates were added to *in vitro* fermentations.

It is apparent that dietary factors are capable of altering ruminal ammonia production. It is suggested by McDonald (1952) that ammonia arising from high quality dietary protein causes a net loss of nitrogen while deamination, on the other hand, may be beneficial to the host when it arises from protein of low quality. Obviously absent in these references, however, is a report on the role that widely different types of rumen microorganisms play in degrading nitrogenous feedstuffs or their ability for producing ammonia. Differences of rumen bacteria and protozoa are of present interest in this regard.

EXPERIMENTAL PROCEDURE AND RESULTS

Rearing Isolated New-Born Orphan Lambs

Methods and procedures

Trial 1. Rearing isolated new-born lambs Ten cross-bred (Cheviot x Columbia), three day old male lambs were obtained in an attempt to raise lambs free of rumen protozoa. These lambs were individually weighed and ear tagged for purposes of record and identification. Lambs were placed in individual pens and after six hours without feed were fed a milk replacer ration modified from Jacobson (1959) as shown in table 25. Individual pens constructed in pairs were placed so that each of five sets of lambs were at least ten feet apart from other lambs. The pens were located in a room which was well insulated, wall and ceiling were aluminum lined, the floor was concrete and it was also equipped with an air conditioner and an exhaust fan. Temperature, humidity and cleanliness were well controlled. This room was equipped with screen doors which were kept locked, except for passage of authorized personnel, feed and equipment, in order to prevent faunation of the lambs by possible protozoa carriers such as flies mice and people. The feces and urine were daily washed down the drains in the floor.

The feeding schedule and amounts fed of the milk replacer are given in table 25. The milk replacer was prepared daily and prewarmed to 35 to 39 degrees centigrade immediately prior to each feeding. Lambs were individually fed from a 24 ounce

Table 25. Trial 1 - composition of milk replacer and schedule of feeding milk replacer to new-born orphan lambs

Ingredient	Percentage[a]
Dried skim milk	40
Dried whole whey	21
Distillers solubles	20
Lard oil	18
Vitamin and antibiotic mix[b]	1

Age of lambs	Feedings per day	Ounces milk replacer per feeding
3 through 6 days	6	2
6 through 10 days	5	4
10 through 14 days	4	6
2 through 4 weeks	3	8 to 10
4 through 6 weeks	2	16 to 24
6 through 8 weeks[c]	2	24 to 16
8 through 10 weeks[d]	1	24 to 12

[a]Mixed into a solution by mixing 5 parts water with 1 part of dry replacer mix.

[b]Composed of vitamin A (31,800 I.U./lb. replacer), vitamin D_2 (1,600 I.U./lb. replacer) and antibiotic (40 mg. aureomycin/lb. replacer).

[c]At 6 weeks of age the amount of milk replacer fed to lambs was reduced as a stimulus for increasing consumption of the starter ration.

[d]Milk replacer removed from lambs diet at 10 to 11 weeks of age.

capacity bottle fitted with a nipple commonly used for raising orphan lambs. After lambs readily accepted the bottle-fed milk replacer, bottles were mounted to the pens so that individual attention was not required in feeding the entire bottle contents to each lamb.

At the beginning, when lambs were 3 to 6 days of age, they

were fed 6 times per day at equally spaced intervals during a 24 hour period and given 2 ounces of milk replacer per feeding. At 6 to 10 days of age the schedule was changed to 5 times per day with 4 ounces of milk replacer per feeding. At 2 weeks of age they were fed 4 times per day and 6 ounces per feeding. Then from 2 to 4 weeks of age they were fed 3 times per day (6:00 a.m., 12:00 noon and 6:00 p.m.) and were given 8 ounces of milk replacer per feeding at the beginning and progressively increased to 10 ounces. From 4 to 6 weeks of age lambs were fed twice daily (7:00 a.m. and 4:00 p.m.) with 16 ounces of milk replacer per feeding at first. This amount was progressively increased to 24 ounces. In order to increase their consumption of starter ration, at 6 to 10 weeks of age the lambs rations were reduced from 24 ounces at first to 16 ounces of milk replacer per twice-a-day feeding. At 8 weeks of age they were fed only 1 time daily (7:00 a.m.) with 24 ounces of milk replacer at first and this amount was progressively reduced to 12 ounces at 10 weeks of age. From 10 to 11 weeks of age the dry mix portion of the milk replacer was proportionally reduced and the water content was increased so that at 11 weeks of age the milk replacer was totally removed from the lambs diet. At 8 weeks of age and thereafter water was made available for free choice consumption.

From 10 to 20 days of age lambs were offered green leafy alfalfa hay which was freshly made available each day. When lambs were about 3 weeks of age, they were offered a mixed

starter ration as given in table 26. This ration was fed twice daily with the refused portion removed before each feeding.

Table 26. Composition of starter rations used for raising isolated new-born lambs

Ingredient	Percentage	
	1st phase[a]	2nd phase[b]
Corn (ground shelled)	35.0	35.0
Alfalfa (ground)	25.0	35.0
Soybean oil meal	20.0	10.0
Oats (rolled)	10.0	10.0
Molasses	9.0	9.0
Dicalcium phosphate	0.4	0.4
Salt	0.6	0.6
Quadrex (gm./100 lb.)[c]	10	7

[a]Ration was fed to lambs from 3 through 8 weeks of age in trial 1 and 2 through 8 weeks of age in trial 2.

[b]Ration was fed to lambs from 8 through 18 weeks of age.

[c]Contains 10,000 I.U. of vitamin A and 1,250 I.U. of vitamin D_2 per gram.

The starter ration for the first phase (3 up to 8 weeks of age) was formulated to give a high degree of palatability, to supply sufficient protein and adequate roughage required to promote rumen development.

From 8 to 18 weeks of age the ration given to lambs was altered as shown in table 25 for the second phase. Protein percentage was decreased and roughage increased. From 12 weeks of age and thereafter lambs were fed only the amount that each would consume prior to the next feeding. When the milk replacer was removed from the lambs diet, consumption of starter

rations and water increased tremendously.

When lambs were 3 weeks of age their tails were cut off and they were testicular castrated. Their tail stubs were tied with sterile nylon string to prevent excess bleeding. Testicles were severed using a burdizzo type pincers, therefore bleeding was no problem.

Following the healing of these operations, lambs were inoculated with perfringens vaccine to prevent Enterotoxemia (overeating disease).

When lambs were 18 weeks of age their ration was changed to a conventional lamb fattening ration which was fed thereafter. The composition of this ration is given in table 28.

Trial 2. Rearing isolated new-born lambs The methods and procedures used in this trial which were common to trial 1 are not repeated in this section. Ten cross-bred (Columbia x Hampshire) male 3 to 4 day old lambs were obtained in a second attempt to raise protozoa-free lambs. In addition to the pre-experimental treatments given to the lambs of trial 1 at the time they were obtained, lambs obtained for trial 2 were injected with 2 milliliters of combiotic (streptomycin and penicillin) per lamb. Before feeding the milk replacer, lambs were starved for 12 hours after which they were fed a small amount of prewarmed milk replacer.

Because of digestive disturbances encountered with the lambs of the first trial, at about 7 to 8 weeks of age the milk replacer formula and feeding schedule were altered somewhat for

lambs in trial 2. The composition of the milk replacer, the feeding schedule and the amounts of replacer fed to lambs in trial 2 are presented in table 27. Dried skim milk and lard

Table 27. Trial 2 - composition of milk replacer and schedule of feeding milk replacer to new-born orphan lambs

Ingredient	Percentage[a]
Dried skim milk	50
Dried whole whey	15
Distillers solubles	14
Lard oil	20
Vitamin and antibiotic mix[b]	1

Age of lambs	Feedings per day	Ounces milk replacer per feeding
7 through 10 days	4	3
10 through 14 days	3	5 to 8
2 through 4 weeks	3	9 to 12
4 through 5 weeks	2	16 to 20
5 through 7 weeks[c]	2	20 to 10
7 through 8 weeks[d]	1	16 to 10

[a]Mixed into a solution by mixing 5 parts of water with 1 part of dry replacer mix.

[b]Composed of vitamin A (31,800 I.U./lb. replacer), vitamin D_2 (1,600 I.U./lb. replacer) and antibiotic (40 mg. aureomycin/lb. replacer).

[c]At 5 weeks of age milk replacer fed to lambs was reduced as a stimulus for increasing consumption of the starter ration.

[d]Milk replacer removed from lambs diet at 8 to 9 weeks of age.

oil were increased from 40 and 18 percent to 50 and 20 percent, respectively. Dried whey and distillers solubles were decreased from 20 and 21 percent to 15 and 14 percent, respectively. The

amount of water and vitamin-antibiotic mix were not changed.

The feeding schedule and amounts of milk replacer fed were altered as compared to trial 1 and are given in table 27. At the beginning, when lambs were 3 to 6 days of age, they were fed 4 times per day at 5:00 a.m., 11:00 a.m., 4:00 p.m. and 10:00 p.m. They were, at the beginning, given 3 ounces of milk replacer per feeding. At 1 through 2 weeks of age they were fed 3 times daily (6:00 a.m., 11:00 a.m. and 4:00 p.m.) with 5 to 8 ounces per feeding of milk replacer. Lambs received 9 to 12 ounces per feeding from 2 to 4 weeks of age. The changes made in the amount of milk replacer fed were progressive. Twice daily feedings (7:00 a.m. and 4:00 p.m.) were conducted from 4 to 7 weeks of age. Milk replacer per feeding was increased from 16 ounces per feeding at the first part of this period to 20 ounces per feeding at 5 weeks of age and was decreased progressively to 10 ounces per feeding at 7 weeks of age. At 7 weeks of age they were fed milk replacer only once daily and the amount was decreased from 16 ounces to 10 ounces at the end of the seventh week of age. From 7 to 8 weeks of age the dry mix portion of the milk replacer was reduced and water content was increased so that at 8 weeks of age the milk replacer was entirely removed from the lambs diet. At 6 weeks of age and thereafter water was made available for free choice consumption.

Green leafy alfalfa hay was offered to lambs when they reached 1 week of age. When lambs were 3 weeks of age, alfalfa

was replaced by a mixed starter ration shown in table 26. The feeding schedule and methods were the same as described for trial 1. Because of its palatable nature, as evidenced from trial 1, no changes were made in the starter ration. When lambs began to consume sizable amounts of this ration, greater reductions were made in the amount of milk replacer fed than were made in the first trial. Milk replacer was removed and starter ration comprised the entire diet at 8 weeks of age which was about three weeks of age sooner than in trial 1.

When lambs were two weeks of age they were injected with 4 milliliters of combiotic (streptomycin and penicillin) per lamb and when 4 weeks of age this was repeated with 5 milliliters per lamb. The purpose of this was to counteract an apparent slight respiratory infection present in the lambs and to prevent any other secondary infections. These treatments appeared very successful.

At 4 weeks of age the tails of these lambs were cut off. Following a 2-week healing period, lambs were inoculated with perphringens vaccine to prevent enterotoxemia.

When the lambs were 18 weeks of age their ration was changed to a conventional lamb fattening ration which was fed thereafter. The composition of this ration is given in table 28.

Results and discussion

Trial 1. Rearing isolated new-born lambs The results

Table 28. Composition of ration fed to lambs in volatile fatty acid and ammonia trials[a]

Ingredient	Percentage
Alfalfa (coarsely ground)	40.0
Corn (ground shelled)	34.0
Corn cobs (medium ground)	7.0
Oats (rolled)	7.0
Molasses	6.0
Soybean oil meal	5.3
Salt	0.5
Dicalcium phosphate	0.2
Quadrex (gm./100 lb.)[b]	5.0

[a]Ration was fed to lambs at 18 weeks of age and thereafter.

[b]Contains 10,000 I.U. of vitamin A and 1,250 I.U. of vitamin D_2 per gram.

of this trial are given in table 29. When the lambs were obtained 2 to 3 days after birth, their weights ranged from 7 to 9 pounds per lamb with an average of 7.5 pounds. Consumption of milk replacer increased from 12 ounces daily at the beginning to about 48 ounces per lamb at 6 weeks of age. As the lambs consumed more starter ration after 6 weeks of age, milk replacer fed per day was decreased progressively. After 12 weeks of age, milk replacer was entirely removed from their diet.

When lambs were about 3 weeks of age they were offered the starter ration (table 26). During the first week they consumed about one-half pound of the starter ration per lamb per day. At 8 weeks of age they were consuming about 1.6 pounds of starter ration per lamb per day. When milk replacer was re-

Table 29. Trial 1 - performance and feed consumption of new-born lambs reared in isolation[a]

Two week periods (age of lambs)	Intake per lamb		Average per lamb				Feed conversion
	Milk replacer[b]	Starter ration	End of period wt.	Daily gain	Daily feed[c]	Daily T.D.N.	
Birth	--	--	7.5	--	--	--	--
0 through 2	17.3	--	13.8	.45	.33	.25	.73
2 through 4	23.7	3.9	20.5	.48	.70	.56	1.46
4 through 6	35.0	15.1	26.4	.49	1.58	1.17	3.22
6 through 8	35.0	23.7	34.4	.57	2.20	1.60	3.88
8 through 10	14.5	30.0	42.0	.54	2.36	1.70	4.37
10 through 12	4.5	33.7	52.0	.71	2.45	1.72	3.38
12 through 14	--	37.6	63.8	.84	2.70	1.89	3.22
Av.	--	--	--	.58	1.75	1.27	2.89

[a]All values are based on the mean response of 8 lambs and are expressed in pound units.

[b]Milk replacer values are expressed as liquid (20% dry matter) volume.

[c]Daily feed consumption values are given on an air-dried basis.

moved from the diet (12 weeks of age) the consumption of starter ration increased greatly. At 14 weeks of age the lambs were eating 2.7 pounds per lamb per day. At 10 weeks of age the lambs were consuming about 5.5 percent of their body weight of air-dried feed. At 12 weeks of age they were consuming about 4.7 and at 14 weeks 4.2 percent of their body weight of air-dried feed daily. Ruminants normally do not consume more than 2.5 to 3.0 percent of their weight of air-dried feed.

These lambs increased from an average per lamb of 7.5 pounds at the beginning to 63.8 at 98 days of age. Average daily gains for the total period were 0.58 pound per lamb. From 10 to 12 weeks, average daily gains were 0.71 pound and from 12 to 14 weeks were 0.84 pound per lamb. With the exception of the 10-week weight, progressive increases in daily gains can be seen. This increase ranged from 0.45 pound per day per lamb at the first 2-week weight to 0.84 at the 14-week weight. The 10-week daily gains dropped slightly rather than increasing. It is thought that this drop was due to the relatively large decrease in milk replacer consumption without a relatively corresponding increase in starter ration.

Feed conversion ranged from 0.73 pound of feed per pound of gain at the 2-week weight to 4.37 pounds of feed per pound of gain at the 10-week weight. After milk replacer was decreased and finally removed from their diet (12 weeks of age), the feed conversion improved giving 3.38 pounds of feed per pound of gain at the 12-week weight and 3.22 pounds of feed per

pound of gain at the 14-week weight. The overall trial average was 2.89 pounds of feed per pound of gain.

Trial 2. Rearing isolated new-born lambs The results of this trial are presented in table 30. At 2 weeks of age the lambs were fed the starter ration (table 26). The ration consumption was less than 0.25 pound at first but increased progressively to about 1.7 pounds per day per lamb when they were 9 weeks of age. With the milk replacer removed when they were 8 to 9 weeks of age, the starter ration consumption increased to about 2.2 pounds daily per lamb at 11 weeks. When the lambs were 15 weeks of age they were consuming 2.5 pounds of starter ration per lamb daily.

The lambs in this trial weighed, on the average, 11.5 pounds each at 1 week of age and at 15 weeks of age they were 60 pounds. The average daily gain per lamb for this period was 0.49 pound. Except for a decrease when the lambs were 9 weeks old, the daily gains progressively increased from 0.39 pound at the beginning to 0.68 pound per lamb for the 13 through 15 week weight. As was apparently true in trial 1, the decrease in daily gains at the 9 week weight was probably due to the decrease in amount of replacer fed without a corresponding relative increase in consumption of the starter ration.

Feed conversion ranged from 1.46 pounds per pound of body weight gain at the 3-week weight to 5.89 pounds at the 9-week weight. After the milk replacer was entirely removed, feed conversion improved from 4.55 pounds of feed required per pound

Table 30. Trial 2 - performance and feed consumption of new-born lambs reared in isolation[a]

Two week periods (age of lambs)	Intake per lamb		Average per lamb				
	Milk replacer[b]	Starter ration	End of period wt.	Daily gain	Daily feed[c]	Daily T.D.N.	Feed conversion
1 week	--	--	11.5	--	--	--	--
1 through 3	18.7	3.0	16.9	.39	.57	.32	1.46
3 through 5	25.0	12.7	23.3	.46	1.33	.98	2.89
5 through 7	28.8	19.5	29.8	.46	1.90	1.39	4.15
7 through 9	7.0	24.9	34.4	.33	1.94	1.38	5.89
9 through 11	--	29.8	41.0	.47	2.14	1.50	4.55
11 through 13	--	34.7	50.2	.66	2.48	1.74	3.76
13 through 15	--	36.8	59.8	.68	2.60	1.82	3.72
Av.	--	--	--	.49	1.85	1.31	3.78

[a]All values are based on the mean response of 8 lambs and are expressed in pound units.

[b]Milk replacer values are expressed as liquid (20% dry matter) volume.

[c]Daily feed consumption values are given on an air-dried basis.

of gain at the 11-week weight to 3.72 pounds at the 15-week weight. The overall average for the trial was 3.78 pounds of feed per pound of gain.

The following discussion pertains to management techniques and general items of interest concerned with one or both of trials 1 and 2.

When lambs of trial 1 reached 8 weeks of age, rumen fluid was obtained via stomach tube and was examined microscopically for the presence of protozoa. These observations showed that 5 of the 8 lambs contained one or more types of rumen protozoa. The lambs were immediately separated according to the presence or absence as well as to types of protozoa in their rumens. The 3 lambs having no protozoa were isolated from each other and also from the other lambs. When these lambs were 10 weeks old similar microscopic observations were repeated on rumen fluid. These observations showed that all 8 lambs harbored one or more types of rumen protozoa.

The lambs harboring only one type of rumen protozoa (Entodinium) were retained isolated from each other and from other faunated lambs. These lambs were subsequently used for studying volatile fatty acid production, ratios of rumen volatile fatty acids as well as ruminal ammonia production. These studies are reported in a following section of this thesis.

The lambs which were faunated with two or more types of rumen protozoa were inoculated with normal protozoal and bacterial populations obtained from a fistulated steer. These lambs

were subsequently used for volatile fatty acid and defaunation studies which are given in following sections.

Since the lambs in trial 1 became faunated, which was opposed to the objective, more rigid precautionary practices were adopted for the rearing of the lambs in trial 2.

In trial 1 the milk replacer was prepared and stored in a laboratory which was used by a number of different researchers and animal caretakers. This may have been the source of protozoa which faunated the lambs in trial 1. For trial 2, all materials and facilities which were used in this trial were located within the room in which lambs were reared. This room excluded flies and mice and the only access door was kept locked except when authorized personnel entered to care for the lambs. The only contact, as far as is known, between the things inside and outside this room was by the caretaker who exercised precaution to prevent rumen protozoa from being brought into contact with the protozoa-free lambs of trial 2.

When lambs began sneezing and coughing while they were eating the starter ration, canvas partitions were placed so that lambs facing each other (in separate pens on opposite sides of the room) could not transfer protozoa, if faunated, via droplet infection. It was later evident that the canvas partitions were needed since they became coated with small saliva-wetted feed particles. Had these canvas partitions not been placed as described, these feed particles would have dropped in the water and feed or on the lamb in the adjacent pen.

When these lambs were 8, 10 and 12 weeks of age, rumen liquid was collected and microscopically examined for the presence or absence of rumen protozoa. Success was obtained in this trial. All observations failed to reveal any protozoa within the rumen of these lambs. All 8 lambs were maintained totally protozoa-free and were subsequently used for studying ratios and production of volatile fatty acids and production of ruminal ammonia. These studies are given in a following section.

Defaunating the Rumen of Lambs Having Protozoal Populations

Methods and results

Becker (1929) defaunated goats and sheep by starving them for 2 to 3 days followed by 2 intraruminal 50 milliliter doses of a 2 percent copper sulfate solution. However, in our laboratory, repeated attempts with Becker's method using 100 pound lambs failed to cause defaunation.

Other approaches for defaunation were attempted. Massive intraruminal doses of lactic and acetic acids were given in hopes of lowering pH enough to kill all protozoa. Even though these acids were administered at various levels and time intervals along with periods of starvation, total defaunation was not obtained. Lambs treated in this way appeared to be free of rumen protozoa immediately following the treatment but within a few days protozoa invariably returned. In many cases there would only be one type of rumen protozoa (usually Entodinium)

that would withstand these treatments.

Defaunation technique using starvation and copper sulfate treatment Fifty to 60-pound lambs were placed in individual pens separated about 10 feet apart. These lambs contained protozoal populations which were considered typical by microscopic study. The lambs were starved for 5 days. Fresh water was accessable during this period. Starvation continued and the lambs were dosed intraruminally, via stomach tube, with 60 milliliters of a freshly prepared 2 percent solution of copper sulfate. Twenty-four hours later this treatment was repeated. About 8 hours after the last copper sulfate treatment, the lambs were offered a small amount of green leafy alfalfa hay. The appetite of the lambs was impaired by this treatment but returned gradually after a few days. Within 7 to 10 days the lambs were consuming nearly a full feed.

About 1 week after lambs resumed normal eating, rumen fluid was collected and examined microscopically for the presence of rumen protozoa. Rumen protozoa were not found in this or subsequent observations. However, within 25 to 30 days after the copper sulfate treatment, 2 of these lambs stopped eating. Numerous rumen inoculations of rumen contents from other ruminants as well as intravenous injections of glucose failed to bring these lambs back on feed. Both lambs died within a few days. Post mortem examination by the veterinary clinic diagnosed the cause of death as copper toxicity. The liver, spleen and kidneys were nearly unrecognizable.

Hematocrit (percent red blood cells) determinations were immediately performed on the blood of two other copper sulfate treated lambs which were still apparently healthy. The hematocrit was about 32 for one of these lambs and about 41 for the other lamb. Normal lamb hematocrit values are about 48. Hemolysis of red blood cells is a symptom of copper toxicity. The lamb with the lowest hematocrit value followed the same path as the previous two and died about 60 days after the copper sulfate was administered.

Research studies in Australia by Dick (1953 and 1954) showed that copper toxicity in sheep, caused by eating forages with high copper content, was prevented by the administration of molybdenum.

In subsequent copper sulfate defaunation trials, molybdenum was administered at the rate of 50 and 100 milligrams per day per lamb both prior to and following copper sulfate treatment. In most cases molybdenum treatment continued for 2 to 3 weeks. Molybdenum was administered as ammonium molybdate and was mixed with finely ground soybean oil meal as a carrier. The molybdenum premix, in measured amounts, was sprinkled once daily on the top of the ration fed to each lamb. There were no cases of copper toxicity observed in any lambs that were treated with copper sulfate accompanied by molybdenum administration. More than 100 different lambs were treated in this manner. Hematocrit values of such treated lambs were very near the normal range (43 to 48). It is believed that ammonium molyb-

date ties up the copper by a complexing reaction.

Defaunation technique using milk replacer as total diet fed to lambs Lambs weighing about 60 pounds in individual pens were used in conducting this experiment. Previous microscopic observations of rumen fluid showed that these lambs contained numerous protozoa of 3 or 4 different types. The lambs were starved for 2 days after which they were fed a liquid diet (milk replacer, trial 2, table 27) as described in the previous section on rearing of new-born isolated lambs. These lambs had previously obtained milk replacer by this method and therefore readily accepted the nippled bottle. They were fed about 36 ounces of this milk replacer twice daily for 2 weeks. The only thing they were allowed to consume in addition to the milk replacer was water.

After 7 or 8 days consumption of this diet, one lamb was treated on 2 consecutive days with 50 milliliters of a 2 percent copper sulfate solution. Molybdenum was administered at levels previously described via the milk replacer. The other lamb was not treated with copper sulfate and molybdenum. At the end of 3 weeks of this experiment neither lamb contained any rumen protozoa. They were maintained defaunated and used for subsequent studies. It was very obvious that these lambs craved roughage in their diet. At every opportunity the lambs chewed and tried to eat anything solid that was within their reach.

Discussion

To study the effects of rumen protozoa upon the *in vivo* production of volatile fatty acids and ammonia it is necessary to use both faunated and defaunated ruminants. Various types of methods to defaunate the rumen of lambs were tried resulting in success with some and failure resulting with other methods. Nearly all methods tried gave almost complete defaunation but within one or two weeks after the treatment, protozoa usually reappeared. In order to obtain and maintain complete defaunation, all protozoa must be entirely killed or removed from the rumen.

Of the many approaches used in this laboratory to defaunate lambs, only two resulted in success. The first was a combination of starvation and copper sulfate treatment. The second was a combination of starvation followed by feeding a milk replacer as the total diet of lambs.

In the earlier defaunation attempts with copper sulfate where success was not achieved, it is believed that one or more of the following factors may have been responsible. In the first place, the lambs were larger and more mature than those used in later attempts when defaunation success was obtained. The starvation period in the previous trials was only 3 days, whereas in the trials where defaunation was obtained, the lambs were starved 7 days. The level of copper administered was higher in the trials where defaunation was obtained than when not obtained. In the latter, two 50 milliliter doses of 2 per-

cent copper sulfate were given as compared to two 60 milliliter doses of 2 percent copper sulfate per lamb where defaunation was successful. The combination of age and size of the lambs and the length of starvation period, as well as level of copper sulfate employed, was probably responsible for the different results obtained.

The other method which resulted in defaunation involved starvation, copper sulfate administration and a milk replacer diet. Since only one of the two lambs was given copper sulfate and since both lambs were completely defaunated, it is questionable whether the starvation and milk replacer diet alone could be relied upon to cause consistent defaunation of faunated lambs. Because of more consistent successes in obtaining protozoa-free lambs with other methods (starvation and copper sulfate and rearing isolated new-born lambs), this method was not repeated. It is realized, however, that if lambs readily consume a liquid diet, this method has good possibilities for successful use.

It appears that three methods can be relied upon to obtain protozoa-free lambs: first, rearing isolated new-born lambs; second, starving lambs 5 to 7 days followed by copper sulfate treatment; and third, starving lambs 3 days followed by a liquid (milk replacer) diet with or without copper sulfate treatment.

Volatile Fatty Acid Production in the Rumen of Faunated, Partially Faunated and Protozoal-Free Lambs

Methods and procedures

Eighteen wether lambs, eight of which were reared from 5 days of age isolated from mature ruminants to prevent faunation and ten of which were obtained from various sources, were used to conduct these studies. To defaunate the rumen of faunated lambs the method using copper sulfate described by Becker (1929) as modified by the author was used. Lambs reared without rumen protozoa as well as lambs reared with rumen protozoa and subsequently defaunated were both used within each comparison herein reported. The possible effects of copper sulfate upon rumen fermentative end products were ruled out by administering it to defaunated lambs and subsequently inoculating them with rumen bacteria and protozoa.

The composition of the ration fed to all lambs in these studies is presented in table 31. This ration remained the same throughout the duration of the entire period of these studies. This ration was fed at a constant level to all lambs individually at 7:00 a.m. and 4:00 p.m. All lambs were nearly the same size and age and all consumed the ration very well. It was therefore believed that by feeding all lambs the amount

Table 31. Ration composition fed to lambs in volatile fatty acid and ammonia studies

Ingredient	Percentage
Alfalfa (coarse ground)	40.0
Corn (ground shelled)	34.0
Corn cobs (medium ground)	7.0
Oats (rolled)	7.0
Soybean oil meal	5.3
Molasses	6.0
Salt	0.5
Dicalcium phosphate	0.2
Quadrex (gm./100 lb.)[a]	5.0

[a]Contains 10,000 I.U. of vitamin A and 1,250 I.U. of vitamin D_2 per gram.

which was normally entirely consumed by all lambs that the level of ration intake was nearly a full feed.

Rumen fluid samples were collected according to the method described by Raun and Burroughs (1962). Results from trials herein reported indicated peak ruminal volatile fatty acids and ammonia were attained 4 to 5 hours after feeding. All subsequent daily rumen fluid collections were therefore made at 11:00 a.m. In studies concerning the diurnal variation of ammonia and volatile fatty acids, rumen fluid collections were made at 7:00 a.m. and every hour thereafter until 4:00 p.m.

At the time of collection, rumen fluid pH was determined with a Beckman pH meter and microbial action was terminated immediately by the addition of saturated mercuric chloride. These collections of rumen fluid were then immediately taken to the laboratory and subsequently analyzed for volatile fatty

acids. When ammonia determinations were made, an additional amount of rumen fluid was collected which supplied an adequate amount for both volatile fatty acid and ammonia analyses.

Collected rumen fluid was taken to the laboratory where microorganisms and solid feed particles were removed by centrifugation. Protein material was removed by precipitation and filtration. Volatile fatty acids were steam distilled after pH was adjusted to 2.2 (McAnally, 1944) and acids were determined with a Markham steam distillation apparatus as described by Markham (1942). The resulting aqueous solutions of volatile fatty acids were then titrated with potassium hydroxide, dried and stored for gas phase chromatographic analysis. Molar ratios of volatile fatty acids were determined by the method described by Ralls (1960) as modified by Raun (1961).

To determine the effect of certain types of rumen protozoa upon volatile fatty acid production, protozoa-free lambs were inoculated individually with either Diplodinium, Entodinium, Isotricha or Ophryoscolex. Techniques used for isolating single types of rumen protozoa followed four different procedures. First, the establishment of Entodinia faunated lambs resulted somewhat accidentally. Protozoa-free lambs, if subjected to fauna sources, normally acquire Entodinia before other types of rumen protozoa become established and, if isolated at this time, will fail to gain any other genera of rumen protozoa. Entodinium faunated lambs may also be obtained by acid or copper sulfate administration intraruminally. Usually

a massive dose will cause all other types of protozoa to disappear leaving only Entodinium.

Second, in vitro culture studies showed that Isotricha remained viable after 96 to 144 hours of incubation in the medium given in table 1 while all other protozoal types disappeared. After various incubation periods, when careful microscopic examination failed to find other types of rumen protozoa, the remaining Isotrichs were inoculated into the rumen of protozoa-free lambs via stomach tube.

Third, Isotrichs were also isolated by means of a gravitational sedimentation technique. Rumen protozoa suspended in a nutrient solution (table 1) were poured into a plastic tube (60 inches long and 1 inch diameter) and after 2 to 4 minutes of settling were drawn off before other types of protozoa settled to the bottom. Ophryoscolex and Diplodinium settled and were at the bottom of the column after 6 to 8 minutes of settling. It was not possible to obtain either of these types individually. However, occasionally when lambs did not contain Isotricha in their rumen, it was possible to obtain Diplodinium with this method.

Fourth, Diplodinium, Ophryoscolex and Isotricha were obtained separately in relatively low numbers by using a microscopically mounted micromanipulator and micropipette apparatus. Rumen fluid was collected from ruminants harboring desired types of protozoa and then washed and the protozoa were suspended in nutrient solution (Quinn, 1962; Quinn et al., 1962).

A drop of this suspension was placed on a glass slide and placed under a light microscope fitted with a micromanipulator and micropipette. Using a 10x objective and the micropipette with the microscope-micromanipulator apparatus, 2 to 4 dozen individuals of a given type of protozoa were obtained. After being drawn into the micropipette, the protozoa were suspended in a nutrient solution previously warmed to 39 degrees centigrade and made anaerobic with carbon dioxide gas. This technique was the most reliable of any tried but was time consuming and tedious. With careful technique, however, one was relatively certain of an uncontaminated supply of a given type of protozoa. The larger sized protozoa were obtained with this method in these studies, but with good technique the smaller sized protozoa could be obtained individually with this method.

Relatively small amounts of single types of protozoa obtained as described were placed in the rumen of protozoa-free lambs via stomach tube. After 1 to 3 weeks, these lambs were used to obtain rumen fluid for volatile fatty acid and ammonia determinations. In some cases, protozoa were well established within a few days after inoculation, while in other cases several days were required before protozoa were established. It should be pointed out that a great many failures in establishing a given type of protozoa in the rumen of protozoa-free lambs occurred. Therefore, persistent attempts with trial and error resulted in sources of establishing lambs harboring rumen populations of Isotricha, Entodinium, Diplodinium and Ophryo-

scolex, all separately. With these somewhat haphazard techniques, it was difficult to obtain enough different lambs at a given time to be statistically sound, but in no case was there less than 2 lambs per comparison used. In most trials there were 4 or more lambs. To offset this weakness of this study, several different trials involving different sets of lambs with determinations made on separate days were conducted.

Results and discussion

Volatile fatty acids, Trial 1 The experimental plan and results of this trial are presented in table 32. An analysis of variance table of data from protozoa-free, faunated and Entodinium comparisons of this trial is given in the appendix. Protozoa-free lambs were different in regards to rumen pH, total volatile fatty acids produced as well as in the ratios of the three major acids (acetate, propionate and butyrate) as compared to faunated lambs. The rumen fluid pH of protozoa-free lambs was 6.5 as compared to a pH of 6.2, 6.1, 6.2, 6.3 and 6.4 for faunated, Entodinium faunated, Diplodinium faunated, Isotricha faunated and Ophryoscolex faunated lambs, respectively.

Total rumen volatile fatty acids produced in protozoa-free lambs was 62.8 micromoles per milliliter of rumen fluid and 72.5 for faunated lambs. Micromoles of total volatile acids produced in the rumen of lambs with separate faunations of Entodinium, Diplodinium, Isotricha and Ophryoscolex were 74.9, 69.0, 68.2 and 65.8, respectively. It can be seen that the

Table 32. Trial 1 - effects of the presence or absence of all or certain types of protozoa upon volatile fatty acid production in the lamb's rumen[a]

Comparison	Rumen pH	Total VFA[b]	Ratio C_2/C_3	Molar percent volatile fatty acids			
				C_2	C_3	C_4	Others
Protozoa-free[c]	6.5	62.8	2.62	60.8	22.4	15.3	1.5
Faunated[c]	6.2	72.5	1.85	50.2	27.1	19.2	3.5
Entodinium[c]	6.1	74.9	2.00	52.8	26.4	17.0	3.8
Diplodinium[d]	6.2	69.0	1.96	48.6	25.1	21.8	4.5
Isotricha[d]	6.3	68.2	2.22	54.9	24.7	18.3	2.1
Ophryoscolex[d]	6.4	65.8	2.10	54.2	25.8	17.4	2.6

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means (labeled [c]) for total VFA and the C_2/C_3 ratio are 6.2 and 0.30, respectively.

[b]Micromoles of volatile fatty acids per milliliter.

[c]Values are based on the mean of 40 determinations, 4 on each of 10 separate days.

[d]Values are based on the mean of 20 determinations, 2 on each of 10 separate days.

amount of volatile acids produced corresponded inversely with the rumen fluid pH in all comparisons. Lambs that were fully faunated produced about 15 percent more total volatile fatty acids in their rumen fluid than defaunated lambs. *Entodinium* faunated lambs produced somewhat more volatile acids than fully faunated lambs. Lambs which were faunated with the other three types of protozoa (*Diplodinium*, *Isotricha* and *Ophryoscolex*)

produced about 8 to 9 percent more volatile fatty acids than the protozoa-free lambs.

Rumen fluid from protozoa-free lambs was found to have a wider acetate-propionate ratio than lambs having all or certain single types of rumen protozoa. The acetate-propionate ratio of protozoa-free lambs was 2.62 and 1.85 for faunated lambs. The acetate-propionate ratios of Entodinium faunated, Diplodinium faunated, Isotricha faunated and Ophryoscolex faunated lambs were 2.00, 1.96, 2.22 and 2.10, respectively.

This shift in acetate-propionate ratio was, at first, somewhat unexpected because rumen protozoa are believed to produce only traces of propionic acid. In order to obtain the change in acetate-propionate ratio as in this trial, greater propionic acid production is necessary. After some deliberation on this, it was postulated that increased propionic acid by the presence of rumen protozoa is indeed possible and, furthermore, is probable. Rumen protozoa normally produce considerable amounts of lactic acid which is readily converted to propionic acid by the rumen bacteria (Johns, 1951a, 1951b and 1952). This is supported by the fact that the growth medium of pure cultures of rumen protozoa contains considerable amounts of lactic acid with very little propionic acid, while, on the other hand, whole rumen fluid contains very little lactic acid but does contain considerable amounts of propionic acid.

The acetate-butyrate ratio also narrowed in rumen contents from faunated lambs as compared to protozoa-free lambs. Proto-

zoa-free lambs showed a rumen acetate-butyrate ratio of about 4 to 1 while faunated lambs exhibited a ratio of 2.62 to 1. The acetate-butyrate ratio ranged from 2.23 for Diplodinium faunated lambs to 3.10 for both Entodinium and Ophryoscolex faunated lambs. Isotricha faunated lambs had an acetate-butyrate ratio of 3.02.

Other acids, such as valeric, iso-valeric, formic, iso-butyric and iso-propionate, were less abundant in protozoa-free lambs than in faunated lambs. These volatile acids from rumen fluid of these lambs comprised only 1.5 percent of the total as compared to 3.5 percent for fully faunated lambs. Rumen fluid from Entodinium and Diplodinium faunated lambs contained 3.8 and 4.5 percent of these acids.

Volatile fatty acids, Trial 2 The experimental plan and summarized results of this trial are given in table 33. An analysis of variance table of data from protozoa-free faunated and Entodinium comparisons of this trial is given in the appendix. Differences which were noted previously in trial 1 were similarly obtained in this trial. Rumen fluid pH ranged from 6.6 in protozoa-free lambs to 6.2 for fully faunated and Entodinium faunated lambs. Rumen fluid pH for Diplodinium and Isotricha faunated lambs was 6.3 and 6.4 for Ophryoscolex faunated lambs.

Total volatile fatty acids (micromoles per milliliter of rumen fluid) for protozoa-free lambs were 64.2 and 75.8 for faunated lambs. For lambs that were separately faunated with

Table 33. Trial 2 - effect of the presence or absence of all or certain types of protozoa upon volatile fatty acid production in the lamb's rumen[a]

Comparison	Rumen pH	Total VFA[b]	Ratio C_2/C_3	Molar percent volatile fatty acids			
				C_2	C_3	C_4	Others
Protozoa-free[c]	6.6	64.2	2.92	61.7	21.1	16.5	0.7
Faunated[c]	6.2	75.8	1.98	52.1	26.4	19.2	2.3
Entodinium[c]	6.2	76.4	1.96	53.0	27.0	17.4	2.6
Diplodinium[d]	6.3	71.2	2.06	50.4	24.8	21.2	3.6
Isotricha[d]	6.3	70.8	2.51	56.8	22.6	18.3	2.2
Ophryoscolex[d]	6.4	67.0	2.30	55.1	24.0	19.4	1.5

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means (labeled c) for total VFA and the C_2/C_3 ratio are 6.2 and 0.30, respectively.

[b]Micromoles of volatile fatty acids per milliliter.

[c]Values are based on the mean of 36 determinations, 4 on each of 9 separate days.

[d]Values are based on the mean of 18 determinations, 2 on each of 9 separate days.

Entodinium, Diplodinium, Isotricha and Orphryoscolex, total volatile fatty acids were 76.4, 71.2, 70.5 and 67.0, respectively. Faunated lambs produced approximately 15 percent more volatile acids than protozoa-free lambs. Lambs faunated with only Entodinium produced similar amounts of acid as did the faunated lambs. Somewhat lesser amounts were produced by Diplodinium faunated, Isotricha faunated and Orphryoscolex faunated lambs.

Similar to trial 1, rumen fluid from lambs in this trial

harboring a so-called normal protozoal population had a narrower acetate-propionate ratio than protozoa-free lambs. The ratio for this comparison was 1.98 and 2.92. respectively, which is about a 32 percent difference. Lambs faunated with Entodinium and others with Diplodinium had rumen fluid acetate-propionate ratios of 1.96 and 2.06, respectively. Somewhat smaller differences were obtained between protozoa-free lambs and either lambs faunated with Isotricha or others faunated with Ophryoscolex. The former was 2.92 as compared to 2.51 and 2.30 acetate-propionate ratios, respectively, for the latter.

The amount of butyric acid in the rumen fluid of protozoa-free lambs was about 14 percent less than in fully faunated lambs. In other lambs faunated with individual types of protozoa (Entodinium, Diplodinium, Isotricha and Ophryoscolex), rumen fluid butyric acid levels were similar to fully faunated lambs. Rumen fluid from Diplodinium faunated lambs contained 21 percent more butyric acid than fully faunated lambs.

Rumen fluid from lambs which were fully faunated or faunated with one type of protozoa contained larger amounts of the so-called minor rumen acids (valeric, iso-valeric, formic, iso-propionic and iso-butyric) than rumen fluid from protozoa-free lambs. These acids comprised less than 1.0 percent of the total rumen acids in protozoa-free lambs and about 3.0 percent in lambs with one or more types of rumen protozoa.

Volatile fatty acids, Trial 3 Summarized results from this trial are presented in table 34. An analysis of variance

Table 34. Trial 3 - effect of the presence or absence of all or certain types of protozoa upon volatile fatty acid production in the lamb's rumen[a]

Comparison	Total VFA[b]	Ratio C_2/C_3	Molar percent volatile fatty acids			
			C_2	C_3	C_4	Others
Protozoa-free[c]	59.8	3.10	64.4	20.8	13.7	1.1
Faunated[c]	79.5	2.83	62.0	21.9	13.9	2.2
Entodinium[c]	76.7	2.49	58.9	23.6	15.1	2.4

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for total VFA and the C_2/C_3 ratio are 6.2 and 0.30, respectively.

[b]Micromoles of volatile fatty acids per milliliters.

[c]Values are based on the mean of 36 determinations, 4 on each of 9 separate days.

table of data from protozoa-free faunated and Entodinium comparisons of this trial is given in the appendix. Three comparisons were conducted in this trial while there were six in trials 1 and 2. Because of the difficulty in obtaining lambs faunated with populations of Diplodinium only, Isotricha only and Ophryoscolex only, this trial was conducted with protozoa-free, Entodinium faunated and fully faunated lambs. It was relatively easy to obtain lambs faunated with Entodinium without any other types of protozoa in the lamb's rumen.

Difference noted in total volatile fatty acids in rumen contents of faunated and protozoa-free lambs in trials 1 and 2 was even greater in this trial. In this trial, rumen fluid

from protozoa-free lambs contained 59.8 micromoles of volatile fatty acids per milliliter while fully faunated and Entodinium faunated lambs had 79.5 and 76.7, respectively. This is a difference of nearly 25 percent while the difference in trials 1 and 2 was about 15 percent.

The narrowing of the acetate-propionate ratio when lambs do not harbor rumen protozoa, as obtained in trials 1 and 2, was also evident in this trial. However, the shift of this ratio in this trial was not as great as in the two previous trials. The acetate-propionate ratio in protozoa-free lambs of this trial was 3.10 as compared to 2.83 and 2.49 for fully faunated and Entodinium faunated lambs.

There were very little differences in the percentage of butyric acid found in rumen fluid of protozoa-free, faunated or Entodinium faunated lambs. For the other acids (valeric, iso-valeric, formic, iso-butyric and iso-propionate), protozoa-free lambs contained slightly less than fully faunated or Entodinium faunated lambs.

Even though there was less of a change in the acetate-propionate shift in trial 3 than in trials 1 and 2, the higher level of total volatile fatty acids in trial 3 would make it so that the gross net energy of the rumen fluid from lambs of these different trials would be nearly equal.

Results of trials 1, 2 and 3 are summarized together and are given in table 35. The average results of these 3 trials show that fully faunated lambs produced about 18 percent more

Table 35. Summary of all trials on the effects of the presence or absence of all or one type of protozoa upon volatile fatty acid production in the lamb's rumen[a]

Comparison	Total VFA[b]	Ratio C_2/C_3	Molar percent volatile fatty acids			
			C_2	C_3	C_4	Others
Protozoa-free[c]	62.3	2.91	62.3	21.4	15.2	1.1
Faunated[c]	75.9	2.23	54.8	24.6	17.4	3.2
Entodinium[c]	76.0	2.14	54.9	25.7	15.8	3.6
Single type protozoa[d]	71.1	2.16	53.9	24.9	18.4	2.8

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means (labeled [c]) for total VFA and the C_2/C_3 ratio are 6.2 and 0.30, respectively.

[b]Micromoles of volatile fatty acids per milliliters.

[c]Comparison values are based on the mean of 3 separate trials involving 112 determinations.

[d]Comparison values are based on the mean of 3 separate trials involving 226 determinations.

total volatile fatty acids than protozoa-free lambs. The overall averages of this trial show that rumen fluid from protozoa-free lambs contains about 10 percent less volatile fatty acids than rumen fluid from lambs harboring a population of a single type of rumen protozoa. It should be pointed out that Entodinium faunated lambs produced levels of total volatile fatty acid similar to fully faunated lambs and had a somewhat narrower acetate-propionate ratio. Total volatile acids were less for lambs faunated with individual populations of Diplodinium,

Isotricha and Ophryoscolex. This indicates that Entodinia may be more beneficial to the host than the other types of rumen protozoa studied.

Volatile fatty acids, Diurnal variation To determine whether or not the differences in total volatile fatty acids between faunated and protozoa-free lambs as found in the 3 previously described trials were present throughout the whole day, diurnation variation studies were conducted. The experimental plan and results of this study are given in table 35 and presented graphically in figure 2. These data are the averages

Table 36. Determination of diurnal variation of volatile fatty acids in the rumen of faunated and protozoal-free lambs[a]

Comparison	Time (hour)[b]							
	7:00	8:00	9:00	10:00	11:00	12:00	2:00	4:00
Faunated[c]	66	75	76	75	80	80	74	65
Protozoa-free[c]	55	67	71	70	72	71	65	60
Entodinium[d]	63	74	75	76	78	78	71	66

[a]Micromoles of volatile fatty acids per milliliter.

[b]Collection began at 7:00 a.m. (after feeding) and ended at 4:00 p.m. (before feeding).

[c]Comparison values are based on the mean of 12 determinations, 6 on each of 2 separate days.

[d]Comparison values are based on the mean of 6 determinations, 3 on each of 2 separate days.

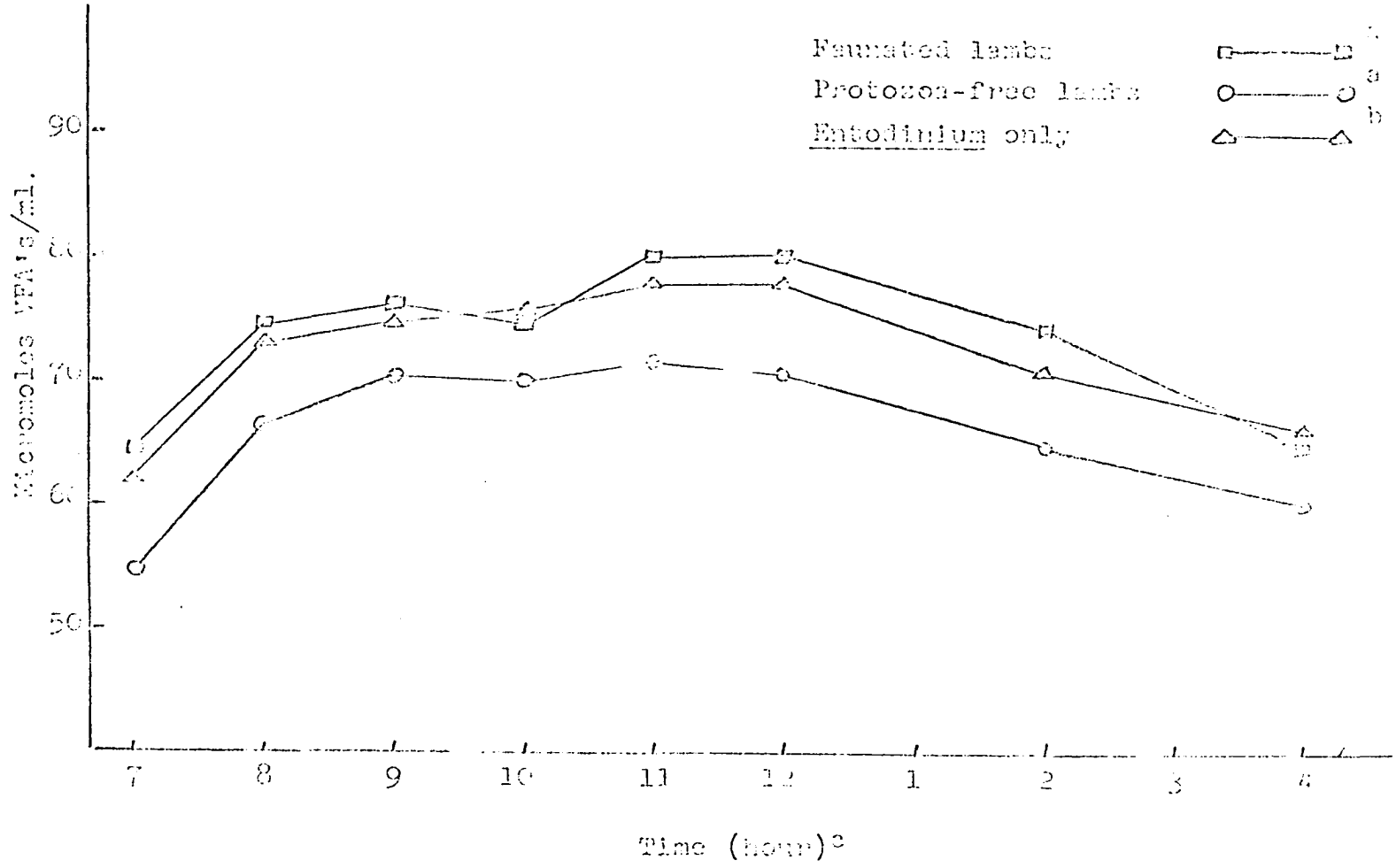
of two separate trials, both of which showed similar results. As can be seen from the data of the table and from the curves of the figure, the differences of total volatile fatty acids

Figure 2. Determination of diurnal variation of volatile fatty acid production in the rumen of faunated and protozoa-free lambs.

[a]Values per point are based on the mean of 12 observations on 2 separate days.

[b]Values per point are based on the mean of 6 observations on 2 separate days.

[c]Collection began at 7:00 a.m. (after feeding) and ended at 4:00 p.m. (before feeding).


Faunated lambs
Protozoa-free lambs
Entodinium only
90
70
50
Micromoles VFA's/ml.
7
8
9
10
11
12
1
2
3
4
Time (hours)

for faunated and protozoa-free lambs were fairly constant throughout the 10 hour period studied. Lambs which were faunated with Entodinium produced somewhat lesser amounts of total volatile fatty acids than fully faunated lambs. However, the overall results were relatively similar.

It is of interest to point out that production of total volatile fatty acids failed to show an increase at the 10:00 a.m. determination. In fact, it was somewhat less than either the 9:00 a.m. or the 11:00 a.m. determinations. It is believed that this decrease was due to a depletion of the readily fermentable carbohydrates coupled with a latent period for the less readily fermentable carbohydrates.

It is noticeable that the level of total volatile acids at 4:00 p.m. failed to drop as far as the low level observed at the 7:00 a.m. determination. It is thought that this was due to the shorter period between a.m. and p.m. feeding than the period between the p.m. and a.m. feeding. There is a 9 hour period for the former and 15 hours for the latter. If these periods were equal, undoubtedly, the low points in the diurnal cycle would be very similar.

Studies by Raun (1961) indicated that peak production of rumen acids was obtained 3 to 4 hours after feeding. Present studies, in general, support this finding. The data from this study indicate peak acid production was obtained 4 hours after feeding. Therefore, collecting rumen fluid to obtain optimal levels of volatile fatty acids was conducted between three and

one-half and four and one-half hours after feeding.

Ruminal Ammonia Production in the Rumen of Faunated, Partially Faunated and Protozoal-Free Lambs

Microbial degradation of proteinaceous feedstuffs results in the production of ammonia, most of which is absorbed through the rumen wall into the blood stream. It is transported to the liver, converted to urea which is recycled via the saliva to the rumen or lost via the urine.

The utilization of protein and non-protein nitrogen by rumen microorganisms is an extremely interesting reactive system of bacteria and protozoa interposed between diet and host in ruminant animals. It is well known that amino acids and especially ammonia produced by microbial fermentations are formed more rapidly than they are utilized by the rumen flora and fauna and, therefore, prior to being absorbed they accumulate in the rumen. MacDonald (1948) has shown that ammonia is absorbed from the rumen and may amount to a considerable loss of ingested nitrogen, especially if the level of free nitrogen in the rumen is relatively high.

Even though dietary protein may be relatively less efficiently utilized by ruminants than non-ruminants, apparent differences in nutritive value of protozoal and bacterial protein cause interest for further study of microbial metabolism of nitrogenous feedstuffs. McNaught et al. (1954), using rat assay techniques, found that the nutritive value of yeast, bac-

terial and protozoal proteins were 74, 84 and 91. respectively. Weller (1957) found that the superior value of protozoal proteins was probably due to a better balance of essential amino acids, particularly lysine. Williams et al. (1960) suggested that rumen protozoa utilize proteinaceous materials very readily.

Methods and procedures

Lambs which were used in conducting volatile fatty acid studies previously described were also used simultaneously in conducting ruminal ammonia production studies. The methods and procedures used for completing the volatile fatty acid research which were also used for ruminal ammonia studies are described in the previous section; therefore, they are not described in this section. The ration fed is given in table 31 and the time of feeding and amounts fed of this ration are described in the previous section.

Rumen fluid samples were obtained in adequate quantity for both volatile fatty acid and ammonia determinations. Generally, this required about 30 milliliters. After pH values were obtained on the total amount of collected rumen fluid, 10 milliliters were taken for ammonia determinations. Ammonia levels of rumen fluid of faunated and defaunated lambs were determined at three and one-half to four and one-half hours after feeding. Diurnal levels were determined at 8:00 a.m. and at hourly intervals thereafter until 4:00 p.m.

Collected rumen fluid was taken to the laboratory where microbial activity was terminated by the addition of saturated aqueous mercuric chloride solution. The amount of ruminal ammonia was then determined by the method of Varner et al. (1953). For a complete description of this method refer to the appendix.

In addition to faunated and protozoa-free lambs, ammonia levels were determined from rumen fluid of lambs faunated with separate populations of Entodinium, Diplodinium, Isotricha and Ophryoscolex. The methods used to obtain lambs faunated with these separate populations of protozoa are described in the previous section.

The first 3 trials of this study involved the determination of ammonia from rumen fluid of all 6 treatment comparisons, namely, protozoa-free, faunated, Entodinium, Diplodinium, Isotricha and Ophryoscolex faunated lambs. Trials 4, 5 and 6, however, involved only three of these treatment comparisons, protozoa-free, faunated and Entodinium faunated lambs. Diurnal levels of ammonia were determined from rumen fluid of lambs which were protozoa-free, fully faunated and Entodinium faunated.

Results and discussion

Ruminal ammonia, Trials 1, 2 and 3 The experimental plan and results of these trials are given in table 36. An analysis of variance table of the data for protozoa-free, fau-

Table 36. The effects of the presence or absence of all or certain types of protozoa upon ammonia production in the lamb's rumen[a]

Comparison	Milligrams percent ammonia Trial 1	Trial 2	Trial 3	Av.
Protozoa-free[b]	7.4	5.2	5.4	6.0
Faunated[b]	17.6	10.8	10.7	13.0
Entodinium[b]	14.4	9.6	11.4	11.8
Diplodinium[c]	12.8	8.6	9.1	10.2
Isotricha[c]	9.7	7.4	8.6	8.6
Ophryoscolex[c]	11.4	8.9	7.2	9.2

[a]A difference (D) of 2.9 required for significance at P = .05 level for comparison of the treatment means (labeled [b]).

[b]Comparison values within each trial are based on the mean of 20 determinations, 4 of each on 5 separate days.

[c]Comparison values within each trial are based on the mean of 10 determinations, 2 of each on 5 separate days.

nated and *Entodinium* comparisons of this trial is given in the appendix. In all three of these trials, protozoa-free lambs consistently produced less ammonia than fully faunated or individual type protozoa faunated lambs. Protozoa-free lambs in these trials produced an average of 6.0 milligrams percent of ruminal ammonia while fully faunated lambs produced an average of 13.0. Lambs faunated separately with *Entodinium* and *Diplodinium* produced similar amounts of ruminal ammonia which was 11.8 and 10.2 milligrams percent, respectively. *Isotricha* faunated and *Ophryoscolex* faunated lambs were somewhat less with

8.6 and 9.2, respectively.

The difference of ruminal ammonia levels between protozoa-free lambs and fully faunated lambs, in these trials, was more than 100 percent (6.0 as compared to 13.0 milligrams percent). Other comparisons were somewhat less with Isotricha faunated lambs giving 8.2 milligrams percent of ammonia which was similar to the protozoa-free lambs. Ophryoscolex faunated lambs had ruminal ammonia levels similar to Isotricha faunated lambs. It is of interest to note that these ruminal ammonia values were relatively comparable to the corresponding comparison of the volatile fatty acid levels.

Ruminal ammonia, Trials 4, 5 and 6 In these trials, comparisons were made with protozoa-free, fully faunated and Entodinium faunated lambs. The results and experimental plan of these trials are given in table 37. An analysis of variance table of the data from protozoa-free, faunated and Entodinium comparisons of this trial is given in the appendix. The same general influence of protozoa upon ruminal ammonia levels shown in the first three trials was also evident in these trials. Protozoa-free lambs produced 6.8 milligrams percent of ruminal ammonia while fully faunated lambs produced 11.3. Entodinium faunated lambs produced 11.7 milligrams percent.

Summarized results of all trials concerning ammonia production are presented in table 38. It can be seen that fully faunated lambs produced essentially 100 percent more ruminal ammonia than protozoa-free lambs. The values obtained were

Table 37. Effects of the presence or absence of protozoa upon ammonia production in the lamb's rumen[a]

Comparison	Milligrams percent ammonia			
	Trial 4	Trial 5	Trial 6	Av.
Protozoa-free[b]	8.3	5.2	6.8	6.8
Faunated[b]	13.8	8.4	11.7	11.3
Entodinium[b]	14.2	10.6	10.2	11.7

[a]A difference (D) of 2.9 required for significance at P = .05 level for comparison of the treatment means.

[b]Comparison values within each trial are based on the mean of 20 determinations, 4 of each on 5 separate days.

6.40 and 12.18 milligrams percent, respectively. These values are based on the mean of 6 separate trials involving 108 different ruminal ammonia determinations. All determinations on lambs faunated with a population of a single type of protozoa showed somewhat lower ruminal ammonia levels than fully faunated lambs, but were still considerably higher than ruminal ammonia levels from protozoa-free lambs.

Ruminal ammonia, Diurnal variation The plan of this experiment and results of the diurnal cycle of ammonia are given in table 39. The results of this study are also shown graphically in figure 3. Since it was previously shown that faunated lambs produced higher ruminal ammonia levels than protozoa-free lambs 3 to 5 hours after feeding, it was of interest to know whether or not this difference existed continuously from one feeding to the next. The purpose of this study was to

Table 38. Summary of all trials on the effects of the presence of absence of all or one type of protozoa upon ammonia production in the lamb's rumen[a]

Comparison	Milligrams percent ammonia
Protozoa-free[b]	6.40
Faunated[b]	12.18
Entodinium[b]	11.75
All single type protozoa trials[c]	10.30

[a]A difference (D) required of 2.9 for significance at P = .05 level for comparison of the treatment means (labeled [b]).

[b]Comparison values are based on the mean of 6 separate trials involving 108 determinations.

[c]Comparison values are based on the mean of 6 separate trials with 4 different types of rumen protozoa involving 198 determinations.

determine the ruminal ammonia diurnal cycle for fully faunated, Entodinium faunated and protozoa-free lambs.

One hour after the a.m. feeding, the ruminal ammonia levels of fully faunated, Entodinium faunated and protozoa-free lambs were not greatly different. However, shortly thereafter differences were obtained and were maximal 5 hours after feeding. Faunated lambs produced about 100 percent higher ammonia levels than protozoa-free lambs. The actual values were 14.2 and 7.8 (milligrams percent of ammonia), respectively. Ruminal ammonia levels from Entodinium faunated lambs were somewhat intermediate with 10.8 milligrams percent.

Table 39. Determination of diurnal variation of ammonia in the rumen of faunated and protozoa-free lambs[a]

Comparison	Time (hour)[b]								
	8:00	9:00	10:00	11:00	12:00	1:00	2:00	3:00	4:00
Faunated[c]	6.3	8.0	12.5	12.6	14.2	12.2	9.7	8.2	7.0
Protozoa-free[c]	6.1	6.3	8.4	8.5	7.8	6.7	6.0	4.2	4.4
Entodinium[d]	5.7	7.5	10.2	12.0	10.8	9.7	8.0	6.5	6.2

[a]Values are reported as milligrams percent.

[b]Collection began at 8:00 a.m. (after feeding) and ended at 4:00 p.m. (before feeding).

[c]Comparison values are based on the mean of 24 determinations, 12 on each of 2 separate days.

[d]Comparison values are based on the mean of 12 determinations, 6 on each of 2 separate days.

From 5 hours after feeding to 9 hours after feeding, ruminal ammonia levels progressively decreased in all comparisons. Even though all comparisons showed much lower ruminal levels at this time, the percentage differences between comparisons were even greater than when maximum differences were observed. Since only 9 hours had elapsed since the a.m. feeding, it is believed that the 15 hour period from p.m. to a.m. feeding minimized the comparison differences that were obtained at the 8:00 a.m. determination. If the time differences between each feeding were equalized, there probably would be similar diurnal cycles between each feeding.

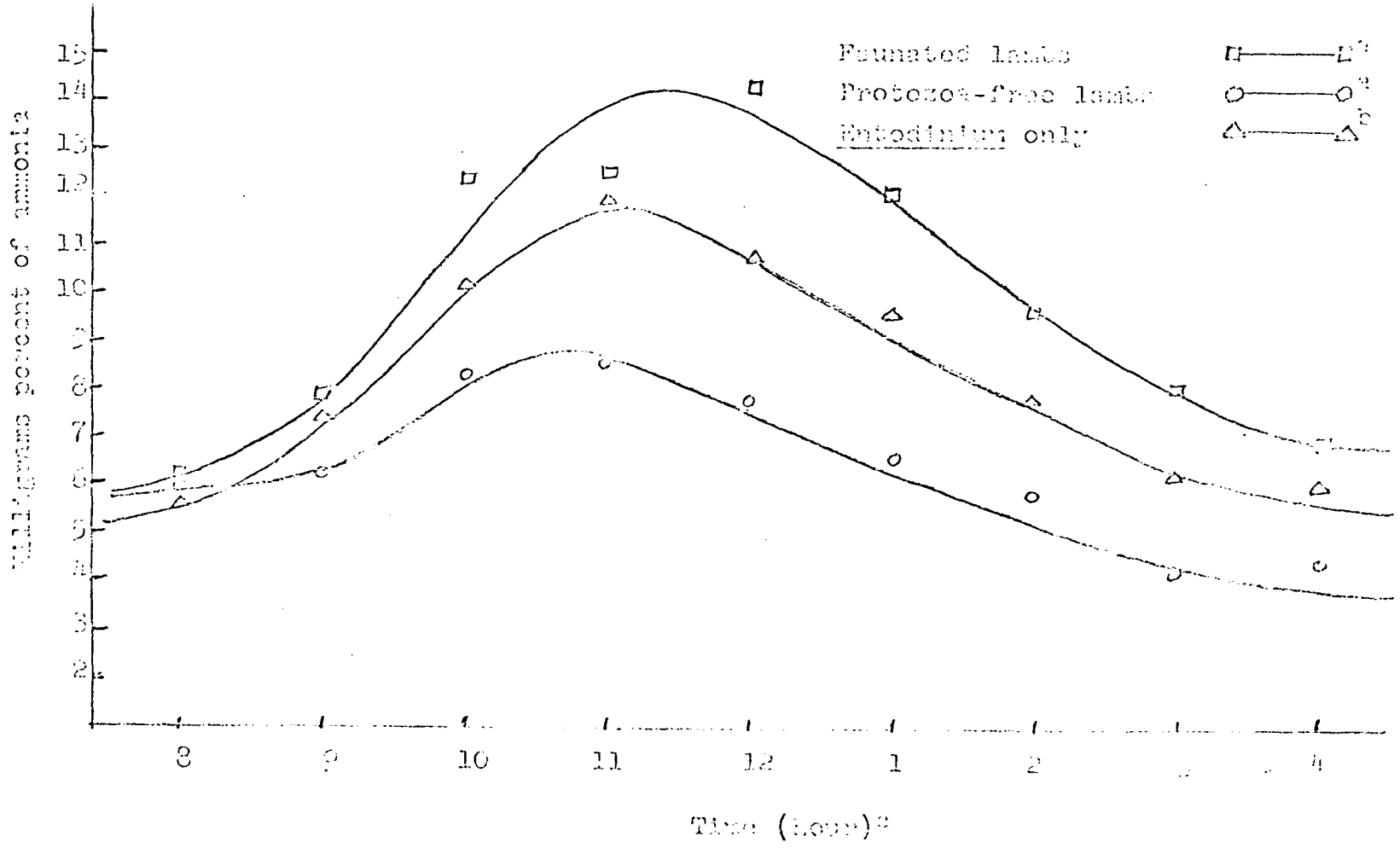
It can be seen that the ruminal ammonia value obtained for

Figure 3. Determination of diurnal variation of ammonia in the rumen of faunated and protozoa-free lambs.

[a]Comparison values are based on the mean of 24 determinations, 12 on each of 2 separate days.

[b]Comparison values are based on the mean of 12 determinations, 6 on each of 2 separate days.

[c]Collection began at 8:00 a.m. (after feeding) and ended at 4:00 p.m. (before feeding).


Faunated lambs
Protozoa-free lambs
Entodinium only
Milligrams percent of ammonia
15
14
13
12
11
10
9
8
7
6
5
4
3
2
8
9
10
11
12
1
2
4
Time (hour)

faunated lambs at the 11:00 a.m. determination may be unduly low. If this value is not correct, highest ammonia levels may be obtained 4 to 5 hours, rather than 5 hours, after feeding. Entodinia faunated and protozoa-free lambs showed highest ruminal ammonia levels 3 to 5 hours after feeding.

The values obtained for the ruminal ammonia diurnal cycle are relatively similar to the values obtained for the diurnal cycle for volatile fatty acid production. The comparisons studied (faunated, Entodinia faunated and protozoa-free lambs) showed that highest levels of volatile fatty acids were at 4 to 5 hours following feeding.

CONCLUSIONS AND SUMMARY

This part of the thesis involves the determination of volatile fatty acid levels, ratios and diurnal cycle and ruminal ammonia levels and diurnal cycle from rumen fluid of lambs fully faunated, partially faunated and free of protozoa. The comparisons studied were as follows: fully faunated, Entodinium faunated, Diplodinium faunated, Isotricha faunated, Ophryoscolex faunated and protozoa-free lambs.

Rumen volatile fatty acid levels were highest for fully faunated lambs or Entodinium faunated lambs. Protozoa-free lambs produced lowest levels of rumen volatile fatty acids. Fully faunated lambs produced about 16 percent more rumen volatile fatty acids than protozoa-free lambs. Lambs which were faunated separately with Diplodinium, Isotricha or Ophryoscolex produced levels of volatile fatty acids which were intermediate to the two extremes.

The determination of acetate-propionate ratios showed that the presence of all or only certain types of rumen protozoa caused a narrowing of this ratio. Protozoa-free lambs produced less propionic acid than any other comparison. Fully faunated and Entodinium faunated lambs produced the highest amount of propionate giving the narrowest acetate-propionate ratio. The other comparisons were intermediate in regard to this ratio.

The acetate-butyrate ratios obtained followed the same general pattern as the acetate-propionate ratio. Highest bu-

tyric acid levels were obtained from fully faunated and Entodinium faunated lambs. The amount of remaining rumen volatile fatty acids (valeric, iso-valeric, formic, iso-butyric and isopropionic) were somewhat higher in rumen fluid from lambs with all or only certain types of rumen protozoa than in rumen fluid from protozoa-free lambs. These acids comprised only a minor portion of the total volatile fatty acids, however.

Relative differences of the diurnal cycle for rumen volatile fatty acids were not greatly affected by the presence or absence of rumen protozoa. The difference which existed at the standard determination time (4 to 5 hours after feeding) remained relatively constant for all time periods studied. This study indicated that peak ruminal activity was 4 to 5 hours after feeding.

Ruminal ammonia levels were highest for fully faunated or Entodinium faunated lambs. Protozoa-free lambs produced lowest ruminal ammonia levels. Fully faunated lambs produced about 100 percent higher ruminal ammonia levels than protozoa-free lambs. Lambs faunated separately with Diplodinium, Isotricha or Ophryoscolex produced intermediate levels of ruminal ammonia.

Relative differences of the diurnal cycle for ruminal ammonia were affected by the presence or absence of rumen protozoa. One hour after feeding ruminal ammonia levels were similar for fully faunated, Entodinium faunated and protozoa-free lambs. However, 4 to 5 hours after feeding, fully faunated

lambs produced about twice the amount of ammonia as compared to protozoa-free lambs. Both comparisons were greatly higher at this determination. Entodinium faunated lambs produced levels of ruminal ammonia intermediate to faunated and protozoa-free lambs, but were somewhat closer to the values of the faunated lambs.

PART III. RATION CHARACTERISTICS INFLUENCING RUMEN PROTOZOA AND THE EFFECT OF PROTOZOA UPON GROWTH PERFORMANCE OF LAMBS

REVIEW OF LITERATURE

It has become increasingly apparent that animal performance is affected when the physical form of the diet is altered. Pelleting rations that are high in roughage has consistently improved rate of gain and feed efficiency. By decreasing the size of particles and increasing the level of consumption of rations, rate of passage of ingesta has been increased. On the other hand, when rations high in concentrates were pelleted, little or no differences have been noted (Rhodes, 1961). When whole dry-shelled and high-moisture shelled corn was full-fed to fattening beef, 20 to 25 percent passed through the gastrointestinal tract totally undigested (Christiansen and Burroughs, 1960). The frequency of feeding rations to ruminants has also been shown to exert effects upon animal performances. Studies are numerous which have dealt with the physical form of diets and its effect upon nutrient digestibility and animal performance. Little has been done, however, in connection with the influence of ration characteristics and their effects upon the rumen microorganisms, particularly protozoa. The main objective of this review is to acquaint the reader with the general effects of physical form, level of intake and other ration characteristics and their effects upon the ruminant and their microbial population.

The physical form of rations has been shown to influence growth, digestibility and certain rumen functions and charac-

teristics of cattle and sheep. Early research by Webb et al. (1957) with pelleted hay rations for cattle showed that gains were improved 2 to 3 times and feed consumption increased 50 percent compared to long hay rations. Meyer et al. (1959a) found when lambs were fed, ad libitum, alfalfa hay pelleted and chopped, their daily feed consumption was 3.25 and 2.48 pounds, respectively, while daily gains were 0.40 and 0.27 pounds, respectively. When feeding steers high roughage rations in three different forms, long, ground and pelleted, Klosterman et al. (1960) found that pelleting and grinding improved gains by 75 and 50 percent, respectively, and both improved feed consumption. Meyer et al. (1959b) found that when alfalfa hay was finely ground and pelleted, feed consumption was increased by 50 and 75 percent, respectively, as compared to the control. Daily gains were improved as feed consumption increased. When feeding rations high in roughages to ruminants, pelleting has consistently resulted in improved performance as compared to feeding the roughage long or coarsely chopped.

When rations high in concentrates are pelleted and fed to ruminants, little or no difference in gains and feed consumption has been noted when compared to non-pelleting. In general, however, feed efficiency has improved somewhat when ruminants are fed pelleted high concentrate rations. Neale (1955) fed lambs three rations, 50, 60 and 70 percent roughage, which were pelleted and non-pelleted. Results showed that 70 percent roughage pelleted rations gave the highest performance

with both feed consumption and gains decreasing as the concentrate level increased in the pelleted rations. Woods and Rhodes (1960) found that grinding or pelleting rations with one-third alfalfa hay and two-thirds concentrate gave superior performances of lambs as compared to long hay rations. In other studies, pelleting high roughage rations improved lamb gains and feed consumption while pelleted rations high in concentrates failed to improve gains and consumption.

Perry et al. (1959) studied roughage to concentrate ratios with cattle utilizing corn cobs as the only source of roughage. These ratios, 75:25, 45:55 and 20:80, resulted in 26 percent increase in gains for the 75 percent roughage while 45 and 20 percent roughage rations gave similar performances when compared to non-pelleted rations. When calves were fed a ration with 80 percent roughage in pelleted and meal form, 0.40 pound increase in daily gains was noted (McCraskey et al., 1960). In other roughage to concentrate ratio studies, Webb et al. (1959), using calves, yearlings and two-year old cattle, found the following results. Highest gains were obtained when roughage-concentrate ratios were 70:30 for calves and 40:60 for the other two types of cattle. Feed efficiency was best for the 80 percent concentrate ration for all groups of cattle. Carcass grade, feed cost and profit margins were, in general, optimal at the 40:60 ratio. It is evident in this review that the physical form of high roughage rations greatly affects the

performance of cattle and sheep; on the other hand, changing a high concentrate ration from a conventional ground mixed to a pelleted form has shown no benefit and in some cases has been detrimental.

With the difference noted in animal performance when ration physical form was changed, the basic reasons for these changes were of interest. Digestion trials were conducted in an attempt to answer why differences were obtained when rations were consumed in different forms. Blaxter et al. (1955), when feeding grass hay, long, ground and pelleted to lambs, found that digestibility was lowest for the pelleted hay. Further studies by Blaxter and Graham (1956), when comparing digestibility of hay rations, long and medium or fine ground pellets, found pelleting lowered digestibility and was further lowered by finer grind of hay in the pellets. Crude fiber digestibility was adversely affected more than other feed components. Clanton et al. (1959), using cattle, observed that dry matter and energy were more digestible in high roughage chopped hay rations than in similar pelleted rations.

Weir et al. (1959) obtained digestibility data with lambs on an alfalfa hay ration and on a 70:30 alfalfa-barley ration. Pelleting or adding barley to the ration caused a decrease in crude fiber digestibility. Digestibility data from Lindahl and Reynolds (1959) demonstrate an increase in "ether extract" and a lowering of the crude fiber content of alfalfa hay after pelleting. Reynolds and Lindahl (1960) studied the digestibility

of alfalfa-grass hay in three forms, long, ground or pelleted. The digestibility of dry matter, crude fiber, nitrogen free extract and gross "energy" was higher for the long hay ration with the differences for crude fiber and dry matter being the largest.

Differences in rate of gain, feed efficiency and consumption of rations caused by changes in physical form of the diet have become common place. More recently, however, research findings indicate that ration changes are causing structural, functional and microbial alterations within the rumen. Rhodes (1961) observed profound changes of the rumen epithelia of lambs fed ground mixed, pelleted and long hay rations. In general, these observations indicate that the higher the concentrate and the finer the particles in a ration, the more the rumen epithelia becomes dark, matted or clumped and the less development of the papillae. Lambs fed long hay rations exhibited rumen epithelia of bright yellowish color, with little or no matting and with uniform and dense papillary development. The condition appears to be rumen "parakeratosis" as described by Jensen et al. (1958). Observations by these workers have indicated an unusually high incidence of parakeratosis in rumens of animals fattened on pelleted feeds as compared to coarse non-pelleted feeds. Studies by Sinclair and Kunkel (1959) have shown that the rumen weight, papillary length and density are significantly correlated with rate of gain. These workers suggested that dietary antibiotics may effect micro-

bial fermentation products and that these products may be responsible for the color changes of the rumen epithelia in cases of parakeratosis.

Warner et al. (1959) when studying various ration factors found that when calves were fed a 90 percent hay ration their rumens were nearly double the volume of rumens of calves fed a 90 percent grain ration. When calves were fed milk, their rumens were smaller than the latter group of calves. Development of the rumen mucosa, including the papillae, was greatest in the high roughage and lowest in the high grain fed calves. Further in vitro studies have shown that rumen papillary development was best when sodium salts of the principal rumen volatile fatty acids were included in the tissue incubation media. It was believed that sodium propionate and sodium butyrate were largely responsible for supporting papillary growth. It is apparent that the diets of ruminants with parakeratosis have been those which, in general, are ground, mixed, high concentrate or pelleted rations. It is not so apparent, however, whether the physical form of the ration alone or its effects upon the microbial fermentations are the specific causes of these changes in rumen structure.

Over the years, cattle and sheep have commonly been fed twice daily. Under pasture or range conditions common practice is to feed once daily or even less. Not only among researchers but also among livestock producers, lack of agreement is evi-

dent on the question of how frequent animals should be fed. Gordon and Tribe (1952) reported a 5 fold increase in rate of gain of ewes fed 8 times daily as compared to once daily. One, two and four feedings per day with dairy heifers were compared by Thomas and Mochrie (1956). Comparisons of relative rate of gain favored feeding at least four times daily.

Moir and Somers (1957) reported on feeding methods and intervals as affecting ration digestibility and certain rumen characteristics. Comparisons were made among the following feeding methods: (1) the total ration fed at 9:00 a.m., (2) half the ration fed at 9:00 a.m. and the remaining part at 5:00 p.m., (3) one-fourth of the ration fed at each of the following times, 9:00 a.m., 11:00 a.m., 1:00 p.m. and 5:00 p.m., (4) concentrate cubes fed at 9:00 a.m. and hay at 5:00 p.m. and (5) hay fed at 9:00 a.m. and concentrate cubes fed at 5:00 p.m. Dry matter digestibility was similar for all methods except (1). The daily ration given as a single feed caused the greatest fall in rumen pH values, bacterial and protozoal counts and the highest ruminal ammonia levels. The best ration utilization was obtained by feeding the concentrate in the morning and the roughage in the afternoon or by feeding the whole ration four times daily.

Frequency of feeding studies by Klosterman (1959) indicated that heifer calves perform similarly when fed three as compared to one time daily. Using dairy heifers, Rakes et al.

(1957) found that feeding ten times as compared to feeding twice daily increased weight gains and decreased feed digestibility. When feeding beef cattle either twice daily or six times daily, Mohrman et al. (1959) found nearly 15 percent increased gains and about a 20 percent increase in feed consumption for the animals fed most frequently. Feed efficiency was similar for both groups. In a number of his studies, more frequent feeding intervals were successful in increasing gains of sheep and cattle. In two separate studies, however, increased benefits were not obtained (Klosterman, 1959; Rakes et al., 1960). In these studies it appears that the nutritional level of diet consumed by the animals or their age may be responsible for the negative results. It is suggested that older animals, by consuming no more than a "maintenance level" of nutrients, will give little or no response to multiple as compared to single daily feeding intervals.

Putnam et al. (1961) studied the effects of frequency of feeding upon rumen volatile acids, protozoal populations and weight gains in Angus heifer calves. Increased frequency of feeding resulted in significantly greater daily gains and in improved feed consumption. As also indicated, increasing frequency of feeding is accompanied by increase in protozoal numbers and production of a higher level of total volatile fatty acids. This work is in agreement with results of Moir and Somers (1956) when studying factors influencing protozoal pop-

ulations in sheep. These data show an increase in numbers of oligotrich protozoa and an increase in daily gains resulted when sheep were fed four times versus one time daily.

Purser (1961), in studying the diurnal cycle for holotrich protozoa of the rumen, has shown that when lambs were fed once daily, the number of holotrichs 20 hours after feeding dropped to one-tenth of the number present at or near feeding. When lambs were fed twice daily, the number of protozoa dropped to about one-half of the number found near time of feeding. This worker suggests that the number of protozoa may be controlled by physical factors such as dilution of the rumen contents caused by eating, drinking and starvation. It is pointed out that there are differences in response of the holotrichs and oligotrichs to different nutritional regimes, even though the factors governing these differences have not been explained. Of interest is the fact that rumen pH follows the same general trend as the number of oligotrich protozoa when feeding regimes and ration characteristics are changed.

Mason (1950) observed that rumen protozoa disappeared when lambs were fed a high level flaked maize ration. But bacterial numbers appeared near normal or were slightly elevated. It was noted that volatile fatty acid proportions were abnormal: acetic acid levels were nearly doubled, propionic and butyric acids were very low. In some cases, propionic was entirely absent. Observations similar to these were reported by Phil-

lipson (1952). In studies involving fatty acids of the rumen of lambs fed flaked maize rations, total acids increased and there was an increase in production of propionic acid. With acetate remaining nearly constant or decreasing, the overall result was a narrowing of the acetate-propionate ratio. High levels of lactic acid also were found. It was not stated by the researcher but it is presumed that, under these conditions, rumen protozoa were entirely absent. When studying certain aspects of volatile fatty acids in sheep, Elsden (1945) reported variations in rumen microorganisms which were related to the feed ingested. Phillipson (1946), studying physiology of digestion in the ruminant, found variability of the microorganisms to be associated with ration differences.

Pounden and Hibbs (1948a and 1948b) found that protozoa and certain types of bacteria failed to become established in milk fed one-month old calves but were easily established in milk and alfalfa hay fed month old calves. They also found that inoculations, using parts of cuds from mature ruminants, assisted in the establishment of these microorganisms in the rumens of calves which were eating either hay alone or hay, grain and milk. Pounden and Hibbs (1950) have shown that calves normally are able to establish a protozoan population at 4 to 5 weeks of age. Young lambs and goats can become faunated at 2 to 4 weeks of age.

Calling to mind the statement of Purser (1961), that rumen oligotrich protozoa may be controlled to a large extent by di-

lution factors of the rumen, it is important to relate the effects of feeding regimes and physical form of rations upon rumen functions. Blaxter and Graham (1956), studying the effect of grinding and cubing upon the utilization of the energy of grasses, and Blaxter et al. (1955), in studies concerned with relationships of rate of passage of ingesta and the digestibility, have demonstrated the following: (1) as ration particle size decreases, the time that the feed stays in the rumen is decreased, (2) as ration consumption increases, the time the feed stays in the rumen decreases, (3) when ration particle size is reduced, the digestibility of the feed is decreased and (4) when ration consumption increases, digestibiltiy decreases. These observations were found using lambs fed grasses in three different physical forms, long, ground and pelleted. In making this review, it has become apparent that research concerned with ration characteristics and their influences upon the rumen microorganisms, particularly protozoa, is very sparce and unrevealing as to how the microbial populations are affected. It is also apparent that high level feeding of finely ground and pelleted rations is usually accompanied by the absence of rumen protozoa and that lower acetate and increased propionate and butyrate also appear to be common occurrences.

EXPERIMENTAL PROCEDURE AND RESULTS

Influence of Level of Feed Intake, Physical Form of Ration and Rate of Passage of Ingesta Upon Rumen Protozoal Populations

The observed disappearance of protozoa from the rumens of sheep that were fed certain rations stimulated interest for this study. Preliminary microscopic observation of rumen contents from lambs that were full-fed pelleted rations has shown that protozoa disappear after lambs consume such a ration for 2 to 3 weeks. It had also been observed by the author that lambs which had lost their rumen protozoa while consuming a full feed of a pelleted ration reestablished a normal protozoal population when the pelleted ration intake was reduced to about two-thirds of the full feed. It had also been observed that when rate of passage of ingesta increased, the number of protozoa decreased. In some cases, total disappearance was observed.

The Tukey test as described by Snedecor (1959) was used to determine a difference required for significance at the 0.05 probability level for comparison of treatment means in each experiment.

Methods and procedures

The influence of level of feed intake upon rumen protozoal populations Wether lambs that weighed about 75 pounds each were selected for this study. The lambs were randomly assigned

to treatments. The percentage composition of the ration fed in this experiment is given in the first column of table 40. Lambs were individually penned and fed separately at 7:00 a.m. and 4:00 p.m. Protozoal numbers were determined according to the method of Moir and Somers (1957) as modified by the author.

In trial 1, four lambs, 2 of which were full-fed and 2 of which were fed two-thirds of a full feed, were used to study the influence of level of feed intake upon rumen protozoal disappearance. After the lambs consumed this ration for 1 week, daily microscopic observations were made on rumen contents until protozoa disappeared within 12 to 15 days.

In trials 2, 3 and 4, a switch-back experimental design was employed to determine the influence of level of feed intake upon the disappearance and subsequent reappearance of pro-

Table 40. Ration composition for all trials concerned with the influence of level of feed intake, physical form of ration and rate of passage of ingesta upon rumen protozoal populations

Ingredients	Level of feed intake trials	Physical form of ration				
		Trial 1		Trial 2	Trial 3[a]	
		Alf-corn	Cob-corn		Basal	H.C.
	(%)	(%)	(%)	(%)	(%)	(%)
Alfalfa hay	32.0	33.0	--	33.0	30.0	25.0
Corn (rolled)	48.5	53.2	48.4	54.5	34.0	60.0
Cobs (ground)	7.0	--	33.0	--	20.0	--
Soybean meal	5.0	5.0	8.5	5.0	8.0	6.0
Molasses	7.0	7.0	7.0	7.0	8.0	8.0
Urea	--	--	0.75	--	--	--
Salt	0.5	0.5	0.5	0.5	0.5	0.5
Dicalcium phosphate	--	--	0.4	--	--	--
Quadrex	0.025	0.02	0.02	0.02	0.024	0.025

[a]H.C. indicates high concentration ration.

tozoa in the rumen. Two lambs were full-fed until disappearance of rumen protozoa was observed, after which they were fed two-thirds of a full feed. Two other lambs were fed two-thirds of a full feed from the beginning of the experiment until the full-fed lambs were switched to two-thirds of a full feed. These lambs were then switched to a full feed until disappearance of rumen protozoa was observed. Using this design, each lamb underwent exposure to both ration-level-of-intake treatments.

Trials 3 and 4 were replicates of trial 2. Other lambs were used for each of these trials. However, it required about 6 weeks for the completion of this experiment.

The influence of physical form of the ration upon rumen protozoal populations

Trial 1 Forty-eight wether lambs that weighed about 70 pounds each were selected for this study. The lambs were randomly assigned to the treatments. The lambs were individually fed the ration which is shown under trial 1 of table 40. Two rations were each fed in 2 physical forms and 2 different proportions of roughages to concentrates. This is shown in table 42. At the end of this trial, rumen fluid samples were collected via stomach tube from each lamb; pH and counts of the numbers of rumen protozoa were determined. Individual lamb weights were determined at 2-week intervals and feed consumption was recorded throughout the trial.

Trial 2 Fifteen wether lambs that averaged about 85 pounds were selected for this study. These lambs were individually fed the ration shown under trial 2 of table 40. This ration was fed in 5 different physical forms which are shown in table 43. Rumen fluid samples were collected via stomach tube from each lamb and pH and protozoal numbers were subsequently determined. Individual lamb weights were determined at 2-week intervals and feed consumption was recorded throughout the trial.

Trial 3 Twenty-four wether lambs that averaged about 70 pounds were selected for this study. These lambs were individually fed the ration shown under trial 3 of table 40. These 2 rations were fed in 2 different physical forms as shown

in table 44. Rumen fluid samples were collected via stomach tube from each lamb and protozoal numbers were subsequently determined. Individual lamb weights were taken at 2-week intervals and feed consumption was recorded throughout the trial.

Trial 4 Fifteen wether lambs that averaged about 85 pounds were selected for this study. The objective of this trial was to determine the influence of feeding a ration in 5 different physical forms upon rate of disappearance of protozoa from the lamb's rumen. These lambs were individually fed ad libitum. The percentage composition of this ration was the same as that given under trial 2 in table 40. Rumen fluid samples were taken from each lamb on the first day of the trial and at weekly intervals thereafter until 5 weeks elapsed. Protozoal numbers were determined on each of these rumen fluid samples immediately following each weekly collection.

The influence of rate of passage of ingesta upon rumen protozoal populations

Trial 1 The objective of this trial was to determine the influence of physical form of the ration upon rate of passage of ingesta and upon rumen protozoal numbers. Nine wether lambs that averaged about 100 pounds were used for this study. The lambs were randomly assigned to treatments. The ration fed was similar to that listed for trial 2 of table 40. The intake of the lambs on all types of rations was equalized for the final 10 days of the trial. For this 10-day period, the lambs were fed at 5:00 a.m. and 5:00 p.m.

At the end of this trial, lambs were slaughtered and the quantity (weight) of ingesta within each separate area of the gastrointestinal tract (rumen-reticulum, omasum, abomasum, small intestine and large intestine) was immediately determined by the method of Weller et al. (1958 and 1962) and Grey et al. (1958). Prior to slaughter, rumen fluid samples were collected and protozoal numbers were determined.

Trial 2 The objective of this trial was to determine the influence of level of feed intake upon rate of passage of ingesta and upon rumen protozoal numbers. Six wether lambs that averaged about 110 pounds were selected for this trial. The ration fed and time of feeding were the same as described in trial 1. Three lambs were full-fed whereas the other 3 lambs were fed two-thirds of this amount.

At the end of the trial the lambs were slaughtered and ingesta quantities were determined as described in trial 1. Prior to slaughter, rumen fluid samples were collected and protozoal numbers were determined.

Results and discussion

The influence of level of feed intake upon rumen protozoal populations The results of these trials are shown in table 41. When lambs were given a full feed of a pelleted ration, protozoa disappeared from their rumens. In trial 1, full-fed lambs lost their rumen protozoa in about 14 days after resuming the full feed. Control lambs, which were fed about two-thirds

Table 41. The influence of level of feed intake upon rumen protozoal populations[a]

First phase[b] Level of intake	Protozoal count (no./ml.)	Second phase Level of intake	Protozoal count (no./ml.)
Trial 1			
Full feed	None	--	--
2/3 full feed	18.3×10^4	--	--
Trial 2			
Full feed	None	2/3 full feed	16.8×10^4
2/3 full feed	22.6×10^4	Full feed	None
Trial 3			
Full feed	None	2/3 full feed	11.2×10^4
2/3 full feed	12.8×10^4	Full feed	1.2×10^4
Trial 4			
Full feed	None	2/3 full feed	8.9×10^4
2/3 full feed	6.9×10^4	Full feed	None

[a]Pelleted ration was fed according to appetite to the full-fed treated lambs and two-thirds of that amount was fed to the lambs of the other treatment.

[b]The same lambs within each trial were used for both first and second phases (switch-back design); only the level of intake was changed. However, different lambs were used for each trial.

of the amount of the full-fed lambs, maintained a so-called normal population of rumen protozoa for the duration of the trial. It was evident from daily microscopic observations on rumen fluid from full-fed lambs that rumen protozoa had decreased in number after lambs had consumed the ration for 7 or 8 days. After 11 days one lamb had lost its protozoa, while all protozoa disappeared from the rumen of the other lamb at about 15 days. The rate of disappearance of the protozoa ap-

peared to follow ration level intake.

Full-fed lambs in trial 2 lost their rumen protozoa and subsequently were repopulated with rumen protozoa when the level of ration intake was reduced to two-thirds of the original intake. The results of this trial were not anticipated and therefore 2 additional trials of this study were conducted.

In trial 3, the full-fed lambs lost their rumen protozoa and subsequently regained them when ration intake was reduced. The two-thirds full-fed lambs maintained relatively high rumen protozoal numbers during the first phase but failed to lose their protozoa when full-fed. This was the only trial in which lambs did not lose their rumen protozoa when given a pelleted high-concentrate ration ad libitum. It was believed that this was due to a relatively low feed consumption. The lambs used in this trial were rejects from another experiment and had poor appetites.

The results of trial 4 were similar to those obtained in trial 2. These lambs appeared very healthy and consumed a high level of the pelleted ration. Daily microscopic observations of rumen fluid indicated that protozoa almost completely disappeared in 7 to 9 days after the trial began. This was also true for the second phase of this trial in which lambs lost their rumen protozoa within 10 days after being switched to a full feed.

The results of these trials indicated that the cause of

the disappearance of rumen protozoa was the increased rate of passage of ingesta due in turn to increased level of ration intake.

The influence of physical form of the ration upon rumen protozoal populations

Trial 1 The results of this trial are summarized in table 42. Lambs which were consuming either of the 2 rations in the mixed form retained relatively high numbers of rumen protozoa. However, lambs consuming the fine ground rations showed lower numbers of rumen protozoa than lambs consuming the coarse-ground rations. All lambs which consumed a pelleted ration lost their rumen protozoa except for those on a coarse-ground, alfalfa-corn ration. It is likely that particle size was larger for this ration than for the coarse-ground, cob-corn ration. When studying the data of table 42, it appeared that protozoal numbers decreased as ration particle size decreased. When these rations were fed in pelleted or finely ground forms, ad libitum, rumen protozoa were consistently absent.

There appears to be little relationship between the number of rumen protozoa and pH, daily gains and feed efficiency.

Trial 2 The results of this trial are summarized in table 43. Microscopic observations on rumen fluid indicated that protozoa were either present in low numbers or entirely absent. Lambs which consumed the basal long hay corn ration showed the highest number of protozoa with 8.1×10^4 per milli-

Table 42. Trial 1 - influence of physical form of the ration upon rumen protozoal populations[a]

Ration treatment[b]	Rumen pH	Protozoal count (no./ml.)	Daily gain	Feed per lb. gain
Mixed rations				
Alfalfa-corn				
Coarse grind	6.6	13.8×10^4	0.63	5.63
Fine grind	6.5	8.2×10^4	0.59	5.67
Cobs-corn				
Coarse grind	6.4	21.8×10^4	0.59	5.97
Fine grind	6.2	12.3×10^4	0.52	6.56
Pelleted rations				
Alfalfa-corn				
Coarse grind	6.5	3.1×10^4	0.57	5.16
Fine grind	6.6	None	0.66	4.86
Cobs-corn				
Coarse grind	6.4	None	0.54	5.68
Fine grind	6.4	None	0.50	6.13

[a]A difference (D) of 3.6×10^4 required for significance at P = .05 level for comparison of protozoal count treatment means.

[b]Values are based on the mean of 6 different lambs.

liter of rumen fluid. Lambs which consumed this mixed ration had 3.2×10^4 protozoa per milliliter of rumen fluid. Similar numbers of protozoa (3.5×10^4) were found in the rumen of lambs fed this ration in which the concentrate portion was pelleted and the roughage was coarsely ground and mixed with the pellets. Total disappearance of the rumen protozoa was observed when lambs were fed either the all pelleted ration or when the roughage portion was pelleted.

Table 43. Trial 2 - influence of the physical form of the ration upon rumen protozoal populations[a]

Ration treatment[b]	Rumen pH	Protozoal count (no./ml.)	Daily gain	Feed per lb. gain
Basal (long hay)	6.4	8.1×10^4	0.31	864
Basal (mixed)	6.1	3.2×10^4	0.34	776
Basal (pelleted)	5.6	None	0.47	563
Basal (concentrate portion pelleted)	6.2	3.5×10^4	0.39	679
Basal (roughage portion pelleted)	6.1	None	0.45	616

[a]A difference (D) of 2.9×10^4 required for significance at P = .05 level for comparison of protozoal count treatment means.

[b]All values are based on the mean of 3 different animals.

These findings indicate that ration particle size and level of feed intake influenced number of rumen protozoa. When particle size of the ration decreased, rumen protozoal numbers decreased. Visual examination of these rations indicated that these rations may be ranked in the following order, beginning with the rations with largest particle: (1) long hay-corn, (2) mixed, (3) concentrate portion pelleted, (4) roughage portion pelleted and (5) total ration pelleted. In general, protozoal numbers followed this same pattern.

It can be seen that rumen pH values were lower for lambs having larger rumen protozoa numbers. In fact, starting from the highest pH values, the rations can be ranked in parallel

according to their particle size. Admittedly, rumen pH may influence rumen protozoal viability.

The amount of ration consumed by the lambs in this experiment may have caused a decrease in protozoal numbers since the pelleted rations were consumed in higher amounts than the non-pelleted rations.

Trial 3 The results of this trial are presented in table 44. The control lambs fed the basal mixed ration maintained a so-called normal supply of rumen protozoa while all other ration treatments caused lambs to retain decidedly fewer, or none, of their protozoa. Lambs fed the basal mixed ration harbored 9.1×10^4 protozoa per milliliter of rumen fluid. The lambs fed the basal pelleted ration contained 2.3×10^4 rumen protozoa per milliliter of fluid. Lambs which consumed the high-concentrate mixed ration showed 4.2×10^4 protozoa per milliliter of rumen fluid. In contrast, lambs consuming this ration in pelleted form lost all their rumen protozoa.

Microscopic observations on rumen fluid from these lambs indicated that the shift in protozoal numbers took place primarily in the first 2 to 3 weeks of the trial.

Trial 4 The results of this trial are summarized in table 45. Determination of protozoal numbers on the first day of this trial indicated that all lambs contained relatively normal numbers of rumen protozoa. Observations also indicated that little change in protozoal numbers had taken place up to

Table 44. Trial 3 - influence of physical form of the ration upon rumen protozoal populations[a]

Ration treatment[b]	Protozoal count (no./ml.)	Daily gain	Feed per lb. gain
Basal mixed	9.1×10^4	0.29	10.39
Basal pelleted	2.3×10^4	0.34	9.72
High-concentrate mixed	4.2×10^4	0.33	8.83
High-concentrate pelleted	None	0.26	9.17

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for the protozoal count is 2.4×10^4 and for daily gain and feed efficiency are 0.15 and 32.5, respectively.

[b]All values are based on the mean of 6 different animals.

the end of the first week of the trial. Second week observations, however, showed that protozoal numbers had greatly decreased in the rumens of lambs on all treatments. Greatest decreases were found in lambs consuming the all-pelleted rations or rations with the roughage portion pelleted. Third week observations showed that protozoa were entirely absent from the rumen fluid of lambs fed the all-pelleted ration. Lambs consuming the ration in which the roughage portion was pelleted showed few rumen protozoa at this time and none at the end of the fourth week.

It can be seen from the data of table 45 that after the third week there was little change in protozoal numbers. It is of interest to note that the level of feed consumption was rel-

Table 45. Trial 4 - influence of physical form of ration and length of time fed upon rumen protozoal numbers[a]

Observation periods	Physical form of rations				
	Conventional	Mixed	Pelleted	Concentrate pelleted	Roughage pelleted
	(x1000)	(x1000)	(x1000)	(x1000)	(x1000)
1st day	14.7	11.5	9.7	12.0	12.5
1st week	17.9	16.3	7.2	11.5	13.5
2nd week	9.2	10.1	2.8	6.9	2.4
3rd week	12.4	5.4	None	5.6	1.3
4th week	10.6	6.4	None	5.2	None
5th week	8.7	5.2	None	4.6	None

[a]Protozoal numbers are based on the mean of 3 determinations.

atively stable during this time.

The results of several trials concerning the influence of physical form of rations upon rumen protozoal populations are summarized in table 46. Lambs which were fed either long hay or ground-and-mixed rations retained relatively high protozoal numbers. Lambs which were fed coarse ground or corn-portion pelleted rations retained intermediate numbers of rumen protozoa. Lambs which were fed rations finely ground retained relatively small numbers of rumen protozoa. In all trials where lambs were fed all pelleted, hay-portion pelleted or reground-pelleted rations, total disappearance of rumen protozoa was observed.

Table 46. Summary of the effects of physical form of ration upon rumen protozoal populations

Physical form of ration	No. of lambs	No. of observations	Protozoal count (no./ml.)
Basal (long hay-corn)	16	48	14.2×10^4
Basal (mixed)	59	140	12.1×10^4
Basal (corn portion pelleted)	3	12	7.5×10^4
Basal (hay portion pelleted)	3	12	None
Basal (coarse ground)	16	32	8.5×10^4
Basal (fine grind)	16	32	4.1×10^4
Basal (pelleted)	59	140	None
Basal (reground pellets)	3	12	None

As was evident from previously described trials, protozoa disappear when lambs consume a full feed of a pelleted high-concentrate ration. Consistent decreases in rumen protozoal numbers also resulted when ration particle size decreased or when ration intake increased. On the contrary, lambs which did not consume relatively high ration levels (2.5 to 3.0 percent of their body weight) had rumen protozoal numbers which were not similarly affected.

The influence of rate of passage of ingesta upon rumen protozoal populations It is evident, when studying the data from the previously described trials, that physical form and level of intake of rations influenced the numbers of rumen protozoa of fattening lambs. The specific factor or factors act-

ing upon rumen protozoa, however, is (are) not directly indicated. There are indirect indications that rate of passage of ingesta may be the primary causal factor for the reduction in number or disappearance of rumen protozoa. This idea is supported by the studies of Blaxter et al. (1956). They found that as the size of ration particles decreased and/or level of feed intake increased, the rate of passage of ingesta increased.

Trial 1 The results of this trial are given in table 47. They indicated that rate of passage of ingesta was greatest when lambs were fed a pelleted ration. When lambs were fed this ration in mixed form, the rate of passage index was 98 as compared to 100 for lambs fed the pelleted ration. The rate of passage index for lambs fed this ration with the hay in long form was 93. These values were not greatly different but supported the proposal that physical form of the ration influences rate of passage of ingesta. Protozoal numbers were 8.1×10^4 per milliliter of rumen fluid for the long hay fed lambs, 2.9×10^4 for mixed ration fed lambs and zero per milliliter for pelleted ration fed lambs.

Trial 2 The results of this trial are presented in table 47. In this trial, lambs that consumed two-thirds of a full feed gave a rate of passage index of 90 and protozoal numbers of 11.2×10^4 per milliliter of rumen fluid. In contrast, lambs which were full-fed lost their rumen protozoa and gave a rate of passage index of 100. These data indicated that

Table 47. The influence of rate of passage of ingesta upon rumen protozoal populations[a]

Ration treatments[b]	Rate of passage index	Protozoal count (no./ml.)
Trial 1		
Basal (long hay-corn)	93	8.1×10^4
Basal (mixed)	98	2.9×10^4
Basal (pelleted)	100	None
Trial 2		
2/3 full feed	90	11.2×10^4
Full feed	100	None

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for trials 1 and 2 are 13.7 and 10.8, respectively.

[b]All values are based on the mean response of 3 animals.

the level of feed intake influenced rate of passage of ingesta and subsequently influenced the number of rumen protozoa of fattening lambs.

Influence of Diethylstilbestrol Upon Rumen Protozoal Numbers and the Effect of Protozoa Upon Growth Performance of Lambs

It is well known that physical forms of rations influence digestibility and growth as well as certain rumen functions of cattle and sheep. It has also been shown that different feeding regimes alter the microbial populations of the rumen. Rumen microorganisms, particularly protozoa, are influenced by the administration of diethylstilbestrol. The rate of gain and feed efficiency of fattening lambs are influenced by rumen pro-

tozoa. The influences upon rumen protozoa by ration physical form and feeding regimes were described in the previous section. The influence of diethylstilbestrol upon rumen protozoa and the effects of rumen protozoa upon daily gains and feed efficiency of fattening lambs are reported in this section.

The Tukey test, as described by Snedecor (1959), was used to determine a difference required for significance at the .05 probability level for comparison of treatment means.

Methods and procedures

The influence of diethylstilbestrol upon rumen protozoal numbers Forty-eight wether lambs that averaged about 65 pounds were selected for this study. The lambs were randomly assigned to treatments. Six lambs were used per treatment. The percentage composition of the 2 different rations fed is given in table 48. The lambs were individually fed at 7:00 a.m. and 3:00 p.m. The alfalfa used in the mixed ration was ground in a hammer-mill using a one inch screen. The alfalfa used in the pelleted ration was ground using a one-quarter inch screen. The 2 rations were fed in 2 physical forms (pelleted and mixed). They contained 2 different proportions of roughages and concentrates.

Diethylstilbestrol (Stilbosol) was tested in both types of rations and in each physical form of the ration. The design of this experiment is shown in table 49. Stilbosol was added to the appropriate rations in amounts which supplied 4 milligrams

Table 48. Ration composition for the trial on the influence of diethylstilbestrol upon rumen protozoal populations

Ingredient	Ration percentage	
	Basal	High concentrate
Alfalfa hay	30.0	25.0
Corn (rolled)	34.0	60.0
Cobs (ground)	20.0	--
Soybean meal	8.0	6.0
Molasses	8.0	8.0
Salt	0.5	0.5
Quadrex	0.025	0.025
Stilbosol[a]		

[a]Stilbosol added to the appropriate rations so that 2.5 pounds daily consumption per lamb supplied 4 milligrams of diethylstilbestrol.

of diethylstilbestrol per 2.5 pounds of ration.

Individual rumen fluid samples were taken from all lambs at 4 and at 5 weeks after the beginning of the experiment. Rumen protozoal numbers were determined on these samples according to the method of Moir and Somers (1957) as modified by the author.

Individual lamb weights were determined at 2 week intervals throughout the experiment. Feed consumption data were also recorded. At the termination of the experiment, lamb weights were taken on 2 consecutive days and averaged for the final weight. Beginning weights were also averaged from 2 consecutive day weights.

The influence of rumen protozoa upon daily gains and feed efficiency of fattening lambs Forty-five wether lambs were used for this study. Three groups of 15 lambs each were used

to study the effects of defaunation upon lamb performance. Since defaunation involved starvation and copper sulfate treatment which may have influenced the results, this was prevented by starving and treating all lambs with copper sulfate.

After the lambs had been weighed (2 consecutive days) and randomly allotted to the treatments, they were starved for 5 days. On the sixth and seventh day of starvation all lambs were treated with 60 milliliter intraruminal doses of a 2 percent copper sulfate solution via stomach tube. About 6 hours after the second copper sulfate dose, the lambs were offered green leafy alfalfa hay. Their appetites were nearly lost, but within 3 to 4 days after the second copper sulfate dose they were eating well. This procedure previously had defaunated the rumen of lambs but failed to do so in this trial. The above described procedure was repeated in hopes of achieving complete defaunation. Success was achieved in the second attempt.

Since the 15 lambs of lot 1 were to serve as controls with faunated rumens, it was necessary to refaunate their rumens. Each of these 15 lambs was inoculated intraruminally with 75 milliliters of rumen liquid which was collected from a fistulated steer. The first inoculation was accomplished 7 days after the copper sulfate treatment and a second inoculation was performed 7 days later.

The ration fed to the lambs of this experiment was composed of alfalfa-grass hay, corn and soybean meal. It was formulated so that it supplied the following percentages when

3 pounds per lamb were consumed (hay, 47 percent; corn, 46 percent; and soybean meal, 5.5 percent). Iodized block salt was supplied free choice. The lambs were group fed in this experiment. The hay (long) was fed once daily at the rate of one and one-half pounds per lamb for the entire trial. The corn supplement mixture was fed twice daily according to appetite.

Copper toxicity was expected to develop in these lambs as a result of the intraruminal doses. This problem is described in the previous part of this thesis. To prevent copper toxicity, ammonium molybdate was mixed with the corn supplement portion of the ration so that 50 milligrams of molybdenum were supplied for each one and one-half pounds. Ammonium molybdate was fed for the first 30 days of the trial. When the lambs were consuming alfalfa only (for 3 days following copper sulfate treatments), 150 milligrams of molybdenum as ammonium molybdate were mixed with soybean meal and fed to prevent copper toxicity early in the trial.

Individual rumen fluid samples were collected at 2-week intervals throughout the trial and examined for presence of protozoa. Lots 2 and 3 were isolated from other ruminants as possible sources for protozoal contamination.

Individual lamb weights were taken at 2-week intervals throughout the experiment. Feed consumption data were also recorded. At the termination of the experiment, lambs weights were taken on 2 consecutive days and these weights were averaged for the final weights.

The influence of dietary copper sulfate upon rumen protozoa, daily gains and feed efficiency of fattening lambs

Forty-five wether lambs were used for this study. Three groups of 15 lambs each were used to study the effects of two levels of dietary copper sulfate upon rumen protozoa and performance of fattening lambs. Lots 2 and 3 (15 lambs each) were fed rations with 125 and 250 parts per million of copper, respectively. The ration fed to the lambs of this experiment was composed of alfalfa-grass hay, corn and soybean meal. This ration composition is described in the previous section. Lot 1 (15 lambs) served as control that received no dietary copper. The lambs were group-fed. The hay was fed once daily at the rate of one and one-half pounds per lamb for the duration of the trial. The corn supplement mixture was fed twice daily according to appetite. About 50 milligrams of molybdenum as ammonium molybdate was fed to each lamb daily to prevent copper toxicity.

Individual rumen fluid samples were collected at 4-week intervals throughout the trial and these were examined for the presence of protozoa. Individual lamb weights were taken at 2-week intervals for the entire experiment. Beginning lamb weights were based on the mean of weights taken on 2 separate days. Final lamb weights were based on the mean of 2-day weights. Feed consumption data were also recorded.

Results and discussion

The influence of diethylstilbestrol upon rumen protozoal numbers and performance of fattening lambs The results of this experiment are summarized in table 49. In preliminary

Table 49. The influence of diethylstilbestrol and physical form of ration upon rumen protozoal populations and performance of fattening lambs[a]

Ration treatment[b]	Protozoal count (no./ml.)	Daily gain	Feed per lb. gain
Basal (mixed)	9.1×10^4	0.29	10.39
Basal (pelleted)	2.3×10^4	0.34	9.72
Basal (mixed + DES)	14.3×10^4	0.42	7.88
Basal (pelleted + DES)	10.6×10^4	0.41	8.09
High concentrate (mixed)	4.2×10^4	0.33	8.83
High concentrate (pelleted)	None	0.26	9.17
High concentrate (mixed + DES)	6.3×10^4	0.46	6.30
High concentrate (pelleted + DES)	3.2×10^4	0.45	5.23

[a]Differences (D) required for significance at P = .05 level for comparison of treatment means for the protozoal count is 2.4×10^4 and for daily gain and feed efficiency are 0.15 and 32.5, respectively.

[b]All treatment values are based on the mean response of 6 lambs.

trials it was found that feeding diethylstilbestrol to lambs that were consuming pelleted high-concentrate rations prevented the disappearance of their rumen protozoa. This experiment was designed to further a study of this finding.

The data from this experiment indicated that protozoa disappear from the rumen of lambs fed pelleted high-concentrate rations. It was also evident that pelleting the basal ration caused a reduction in protozoal numbers. All comparisons where lambs consumed the high-concentrate ration as compared to the basal showed large reductions of rumen protozoal numbers.

Diethylstilbestrol (DES) caused an increase in rumen protozoal numbers on all rations studied. Diethylstilbestrol prevented the disappearance of rumen protozoa when lambs were fed a high-concentrate pelleted ration. Even though lambs fed the basal pelleted ration did not lose their rumen protozoa, diethylstilbestrol greatly increased protozoal numbers in lambs fed the basal ration. The comparisons are as follows: (1) basal, "9.1×10^4" compared to basal plus DES, "14.3×10^4"; (2) basal pelleted, "2.3×10^4" compared to basal pelleted plus DES, "10.6×10^4"; (3) high-concentrate mixed, "4.2×10^4" compared to high-concentrate mixed plus DES, "6.3×10^4"; and (4) high-concentrate pelleted, "none" compared to "3.2×10^4" for lambs fed the high-concentrate ration plus DES.

After sixty days of diethylstilbestrol consumption, a number of lambs exhibited difficulty in urinating. This condition exhibited symptoms similar to urinary calculi. Post mortem examination by the veterinary clinic revealed that urinary calculi were not present. It did, however, show that the male accessory sexual glands (particularly the prostate) were

hyper-vascularized. The excessive swelling caused blockage of the urethra.

Diethylstilbestrol was removed from the ration and within 2 to 3 days all urinating difficulties were absent. For the remainder of the trial, diethylstilbestrol was fed intermittently (7 days fed followed by 7 days not fed). No further urinary difficulties were encountered in the experiment. Diethylstilbestrol was removed from the ration 7 days prior to slaughter. Veterinary examination of secondary male sexual glands at time of slaughter revealed no abnormalities in diethylstilbestrol treated lambs.

Rate of gain and feed efficiency were improved by diethylstilbestrol. Daily gains were stimulated by about 30 percent in lambs fed the basal mixed ration and by about 25 percent when lambs were fed the basal pelleted ration. About 30 percent daily gain increase was obtained from supplementation with diethylstilbestrol in lambs fed the high-concentrate mixed ration. Lambs that consumed a pelleted high-concentrate ration showed a 45 percent increase in daily gains when fed diethylstilbestrol. Feed efficiency was improved by a greater magnitude than daily gains in all comparisons where diethylstilbestrol was fed.

The influence of rumen protozoa upon daily gains and feed efficiency of fattening lambs The results of this experiment are summarized in table 50. The data from this experiment

Table 50. The influence of rumen protozoa upon daily gains and feed efficiency of fattening lambs[a]

Treatment[b]	No. of lambs	Average daily gain	Last 14 day gain	Feed per lb. gain
Faunated lambs	15	0.56	0.64	4.21
Defaunated lambs	15	0.42	0.36	5.55
Defaunated lambs	15	0.40	0.36	5.72

[a]Difference (D) required for significance at P = .05 level for comparison of treatment means for daily gains is 0.20.

[b]All treatment values are based on the mean of 15 lambs.

indicate that rumen protozoa improved daily gains and feed efficiency of lambs.

For a period of 84 days, fully faunated lambs gained an average of 0.56 pound per lamb daily. "Defaunated lambs" (2 separate lots of 15 per lot) gained 0.42 and 0.40 pound per lamb daily. This is approximately a 28 percent increase in daily gains.

The defaunated lambs were treated somewhat differently than the faunated control lambs. The defaunated lambs were isolated from other ruminants and fed by a different person than the control lambs in order to maintain defaunation. It is not believed, however, that these differences in location and animal care influenced the results of this experiment.

Feed efficiency was also enhanced in the faunated lambs. The faunated lambs gained a pound of body weight for each 4.21

pounds of ration consumed. The defaunated lambs consumed 5.63 pounds of feed for each pound increase in weight. This was about a 34 percent increase in feed efficiency.

The influence of two levels of dietary copper sulfate upon rumen protozoa, daily gains and feed efficiency of fattening lambs The results of this experiment are presented in table 51. Microscopic observations indicate that 125 or 250 parts

Table 51. The influence of two levels of dietary copper sulfate upon daily gain and feed efficiency of fattening lambs

Treatment	No. of lambs	Average daily gain	Last 14 day gain	Feed per lb. gain
Basal	15	0.64	0.64	4.33
Basal + 125 ppm Cu	15	0.62	0.50	4.48
Basal + 250 ppm Cu	15	0.56	0.21	4.92

per million of copper had little or no effect on the numbers of rumen protozoa. All lambs of this experiment contained relatively high numbers of rumen protozoa.

For about 70 days of this experiment, copper sulfate had little or no effect upon rate of gain and feed efficiency. After 70 days, however, copper sulfate appeared to reduce rate of gain, particularly at the ration level of 250 parts per million. The control lambs gained 0.64 pound per lamb daily while lambs consuming rations with 125 and 250 parts per million gained 0.50 and 0.21, respectively, for the last 14 days

of the trial. The overall experimental results were, however, not greatly different. For 84 days gains ranged from 0.64 to 0.56 pound per lamb daily.

Feed efficiency corresponded directly with rate of gain. The basal group consumed 4.33 pounds of feed per pound gain, while lambs eating rations with 125 and 250 parts per million copper consumed 4.48 and 4.92, respectively.

Since ammonium molybdate was fed to these lambs as a copper toxicity preventative, it is of interest to speculate whether the copper was tied up by the molybdenum before it had time to exhibit lethal effects upon the rumen protozoa.

CONCLUSIONS AND SUMMARY

The data from these studies indicated that ration characteristics influence rumen protozoal populations. These characteristics also influence rate of gain and feed efficiency of lambs. Further, the data indicated that rumen protozoa influenced the rate of gain and feed efficiency of lambs.

When wether feeder lambs were fed a full feed of a pelleted ration, their rumen protozoa disappeared. When the level of this ration was at about two-thirds of a full feed, protozoa remained in the lamb's rumen in relatively high numbers. When lambs lost their rumen protozoa while consuming a full feed of a pelleted ration and subsequently were given two-thirds of a full feed, rumen protozoa reappeared.

Pelleted rations without DES fed to lambs resulted in the disappearance of rumen protozoa. As the particle size of the ration decreased, the number of rumen protozoa also decreased. Lambs fed all-mixed rations had fewer rumen protozoal numbers than lambs fed rations containing long hay. Lambs fed pelleted high roughage rations had fewer rumen protozoa than lambs fed an all-mixed ration. And finally, lambs fed a pelleted high-concentrate ration lost their protozoa after 2 to 3 weeks feeding. Lambs consuming reground pelleted rations, either high or low concentrate, also lost their rumen protozoa.

When studying the pattern of rumen protozoal disappearance or reduction in number, it was observed that the majority of

the changes took place 2 to 3 weeks after the ration regime was changed.

Determinations of rate of passage of ingesta indicated that lambs consuming a long hay and corn ration had a rate of passage index of 93 as compared to an index of 100 for lambs consuming a pelleted ration. Lambs fed this same ration in a mixed form had an index of 98. When the pelleted ration was fed at two-thirds of a full feed, the rate of passage index was 93. Rumen protozoal numbers were normal in lambs with indices of 90 and 93. Lambs with rate of passage indices of 98 contained only about 30 percent of a normal protozoal population, while those with indices of 100 lost all their rumen protozoa.

Diethylstilbestrol feeding at about 4 milligrams per lamb daily prevented total disappearance of rumen protozoa in lambs fed pelleted rations. Diethylstilbestrol also greatly aided in the maintenance or increase of ruman protozoal numbers in lambs fed 2 rations in 2 different physical forms. Daily gains and feed efficiency were greatly improved by diethylstilbestrol in finishing lambs.

Defaunation of lambs caused a reduction of about 25 percent in daily gains. Feed efficiency was reduced by more than 30 percent by the absence of rumen protozoa.

The data from these studies suggested that protozoa served a beneficial overall function in the rumen of finishing lambs. This benefit was the improvement of daily gains and feed effi-

ciency. Specific influences by rumen protozoa which may have contributed to improved lamb performance were: (1) increased total rumen fatty acids, (2) greater proportion of more efficiently utliizable fatty acids and (3) synthesis of proteins of superior nutritional value.

GENERAL DISCUSSION

It appears that one of the most significant findings of this research was the demonstration that rumen protozoa contribute to the overall nutrition of ruminants which serve as their host. This was demonstrated by more rapid liveweight gains and improved feed conversion in faunated as compared to defaunated lambs receiving a conventional finishing ration. Superior body development and feed conversion was also exhibited by faunated baby lambs as compared to baby lambs without fauna. The significance of this finding becomes more evident when it is realized that investigators since the early classical research of Becker (1929) have largely failed in demonstrating unique physiological influences of the large mass of protozoa known to be harbored within the digestive tract of ruminants.

Another significant finding of this research appears to be the demonstration that protozoa are capable of altering the amounts and kinds of acids produced by rumen fermentation. Faunated lambs as compared to protozoal-free lambs consistently produced more total rumen acids as evidenced by total fatty acid analysis and by lower rumen pH under comparable feeding conditions. The acetate-propionate as well as the acetate-butyrate ratios were narrower in faunated as compared to protozoa-free lambs. The lower pH and greater amounts of total acids, particularly propionate, were in part due to a greater

production of lactic acid. Johns (1951a and 1952) showed that Veillonella gazogenes and Clostridium propionicum readily ferment lactic acid which resulted in the formation of propionic acid. It is reasonable to assume that greater amounts of propionate and butyrate should improve growth performance of ruminants. Propionate and butyrate are universally considered to yield greater amounts of net energy, relatively, than acetate. The heat increment of acetate exceeds that of propionate or butyrate, particularly in fattening ruminants.

However, the significance of this altered rumen acid production by protozoa may be beyond the scope of this research. The possibility exists, none the less, that this altered rumen acid production may be the physiological basis whereby protozoa contribute to the nutrition of ruminants. Most previous research has attempted without success to ascribe a beneficial influence of protozoa to increased digestion within the alimentary tract of lambs or other ruminants.

A further significant finding of this research appears to be the evidence supporting the theory that feeding conditions influence the maintenance of sizable rumen protozoal populations by favoring or inhibiting protozoal life-cycle multiplication. One of the demonstrated favorable feeding conditions in this respect was the use of coarse textured feeds, such as long hay, which slow down the rate of passage of ingesta of the rumen, thus allowing protozoa more time for multiplication by

cell division. Pelleted high-concentrate rations pass through the rumen more rapidly which discourages protozoal proliferation. Another demonstrated favorable feeding condition was the including of diethylstilbestrol in the ration which presumably enhanced protozoal populations through their reproductive processes. Still another feeding condition favoring protozoal proliferation was a reduced level of feeding.

The significance of this proposed theory appears to lie in the newly proposed approach to a fuller understanding of conditions favorable to maximum protozoal development and the benefits which thereby contribute to the ruminant host. Previously, very little information has been available concerning influencial rumen factors favoring protozoal development.

Two research techniques for the study of rumen protozoa were developed during the course of this research which may prove particularly useful in furthering knowledge in this area of research. One technique developed which is believed suitable for extensive future research with rumen protozoa is the in vitro technique for studying fatty acid production. The metabolism of rumen protozoa in the absence of bacteria can be studied in detail in the laboratory. This technique appears to be valuable in studying mechanisms and influences of various factors upon protozoa with respect to the end product acids produced which are beneficial to the ruminant host. Most previous studies of this type have been confounded by fermenta-

tions resulting from mixed bacterial-protozoal cultures.

Other techniques developed in this research with rumen protozoa were the obtaining of protozoal-free lambs for subsequent research. First, a modification of Beckers (1929) method of defaunation which employed a prestarvation period followed by the administration of intraruminal copper sulfate and dietary molybdenum. This method, although not completely satisfactory, is effective in freeing lambs of their rumen protozoa. A second technique involved the rearing of new-born lambs isolated from all possible sources of rumen protozoa. This method proved successful in obtaining protozoa-free lambs but has the disadvantage of a time period which is required for lambs to reach a usable age.

Before concluding this discussion, one additional observation was made in this research which is believed to have considerable significance but which was outside the scope of the original objectives. This observation was the unusually good growth and feed efficiency performance of lambs reared from birth by hand feeding in isolated conditions. These lambs not only made excellent liveweight gains but their feed required (about 3 pounds) per pound gain was less than half of that required under practical feedlot conditions. Their efficiency was rather similar to good feed efficiencies achieved by young non-ruminant species of farm animals. The only known recorded efficiency of feed utilization in ruminants which approached

this lamb performance was that obtained by Phillips (1961) with 2 bull calves fed a roughage-free diet from birth to about 950 pounds liveweight.

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APPENDIX

A. Collection and Steam Distillation of Ruminal VFA's

1. Withdraw 20 ml. of rumen fluid using a suction strainer coupled to a 50 ml. syringe.

2. Add 1 ml. of saturated $HgCl_2$ to kill the microorganisms to prevent any alteration of *in vivo* ruminal VFA ratios.

3. Centrifuge raw rumen juice in a high speed angle centrifuge for 40' to remove microorganisms and solid feed particles.

4. Decant or pipette off *clear* amber colored supernatant.

5. Place 5 ml. of this centrifuged rumen juice in a 100 ml. beaker.

6. Add 5 ml. of H_2O.

7. Add 10 ml. of McAnally reagent; i.e. solution saturated with $MgSO_4$ and containing $2\frac{1}{2}\%$ H_2SO_4.

8. Filter through Whatman #40 into a 50 ml. volumetric.

9. Adjust pH to 2.3 with 2N NaOH and 10N H_2SO_4 using bromphenol blue indicator; then dilute to volume (50 ml.).

10. Place 10-20 ml. aliquot (i.e. VFA's from 1-2 ml. of rumen juice) into Markham steam distillation apparatus.

11. Collect 150 ml. of distillate.

12. Using phenol red as indicator, titrate with N/10 KOH while maintaining CO_2 free atmosphere (CO_2 free air bubbled through distillate). Use a 5 ml. buret, preferably one modified as an alkali and a filling buret in order that the buret need not be cleaned after each day's use and in order to facilitate easy filling.

13. After end point is reached, add 10% extra KOH.

14. Place aqueous potassium salts in 100°C oven, take to dryness of near dryness.

15. If necessary, dissolve in H_2O. Transfer to 5 dram snap cap opticlear vials or any other type of wide mouth vial of similar size. Take to dryness in 100°C drying oven. Store in dessicator. Do not store potassium salts of VFA's for extended length of time in 100°C oven.

CAUTION: It is imperative that an acid free atmosphere be maintained in the drying oven. If any acid is present (for example acetic acid), the extra base present in the dried sample will absorb the acid, resulting in completely distorted VFA ratios. Thus to insure complete protection against contamination by extraneous acids present in the oven atmosphere, it is advisable to place only titrated VFA samples in a particular oven.

B. Gas Phase Chromatographic Technique

1. Instrument

   a. Aerograph 110 C connected to a Brown-Honeywell 1 mv recorder.
   b. Column: stainless steel, 10 foot, $\frac{1}{4}$ inch diameter packed with silicone stearate (non-polar).

2. Materials

   a. 3.1 mm. x 11.5 cm. pyrex capillary tubes; 120° bend 1 1/8" from end.
   b. High boiling point oil
   c. $C_2H_5SO_4K$
   d. Filling funnels (medicine dropper with a rubber tube splicing unit).

3. Preparation of $C_2H_5SO_4K$

   a. Mix 27.1 cc. of absolute alcohol with 25.0 cc. of reagent grade H_2SO_4. Maintain at 20°C during addition of acid to the alcohol. Allow reaction to proceed for 3 hours at room temperature.
   b. At end of three hour period, neutralize the reaction mixture with an aqueous suspension of $CaCO_3$ (35-40 gm. of $CaCO_3$ required). As the equiligrium in the reaction of C_2H_5OH and H_2SO_4 is very far to the right, no danger exists in

adding the aqueous $CaCO_3$. It must be added in the aqueous form in order that the precipitated $CaSO_4$ may be filtered out. Continue adding the aqueous $CaCO_3$ until effervescence discontinues. Filter out the $CaSO_4$ and wash with H_2O.

c. Next add slowly a K_2CO_3 solution until precipitation of $CaCO_3$ is complete. Digestion of the reaction mixture will aid in the precipitation of the $CaCO_3$ and will facilitate detection of the end point. Filter off the $CaCO_3$. Evaporate the filtrate ($C_2H_5SO_4K$ solution) to dryness. A yield of approximately 25 gm. should be obtained.

4. Preparation of Samples

CAUTION: With exception of weighing the $C_2H_5SO_4K$, perform all scraping, mixing and capillary tube filling in front of the oven as the potassium salts of the VFA's are extremely hygroscopic. Wearing of cotton gloves will facilitate handling of hot vials, spatulas, funnel and capillary tubes which must be kept in the oven to insure their complete dryness.

a. Place dried samples (K salts of VFA's) in 100°C drying oven.
b. Using a spatula, scrape the dried salts away from the botton of the container. Place scraped sample back in oven.
c. Using the titration data and assuming an average molecular weight of 110 for the potassium salts of the VFA's, add an <u>equal weight</u> of $C_2H_5SO_4K$ to the potassium salts.
d. Thoroughly mix the potassium salts and $C_2H_5SO_4K$ with a small spatula. Place mixed sample back in oven.
e. While standing in front of oven, connect filling funnel and capillary tube. Hold this unit and the wide mouthed vial in one hand and then place approximately 10 mg. of the reaction mixture in the capillary tube. It is not necessary to accurately weigh out a prescribed amount. A visual gauging of the amount in the capillary tube is sufficiently accurate. Gently tap the capillary tube to vibrate the mixture to the bottom of the tube. If filling is done rapidly and if the reaction mixture was perfectly dry prior to filling, very little of the reaction mixture will cling to the sides of the capillary tube. Store capillary tube in boen until ready to be inserted

in injection port of gas phase chromatograph. Mixed samples should be analyzed a few hours after initial mixing of the salts and $C_2H_5SO_4K$.

f. Do not store $C_2H_5SO_4K$in 100°C drying oven. Extended storage at this temperature degrades the $C_2H_5SO_4K$. Store in a closed container or dessicator. Any moisture present in the $C_2H_5SO_4K$ will be removed when the mixture of potassium salts of VFA's and $C_2H_5SO_4K$ are placed back in the oven after mixing.

5. Qualitative analysis in gas phase chromatograph

   a. Operating conditions
      1) Oven temperature - 85-90°C
      2) Gas flow (helium) - 35-40 ml. per minute
      3) Injector and collector temperatures - 210-225°C
      4) Filament - 250 ma.
      5) Sensitivity - 64X
   b. Place a silicone collector gasket in the injection port compression nut.
   c. Allow machine to stabilize and sufficiently warm up (several hours) with an empty capillary tube inserted into the injection port and with the compression nut tightened only tight enough to prevent gas leakage.
   d. When the machine has stabilized, remove charged capillary tube from oven, cover end with dry tissue, loosen injector compression nut slightly, remove old capillary tube and immediately insert new one. Retighten compression nut.
   e. As some air is introduced into the chromatograph, allow 5-10 minutes for the machine to restabilize. In our case, this is indicated by the recording needle being on the base line without the benefit of any manual fine adjustment.
   f. Heat a pyrex tube containing Dow-Corning 550, or any other high boiling point oil, til the oil reaches 325°C. Amount of oil in the tube should be adjusted so that the tube is nearly full at 325°C.
   g. Through use of this pyrex tube containing the 325°C oil, immerse the capillary tube as completely as possible in the oil. The following reaction takes place spontaneously 6-8 sec. after heating is commenced:

$$\underset{O}{RCOK} - C_2H_5SO_4K \quad 325^{\circ}C \quad K_2SO_4 - \underset{O}{RCOC_2H_5}$$

As the temperature of the oil and the injection port is far above the boiling point of these ethyl esters, they are volatilized into the chromatograph while K_2SO_4 is left residually deposited in the capillary tube. Continue immersion of the capillary tube for 30 sec., then remove the tube of hot oil.

h. After 25-30', all VFA's through n-valeric will have passed through the column.

i. The chromatograph is now ready for recharging with a new sample.

CAUTION: It is absolutely mandatory that the capillary tube and contained reaction mixture be completely free of any traces of H_2O to minimize trailing of peaks and to achieve optimal separation and sharpness of peak patterns. Thus, the foregoing procedures and precautions must be observed in order to achieve this end.

j. As the area under each peak is proportional to the molar amount of each acid, the relative ratios of the VFA's can therefore be obtained. Through reference to the total VFA level determined by titration, the absolute amount of each acid can be ascertained if desired.

If each peak reaches the base line before the next peak commences, the disc integrator recording will give directly the area under each peak. If not, some extrapolation will have to be performed and the adjusted peak area for each component may be measured through use of a planimeter.

C. Determination of Ammonium Nitrogen in Ruminal Ingesta

1. Transfer 5 ml. of rumen fluid and 20 ml. of distilled H_2O into a 100 ml. round bottom flask.
2. Approximately 10 ml. of borate buffer is added to adjust pH to 9.2.
3. Flask is immersed in a water bath (50-55°C) and connected to a water cooled condenser to which is attached a 125 ml. Erlenmeyer flask containing 25 ml. of a 2 percent solution of boric acid.
4. The system is closed and the water aspirator is turned on.
5. A small stream of air is drawn through the sample to prevent excess bumping.

6. Distillation should be complete in 10 minutes.
7. Add 1 drop of indicator and titrate the ammonia in boric acid with a standard solution of HCl.

Table 52. Trial 1 - analysis of variance of the influence of pH upon *in vitro* fatty acid production by rumen protozoa

Source of variation	Degrees of freedom	Mean square
Total	23	
Treatment	7	1.432
Error	16	0.013

Table 53. Trial 1 - analysis of variance of the influence of substrate and protozoal concentration upon *in vitro* fatty acid production

Source of variation	Degrees of freedom	Mean square
Total	47	
Treatment		
A(protozoal concentrations)	3	1.211
B(substrate level)	3	0.597
A x B	9	0.034
Replication	2	0.004
Error	30	0.034

Table 54. Trial 1 - analysis of variance of the influence of sodium upon *in vitro* fatty acid production by rumen protozoa

Source of variation	Degrees of freedom	Mean square
Total	47	
Treatment		
A(NaCl levels)	3	4.091
B(Na acetate levels)	3	5.323
A x B	9	0.000
Replication	2	0.175
Error	30	0.036

Table 55. Analysis of variance of the influence of sodium and potassium upon *in vitro* fatty acid production by rumen protozoa

Source of variation	Degrees of freedom	Mean square
Total	74	
Treatment		
A(NaCl levels)	4	1.080
B(KCl levels)	4	28.921
A x B	16	1.408
Replication	2	0.055
Error	48	0.043

Table 56. Trial 1 - analysis of variance of the influence of potassium upon *in vitro* fatty acid production by rumen protozoa

Source of variation	Degrees of freedom	Mean square
Total	27	
Treatment		
A(KCl levels)	2	0.055
B(K acetate levels)	2	6.645
A x B	4	4.872
Replication	2	0.110
Error	17	0.041

Table 57. Trial 1 - analysis of variance of the influence of phosphorus upon *in vitro* fatty acid production by rumen protozoa

Source of variation	Degrees of freedom	Mean square
Total	47	
Treatment		
A(Na_2HPO_4 levels)	3	1.135
B(KH_2PO_4 levels)	3	2.475
A x B	9	2.133
Replication	2	0.074
Error	30	0.032

Table 58. Trials 1, 2 and 3 - analysis of variance of the influence of rumen protozoa upon the production of volatile fatty acids

Source of variation	Degrees of freedom	Mean squares	
		Total VFA/ml.	C_2/C_3
Treatment	2	7695	1.78
Lambs/treatment (Error A)	9	43	0.114
Trials	2	309	12.28
Trials x treatment	4	194	1.23
Trial x lambs/treatment (Error B)	18	58	0.121

Table 59. Trials 1, 2, 3, 4, 5 and 6 - analysis of variance of the influence of rumen protozoa upon ammonia production

Source of variation	Degrees of freedom	Mean square
Treatment	2	1246
Lambs/treatment (Error A)	9	3.67
Trials	5	251
Trials x treatment	10	35.80
Trials x lambs/treatment (Error B)	45	4.64

Table 60. Analysis of variance of the influence of physical form and level of intake of rations upon rate of passage of ingesta

Source of variation	Degrees of freedom	Mean square
Trial 1		
Total	8	34
Treatments	2	39
Lambs/treatment	6	33
Trial 2		
Total	5	32
Treatments	1	36
Lambs/treatment	4	28

Table 61. Analysis of variance of the influence of DES and physical form of ration upon rumen protozoal populations

Source of variation	Degrees of freedom	Mean square
Total	41	0.189
Treatments	6	1.230
Lambs/treatment	35	0.104

Table 62. Analysis of variance of the influence of DES and physical form of ration upon daily gains and feed efficiency of lambs

Source of variation	Degrees of freedom	Mean squares	
		Daily gains	Feed conversion
Total	47	0.107	5510
Treatments	7	0.339	1918
Lambs/treatment	40	0.066	312

Table 63. Analysis of variance of the influence of rumen protozoa upon daily gains of lambs

Source of variation	Degrees of freedom	Mean square
Total	44	0.102
Treatments	2	1.140
Lambs/treatment	42	0.053