UNIDENTIFIED FACTORS STIMULATORY TO CELLULOSE DIGESTION BY RUMEN MICROORGANISMS

by

Orbie Glen Hall

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Animal Nutrition

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College

1955

TABLE OF CONTENTS

INTRODUCTION

The recent concept that the first step in ruminant nutrition is microbial nutrition has prompted numerous investigations during the past 10 to 15 years concerning the factors which enhance rumen microbial activity. Such studies are particularly important from the standpoint of roughage utilization by ruminants. Ruminants can utilize large quantities of high cellulosic materials only because of extensive microbial action in the rumen. These microorganisms secrete cellulolytic enzymes which in turn act on roughage materials converting them to products usable by the animal.

More complete knowledge of the factors which influence the cellulolytic activity of rumen microorganisms would be of interest from the academic point of view and would in all probability be of great practical importance. More extensive and efficient utilization of the vast quantities of roughages (particularly low quality roughage) is certainly one way of producing beef and lamb more economically. The efficient utilization of such materials is in turn dependent upon maximum rumen microbial activity.

Studies with rumen microorganisms <u>in vitro</u> have demonstrated that certain nutrients are required for maximum

cellulolytic activity. In addition to specific chemically defined nutrients, factors, as yet chemically unidentified, have been shown to exert a marked stimulatory action on microbial cellulose digestion. These unidentified stimulatory factors (cellulolytic factors) have been shown to occur in highest concentration in materials rich in either protein or in non-protein nitrogen. Furthermore, many of the materials exhibiting rumen microbial stimulatory activity contain an abundance of the B-complex vitamins or specific carbohydrates which can serve as readily available energy for rumen microorganisms.

In addition to the chemical identification of these stimulatory factors, another area in which information is lacking is their general effects upon the performance of the animal. In this regard, the development of a potent source of the factors which would be available in large quantities is needed. If such materials do have a beneficial influence upon the utilization of roughages by ruminants, a source of them which could be produced economically and in large quantities would be particularly valuable.

This study was initiated to obtain more information concerning unidentified factors stimulatory to cellulose digestion by rumen microorganisms. In order to facilitate such studies, a more sensitive and convenient laboratory

assay technique than those currently used was needed. One phase of this study consisted of the development of such a technique as well as a synthetic medium containing all the chemically defined nutrients known to be required by rumen microorganisms. Equally important phases of the study included (1) determining if known B-vitamins or certain carbohydrates were the active unidentified stimulatory factors, (2) the development of a potent source of the factors which might be produced economically and in large quantities, (3) determining the significance of the association of unidentified cellulolytic stimulatory factors with materials rich in either protein or non-protein nitrogen, and (4) determining the influence of certain sources of these cellulolytic factors upon body weight gains and feed consumption by lambs. It was hoped that such studies would contribute information which would eventually lead to greater and more efficient utilization of low quality roughages by cattle and sheep.

REVIEW OF LITERATURE

Nutrient Requirements of Rumen Microorganisms

Three classes of chemically defined nutrients have been shown to be required by rumen microorganisms for maximum activity. These include (1) a nitrogen source--urea, ammonium salts, proteins, etc., (2) minerals--major and minor, and (3) a source of readily available energy--primarily soluble carbohydrates. Much of the information concerning the nutrient influences upon rumen microbial activity has been obtained indirectly by observing the performance of cattle and sheep on different feeding regimes. Additional information has been obtained more directly by studying the nutrient requirements of rumen microorganisms in vitro.

The importance of protein or nitrogen to cellulose digestion by rumen microorganisms was first noted in studies in which the feeding value of corncobs was being investigated. Burroughs and Gerlaugh (15) obtained 14 and 17 per cent increases in the dry matter digestibility of corncobs and timothy hay, respectively, when soybean oil meal was added to fattening rations containing these roughage materials. It was suggested that factors in soybean oil meal in addition to protein might have contributed to its favorable

influence. In further work by Burroughs et al. (17), the effects of skim milk additions upon roughage utilization were studied. Protein levels of rations containing corncobs or clover hay were varied by substituting dried skim milk for mineralized starch. Improvements in digestion of corncobs and clover hay were obtained when skim milk was added to rations containing some starch. Skim milk additions to rations without starch resulted in little improvement in roughage digestion. Similar results were obtained in another experiment by the Ohio group (14) when casein was added to roughage rations containing starch. In this study, roughage digestion was shown to be very good when a 3.4 per cent crude protein ration containing no starch was fed to cattle. These data indicated that the protein requirement of microorganisms for maximum roughage digestion is lower than the protein requirement for body needs. It was also apparent that inclusion of starch in cattle rations increased the need for protein or nitrogen by cellulose digesting bacteria.

An artificial rumen technique was used by Burroughs et al. (20) to determine the influence of various protein-rich feeds and cereal grains upon cellulose digestion by rumen microorganisms. Their results showed that many feeds influenced cellulose digestion by rumen microorganisms in

vitro. Additions of distillers' dried solubles, soybean oil meal, or linseed oil meal increased cellulose digestion markedly, while various cereal grains and animal by-products feeds had little effect. In another series of *in vitro* experiments by Burroughs *et al.* (18), cellulose digestion in high and low quality roughages was studied. Nitrogen and minerals improved the breakdown of cellulose in the poor quality roughages, while these nutrients had very little effect upon cellulose digestion when the roughage used was of high quality. The authors suggested, in this report, that rumen microorganisms require three classes of nutrients. These include (1) energy, (2) nitrogen, and (3) minerals.

Clark and Quinn (22) supplemented poor quality grass hay with urea and molasses in wintering rations for sheep. Animals receiving these supplementations maintained their weight better and consumed more hay than sheep fed only poor quality grass hay. More total cellulose was digested in the supplemented group but the percentage of cellulose digested was not affected.

An extensive *in vitro* study was conducted by Belasco (4) to evaluate various non-protein nitrogen compounds as sources of nitrogen for rumen bacteria. Cellulose digestion, release of ammonia, and bacterial growth were used as criteria to determine the effectiveness of the compounds as

nitrogen sources in these experiments. The addition of either urea or various ammonium salts to the fermentation flasks resulted in cellulose digestion coefficients of 75-80 per cent as compared to only 25 per cent for flasks without an added nitrogen source. Certain urea derivatives were found to be very poor sources of nitrogen for bacteria. The cellulolytic response from such compounds as formamide, acetamide, propionamide and n-butyramide was generally less than that observed with urea. Glycine was ineffective as a nitrogen source for bacteria, whereas the amide of glycine was very effective. The nitrogen from various organic and inorganic ammonium salts was highly available. Ammonium succinate and lactate were especially good nitrogen sources. The author postulated that the organic fragments of these nitrogen salts might have stimulated rumen microbial activity in some fashion. Various amidines such as creatine, creatinine, and salts of guanidine proved to be effective nitrogen sources. Purines such as uric acid and allantoin were utilized by rumen bacteria as nitrogen sources. The importance of nitrogen to rumen microorganisms was amply demonstrated in these studies. It was also shown that a wide variety of non-protein nitrogen compounds were very effective nitrogen sources for rumen bacteria.

In regard to the nitrogen requirement of rumen bacteria, both Burroughs et al. (12) and McDonald (48) suggested that ammonia is the primary intermediate and that the nitrogen requirement does not involve the more complex forms of nitrogen such as amino acids or protein per se. The stimulatory effects upon rumen microorganisms by certain complex protein materials is explained partially on the basis of accompanying nutrients such as minerals and energy yielding carbohydrates.

The stimulatory effect of soluble carbohydrates upon rumen microbial activity has been observed by a number of workers. The earlier studies with carbohydrates were conducted to determine the influence of such materials upon non-protein nitrogen utilization by rumen microorganisms. In an experiment using one fistulated cow, Mills et al. (53) found that the addition of starch to a timothy hay-urea ration improved the utilization of urea markedly. The protein content of the rumen was increased by 57 per cent when starch was included in the diet. The authors stated that starch served as a readily available energy source for the microorganisms enabling them to build new protoplasm in which the nitrogen from the urea was incorporated. The Australian worker, Pierce (59), observed that the addition of potato starch to a ration low in protein, but containing

urea, improved the utilization of the urea as evidenced by the increased wool production of Merino sheep.

The *in vitro* experiments by Pearson and Smith (55) and McNaught (49) showed that a readily available source of energy is essential for the conversion of non-protein nitrogen to bacterial protein. Starch, dextrin, maltose, glucose, lactose, L(+)-arabinose, D(+)-xylose, D(-)-fructose, raffinose, inulin, cellobiose, and D(+)-mannose were shown to increase the conversion of non-protein nitrogen to protein nitrogen.

Using the breaking strength of cotton string which had been incubated in ruminal ingesta as a criterion of rumen microbial activity, Hoflund *et al.* (34) found that small amounts of sugar increased cellulose digestion and appetite in sheep. Excessive amounts of sugar depressed cellulose digestion.

Arias *et al.* (2) studied the influence of six sources of energy upon rumen microorganisms in the artificial rumen. The substances studied were dextrose, cane molasses, sucrose, starch, cellulose, and ground corncobs. Cellulose digestion and urea utilization were used as indices of microbial activity. It was observed that small additions of each of the energy sources increased urea utilization. High levels of the readily available carbohydrates decreased cellulose

digestion. These workers suggested that some readily available energy is needed during the time cellulose is being broken down to a form utilizable by the bacteria. They also postulated that if too much soluble carbohydrate is supplied, the energy requirement of the bacteria will be met and they no longer need to break down cellulose for energy.

The synthesis of riboflavin, niacin, and pantothenic acid by rumen bacteria was stimulated by the presence of starch in the medium as shown in the experiments conducted by Hunt *et al.* (40). The synthesis of vitamin B_{12} was not significantly increased by the starch. Urea utilization was increased but cellulose digestion was depressed in the presence of starch.

The mineral requirement of ruminants (particularly for trace minerals) is generally assumed to be greater than for monogastric animals because of additional mineral needs for bacterial activity in the rumen. Using the diminution of non-protein nitrogen in bovine rumen liquid when incubated *in vitro* as an index of bacterial growth, McNaught *et al.* (50) reported that iron stimulated the utilization of non-protein nitrogen by rumen bacteria. Toxic levels of iron, copper, cobalt, and molybdenum were reported as being 1000, 25, 1000, and 2000 parts per million, respectively.

Protein synthesis was very poor when the iron content of the medium was less than 1 part per million. A level of 1-2 parts per million of iron supported good bacterial growth. The use of chelating agents as demonstrated in this work appears to be one possible method of determining some of the quantitative mineral requirements of rumen bacteria.

Burroughs et al. (19) found that molasses ash, phosphorus, and iron stimulated cellulose digestion by rumen microorganisms in vitro. These workers suggested that elements other than iron and phosphorus were involved in microbial physiology in addition to sodium, potassium, calcium, magnesium, chlorine, and sulfur. The latter elements were routinely used in their artificial rumen studies, but an actual requirement for them by rumen bacteria was not established in their studies. Gall et al. (29) reported a marked difference in types and a reduction in the number of rumen bacteria in cobalt deficient sheep. Various forms of sulfur, i.e., disodium sulfate, methionine, and cystine stimulated cellulose digestion, urea utilization, and synthesis of riboflavin and vitamin B_{12} in the artificial rumen (Hunt et al., 40).

Occurrence of Unidentified Factors Stimulatory to Rumen Microbial Activity

In addition to minerals, soluble carbohydrates, and a nitrogen source, many natural substances are known to stimulate the activity of rumen microorganisms. Attempts to explain the stimulatory properties of such substances on the basis of known chemical composition have not been entirely successful.

Rumen liquid has been shown to be one of the best sources of these unidentified stimulatory factors. Hungate (39) and Bryant (10) used rumen liquid in their media for culturing pure strains of rumen bacteria. Growth of cellulolytic rumen bacteria was speeded up when rumen liquid was present in the culture medium; some strains were found to require it. Doetsch *et al.* (26) suggested that rumen liquid contains unknown substances essential for bacterial growth not included in commercial media. Burroughs *et al.* (13) obtained large increases in cellulose digestion in the artificial rumen by adding autoclaved rumen liquid. This material was found to restore the cellulose-digesting ability of rumen bacteria after they had lost it due to prolonged incubation *in vitro*. Cellulose digestion by washed rumen microorganisms was increased 25-30 per cent by rumen liquid

in experiments reported by Garner *et al.* (31). In addition to stimulating cellulose digestion, rumen liquid was also found to inhibit the growth of undesirable microorganisms sometimes found in the rumen.

Other natural substances have been reported to enhance cellulose digestion by rumen microorganisms *in vitro*. Numerous studies (37, 63, 60, 61, 51, 5) have shown yeast extract to be an exceptionally good source of these unidentified cellulolytic stimulatory factors. Water extracts of manure were found to increase cellulose digestion in low quality roughages in the artificial rumen (18). Cellulose digestion in high quality roughages was not affected by the manure extract supplementation. On the basis of these results it was concluded that these unidentified cellulolytic nutrients were present in ample quantities in certain high quality roughages and were present in inadequate amounts in certain low quality roughages.

That some high quality roughages contain factors which stimulate cellulose digestion, was shown in subsequent experiments by Burroughs *et al.* (19), Ruf (61), and Bentley *et al.* (5). Water extracts of alfalfa leaf meal, fresh-cut ladino clover, and good timothy hay stimulated cellulose digestion by rumen microorganisms in the artificial rumen.

An extensive study of these unidentified factors and their occurrence was made by Ruf (61). He obtained marked increases in cellulose digestion from soybean oil meal, linseed oil meal, cottonseed oil meal, and distillers' dried solubles in addition to rumen liquid, yeast extract, and various other complex materials. The effects of the different sources of these factors were found not to be additive. This indicates that the stimulatory properties of these substances may be due to common factors or nutrients.

On the basis of these studies it is apparent that the distribution of these unidentified stimulatory factors is widespread. Protein-rich feeds and other materials rich in non-protein nitrogen appear to be very good sources of them. Rumen liquid, yeast extract, and various forage extracts are perhaps the most potent sources of those unidentified stimulatory factors.

Some Chemical and Physical Properties of Unidentified Cellulolytic Stimulatory Factors

A few studies have been conducted in which attempts have been made to ascertain the chemical identity of unidentified cellulolytic factors. One of the more extensive studies of this subject was conducted by Ruf *et al.* (62).

Manure extracts, yeast extract, and soybean oil meal were fractionated in an attempt to isolate the active principles in these materials. The factors were not isolated but some of their properties were determined. The stimulatory factors were found to be heat stable, water soluble, and soluble in dilute ethanol. They were absorbed on Norite and were eluted with acetone and ethanol. However, the eluates were not as active as the original material. Since these factors were not absorbed on ion-exchange resins and ashing was found to destroy them, it was concluded that the factors were organic rather than mineral. Precipitation of the proteins in water extracts of manure and yeast with hydrochloric acid or alkaline-zinc sulfate did not remove the active principles indicating they were non-protein.

Garner et al. (30) reported that the stimulatory factors in rumen liquid were stable to autoclaving at a pH between 7 and 10. Other studies (51) showed that prolonged heating of rumen liquid at a pH of 2.0 or 11.0 decreased its stimulatory properties. Rumen liquid treated in this fashion supported the growth of only a few types of rumen bacteria. The factors in rumen liquid were found to have some affinity for Norite and were eluted with ethanol, thus, confirming the results of the previous study. The eluates were

less active than the original starting material as was found by other workers.

Bentley et al. (5) concentrated an active fraction from rumen liquid by lead acetate precipitation. The affinity of these factors for Norite at a pH of 5, or above, and their subsequent elution with ethanol were further confirmed in these studies. More recently, Bentley et al. (6) reported that the active factors were obtained from acidified rumen liquid by steam distillation. Steam distillates of alkaline rumen liquid were found to be inactive. This indicated that the active principles were volatile fatty acids. Subsequent tests with fatty acids in the artificial rumen showed that valeric acid was just as active in stimulating cellulose digestion as was the steam distillate of acidified rumen liquid. Isobutyric, isovaleric, and caproic acids were also found to have some activity. The active factors were not steam distillable from water extracts of dried distillers' solubles, alfalfa meal, or yeast extract. The author suggested that the cellulolytic factors in these materials may be amino acids which could act as precursors of the five and six carbon fatty acids.

That short-chain fatty acids stimulate the activity of cellulolytic rumen bacteria was confirmed by the studies of Bryant and Doetsch (11). Using a pure culture of a

cellulolytic bacterium, Bacteroides succinogenes, they found that rumen liquid was an absolute requirement for the growth of this rumen microbe. The required factors in rumen liquid were shown to be stable to heat, acid, alkali, and drying. They were found not to be a known B-vitamin, amino acid, peptide, purine, pyrimidine, oleic acid, or mineral. Ether extracts of rumen liquid at a pH of 2.0 completely removed the active principles. Steam distillation of acidified rumen liquid also removed the stimulatory factors. When the active steam distillate fraction was chromatographed, most of the activity was found to be present in the valeric acid fraction. Further work showed that the active cellulolytic factors were a mixture of straight and branched-chain fatty acids. The active branched-chain acids were postulated as being isobutyric, isovaleric, and DL-α-methyl-n-butyric acids. Either n-caproic acid or n-valeric acid was the straight-chained component. (All of these fatty acids have been isolated from rumen contents by other workers. 28, 1). Both structural types of acids were required for activity. This is in contrast with the Ohio group findings in that either the branched or straight-chain acid was active under their conditions. However, in the latter study, a mixed population of rumen bacteria was used. Some species may have been present which could synthesize either one or the

other types of acids but not both types. Another possibility is that the fatty acids used by these workers may have been impure and actually contained both structural types of acids.

Recently, diethylstilbestrol, cholesterol, and estrone have been found to be active cellulolytic factors. Brooks et al. (9) found that these compounds stimulated cellulose digestion by rumen microorganisms in the artificial rumen. Cellulose digestion in sheep was also significantly increased when diethylstilbestrol was added to a high roughage ration. Further work is needed to confirm these findings.

To what extent these steroid compounds and volatile fatty acids may explain the cellulolytic properties of rumen liquid and other materials needs further study. There is some indication that factors other than fatty acids may be involved in the case of yeast extract and various plant extracts. Whether these additional factors are steroid compounds is not known.

Effects of the Unidentified Stimulatory Factors upon the Performance of Beef Cattle and Sheep

The influence of many sources of the unidentified stimulatory factors upon rumen microbial activity has been

determined rather extensively *in vitro*. However, the activity of only a few of these materials that contain these stimulatory factors has been noted in the live animal. A number of studies have been conducted to determine the influence of adding alfalfa meal fractions to low quality roughage rations upon the performance of cattle and sheep.

In three series of digestion trials with steers, Burroughs *et al.* (16) found that either water extracts or the ash of alfalfa meal improved the digestion of corncobs markedly. Each of the meal fractions was fed at a level equivalent to 4 pounds of the original meal. Swift *et al.* (67) obtained similar results with sheep when alfalfa ash was added to rations containing 40.4 per cent corncobs. Calorimetric measurements were made in conjunction with the digestion trials in the latter study and showed that the total digestible nutrients of the basal ration were increased by the ash addition.

Beeson and Perry (3) have reported a beneficial effect from adding alfalfa meal to low quality roughage rations for calves and yearling steers. Likewise, Klosterman *et al.* (44) obtained a significantly higher rate of gain in cattle when one-half of the soybean oil meal in the ration was replaced by alfalfa meal. In these studies the ash from 1.75 pounds of alfalfa meal when fed to cattle resulted in

an average daily gain per head of 2.19 pounds as compared to only 1.75 pounds per head daily for cattle not receiving the ash in their ration. These workers concluded that the stimulatory factors in alfalfa meal were accounted for entirely by the mineral fraction.

Experiments by Oklahoma workers (70, 21) have shown that the digestibility of cottonseed hulls and corncobs by sheep is improved materially by adding alfalfa meal ash to the rations. Sheep receiving the ash in their ration were noted to have better appetites than the control animals. Additional work by this group of workers (35) showed that alfalfa ash significantly increased the synthesis of riboflavin, nicotinic acid, and pantothenic acid in the rumen of sheep. This increased B-vitamin production may account for the improved appetites of the ash supplemented sheep.

Bentley et al. (7) reported the results of two feeding trials with steer calves in which the effects of alfalfa ash and trace minerals upon weight gains, feed consumption, feed utilization, and vitamin B_{12} synthesis were observed. Steer calves were fed a ration of mature timothy hay, ground ear corn, urea, cerelose, calcium, phosphorus, iodized salt, and vitamin A. They found that supplementing this ration with alfalfa ash or a trace mineral mixture (cobalt, manganese, zinc, iron, and copper) significantly increased the

average daily gains of the steers. Their results indicated that cobalt was the primary limiting trace mineral in this ration. Feeding cobalt, or a mineral supplement containing cobalt, increased vitamin B_{12} synthesis as indicated by the increased liver content of vitamin B_{12} and the amount of this vitamin excreted in the feces. The apparent digestibility of the ration appeared not to be affected by the mineral supplementation. However, feed consumption was increased.

The experiments by Pfander et al. (58) demonstrated that the stimulatory activity of alfalfa hay ash was highly correlated with the fertility of the soil on which the hay was grown. Ash from alfalfa hay grown on fertile soil increased cellulose digestion, volatile fatty acid production, and live weight gains in sheep when added to a low quality roughage ration. Ash from alfalfa hay grown on low-fertility soils was not active as demonstrated in their studies.

Some studies have been conducted to determine the effects of adding yeast to cattle and sheep rations. The results of these experiments have been variable. Some early German work by Klein and Müller (43) indicated that feeding a nectar yeast, Anthomyces reukaufii, to sheep resulted in improved utilization of non-protein nitrogen.

The yeast-fed sheep were reported to have had better appetites and went off feed less often than the control animals. Since this yeast is commonly found in the nectar of flowers, the authors felt that little benefit would be derived from feeding it to grazing animals. Further work by Müller (54) indicated that feeding a thermophilic yeast to calves improved the utilization of the non-protein nitrogen in alfalfa silage.

Beeson and Perry (3) obtained an apparent increase in daily gains in cattle by adding live cell yeast to a ration of low quality roughage and Purdue Supplement A. However, the increase in gains was not statistically significant. In another experiment conducted by Perry et al. (56), the addition of live cell yeast to a wintering ration for cattle improved the daily gains from 1.33 to 1.53 pounds per head daily. However, no improvement in gains was obtained by feeding the live cell yeast when the experiment was repeated the following year (57). Artificial rumen studies (61) have indicated that live cell yeast may compete with rumen bacteria for essential nutrients. This may explain the lack of improvement in cattle performance when this product is fed.

Very good results were obtained with yeast in two series of experiments conducted in England. Tosic (72)

studied the effect of small quantities of a yeast preparation on the recovery of appetite in sheep. A water extract of bakers' yeast containing 2.3 grams of dry matter and 247 milligrams of nitrogen was introduced into the rumen of a sheep which had lost its appetite and considerable weight as well. Soon after the yeast treatment, this sheep almost doubled its feed intake and regained its body weight. Two other sheep in a similar condition were also treated with equal effectiveness. However, attempts to experimentally induce a loss of appetite in sheep so that this problem could be studied more extensively failed.

Thomson and Tosic (69) studied the effects of feeding "fodder yeast" upon feed intake and body weight increases in twelve lambs. All lambs were fed a low quality hay containing only 4.56 per cent crude protein. One group of four lambs received 40 grams of "fodder yeast", whereas four other lambs received an equivalent amount of nitrogen in the form of casein. A third group of lambs served as the unsupplemented controls. Body weight gains and feed consumption were increased significantly over the other groups by the yeast supplementation as determined over a 53 day feeding period. The average hay intake in the yeast group was increased from 700 to 1250 grams per animal

daily. The increased gains were entirely accounted for by the increase in feed consumption.

Similar results with yeast were obtained in an experiment conducted by Ruf *et al.* (62). Two groups of four lambs each were individually fed a semi-purified ration. The treatment group received 5 per cent torula yeast (wood yeast) in their diet while the control lambs were fed an equivalent amount of nitrogen in the form of casein. The results of a 56 day feeding test showed that the yeast group of lambs consumed an average of 2.24 pounds of feed per lamb daily, whereas the control lambs ate only 1.58 pounds of feed per head daily. Sheep receiving yeast in their diet gained an average of 0.21 pound per head daily as compared to 0.05 pound per head for the controls. The authors pointed out that the good results obtained with yeast *in vivo* in their studies paralleled those obtained with yeast *in vitro*.

EXPERIMENTAL

A Method for the Study of Cellulose Digestion by Washed Suspensions of Rumen Microorganisms

Several methods have been developed for the study of *in vitro* cellulose digestion of the ruminant. Marston (52) used a procedure which appeared to simulate closely the natural environment in the rumen. His method was later improved by Louw *et al.* (47) by incubating rumen liquid in a semi-permeable bag suspended in a large volume of aqueous growth medium. This improved method prevents the accumulation of metabolic end products such as volatile fatty acids in the bag so that the rate of digestion will not be inhibited. A similar method, but on a smaller scale, was devised by Huhtanen *et al.* (38).

An artificial rumen technique was developed by Burroughs *et al.* (13) for the study of cellulose digestion. In this method the rumen inoculum (whole rumen liquid) was diluted each day so that the nutrients originally present in the rumen liquid were gradually exhausted. This method was used to evaluate the comparative merits of different feeds as sources of unidentified factors and was shown to be very sensitive to these factors (62). One limitation

of this technique, however, was the time required (5 to 10 days) to complete a fermentation test. During this long incubation period *in vitro*, the rumen microorganisms might become so modified that they would not represent a typical sample of the normal rumen flora.

The purpose of this phase of the study was to develop a method which would not only be sensitive enough to detect the effect of small amounts of unidentified factors and other nutrients stimulatory to cellulose digestion, but at the same time would require a short fermentation period.

Materials and methods

Rumen contents were obtained from a 1000-pound fistulated steer receiving a ration consisting of 6 pounds of corn, 2 pounds of soybean oil meal, 12 pounds of corn silage, and hay *ad libitum*. The liquid portion of the rumen contents was strained through four layers of number 50 grade cheesecloth and collected in thermal-neutral containers. This constituted the first step in obtaining rumen inoculum in liquid form. In a typical experiment 1200 milliliters of rumen liquid were collected. The strained rumen fluid was next centrifuged in a small Servall angle centrifuge at a speed of about 1000 r.p.m. for 1 minute. This process

sedimented partially digested feed particles and protozoa which were not removed by straining the rumen liquid through cheesecloth. These were discarded. The supernatant was then centrifuged again at a speed of 5000 r.p.m. for 20 minutes. The resulting sediment consisting principally of rumen bacteria was suspended in 360 milliliters of distilled water saturated with carbon dioxide gas. A Waring Blendor was used to suspend the cells in the washing solution so that clumps of cells would be dispersed. This bacterial suspension was centrifuged again for 20 minutes at 5000 r.p.m. and the washing process repeated. The final sediment obtained from the third high-speed centrifugation was suspended in 600 milliliters of a nutrient solution prepared according to a formula modified from Burroughs et al. (18) and shown in Table 1.

Three grams of a purified wood cellulose, Solka-floc, were added to the bacterial suspension making the concentration of this insoluble cellulose 0.5 per cent. A stream of carbon dioxide gas was directed through the suspension for 10 minutes after which time the pH was adjusted to 7.0 by the addition of a saturated solution of sodium carbonate. Aliquots of 20 milliliters each were pipetted into 75-milliliter pyrex centrifuge tubes. Triplicate samples of 20 milliliters each were also saved for cellulose

Table 1. Composition of nutrient solution

Constituent	Amount gm./liter
Na_2HPO_4	0.316
KH_2PO_4	0.152
$NaHCO_3$	2.620
KCl	0.375
NaCl	0.375
$MgSO_4$	0.112
$CaCl_2$	0.038
$FeSO_4 \cdot 7H_2O$	0.008
$MnSO_4$	0.004
$ZnSO_4 \cdot 7H_2O$	0.004
$CuSO_4 \cdot 5H_2O$	0.002
$CoCl_2$	0.001
Urea	1.000

determination. Each of these fermentation tubes was fitted with a rubber stopper and inlet and outlet glass tubings. A constant flowing stream of carbon dioxide gas was passed through the tubes to establish and maintain anaerobiosis as well as to agitate the fermenting suspension. The tubes were incubated in a constant temperature water bath thermostatically controlled at 39° Centigrade. The apparatus used in these studies is illustrated in Figure 1. At the end of 24 hours, the fermentation was terminated and cellulose was determined on the entire contents of each tube using the procedure of Crampton and Maynard (24) with slight modification.

In studying factors influencing cellulose digestion by this method, it was necessary only to add such factors into respective tubes, and compare the degree of cellulose digestion obtained with cellulose digestion in control tubes without the added factors. Usually, all the treatments, including the control, were triplicated. Where 0.5 per cent cellulose was placed in the medium, approximately 60 per cent digestion was obtained in the control tubes by the end of 24 hours.

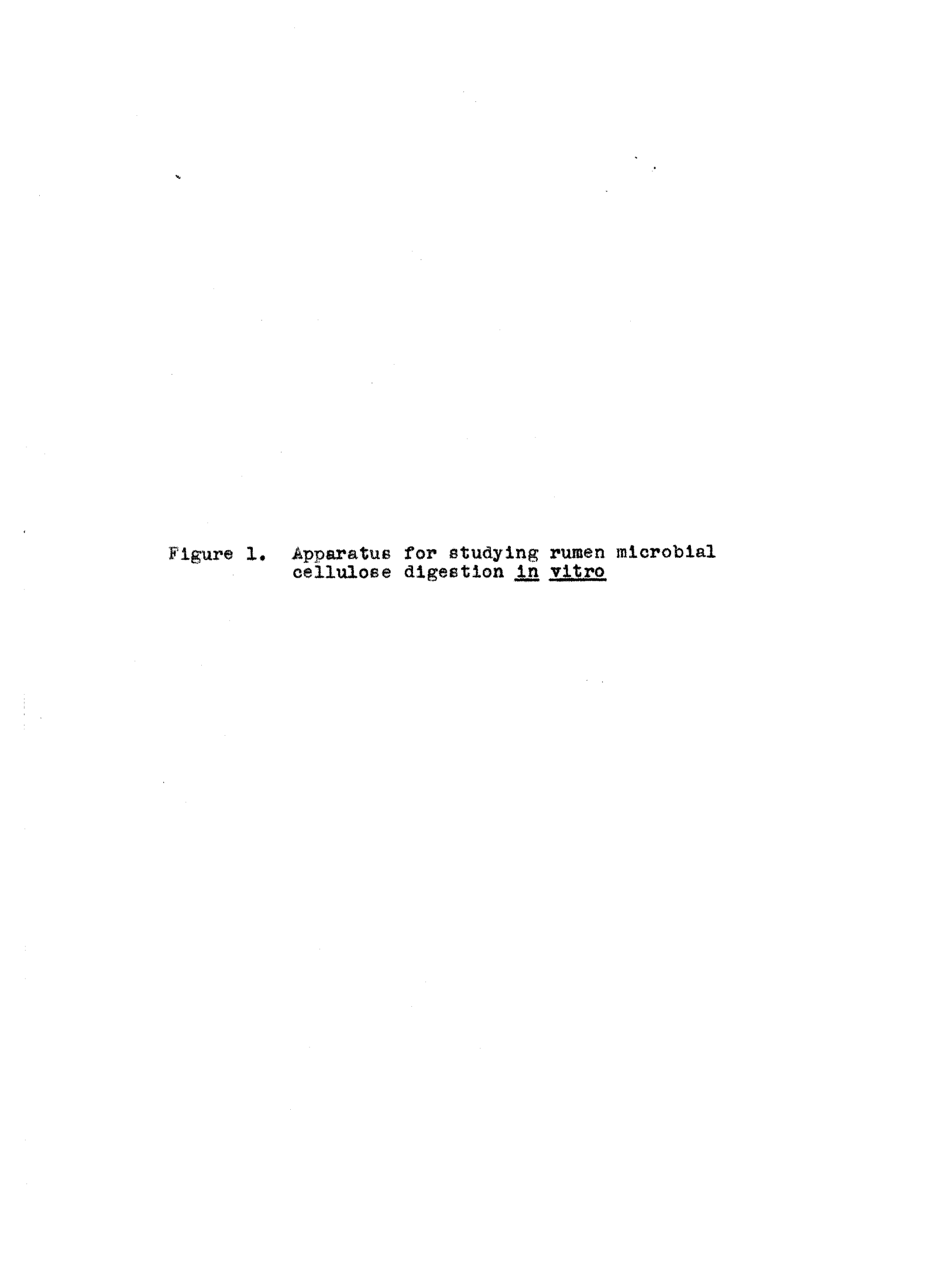
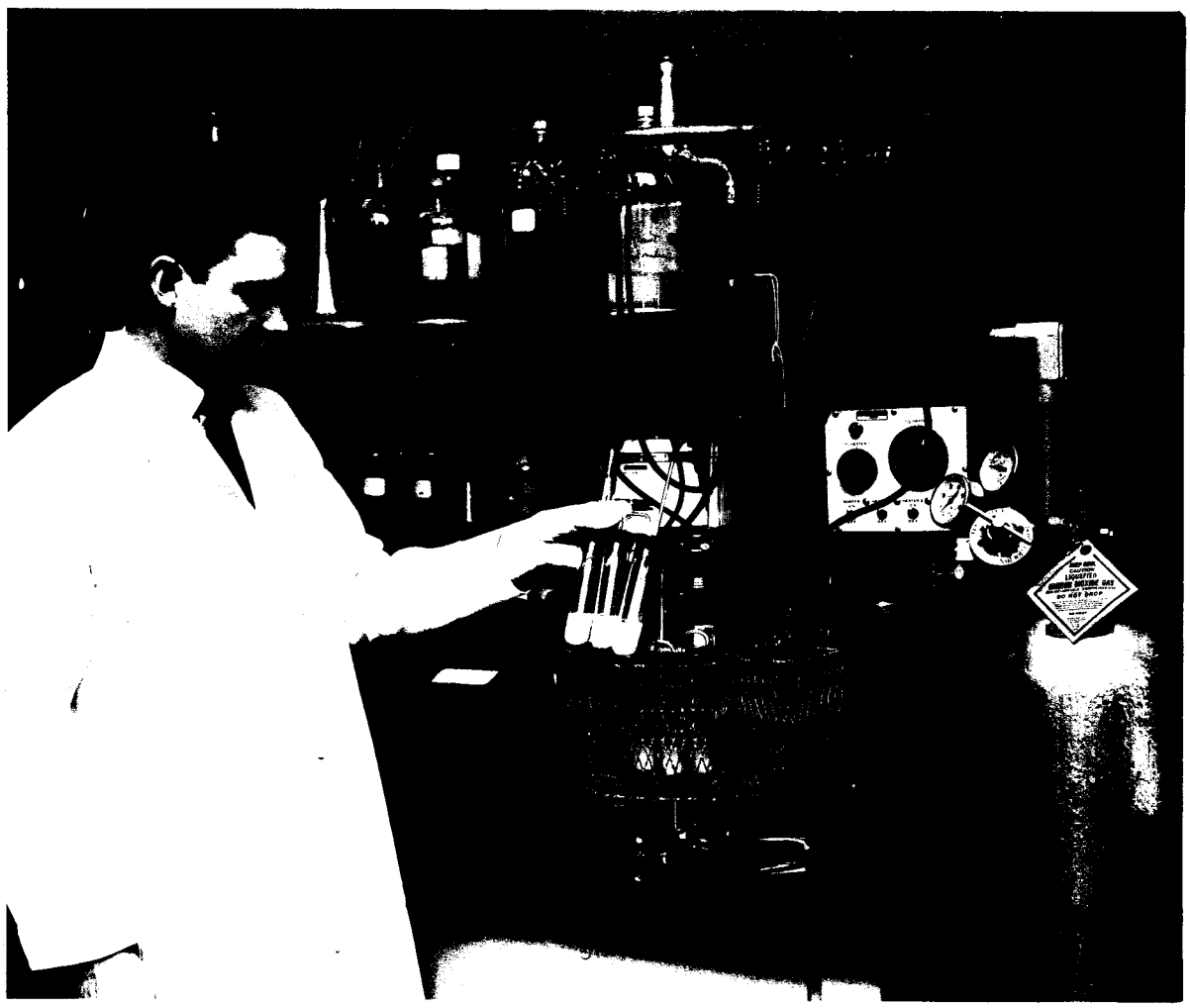


Figure 1. Apparatus for studying rumen microbial cellulose digestion *in vitro*



Results and discussion

There are certain conditions which must be maintained in the fermentation tubes if maximum cellulose digestion is to occur. The influence of the initial pH of the medium upon cellulose digestion is shown in Table 2 and is illustrated graphically in Figure 2. The desired pH in the different groups was obtained by the addition of either

Table 2. The influence of pH upon cellulose digestion by rumen microorganisms *in vitro*

pH	Cellulose digested[a]	
	gm.	%
5.6	0.0282	30.3
6.0	0.0443	47.8
6.4	0.0511	55.1
6.8	0.0599	64.5
7.2	0.0607	65.4
7.6	0.0586	63.2
8.0	0.0527	56.8
8.4	0.0488	52.5

[a]The initial amount of cellulose in each tube was 0.0928 gm.

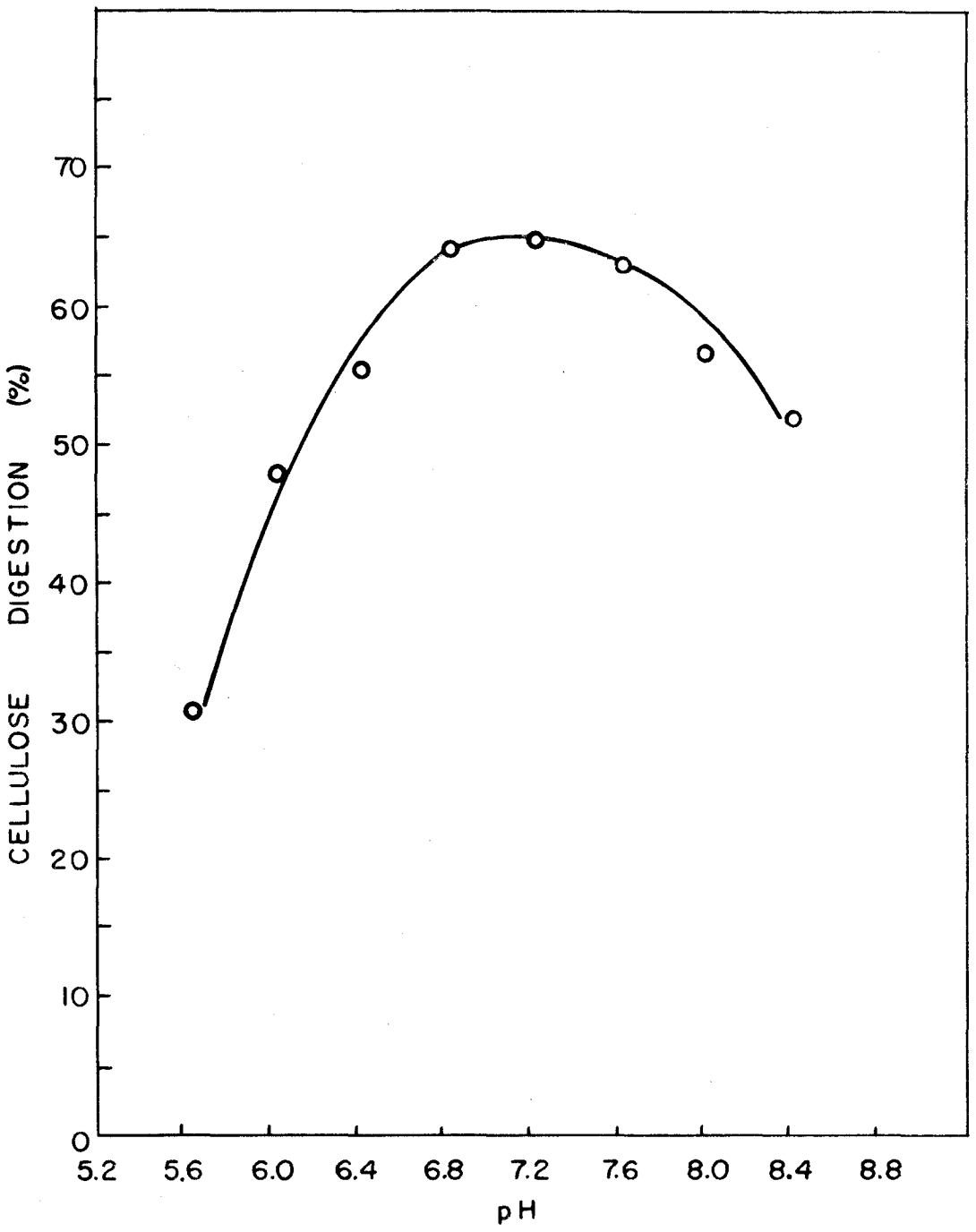


Figure 2. The influence of pH upon cellulose digestion by rumen microorganisms <u>in vitro</u>

sodium carbonate or hydrochloric acid. When the percentage of cellulose digested is plotted against pH, it is seen that maximum cellulose digestion occurred over a pH range of 6.8-7.6. Only when the initial pH was lower than 6.4 or higher than 8.0 was cellulose digestion reduced to a considerable extent. The rumen microorganisms appeared to be able to tolerate an alkaline environment better than an acidic one. This may be expected since the end products of cellulose degradation by rumen microorganisms are acidic. The formation of such products in an already acid environment would tend to aggravate the condition. At the end of the 20-hour incubation period, the pH of all the tubes having an initial pH between 6.8 to 8.4 remained between 6.7 to 7.1. Since this pH range is still adequate for maximum cellulose digestion, it was not necessary to readjust the hydrogen-ion concentration during the course of an experiment.

Most of the cellulose digestion experiments were terminated at the end of 20-24 hours. With 0.5 per cent cellulose in the medium, vigorous digestion occurred between the sixteenth and twentieth hours of incubation. The rate of cellulose digestion for 4-hour intervals is shown in Table 3 and Figure 3. A marked decrease in the rate of cellulose digestion occurred between the fourth and eighth

Table 3. The influence of time of incubation upon cellulose digestion by rumen microorganisms *in vitro*

Incubation time hrs.	Cellulose digested[a]		Rate of digestion mg./4 hrs.
	mg.	%	
0	0	0	0
4	102	9.6	102
8	142	13.4	40
12	295	27.7	153
16	519	48.9	224
20	726	68.4	207
24	824	77.7	98

[a]The initial amount of cellulose in each tube was 1060 mg.

hour of the incubation period. This may correspond to the lag period which often occurs when bacteria are transferred into a new environment. Apparently, the presence of nutrients in the cytoplasm permit a high rate of cellular activity in a new environment for a short period of time. After these nutrients are exhausted, the cells require a period of adjustment to their new medium before normal activity can be resumed.

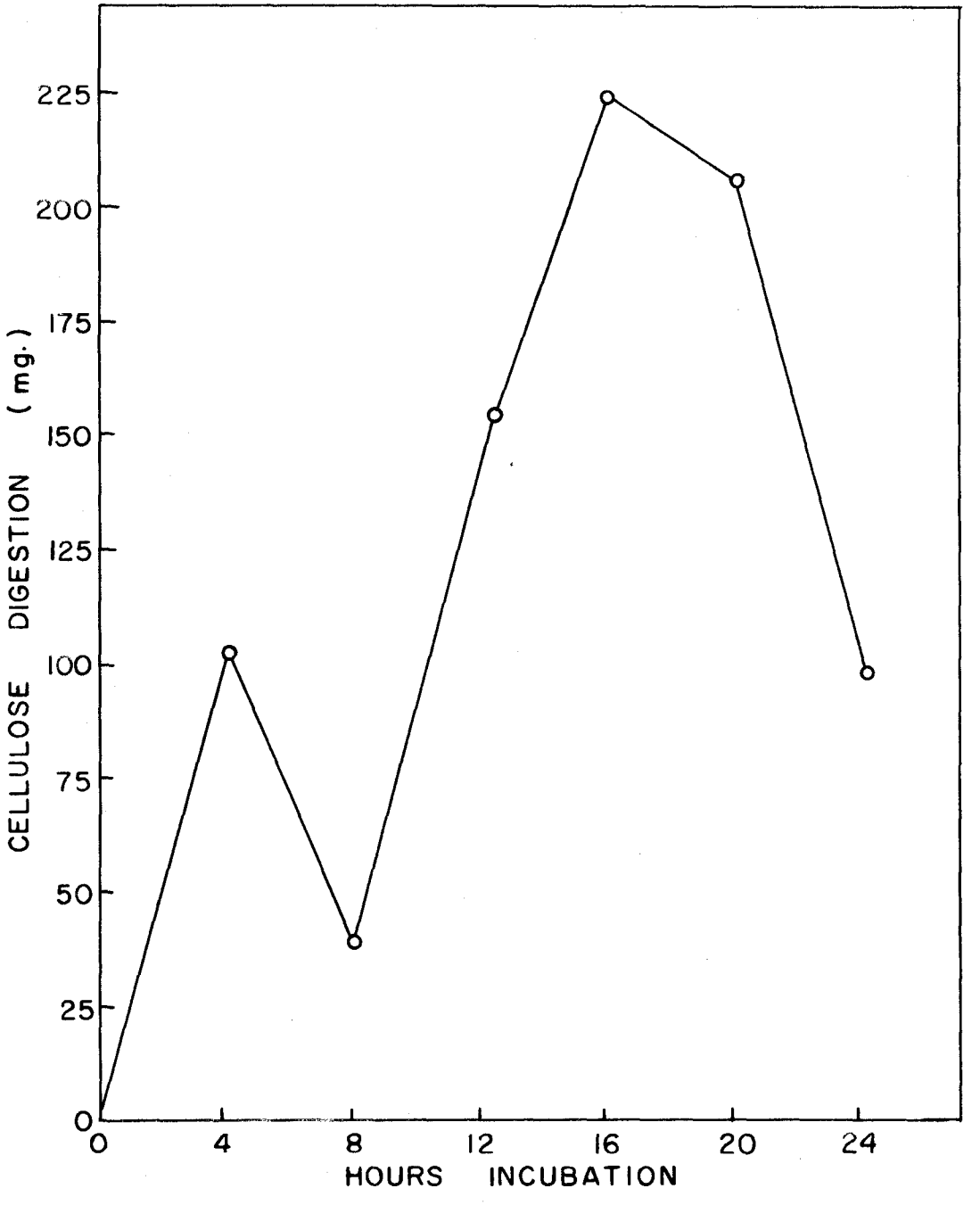


Figure 3. The influence of time upon cellulose digestion by rumen microorganisms <u>in vitro</u>

The concentration of cellulose used in the medium also affected the amount that was digested in 24 hours. As shown in Table 4, the greater amount of cellulose was digested when a concentration of 1 per cent was used. However, on a percentage basis, a greater digestion coefficient was obtained when the concentration of cellulose in the medium was less than 1 per cent. Thus, it is seen that, in general, the less the concentration of cellulose is in the medium the more complete is its digestion by the microorganisms where the size of the inoculum is held constant.

The rate of cellulose digestion was also affected by the size of the inoculum or the concentration of rumen

Table 4. The influence of substrate concentration upon cellulose digestion by rumen microorganisms *in vitro*

Cellulose in nutrient medium %	Initial wt. of cellulose gm.	Cellulose digested	
		gm.	%
0.25	0.0432	0.0327	75.7
0.50	0.0812	0.0466	57.3
0.75	0.1351	0.0857	64.6
1.00	0.1838	0.0984	53.6
1.25	0.2260	0.0862	38.1

microorganisms used per unit volume of medium. Most of the experiments reported in this study were based on the use of 20 milliliters washed suspension of rumen microorganisms equivalent to 40 milliliters of original rumen liquid taken from the steer. In other words, each 20-milliliter aliquot of basal medium containing 0.5 per cent cellulose was inoculated with the washed rumen bacteria removed from 40 milliliters of rumen liquid. Table 5 shows the percentage of cellulose digested as influenced by the amounts of

Table 5. The influence of the concentration of rumen microorganisms upon cellulose digestion <u>in vitro</u>

Rumen liquid ml.[a]	Initial amount of cellulose gm.	Cellulose digested	
		gm.	%
10	0.0894	0.0247	27.7
20	0.0932	0.0614	65.9
40	0.0961	0.0890	92.8
60	0.0960	0.0901	93.2
80	0.0948	0.0903	95.3

[a]Amount of rumen liquid used to prepare 20 ml. of a washed suspension of bacteria.

original rumen liquid used. The bacteria removed from each of the amounts of rumen liquid listed were used to inoculate one 20-milliliter aliquot of basal medium. These results show that 40 milliliters of rumen liquid were adequate to produce maximum digestion when the concentration of cellulose in the medium was approximately 0.5 per cent. Since the rumen liquid used was taken from one animal receiving a specific ration, these results cannot be interpreted too broadly. It is very likely that with different animals fed other rations the activity of the rumen micro-flora would be altered to some extent. Therefore, the amount of rumen liquid required to give an optimum cellulose digestion rate could conceivably vary with different conditions.

One of the more important advantages of using washed microbial suspensions over the unwashed rumen liquid is that in the former method the unknown factors which are present in the rumen liquid are washed out. The washed suspension is also comparatively free from any metabolic end products that were produced by the microorganisms while they were in the rumen. Therefore, when an adequate nutrient solution is provided together with a substrate, in this case an insoluble cellulose, the enzymes of the microorganisms begin to act on the substrate during the period of incubation. Any influences from unknown sources are reduced

to a minimum by repeated washings of the rumen microorganisms. However, unnecessary repeated manipulations of them in the laboratory would decrease their activity.

The data in Table 6 show that the reduction in cellulose digestion due to a second or even a third washing of the rumen microorganisms was relatively small when a solution adequate in minerals was used. However, the effect of thrice washing as compared to only one was to decrease cellulose digestion markedly when the nutrient solution was

Table 6. The influence of washing rumen microorganisms upon cellulose digestion *in vitro*

	Reduced mineral solution[a]		Adequate mineral solution[b]	
	Cellulose digested		Cellulose digested	
	gm.	%	gm.	%
Washed once	0.0404	42.6	0.0658	69.5
Washed twice	0.0286	30.7	0.0613	62.4
Washed thrice	0.0155	15.8	0.0534	54.6

[a]The concentrations of chemicals, except those of phosphate and urea were reduced to one-tenth of those shown in Table 1.

[b]The concentrations of minerals are given in Table 1.

inadequate in regards to minerals. Apparently, this washing procedure is very effective in removing minerals required by bacteria from rumen liquid. Thus, the bacteria are very sensitive to the concentration of minerals supplied in the basal medium. It appears that this washed suspension technique would be very suitable for the in vitro study of mineral requirements of rumen microorganisms.

The use of washed suspensions of rumen microorganisms in the study of their metabolic activity has furnished much valuable information regarding the reduction of nitrate to nitrite and ammonia (46), the mechanism of the decarboxylation of succinic acid (41), the fermentation of glucose and cellobiose (64), and the catabolic reactions of various compounds (25). The technique herein described appears to be suitable for the study of various factors influencing cellulose digestion as well as the conditions necessary for maximum utilization of cellulose by rumen microorganisms. While in vitro studies may not yield results which are directly applicable to in vivo conditions, it is, nevertheless reasonable to suggest that such may be the necessary conditions that are likely to meet the needs of cellulolytic microorganisms in the rumen of live animals.

Summary

The technique finally adapted in this study consisted of separating the rumen microorganisms from rumen liquid by a process of differential centrifugation. The bacterial cells were washed twice before being used to inoculate a chemically defined nutrient solution containing cellulose. When washed rumen microorganisms were suspended in 20 milliliters of a mineral-nutrient solution containing 0.5 per cent cellulose, approximately 60 per cent of the cellulose was digested during an incubation period of 24 hours.

The optimum pH range for cellulose digestion by washed rumen microorganisms was found to be 6.8-7.2. Maximum cellulose digestion was obtained when the concentration of cellulose in the medium did not exceed 0.5 per cent. The concentration of rumen microorganisms as well as the length of the incubation period to use was determined. This technique appears to be very sensitive for the *in vitro* study of factors influencing cellulose digestion by rumen microorganisms.

The Effects of Some Minerals and Nitrogen upon Cellulose Digestion by Rumen Microorganisms *in vitro*

Minerals have been shown to influence the activity of rumen microorganisms materially both *in vitro* and in the live animal. In spite of numerous investigations and observations that have been reported on this subject, quantitative mineral requirements for rumen microorganisms are still quite obscure.

Burroughs *et al.* (13, 18, 20, 19) studied the effects of minerals upon roughage digestion by rumen microorganisms in the artificial rumen. They found that cellulose digestion was favored by the addition of a mineral mixture to the fermentation flasks. Iron and phosphorus were found to be very important. These workers reported that rumen microorganisms require sodium, potassium, calcium, magnesium, sulfur, and chlorine in addition to phosphorus and iron. However, a quantitative requirement for these minerals was not determined.

The amount of iron needed by rumen bacteria for growth *in vitro* was determined by McNaught *et al.* (50) When the chelating agent, α-α-dipyridyl, was added to the medium, bacterial growth was inhibited. The inhibition was reversed by the addition of iron. In this way it was found that

rumen bacteria need about two parts per million of ferrous iron for growth *in vitro*.

The preparation of an adequate synthetic medium necessitated the determination of the quantitative requirements of washed rumen bacteria for minerals as well as for the other chemically known required nutrients such as nitrogen. This was important since the use of a chemically defined basal medium would help rule out any possible influences of such nutrients in the various sources of the unidentified factors. It was the purpose of this study to determine the optimum range in the amounts of minerals and nitrogen that would permit the maximum amount of cellulose digestion by washed rumen microorganisms.

Materials and methods

The washed suspension technique as described in the previous section was used in these studies. Rumen microorganisms were obtained from a fistulated steer, separated from the rumen liquid, and washed twice before being added to the basal medium. The composition of the basal medium is shown in Table 1 of the previous section. This mineral and nitrogen mixture has been shown to support rapid cellulose digestion by rumen microorganisms incubated at 39°

Centigrade for 24 hours. Each 20-milliliter aliquot of the basal medium was inoculated with washed rumen microorganisms obtained from 40 milliliters of strained rumen liquid.

To study the requirements of different minerals, a medium free from one particular mineral was prepared. In the case of sodium-free medium, the sodium chloride normally present was omitted while other sodium salts in the medium were replaced by potassium salts. Similarly, to study the sulfur requirement all the sulfate salts in the medium were replaced by chlorides. Various levels of the mineral omitted from the basal medium were added to the fermentation tubes and the per cent cellulose digested in the different groups over a 20 to 24 hour incubation period was determined. The nitrogen requirement of rumen microorganisms was studied in a similar way.

Relative activity values were calculated for each group of an experiment in addition to cellulose digestion coefficients. These values were obtained by arbitrarily assigning a value of 100 to the mean cellulose digestion coefficient of the control group (tubes without an added amount of the element being studied). The coefficients for the treatment groups were adjusted proportionally to their respective control.

All groups in every experiment were triplicated, and the least significant mean difference (L.S.D.) at the five per cent level of probability was determined for each experiment. Cellulose digestion coefficients for the individual tubes were used for the statistical analysis.

Results and discussion

The results in Table 7 show that many minerals as well as nitrogen are essential for high cellulolytic activity by rumen microorganisms. Sodium, potassium, and sulfur appear to be extremely important as indicated by the low cellulose digestion coefficients which resulted when these nutrients were omitted from the basal medium. Additions of phosphorus, magnesium, manganese, or iron resulted in increases in cellulose digestion which were highly significant. Added amounts of calcium, copper, zinc, or cobalt did not increase cellulose digestion by washed rumen microorganisms. The lack of response from cobalt is somewhat puzzling in view of the role of rumen microorganisms in the synthesis of vitamin B_{12}. However, these results appear to be correct since ten additional experiments failed to show a beneficial effect from adding cobalt to a basal medium lacking an added source of this mineral.

Table 7. The influence of minerals and nitrogen upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Element	Form added	Amount added p.p.m.	Cellulose digested %	Relative activity	L.S.D.[a] %
1	Phosphorus	Na_2HPO_4 and KH_2PO_4	0	47.8	100	
			10	51.4	108	
			50	54.8	115	
			100	55.9	117	
			200	54.1	113	5.69
2	Sodium	NaCl	0	8.0	100	
			1.5	13.4	168	
			75	13.5	169	
			750	37.0	463	
			1500	36.0	450	3.68
3	Potassium	KCl	0	13.5	100	
			2	22.3	165	
			10	47.5	352	
			20	50.8	376	
			100	64.5	478	
			200	65.8	487	
			1000	58.2	431	
			2000	58.7	435	8.28

[a] $p = 0.05$.

Table 7. (Continued)

Experiment number	Element	Form added	Amount added p.p.m.	Cellulose digested %	Relative activity	L.S.D.[a] %
4	Sulfur	Na_2SO_4	0	32.5	100	
			0.07	37.1	114	
			0.14	33.2	102	
			0.7	33.4	103	
			1.4	34.2	105	
			7	49.0	151	
			14	55.0	169	
			70	52.5	162	10.62
5	Calcium	$CaCl_2$	0	85.1	100	
			1	84.7	100	
			3	85.6	101	
			7	84.3	99	
			10	86.8	102	
			28	85.7	101	
			70	86.1	101	
			140	86.0	101	---
6	Magnesium	$MgSO_4$	0	35.5	100	
			2	42.6	120	
			10	47.1	133	
			20	51.2	144	
			30	52.5	148	
			40	52.5	148	3.97

Table 7. (Continued)

Experiment number	Element	Form added	Amount added p.p.m.	Cellulose digested %	Relative activity	L.S.D.[a] %
7	Manganese	$MnSO_4$	0	47.0	100	
			0.01	45.9	98	
			0.07	55.9	119	
			0.3	54.5	116	
			8	50.1	107	
			15	48.3	103	
			30	50.4	107	3.16
8	Copper	$CuSO_4 \cdot 5H_2O$	0	54.8	100	
			0.3	59.4	108	
			0.6	56.2	103	
			1	50.8	93	
			2	46.0	84	
			5	15.3	28	14.50
9	Iron	$FeSO_4 7H_2O$	0	61.4	100	
			0.15	64.7	105	
			0.7	67.0	109	
			3	68.3	111	
			5	67.8	110	
			6	69.3	113	
			8	69.2	113	3.36

Table 7. (Continued)

Experiment number	Element	Form added	Amount added p.p.m.	Cellulose digested %	Relative activity	L.S.D.[a] %
10	Cobalt	$CoCl_2$	0	38.7	100	
			0.0005	39.5	102	
			0.005	40.6	105	
			0.05	38.5	99	
			0.5	38.1	98	
			5	22.2	57	6.13
11	Zinc	$ZnSO_4$	0	54.1	100	
			0.001	54.2	100	
			0.008	51.8	96	
			0.2	52.2	96	
			2	50.0	92	
			8	49.5	91	
			16	3.8	7	6.25
12	Nitrogen	Urea	0	53.3	100	
			117	69.9	131	
			234	71.7	135	
			468	72.3	136	
			702	58.6	110	
			1170	41.0	77	
			1404	15.3	29	7.90

The lack of a stimulatory response from these minerals does not necessarily mean that they are not required by rumen microorganisms. The technique used may not have been critical enough to reduce their concentration in the washed bacterial suspension to a point that would adversely affect cellulose digestion. It has been shown (50) that some of these minerals are concentrated within the bacterial cell, thus, some means other than washing would have to be used to eliminate this source of the mineral. Furthermore, it is possible that the requirement for some of these minerals was so small that the minute amounts present as impurities in other reagent grade chemicals used in the medium might have furnished sufficient amounts for the rumen microorganisms.

It is apparent from these results that washed suspensions of rumen microorganisms can tolerate a wide range of mineral and nitrogen concentrations. However, very high levels of zinc, cobalt, and copper were toxic to rumen bacteria. Toxic levels of other minerals have not been determined. The range in the amounts of minerals and nitrogen required and the level at which cellulose digestion was inhibited by some elements are shown in Table 8.

It is interesting to note that on the parts per million basis the requirement for sulfur ranged from 7 to 70 and

Table 8. Mineral and nitrogen requirements of washed rumen microorganisms

Element	Optimum range p.p.m.	Toxic level p.p.m.
Phosphorus	50-200	---
Sodium	750-1500	---
Potassium	10-2000	---
Iron	1-8	---
Magnesium	2-40	---
Manganese	0.07-8	---
Copper	---	5
Cobalt	---	5
Zinc	---	16
Sulfur	7-70	---
Nitrogen	117-702	1170

that of nitrogen from 117 to 702. The ratio of sulfur to nitrogen required by the washed rumen microorganisms as determined in these studies ranged between 1:10 to 1:17. Since most proteins have a ratio of sulfur to nitrogen of 1:15, which falls in the range of 1:10 to 1:17, it would suggest that synthesis of microbial protein was occurring,

and that the increase in cellulose digestion was due to an increased number of bacteria.

Summary

The mineral and nitrogen requirements of rumen microorganisms for optimum cellulose digestion were determined by using the washed suspension technique. Among the 11 mineral elements tested, calcium, zinc, copper, and cobalt did not appear to be needed by cellulolytic rumen bacteria under the conditions of these experiments. Levels of 5, 5, and 16 parts per million of cobalt, copper, and zinc, respectively, inhibited cellulose digestion. The optimum ratio between sulfur and nitrogen required by rumen microorganisms was found to be in the range of 1:10 to 1:17.

The Effects of B-Vitamins and Certain Carbohydrates upon Cellulose Digestion by Rumen Microorganisms *in vitro*

Numerous investigators have reported the presence of unidentified factors in certain feedstuffs and other materials which enhance rumen microbial activity *in vitro* (18, 19, 39, 26, 37, 62, 51, 5). Yeast extract was found to be one of the best sources of these factors by Ruf *et*

al. (62) and Bentley et al. (5). The stimulatory effect of yeast extract upon cellulose digestion has been confirmed by Hall et al. (32) using a washed suspension of rumen microorganisms.

Since yeast extract is one of the best sources of the B-vitamins (excluding B_{12}), it is possible that a particular B-vitamin or a combination of them might be the cellulolytic factors in yeast extract. One objective of this study was to determine some of the effects of the B-vitamins upon cellulose digestion by rumen microorganisms.

In addition to B-vitamins, many of the materials which exhibit cellulolytic stimulatory activity have a high content of free sugars or compounds which yield sugars upon hydrolysis. Therefore, a second objective of this study was to determine to what extent these unidentified cellulolytic factors could be explained by compounds which supply readily available energy to rumen bacteria.

Materials and methods

Rumen microorganisms used in these studies were obtained from a 1400-pound fistulated Shorthorn steer receiving a ration consisting of corn, soybean oil meal, and poor quality hay. The steer was fed twice daily.

Rumen contents were removed either before the morning feeding at approximately eight o'clock or around one o'clock in the afternoon. The washed suspension technique as previously described was used in these studies.

A new basal medium was formulated on the basis of the information obtained from the experiments in which the mineral and nitrogen requirements were studied. This new basal medium (see Table 9) was used in these experiments. This medium supplied the microorganisms with minerals and nitrogen in amounts which fell within the optimum required range as determined in the previous experiments. Small amounts of copper and zinc were included although these elements did not beneficially influence cellulose digestion in the preceding experiments.

All the B-vitamins used were crystalline products, and each of them was added to fermentation tubes at levels ranging from 0 to 100 micrograms. Generally, only one vitamin was studied in any one experiment. Cellulose digestion in tubes containing a B-vitamin was compared to that in negative control tubes (tubes without an added amount of the vitamin). A positive control group (tubes containing 50 milligrams of yeast extract) was included in most of the experiments. After all the B-vitamins were tested individually, various combinations were tried.

Table 9. Composition of basal medium

Constituent	Amount gm./2 liters
Cellulose[a]	12.00
Urea	2.00
$Na_2HPO_4 \cdot H_2O$	1.20
KH_2PO_4	0.60
$NaHCO_3$	3.50
KCl	0.75
NaCl	0.75
$MgSO_4$	0.15
$FeSO_4 \cdot 7H_2O$	0.075
$CuSO_4 \cdot 5H_2O$	0.002
$CoCl_2$	0.002
$MnSO_4$	0.0004
$ZnSO_4 \cdot 7H_2O$	0.00008

[a]Solka-floc--Brown Company, Berlin, New Hampshire.

The influence of certain carbohydrates upon cellulose digestion *in vitro* was determined in a manner similar to that described for the B-vitamins. All sugars used were chemically pure compounds, and levels ranging from 0 to 10 milligrams were tested. Combinations of these sugars were not tried, however.

Each experiment was terminated at the end of a 20 to 24 hour incubation period, and cellulose digestion coefficients as well as relative activity values were calculated for each experimental group. The standard deviation of the individual observations was calculated for each experiment or for a group of related experiments.

Results and discussion

In Table 10 are recorded the cellulolytic responses obtained from the B-vitamins when they were added individually to the fermentation tubes. It will be noted that addition of either vitamin B_{12}, pseudovitamin B_{12}, biotin, pyridoxine, folic acid, or para-aminobenzoic acid resulted in a considerable increase in cellulose digestion by rumen microorganisms. Riboflavin appeared to have some cellulolytic activity although it was not as active as some of the other B-vitamins. The effective levels ranged from

Table 10. The influence of B-vitamins upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	B-vitamin	Amount added per tube mcg.	Cellulose digested %	Relative activity	Std. dev. %
1	B_{12}	0	62.9	100	
		1	68.3	109	
		5	73.9	124	
		10	71.9	116	1.54
2	Pseudo B_{12}	0	29.3	100	
		1	33.6	107	
		5	37.5	119	
		10	35.6	113	
		25	39.6	126	
		50	41.6	132	
		100	40.0	127	1.97
3	Biotin	0	39.4	100	
		0.1	48.6	123	
		1	48.1	122	
		10	48.8	124	2.15
4	Folic acid	0	20.3	100	
		1	24.5	121	
		10	27.6	136	
		100	28.8	142	

Table 10. (Continued)

Experiment number	B-vitamin	Amount added per tube mcg.	Cellulose digested %	Relative activity	Std. dev. %
	Pyridoxine	0	20.3	100	
		1	24.1	119	
		10	24.2	119	
		100	29.5	145	2.49
5	para-Amino-benzoic acid	0	46.2	100	
		1	50.5	109	
		10	53.2	115	
		100	56.3	122	2.75
6	Riboflavin	0	47.7	100	
		0.1	52.3	110	
		1	50.0	105	
		100	52.8	111	2.21
7	Nicotinic acid	0	44.4	100	
		1	40.5	91	
		10	45.3	102	
		100	44.9	101	1.80

Table 10. (Continued)

Experiment number	B-vitamin	Amount added per tube mcg.	Cellulose digested %	Relative activity	Std. dev. %
8	Pantothenic acid	0	59.1	100	
		1	58.4	99	
		10	60.2	102	
		100	59.7	101	1.73
9	Inositol	0	60.3	100	
		1	64.2	106	
		10	58.0	96	
		100	58.5	97	2.44
10	Thiamine	0	21.4	100	
		1	20.6	96	
		10	21.1	99	
		100	21.0	98	2.40
11	Choline chloride	0	45.8	100	
		1	44.5	97	
		10	43.7	95	
		100	40.3	88	2.65

0.1 to 100 micrograms per 20 milliliters of washed suspension of rumen microorganisms. Folic acid, pyridoxine, para-aminobenzoic acid, and pseudovitamin B_{12} were most active at levels of 50 or 100 micrograms per tube, while biotin and vitamin B_{12} were most active at levels of 1 and 5 micrograms, respectively.

Additions of nicotinic acid, pantothenic acid, inositol, thiamine, and choline chloride did not significantly increase cellulose digestion in these experiments. These data indicate that either washed cellulolytic rumen bacteria can synthesize sufficient quantities of these B-vitamins to meet their needs under the conditions of these experiments, or that these vitamins were not required for cellulose digestion.

A combination of vitamin B_{12} and biotin was found to stimulate microbial cellulose digestion more than did either of these vitamins alone. This synergistic effect of vitamin B_{12} and biotin is shown in Table 11. It is further shown in Table 11 that yeast extract was more stimulatory to cellulose digestion than the combination of biotin and vitamin B_{12}. However, the addition of these two vitamins and yeast extract to the fermentation tubes did not result in any more cellulose digestion than was obtained when yeast extract was added alone. This would indicate that

Table 11. The influence of biotin, vitamin B_{12}, and yeast extract upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	51.0	100	
	5 mcg. vitamin B_{12}	58.5	115	
	1 mcg. biotin	58.0	112	
	5 mcg. vitamin B_{12} + 1 mcg. biotin	64.6	127	1.06
2	None	55.0	100	
	5 mcg. vitamin B_{12} + 1 mcg. biotin	68.0	124	
	50 mg. yeast extract (Difco)	81.6	148	
	50 mg. yeast extract + 5 mcg. vitamin B_{12} + 1 mcg. biotin	81.4	148	2.11
-	None	43.6[a]	100	
	5 mcg. vitamin B_{12} + 1 mcg. biotin	56.4[a]	129	
	None	45.3[b]	100	
	50 mg. yeast extract (Difco)	65.7[b]	145	---

[a]Average of 24 experiments.

[b]Average of 34 experiments.

yeast extract contains cellulolytic factors in addition to the B-vitamins. Since yeast extract and the combination of vitamin B_{12} and biotin were often used as positive controls, a large number of experiments were conducted in which the effects of these materials were observed. The combination of vitamin B_{12} and biotin increased cellulose digestion by 29 per cent as determined by 24 different experiments, while yeast extract resulted in an average increase of 45 per cent in 34 different experiments (Table 11).

Additions of other B-vitamins to the combination of vitamin B_{12} and biotin did not result in any additional increases in cellulose digestion. Various levels of each of the B-vitamins shown in Table 12 were tested, but only the particular levels which resulted in the greatest amount of cellulose digested are recorded. It is possible that in the presence of vitamin B_{12} and biotin, the microorganisms were able to synthesize other B-vitamins in sufficient quantities to supply their needs.

Many additional experiments were conducted in an effort to determine if other groups of B-vitamins were more active than vitamin B_{12} and biotin. Some of these B-vitamin combinations and the level at which they were found to be most effective are recorded in Table 13. The combination

Table 12. The influence of adding certain B-vitamins to the combination of vitamin B_{12} and biotin upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	B-vitamin addition per tube	Cellulose digested %	Relative activity
1	None[a]	65.4[b]	100
	10 mcg. folic acid	62.8	96
	100 mcg. pyridoxine	60.4	92
2	None	69.2	100
	1 mcg. inositol	70.1	101
3	None	65.4	100
	0.1 mcg. riboflavin	63.5	97
4	None	66.4	100
	100 mcg. para-aminobenzoic acid	62.6	94
5	None	52.6	100
	1 mcg. each of B_1, choline, niacin, folic acid, B_6, and pantothenic acid	51.5	98
6	None	42.2	100
	10 mcg. pyridoxine + 100 mcg. para-aminobenzoic acid	36.8	87
7	None	54.4	100
	50 mcg. pseudovitamin B_{12}	53.8	99

[a]Basal medium contained 5 mcg. vitamin B_{12} and 1 mcg. biotin per 20 ml.

[b]Standard deviation of the individual observations = 2.42%.

Table 13. The influence of some additional combinations of B-vitamins upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	B-vitamin addition per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	44.0	100	
	50 mcg. pseudo B_{12} + 5 mcg. B_{12}	53.6	122	
	50 mcg. pseudo B_{12} + 1 mcg. biotin	53.5	122	
	5 mcg. B_{12} + 1 mcg. biotin	54.4	124	1.73
2	None	53.6	100	
	5 mcg. B_{12} + 1 mcg. choline	64.0	119	
	5 mcg. B_{12} + 100 mcg. folic acid	65.6	122	
	5 mcg. B_{12} + 1 mcg. biotin	66.7	124	1.42

Table 13. (Continued)

Experiment number	B-vitamin addition per tube	Cellulose digested %	Relative activity	Std. dev. %
3	None	42.8	100	
	1 mcg. biotin + 1 mcg. niacin	50.4	118	
	1 mcg. biotin + 10 mcg. thiamine	50.9	119	
	5 mcg. B_{12} + 1 mcg. biotin	61.0	143	3.65
4	None	51.2	100	
	100 mcg. pyridoxine + 1 mcg. B_{12}	58.0	113	
	5 mcg. B_{12} + 1 mcg. biotin	57.9	113	3.67
5	None	63.0	100	
	100 mcg. para-aminobenzoic acid + 1 mcg. pyridoxine	72.8	116	
	5 mcg. B_{12} + 1 mcg. biotin	76.8	122	2.40

of vitamin B_{12} and biotin appeared to be equally as active as any other combination of vitamins tested. The more active combinations usually contained vitamin B_{12} or biotin.

In Table 14 are summarized the results of adding certain carbohydrates to the fermentation tubes. Some readily available energy was needed by cellulolytic rumen bacteria as indicated by these results. Small amounts of xylose, arabinose, ribose, rhamnose, sorbose, and maltose produced significant increases in cellulose digestion. High levels of many of these sugars depressed cellulose digestion. The 10-milligram level of mannose was particularly depressing. The inhibition of cellulose digestion by these compounds at high levels was probably due to their preferential use by the bacteria as a source of energy. Small amounts of carbohydrates such as dextrose, lactose, levulose, dextrin, and starch had little influence upon cellulose digestion under the conditions of these experiments.

Summary

The washed suspension technique was used to study the influence of B-vitamins and certain carbohydrates upon cellulose digestion *in vitro*. Several B-vitamins were

Table 14. The influence of certain carbohydrates upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Carbohydrate	Amount added per tube mg.	Cellulose digested %	Relative activity	Std. dev. %
1	d(+)-Xylose	none	58.0	100	
		0.1	56.8	98	
		0.5	68.4	118	
		1.0	64.6	111	
		10.0	52.3	90	
	d(+)-Arabinose	none	58.0	100	
		0.1	65.8	113	
		0.5	64.0	110	
		1.0	64.8	112	
		10.0	52.1	90	2.28
2	d(+)-Ribose	none	53.3	100	
		1.0	55.2	104	
		5.0	60.5	114	
		10.0	60.5	114	
	l(-)-Rhamnose·H_2O	none	53.3	100	
		1.0	55.3	104	
		5.0	58.7	110	
		10.0	60.8	114	2.54

Table 14. (Continued)

Experiment number	Carbohydrate	Amount added per tube mg.	Cellulose digested %	Relative activity	Std. dev. %
3	d(+)-Mannose	none	29.9	100	
		1.0	27.8	93	
		5.0	28.3	95	
		10.0	19.4	65	2.21
	l-Sorbose	none	29.9	100	
		1.0	34.8	116	
		5.0	34.6	116	
		10.0	35.8	120	2.21
4	d(+)-Dextrose	none	59.4	100	
		0.1	58.3	98	
		0.5	61.0	103	
		1.0	57.9	97	1.45
5	d(-)-Levulose	none	50.3	100	
		1.0	46.7	93	
		5.0	48.6	97	
		10.0	45.5	90	2.60

Table 14. (Continued)

Experiment number	Carbohydrate	Amount added per tube mg.	Cellulose digested %	Relative activity	Std. dev. %
6	d(+)-Maltose	none	59.2	100	
		5.0	65.1	110	
		10.0	64.7	109	2.27
7	Lactose	none	61.4	100	
		1.0	59.3	97	
		5.0	60.5	99	
		10.0	58.2	95	2.73
8	Dextrin	none	56.0	100	
		1.0	54.8	98	
		5.0	51.3	92	
		10.0	50.2	90	
	Starch	none	56.0	100	
		1.0	55.0	98	
		5.0	56.6	101	
		10.0	53.4	95	2.06

found to enhance cellulose digestion when added individually to the fermentation tubes. These were: pseudovitamin B_{12}, biotin, folic acid, pyridoxine, para-aminobenzoic acid, and riboflavin. A combination of vitamin B_{12} and biotin stimulated microbial cellulose digestion more than either of these vitamins alone. In 24 experiments vitamin B_{12} and biotin increased cellulose digestion an average of 29 per cent. This combination of vitamins was equally as active as any other combination of vitamins studied.

No single vitamin or combination of B-vitamins was found which increased microbial cellulose digestion to the same extent as did yeast extract. A yeast extract addition of 50 milligrams per tube resulted in an average increase in cellulose digestion of 45 per cent as determined in 34 different experiments. This substance apparently contains cellulolytic factors in addition to the B-vitamins.

Various sugars were found to stimulate cellulose digestion by rumen microorganisms to a small degree. High levels of some of these compounds depressed cellulose digestion.

The Effects of Fractions of Yeast Extract and Other Yeast Products upon Cellulose Digestion by Rumen Microorganisms *in vitro*

The use of spent sulfite liquor for the production of *Torula utilis*, a wood yeast, has created a large potential feed supplement for ruminants as well as livestock in general. In view of the finding that yeast extract is a good source of unidentified stimulatory factors for rumen bacteria, it appeared that the presence of such factors in other types of yeast was worthy of investigation. The development of a torula yeast product which contains a high concentration of these factors would be especially important since sufficient quantities of such a product could possibly be produced for immediate incorporation into ruminant rations.

In this phase of the study a large number of torula and *Saccharomyces* yeast products were screened in an effort to find an economical source of these microbial stimulatory factors which could be produced in large quantities. A limited number of fractionation studies were undertaken to determine some of the properties of the unidentified factors in yeast extract.

Materials and methods

A Difco yeast extract product was used in the fractionation studies. This product consists of the water soluble fractions of yeast cells and was found to contain a high concentration of the unidentified stimulatory factors. Five-hundred milliliters of a 10 per cent water solution of yeast extract were treated with 1000 milliliters of methanol in order to precipitate the methanol-insoluble protein fraction. A large quantity of protein material was precipitated by this procedure and was removed by decantation and filtration. Methanol was removed from the filtrate by evaporation over a steam plate. Samples of the resulting solution were saved for assay.

The pH of 400 milliliters of the methanol-treated yeast extract solution was adjusted to 5.0 with hydrochloric acid. This acidified solution was added to 10 grams of Norite A, heated to 80° Centigrade, and agitated for 10 minutes. Norite A was separated from the liquid by filtration and saved. The filtrate was treated as above for four additional times and each of the 10-gram Norite A fractions saved. All of the used Norite A was placed in a beaker and mixed thoroughly with 400 milliliters of 70 per cent ethanol. The pH was adjusted to 7.0 with sodium hydroxide. After about

10 minutes, the mixture was filtered and the Norite A residue treated again with 400 milliliters of 70 per cent ethanol. The two ethanol fractions were combined and the alcohol was removed by evaporation over a steam plate. After removing the alcohol, distilled water was added to adjust the volume to 400 milliliters.

To determine if the unidentified factors in yeast extract were dialyzable, 100 milliliters of a 5 per cent yeast extract water solution were placed in a semi-permeable cellophane bag and immersed into 800 milliliters of distilled water. After refrigeration for 48 hours, the water was removed and the volume reduced to 100 milliliters by heating over a steam plate. Both the residue (solution remaining inside the cellophane bag) and the dialyzate were tested.

A number of torula and Saccharomyces yeast products were supplied by the Red Star Yeast and Products Company of Milwaukee, Wisconsin, and assayed for rumen microbial stimulatory factors. The first of these were separator and press yeast liquors. In order to describe these samples adequately it is necessary to relate very briefly some of the processes involved in yeast production.

After a yeast fermentation has been completed, the contents are removed from the fermenter and passed through a series of separators for removal of a considerable amount

of liquid. The effluent from the separators is called separator liquor and is usually discarded. Samples of separator liquor from Saccharomyces yeast production were obtained and concentrated to 23 per cent dry matter by heating over a boiling water bath.

The residual yeast material in the separators contains about 14 to 17 per cent solids and is termed a cream. The yeast cream is further concentrated by expressing the liquid from it by a series of presses. This liquid is the press yeast liquor used in these studies. The press yeast liquor samples contained 2.3 per cent solids. Samples of both the press and separator yeast liquors were dried at 100° Centigrade and the solids redissolved in water to determine the effect of heating upon the stimulatory activity of these products. Press liquor samples from Saccharomyces yeast used to make compressed yeast as well as from strains used to make Active Dry Yeast were assayed.

In addition to yeast liquors, a variety of other yeast products were assayed. These products were prepared as follows:

3102 F.V.D. - Active Dry Yeast (ADY) press liquor concentrated by freezing, then drum dried under vacuum.

3109 B - Equal weight of Active Dry Yeast wet cake and water mixed, boiled for one-half hour, centrifuged, supernate brought to dryness.

3109 P - Active Dry Yeast press wet cake treated with 10 per cent of its weight of sodium chloride, centrifuged, and supernate evaporated to dryness.

F-31 - Bakers Yeast cream autolyzed for 8 hours at 114° F., drum dried.

F-53 - Active Dry Yeast cream autolyzed as above.

3090 LS - Active Dry Yeast with 5.7 per cent moisture extracted with six volumes of ice cold water for one and one-half hours, centrifuged, supernate boiled to dryness.

Sample No. 1 - Active Dry Yeast ground to 20 mesh in a Wiley mill.

Sample No. 2 - Drum dried Active Dry Yeast cream.

Sample No. 3 - Made by the addition of 10 per cent sodium chloride (based on wet weight of yeast) to Active Dry Yeast cream, drum dried.

Sample No. 4 - Made in pilot plant by growing torula yeast on molasses mash, harvested, drum dried.

Sample No. 5 - Molasses torula press cake plasmolyzed with 10 per cent sodium chloride, centrifuged, supernate dried.

Sample No. 6 - Same as Sample No. 5 except that cells and supernate were not separated before drying.

Sample No. 7 - Made in pilot plant by growing torula yeast in concentrated sulfite liquor, harvested, drum dried.

Sample No. 8 - Sulfite torula press cake plasmolyzed with 10 per cent sodium chloride, centrifuged, supernate dried.

Sample No. 9 - Same as Sample No. 8 except that cells and supernate were not separated before drying.

To determine whether the activity in plasmolyzed torula yeast was consistent from batch to batch, a series of samples were obtained on different dates and assayed. Three of these samples were produced on a pilot plant scale while the fourth was produced on a commercial or large scale

basis. These products were all prepared by adding 10 per cent by weight of salt to torula yeast cream which had been produced in a sulfite liquid medium. After one to two hours the material was drum dried.

In this series of studies inoculum was obtained from the same fistulated steer used in the previous studies. However, to prevent the introduction of unidentified factors to the tubes via the inoculum, a ration low in these factors was fed. This ration consisted of 20 per cent corn, 70 per cent corncobs, and 10 per cent supplement. The supplement consisted of soybean oil meal, urea, steamed bone meal, limestone, dried molasses, and vitamins A and D.

After removing the coarse feed particles and protozoa from the rumen liquid with a small Servall angle centrifuge, the bacteria were separated by centrifuging in a Sharples super-centrifuge at a speed of 25,000 r.p.m. The bacteria and 50 milliliters of the liquor draining from the barrel of the centrifuge were suspended in 1000 milliliters of warm distilled water saturated with carbon dioxide gas. The bacterial suspension was passed through the Sharples super-centrifuge once more to sediment the bacteria. The resulting sediment along with 10 milliliters of the liquor draining from the barrel of the centrifuge were used for the inoculum.

The basal medium used in this series of studies is shown in Table 9. In addition to these constituents, vitamin B_{12} and biotin were added so that each 20-milliliter aliquot of washed bacterial suspension contained 5 micrograms of vitamin B_{12} and 1 microgram of biotin. Xylose was also added at a level of 0.5 milligram per 20 milliliters of bacterial suspension.

Cellulose digestion coefficients, relative activity values, and the standard deviations were calculated in a manner previously described. In some of these experiments only two tubes were used per treatment rather than three.

Results and discussion

Removing the methanol-insoluble protein fraction did not affect the stimulatory cellulolytic activity of yeast extract as shown in Table 15. The methanol-treated yeast extract was just as active as the untreated material. Slightly acidified yeast extract which had been treated with Norite A was not as active as untreated yeast extract indicating that some of the activity was adsorbed on Norite A. Some activity was eluted from Norite A with 70 per cent ethanol but this fraction was not as active as the untreated

Table 15. The influence of fractions of yeast extract (Difco) upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	45.3	100	
	1.0 ml. methanol-treated yeast extract solution	77.0	170	
	1.0 ml. Norite-treated yeast extract solution	61.6	136	
	1.0 ml. Norite eluate (70% ethanol)	66.9	148	
	1.0 ml. 5% yeast extract solution	76.5	169	1.99
2	None	57.0	100	
	0.5 ml. yeast extract dialyzate[a]	71.5	125	
	0.5 ml. dialyzed yeast extract[b]	65.6	115	
	1.0 ml. 5% yeast extract solution	79.3	139	0.87

[a]Solution containing the material which passed through the semi-permeable membrane.

[b]Solution remaining inside the cellophane bag.

material. These results with Norite A paralleled those reported in earlier studies (61, 51, 5).

It is further shown in Table 15 that an active fraction was obtained by dialyzing a yeast extract solution for 48 hours. However, considerable activity remained in the residue. It is possible that a longer period of dialysis and a larger volume of water would have completely removed the stimulatory factors in yeast extract. These results indicate that the active factors are relatively small molecules since they passed through a semi-permeable barrier.

In Table 16 are recorded the results obtained from Saccharomyces yeast liquors. These results show that press yeast liquor was extremely active in stimulating rumen microbial cellulose digestion. Separator yeast liquor stimulated cellulose digestion but not to the same extent as press liquor. Additional work showed that drying press yeast liquor at 100° Centigrade and redissolving the solids in water before testing did not deleteriously affect its stimulatory activity. A similar treatment of the separator liquor resulted in a considerable loss in activity. For this reason further work with this product was discontinued. Methanol-treated press yeast liquor was equally as active as untreated press liquor. Both samples resulted in 100 per cent increases in cellulose digestion over a 24-hour

Table 16. The influence of separator and pressed yeast liquors upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	13.4	100	
	0.5 ml. press yeast liquor	43.8	327	
	1.0 ml. press yeast liquor	56.2	419	
	0.5 ml. separator yeast liquir	39.5	295	
	1.0 ml. separator yeast liquor	42.5	317	1.89
2	None	32.6	100	
	2.0 ml. press yeast liquor	51.2	157	
	2.0 ml. heated press yeast liquor	53.5	164	
	2.0 ml. heated separator yeast liquor	39.6	121	1.96
3	None	31.5	100	
	2.0 ml. press yeast liquor	65.8	209	
	2.0 ml. methanol-treated press yeast liquor	62.9	200	1.10
4	None	41.4	100	
	1.0 ml. press yeast liquor (1100 P)[a]	49.6	120	
	2.0 ml. press yeast liquor (1100 P)	52.8	128	
	1.0 ml. press yeast liquor (3090)[b]	60.2	145	
	2.0 ml. press yeast liquor (3090)	67.6	163	1.78

[a]Press liquor from yeast used to make compressed yeast.

[b]Press liquor from yeast used to make Active Dry Yeast.

incubation period. The type of yeast from which press liquor is obtained was very important in regard to the stimulatory activity exhibited by press liquor. Press liquor from Saccharomyces yeast used to make compressed yeast was found to be much less active than press liquor from cultures used to make Active Dry Yeast.

Perhaps the most significant finding in the studies summarized in Table 17 was that torula yeast products were more active than the Saccharomyces yeast products. This was particularly true when a concentrated sulfite liquor was used as the nutrient medium in which to grow the torula yeast. Another important point demonstrated in these results is that the activity of these yeast products was increased considerably by plasmolyzing the yeast cream with sodium chloride. Apparently this process resulted in the concentration of the active factors outside of the yeast cells making the factors more accessible to cellulolytic rumen bacteria.

Another good feature of the torula yeast plasmolyzate is demonstrated in the experiments reported in Table 18. These results show that the activity of this product was consistently present in different production batches. The first three samples used in these experiments were produced on a pilot plant scale while the fourth came from a

Table 17. The influence of yeast products upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	52.5	100	
	20 mg. 3102 F.V.D.	64.1	122	
	40 mg. 3102 F.V.D.	67.4	128	
	20 mg. 3109-B	54.9	105	
	40 mg. 3109-B	56.5	108	
	20 mg. 3109-P	59.1	113	
	40 mg. 3109-P	64.9	124	
	20 mg. F-31 autolysate	49.6	94	
	40 mg. F-31 autolysate	33.8	64	
	20 mg. F-53 autolysate	51.2	98	
	40 mg. F-53 autolysate	47.6	91	
	20 mg. 3090 L.S.	57.6	110	
	40 mg. 3090 L.S.	53.1	101	1.61

Table 17. (Continued)

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
2	None	31.5	100	
	25 mg. ADY 3161 (Sample No. 1)	33.4	106	
	50 mg. ADY 3161 (Sample No. 1)	34.5	110	
	25 mg. ADY 3161 dried cream (Sample No. 2)	35.8	114	
	50 mg. ADY 3161 dried cream (Sample No. 2)	36.5	116	
	25 mg. plasmolyzed ADY 3161 cream (Sample No. 3)	39.5	125	
	50 mg. plasmolyzed ADY 3161 cream (Sample No. 3)	46.8	149	
	25 mg. molasses torula yeast (Sample No. 4)	33.8	107	
	50 mg. molasses torula yeast (Sample No. 4)	32.8	104	
	50 mg. plasmolyzed torula yeast (Sample No. 5)	59.0	187	
	50 mg. plasmolyzed torula yeast (Sample No. 6)	43.2	137	
	25 mg. sulfite torula yeast (Sample No. 7)	45.0	143	
	50 mg. sulfite torula yeast (Sample No. 7)	45.4	144	
	50 mg. plasmolyzed torula yeast (Sample No. 8)	60.5	192	
	50 mg. plasmolyzed torula yeast (Sample No. 9)	55.5	176	2.43

Table 18. The influence of plasmolyzed torula yeast cream upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	39.3	100	
	100 mg. plasmolyzed torula yeast cream (Batch No. 1)	66.6	169	2.23
2	None	45.4	100	
	100 mg. plasmolyzed torula yeast cream (Batch No. 2)	64.9	143	
	100 mg. plasmolyzed torula yeast cream (Batch No. 3)	68.7	151	1.72
3	None	32.6	100	
	5 mg. plasmolyzed torula yeast cream (Batch No. 4)	34.3	105	
	10 mg. plasmolyzed torula yeast cream (Batch No. 4)	40.6	125	
	20 mg. plasmolyzed torula yeast cream (Batch No. 4)	45.9	141	
	40 mg. plasmolyzed torula yeast cream (Batch No. 4)	54.0	166	2.92

commercial production source. Each of these four samples was obtained from a separate fermentation.

Summary

Fractionation studies of yeast extract showed that the stimulatory factors were not associated with the methanol-insoluble protein fraction. Furthermore, some activity was removed by treating slightly acidified yeast extract with Norite A. A fraction with some activity was eluted from Norite A with 70 per cent ethanol. Neither of the Norite A fractions was as active as untreated yeast extract. The finding that the active factors are dialyzable indicated that they are relatively small molecules.

Press yeast liquor (ADY), a product which is normally discarded by the yeast industry, was found to be an excellent source of the unidentified stimulatory factors. The stimulatory factors in press yeast liquor were found to be heat stable, and they were not associated with the methanol-insoluble protein fraction.

Torula yeast grown in sulfite liquor was more active in enhancing cellulose digestion than either Saccharomyces yeast or torula yeast grown in a molasses mash.

Plasmolyzing the torula yeast cream with sodium chloride increased its stimulatory property materially. This process apparently concentrated the active factors outside the yeast cells.

The results of these experiments have led to the development of a torula yeast product which can be produced on a commercial basis. This product is made by adding 10 per cent salt to torula yeast cream. After a short period of time (one to two hours) the entire contents are drum dried. The resulting product contains the active factors formerly present inside the yeast cells as well as those in the press liquor. This product was found to be a very good source of cellulolytic factors for rumen bacteria *in vitro*.

The Effects of Partial Acid Hydrolyzates of Certain Protein Materials upon Cellulose Digestion by Rumen Microorganisms *in vitro*

Numerous investigators have reported the unidentified rumen microbial stimulatory factors to be water soluble and that they occur most abundantly in materials rich in either protein or in non-protein nitrogen. Since the unidentified factors appear to be closely linked with the protein

molecule, attempts were made in this phase of the study to alter the structure of water insoluble protein materials so that these factors could be produced at will in the laboratory. It was thought that such studies might aid in the elucidation of the chemical identity of these factors.

Materials and methods

Vitamin-free casein, feather meal, hog hair, soybean oil meal, a purified soybean protein (drackett protein), and gelatin were partially hydrolyzed with hydrochloric acid. The feather meal was a special product obtained from the Carroll Rendering Works of Carroll, Iowa. Chicken feathers containing 10 per cent packing-house fat were pressure-cooked for four hours, removed from the cooker, and ground. This product contained about 80 per cent protein as determined by the Kjeldahl method.

Generally, 2 grams of protein material were added to 100 milliliters of a one normal or two normal hydrochloric acid solution. This mixture was autoclaved at 15 p.s.i. for varying lengths of time (one to four hours was used most often), removed from the autoclave, and cooled. The pH of the acidified hydrolyzates was adjusted to 6.5-6.8

with 25 per cent sodium hydroxide. The insoluble material was removed by centrifugation and filtration.

The source of the inoculum, manner of processing the bacteria, and the basal medium used were the same as those described in the preceeding section.

Results and discussion

Untreated vitamin-free casein exhibited little if any stimulatory activity on cellulose digestion by washed rumen microorganisms (Table 19). Cellulolytic activity of the bacteria was depressed when a level of 100 milligrams of this substance was added to the fermentation tubes. A partial acid hydrolyzate of this same substance, on the other hand, had a pronounced stimulatory effect. Cellulose digestion was increased 48 per cent when 10 milligrams of the hydrolyzate were added to the fermentation tubes. This hydrolyzate was prepared by autoclaving a 2 per cent casein suspension in one normal hydrochloric acid for 1 hour. It is further shown in Table 19 that a commercial acid hydrolyzate of casein had very little cellulolytic stimulatory activity. This is particularly interesting since this casein preparation was completely hydrolyzed. The finding that untreated casein and completely hydrolyzed

Table 19. The influence of casein and acid hydrolyzates of casein upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	29.5	100	
	5 mg. vitamin-free casein	31.6	107	
	50 mg. vitamin-free casein	33.0	112	
	100 mg. vitamin-free casein	23.7	80	
	5 mg. vitamin-free casein hydrolyzate (1N HCl-1 hr.)	33.3	113	
	10 mg. vitamin-free casein hydrolyzate (1N HCl-1 hr.)	43.6	148	3.25
	None	67.1	100	
	10 mg. commercial casein hydrolyzate (completely hydrolyzed)	74.3	111	
	20 mg. commercial casein hydrolyzate (completely hydrolyzed)	72.5	108	1.99
-	None	32.8[a]	100	
	Casein hydrolyzate	49.9[b]	152	---

[a]Average of 14 experiments.

[b]Average activity for optimum levels of casein acid hydrolyzates from 14 experiments.

casein were practically inactive, whereas a partial hydrolyzate of casein was highly active, suggests that the stimulatory factors in the partial hydrolyzate of casein are intermediate breakdown products of protein molecules.

The concentration of acid and time of hydrolysis both had a marked effect upon the stimulatory activity of casein hydrolyzate as shown in Figure 4. A 2-milligram level was used in all these studies so the increases in cellulose digestion were not as high as might have occurred had a higher level been used. Using 100 as the relative activity value for cellulose digestion in the tubes containing untreated casein, it will be noted that maximum activity was obtained after autoclaving acid suspensions of casein for four to six hours. Autoclaving a 2 per cent casein suspension in two normal hydrochloric acid for 4 or 6 hours resulted in much more activity than a similar sample autoclaved for the same length of time in one normal hydrochloric acid. However, considerable activity was lost when casein in two normal hydrochloric acid was autoclaved for 12 hours.

In Table 20 are recorded the results obtained from other protein hydrolyzates. The concentration of acid used and the length of time these materials were autoclaved are given in parentheses after each treatment group. A 2 per cent suspension was used in every instance. These results

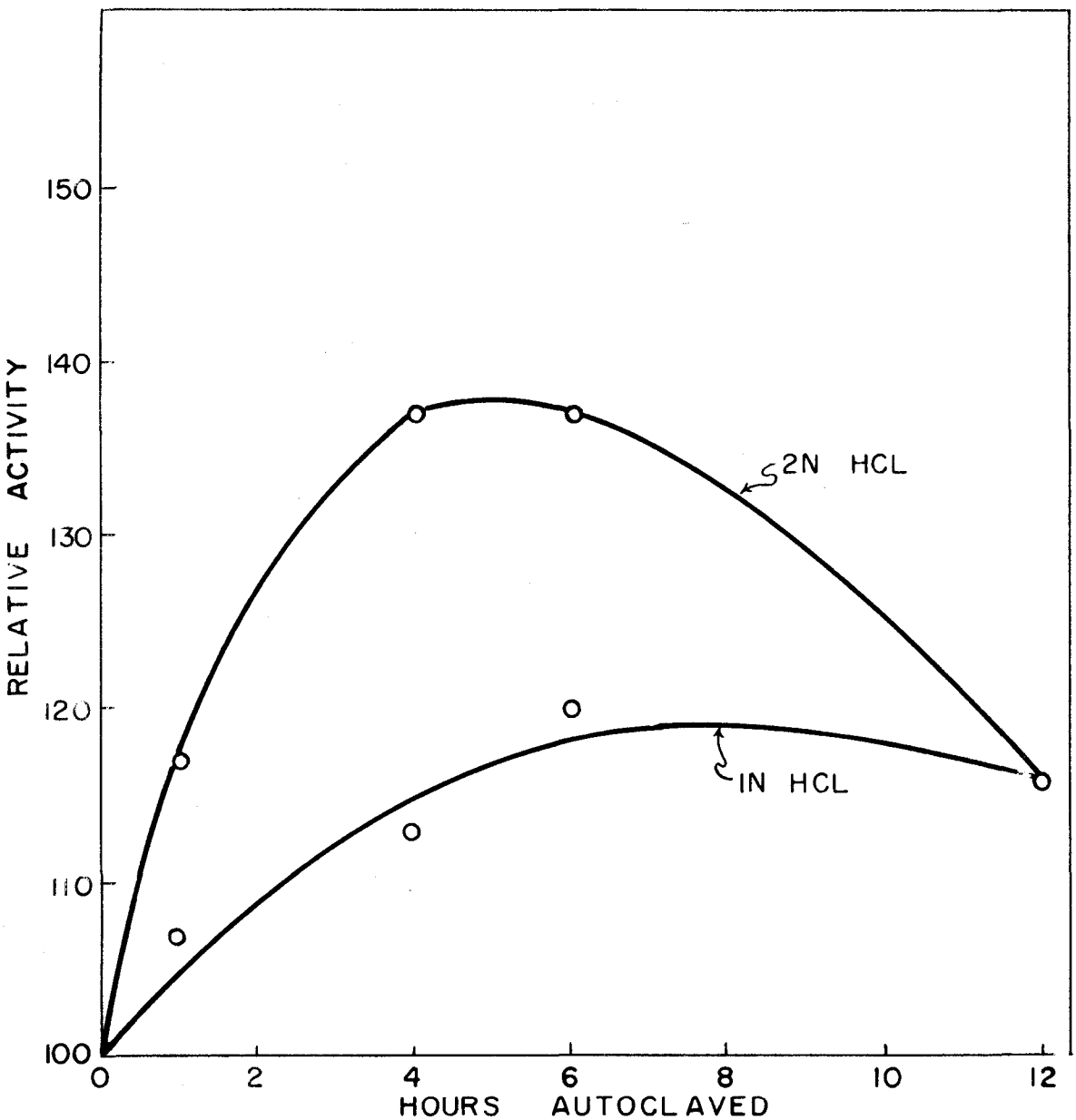


Figure 4. The influence of acid concentration and time of hydrolysis upon the cellulolytic stimulatory activity of casein hydrolyzate

Table 20. The influence of partial acid hydrolyzates of certain protein materials upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	17.7	100	
	1 mg. feather meal hydrolyzate (2N HCl-4 hrs.)	24.6	139	
	7 mg. feather meal hydrolyzate (2N HCl-4 hrs.)	38.8	219	
	1 mg. hair hydrolyzate (2N HCl-4 hrs.)	22.5	127	
	7 mg. hair hydrolyzate (2N HCl-4 hrs.)	28.5	161	
	7 mg. casein hydrolyzate (2N HCl-4 hrs.)	31.4	177	3.53
2	None	51.8	100	
	1 mg. soybean oil meal hydrolyzate (1N HCl-1 hr.)	57.3	111	
	7 mg. soybean oil meal hydrolyzate (1N HCl-1 hr.)	60.9	118	
	15 mg. soybean oil meal hydrolyzate (1N HCl-1 hr.)	60.9	118	
	1 mg. drackett protein hydrolyzate (1N HCl-1 hr.)	58.6	113	
	7 mg. drackett protein hydrolyzate (1N HCl-1 hr.)	63.7	123	
	15 mg. drackett protein hydrolyzate (1N HCl-1 hr.)	66.1	128	
	7 mg. casein hydrolyzate (1N HCl-1 hr.)	69.1	133	2.23

Table 20. (Continued)

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
3	None	40.5	100	
	2 mg. gelatin hydrolyzate (2N HCl-4 hrs.)	46.3	114	
	10 mg. gelatin hydrolyzate (2N HCl-4 hrs.)	55.7	138	
	15 mg. gelatin hydrolyzate (2N HCl-4 hrs.)	58.1	143	
	10 mg. feather meal hydrolyzate (2N HCl-4 hrs.)	66.8	165	4.54
-	None	32.5[a]	100	
	feather meal hydrolyzate	60.4[b]	186	---

[a]Average of 14 experiments.

[b]Average activity for optimum levels of feather meal hydrolyzate from 14 experiments.

show that feather meal hydrolyzate was an extremely potent source of cellulolytic stimulatory factors for rumen microorganisms. The increase in cellulose digestion resulting from feather meal hydrolyzate additions in all the experiments in which this material was used averaged 86 per cent. In some experiments cellulose digestion was more than doubled by feather meal hydrolyzate. Hair and gelatin hydrolyzates were quite active but did not increase cellulose digestion as much as feather meal hydrolyzate. Acid hydrolyzates of soybean oil meal and drackett protein were only slightly active.

The results in Table 20 might indicate that the sulfur amino acids are constituents or are in some manner related to the unidentified stimulatory factors in these hydrolyzates. Gelatin, which has only a trace of the sulfur amino acids, was inferior to feather meal as a source of these cellulolytic stimulatory factors. Feathers have an extremely high content of cystine and an appreciable amount of methionine. Further work is needed to clarify this point.

The results in Table 21 show that untreated feather meal was only slightly active, whereas a partial acid hydrolyzate of this same material stimulated cellulose digestion equally as much as did autoclaved rumen liquid. A combination of rumen liquid and feather meal hydrolyzate

Table 21. The influence of feather meal, feather meal hydrolyzate, autoclaved rumen liquid, and torula yeast plasmolyzate upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	24.7	100	
	1 mg. feather meal	28.2	114	
	5 mg. feather meal	28.3	114	
	10 mg. feather meal	25.5	103	
	1 mg. feather meal hydrolyzate (2N HCl-4 hrs.)	34.5	140	
	5 mg. feather meal hydrolyzate (2N HCl-4 hrs.)	48.9	198	
	10 mg. feather meal hydrolyzate (2N HCl-4 hrs.)	57.0	231	1.40
2	None	19.5	100	
	5 mg. feather meal hydrolyzate	55.5	285	
	10 mg. feather meal hydrolyzate	63.4	325	
	15 mg. feather meal hydrolyzate	65.4	337	
	3 ml. autoclaved rumen liquor	56.9	292	
	5 ml. autoclaved rumen liquor	64.3	330	
	6 ml. autoclaved rumen liquor	65.6	336	
	50 mg. torula yeast plasmolyzate	49.4	253	
	5 ml. autoclaved rumen liquid + 10 mg. feather meal hydrolyzate	74.0	379	
	50 mg. torula yeast plasmolyzate + 10 mg. feather meal hydrolyzate	72.4	371	1.23

stimulated microbial cellulose digestion in these experiments more than did either of these materials alone. Similar results were obtained with a combination of feather meal hydrolyzate and torula yeast plasmolyzate. One possible explanation for these results is that the active factors in feather meal hydrolyzate may differ from those in rumen liquid and torula yeast plasmolyzate.

Various fractionation studies of feather meal hydrolyzate were made in an attempt to find out more about the nature of the active factors in this material. The results of these studies are summarized in Table 22. Drying feather meal hydrolyzate by heating to 100° Centigrade did not result in any loss of activity so apparently the factors are relatively stable to heat. Steam distillation of acidified feather meal hydrolyzate did not remove the factors indicating that the volatile fatty acids are not involved. Repeated extraction of acidified or alkaline feather meal hydrolyzate with ether was not effective in removing an active fraction. The extracts did not show any activity while the extracted hydrolyzate was just as active as the untreated material.

An active fraction was obtained from feather meal hydrolyzate by repeated extraction with 70 per cent ethanol. This fraction was obtained by adding 100 milliliters of 70

Table 22. The influence of fractions of feather meal hydrolyzate upon cellulose digestion by rumen microorganisms *in vitro*

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	47.9	100	
	5 mg. heated feather meal hydrolyzate	66.9	140	
	10 mg. heated feather meal hydrolyzate	73.6	154	
	10 mg. feather meal hydrolyzate	73.9	154	1.65
2	None	27.0	100	
	0.5 ml. steam distillate of acidified feather meal hydrolyzate	30.6	113	
	1.0 ml. steam distillate of acidified feather meal hydrolyzate	27.4	101	
	0.5 ml. steam distilled feather meal hydrolyzate	44.2	164	
	1.0 ml. steam distilled feather meal hydrolyzate	53.6	199	
	10 mg. feather meal hydrolyzate	50.4	187	1.35

Table 22. (Continued)

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
3	None	61.9	100	
	0.5 ml. ether extract of acidified feather meal hydrolyzate	57.6	93	
	1.0 ml. ether extract of acidified feather meal hydrolyzate	60.6	98	
	1.5 ml. ether extract of acidified feather meal hydrolyzate	62.5	101	
	0.5 ml. ether extracted feather meal hydrolyzate	79.9	129	
	1.0 ml. ether extracted feather meal hydrolyzate	84.2	136	
	1.5 ml. ether extracted feather meal hydrolyzate	88.3	143	
	15 mg. feather meal hydrolyzate	86.7	140	2.82
4	None	37.2	100	
	0.5 ml. ether extract of alkaline feather meal hydrolyzate	36.0	97	
	1.0 ml. ether extract of alkaline feather meal hydrolyzate	35.1	94	
	0.5 ml. ether extracted feather meal hydrolyzate	53.7	144	
	1.0 ml. ether extracted feather meal hydrolyzate	62.0	167	

Table 22. (Continued)

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
	0.5 ml. ethanol extract of feather meal hydrolyzate	55.8	150	
	1.0 ml. ethanol extract of feather meal hydrolyzate	62.1	167	
	1.0 ml. ethanol extracted feather meal hydrolyzate	58.0	156	
	1.0 ml. ethanol precipitate	58.5	157	1.10
5	None	44.7	100	
	0.5 ml. hydrolyzed feather meal dialyzate (1st 24 hrs.)	66.6	149	
	0.5 ml. hydrolyzed feather meal dialyzate (2nd 24 hrs.)	52.2	117	
	0.5 ml. hydrolyzed feather meal dialyzate (3rd 24 hrs.)	45.5	102	
	0.5 ml. dialyzed feather meal hydrolyzate	46.6	104	
	1.0 ml. dialyzed feather meal hydrolyzate	44.8	100	
	10 mg. feather meal hydrolyzate	69.2	155	1.35

per cent ethanol to 100 milliliters of feather meal hydrolyzate. The mixture was agitated vigorously for about 10 minutes. The alcohol fraction was removed by adding 50 milliliters of ether to the mixture. The water layer was removed from the alcohol-ether layer with the aid of a separatory funnel. This extraction with ethanol was repeated until no more colored material was obtained. The ethanol-ether fractions were combined and reduced to dryness over a steam plate. The solids were redissolved in 100 milliliters of distilled water and assayed. As shown in Table 22 this fraction increased cellulose digestion by 67 per cent. However, the extracted feather meal hydrolyzate was also active indicating that the extraction was not successful in removing all the stimulatory factors. When equal volumes of 70 per cent ethanol and feather meal hydrolyzate were mixed and allowed to stand for 24 hours a precipitate was formed. This precipitate, when redissolved in distilled water and assayed, showed some stimulatory activity.

In another phase of the fractionation studies an attempt was made to remove the active factors by dialysis. One-hundred milliliters of feather meal hydrolyzate were placed in a semi-permeable cellophane bag and immersed into 800 milliliters of distilled water. After refrigeration

for 24 hours the water was changed. This was repeated for six consecutive 24-hour periods. Each of the water fractions was reduced to 50 milliliters over a steam plate and assayed. The hydrolyzate was dialyzed against running water for three additional days. The results from these studies (Table 22) show that the factors were completely removed by this procedure indicating that the active factors are relatively small molecules.

Bentley et al. (6) and Bryant and Doetsch (11) reported that five and six carbon fatty acids accounted for most of the rumen microbial stimulatory factors in rumen liquid. It was also reported that certain amino acids were active since they could act as precursors for these volatile fatty acids. Consequently, these fatty acids as well as a steam distillate of acidified rumen liquid were assayed to determine if these compounds were active under the conditions of these experiments.

The results of these experiments (Table 23) show that neither n-valeric acid, n-caproic acid nor a mixture of n-valeric and isovaleric acids enhanced microbial cellulose digestion when tested over a wide range of levels. Even more significant was the failure to obtain a cellulolytic stimulatory response from steam distillates of acidified rumen liquid. These findings along with the fractionation

Table 23. The influence of some volatile fatty acids and steam distillate of acidified rumen liquid upon cellulose digestion by rumen microorganisms *in vitro*[a]

Experiment number	Treatment per tube	Cellulose digested %	Relative activity	Std. dev. %
1	None	39.3	100	
	1.0 ml. steam distillate of acidified fresh rumen liquid	39.5	100	
	2.0 ml. steam distillate of acidified fresh rumen liquid	40.8	104	2.07
2	None	36.4	100	
	0.1 mg. n-valeric acid	37.7	104	
	0.5 mg. n-valeric acid	30.4	84	
	1.0 mg. n-valeric acid	31.2	86	
	0.1 mg. n-caproic acid	35.8	98	
	0.5 mg. n-caproic acid	35.0	96	
	1.0 mg. n-caproic acid	30.6	84	
	10 mg. feather meal hydrolyzate (2N HCl-4 hrs.)	71.8	197	2.47
3	None	24.6	100	
	1 mcg. n-valeric acid + 0.5 mcg. isovaleric acid	25.1	102	
	10 mcg. n-valeric acid + 5 mcg. isovaleric acid	24.6	100	
	30 mcg. n-valeric acid + 15 mcg. isovaleric acid	22.1	90	
	1 mg. isocaproic acid	26.1	106	
	10 mg. feather meal hydrolyzate (2N HCl-4 hrs.)	46.9	191	5.88

[a]para-Aminobenzoic acid (100 mcg. per 20 ml. of basal medium) was used in place of vitamin B_{12} in these experiments.

studies of feather meal hydrolyzate indicate that the volatile fatty acids are not the stimulatory factors in the protein hydrolyzates. Apparently, under the conditions of these experiments other microbial stimulatory factors are involved.

Summary

Partial acid hydrolyzates of various protein materials were found to be very good sources of cellulolytic stimulatory factors for rumen microorganisms. Hydrolyzates of feather meal, vitamin-free casein, hair, gelatin, soybean oil meal, and drackett protein all showed some activity. Of the conditions of hydrolysis studied, a two normal hydrochloric acid solution and an autoclaving time of 4 or 6 hours appeared to be best when a 2 per cent protein suspension was hydrolyzed. Untreated vitamin-free casein, feather meal, and completely hydrolyzed vitamin-free casein exhibited only slight, if any, stimulatory activity. Feather meal hydrolyzate was equally as active as autoclaved rumen liquid, and a combination of these two materials was more active than either alone as determined under the conditions of these experiments. The active factors in feather meal hydrolyzate were found to be relatively heat stable and

dialyzable. They were not removed from an acidified solution by steam distillation or ether extraction. Some activity was removed from aqueous solution by repeated extractions with 70 per cent ethanol.

Additions of either n-valeric acid, n-caproic acid, a mixture of n-valeric and isovaleric acids, or a steam distillate of acidified rumen liquid failed to stimulate cellulose digestion by rumen microorganisms under the conditions of these experiments. These results and those obtained from partial acid hydrolyzates suggest that water soluble peptides may be involved in rumen microbial cellulose digestion *in vitro*.

The Effects of Protein Hydrolyzates and a Press Yeast Liquor Concentrate upon Body Weight Gains and Feed Consumption by Lambs

Artificial rumen studies using the washed suspension technique demonstrated that partial protein hydrolyzates and press yeast liquor contain factors which enhance the cellulolytic activity of rumen microorganisms. The addition of these substances to fermentation tubes containing all the known chemically defined nutrients required by rumen

microorganisms increased cellulose digestion considerably in several experiments.

The purpose of these experiments was to determine the effects of protein hydrolyzates and a press yeast liquor concentrate upon body weight gains and feed consumption by lambs fed semi-purified rations.

Materials and methods

These studies consisted of two individual feeding experiments. Experiment I was conducted during the summer of 1954 and Experiment II was conducted during the winter of 1954-55. Thirty-two California feeder lambs (ewes) weighing approximately 65 pounds each were obtained for use in the first experiment. The lambs were vaccinated against enterotoxemia and allowed a rest period of one week following their arrival in Ames. During this period the lambs were fed mixed hay, corn, and soybean oil meal.

After the rest period all lambs were placed in individual feeding pens and fed ad libitum the basal semi-purified rations shown in Table 24. One group of 18 lambs was fed Ration I while the second group of 14 lambs received Ration II. Individual daily feed consumption data for both groups of lambs were collected for a period of 14 days.

Table 24. Experimental basal rations individually fed to lambs in Experiments I and II[a]

(Expressed in pounds)

Constituent	Basal ration	
	I	II
Corncobs	----	100.0
Wheat straw	20.0	----
Wood cellulose (Solka-floc)	40.0	30.0
Corn starch	61.8	22.8
Cerelose	48.0	20.0
Lard	6.0	5.0
Urea	6.0	6.0
Crude casein	10.0	8.0
Minerals[b]	7.0	7.0
Methionine	1.0	1.0
Choline	0.2	0.2
Total	200.0	200.0

[a]To each ration (per 200 lbs.) were added 250,000 units synthetic vitamin A, 30,000 units of vitamin D as Delsterol, and 1500 mg. of vitamin E as Alpha Tocopherol.

[b]The mineral mixture was made up as follows: monocalcium phosphate 9600 gm., potassium carbonate 8000 gm., sodium chloride 2000 gm., magnesium sulfate 1200 gm., ferric citrate 421 gm., manganese sulfate 113 gm., copper sulfate 15 gm., zinc chloride 11 gm., calcium fluoride 8 gm., cobalt sulfate 2 gm., potassium iodide 1 gm.

Following this pre-experimental period, two lambs in each group were discarded while the remaining lambs of each group were placed in outcome groups on the basis of total feed consumption during the 14-day period and randomly assigned to treatments. Four groups of four lambs each were fed Ration I. Group I received the basal ration only, Group II and Group III lambs were fed the basal ration plus 100 and 200 milliliters of casein hydrolyzate per pound of ration, respectively, and Group IV received the basal ration plus 0.025 pound of a press yeast liquor concentrate per pound of ration.

Three groups of four lambs each were fed a semi-purified ration containing 50 per cent finely ground corncobs (Ration II). Group I served as the unsupplemented control, Group II received 100 milliliters of casein hydrolyzate per pound of ration, and Group III received 0.025 pound of a press yeast liquor concentrate per pound of ration.

Thirty wether lambs averaging 55 pounds each were obtained from South Dakota and used in the second feeding experiment conducted during the winter of 1954-55. These lambs were handled in a manner similar to that used with the lambs in the first experiment. However, since these lambs appeared to be infected with parasites, they were

drenched with a phenothiazine-lead arsenate mixture three days after their arrival and again the day they were placed on the experimental treatments. The lambs were placed in outcome groups on the basis of total feed consumption of the basal ration during a 12-day pre-experimental period and randomly allotted to treatments. Only semi-purified Ration I (Table 24) was used in this experiment.

In the second feeding experiment there were four groups of six lambs each. The treatments consisted of varying levels of feather meal hydrolyzate. Groups I, II, III, and IV received 0, 10 milliliters, 20 milliliters, and 40 milliliters of feather meal hydrolyzate per pound of ration, respectively.

All lambs in both experiments were kept in individual pens and bedded with wood shavings. The lambs were individually fed ad libitum with water and trace-mineralized salt being available at all times. Daily feed consumption and 14-day weights were recorded throughout the experimental periods. In the first experiment, lambs were fed for 56 days with the exception of lambs receiving the press yeast liquor concentrate. Due to a shortage of this material, the feeding period was terminated at the end of 42 days for the lambs on Ration I and at 35 days for

those on the corncob ration. In the second experiment, all lambs were fed the experimental rations for 60 days.

Casein hydrolyzate was prepared by hydrolyzing a 2 per cent suspension of vitamin-free casein with two normal hydrochloric acid. This mixture was autoclaved for 4 hours, cooled, and the pH adjusted to 3.0 with 25 per cent sodium hydroxide. The insoluble material was then removed by centrifugation and filtration. The pH of the filtrate was adjusted to 6.5-6.8 with further additions of sodium hydroxide. This solution was prepared each day throughout the feeding period to prevent any possibility of contamination. One liter of casein hydrolyzate was added to 0.5 pound of ground wheat straw in a shallow pan and dried in a forced-air oven at 60° Centigrade. Sufficient quantities of the straw containing the dried hydrolyzate was added to the basal ration to give the desired level of casein hydrolyzate. Casein hydrolyzate was added to the semi-purified ration containing corncobs by mixing 100 milliliters of the solution with each pound of basal ration. The material was not dried prior to feeding.

The press yeast liquor concentrate was prepared by the Red Star Yeast and Products Company. This material consisted of press yeast liquor obtained from Active Dry Yeast cream which had been concentrated over a boiling water bath. An

analysis of this product showed that it contained approximately 40 per cent dry matter and about 9-10 per cent protein on a wet basis. Press yeast liquor concentrate was mixed with the basal ration each day prior to feeding.

The feather meal hydrolyzate used in the second experiment was prepared by hydrolyzing a 5 per cent feather meal suspension with three normal hydrochloric acid (feather meal was obtained from the Carroll Rendering Works). This mixture was autoclaved for 4 hours, cooled, and the pH adjusted to 6.5-6.8 by adding solid technical grade sodium hydroxide. The solution was strained through six layers of cheesecloth after neutralization. Feather meal hydrolyzate was dried on finely ground wheat straw at 60° Centigrade and added to the rations in this form.

The basal rations for the control groups of lambs are shown in Table 24. These semi-purified rations are similar to that used by Ruf *et al.* (62). In the case of the ration for the treatment groups, minute amounts of casein and starch were omitted so that the protein content of all rations fed were equal. The protein content of the rations was 12.2 per cent with about 65 per cent of the total nitrogen being supplied by urea. Since considerable salt was present in the protein hydrolyzates, additional amounts of sodium chloride were added so that the salt content of

all rations was equal to that ration which contained the highest level of protein hydrolyzate.

Results and discussion

The results of the first experiment are summarized in Table 25. Considering the results with Ration I, it will be noted that lambs fed a semi-purified ration supplemented with either casein hydrolyzate or a press yeast liquor concentrate consumed more feed and made larger gains than lambs fed the unsupplemented ration. The addition of casein hydrolyzate (100 milliliters per pound of ration) resulted in a 50 per cent increase in rate of gain and a 11 per cent increase in feed consumption; whereas the increases in gains and feed consumption by lambs receiving press yeast liquor were even more pronounced, being 68 per cent and 19 per cent, respectively.

Lambs consuming a semi-purified ration containing 50 per cent corncobs and supplemented with casein hydrolyzate consumed 35 per cent more feed than unsupplemented lambs. However, the rate of gain was increased by only 27 per cent. These results, as well as the appearance of these lambs, indicated that the corncob ration was poorly utilized by the lambs. Many of the lambs in Group II had a "paunchy"

Table 25. Rate of gain and feed consumption by lambs fed either casein hydrolyzate or a press yeast liquor concentrate

Ration	Group number and treatment	Average daily gain lbs.	Average daily feed consumed lbs.
I	I Basal ration	0.22	2.46
	II Basal ration plus 100 ml. casein hydrolyzate per lb. of ration	0.33	2.73
	III Basal ration plus 200 ml. casein hydrolyzate per lb. of ration	0.30	2.69
	IV Basal ration plus 0.025 lb. press yeast liquor concentrate per lb. of ration	0.37	2.92
II	I Basal ration	0.22	2.81
	II Basal ration plus 100 ml. casein hydrolyzate per lb. of ration	0.28	3.64
	III Basal ration plus 0.025 lb. press yeast liquor concentrate per lb. of ration	0.29	2.91

appearance. Some of the increased gains of this group were probably due to extra fill. Apparently, 50 per cent corncobs was too high a concentration of this poor quality roughage material to include in lamb rations as supplemented in this experiment.

The press yeast liquor concentrate addition to Ration II resulted in an increased daily gain but very little increase in feed consumption. However, this material was fed only 35 days so these results are not conclusive.

These results of the first feeding experiment have to be accepted with certain reservations since none of the differences in either rate of gain or feed consumption were statistically significant. Since only four lambs were used for each treatment and the variation among lambs on the same treatment was very great, an extremely large difference would have been necessary for statistical significance.

The results obtained from feather meal hydrolyzate in Experiment II (Table 26) paralleled those obtained with casein hydrolyzate in the first experiment. A feather meal hydrolyzate addition to the basal ration (40 milliliters per pound) resulted in a 35 per cent increase in average daily gains and a 21 per cent increase in feed consumption. This level of feather meal hydrolyzate is approximately equivalent to the 100 milliliters of casein hydrolyzate

Table 26. Rate of gain and feed consumption by lambs fed feather meal hydrolyzate

Group number and treatment	Average daily gain lbs.	Average daily feed consumption lbs.
I Basal ration	0.23 (0.23)[a]	1.79 (1.81)
II Basal ration plus 10 ml. feather meal hydrolyzate per pound of ration	0.23 (0.26)[b]	1.79 (1.88)
III Basal ration plus 20 ml. feather meal hydrolyzate per pound of ration	0.31 (0.36)[b]	2.16 (2.45)
IV Basal ration plus 40 ml. feather meal hydrolyzate per pound of ration	0.29 (0.33)[b]	2.06 (2.20)

[a]Average of five lambs.

[b]Average of four lambs.

per pound of ration used in Experiment I. There was no apparent increase in rate of gain and feed consumption when the ration was supplemented with only 10 milliliters of feather meal hydrolyzate per pound of ration. Lambs receiving 40 milliliters of feather meal hydrolyzate per pound of ration did not perform any better than lambs receiving the intermediate level.

All lambs used in Experiment II were drenched with a phenothiazine-lead arsenate mixture on the same day they were started on the experimental rations. After drenching, one lamb in the control group and two lambs in each of the three remaining groups failed to eat any feed for a period of 10-14 days. This loss in appetite was apparently due to the strong drenching solution. When it became apparent that these lambs were not going to start eating on their own volition, an attempt was made to restore the appetite of these lambs with a yeast solution. A 5 per cent yeast solution was prepared which contained equal amounts of yeast extract (Difco) and plasmolyzed torula yeast. Each of the seven lambs were drenched with 100 milliliters of this solution for three consecutive days. In every case there was a complete and rapid recovery of appetite.

These lambs were continued on the experimental rations although some of them had lost considerable weight during

the time they were off feed. As shown by the values in parentheses in Table 26, the results from the feather meal hydrolyzate additions were improved when the values for these lambs were omitted.

The variation in feed consumption and rate of gain among lambs treated alike was also very large in this experiment. As a result, none of the differences in either rate of gain or feed consumption were statistically significant. However, these results, as well as those obtained in the first experiment, while not conclusive, are a good indication that these protein hydrolyzates and press yeast liquor contain factors which will improve the rate of gain and feed consumption by lambs fed semi-purified rations. The results further indicate that yeast products contain factors which are associated with appetite in lambs.

Summary

Two feeding experiments were conducted in which the effects of protein hydrolyzates and a press yeast liquor concentrate upon the body weight gains and feed consumption by lambs fed semi-purified rations were studied. Rate of gain and feed consumption were increased considerably in both experiments by these materials. Although the

differences in gains and feed consumption were not statistically significant, they are suggestive that protein hydrolyzates and press yeast liquor contain factors which are needed by lambs for maximum performance. It was also found that a solution of yeast products, when given orally, restored appetites in lambs that had been off feed for periods of time ranging from 10-14 days. However, since no control lambs were used, more extensive studies are being conducted to confirm this observation.

GENERAL DISCUSSION

The results of the experiments reported in this thesis show that washed rumen microorganisms have specific nutrient requirements for maximum cellulose digestion. These requirements were shown to consist of chemically known nutrients as well as factors of an unknown nature. In addition to enhancing cellulose digestion *in vitro*, the addition of potent sources of these unidentified factors to semi-purified rations resulted in large apparent increases in daily gains and feed consumption by lambs. These results are suggestive that these factors may be of importance to ruminants generally in so far as maximum and most efficient performance on a given ration is concerned. In this regard it appears likely that the best results would be obtained if these factors were to be added to rations containing large amounts of low quality roughages and rations in which a non-protein nitrogen source supplies a major part of the total nitrogen. Many of the high quality roughages and protein concentrates commonly fed to cattle and sheep contain these factors, therefore, the inclusion of additional amounts of them in such rations may not be beneficial. However, the possibility that these factors may have a beneficial influence

when added to such rations cannot be excluded on the basis of the information available at the present time.

These studies were not successful in elucidating the chemical identity of the unidentified cellulolytic factors although additional information concerning their nature was obtained. The finding that certain B-vitamins were stimulatory to microbial cellulose digestion in vitro would indicate that the stimulatory activity of yeast extract, rumen liquid, and possibly other sources of these factors may be due in part to the B-vitamin content of these materials. In this regard the results reported in this thesis confirm earlier studies (32, 5) which showed that certain B-vitamins stimulated cellulose digestion by rumen microorganisms. It was also reported in these studies that the stimulatory action of the B-vitamins was not equal to that exhibited by such materials as yeast extract or rumen liquid.

It is not yet known whether the addition of these B-vitamins which stimulated cellulose digestion in vitro to ruminant rations would increase rumen microbial activity. There are a few reports in the literature which indicate they do not improve the performance of sheep. Head (33) reported that a mixture of the B-complex vitamins did not improve the digestibility of the dry matter by sheep on

hay rations. Stitt *et al.* (66) found no beneficial effects upon the performance of pregnant ewes or the lambs produced by adding either vitamin B_{12} or a mixture of vitamin B_{12}, thiamine, pyridoxine, choline chloride, folic acid, and biotin to corncob rations. The results of these experiments are in agreement with the generally accepted concept that under normal conditions rumen microorganisms synthesize sufficient quantities of the B-vitamins to meet their needs as well as that of their host. It is conceivable that these B-vitamins required by cellulolytic rumen bacteria *in vitro* may be supplied by the microbial synthesis of other types of rumen bacteria inasmuch as symbiotic relationships undoubtedly exist in the normal rumen.

The data concerning the influence of partial hydrolyzates of certain protein materials upon microbial cellulose digestion suggest that intermediate breakdown products of the protein molecule may be the active principles in these substances. It was shown that untreated casein and a commercial hydrolyzate of casein which was completely hydrolyzed exhibited only slight activity, whereas a partial acid hydrolyzate of casein was very active. Untreated feather meal was inactive while a partial acid hydrolyzate of this same material stimulated cellulose digestion considerably in numerous experiments. Similar results were obtained with

other protein materials. While these findings are suggestive that water soluble peptides may be the active factors, no definite conclusions can be drawn at this time. The possibility that the activity is due to some contaminant which is released from the protein material during the acid hydrolysis cannot be excluded, although this seems unlikely.

That peptides may be growth factors for bacteria has been demonstrated in numerous reports. Sprince and Woolley (65) found that a partial hydrolyzate of casein contained factors required for growth by certain hemolytic streptococci and the bacterium, Lactobacillus casei. These workers called this growth factor strepogenin and suggested that it was a peptide. In later work (73), synthetic oxytocin (an octapeptide) was found to have a high strepogenin activity. These results definitely excluded the possibility of a contaminant being involved since a synthetic peptide was used. In this same experiment, it was reported that the cystine residue in the peptide was essential for activity. These results are interesting in view of the findings in this thesis that a feather meal hydrolyzate was more active than a similar hydrolyzate of gelatin for rumen microorganisms. Feathers have a high content of the sulfur amino acids, particularly cystine, while gelatin contains only a trace of these compounds.

Kihara *et al.* (42) isolated peptide fractions from a purified soybean protein hydrolyzate which possessed considerable growth promoting activity for *Lactobacillus casei*. Coleman (23) separated and partially purified a growth factor for this same organism from yeast. This factor appeared to be a peptide. Dunn *et al.* (27) reported that a partial acid hydrolyzate of casein stimulated the growth of *Lactobacillus casei*. An enzymatic hydrolyzate of casein was found to be highly stimulatory to cellulose digestion by rumen microorganisms by Huhtanen (36). These experimental results and those reported in this thesis suggest that peptides may be very important in microbial metabolism. While the mode of action of these materials is not known, it is believed that they may be involved in protein synthesis.

The failure of either steam distillates of acidified rumen liquid or short-chain fatty acids to stimulate cellulose digestion in these experiments does not agree with the results reported by Bentley *et al.* (6). There are several possible reasons which might account for this discrepancy. Due to the varied conditions and techniques used in the assay procedure, it is very unlikely that the same strains or types of rumen bacteria were used in both studies. Rumen bacteria used in the experiments reported in this thesis were obtained from an animal on a high corncob

ration, whereas the Ohio workers obtained their bacteria from an animal on good quality alfalfa hay. Also, slightly different procedures were used in processing the bacteria. It is possible that strains of rumen bacteria were present in the inocula used in the experiments reported herein which synthesized these fatty acids. The fact that these fatty acids have been isolated from the rumen is an indication that they may be produced by certain types of rumen microorganisms.

The preliminary observations on the effects of yeast products upon the recovery of appetite in lambs merits further study. If these results can be verified with a larger number of animals, it is felt that this would be very significant. A product such as yeast plasmolyzate which would effectively stimulate appetite in animals off feed due to disease or various other causes, would have wide application. It is possible that such a product, if included in ruminant rations, would help prevent animals from going off feed, as well as to facilitate the adaptation of the rumen flora to changes in feeding regimes such as occur when animals are changed from a high roughage to a high grain diet.

If future experiments show that these unidentified factors will definitely improve gains and/or feed utilization

by cattle and sheep, then, potent sources of them which would be available in large quantities will be needed. Chicken feathers which are a farm waste product at the present time and which are available in large amounts may be one of the better potential sources providing that commercial processing and handling of large quantities of liquid are not prohibitive in so far as cost is concerned. Hydrolytic agents other than acid may be more applicable for industrial use since acids would be detrimental to metal cookers. Preliminary studies with sodium hydroxide and the proteolytic enzyme, trypsin, indicated that these materials may be effective hydrolytic agents. Such materials could be used in metal containers without any major destructive effects.

The inclusion of large quantities of liquid such as a protein hydrolyzate in feed supplements might be a problem although not a major one. One possible way would be to first mix the hydrolyzate with molasses. The resulting liquid mixture could then be mixed with supplements containing a large amount of corncobs or other suitable liquid carriers.

Another very good potential source of these factors which could be added to cattle and sheep feeds is torula yeast plasmolyzate. This product which is produced from

sulfite waste liquor is available in large quantities. In addition to containing a high concentration of unidentified microbial stimulatory factors, this product also contains other valuable nutrients such as protein and the B-vitamins. It also has the added advantage of being in a very convenient form for handling and mixing into rations.

The identity of the stimulatory factors in yeast products is not known. The finding that a combination of feather meal hydrolyzate and yeast plasmolyzate was more stimulatory than the hydrolyzate alone, suggests that the yeast product contains additional factors other than those in the feather meal hydrolyzate. It is conceivable that some of the activity in yeast products might be due to the highly unsaturated hydrocarbon, squalene. This compound has been found to occur in yeast in a relatively high concentration (68). More recently, studies have been reported which showed that squalene was a precursor of cholesterol (45, 71). In view of the Missouri work (9) showing that cholesterol stimulated microbial cellulose digestion, it is possible that a precursor of cholesterol, such as squalene, may also be active cellulolytically. Experimental data are needed to either refute or confirm this supposition. It is of interest that isovalerate which has been shown to be used in the biosynthesis of cholesterol (8) has also

been found to stimulate cellulose digestion by rumen microorganisms (11, 6). Whether these results are directly related or purely coincidental is not ascertainable at the present time.

One seeming limitation in these thesis data is the fact that only one animal on a specific ration was used as the source of rumen microorganisms throughout these studies. The use of a ration which had a low concentration of unidentified factors and a large amount of poor quality roughage would appear to be justified considering the objectives of this study. In order to study the factors influencing low quality roughage utilization, it was felt that rumen cellulolytic bacteria should be obtained from an animal receiving this type of ration. The results obtained in *in vitro* studies with these types of bacteria should have more direct application to *in vivo* experiments.

Factors found to influence rumen microbial activity *in vitro* may or may not be beneficial when added to cattle and sheep rations. Thus, results of *in vitro* studies with rumen microorganism will necessarily need be corroborated with the live animal. At best then, *in vitro* techniques are a screening tool for studying many factors which might have application in feeding practices. Regardless of these limitations, it is believed that *in vitro* studies with

rumen microorganisms have and will continue to contribute much valuable information concerning the nutrition of cattle and sheep.

SUMMARY

A technique employing washed suspensions of rumen microorganisms was developed for the study of factors influencing cellulose digestion *in vitro*. The technique consisted of separating the bacteria from strained rumen liquid by differential centrifugation. The bacterial cells were washed twice before being used to inoculate a chemically defined nutrient solution containing purified wood cellulose. When washed rumen microorganisms were suspended in 20 milliliters of a nutrient solution containing 0.5 per cent cellulose, approximately 40-60 per cent of the cellulose was digested during an incubation period of 24 hours. Conditions required for optimum cellulolytic activity, such as pH, concentration of the substrate, concentration of rumen bacteria, and the duration of incubation were determined.

The mineral and nitrogen requirements of rumen microorganisms for maximum cellulose digestion were determined by using the washed suspension technique. Among the 11 mineral elements tested, calcium, zinc, cobalt, and copper did not significantly increase microbial cellulose digestion. Levels of 5, 5, and 16 parts per million of cobalt, copper, and zinc, respectively, inhibited cellulose

digestion. The optimum ratio of sulfur to nitrogen required by rumen microorganisms was found to be in the range of 1:10 to 1:17.

Several of the B-vitamins were found to enhance cellulose digestion by rumen microorganisms when added individually to fermentation tubes. These vitamins were: pseudovitamin B_{12}, vitamin B_{12}, biotin, folic acid, pyridoxine, para-aminobenzoic acid, and riboflavin. A combination of vitamin B_{12} and biotin stimulated microbial cellulose digestion more than did either of these vitamins alone. This combination of vitamins was equally as active as any other combination of vitamins studied. No single B-vitamin or combination of B-vitamins was found which increased microbial cellulose digestion to the same extent as did yeast extract.

Of the carbohydrates studied, xylose, arabinose, ribose, rhamnose, sorbose, and maltose stimulated cellulose digestion only slightly. High levels of many of these compounds depressed cellulose digestion by rumen microorganisms.

Fractionation studies of different types of yeasts and yeast fermentation by-products showed that plasmolyzed torula yeast cream stimulated cellulose digestion <u>in vitro</u> by rumen microorganisms markedly. The plasmolysis apparently concentrated the active cellulolytic factors outside the yeast cells making them more accessible to rumen bacteria.

A partial acid hydrolyzate of feather meal was shown to be equally as active as autoclaved rumen liquid in stimulating cellulose digestion *in vitro* by rumen microorganisms. Partial acid hydrolyzates of casein, hair, soybean oil meal, gelatin, and drackett protein were also active. Untreated vitamin-free casein, feather meal, and completely hydrolyzed vitamin-free casein exhibited only slight if any activity.

The active factors in feather meal hydrolyzate were found to be relatively stable to heat and dialyzable. They were not removed from an acidified solution by steam distillation or ether extraction. Some activity was removed from aqueous solution by repeated extractions with 70 per cent ethanol.

Additions of either n-valeric acid, n-caproic acid, a mixture of n-valeric and isovaleric acids, or a steam distillate of acidified rumen liquid failed to stimulate cellulose digestion by rumen microorganisms under the conditions of these experiments. These results and those obtained from partial acid hydrolyzates of various protein materials suggest that water soluble peptides may be involved in rumen microbial cellulose digestion *in vitro*.

Two feeding experiments were conducted in which the effects of protein hydrolyzates and a press yeast liquor concentrate upon the body weight gains and feed consumption

by lambs fed semi-purified rations were studied. Rate of gain and feed consumption were increased considerably in both experiments by these materials. Although the differences in gain and feed consumption were not statistically significant, the results of these experiments suggest that protein hydrolyzates and press yeast liquor contain factors which are needed by lambs for maximum performance. It was also found that a solution of yeast products, when given orally, restored appetites in lambs that had been off feed for periods of time ranging from 10-14 days.

REFERENCES CITED

1. Annison, E. F. Some observations on volatile fatty acids in the sheep's rumen. Biochem. J. 57:400-405. 1954.

2. Arias, C., Burroughs, W., Gerlaugh, P. and Bethke, R. M. The influence of different amounts and sources of energy upon *in vitro* urea utilization by rumen microorganisms. J. Animal Sci. 10:683-692. 1951.

3. Beeson, W. M. and Perry, T. W. Balancing the nutritional deficiencies of roughages for beef steers. J. Animal Sci. 11:501-515. 1952.

4. Belasco, I. J. New nitrogen feed compounds for ruminants--a laboratory evaluation. J. Animal Sci. 13:601-610. 1954.

5. Bentley, O. G., Johnson, R. R., Vanecko, S. and Hunt, C. H. Studies on factors needed by rumen microorganisms for cellulose digestion *in vitro*. J. Animal Sci. 13:581-593. 1954.

6. Bentley, O. G., Lehmkuhl, A., Johnson, R. R. and Moxon, A. L. The stimulatory effect of certain fatty acids on *in vitro* cellulose digestion by rumen microorganisms. (Abstract) J. Animal Sci. 13:61. 1954.

7. Bentley, O. G., Moinuddin, M., Hershberger, T. V., Klosterman, E. W. and Moxon, A. L. The effect of trace minerals on growth performance and vitamin B_{12} synthesis of steers. J. Animal Sci. 13:789-801. 1954.

8. Brady, R. O. and Gurin, S. The synthesis of radioactive cholesterol and fatty acids *in vitro*. J. Biol. Chem. 189:371-377. 1951.

9. Brooks, C. C., Garner, G. B., Muhrer, M. E. and Pfander, W. H. Effect of some steroid compounds on bovine rumen function. Science 120:455-456. 1954.

10. Bryant, M. P. The isolation and characterization of a spirochaete from the bovine rumen. J. Bacteriol. 64:325-335. 1952.

11. Bryant, M. P. and Doetsch, R. N. Factors necessary for the growth of Bacteroides succinogenes in the volatile acid fraction of rumen fluid. Science 120:944-945. 1954.

12. Burroughs, W., Arias, C., DePaul, P., Gerlaugh, B. and Bethke, R. M. In vitro observations upon the nature of protein influences upon urea utilization by rumen micro-organisms. J. Animal Sci. 10:672-682. 1951.

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

14. Burroughs, W., Gall, L. S., Gerlaugh, P. and Bethke, R. M. The influence of casein upon roughage digestion in cattle with rumen bacteriological studies. J. Animal Sci. 9:214-220. 1950.

15. Burroughs, W. and Gerlaugh, P. The influence of soybean oil meal upon roughage digestion in cattle. J. Animal Sci. 8:3-9. 1949.

16. Burroughs, W., Gerlaugh, P. and Bethke, R. M. The influence of alfalfa ash and fractions of alfalfa meal upon the digestion of ground corncobs. J. Animal Sci. 9:207-213. 1950.

17. Burroughs, W., Gerlaugh, P., Edington, B. H. and Bethke, R. M. The influence of cornstarch upon roughage digestion in cattle. J. Animal Sci. 8:271-278. 1949.

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

19. Burroughs, W., Latona, A., DePaul, P., Gerlaugh, P. and Bethke, R. M. Mineral influences upon urea utilization and cellulose digestion by rumen microorganisms using the artificial rumen technique. J. Animal Sci. 10:693-705. 1951.

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

21. Chappel, C. F., Sirny, R. J., Whitehair, C. K., and MacVicar, R. Effect of mineral supplements on digestibility of a corncob ration by sheep. J. Animal Sci. 14:153-159. 1955.

22. Clark, R. and Quin, J. I. Studies on the alimentary tract of the Merino sheep in South Africa. 23. The effect of supplementing poor quality grass hay with molasses and nitrogenous salts. Onderstepoort J. Vet. Res. 25:93-103. 1951.

23. Coleman, I. W. A study by means of paper chromatography of the growth stimulating properties of liver extract, yeast solubles, and trypsin digest of casein for Lactobacillus casei. Canad. J. Med. Sci. 29:151-158. 1951.

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

25. Doetsch, R. N., Robinson, R. Q., Brown, R. E. and Shaw, J. C. Catabolic reactions of mixed suspensions of bovine rumen bacteria. J. Dairy Sci. 36:825-831. 1953.

26. Doetsch, R. N., Robinson, R. I. and Shaw, J. C. Techniques employed in cultural investigations of the bacteriology of bovine rumen contents. J. Animal Sci. 11:536-544. 1952.

27. Dunn, M. S. and McClure, L. E. The response of Lactobacillus casei to partial hydrolyzates of proteins. J. Biol. Chem. 184:283-288. 1950.

28. El-Shazly, K. Degradation of protein in the rumen of the sheep. 2. The action of rumen micro-organisms on amino-acids. Biochem. J. 51:647-652. 1952.

29. Gall, L. S., Smith, S. E. and Becker, D. E. Rumen bacteria in cobalt-deficient sheep. Science 109:468-469. 1949.

30. Garner, G. B., Muhrer, M. E., Ellis, W. C. and Pfander, W. H. Antibiotic-like substance and cellulose digestion stimulator found in rumen fluid. Science 120:435-436. 1954.

31. Garner, G. B., Muhrer, M. E. and Pfander, W. H. Rumen microorganisms growth regulating factor(s) found in extracts of rumen contents and of some ruminant feeds. (Abstract) J. Animal Sci. 13:29. 1954.

32. Hall, G., Cheng, E. W. and Burroughs, W. B-vitamins and other factors stimulatory to cellulose digestion by washed suspensions of rumen microorganisms. (Abstract) J. Animal Sci. 12:918. 1953.

33. Head, M. J. The effect of quality and quantity of carbohydrate and protein in the ration of the sheep on the digestibility of cellulose and other constituents of the ration with a note on the effect of adding vitamins of the B-complex on the digestibility and retention of the nutrients of a hay ration. J. Agr. Sci. 43:281-293. 1953.

34. Hoflund, S., Quin, J. I. and Clark, R. Studies on the alimentary tract of the Merino sheep in South Africa. 15. The influence of different factors on the rate of cellulose digestion (a) in the rumen and (b) in ruminal ingesta as studied in vitro. Onderstepoort J. Vet. Res. 23:395-409. 1949.

35. Hollis, L., Chappel, C. F., MacVicar, R. and Whitehair, C. K. Effect of ration on vitamin synthesis in rumen of sheep. J. Animal Sci. 13:732-738. 1954.

36. Huhtanen, C. N., National Dairy Research Laboratories, Inc., Oakdale, Long Island, N. Y. Information concerning the effects of enzyme-hydrolyzed casein upon cellulose digestion by rumen microorganisms. (Private communication.) 1954.

37. Huhtanen, C. N., Rogers, M. R. and Gall, L. S. Improved techniques for isolating and purifying rumen organisms. J. Bacteriol. 64:17-23. 1952.

38. Huhtanen, C. N., Saunders, R. K. and Gall, L. S. Fiber digestion using the miniature artificial rumen. J. Dairy Sci. 37:328-335. 1954.

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

40. Hunt, C. H., Bentley, O. G., Hershberger, T. V. and Cline, J. H. The effect of carbohydrates and sulfur on B-vitamins synthesis, cellulose digestion, and urea utilization by rumen microorganisms in vitro. J. Animal Sci. 13:570-580. 1954.

41. Johns, A. T. Isolation of a bacterium, producing propionic acid, from the rumen of sheep. J. Gen. Microbiol. 5:317-325. 1951.

42. Kihara, H., McCollough, W. G. and Snell, E. E. Peptides and bacterial growth. 1. Purification of a peptide factor required for growth of Lactobacillus casei. J. Biol. Chem. 197:783-789. 1952.

43. Klein, W. and Müller, R. Das Eiweizminimum die Zymogene Symbiose und die Erzengung von Microbeneiweiz im Pansen aus Stickstoff Verbindungen nicht eiweizartiger Natur. Ztschr. F. Tierzüch und Züchtungsbiol. 48:225-276. 1941.

44. Klosterman, E. W., Kunkle, L. E., Bentley, O. G. and Burroughs, W. Supplements to poor quality hay for fattening cattle. Ohio Agr. Exp. Sta. Res. Bul. 732. 1-20. 1953.

45. Langdon, R. G. and Bloch, K. The utilization of squalene in the biosynthesis of cholesterol. J. Biol. Chem. 200:135-144. 1953.

46. Lewis, D. The metabolism of nitrate and nitrite in the sheep. 1. The reduction of nitrate in the rumen of sheep. Biochem. J. 48:175-180. 1951.

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

48. McDonald, I. W. The role of ammonia in ruminal digestion of protein. Biochem. J. 51:86-90. 1952.

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

50. McNaught, M. L., Owen, E. C. and Smith, J. A. B. The utilization of non-protein nitrogen in the bovine rumen. 6. The effect of metals on the activity of the rumen bacteria. Biochem. J. 46:36-43. 1950.

51. McNeill, J. J., Doetsch, R. N. and Shaw, J. C. Some nutritional requirements of bovine rumen bacteria. J. Dairy Sci. 37:81-88. 1954.

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

53. Mills, R. C., Booth, A. N., Bohstedt, G. and Hart, E. B. The utilization of urea by ruminants as influenced by the presence of starch in the ration. J. Dairy Sci. 25:925-929. 1942.

54. Müller, R. Untersuchungenüber den Eiweizwert von Luzeresilage (Ein Beitrag zur Frage der Eiweizund Amidwertung beim Weiderkäuer). Ztschr. F. Tierzücht. und Züchlungsbiol. 49:279-305. 1941.

55. Pearson, R. M. and Smith, J. A. B. The utilization of urea in the bovine rumen. 3. The synthesis and breakdown of protein in rumen ingesta. Biochem. J. 37:153-164. 1943.

56. Perry, T. W., Beeson, W. M. and Mohler, M. T. Testing modifications of Purdue Supplement A. Purdue Univ., Animal Husbandry Dept., No. A. H. 102. (Mimeo.) 1953.

57. Perry, T. W., Beeson, W. M. and Mohler, M. T. The use of antibiotics, molasses solubles and yeast in cattle feed supplements. Purdue Univ., Animal Husbandry Dept., No. 124. (Mimeo.) 1954.

58. Pfander, W. W., Comfort, J. E. and Dyer, A. J. Ruminant nutrition. Missouri Livestock Feeders' Day Report. Columbia, Mo. April 9, 1954. p. 1-6. 1954.

59. Pierce, A. W. The influence of the amount of starch on the utilization of urea by sheep. Austral. J. Sci. Res. 2:447-456. 1951.

60. Quinn, L. Y. Isolation and morphological study of a thermophilic cellulose digesting organism. Unpublished Ph.D. Thesis. Lafayette, Ind., Purdue Univ. Library. 1949.

61. Ruf, E. W. In vitro study of unidentified factor(s) in cattle feeds which favorably influence cellulose digestion by rumen micro-organisms. Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1952.

62. Ruf, E. W., Hale, W. H. and Burroughs, W. Observations upon an unidentified factor in feedstuffs stimulatory to cellulose digestion in the rumen and improved liveweight gains in lambs. J. Animal Sci. 12:731-739. 1953.

63. Sijpesteijn, A. K. Cellulose-decomposing bacteria from the rumen of cattle. Thesis, Leiden. 1948.

64. Sijpesteijn, A. K. and Elsden, S. R. The metabolism of succinic acid in the rumen of sheep. Biochem. J. 52:41-45. 1952.

65. Sprince, H. and Woolley, D. W. Relationship of a new growth factor required by certain hemolytic streptococci to growth phenomena in other bacteria. J. Exp. Med. 80:213-217. 1944.

66. Stitt, W. A., Perry, T. W. and Beeson, W. M. Supplementation of farm roughages for pregnant ewes in dry lot. J. Animal Sci. 13:234-241. 1954.

67. Swift, R. W., Cowan, R. L., Barron, G. P., Maddy, K. H. and Grose, E. C. The effect of alfalfa ash upon roughage digestion in sheep. J. Animal Sci. 10:434-438. 1951.

68. Täufel, K., Thaler, H. and Schreyegg, N. Fat of yeast. Z. Untersuch Lebensm. 72:394-404. 1936. (Original not available; abstracted in Chem. Abstr. 31:6277. 1937.)

69. Thomson, W. and Tosic, J. "Fodder yeast" as a dietary supplement for sheep maintained on poor-quality hay. J. Agr. Sci. 39:283-286. 1949.

70. Tillman, A. B., Sirny, R. J. and MacVicar, R. The effect of alfalfa ash upon the digestibility and utilization of cottonseed hulls by sheep. J. Animal Sci. 13:726-731. 1954.

71. Tomkins, G. M., Dauben, W. G., Sheppard, H. and Chaikoff, I. L. Squalene as a precursor of cholesterol in liver. J. Biol. Chem. 202:487-489. 1953.

72. Tosic, J. Effect of small quantities of a yeast preparation on the recovery of appetite in sheep. Brit. J. Nutr. 3:234-241. 1949.

73. Woolley, D. W. and Merrifield, R. B. High strepogenin potency of synthetic oxytocin and of purified vasopressin. J. Am. Chem. Soc. 76:316. 1954.

ACKNOWLEDGMENTS

The author wishes to express his deep appreciation to Dr. Wise Burroughs for his helpful guidance and suggestions throughout the course of this study. He is grateful to Dr. Edmund Cheng and Dr. William Hale for their valuable assistance and constructive criticism of various phases of the experimental work, and to Professor Gordon Ashton for his suggestions in analyzing the data.

The author particularly wishes to thank his wife for her encouragement and cooperation which helped him greatly in completing this research.

The furnishing of experimental materials by the Red Star Yeast and Products Company, Milwaukee, Wisconsin, is very much appreciated.