STUDIES ON THE IN VITRO DIGESTION OF CELLULOSE BY RUMEN MICROORGANISMS

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ROBERT LAWRENCE SALSBURY

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Dairy

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Four methods of incubating rumen fluid for the purpose of studying cellulose digestion in vitro were examined. Under the conditions used, it was found that the maximum cellulose digestion and most reproducible results were obtained when a semipermeable sac was used and the inoculum: mineral-solution ratio was small. When CO_2 was passed through the fermentation mixture during incubation, the percentage cellulose digested was slightly lower than when CO_2 was used very briefly at the start of the fermentation period. A modification of the "artificial saliva" of McDougall (1948) was adopted as a source of mineral nutrients.

Cellulose digestion was increased by addition of urea up to 0.1% when no readily available carbohydrate was added but decreased by additions of 0.15 or 0.20%. Addition of glucose up to 0.2% did not appreciably affect the amount of cellulose digested.

Addition of large amounts of cobalt (up to 200 ppm.) depressed cellulose digestion, the amount of depression being greater as the concentration of the cobalt increased. Addition of vitamin B_{12} (up to 50 micrograms per liter) had no appreciable effect on cellulose digestion.

Inocula obtained from a steer receiving high levels of cobalt had a decreased ability to digest the cellulose in a wood cellulose, alfalfa leaf meal and timothy hay when incubated without the addition of urea. When urea was added before incubation the inocula had a decreased ability to digest the cellulose in the wood cellulose and timothy hay but the digestion of the cellulose in alfalfa leaf meal was essentially unchanged.

A comparison of rates of cellulose digestion in vitro showed that

the cellulose in alfalfa leaf meal was digested at a relatively greater rate than the wood cellulose or the cellulose of cotton linters when compared on the basis of percentage of 24-hour digestion.

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INTRODUCTION

The digestive tract of the ruminant is uniquely adapted to the digestion of fibrous plant material. The key to the ruminant's ability to digest material which is unavailable to monogastric animals is its possession of a fore-stomach or reticulo-rumen. This organ functions as a large fermentation vat in which a more or less stable microbial population attacks ingested food. Products of the microbial action become available to the animal and in passing along the digestive tract many of the microorganisms themselves are digested by the enzymes of the animal. Consequently, constituents of the feed which would otherwise not be utilized by the animal are made available to it. One such constituent is cellulose.

The importance of understanding the mechanism of the digestion of cellulose and other materials in the rumen now becomes obvious. Not only should such an understanding be of great value in choosing the most suitable ration, but it could lead to the modification of some of the processes to permit more efficient utilization of feed.

The use of fistulated animals has helped considerably in the study of rumen processes. In vivo studies, however, have been complicated by the fact that the investigator has to deal with a dynamic system. There is the frequent passage of material out of the reticulo-rumen into the omasum, the loss of some constituents through the rumen wall, and unless feeding is carefully controlled, the intermittent introduction of feed, water, and saliva.

There has been a tendency in recent years to use *in vitro* methods in the study of rumen microorganisms. This avoids the complications mentioned above but introduces others, notably the lack of assurance that the *in vitro* fermentation simulates that occurring in the rumen.

REVIEW OF THE LITERATURE

Fermentation Methods

Marston (1948) was one of the first to attempt to simulate rumen conditions in studying cellulose digestion *in vitro*. In attempting the experimental imitation of the conditions of the rumen he considered the low partial pressure of O_2 and the high CO_2 tension, the supply of phosphate and inorganic nitrogen from the saliva, the buffering capacity of the saliva, the temperature of approximately 40°C, and the residual contents of the rumen which provide a massive inoculum of organisms already selected by the environment. In the method used, cellulose prepared from beech wood or filter paper was suspended in buffer solution and inoculated with a suspension of rumen microorganisms which had been separated from 3 to 5 liters of rumen fluid. Anaerobic conditions were maintained by passing pure N_2 over the fermentation mixture.

Louw *et al.* (1949) modified the method of Marston (1948) by introducing the use of a semipermeable bag to permit the diffusion of non-volatile end products out of the fermentation mixture. He used a large inoculum (700 ml.) of strained rumen fluid inside the bag with ground filter paper and buffer, and suspended the bag in a bath of the mineral solution. Nitrogen containing 5% of CO_2 was passed over the fermentation mixture during incubation. The somewhat greater cellulose digestion obtained with this method was attributed to the use of the sac.

The method used by Burroughs *et al.* (1950) was also adapted from that of Marston (1948). The procedure involved the use of a glass fermentation

vessel without any semipermeable bag. Stirring and anaerobiosis were maintained by a stream of CO_2 passed through the fermentation mixture during incubation. Generally a series of incubations was performed, half the fermentation mixture at the end of each 24-hour incubation period serving as the inoculum for the succeeding 24-hour fermentation.

The method used by Wasserman et al. (1952) combined elements of each of the methods previously mentioned. The inoculum and cellulose were suspended in a bath of nutrient mineral solution. CO_2 was passed into the sac and the outer liquid was stirred mechanically during incubation.

In the method of Huhtanen et al. (1954) the size of the apparatus was considerably reduced, a small semipermeable sac being used within a 4-ounce bottle. No CO_2 was introduced during fermentation and no mechanical stirring was used. This method involves less manipulation than the methods discussed above and permits the simultaneous incubation of a large number of samples.

The method of McBee (1953) takes a somewhat different approach. Gas evolution when rumen fluid and cellulose or other substrates were incubated in a Warburg apparatus was measured. The method is based on the assumption that the rate of fermentation of a substrate is proportional to the total activity of all the organisms in the rumen capable of attacking that substrate. An advantage of this method is the relatively short time required in vitro, thus increasing the likelihood of results representative of rumen fermentation.

Henderson et al. (1954) used a simplified method for measuring cellulose digestion. Frozen rumen fluid was blended with a warmed nutrient solution containing a number of salts and the mixture incubated with

weighed parchment strips. The loss in dry weight of the parchment strips was considered to represent cellulose digestion. In obtaining the inoculum, fresh rumen contents were mixed with dry ice. According to the authors this procedure not only accomplished rapid freezing but maintained anaerobic conditions during the freezing.

Doetsch et al. (1953) used an approach somewhat similar to that of McBee (1953). The inoculum used was a suspension of washed cells. A number of substrates were used and the amount of gas produced with each substrate was determined by using a Warburg apparatus. Volatile fatty acids were also determined.

Methods for the Determination of Cellulose

The determination of cellulose is complicated by the fact that the precise structure of the cellulose molecule is not known. It is generally accepted that the cellulose molecule is a linear polymer of anhydroglucose units linked through a 1,4-beta-glucosidic linkage, but there is experimental evidence that other linkages may occur. Moreover, according to Pigman and Goepp (1948), it would appear that celluloses from different sources may have different degrees of polymerization. According to Norman (1937) the possibility exists that the cellulose chain may include xylose residues.

Methods for the determination of cellulose fall into two main classes. In the first of these, the cellulose is hydrolyzed and increase in reducing power due to the release of the glucose units is determined. These methods are not applicable to the present study because of the heterogeneous nature of rumen fluid and the probability of the development of interfering substances during the hydrolysis and hence will not be discussed

here. In the second class of determinations, non-cellulosic materials are removed, leaving a cellulose residue.

None of the methods of cellulose determination is entirely satisfactory but there are some in the second class mentioned which give reproducible results and which are applicable to the type of study presented here. In the method of Cross and Bevan (1911), the sample is treated with boiling 1% sodium hydroxide solution followed by alternate chlorination and boiling with sodium sulfite until there is no red color indicative of lignin when sulfite is added. The preliminary treatment with alkali has been the subject of some criticism and in some later modifications of the methods this step has been omitted. Norman and Jenkins (1933) pointed out that the alkali treatment dissolves an appreciable portion of the cellulosans. Moreover, a large number of alternate chlorinations and treatments with sodium sulfite may be necessary to remove the lignin of some materials. Consequently, these authors eliminated the alkali treatment and used sodium hypochlorite solution instead of gaseous chlorine. Their method involves two treatments with neutral hypochlorite solution then three or more with acid hypochlorite, each followed by boiling with sodium sulfite. Matrone et al. (1946) proposed a modification of this method. They treated the ethanol-benzene-extracted sample with neutral sodium sulfite, 0.25 normal sodium hydroxide in approximately 60% ethanol, then with neutral sodium hypochlorite solution. This sequence was repeated using acid sodium hypochlorite instead of the neutral solution until no test for lignin was obtained on addition of the sodium sulfite solution.

Ritter and Kurth (1933) described a method for the determination of the total carbohydrate of the extractive-free material by alternate chlorinations and extraction with alcohol-pyridine solution.

The method of Kürschner and Hanak (1930) consisted of oxidizing the non-cellulosic constituents of the sample with a mixture of 10 parts of acetic acid and 1 part of concentrated nitric and washing the residue with water. Crampton and Maynard (1938) used this method. They found, however, that by using alcohol for washing the cellulose they could perform this step by centrifugation, a modification which considerably facilitated the washing operation.

The main difference between the results obtained using the Norman and Jenkins (1933) method and those obtained using the method of Crampton and Maynard (1938) appears to be that the former include cellulosans. Ferguson (1942) used both the Norman and Jenkins (1933) and the Kürschner and Hanak (1930) method to determine the cellulose (as percentage of dry matter) of wheat straw and wheat straw pulp. With the untreated straw, he obtained values of 54.21% for "crude" cellulose and 35.28% for "true" cellulose using the Norman and Jenkins (1933) method. The Kürschner and Hanak (1930) method gave values of 39.45% for "crude" cellulose and 35.00% for "true" cellulose. When wheat straw pulp was used, 63.87% of "crude" cellulose and 43.78% of "true" cellulose was found by the Norman and Jenkins (1933) method while the Kürschner and Hanak (1930) method gave a value of 52.18% for "crude" cellulose and 43.18% for "true" cellulose.

Druce and Wilcox (1949) suggested a method involving an oxidation with acid potassium dichromate followed by an alkaline hydrogen peroxide oxidation. According to Hägglund (1951), however, such a procedure would

be liable to form oxycellulose or other degradation products which would subsequently dissolve in the alkali.

Digestion of Cellulose by the Ruminant

A number of reviews on the subject of ruminant digestion are available and the reader is referred to papers by Marston (1939), Goss (1943), McAnally and Phillipson (1944), Baker and Harriss (1947), McNaught and Smith (1947), Phillipson (1947), Elsden (1948), Owen (1951), Jarrige (1953), and Huffman (1953) for more detailed information.

There is abundant evidence that the cellulose digestion accomplished in the rumen is due to the action of microorganisms. Mangold (1934) and Marston (1948) have cited some of the earlier work supporting this view. Baker and Harriss (1947) reviewed the direct microscopic evidence for the microbiological breakdown of cellulose in the rumen, basing their evidence largely on the appearance of "corrosion" figures and enzymatic cavities in the cellulosic material and on the loss of birefringence by the cellulose. The in vitro fermentation methods already mentioned have also contributed strong evidence that the digestion of cellulose in the rumen is due to the microorganisms present. This positive evidence together with the failure to detect any cellulase in the digestive secretions of mammals and the fact that action of plant cellulases in the rumen is unlikely, has brought about acceptance of the view that the digestion of cellulose by ruminants is due to microbial action.

Mechanism of cellulose breakdown. According to Baker and Harriss (1947) rumen microorganisms reach susceptible plant structures by invading the middle lamella of adjoining cells, penetrating into the lumen of fibers, and invading the fractures in the plant material caused by

prehension and rumination. Disintegration of plant material then takes place adjacent to the microorganisms with the result that cavities are formed. At first these cavities generally follow the form of the microorganisms but as digestion proceeds, the cavities enlarge and form "corrosion figures" which lack the birefringence shown by the neighboring plant material. These "corrosion figures" do not follow the orientation of the cellulose fibers to the same extent as those produced by fungi but this may be due to the displacement caused by the rapid growth of the bacteria. In some of the photomicrographs shown by Baker and Harriss the cavities are outlined by bacteria which appear to be closely associated with the undisintegrated tissue and this would tend to support the view held by some workers that cellulase is tightly adsorbed on the surface of the bacterial cell. According to this view, direct contact between the cell and the cellulose would be required. The appearance of many of the cavities, however, suggests the activity of an extracellular enzyme. This seems the more plausible in view of the suggestion made by Hungate (1944) that the apparent requirement of close proximity of the cellulose digesting cell to its substrate could be explained by the secretion of an exocellular cellulase. The close association of cell and substrate would be required for the organisms to benefit from the breakdown products of the cellulose. He described a cellulolytic organism, Clostridium cellobioparus, isolated from the rumen, which was motile but remained in the cellulose sediment of liquid media until most of the cellulose was utilized. In any case, there is no doubt that the degradation of cellulose to diffusible intermediates must take place outside of the cell.

One difficulty in assessing available information on the mechanism of cellulose breakdown is presented by the number of cellulose substrates that have been used by different workers. These range from "native" cellulose to substituted soluble cellulose derivatives. Hermans (1949) uses the term "native cellulose" to include fiber obtained from natural products such as wood and straw "without excessive interference". This would presumably include materials such as filter paper, mercerized cotton, or cotton swollen with phosphoric acid. However, there are differences between cultivated fibers and those which have received even a mild chemical treatment. Hermans (1949) points out that mercerization alters the X-ray pattern of the crystalline portion of cellulose from that of Cellulose I to that of Cellulose II. According to Walseth (1948), cotton linters swollen with phosphoric acid were acted upon much more rapidly by an enzyme preparation than were dewaxed cotton linters that had not been treated with phosphoric acid. Reese and Levinson (1952) found that certain fungi which did not bring about loss in tensile strength of cotton duck were able to grow in shake flasks containing wood cellulose and that the loss in weight of the wood cellulose far exceeded the amount of pentosan present. These same organisms could not grow in shake flasks containing ground dewaxed cotton fiber, purified cotton linters, ground duck, cotton linters swollen with 35% sodium hydroxide solution, or wood cellulose low in pentosan. Consequently, the term "native cellulose" will be used here to refer to plant cellulose in situ or cotton linters that have received no chemical treatment other than extraction to remove wax.

The form of the crystalline lattice is altered by mercerization.

Hermans (1949) reported that in some cases even mechanical grinding causes loss of the crystalline pattern (Cellulose I) which returns in an altered form (Cellulose II) when the ground material comes into contact with water.

There is also the possibility that even mild chemical treatment may bring about some chemical change in "native" cellulose. Stillings and Van Nostrand (1944) and Heuser and Chamberlin (1946) found that ultraviolet irradiation, even in the absence of oxygen, brought about a decrease in the degree of polymerization and alpha-cellulose content of cotton linters, a reaction which was accelerated by the presence of oxygen. Pigman and Goepp (1948) stated that, in the absence of oxygen, "native" cellulose may have a degree of polymerization as high as 15,000. It seems quite possible, therefore, that even mild conditions for the isolation of cellulose may result in a product somewhat different chemically than the original material.

In view of the above discussion, some credence can be given to the tentative scheme for the enzymic breakdown of cellulose advanced by Siu (1951). He visualized the first step as the rupture of Van der Waal's forces in the amorphous region of the cellulose and of the hydrogen bonds in the crystalline region.

The next step would be the rupture of the cross linkages which are assumed to join the linear chains of anhydroglucose units. The supposition of an exocellular enzyme catalyzing this step leads to difficulty in conceiving how the organism would immediately benefit from the reaction. However, if the reaction were an oxidative one, energy would be released and possibly transferred into the cell by some mechanism

suggested by the demonstration of dehydrogenases on the surface of cells by Quastel (1926) and Quastel and Wooldridge (1927).

For the next step, the breakdown of the linear polysaccharide into units which can diffuse into the cell, Siu (1951) postulated a separate enzyme, C_x. He reserves the term "cellulase" for the enzyme bringing about the initial change in the "native" cellulose molecule. The postulation of a "C_x" enzyme is based on the observation made by Reese et al. (1950) that carboxymethyl cellulose was attacked not only by filtrates from cellulolytic microorganisms but also by those from non-cellulolytic fungi. Ability to hydrolyze cellulose was determined in this work by action of the filtrate on ground, dewaxed cotton linters.

According to Siu (1950, 1951) the linear polysaccharide is degraded by the C_x enzyme directly into glucose without the formation of cellobiose as an intermediate product. This view was also held by Reese et al. (1950). In a later report from the same laboratory, however, Levinson et al. (1951) stated that cellobiose was the principal product of cellulose degradation. These workers used glucose oxidase (notatin) for the determination of glucose and beta-glucosidase followed by the determination of the glucose formed with glucose aerodehydrogenase for the estimation of cellobiose. Results were confirmed by paper-partition chromatography. Cellobiose and glucose were the only breakdown products obtained when cellulose or alkali cellulose was the substrate. When cellulose sodium sulfate was used as the substrate however, reducing substances which moved more slowly on the chromatogram were observed. The authors suggest that these slow-moving components may be substituted glucose or substituted cellobiose rather

than cellotriose or cellotetraose.

Kitts and Underkofler (1954) studied the products of degradation of carboxymethyl cellulose by extracts of rumen bacteria, using paper chromatography to separate and identify the products. They found that only glucose was present in the early hours of incubation but that after 20 and 24 hours traces of xylose appeared. The presence of xylose was explained by the assumption that a small amount of xylan was present in the carboxymethyl cellulose and that the cell-free filtrates used contained an enzyme capable of hydrolyzing xylan to xylose. Cellobiose was not detected as a degradation product of carboxymethyl cellulose. Subsequent tests with the cell-free filtrates showed that a cellobiase was present that degraded cellobiose to glucose at a much faster rate than that obtained for the degradation of carboxymethyl cellulose.

Reese et al. (1952a) have presented evidence to support the contention that cellobiose is the principal product of the action of the C_x enzyme upon cellulosic materials. They found that cellobiose inhibited the hydrolysis of cellulose by the filtrates of most of the organisms tested. This phenomenon was interpreted as the action of an end product inhibiting enzymatic activity in a manner similar to the inhibition of the hydrolysis of starch alpha-amylase by maltose. With filtrates from 7 of the 36 organisms tested, hydrolysis of carboxymethyl cellulose was stimulated by low concentrations of cellobiose although with these filtrates too, higher concentrations inhibited the hydrolysis. The authors explain this observation by assuming that the C_x enzyme of these filtrates was of a somewhat different configuration. As a result, the hydrolytic product (cellobiose) of the hydrolysis of carboxymethyl cellulose would not be as

readily removed from the active sites of the enzyme molecule. It was thought possible that cellobiose, by competing for the same areas, would aid in the removal of the products. However, this does not explain the increased stimulation at a lower pH. Nor does the alternative explanation given, that of a protective action exerted by cellobiose on the C_x enzyme, account for the lack of stimulation obtained when unsubstituted celluloses were used.

Reese and Gilligan (1953) were able to separate C_x enzymes into as many as three components by differential adsorption onto cellulose and kaolin. The number of components varied depending upon the species of microorganism used and upon the conditions of growth. No conclusion could be reached as to whether these components represented individual enzymes or were due to interaction of a single enzyme with other elements of the system.

The results obtained by Whitaker (1953) are not in agreement with the theory of a multienzyme system for the degradation of cellulose. Whitaker was able to purify the cellulase of Myrothecium verrucaria by concentration, precipitation with ammonium sulfate, fractionation with ethanol, and precipitation with polymethacrylic acid. The enzyme obtained in this manner was electrophoretically homogeneous at 3 pH's and was also homogeneous in the ultracentrifuge. Moreover, when its activity toward untreated cotton linters, swollen cotton linters, cellulose degraded by acid hydrolysis, carboxymethyl cellulose, and cellobiose was determined, it was found that the enrichment in activity was approximately the same for each substrate. Whitaker therefore postulated that cellulolytic organisms differ from non-cellulolytic organisms, not because they possess

a C_1 enzyme, but because the cellulase produced by them is capable of adsorption onto the insoluble substrate while that produced by the non-cellulolytic organisms is not capable of this adsorption.

During his work on the purification of the cellulase of Myrothecium verrucaria, Whitaker (1952) found that proteins and proteoses produced a marked stimulation in the cellulase activity of the enzyme. No stimulation was observed when cellobiose or carboxymethyl cellulose was used as the substrate. The extent of stimulation varied with pH but this change did not appear to depend on the isoelectric point of the protein. The stimulation was greater at 27°C than at 37°C and increased with increasing substrate concentration. An increase in the enzyme concentration decreased the stimulation by added protein, possibly due to a sparing effect from the non-cellulase protein present in the enzyme preparation. The author suggests that the stimulation by protein is due to its adsorption onto the substrate.

In a later report from the same laboratory, Basu and Whitaker (1953) described the inhibition of the activity of Myrothecium verrucaria cellulase by various reagents. Reversible inhibition by para-chloromercuribenzoate and by salts of heavy metals was interpreted as indicating that thiol groups were essential for activity. A negative nitroprusside test for thiols given by the undenatured enzyme preparation, together with the weak inhibitory effect of ferricyanide, was taken to indicate that essential thiol groups were of the sluggish type. The inhibition of cellulase activity by acid dyes at low pH levels and by basic dyes at high pH levels, and the stimulation by basic dyes at low pH levels were attributed to the adsorption of the dye onto the substrate. The weak stimulatory effect

obtained with a protein or with a basic dye when carboxymethyl cellulose was the substrate was not considered to be contradictory to this view, since some binding might be expected by this substrate even though it is soluble.

Jermyn (1952) found that the culture filtrate from Aspergillus oryzae contained enzymes capable of depolymerizing sodium carboxymethyl cellulose and of splitting a number of beta-glucosides. It is interesting to note that whereas Reese et al. (1950) believed that the C_x enzyme was produced only in response to the presence of the beta-glucoside linkage, Jermyn found that with A. oryzae it was produced even in sucrose-tartrate media. The activities found, however, were much lower with sucrose as the substrate than with sodium carboxymethyl cellulose. The filtrate attacked sodium carboxymethyl cellulose and cellodextrin vigorously but there was little or no attack on native or insoluble derived cellulose. A number of compounds were examined for inhibition or stimulation of enzyme activity. Dithionite totally inhibited activity while permanganate gave a four-fold increase in activity.

In a later paper, Jermyn (1952a), using filter paper electrophoresis, showed that the beta-glucosidase of A. oryzae which attacked sodium carboxymethyl cellulose consisted of a number of components. He found that in the crude enzyme preparation there were at least 7 components capable of splitting both simple glucosides and sodium carboxymethyl cellulose. An eighth component could attack para-nitrophenyl-beta-glucoside but not sodium carboxymethyl cellulose. Because, with the one exception, there seemed to be no qualitative difference between the enzymes breaking down monomeric beta-glucosides and those breaking down polymeric beta-glucosides,

he suggested that there is no need to assume the existence of a cellulase or a C_x enzyme to explain the breakdown of long chain cellulose derivatives in solution. He also pointed out that neither of the alternatives suggested by Veibel (1950), a battery of specific beta-glucosidases or a single non-specific glucosidase, are tenable for A. oryzae.

Later, Jermyn (1953) found that Stachybotrys atra could produce enzymes which degraded sodium carboxymethyl cellulose even when grown on media which contained no beta-glucoside. He reported that the production of enzyme capable of degrading solium carboxymethyl cellulose apparently proceeded in bursts, being greatest at or just after germination of the spores and showing a second increase in production when maximum mycelial growth was obtained. He suggested that the C_x enzyme, rather than being wholly adaptive as reported by Reese et al. (1950), is partially adaptive since large amounts can be produced in the absence of cellulose derivatives but in general somewhat higher levels are obtained when cellulose derivatives are present.

Amount of cellulose digested in the rumen. Hale et al. (1940), by means of the lignin ratio method, determined rumen digestion coefficients for alfalfa alone at three different levels. The Crampton and Maynard (1938) method was used for the determination of cellulose. They found a rumen digestion coefficient for cellulose of 38.1 when 10 lb. of alfalfa hay per day were fed, 38.3 when 20 lb. were fed and 47.4 when 30 lb. were fed. The total apparent cellulose digestion, determined by digestion trials, was 44.1% at the 10 lb. level, 51.4% at the 20 lb. level, and 50.9% at the 30 lb. level.

Louw (1941) determined the total apparent digestion of cellulose by sheep of a mixture of veld grasses after 1, 2, 3 and 4 months of growth.

The Norman and Jenkins (1933) method for cellulose determination was used. Coefficients of digestibility of "natural" cellulose were 75.0, 68.2, 63.9, and 58.1 and of "true" cellulose were 75.9, 69.1, 64.8, and 60.9 for the 1-, 2-, 3-, and 4-month old grass, respectively.

McAnally (1942) determined the digestibility of the cellulose of wheat straw, oat straw, and filter paper using both in vitro and in vivo methods. The method for the determination and isolation of cellulose was that of Norman and Jenkins (1933). The digestibility in vivo was determined by placing the samples in small silk squares tied up with silk and suspending them in the rumen of the sheep. The in vitro experiments were performed by adding strained rumen fluid or strained rumen fluid plus inorganic salt solution to the cellulosic material. In vivo, wheat straw showed a cellulose digestion of 20, 36, 36, and 52% in 1, 2, 3, and 4 days, respectively, oat straw showed a cellulose digestion of 25, 33, 50 and 62% in 1, 2, 3 and 4 days, respectively, and filter paper showed a cellulose digestion of 26, 64 and 93% in 1, 2 and 3 days, respectively. In the experiments conducted in vitro, 56 and 100% of the cellulose was digested in 2 and 4 days, respectively, when filter paper was the substrate and 50 and 87% was digested in 2 and 4 days, respectively, when cellulose isolated from oat straw was used as the substrate. The digestibility of the cellulose in the wheat and oat straws could be increased by pretreatment with alkali.

Ferguson (1942) conducted digestion trials with sheep to determine the digestibility of wheat straw and wheat straw pulp. The digestibility coefficient of crude cellulose was 62.4 for the wheat straw and 87.7 for the wheat straw pulp, and the digestibility coefficient of "true" cellulose was 62.1 for wheat straw and 86.7 for wheat straw pulp.

Gray (1947) determined the cellulose content of ingesta taken from successive levels of the alimentary tracts of slaughtered sheep. Cellulose was determined by the method of Norman and Jenkins (1933). Approximately 40% of the cellulose of the fodder was digested in the rumen. This amounted to 70% of the cellulose digested in the entire digestive tract. A further 17% of the digestible cellulose was digested in the caecum, and 13% was digested in the colon.

Hale et al. (1947) used the lignin ratio method to determine the rumen digestion coefficient of cellulose in alfalfa hay. Cellulose was determined by the method of Crampton and Maynard (1938). For one cow, the rumen digestion coefficients of cellulose were 12.8 and 43.6 at 6 and 12 hours, respectively, and the fecal digestion coefficient of cellulose was 63.1. For another cow, the rumen digestion coefficients of cellulose were 39.3 and 42.9 at 14 and 24 hours, respectively. When 8 trials were averaged, the rumen digestion coefficient of cellulose was 42.1 for 12 to 14 hours and 43.4 when corrected for lignin digestion. The average fecal digestion coefficient of cellulose was 55.0 for these 8 trials. The results obtained by these authors indicate that the greater part of the fodder cellulose was digested in the rumen and in the second 6 hours after ingestion.

Marston (1948) referred to unpublished observations indicating that a digestion mass of approximately 5 liters in the rumen of a sheep fermented from 400 to 600 g. of mixed carbohydrates, at least half of which was cellulose, in 8 hours.

Umezu et al. (1951) determined the rate of digestion of the cellulose of filter paper in a four-months-old kid, using the in vivo silk bag method of McAnally (1942). Cellulose was estimated by determining the

loss in weight gravimetrically after washing with dilute acid, dilute alkali and then water. This experiment differs from similar in vivo determinations in that a non-fistulated animal was used, the silk bag being inserted into the rumen through the esophagus. It was found that approximately 10% of the cellulose of the filter paper was digested in 10 hours, 40% in 15 hours, and 65% in 20 hours. These values are considerably higher than the 26% digestion of the cellulose of filter paper which McAnally (1942) obtained in sheep over a 24-hour period using the silk bag technic.

Rate of cellulose digestion in the rumen. The values given in the report by McAnally (1942) mentioned above, in which the rate of cellulose digestion in the rumen of sheep was determined by the silk bag technic, probably do not represent the actual rate of digestion of cellulose in the rumen. He found that two days were required to attain a 36% digestion of the cellulose in wheat straw, a 33% digestion of the cellulose in oat straw, or a 64% digestion of the cellulose in filter paper. The results given by Hale et al. (1947), in which 12.8% of the fodder cellulose was digested in the first 6-hour period, are probably more representative of the actual rates. Umezu et al. (1951) also found an increased rate of cellulose digestion after filter paper had been in the rumen several hours. In the first 10 hours in the rumen only 10% of the cellulose was digested while in the second 10 hours an additional 55% was digested.

Hoflund et al. (1948) used the decrease in the breaking strength of cotton threads suspended in the rumen of sheep to study factors affecting the rate of cellulose digestion. Determinations of the actual amounts of cellulose digested in the rumen were not made. They did find, however,

that under the conditions of their experiment the rate of cellulose digestion *in vitro* was similar to the rate *in vivo*.

Balch and Johnson (1950) investigated the rate of cellulose digestion in the rumen by determining the loss in weight of cotton thread. With a cow on a hay and concentrates diet, they found that in the ventral sac approximately 37% of the cellulose was digested in 24 hours but that in the mid-rumen approximately 35 hours were required for the digestion of this amount of cellulose. In the dorsal sac approximately 15% was digested in 70 hours. With a cow on a hay diet they found that in the ventral sac 16 and 63% were digested in 24 and 48 hours, respectively, in the mid-rumen 8 and 32% in 24 and 48 hours, respectively, and in the dorsal sac 4 and 17% in 24 and 48 hours, respectively.

Miles (1951), using the silk bag technic to study the digestibility of alfalfa hay *in vivo*, found that apparent digestion of dry matter, cellulose and pentosans was more rapid in the ventral sac than in the dorsal part of the rumen. Apparent digestion increased rapidly in the periods through 36 hours, slowly from 36 to 72 hours, and there was no notable increase after 72 hours. The dry matter, cellulose and pentosans of beet pulp were digested the most rapidly, those of alfalfa hay next, and those of corncobs much more slowly.

Effect of dry matter concentration on cellulose digestion. Apparently the percentage of dry matter in the rumen has some effect on the rate of cellulose digestion. Balch (1950) and Balch and Johnson (1950) showed that cellulose was digested more rapidly in the more fluid portions of the ingesta. They found that about 30 hours were required for 50% loss in weight of cotton thread suspended in the ventral sac where the dry matter

concentration was 5 to 6%. When the dry matter concentration in the ventral sac had been increased to 10% by feeding ground hay, about 50 hours were required to attain a 50% loss in weight of the cotton thread.

Effect of soluble carbohydrate on cellulose digestion. Feeding of large amounts of soluble carbohydrates apparently depresses the digestion of cellulose in the rumen. Mitchell et al. (1940) found that the addition of 1200 g. of glucose per day to rations of growing Hereford calves decreased the digestibility of crude fiber. Hamilton (1942) confirmed this by adding 150 to 200 g. of glucose to the daily ration of sheep with a resulting depression of the digestibility of crude fiber. Johnson et al. (1942) found that when a ration containing 20% corn molasses plus added urea was fed to lambs, urea utilization was improved but cellulose digestion was depressed. Swift et al. (1947) found a depression of crude fiber digestibility when starch or corn sugar was added to rations of sheep. Hoflund et al. (1948) found that although large amounts of sucrose added to the ration of sheep depressed cellulose digestion, the addition of smaller amounts (1 and 3%) had a stimulating effect on cellulose digestion. In vitro experiments showed a stimulating effect with small amounts of glucose and a depressing effect when larger amounts were added. In this case, stimulation was obtained when the glucose concentration was 0.1 to 0.2%. A glucose concentration of 0.4 percent had an inhibitory effect on cellulose digestion. Burroughs et al. (1949) noted a decrease in roughage dry matter digestion when 2 to 4 lbs. of starch were added to the rations of steers in which corncobs or corncobs and limited alfalfa hay made up the roughage part of the ration. The in vitro experiments performed by Arias et al. (1951) are in agreement with the results obtained by

Hoflund et al. (1948). Using an in vitro method essentially the same as that described by Burroughs et al. (1950), Arias and his associates obtained, as an average percentage cellulose digestion for three successive fermentation periods, 48% for cellulose alone, 65% when 0.1 % glucose was added, 57% when 0.2% glucose was added, and 47% when 0.3% glucose was added. The corresponding results for sucrose and starch were 53, 57, 48, and 47% and 54, 62, 55, and 55%, respectively.

Effect of nitrogen source on cellulose digestion. The literature pertaining to nitrogen metabolism in the rumen will not be reviewed here in detail. The reader is referred to the excellent reviews of McNaught and Smith (1947) and Reid (1953) for a more general coverage.

Hoflund et al. (1948) found that addition of casein to the ration of sheep prevented the depressing effect of added readily available carbohydrate on cellulose digestion. When the casein was removed from the ration, cellulose digestion was depressed but could be restored to the original level by again adding casein.

Pearson and Smith (1943) showed that there was sufficient urease activity in bovine rumen ingesta to convert any amount of urea likely to be fed, even to a high-yielding cow in lactation, to ammonia in one hour. They suggested that urea is utilized in the rumen by being first converted to ammonia. Later (1943a) they reported that in the presence of 0.3% of starch, maximum protein synthesis was obtained when the urea nitrogen concentration was 0.075 to 0.1%. The optimum concentration for cellulose digestion was not determined.

Hudman and Kunkel (1953) obtained the highest rate of protein synthesis when 0.9% of urea was used with approximately 1% glucose and 1%

cellulose. In these experiments also, cellulose digestion was not determined.

The results obtained by McDonald (1952) with zein and casein indicate that protein is utilized by bacteria in the rumen by first being converted into ammonia. He suggested that the less soluble zein was more efficiently utilized, since excess ammonia was not formed.

Loosli et al. (1949) showed that essential amino acids were synthesized in the rumen of sheep when urea was the main source of nitrogen in the ration. However, Gall et al. (1951) reported that the number and kinds of bacteria found in the rumen when this ration was fed were very different than those found when a normal ration was fed.

Burroughs et al. (1950a) obtained an increase in the percentage cellulose digestion when certain cereal grains and protein-rich feeds were present in the fermentation mixture. In a later paper Burroughs et al. (1951) showed that addition of casein or gelatin to the fermentation mixture increased cellulose digestion slightly. They suggested that this increase may be due to minerals in the protein.

Belasco (1954) made a comparative study of different nitrogen compounds as sources of nitrogen for the digestion of cellulose by rumen microorganisms. An in vitro fermentation method similar to that described by Burroughs et al. (1950) was used. The amount of each nitrogenous compound added was chosen so that the amount of nitrogen added was 187 mg. per 900 ml. of fermentation mixture. The urea derivatives used gave poor cellulose digestion and some were even inhibitory. Of the amides, urea gave the most cellulose digestion with propionamide giving 92% of the amount obtained when urea was used. Of the amidines, guanidine acetate proved to

be as good a source of nitrogen as urea when judged by cellulose digestion. A number of ammonium salts were also examined. Ammonium alpha-ketoglutarate and ammonium formate gave slightly higher cellulose digestion than did urea, while ammonium succinate and ammonium lactate gave somewhat lower cellulose digestion. However, all four ammonium salts showed better nitrogen utilization than did urea.

Later, Belasco (1954a) showed that when urea was used as the source of nitrogen in vitro, a greater percentage cellulose digestion was obtained than when soybean, cottonseed, linseed or corn gluten meal was used as the source of nitrogen. He also showed (1954b) that with urea higher levels of propionic acid and lower levels of butyric and valeric acids were formed. The levels of acetic acid and total volatile acids were unaffected by the type of nitrogen source used.

Other factors affecting cellulose digestion. Burroughs et al. (1950) showed that the addition of autoclaved manure extract or autoclaved rumen fluid helped to maintain cellulose digestion over successive fermentation periods. Ruf et al. (1953) reported that a factor or factors occurred in several of the more common feeds fed to cattle and sheep that stimulated cellulose digestion in vitro and stimulated appetite and increased liveweight gains in lambs. Manure extract and yeast were particularly rich sources of the material. The material was found to be heat stable, soluble in water and destroyed by ashing. It could be adsorbed on Norite and eluted with acetone and ethanol.

Bentley et al. (1954), using cells obtained by centrifuging strained rumen fluid, found that the addition of autoclaved rumen juice, extracts of various plant materials, molasses, or yeast extract markedly increased the

rate of cellulose digestion. Biotin, vitamin B_{12}, para-aminobenzoic acid, xanthine, uracil, guanine and adenine improved cellulose digestion but not to the same extent as did rumen juice supernatant, water extract of alfalfa, yeast or molasses. An active substance could be precipitated with lead acetate, extracted with alcohol, adsorbed on Norite and eluted with dilute, aqueous alkali solution. The eluate was less active than the original rumen liquid.

According to Burroughs et al. (1951a) the cellulolytic organisms of the rumen require sodium, potassium, calcium, magnesium, iron, phosphorus, sulfur and chlorine in varying amounts. They suggested that other minerals also may be required.

Cardon (1953) found that high levels of sodium chloride had no effect on cellulose digestion either in vitro or in vivo.

Hunt et al. (1954) reported that inorganic sulfur as Na_2SO_4 and organic sulfur as methionine both stimulated cellulose digestion in vitro. The stimulation obtained from each of these sulfur compounds was substantially the same. A somewhat lower stimulation was obtained when cystine was used as the source of sulfur and an even lower stimulation when elemental sulfur was used. Ethionine had an inhibitory effect.

Bentley et al. (1954a) found that n-valeric acid, caproic acid, isobutyric acid or isovaleric acid stimulated cellulose digestion by centrifuged rumen microorganisms. Valine, proline or valine plus proline also had a stimulatory effect.

Bryant and Doetsch (1954) found that 3 micromoles of n-valeric acid plus 1.5 micromoles of isovaleric acid could replace rumen fluid in permitting good growth of Bacteroides succinogenes.

According to Brooks et al. (1954), stilbesterol, estrone or cholesterol increased cellulose digestion by ovine rumen microorganisms both in vivo and in vitro, and Brooks et al. (1954a) reported that addition of corn oil reduced cellulose digestion both in vivo and in vitro.

There is little direct evidence relating to the effect of cobalt on cellulose digestion. Bowstead and Fredeen (1948) found that when cows being fed urea went off feed, appetite could be restored in some cases by the addition of cobalt to the diet. Becker and Smith (1951) found that cobalt-deficient lambs showed a higher apparent coefficient of digestion for crude fiber than did cobalt-supplemented lambs. They attributed this to the lower dry matter intake of the cobalt-deficient lambs. Ruf et al. (1953) found that the addition of approximately one microgram of vitamin B_{12} per 100 ml. did not stimulate cellulose digestion in vitro. However, Bentley et al. (1954) reported that vitamin B_{12} together with various combinations of other B vitamins stimulated cellulose digestion in vitro when cells centrifuged from rumen fluid were used as the inoculum. Jermyn (1952) obtained a slight increase in the degradation of sodium carboxymethyl cellulose by unpurified Aspergillus oryzae enzymes when cobalt was added to give a 10^{-3} molar concentration.

EXPERIMENTAL PART

SECTION I - PRELIMINARY EXPERIMENTS

The experiments included in this section were undertaken in an attempt to ascertain some of the factors affecting *in vitro* cellulose digestion. Because the results obtained were affected by the methods used, it was felt that they should be included in a separate section.

EXPERIMENTAL

Animals used. Inocula for use in these studies were obtained from two steers, 707 and 741, and one cow, A-55. Of the steers, 707 was a four-year old Guernsey and 741 was a four-year old Holstein. The cow was a Holstein and was nine years old at the time of these experiments. All three animals had been fistulated and the fistulae were fitted with screw-cap lucite plugs similar in construction to the one described by Hentschl *et al.* (1954). The plugs used in the steers were somewhat smaller than the one described.

Collection of samples. In experiment No. 2, 3, and 4 the method of sample collection described by Wasserman *et al.* (1952) was used. In later experiments it was found more expedient to use an insulated jug and to eliminate the passage of CO_2 through the rumen fluid during the collection of the sample. The procedure finally adopted was as follows:- The insulated jug was pre-warmed by filling it with hot water and allowing it to stand for half an hour or longer in the laboratory. The water was then removed and the air flushed out of the jug with CO_2. At the barn, a

large, long-stemmed funnel was inserted into the mouth of the jug. This served the double purpose of facilitating the introduction of the rumen fluid into the jug and of reducing the diffusion of CO_2 from the jug. A sufficient amount of the rumen contents was then removed from the animal through the fistula to permit the introduction of a half-pint milk bottle into the ventral sac of the rumen. Usually there was enough fluid in the rumen to fill the bottle rapidly. If the rumen contents were so dry that this was not the case, the bottle could be filled by holding it in position until the next rumen contraction occurred. The rumen fluid was then squeezed into the insulated jug through one layer of cheesecloth. In most cases, sufficient fluid was collected to fill the jug, but this did not appear to be an important factor in the cellulose digestion.

Fermentation procedures. In experiment No. 2, 3, and 4 the method of Wasserman et al. (1952) was used. The volume of Complex No. 1 used outside the sac in each of these experiments was approximately 1800 ml.

In the remainder of the experiments reported in this section the fermentation procedure used was similar to that described by Huhtanen et al. (1954). The inocula used consisted of 25 ml. of rumen fluid except in four cases in experiment No. S-4 where 10 ml. inocula were used in order to increase the percent cellulose present in the fermentation mixture. Larger bottles than the prescribed 4-ounce size were used in experiment No. S-2 to S-7. In subsequent experiments the 4-ounce bottles were used. Complex No. 1 was used for experiment No. S-2 and S-3, and Complex No. 2 for experiment No. S-4 to S-11.

"Visking" cellulose casing[1] of 1 inch diameter was used for all

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[1] Obtainable from the Visking Corporation, Chicage, Ill.

experiments involving the use of the fermentation procedure of Huhtanen et al. (1954). The substrates used in the experiments described in this section were Whatman No. 1 filter paper, ground to pass through the medium screen of a Wiley mill, and alfalfa leaf meal.[2] The composition of the mineral mixtures used is shown in Table 1.

Determination of cellulose. Cellulose was determined by the method of Crampton and Maynard (1938). In the experiments of this section the mixture from within the sacs was transferred to tared evaporating dishes of appropriate size and the dry matter determined by drying to constant weight in a forced air oven at 60°C. In experiment No. 2, 3, and 4 the dried material was extracted with ethyl ether in a Soxhlet apparatus and the percentage cellulose determined on the fat-free dry matter. Since the results are expressed as percentage of the cellulose originally present that is digested, the calculation on a fat-free basis was considered to be superfluous and the extraction step was eliminated in subsequent experiments.

As will be shown in Section II, it was later found possible to eliminate the dry matter determination thus increasing the reproducibility of the results and reducing considerably the time required for the analyses.

RESULTS

Table 2 shows the percentage cellulose digested when Complex No. 1 or a solution containing 2.5 g. $(NH_4)_2SO_4$ per liter was used outside the sac. At the beginning of the fermentation period each sac contained

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[2] Lot No. 28-3MA-131 obtained from the Cerophyl Laboratores Inc., 2438 Broadway, Kansas City 10, Missouri.

TABLE 1

COMPOSITION OF THE MINERAL MIXTURES USED IN THE PRELIMINARY EXPERIMENTS

Salt	Complex No. 1	Complex No. 2
	g./l.	g./l.
$(NH_4)_2SO_4$	2.5	2.5
$NaH_2PO_4.H_2O$	2.5	2.5
$NaHCO_3$	1.6	1.6
$MgSO_4.7H_2O$	0.15	0.15
KCl	0.75	0.75
NaCl	0.30	0.30
$CaCl_2.2H_2O$	0.05	0.01
$FeSO_4.7H_2O$	0.002	0.002
$CoCl_2.6H_2O$	0.002	0.002
$ZnSO_4.7H_2O$	0.002	0.002
$CuSO_4.5H_2O$	0.001	0.001
$MnSO_4.H_2O$	0.001	0.001

TABLE 2

THE AMOUNTS OF CELLULOSE DIGESTED WHEN A SOLUTION OF AMMONIUM SULFATE OR COMPLEX NO. 2 WAS PLACED OUTSIDE OF THE SAC[1]

Experiment	Material outside sac	Cellulose present		Cellulose digested	
		At start	At finish		
no.		g.	g.	g.	%
2 - 1	$(NH_4)_2SO_4$[2]	3.724	4.014	-0.290	
2 - 2	$(NH_4)_2SO_4$[2]	3.724	3.597	0.127	3.4
2 - 3	Complex No. 1	3.724	2.005	1.719	46.2
2 - 4	Complex No. 1	3.724	2.308	1.416	38.0
2 - 5	Complex No. 1	3.724	2.623	1.101	29.6
2 - 6	Complex No. 1	3.724	2.229	1.495	40.1

[1] Fermentation method - Wasserman *et al.* (1952)
Inoculum - 200 ml. of strained rumen fluid from cow No. A-55
Substrate - 4 g. ground filter paper
Incubation - 30 hours at 40°C.

[2] 2.5 g. per liter

200 ml. of strained rumen fluid from cow No. A-55 and 4 grams of ground filter paper. Incubation was maintained for 30 hours at 40°C. The percentage cellulose digested ranged from 29.6 to 46.2 when complex mineral solution was used and was either negative or slightly positive when $(NH_4)_2SO_4$ was used alone.

The effect of adding small amounts of indole-3-acetic acid and the three related compounds ethyl ester indole-3 acetic acid, 2-methyl indole-3-acetic acid, and indole-3-pyruvic acid early in the digestion period is shown in Table 3. This experiment was similar to the one above except ground filter paper (3 g.) was used as the substrate. Incubation was maintained at 40°C for 24 hours and the compounds to be tested were added within the sac after 2 hours of incubation. The percentage cellulose digested in the presence of ethyl ester indole-3-acetic acid and of indole-3-pyruvic acid was 52.7 and 51.3, respectively, as compared to 53.7% and 56.9% for the controls. The percentage cellulose digested in the presence of indole-3-acetic acid and of 2-methyl indole-3-acetic acid was 48.5 and 44.2, respectively, slightly lower than the digestion obtained in the controls. These differences are probably less than the accuracy of the method.

Table 4 shows the effect of added starch and/or lactose and of dried corn steep liquor on cellulose digestion. In this experiment, 75 ml. of strained rumen fluid from cow No. A-55 were added to 2.3 grams of ground filter paper in each sac. The test materials were then washed into the sacs with 75 ml. of distilled water, bringing the total volume within each sac to 150 ml. The volume within each sac of the controls was also brought to 150 ml. by adding 75 ml. of distilled water. Complex No. 1 was used

TABLE 3

AMOUNTS OF CELLULOSE DIGESTED WHEN INDOLE-3-ACETIC ACID OR SOME RELATED COMPOUNDS WERE ADDED TO THE FERMENTATION MIXTURE[1]

Experiment	Compound tested	Amount added per sac	Cellulose present		Cellulose digested	
			At start	At finish		
no.		ml.	g.	g.	g.	%
3 - 1	None - control	-	3.026	1.400	1.626	53.7
3 - 2	None - control	-	3.026	1.304	1.722	56.9
3 - 3	Indole-3-acetic acid[2]	1	3.026	1.558	1.468	48.5
3 - 4	Ethyl ester indole-3-acetic acid[2]	1	3.026	1.432	1.594	52.7
3 - 5	2-methyl indole-3-acetic acid[2]	1	3.026	1.690	1.337	44.2
3 - 6	Indole-3-pyruvic acid[2]	1	3.026	1.475	1.552	51.3

1 Fermentation - Wasserman et al. (1952)
Inoculum - 200 ml. of strained rumen fluid from cow No. A-55
Substrate - 3 g. ground filter paper
Incubation - 24 hours at 40°C.

2 Added as a solution containing 0.013 mg. per ml.

TABLE 4

THE AMOUNTS OF CELLULOSE DIGESTED WHEN LACTOSE, STARCH, OR CORN STEEP LIQUOR WERE ADDED TO THE FERMENTATION MIXTURE[1]

Experiment	Material added to sac	Cellulose present		Cellulose digested	
		At start	At finish		
no.		g.	g.	g.	%
4 - 1	Nil - control	2.035	1.224	0.811	39.9
4 - 2	Lactose, 0.6 g.	2.035	1.472	0.563	27.7
4 - 3	Lactose, 0.6 g. plus starch, 3 g.	2.035	2.117	-0.082	-
4 - 4	Nil - control	2.035	1.047	0.988	48.6
4 - 5	Starch, 3 g.	2.035	1.980	0.055	5.2
4 - 6	Corn steep liquor, dried, 0.6 g.	2.035	1.015	1.020	50.1

[1] Fermentation method - Wasserman *et al.* (1952)
Inoculum - 75 ml. of strained rumen fluid from cow No. A-55
Substrate - 2.3 g. ground filter paper
Incubation - 28 hours at 40°C.

outside the sacs. Incubation was maintained at 40°C for 28 hours. The percentage cellulose digested in the controls was 39.9 and 48.6. Addition of 0.6 grams of lactose depressed the cellulose digestion to 27.7% and addition of 3 grams of starch or 3 grams of starch plus 0.6 grams of lactose effectively inhibited cellulose digestion. The value obtained when 0.6 grams of corn steep liquor had been added was 50.1% substantially the same as in the controls.

The effect of changing the concentration of cobalt in the outer liquid is shown in Table 5. The bottles used were of 400 ml. capacity except in the case of S-2-13 where a 625 ml. bottle was used. The purpose of using the larger bottles was to bring the total cobalt concentration closer to the amount added, by equilibration across the membrane. The cobalt was added as a concentrated solution of $CoCl_2.6H_2O$. Examination of the percent cellulose digested when 1 ppm. of cobalt was added reveals a satisfactory agreement among replicates. In common with later results in this section, however, the values obtained were quite low. When 1000 ppm. of cobalt were added, 7.6% of the cellulose was digested and when 100 ppm. were added 10.8% of the cellulose was digested. This may have been due to precipitation of a large part of the cobalt by the formation of carbonate or of complexes with the constituents of the outer liquid, since a voluminous precipitate was observed in these bottles. Addition of 2, 4, and 10 ppm. of cobalt gave negative values for percent cellulose digested. Addition of 0.5 ppm. gave a cellulose digestion of 5.7% and 1.4% was digested when no addition was made.

Table 6 shows the percentage cellulose digested after 4, 8, 12, and 24 hours incubation at 40°C. In this experiment both ground filter paper

TABLE 5

THE AMOUNTS OF CELLULOSE DIGESTED WHEN DIFFERENT AMOUNTS OF COBALT WERE ADDED[1]

Experiment	Cobalt added	Cellulose present		Cellulose digested	
		At start	At finish		
no.	ppm.	g.	g.	g.	%
S-2-1	1	0.7788	0.7244	0.0544	6.99
S-2-2	1	0.7857	0.7150	0.0707	9.00
S-2-3	1	0.7741	0.7175	0.0566	7.31
S-2-4	1	0.7753	0.7233	0.0520	6.71
S-2-5	1	0.7692	0.7119	0.0573	7.45
S-2-6	1000	0.7628	0.7048	0.0580	7.60
S-2-7	100	0.7665	0.6835	0.0830	10.83
S-2-8	10	0.7602	0.7874	-0.0272	-3.58
S-2-9	4	0.7627	0.7719	-0.0092	-1.21
S-2-10	2	0.7933	0.8048	-0.0115	-1.45
S-2-11	0.5	0.7585	0.7153	0.0432	5.70
S-2-12	0	0.7559	0.7450	0.0109	1.44

[1] Fermentation method - Huhtanen *et al.* (1954) using 400 ml. glass bottles for S-2-1 to S-2-11 and a 625 ml. bottle for S-2-12
Inoculum - 25 ml. of strained rumen fluid from steer No. 707
Substrate - ground filter paper
Incubation - 24 hours at 40°C
Cobalt added as the chloride

TABLE 6

THE AMOUNTS OF CELLULOSE DIGESTED IN DIFFERENT INCUBATION PERIODS[1]

Experiment no.	Incubation hr.	Cellulose present At start g.	Cellulose present At finish g.	Cellulose digested g.	Cellulose digested %	Average %
		Substrate - ground filter paper				
S-3-1	4	0.7935	0.7915	0.0020	0.25	
S-3-2	4	0.7947	0.7902	0.0045	0.57	
S-3-3	4	0.7965	0.7814	0.0151	1.90	0.91
S-3-4	8	0.8242	0.7645	0.0597	7.24	
S-3-5	8	0.7924	0.7701	0.0223	2.81	
S-3-6	8	0.7913	0.7448	0.0425	5.37	5.14
S-3-7	12	0.7736	0.7438	0.0298	3.85	
S-3-8	12	0.8430	0.8301	0.0129	1.53	
S-3-9	12	0.7759	0.7418	0.0341	4.39	3.26
S-3-10	24	0.8035	0.7560	0.0475	5.91	
S-3-11	24	0.7828	0.7261	0.0567	7.24	
S-3-12	24	0.8127	0.7662	0.0465	5.72	6.29
		Substrate - alfalfa leaf meal				
S-3-13	4	0.2190	0.2110	0.0080	3.65	
S-3-14	4	0.2175	0.2011	0.0164	7.54	
S-3-15	4	0.2213	0.2180	0.0033	1.49	4.23
S-3-16	8	0.2252	0.2145	0.0107	4.75	
S-3-17	8	0.2168	0.1936	0.0232	11.22	
S-3-18	8	0.2341	0.2146	0.0195	8.33	8.10
S-3-19	12	lost				
S-3-20	12	0.2230	0.2062	0.0168	7.53	
S-3-21	12	0.2174	0.1962	0.0212	9.75	8.64
S-3-22	24	0.2155	0.1724	0.0431	20.00	
S-3-23	24	0.2155	0.1672	0.0483	22.41	
S-3-24	24	0.2157	0.1740	0.0417	19.33	20.58

[1] Fermentation method - Huhtanen *et al.* (1954) using 8-ounce bottles
Inoculum - 25 ml. of strained rumen fluid from steer No. 707
Incubation - at 40°C.

and ground alfalfa leaf meal were used as substrates. With ground filter paper as the substrate, the percentage cellulose digested in 24 hours was approximately 20% greater than the percentage digested in 8 hours. When alfalfa leaf meal was used as the substrate, the corresponding difference was 60%. Moreover, when the percentage cellulose digested at the end of each incubation period using alfalfa leaf meal as the substrate is compared with the corresponding value for the filter paper substrate, it can be seen that the alfalfa leaf meal gave a higher percentage digestion. This may indicate that the alfalfa leaf meal cellulose was more easily digested or that the alfalfa leaf meal supplied some factor that was lacking in the filter paper.

Experiment No. S-7 was designed to determine whether centrifuging the inoculum would improve agreement among replicates. The centrifuged inoculum was obtained by centrifuging strained rumen fluid from steer No. 741 at approximately 1500 rpm. for 10 minutes. This operation was performed in a walk-in incubator to avoid cooling the sample. A compact layer of plant material separated out with a thin, white layer immediately above it. The white layer was mixed with the turbid supernatant liquid by gentle swirling without visibly disturbing the compact bottom layer. Ten replicates using the centrifuged inoculum and ten replicates using the uncentrifuged inoculum were incubated at 40°C for 24 hours with the results shown in Table 7. Ground filter paper was used as the substrate. The centrifuged inoculum gave the better agreement among replicates but the percentage cellulose digested was somewhat lower than that obtained using the uncentrifuged inoculum. The replicates inoculated with the centrifuged rumen fluid averaged 7.8% cellulose digested with a range from

TABLE 7

THE EFFECT OF CENTRIFUGED AND UNCENTRIFUGED INOCULA ON CELLULOSE DIGESTION[1]

Experiment no.	Cellulose present At start	Cellulose present At finish	Cellulose digested	
	g.	g.	g.	%
Centrifuged inoculum				
S-7-1	0.3815	0.3612	0.0203	5.32
S-7-2	0.3723	0.3404	0.0319	8.57
S-7-3	0.3762	0.3486	0.0276	7.34
S-7-4	0.3884	0.3682	0.0202	5.20
S-7-5	0.3819	0.3493	0.0326	8.54
S-7-6	0.3734	0.3415	0.0319	8.54
S-7-7	0.3679	0.3322	0.0357	9.70
S-7-8	0.3710	0.3417	0.0293	7.90
S-7-9	0.3718	0.3427	0.0291	7.83
S-7-10	0.3694	0.3371	0.0323	8.74
			Average	7.77
Uncentrifuged inoculum				
S-7-11	0.3894	0.3527	0.0367	9.42
S-7-12	0.3890	0.3405	0.0485	12.47
S-7-13	0.3926	0.3554	0.0372	9.48
S-7-14	0.4082	0.3720	0.0362	8.87
S-7-15	0.3878	0.3438	0.0440	11.35
S-7-16	0.3928	0.3204	0.0720	18.43
S-7-17	0.3884	0.3429	0.0455	11.71
S-7-18	0.3867	0.3571	0.0296	7.65
S-7-19	0.3867	0.3446	0.0421	10.89
S-7-20	0.3881	0.3549	0.0332	8.55
			Average	10.88

[1] Fermentation procedure - Huhtanen *et al.* (1954) using 8-ounce bottles
Inoculum - 25 ml. of centrifuged or uncentrifuged rumen fluid from steer No. 741
Substrate - ground filter paper
Incubation - 24 hours at 40°C

5.2 to 8.7%. Those inoculated with uncentrifuged rumen fluid averaged 10.9% with a range from 7.7 to 18.4%.

Tables 8, 9, and 10 show the effect of changing the ratio of the substrate to the inoculum on the percentage cellulose digested. In these experiments 8-ounce bottles were used. In Table 8 the percentage cellulose digested when two different substrates were used is shown. The inoculum was uncentrifuged rumen fluid from steer No. 741. When ground filter paper was used as the substrate no appreciable difference in cellulose digestion was obtained. This may have been due to the high proportion of cellulose in the fermentation mixture, since the percentage cellulose present at the start of the incubation period ranged from 7.6 to 0.7%.

The results obtained in experiment No. S-8 are shown in Table 9. This experiment was essentially a repetition of experiment No. S-4 with ground filter paper as the only substrate and using both centrifuged and uncentrifuged inocula. The percentage cellulose present at the start of the incubation was lower than in experiment S-4 and ranged from 0.22 to 2.9% for the centrifuged inoculum and from 0.25 to 3.2% for the uncentrifuged inoculum. Differences between duplicates were considerable, particularly with the uncentrifuged inoculum.

Experiment No. S-9 was a repetition of the above experiment with the difference that alfalfa leaf meal was used as the substrate. Both centrifuged and uncentrifuged rumen fluid were used as inocula. The results obtained are shown in Table 10. The cellulose present in the fermentation mixture at the start of the incubation period ranged from 0.24 to 0.79% for the centrifuged inoculum and from 0.23 to 0.76% for the uncentrifuged

TABLE 8

THE EFFECT OF CHANGING THE RATIO OF SUBSTRATE TO INOCULUM ON CELLULOSE DIGESTION[1]

Experiment no.	Cellulose present At start g.	Cellulose present At finish g.	Cellulose digested g.	Cellulose digested %	Average %
		Substrate - ground filter paper			
S-4-1	0.7492	0.6855	0.0637	8.38	
S-4-2	0.7604	0.6938	0.0666	8.76	8.57
S-4-3	0.7587	0.6832	0.0755	9.95	
S-4-4	0.7577	0.7128	0.0449	5.93	7.94
S-4-5	0.3756	0.3598	0.0158	4.21	
S-4-6	0.3804	0.3617	0.0187	4.92	4.57
S-4-7	0.1912	0.1804	0.0104	5.44	
S-4-8	0.1889	0.1729	0.0160	8.47	6.96
		Substrate - alfalfa leaf meal			
S-4-9	0.1841	0.1925	-0.0084		
S-4-10	0.1830	0.2052	-0.0222		
S-4-11[2]	0.2014				
S-4-12	0.1831	0.1616	0.0215	11.74	
S-4-13	0.0922	0.0727	0.0195	21.15	
S-4-14	0.0915	0.0691	0.0224	24.48	22.82
S-4-15	0.0555	0.0387	0.0168	30.27	
S-4-16	0.0549	0.0394	0.0155	28.23	29.25

1 Fermentation procedure - Huhtanen et al. (1954) using 8-ounce bottles
Inoculum - 25 ml. of strained rumen fluid from steer No. 741 except in the case of S-4-1, S-4-2, S-4-9, and S-4-10, each of which received 10 ml.
Incubation - 24 hours at 40°C

2 Oxidation with nitric and acetic acids not complete

TABLE 9

EFFECT OF CHANGING THE RATIO OF SUBSTRATE TO INOCULUM ON CELLULOSE DIGESTION[1]

Experiment no.	Cellulose present At start	Cellulose present At finish	Cellulose digested	
	g.	g.	g.	%
		Centrifuged inoculum		
S-8-1	0.0542	0.0572	-0.0030	-5.54
S-8-2	0.0550	0.0540	0.0010	1.82
S-8-3	0.1100	0.1081	0.0019	1.73
S-8-4	0.1070	0.1044	0.0026	2.43
S-8-5	0.1860	0.1726	0.0134	7.20
S-8-6	0.1799	0.1682	0.0117	6.50
S-8-7	0.2646	0.3312	0.0334	9.16
S-8-8	0.3628	0.3743	-0.0105	-2.89
S-8-9	0.5435	0.5255	0.0180	3.31
S-8-10	0.5384	0.4663	0.0721	13.39
S-8-11	0.7280	0.6379	0.0901	12.38
S-8-12	0.7241	0.6841	0.0400	5.52
		Uncentrifuged inoculum		
S-8-13	0.0613	0.0715	-0.0102	-16.64
S-8-14	0.0623	0.0663	-0.0040	-6.42
S-8-15	0.1210	0.1109	0.0101	8.35
S-8-16	0.1293	0.1232	0.0041	3.17
S-8-17	0.2123	0.1984	0.0139	6.55
S-8-18	0.2029	0.1796	0.0233	11.48
S-8-19	0.4037	0.3602	0.0435	10.78
S-8-20	0.4027	0.3676	0.0451	10.93
S-8-21	0.6014	0.5259	0.0755	12.55
S-8-22	0.6091	0.5322	0.0769	12.63
S-8-23	0.8093	0.6909	0.1184	14.63
S-8-24	0.8045	0.7116	0.0929	11.55

[1] Fermentation procedure - Huhtanen *et al.* (1954)
Inoculum - 25 ml. of centrifuged or uncentrifuged rumen fluid from steer No. 741
Substrate - ground filter paper
Incubation - 24 hours at 40°C

TABLE 10

EFFECT OF CHANGING THE RATIO OF SUBSTRATE TO INOCULUM ON CELLULOSE DIGESTION[1]

Experiment no.	Cellulose present At start g.	Cellulose present At finish g.	Cellulose digested g.	Cellulose digested %	Average %
		Centrifuged inoculum			
S-9-1	0.0601	0.0456	0.0145	24.13	
S-9-2	0.0606	0.0466	0.0140	23.10	
S-9-3	0.0607	0.0449	0.0158	26.03	24.42
S-9-4	0.0991	0.0685	0.0306	30.88	
S-9-5	0.0996	0.0731	0.0265	26.61	28.75
S-9-6	0.1583	0.1229	0.0354	22.36	
S-9-7	0.1578	0.1160	0.0418	26.49	24.43
S-9-8	0.1983	0.1512	0.0471	23.75	
S-9-9	0.1985	0.1606	0.0379	19.09	21.42
		Uncentrifuged inoculum			
S-9-10	0.0576	0.0577	-0.0001	-0.17	
S-9-11	0.0575	0.0470	0.0105	18.26	
S-9-12	0.0574	0.0516	0.0058	10.10	9.39
S-9-13	0.0956	0.0764	0.0192	20.08	
S-9-14	0.0953	0.0761	0.0192	20.15	20.12
S-9-15	0.1524	0.1200	0.0324	21.26	
S-9-16	0.1522	0.1142	0.0380	24.97	23.12
S-9-17	0.1910	0.1596	0.0314	16.44	
S-9-18	0.1907	0.1616	0.0291	15.26	15.85

[1] Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of centrifuged or uncentrifuged rumen fluid from steer No. 741
Substrate - alfalfa leaf meal
Incubation - 24 hours at 40°C

inoculum. No consistent difference in the percentage cellulose digested was observed.

In Table 11 the results of a re-examination of the effect of different cobalt concentrations are presented. It had been found that by thoroughly gassing Complex No. 2 with CO_2 before the addition of the cobalt stock solution as much as 200 ppm. of the cobalt could be held in solution as judged by visual examination. Consequently, the quantities of stock solution were chosen so as to give a range of cobalt added from 2 to 200 ppm. The salt used was $CoCl_2.6H_2O$. Both centrifuged and uncentrifuged inocula were used. Incubation with the centrifuged inoculum showed little variation with the change in cobalt concentration. When the uncentrifuged inoculum was used, however, the percentage cellulose digested in the presence of 200 ppm. and 100 ppm. was negative or slightly positive.

TABLE 11

THE EFFECT OF ADDED COBALT ON CELLULOSE DIGESTION[1]

Experiment no.	Cobalt added ppm.	Cellulose present At start g.	Cellulose present At finish g.	Cellulose digested g.	Cellulose digested %	Average %
		Centrifuged inoculum				
S-10-1	200	0.4786	0.4588	0.0198	4.14	
S-10-2	200	0.4952	0.4785	0.0167	3.37	3.76
S-10-3	100	0.4749	0.4600	0.0149	3.14	
S-10-4	100	0.4923	0.4812	0.0111	2.25	2.65
S-10-5	50	0.4874	0.4698	0.0176	3.61	
S-10-6	50	0.4949	0.4921	0.0028	0.57	2.09
S-10-7	25	0.4804	0.4671	0.0133	2.77	
S-10-8	25	0.4819	0.4587	0.0232	4.81	3.79
S-10-9	10	0.4831	0.4705	0.0126	2.61	
S-10-10	10	0.4873	0.4618	0.0255	5.23	3.92
S-10-11	2	0.4772	0.4599	0.0173	3.63	
S-10-12	2	0.5113	0.4822	0.0291	5.69	4.66
		Uncentrifuged inoculum				
S-10-13	200	0.5112	0.5062	0.0050	0.98	
S-10-14	200	0.5350	0.5352	-0.0002	-0.04	0.47
S-10-15	100	0.5125	0.5390	-0.0265	-5.17	
S-10-16	100	0.5050	0.4814	0.0236	4.67	-0.25
S-10-17	50	0.5112	0.4830	0.0282	5.52	
S-10-18	50	0.5106	0.4789	0.0317	6.21	5.87
S-10-19	25	0.5102	0.4666	0.0755	14.80	
S-10-20	25	0.5131	0.4814	0.0317	6.18	10.49
S-10-21	10	0.5216	0.4976	0.0240	4.60	
S-10-22	10	0.5064	0.5094	-0.0030	-0.59	2.01
S-10-23	2	0.5204	0.4940	0.0264	5.07	
S-10-24	2	0.5180	0.4697	0.0483	9.32	7.20

1 Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. centrifuged or uncentrifuged rumen fluid from steer No. 741
Substrate - ground filter paper
Incubation - 24 hours at 40°C
Cobalt added as the chloride

SECTION II - EXAMINATION OF THE METHODS

The results obtained in the preliminary experiments (Section I) are subject to two obvious criticisms. First, the analytical procedure, in which the percentage cellulose was based on dry matter, involved too many manipulations which contributed to the inaccuracy of the results. The substrate was weighed into the sacs as a dry sample, necessitating the use of separately weighed samples as controls, and the possibility of differences in mixing affecting the percentage cellulose digested must be added to the likelihood of variation in composition and of inaccuracy in weighing and transferring associated with small samples. The transfers involved in the dry matter determination contributed to the inaccuracy of the method and the hygroscopic nature of the dried fermentation mixture made accurate weighing difficult. Moreover, the possibility existed that unequal loss of non-cellulosic constituents from the fermentation mixture during the drying process may have increased the error. The negative values for percentage cellulose digested which were obtained in some cases strengthen the possibility that there was greater loss from the fermented samples than from the unfermented controls.

The second criticism is that the percentage cellulose digested when the fermentation procedure of Huhtanen *et al.* (1954) was used was very low. This would indicate that optimum or even good conditions for cellulose digestion were not attained. The possibility existed, therefore, that digestion might be limited by some deficiency in the method in such a way as to conceal or distort the effect of the factors being studied on cellulose digestion.

The object of the experiments reported in this section was the reduction of the analytical error and the attainment of a fermentation procedure which would result in satisfactory digestion of the cellulose.

EXPERIMENTAL

The inocula for the experiments reported in this section were obtained from steer No. 741 and from cow No. A-55. The method of collecting samples was the same as that used in Section I. The substrates used were Solka-Floc[1], Whatman No. 1 filter paper ground to pass through the medium screen of a Wiley mill, and alfalfa leaf meal[2].

Fermentation procedures. The method used for experiment No. B-1, B-8, B-18, and B-20 was similar to the one described by Burroughs et al. (1950). Brown glass reagent bottles of 625 ml. capacity were used. In experiment B-1 the substrate and the complex mineral solution were introduced and the volume adjusted to 210 ml. The bottles were placed in a water bath at 40°C. and CO_2 was bubbled through while the inoculum was being obtained. Sufficient rumen fluid was added to bring the volume in the bottles to 350 ml. Samples for cellulose determinations were removed by means of a 25 ml. pipette while the mixture was being stirred vigorously with CO_2. The passage of CO_2 was then continued at a much slower rate while the mixtures were being incubated at 40°C. for 24 hours. At the end of this period the rate of CO_2 passage was again increased and 25 ml. portions removed for cellulose determination. A 350 ml. portion of the

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1 A wood cellulose product manufactured by the Brown Company, Berlin, New Hampshire.

2 Lot No. 28-3MA-131, obtained from the Cerophyl Laboratories Inc., 2438 Broadway, Kansas City 10, Missouri.

remainder was added to another 625 ml. bottle containing approximately one gram of ground filter paper and the required volume of mineral solution which had been brought to 210 ml. by the addition of distilled water. Samples were then taken for the determination of the cellulose present at the start of the second period. In this way, fermentation was continued for three successive periods. In the other experiments reported in this section, fermentation was limited to one 24-hour period.

The concentrations of the different salts present in the fermentation mixture when the various mineral solutions were used in experiment No. B-1 are shown in Table 16. These concentrations do not take into account the salts already present in the rumen fluid.

In experiment No. B-8 the fermentation procedure was similar to that described above, except that 350 ml. of complex mineral solution and 200 ml. of rumen fluid were used. In experiment No. B-18 and B-20, 200 ml. portions of mineral solution and of rumen fluid were used. The mineral solution used in these three experiments contained the ingredients suggested by McDougall (1948) but at one-half the concentrations given and with the following additional salts per liter:- $FeSO_4.7H_2O$, 40 mg.; $CoCl_2.6H_2O$, 2 mg.; $ZnSO_4.7H_2O$, 2 mg.; $CuSO_4.5H_2O$, 1 mg.; $MnSO_4.H_2O$, 1 mg.

In experiment No. S-16 and S-28, no sacs were used and incubation was conducted in 2-ounce, brown glass reagent bottles using 25 ml. of rumen fluid as the inoculum. When mineral solution was used, it was evaporated to dryness at 105°C in the bottles in which the fermentation was to take place, in order to avoid any effects caused by dilution of the inoculum.

In experiment No. S-24 and S-37, the fermentation procedure was similar to that of Huhtanen et al (1954) except that bottles of different sizes

were used in order to test the effect of changing the ratio of the volume of the inoculum to that of the outer liquid. The mineral mixture used was the modification of the "artificial saliva" of McDougall (1948) described earlier in this section. This solution was also used in experiment No. 8 and S-15 and for the controls in experiment No. S-19.

In experiment No. 8 the fermentation procedure of Wasserman et al. (1952) was used along with modifications permitting the passage of CO_2 into the outer liquid or eliminating altogether the passage of CO_2 during the fermentation. No mechanical stirring was used for these modifications.

The fermentation procedure of Huhtanen et al (1954) was used for experiment No. S-5, S-6, S-15 and S-19. The mineral solution used in experiment No. S-5 and S-6 was Complex No. 2 with the $(NH_4)_2SO_4$ omitted.

The following procedure was used for inoculation in those experiments in which sacs were used:-

Sufficient substrate to give a final concentration of approximately one percent was added to a brown glass bottle of the appropriate size. The required amount of strained rumen fluid was added and CO_2 bubbled through the mixture until the substrate appeared to be uniformly distributed throughout. While the mixture was being stirred vigorously with CO_2, 25 ml. portions were pipetted into the sacs or fermentation bottles. Samples were also taken at this time for the determination of cellulose in order to ascertain how much cellulose was present at the start of the fermentation. The pipette used for sampling was one from which the fine point had been removed so that it could be filled and emptied rapidly. As soon as each sac was filled it was placed in its bottle which had been standing in a water bath at 40°C and already contained the mineral

solution which had been gassed with CO_2. The bottle was capped tightly and replaced in the water bath where it was held until inoculation had been completed for the whole experiment. All the bottles were then placed in an incubator at 39°C. At the end of the incubation period the contents of each sac were washed into a beaker or a centrifuge bottle for the cellulose determination.

Determination of cellulose. Since the Druce and Wilcox (1949) method of determining cellulose has been reported to give a product composed mainly of hexosan, it was compared with the Crampton and Maynard (1938) method. The results obtained did not appear to justify further work on the Druce and Wilcox method and subsequent experiments were therefore directed toward devising a satisfactory modification of the Crampton and Maynard method.

The Crampton and Maynard (1938) method of determining cellulose requires a one-gram, air-dry sample. Hence the calculations must be based on a dry matter determination with its concomitant disadvantages discussed earlier in this section. Moreover, the use of a one-gram sample of filter paper or Solka-Floc frequently results in serious bumping. For these reasons, and also to eliminate the use of a reflux condenser and round-bottom flask for the acid digestion, the following procedure was used:-

A 25 ml. sample of the fermentation mixture was evaporated to dryness in a 250 ml. centrifuge bottle and the residue digested with the acid mixture in this same bottle by placing it on a hot plate for 23 minutes. A microscope slide was added to prevent bumping which was usually not serious unless the amount of cellulose present in the sample was over 0.2 gram. In the earlier experiments the sample was evaporated to dryness

on a hot plate, a stream of air being drawn from the bottle by suction during the drying process. Because of the loss experienced from overheating when this method was used, the bottle containing the wet sample was placed in a constant temperature oven at 105°C overnight in later experiments. It was found that when this method was used, evaporation took place too slowly because of the height of the centrifuge bottles. Consequently, in the method finally adopted, the 25 ml. sample was placed in a 50 or 100 ml. beaker, dried at 105°C overnight, then refluxed with the acid mixture by using a round-bottom flask filled with cold water to cover the beaker while it was being heated on the hot plate.

To ascertain the reproducibility of the results obtained when the samples for the determination of cellulose were pipetted from a suspension as described above, approximately 5 grams of Solka-Floc were weighed and transferred to a 625 ml. bottle. A 550 ml. portion of Complex No. 2 was added, CO_2 bubbled through the mixture, and twelve successive 25 ml. samples pipetted into centrifuge bottles for cellulose determinations. In another experiment 5 grams of Solka-Floc were weighed and transferred to a 625 ml. bottle, 350 ml. of Complex No. 2 plus 200 ml. of rumen fluid added, and twelve successive 25 ml. samples obtained as before.

RESULTS

Determination of cellulose. The comparison of the Crampton and Maynard (1938) and the Druce and Wilcox (1949) methods of cellulose determination gave the results shown in Table 12. It can be seen that the Druce and Wilcox method gave a much lower value for the percentage cellulose. This may have been due, at least in part, to unfamiliarity with the method. The filtrations involved were generally slow.

TABLE 12

THE PERCENTAGE CELLULOSE IN TWO HAYS AND IN FILTER PAPER AS DETERMINED BY TWO DIFFERENT METHODS

Material	Method of Crampton and Maynard[1]		Method of Druce and Wilcox[2]	
	Replicates	Average	Replicates	Average
	%	%	%	%
Hay No. K-2	31.18		19.0	
Hay No. K-2	31.11	31.15	19.3	19.15
Hay No. K-3	26.70		18.2	
Hay No. K-3	26.67	26.69	18.3	18.25
Filter paper	88.2		43.5	
Filter paper	88.7		44.9	
Filter paper	89.7	88.9	45.5	44.6

1 J. Nutrition 15, 383 - 395, 1938

2 Jour. Agric. Sci. 39, Part 2, 145 - 152, 1949

Table 13 shows the results of the experiments to test the reproducibility of the method of sampling. Replicate 25 ml. samples were pipetted from the fermentation mixture while it was being stirred with a stream of CO_2 as previously described. The results shown include any error due to the method of cellulose determination as well as that due to sampling. Since the method of cellulose determination used here was the one described previously in which centrifuge bottles were used, results from the controls of later experiments where beakers were used in the analysis are also included in the table. In experiment No. C-47 the average amount of cellulose per 25 ml. sample was 211.6 mg., with a range from 194.1 to 225.5 mg. The standard deviation was 8.9 mg. and the 5% fiducial limits 206.0 to 217.2 mg. In experiment No. C-48 the average amount of cellulose per 25 ml. sample was 196.2 mg., with a range from 182.4 to 216.0 mg. The standard deviation was 6.8 mg. and the 5% fiducial limits 191.9 to 200.5 mg. The results shown for the examples taken from experiment No. S-25 and S-30 indicate that the method finally adopted had a somewhat greater accuracy. This was probably due to the fact that overheating was avoided by evaporation in the oven, very little vapor was lost during refluxing, and bumping could be avoided when breakers were used. The controls from S-25 gave an average of 272.3 mg. of cellulose per 25 ml. sample, and ranged from 268.1 to 276.5 mg. The standard deviation was 2.7 mg. and the 5% fiducial limits were 270.3 and 274.3 mg.

Alfalfa leaf meal was used in experiment No. S-30. When the controls from this experiment were analysed, an average of 242.4 mg. of cellulose per 25 ml. was obtained and the range was from 239.1 to 246.7 mg. The standard deviation was 2.6 mg. and the 5% fiducial limits were 240.5 and 244.3 mg.

TABLE 13

THE AMOUNTS OF CELLULOSE FOUND IN SUCCESSIVE ALIQUOTS FROM THE SAME SUSPENSION

Experiment no.	Cellulose mg.	Experiment no.	Cellulose mg.
Solka-Floc		**Alfalfa leaf meal**	
C-47-1	225.5	S-30-0-1	246.7
C-47-2	219.7	S-30-0-2	239.1
C-47-3	209.0	S-30-0-3	242.2
C-47-4	222.0	S-30-0-4	243.4
C-47-5	217.6	S-30-0-5	240.4
C-47-6	194.1	S-30-0-6	242.5
C-47-7	206.2	Average	242.4
C-47-8	216.5		
C-47-9	209.4		
C-47-10	208.0	**Pith from bagasse**	
C-47-11	206.3		
C-47-12	204.3	S-35-0-13	111.4
Average	211.6	S-35-0-14	114.2
		S-35-0-15	113.9
C-48-1	197.4	Average	113.2
C-48-2	191.0		
C-48-3	193.9		
C-48-4	201.7	**Extracted cotton linters**	
C-48-5	198.4		
C-48-6	192.9		
C-48-7	182.4	S-34-0-7	247.4
C-48-8	199.3	S-34-0-8	244.9
C-48-9	196.1	S-34-0-9	244.0
C-48-10	216.0	Average	245.4
C-48-11	190.7		
C-48-12	194.4		
Average	196.2	**Untreated cotton linters**	
S-25-0-1	271.0		
S-25-0-2	272.2	S-34-0-4	231.0
S-25-0-3	276.5	S-34-0-5	234.7
S-25-0-4	268.1	S-34-0-6	236.9
S-25-0-5	273.2	Average	234.2
S-25-0-6	272.8		
Average	272.3		

Results from some of the controls used in experiment No. S-34 and S-35 are shown to illustrate the repeatability of the determination when other substrates were used. Since only three replicates were used, no statistical treatment was attempted. It can be seen, however, that although suspensions of pith or cotton linters are difficult to sample, satisfactory agreement among replicates was obtained.

Effect of cysteine.HCl and sodium thioglycollate with Complex No. 2.

Quastel and Stephenson (1926a) found that cysteine and other -SH compounds could be used to lower the oxidation-reduction potential of media in order to facilitate the growth of anaerobic organisms. To test the possibility that the poor cellulose digestion heretofore obtained in the experiments in which the fermentation procedure of Huhtanen et al. (1954) was used was due to lack of anaerobic conditions, cysteine hydrochloride or sodium thioglycollate was added to the Complex No. 2 used outside the sac. The results obtained using filter paper as the substrate are shown in Table 14. The average cellulose digestion in the controls was 5.2%. When cysteine hydrochloride or sodium thioglycollate was added to the outer liquid, the percentage cellulose digested was 3.9 and 3.0 respectively. When $(NH_4)_2SO_4$ was omitted from the mineral solution, only 1.8% of the cellulose was digested.

Table 15 shows the effect of changing the concentration of $(NH_4)_2SO_4$ in the mineral solution and of adding cysteine hydrochloride or sodium thioglycollate when the substrate was alfalfa leaf meal. On the assumption that the alfalfa leaf meal might provide sufficient readily available nitrogen, $(NH_4)_2SO_4$ was omitted from the complex when cysteine hydrochloride or sodium thioglycollate was added. The results indicated that added

TABLE 14

THE AMOUNTS OF CELLULOSE DIGESTED WHEN CYSTEINE HYDROCHLORIDE OR SODIUM THIOGLYCOLLATE WAS ADDED TO THE OUTER LIQUID[1]

Experiment no.	Additions to outer liquid	Cellulose present At start	Cellulose present At finish	Cellulose digested		Average
		g.	g.	g.	%	%
S-5-3	None - control	0.7386	0.7097	0.0289	3.91	
S-5-4	None - control	0.7947	0.7362	0.0585	7.36	
S-5-5	None - control	0.6793	0.6493	0.0300	4.42	5.23
S-5-6	Cysteine.HCl[2]	0.6479	0.6236	0.0243	3.75	
S-5-7	Cysteine.HCl[2]	0.8183	0.7833	0.0350	4.28	
S-5-8	Cysteine.HCl[2]	0.5796	0.5576	0.0220	3.80	3.94
S-5-9	Na thioglycollate[3]	0.6223	0.6078	0.0145	2.33	
S-5-10	Na thioglycollate[3]	0.6735	0.6472	0.0263	3.90	
S-5-11	Na thioglycollate[3]	0.6067	0.5894	0.0173	2.85	3.03
S-5-12	Cysteine.HCl[2,4]	0.7661	0.7486	0.0175	2.28	
S-5-13	Cysteine.HCl[2,4]	0.7103	0.7010	0.0093	1.31	1.80
S-5-14	None[4]	0.7428	0.7454	-0.0026	-0.35	
S-5-15	None[4]	0.7644	0.7449	0.0195	2.55	1.10

1 Fermentation procedure - Huhtanen et al. (1954), using 8-ounce bottles
Inoculum - 25 ml. of centrifuged rumen fluid from steer No. 741
Substrate - ground filter paper
Incubation - 24 hours at 40°C

2 To a concentration of 0.13 g. per l.

3 To a concentration of 0.12 g. per l.

4 $(NH_4)_2SO_4$ omitted from the mineral solution

TABLE 15

THE AMOUNTS OF CELLULOSE DIGESTED WITH DIFFERENT LEVELS OF AMMONIUM SULFATE AND WHEN CYSTEINE HYDROCHLORIDE OR SODIUM THIOGLYCOLLATE WAS ADDED TO THE OUTER LIQUID[1]

Experiment	Additions to outer liquid[2]	Cellulose present		Cellulose digested		
		At start	At finish			Average
no.		g.	g.	g.	%	%
S-6-1	$(NH_4)_2SO_4$ doubled	0.1530	0.1130	0.0400	26.14	
S-6-2	$(NH_4)_2SO_4$ doubled	0.1520	0.1185	0.0335	22.04	24.09
S-6-3	None - control	0.1623	0.1247	0.0376	23.17	
S-6-4	None - control	0.1522	0.1252	0.0270	17.74	20.46
S-6-5	$(NH_4)_2SO_4$ halved	0.1523	0.1463	0.0060	3.94	
S-6-6	$(NH_4)_2SO_4$ halved	0.1520	0.1344	0.0176	11.58	7.76
S-6-7	$(NH_4)_2SO_4$ omitted	0.1533	0.1413	0.0120	7.83	
S-6-8	$(NH_4)_2SO_4$ omitted	0.1520	0.1486	0.0034	2.24	5.04
S-6-9	Cysteine.HCl[3]	0.1523	0.1375	0.0148	9.72	
S-6-10	Cysteine.HCl[3]	0.1526	0.1214	0.0312	20.45	15.09
S-6-11	Na thioglycollate[4]	0.1527	0.1337	0.0190	12.44	
S-6-12	Na thioglycollate[4]	0.1527	0.1393	0.0134	8.78	10.61

[1] Fermentation procedure - Huhtanen et al. (1954), using 8-ounce bottles
Inoculum - 25 ml. of centrifuged rumen fluid from steer No. 741
Substrate - alfalfa leaf meal
Incubation - 24 hours at 40°C

[2] Complex No. 2

[3] To a concentration of 0.13 g. per l.

[4] To a concentration of 0.12 g. per l.

nitrogen was required, since in the absence of added nitrogen only 5% of the cellulose was digested as compared to 24.1% for the highest level of added nitrogen. However, when cysteine hydrochloride was added in the absence of $(NH_4)_2SO_4$, 15.1% of the cellulose was digested and when sodium thioglycollate was added, 10.6% was digested.

Effect of different mineral solutions. In experiment No. B-1, the relative effects of different mineral solutions on the percentage cellulose digested was examined. The results are shown in Table 17. Urea was added to each fermentation bottle to give a final concentration of 0.1%. Glucose was added to B-1-9, B-1-10, B-1-11, and B-1-12, also at the 0.1 level. Each bottle received an inoculum of 140 ml. of strained rumen fluid, except B-1-11 which received 70 ml. of strained rumen fluid and B-1-10 and B-1-12 which received 140 ml. and 70 ml., respectively, of centrifuged rumen fluid. The salt concentrations attained in the fermentation mixture, exclusive of the amounts already present in the rumen fluid, are given in Table 16. Except for Complex No. 2 (with $(NH_4)_2SO_4$ omitted), the mineral solutions used in this experiment are identified in Tables 16 and 17 by the publications in which they were reported. The "artificial saliva" suggested by McDougall (1948) was used at full strength and also at half the concentration and one-third the concentration given in his report.

From the values given in Table 17, it can be seen that the cellulose digestion was much greater than that which had been achieved in previous experiments using the method of Huhtanen et al. (1954). If the digestion obtained in the first 24-hour period is considered alone, none of the solutions used seem to show any great advantage over the others. The digestion obtained using Complex No. 2 (with $(NH_4)_2SO_4$ omitted), although much

TABLE 16

THE SALT CONCENTRATIONS ATTAINED IN THE FERMENTATION MIXTURE WHEN DIFFERENT MINERAL SOLUTIONS WERE USED IN EXPERIMENT B-1

Salt	Bur-roughs[1]	Arias[2]	Hudman & Kunkel[3]	McDoug-all[4]	McDoug-all[5]	McDoug-all[6]	McDoug-all[7]	Complex No. 2[8]
	g./l.	g./l.	g./l.	g./l.	g./l.	g./l.	g./l.	g./l.
$NaHCO_3$	3.75	3.75	4.14	5.88	2.94	1.96	5.88	0.96
$NaH_2PO_4.H_2O$	3.75	3.75						1.5
$Na_2HPO_4.12H_2O$				5.64	2.82	1.88	5.64	
$NaCl$	0.53	0.53	0.59	0.29	0.14	0.10	0.29	0.18
KCl	0.53	0.53	0.59	0.35	0.17	0.12	0.35	0.45
$CaCl_2.2H_2O$	0.54	0.54		0.021	0.016	0.007	0.021	0.006
$CaCl_2$			0.06					
$MgCl_2.6H_2O$				0.077	0.038	0.026	0.077	
$MgSO_4.7H_2O$	0.16	0.16	0.18					0.09
$FeSO_4.7H_2O$	0.0114		0.10				0.048	0.0012
$CoCl_2.6H_2O$	0.0114						0.0048	0.0012
$ZnSO_4.7H_2O$	0.0057						0.0024	0.0012
$CuSO_4.5H_2O$	0.0028						0.0012	0.0006
$MnSO_4.H_2O$	0.0057						0.0012	0.0006
Na_2SO_4			0.23					
$CoCl_2$			0.001					
NaH_2PO_4			4.93					

1 J. Nutrition 40, 9 - 24, 1950
2 Jour. An. Sci. 10, 683 - 692, 1951
3 Agric. Food Chem. 1, 1060 - 1062, 1953
4 Biochem. J. 43, 99 - 109, 1948
5 "Artificial saliva" of McDougall (1948) diluted to half strength
6 "Artificial saliva" of McDougall (1948) diluted to one-third strength
7 "Artificial saliva" of McDougall (1948) with trace minerals added
8 $(NH_4)_2SO_4$ omitted

TABLE 17

THE AMOUNTS OF CELLULOSE DIGESTED WHEN DIFFERENT MINERAL SOLUTIONS WERE USED OUTSIDE OF THE SAC[1]

Experiment	Mineral solution	Inoculum	Cellulose digested			
			First period	Second period	Third period	Average
no.		ml.	%	%	%	%
B-1-1	Burroughs et al. (1950)	140	59.2	67.7	47.1	58.0
B-1-2	Arias et al. (1951)	140	64.0	68.1	51.7	61.3
B-1-3	Hudman & Kunkel (1953)	140	66.5	51.8	44.9	54.4
B-1-4	McDougall (1948)	140	70.1	47.8	39.0	52.3
B-1-5	McDougall (1948)[2]	140	69.3	67.8	44.2	60.4
B-1-6	McDougall (1948)[3]	140	62.5	61.9	53.0	59.1
B-1-7	Complex No. 24	140	52.7	55.5	53.0	53.7
B-1-8	McDougall (1948)[5]	140	67.3	46.5	31.1	48.3
B-1-9	Arias et al. (1953)	140	61.2	65.2	53.8	60.1
B-1-10	Arias et al. (1953)	140[6]	56.2	70.1	68.3	64.8
B-1-11	Arias et al. (1953)	70	66.7	69.1	58.8	64.9
B-1-12	Arias et al. (1953)	70[6]	49.6	60.5	56.9	55.7

1 Fermentation procedure - Burroughs et al. (1950)
Inoculum - rumen fluid from cow No. A-55
Substrate - Solka-Floc
Non-protein nitrogen - 0.1% urea
Readily available carbohydrate - 0.1% glucose added to B-1-9, B-1-10, B-1-11 and B-1-12
Incubation - 40°C for 24 hours

2 Half strength

3 One-third strength

4 $(NH_4)_2SO_4$ omitted

5 Trace minerals added

6 Centrifuged inoculum

higher than had been obtained in most of the earlier experiments, was lower than that obtained with the other mineral solutions. This suggested the possibility that the use of Complex No. 2 may have contributed to the low values for cellulose digestion obtained previously.

It can be seen that with the solutions used by Burroughs et al. (1950) and by Arias et al. (1951) a greater percentage of the cellulose was digested in the second period than in the first. This may indicate an adaptation of the microorganisms to the conditions used.

When the inoculum was centrifuged and glucose added, the percentage cellulose digested was maintained at a somewhat higher level than when glucose was added and an uncentrifuged inoculum was used. When the inoculum was reduced to 70 ml. and glucose added, the percentage cellulose digested was maintained at a higher level by using an uncentrifuged inoculum than by a centrifuged inoculum.

Effect of rate of passage of CO_2. Hudman and Kunkel (1953) have reported that, at low levels of urea concentration, somewhat more protein was synthesized by rumen microorganisms in vitro when a high CO_2 tension was maintained than in an atmosphere of nitrogen. Cellulose digestion was not determined. In the fermentation procedure of Burroughs et al. (1950) it is difficult to control rigidly the rate of passage of CO_2 through the mixture. It was of interest, therefore to determine whether the rate of passage of CO_2 affected the extent of cellulose digestion under the conditions used. In experiment No. B-18 and B-20 the CO_2 was passed through the fermentation mixtures at different rates. The results obtained are shown in Table 18. Urea was added to a final concentration of 0.1% in both experiment No. B-18 and experiment No. B-20. However, glucose was added only in experiment No. B-18.

TABLE 18

THE EFFECT OF RATE OF PASSAGE OF CO_2 ON CELLULOSE DIGESTION[1]

Experiment no.	Rate of passage of CO_2	Cellulose digested Replicates %	Average %
B-18-1	Fast	35.2	
B-18-2	Fast	36.5	35.9
B-18-3	Medium	37.7	
B-18-4	Medium	41.7	39.7
B-18-5	Slow	44.6	
B-18-6	Slow	43.3	44.0
B-20-1	Fast	60.6	
B-20-2	Fast	56.9	58.8
B-20-3	Medium	60.7	
B-20-4	Medium	58.8	59.8
B-20-5	Slow	63.5	
B-20-6	Slow	67.9	65.7
B-20-7	Nil	68.5	
B-20-8	Nil	67.0	67.8

[1] Fermentation procedure - Burroughs et al. (1950)
Inoculum - 200 ml. of strained rumen fluid from cow No. A-55
Mineral solution - "artificial saliva" of McDougall (1948), diluted to half strength and trace minerals added
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea
Readily available carbohydrate - 0.1% glucose in experiment No. B-18. Not added in experiment No. B-20
Incubation - 24 hours at 40°C

Since no flow meter was used, the rates are given as "fast", "medium", and "slow". It can be seen that as the rate of passage of CO_2 was decreased the percentage of the cellulose digested increased. The replicates where the rate of passage of CO_2 is described as "nil" received CO_2 only while being sampled. The percentage cellulose digested in experiment No. B-20 was considerably higher than that digested in experiment No. B-18. This may have been due to the presence of glucose in experiment No. B-18, for although the quantity added (0.1%) was not excessive, it is possible that differences in the ration used and the time elapsed since feeding may have produced different levels of readily available carbohydrate in the two samples of rumen fluid used for inoculation.

Effect of manner of introduction of CO_2. Since the continuous passage of CO_2 through the fermentation mixture appeared to have a depressing effect on cellulose digestion, an experiment was conducted using the fermentation procedure of Wasserman et al. (1952) to determine whether the manner of introduction of the CO_2 was responsible for the rather low cellulose digestion obtained in previous experiments with this method. The results obtained are shown in Table 19. Three sets of duplicate fermentations were run; one which followed the method described by Wasserman et al. (1952), one in which the CO_2 was introduced outside the sac, and one in which no CO_2 was used and in which the sac was held in place by tightly capping the bottle. Introduction of CO_2 within the sac gave the greatest percent cellulose digestion and the elimination of CO_2 passage gave the least. It should be noted that the efficiency of mixing was greatest in experiment No. 8-1 and 8-2 where CO_2 was introduced within the sac and the liquid outside the sac was stirred mechanically. In experiment 8-3 and 8-4

TABLE 19

THE AMOUNTS OF CELLULOSE DIGESTED WHEN CO_2 WAS PASSED INSIDE OR OUTSIDE OF THE SAC OR WHEN CO_2 WAS NOT USED[1]

Experiment no.	CO_2 added	Cellulose digested %	Average %
8-1	Inside sac	48.5	
8-2	Inside sac	44.5	46.5
8-3	Outside sac	44.0	
8-4	Outside sac	42.8	43.4
8-5	Not added	40.3	
8-6	Not added	45.4[2]	

1 Fermentation procedure - Wasserman et al. (1952) for 8-1 and 8-2. Modified for 8-3 to 8-6
Inoculum - 100 ml. of strained rumen fluid from cow No. A-55
Mineral solution - "artificial saliva" of McDougall (1948) diluted to half strength and trace minerals added
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea added to inoculum and to mineral solution
Readily available carbohydrate - not added
Incubation - 24 hours at 40°C

2 Some loss during transfer from sac, resulting in a high value for percent cellulose digested

mixing was accomplished in the outer liquid only by the stream of CO_2. There was no mixing in experiment No. 8-5 and 8-6. Consequently, the differences in percent cellulose digested could be interpreted as arising from differences in mixing rather than from differences in the manner of introducing the CO_2.

Effect of increasing the calcium content of Complex No. 2. The percentage cellulose digested in experiment No. 2, 3, and 4 (Section I, Tables 2, 3, and 4) where Complex No. 1 was used, was considerably higher than in the experiments where Complex No. 2 was used. To investigate the possibility that this was due to the lower calcium content of Complex No. 2, an experiment was conducted using the fermentation procedure of Huhtanen et al. (1954). The mineral solution used for the controls was the "artificial saliva" of McDougall (1948) diluted to half strength and with trace minerals added. Complex No. 2 (with $(NH_4)_2SO_4$ omitted) was used for the other fermentations and calcium chloride solution added to give a range of calcium concentration in the solution from 1.3 to 13.8 ppm. The results are shown in Table 20. The percentage cellulose digested was much higher than had been obtained with Complex No. 2 in previous experiments but considerably lower than that obtained in the controls. Differences in calcium concentration in Complex No. 2 had no appreciable effect on the percentage cellulose digested.

Fermentation without a sac. Table 21 shows the results of three experiments conducted to compare the fermentation method of Huhtanen et al. (1954) with one in which the inoculum was placed in a 2-ounce bottle with the substrate and the bottle capped and incubated. When the sac was used, 82% of the cellulose was digested in the absence of added glucose and 80%

TABLE 20

THE EFFECT OF INCREASING THE CALCIUM CONCENTRATION IN COMPLEX NO. 2 ON CELLULOSE DIGESTION[1,4]

Experiment no.	Solution used outside of sac	Concentration of Ca outside of sac	Cellulose digested	Average
		ppm.	%	%
S-19-1	Complex No. 2	1.3	54.8	
S-19-2	Complex No. 2	1.3	51.5	
S-19-3	Complex No. 2	1.3	56.8	54.4
S-19-4	Complex No. 2 plus Ca	2.6	47.0	
S-19-5	Complex No. 2 plus Ca	2.6	53.0	
S-19-6	Complex No. 2 plus Ca	2.6	51.0	50.3
S-19-7	Complex No. 2 plus Ca	7.6	57.1	
S-19-8	Complex No. 2 plus Ca	7.6	55.4	
S-19-9	Complex No. 2 plus Ca	7.6	54.9	55.8
S-19-10	Complex No. 2 plus Ca	13.8	53.0	
S-19-11	Complex No. 2 plus Ca	13.8	53.8	
S-19-12	Complex No. 2 plus Ca	13.8	51.2	52.7
S-19-13	McDougall (1948)[2]	14	83.8	
S-19-14	McDougall (1948)[2]	14	82.9	
S-19-15[3]	McDougall (1948)[2]	14	78.7	81.8

1 Fermantation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from cow No. A-55
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea added to the inoculum bottle
Readily available carbohydrate - not added
Incubation - 24 hours at 39°C

2 Diluted to half strength and trace minerals added

3 8-ounce bottle used

4 $(NH_4)_2SO_4$ omitted from Complex No. 2

TABLE 21

CELLULOSE DIGESTION WITH AND WITHOUT A SAC[1]

Experiment no.	Glucose added %	Mineral solution	Cellulose digested %	Cellulose digested Average %
		With sac[2]		
S-15-1	None	McDougall[3]	86.2	
S-15-2	None	McDougall[3]	83.0	
S-15-3	None	McDougall[3]	76.8	82.0
S-15-4	0.1[4]	McDougall[3]	77.6	
S-15-5	0.1[4]	McDougall[3]	81.7	
S-15-6	0.1[4]	McDougall[3]	81.3	80.2
		Without sac[5]		
S-16-1	None	None	57.1	
S-16-2	None	None	56.0	
S-16-3	None	None	56.3	56.5
S-16-4	0.1	None	65.0	
S-16-5	0.1	None	65.6	
S-16-6	0.1	None	61.4	64.0
S-16-7	None	McDougall[3,6]	-5.3	
S-16-8	None	McDougall[3,6]	0.2	
S-16-9	None	McDougall[3,6]	0.5	-1.5
S-16-10	0.1	McDougall[3,6]	11.5	
S-16-11	0.1	McDougall[3,6]	7.2	
S-16-12	0.1	McDougall[3,6]	2.0	6.9
S-28-1	None	None	47.0	
S-28-2	None	None	34.5	
S-28-3	None	None	34.6	38.7
S-28-4	0.1	None	30.1	
S-28-5	0.1	None	24.1	
S-28-6	0.1	None	19.2	24.5
S-28-7	None	Complex #2[6,7]	50.7	
S-28-8	None	Complex #2[6,7]	53.4	
S-28-9	None	Complex #2[6,7]	40.6	48.2
S-28-10	0.1	Complex #2[6,7]	51.3	
S-28-11	0.1	Complex #2[6,7]	44.4	
S-28-12	0.1	Complex #2[6,7]	31.7	42.5

1 Inoculum - 25 ml. of strained rumen fluid from cow No. A-55
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea added to inoculum
Incubation - 24 hours at 39°C
2 Fermentation procedure - Huhtanen et al. (1954)
3 McDougall (1948)- 1/2 strength plus trace minerals
4 0.1% in inoculum; not added to outer liquid
5 Fermentation procedure - 2-ounce bottles, no sac
6 Residue from 25 ml.
7 $(NH_4)_2SO_4$ omitted

in the presence of glucose. The glucose was added (to give a concentration of 0.1%) to the bottle from which the sacs were inoculated. It is unlikely that the concentration would be maintained as long or at as high a level as would be the case if the glucose had also been added outside the sac.

Values for the percentage cellulose digested when no sac was used were considerably lower. The mineral mixture used in experiment No. S-16 was the residue from "artificial saliva" of McDougall (1948) which had been diluted to half strength and to which trace minerals had been added. When the dry matter from 25 ml. of this solution was added, cellulose digestion ceased or was greatly reduced. In experiment No. S-28, when the residue from 25 ml. of Complex No. 2 (with $(NH_4)_2SO_4$ omitted) was added, cellulose digestion was increased. Addition of 0.1% glucose resulted in an increase in the percentage cellulose digested in experiment No. S-16 and a decrease in experiment No. S-28. Thus, under these conditions, the inoculum appears to be a factor in the effect of glucose on the percentage cellulose digested.

Effect of ratio of inoculum to mineral solution. Examination of Tables 19 and 21 shows that a much higher percentage of the cellulose was digested in experiment No. S-15 than in experiment No. 8. The most obvious differences between the conditions used for experiment No. 8-5 and 8-6 and those used for experiment No. S-15 were the amounts of mineral solution used, the size of the sacs, and the volume of the fermentation mixture. The possibility existed that the larger sacs which were used in experiment No. 8, and which appeared to be of a somewhat thicker material than the small sacs used in experiment No. S-15, retarded the diffusion of some nutrient or end product. Another possibility was that the ratio of the volume of the fermentation mixture to the surface exposed for dialysis might have been different for

one experiment than for the other. If the lower percentage cellulose digestion obtained in experiment No. 8 was due to the larger volume of mineral solution used, it might indicate that the resulting dilution had produced a deficiency in a dialyzable nutrient.

Two experiments were conducted to test the possibility that the change in the ratio of the volume of the inoculum to the volume of the mineral solution was responsible for the difference in cellulose digestion. The results are shown in Table 22. The ratios of the volume of the inoculum to the volume of the mineral solution ranged from 0.0132 to 0.25. The small sacs were used. To keep conditions as uniform as possible, it was necessary to add sufficient urea to both inoculum and mineral solution to bring the concentration to 0.1%. For comparison with previous work, controls were used in which urea was added only to the inoculum. In experiment No. S-37 there was a consistent decrease in percentage cellulose digested as the ratio of the inoculum to the mineral solution decreased. The results obtained in experiment No. S-24 were more erratic but the trend was in the same direction. The conditions in experiment No. S-24 were not as well controlled as those in experiment No. S-37, and this may account for the greater variation in the results.

TABLE 22

THE EFFECT OF THE INOCULUM:MINERAL-SOLUTION RATIO ON CELLULOSE DIGESTION[1]

Experiment	Bottle size	Volume outside sac	Inoculum: mineral-solution ratio	Cellulose digested	Average
no.		ml.		%	%
S-24-1	4 oz.	100	0.25	59.2	
S-24-2	4 oz.	100	0.25	65.9	
S-24-3	4 oz.	100	0.25	67.2	64.1
S-24-4	8 oz.	200	0.125	7.7	
S-24-5	8 oz.	200	0.125	7.2	
S-24-6	8 oz.	200	0.125	10.3	8.4
S-24-7	500 ml.	450	0.0556	47.9	
S-24-8	500 ml.	450	0.0556	45.4	
S-24-9	500 ml.	450	0.0556	35.8	43.0
S-24-10	750 ml.	700	0.0357	53.7	
S-24-11	750 ml.	700	0.0357	65.0	
S-24-12	750 ml.	700	0.0357	40.6	53.1
S-24-13	2000 ml.	1900	0.0132	50.1	
S-24-14	2000 ml.	1900	0.0132	39.5	
S-24-15	2000 ml.	1900	0.0132	39.4	43.0
S-24-16	4 oz.	100[2]	0.25	58.3	
S-24-17	4 oz.	100[2]	0.25	65.3	
S-24-18	4 oz.	100[2]	0.25	60.7	61.4
S-37-1	4 oz.	100	0.25	59.6	
S-37-2	4 oz.	100	0.25	71.1	
S-37-3	4 oz.	100	0.25	52.8	61.2
S-37-4	8 oz.	200	0.125	55.8	
S-37-5	8 oz.	200	0.125	47.6	
S-37-6	8 oz.	200	0.125	57.7	53.7

TABLE 22 (CONCLUDED)

Experiment	Bottle size	Volume outside sac	Inoculum: mineral solution ratio	Cellulose digested	Cellulose digested Average
no.		ml.		%	%
S-37-7	500 ml.	450	0.0556	48.6	
S-37-8	500 ml.	450	0.0556	47.2	
S-37-9	500 ml.	450	0.0556	43.2	46.3
S-37-10	750 ml.	700	0.0357	44.5	
S-37-11	750 ml.	700	0.0357	39.0	
S-37-12	750 ml.	700	0.0357	42.7	42.1
S-37-13	2000 ml.	1900	0.0132	37.2	
S-37-14	2000 ml.	1900	0.0132	38.7	
S-37-15	2000 ml.	1900	0.0132	44.3	40.1
S-37-16	4 oz.	100[2]	0.25	70.7	
S-37-17	4 oz.	100[2]	0.25	74.1	
S-37-18	4 oz.	100[2]	0.25	73.1	72.6

[1] Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. strained rumen fluid from steer No. 741
Mineral solution - "artificial saliva" of McDougall (1948) diluted to half strength and trace minerals added
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea
Readily available carbohydrate - not added
Incubation - 24 hours at 39°C

[2] Urea not added to outer liquid

SECTION III - FACTORS AFFECTING CELLULOSE DIGESTION

It was felt at this point that the fermentation procedure of Huhtanen et al. (1954), with the modifications adopted (including the use of the "artificial saliva" of McDougall (1948) diluted to half strength and with trace minerals added), was sufficiently reliable to justify its use in continuing the study of some of the factors which affect cellulose digestion. Certain of the experiments reported in this section were re-examinations of results obtained in the preliminary experiments.

EXPERIMENTAL

Inocula for the experiments described in this section were obtained from cow No. A-55 and steer No. 741. The methods used in collecting the samples were the same as those used in Section I.

Fermentation procedure. The fermentation procedure used in most of the experiments of this section was similar to that described by Huhtanen et al. (1954). High-form, screw-capped bottles of 4-ounce capacity were used. The sacs were made from "Visking" cellophane tubing of 1 inch diameter. The mineral solution used was patterned after the "artificial saliva" suggested by McDougall (1948) and was designated as Complex No. 3. Its composition is shown in Table 23.

A large flask or 10-liter bottle of the mineral solution was gassed vigorously with CO_2 while the inoculum was being obtained from the animal. The solution was kept in a water bath at 45°C while it was being gassed. The 4-ounce bottles to be used for incubation were filled

TABLE 23

COMPOSITION OF COMPLEX NO. 3

Salt	Concentration
	g./l.
$NaHCO_3$	4.9
$Na_2HPO_4.12H_2O$	4.7
NaCl	0.24
KCl	0.29
$CaCl_2.2H_2O$	0.026
$MgCl_2.6H_2O$	0.064
$FeSO_4.7H_2O$	0.04
$CoCl_2.6H_2O$	0.004
$ZnSO_4.7H_2O$	0.002
$CuSO_4.5H_2O$	0.001
$MnSO_4.H_2O$	0.001

with the warm, gassed Complex No. 3, tightly capped, and placed in a water bath at 40°C. The substrate was weighed into a bottle of the appropriate size, the required amount of strained rumen fluid added, and the bottle with its contents placed in the water bath at 40°C. Usually, when one concentration of some other additive was desired, this was also added to the inoculum bottle but to obtain a range of concentrations, additions were made to both the sac and the outer liquid. Non-protein nitrogen was usually supplied as urea and added at the 0.1% level to the inoculum bottle only.

The fermentation mixture in the inoculum bottle was stirred vigorously by a stream of CO_2 until the substrate was wet and the suspension appeared to be uniform. The time required for this varied considerably. Solka-Floc became wet almost immediately but untreated cotton linters required several minutes and it was necessary to rotate the bottle to keep the linters in contact with the rumen fluid.

When the suspension appeared to be uniform, 25 ml. portions were removed for cellulose determinations and for inoculation of the sacs. The pipette used for this purpose was one from which the fine point had been broken, permitting rapid filling and delivery. Whenever possible, the removal of the samples for the inoculation of each set of replicates was alternated with the removal of a sample for cellulose determination in order to compensate for any differences in the settling of the substrate due to the decreasing volume. This precaution appeared to be unnecessary if the volume of rumen fluid used sufficiently exceeded the volume required for inoculation and if the substrate did not settle rapidly.

As each sac was inoculated, it was transferred to the appropriate 4-ounce bottle. The bottle was again tightly capped and returned to the water bath until inoculation and sampling were completed. All replicates were then transferred to an incubator and held at 39°C for the incubation period.

In experiment No. B-5, B-6, B-8, B-10 and B-11 the fermentation procedure was that of Burroughs et al. (1950) except that 350 ml. of Complex No. 3 and 200 ml. of strained rumen fluid were used in each bottle. Experiment No. B-14 and B-15 were also incubated according to the procedure of Burroughs et al. (1950) but equal volumes of Complex No. 3 and strained rumen fluid were used. Experiment No. B-11 was continued for two 24-hour periods.

Determination of cellulose. The procedure finally adopted for the determination of cellulose was as follows:-

If the sample was obtained from the inoculum bottle or from the bottle used in the fermentation procedure of Burroughs et al. (1950), 25 ml. were pipetted into a 50 or a 100 ml. beaker and evaporated to dryness by leaving in a constant temperature oven at 105°C overnight. If the sample was from a sac, the entire contents of the sac were washed into a 100 or 150 ml. beaker and evaporated to dryness.

When the mixture was dry, 15 ml. of 80% acetic acid and 1.5 ml. of concentrated nitric acid were added. When a 50 or 100 ml. beaker was used, it was necessary to add a large glass bead to control bumping if the amount of cellulose expected was much over 0.2 g. This was usually unnecessary when a 150 ml. beaker was used.

The beaker and contents were placed on a Lindberg hot plate set at "medium", covered with a 150 ml. round-bottom flask of cold water, and the mixture boiled gently for 20 minutes. At the end of this period the mixture was cooled, washed into a 250 ml. centrifuge bottle with ethyl alcohol, and centrifuged for 10 minutes. The supernatant was decanted through a Selas porcelain filter crucible of XF porosity. The residue was resuspended in ethyl alcohol and poured onto the crucible, the last portions being washed out of the centrifuge bottle with a stream of ethyl alcohol from a wash bottle with the aid of a rubber policeman. The crucible was dried at 105°C overnight, cooled in a desiccator and weighed. It was then placed in a muffle furnace at 700°C for 2 hours, cooled in a desiccator, and weighed. The difference in weight was recorded as the weight of the cellulose in the sample.

RESULTS

Effect of 2,4-D on cellulose digestion. Neely et al. (1950) have shown, in the tumorous tissue resulting from the application of 2,4-D to red kidney bean plants, that the pectin methoxylase activity is about twice that observed in untreated tissue. This suggested the possibility of 2,4-D having a similar effect in the rumen. It was thought that if the pectin methoxylase activity could be stimulated by 2,4-D pectic material might be digested more rapidly, increasing the accessibility of cellulose and thus permitting it to be more rapidly digested also. Table 24 shows the results of an experiment to test this possibility. Alfalfa leaf meal was used as the substrate, since a plant species with a relatively high content of pectic material was

TABLE 24

THE AMOUNTS OF CELLULOSE DIGESTED IN THE PRESENCE OF ADDED 2,4-D[1]

Experiment no.	2,4-D added mg./ml.	Cellulose digested %	Cellulose digested Average %
S-20-1	0.0028	49.0	
S-20-2	0.0028	50.0	
S-20-3	0.0028	52.1	50.4
S-20-4	0.0056	53.5	
S-20-5	0.0056	53.7	
S-20-6	0.0056	52.1	53.1
S-20-7	0.014	53.3	
S-20-8	0.014	51.5	
S-20-9	0.014	51.8	52.2
S-20-10	0.028	54.2	
S-20-11	0.028	53.8	
S-20-12	0.028	53.7	53.9
S-20-13	0.056	54.1	
S-20-14	0.056	53.4	
S-20-15	0.056	54.1	53.9
S-20-16	0.112	53.0	
S-20-17	0.112	52.8	
S-20-18	0.112	52.2	52.7
S-20-19	None	47.4	
S-20-20	None	48.8	
S-20-21	None	47.2	47.7
S-20-22	0.28	46.5	
S-20-23	0.28	49.7	
S-20-24	0.28	50.4	48.9
S-20-25	0.56	51.5	
S-20-26	0.56	47.5	
S-20-27	0.56	50.5	49.8

1 Fermentation procedure - Huhtanen et al.(1954)
Inoculum - 25 ml. strained rumen fluid from cow No. A-55
Substrate - 4% alfalfa leaf meal
Mineral solution - Complex No. 3
Non-protein nitrogen - 0.1% urea added to inoculum
Readily available carbohydrate - not added
Incubation - 24 hours at 39°C

required. The 2,4-D was added both to the sac and to the outer liquid, so that the concentrations given were not lowered by diffusion from the sac. The results obtained show that the percentage cellulose digested was not affected by the presence of 2,4-D under the conditions used. The slightly lower percentage cellulose digested in experiment No. S-20-19 to S-20-27 may have been due to a higher initial cellulose concentration, since it was necessary to use a separate inoculum bottle for their inoculation. The cellulose level in this bottle was 15% higher than in the one used for inoculating experiment No. S-20-1 to S-20-18 (144.2 as compared to 124.8 mg. per 25 ml.).

Effect of certain treatments on digestion of cellulose in corn fiber. In the commercial preparation of corn starch, one of the byproducts is fiber from the kernel hulls. The question arose as to the suitability of this material for use in the feeding of cattle. A sample of the fiber was made available[1] and also a sample of the same fiber which had been treated with lime and a sample which had been treated with lime and then extracted with water. Experiments were conducted to determine the amounts of the cellulose of each sample that could be digested *in vitro*. The fermentation procedure of Burroughs *et al*. (1950) was used. The results of these experiments are shown in Table 25. The cellulose of the extracted fiber was found to be digested to a greater extent than that of the untreated fiber. In experiment No. B-5, the treated corn fiber also gave a high percentage cellulose digestion but the low value obtained when Solka-Floc was used as the substrate in this experiment

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[1] By the Corn Products Refining Co., Argo, Ill.

TABLE 25

THE AMOUNTS OF CELLULOSE DIGESTED WHEN UNTREATED, TREATED, AND EXTRACTED CORN FIBER WERE USED AS SUBSTRATES[1]

Experiment no.	Substrate	Cellulose digested, based on Cellulose at start %	Cellulose digested, based on Solka-Floc digestion %
B-5-1	Untreated corn fiber[2]	53.5	192
B-5-2	Treated corn fiber[3]	73.5	264
B-5-3	Extracted corn fiber[4]	70.9	255
B-5-4	Solka-Floc	27.8	100
B-6-1	Untreated corn fiber[2]	32.2	40
B-6-2	Treated corn fiber[3]	23.8	30
B-6-3	Extracted corn fiber[4]	81.2	101
B-6-4	Solka-Floc	79.5	100
B-8-1	Untreated corn fiber[2]	28.3	33
B-8-2	Treated corn fiber[3]	31.0	36
B-8-3	Extracted corn fiber[4]	73.0	84
B-8-4	Solka-Floc	86.8	100

1 Fermentation procedure - Burroughs *et al.* (1950)
Inoculum - 200 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Non-protein nitrogen - 0.1% urea
Readily available carbohydrate - 0.1% glucose
Incubation - 40°C for 24 hours

2 From kernel hulls

3 Treated with lime

4 Treated with lime then extracted with water

suggests that the fermentation was not a normal one.

The difference in the digestion of the cellulose in different substrates. Table 26 shows the results of an experiment to determine the relative digestion of the cellulose in different substrates. Solka-Floc was used as the control substrate to permit comparison with other experiments. Cotton linters[1] were used since they are a source of relatively pure "native" cellulose. Both untreated linters and linters that had been extracted in a Soxhlet apparatus with a 1:2 benzene:alcohol mixture were used. A sample of pith[2] obtained from bagasse was also included because of its possible use as a cattle feed. Alfalfa leaf meal and a sample of timothy hay grown on unfertilized soil served as examples of good and poor quality roughages.

It can be seen that the percentage cellulose digested when Solka-Floc was used as the substrate was again rather low. The percentage cellulose digested when extracted cotton linters were the substrate was considerably greater than when the untreated linters were used. The cellulose of the alfalfa leaf meal was digested to a much greater extent than that of the unfertilized timothy hay, the values obtained being 47.4 and 25.9%, respectively. Forty-three percent of the cellulose of the pith was digested.

Effect of urea concentration on cellulose digestion. Table 27 shows the percentage cellulose digested when different concentrations of urea were present at the start of the fermentation. The amount of

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1 Supplied by L. V. Curtin, Buckeye Cotton Oil Co., Cincinnati 17, Ohio.

2 A research product from the Northern Regional Research Laboratory, Peoria, Ill.

TABLE 26

THE AMOUNTS OF CELLULOSE DIGESTED WHEN DIFFERENT SUBSTRATES WERE USED[1]

Experiment no.	Substrate	Glucose added %	Cellulose digested, based on Cellulose at start %	Cellulose digested, based on Solka-Floc digestion %
B-10-1	Solka-Floc	0.1	44.9	100.0
B-10-2	Untreated cotton linters	0.1	42.3	94.2
B-10-3	Extracted cotton linters	0.1	53.7	119.6
B-10-4	Alfalfa leaf meal	None	47.4	105.6
B-10-5	Unfertilized hay No. 6535	0.1	25.9	57.7
B-10-6	Pith from bagasse	0.1	43.0	95.8

[1] Fermentation procedure - Burroughs et al. (1950)
Inoculum - 200 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Non-protein nitrogen - 0.1% urea
Readily available carbohydrate - 0.1% glucose
Incubation - 40°C for 24 hours

TABLE 27

THE EFFECT OF DIFFERENT CONCENTRATIONS OF UREA ON CELLULOSE DIGESTION[1]

Experiment no.	Urea added %	Cellulose digested %	Cellulose digested Average %
S-26-1	Nil	60.7	
S-26-2	Nil	42.1	
S-26-3	Nil	65.6	56.1
S-26-4	0.01	63.6	
S-26-5	0.01	57.9	
S-26-6	0.01	64.9	62.1
S-26-7	0.05	67.4	
S-26-8	0.05	67.8	
S-26-9	0.05	65.2	66.8
S-26-10	0.10	76.7	
S-26-11	0.10	74.6	
S-26-12	0.10	72.0	74.4
S-26-13	0.15	7.5	
S-26-14	0.15	6.5	
S-26-15	0.15	5.2	6.4
S-26-16	0.20	4.1	
S-26-17	0.20	4.0	
S-26-18	0.20	5.1	4.4

[1] Fermentation procedure - Huhtanen *et al.* (1954)
Inoculum - 25 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Substrate - 1% Solka-Floc
Readily available carbohydrate - not added
Incubation - 39°C for 24 hours

urea added ranged from 0.01% to 0.20%. No readily available carbohydrate was added. Under these conditions, a small amount of urea increased the percentage cellulose digested but excessive amounts effectively inhibited digestion. The average percentage cellulose digested increased progressively from 56.1% when no urea was added to 74.4% when 0.10% was added. When the initial urea concentration was 0.15% the percentage cellulose digested dropped sharply to 6.4% and was even less (4.4%) when the initial urea concentration was 0.20%. Replicates showed a wider range of variation when urea was not added or when the concentration was 0.01% than at the higher levels of added urea.

Effect of glucose concentration on cellulose digestion. The results obtained when different amounts of glucose were added are shown in Table 28. Since somewhat erratic results had been obtained in previous experiments when 0.1% glucose had been added, the range chosen was quite low, 0.00 to 0.20%. In general, the percentage cellulose digested in the presence of glucose was slightly higher than in the controls to which glucose had not been added. The gradual increase in percentage cellulose digested as the glucose concentration was increased from 0.005 to 0.15% may indicate a general trend, but the differences obtained were too small to be regarded as anything more than a possible indication. The agreement among replicates was best when no glucose was added and when 0.20% was added. This may indicate that the glucose added at the other levels was not evenly distributed because of the small volume of stock solutions used (0.25 to 0.75 ml.).

TABLE 28

THE EFFECT OF DIFFERENT CONCENTRATIONS OF GLUCOSE ON CELLULOSE DIGESTION[1]

Experiment no.	Glucose added %	Cellulose digested %	Cellulose digested Average %
S-29-1	Nil	68.5	
S-29-2	Nil	68.2	
S-29-3	Nil	65.5	67.4
S-29-4	0.005	74.7	
S-29-5	0.005	63.1	
S-29-6	0.005	70.8	69.5
S-29-7	0.01	72.1	
S-29-8	0.01	71.0	
S-29-9	0.01	71.8	71.6
S-29-10	0.05	69.6	
S-29-11	0.05	61.3	
S-29-12	0.05	61.3	64.1
S-29-13	0.10	65.5	
S-29-14	0.10	74.5	
S-29-15	0.10	77.0	72.3
S-29-16	0.15	74.4	
S-29-17	0.15	67.2	
S-29-18	0.15	77.3	73.0
S-29-19	0.20	68.4	
S-29-20	0.20	69.9	
S-29-21	0.20	66.4	68.2

1 Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from steer No. 741
Mineral solution - Complex No. 3
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea added to the inoculum bottle
Incubation - 39°C for 24 hours

Effect of cobalt and of vitamin B_{12} on cellulose digestion. Cobalt is known to stimulate the appetite in some cases of bovine anorexia. Because of the connection between cellulose digestion, rate of passage, and appetite, the possibility existed that cobalt might have some stimulatory effect on cellulose digestion. Although negative results had been obtained in previous experiments (see Section I), it was thought that the methods being used in this section might bring out differences not discernible in the earlier work.

Table 29 shows the percentage cellulose digested when different concentrations of cobalt were added. The fermentation procedure used was that of Burroughs et al. (1950) and urea and glucose were each added at the 0.1% level. Incubation was maintained at 40°C for two 24-hour periods. The results show a consistent decrease in the percentage cellulose digested as the amount of cobalt added was increased from 0.25 to 91 parts per million, but the decrease was not as great as was expected. In the first period, the percentage cellulose digested dropped from 61.8 when 0.25 part per million was added to 52.3 when 91 parts per million were added. In the second period, the corresponding values were 38.5 and 24.4%, respectively. Since urea and glucose were present, it is possible that one or both of these modified the effect of the higher levels of cobalt on the digestion of the cellulose.

Table 30 shows the percentage cellulose digested when different levels of cobalt were added in the absence of glucose. The fermentation procedure of Huhtanen et al. (1954) was used and 0.1% of urea was added to the inoculum bottle. The amount of added cobalt ranged from 1 to 200 parts per million. Again it was found that the percentage cellulose

TABLE 29

THE EFFECT OF DIFFERENT COBALT CONCENTRATIONS ON CELLULOSE DIGESTION[1]

Experiment	Cobalt added	Cellulose digested		
		First period	Second period	Average
no.	ppm	%	%	%
B-11-1	0.25	61.8	38.5	50.2
B-11-2	2.5	63.7	33.9	48.8
B-11-3	12.5	60.8	28.3	44.6
B-11-4	23.6	59.9	23.4	41.7
B-11-5	91	52.3	24.4	38.4

[1] Fermentation procedure - Burroughs et al. (1950)
Inoculum - 200 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3 with cobalt omitted
Substrate - 1% Solka - Floc
Non-protein nitrogen - 0.1% urea
Readily available carbohydrate - 0.1% glucose
Incubation - 40°C for 24 hours
Cobalt added as a solution of the chloride

TABLE 30

THE EFFECT OF DIFFERENT COBALT CONCENTRATIONS ON CELLULOSE DIGESTION[1]

Experiment no.	Cobalt added ppm	Cellulose digested %	Average %
S-22-1	1	61.5	
S-22-2	1	57.5	
S-22-3	1	52.3	57.1
S-22-4	2	55.1	
S-22-5	2	53.9	
S-22-6	2	52.5	53.8
S-22-7	12	44.7	
S-22-8	12	49.3	
S-22-9	12	46.1	46.8
S-22-10	25	41.4	
S-22-11	25	39.6	
S-22-12	25	41.4	40.8
S-22-13	100	24.0	
S-22-14	100	13.2	
S-22-15	100	18.0	18.4
S-22-16	200	7.9	
S-22-17	200	10.3	
S-22-18	200	8.0	8.7

[1] Fermentation procedure - Huhtanen *et al.* (1954)
Inoculum - 25 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3 with cobalt omitted
Substrated - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea added to inoculum bottle
Incubation - 39°C for 18 hours
Cobalt added as $CoCl_2.6H_2O$

digested decreased as the cobalt added was increased, but in this experiment the decrease was much greater. The percentage cellulose digested ranged from 57.1 when 1 part per million of cobalt was added, to 8.7 when 200 parts per million were added. When 100 parts per million of cobalt were added, only 18.4% of the cellulose was digested, much less than the 52.3% digested when 91 parts per million of cobalt were added in experiment No. B-11. It should be noted that in experiment No. S-22 incubation was maintained at 39°C for 18 hours. The shorter incubation period was used on the assumption that any stimulatory action of cobalt on cellulose digestion would be an effect on rate of digestion. It was thought advisable to avoid any possible limitation of digestion by the substrate which could have a leveling effect on the percentage cellulose digested if the incubation period were prolonged.

The results of these experiments indicated that cellulose digestion was not stimulated by added cobalt under the conditions used. It is known, however, that rumen microorganisms can convert cobalt to vitamin B_{12}. If it were assumed that in the <u>in vitro</u> fermentation this conversion was not equal to that obtained <u>in vivo</u>, the possibility that vitamin B_{12} has a stimulatory effect was not excluded. Consequently, an experiment was conducted to determine the effect of different amounts of added vitamin B_{12} on the percentage cellulose digested. The results obtained are shown in Table 31. An 18-hour fermentation was again used to avoid any limiting effect due to substrate depletion. The amounts of vitamin B_{12} added ranged from 0.05 to 50 micrograms per ml. The percentage cellulose digested in the fermentation mixtures receiving vitamin B_{12} was substantially the same as in the controls.

TABLE 31

THE EFFECT OF DIFFERENT VITAMIN B_{12} CONCENTRATIONS ON CELLULOSE DIGESTION[1]

Experiment no.	Vitamin B_{12} added ug./ml.	Cellulose digested %	Cellulose digested Average %
S-23-1	50	49.8	
S-23-2	50	56.8	
S-23-3	50	63.3	56.6
S-23-4	5	60.3	
S-23-5	5	58.7	
S-23-6	5	71.3	63.4
S-23-7	0.5	55.7	
S-23-8	0.5	60.9	
S-23-9	0.5	58.4	58.3
S-23-10	0.05	61.8	
S-23-11	0.05	61.4	
S-23-12	0.05	54.5	59.2
S-23-13	None	61.8	
S-23-14	None	56.0	
S-23-15	None	64.3	60.7

[1] Fermentation procedure - Huhtanen *et al.* (1954)
Inoculum - 25 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea added to inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 18 hours

In the experiments just discussed, the cobalt or vitamin B_{12} additions were made to the *in vitro* fermentations. It is possible that the action of cobalt in the rumen of the animal may be different than in the fermentations conducted in the laboratory. It was of interest, therefore, to determine the extent of cellulose digested *in vitro* when the animal from which the inocula were obtained was receiving a ration which contained a high level of cobalt. Table 32 shows the results of a series of experiments in which daily additions of cobalt sulfate were made to the rumen of the animal from which the inocula were obtained. A control experiment was inoculated with a sample of rumen fluid obtained from steer No. 741 on June 3, 1954 before cobalt feeding had begun. A sample was also obtained on July 19. At this time the animal had received 2.5 g. of cobalt (as cobalt sulfate) per day for 7 days, 5 g. per day for 18 days, and 7 g. per day for 5 days. The level of cobalt fed was increased to 10g. daily on July 24 and another sample was taken on July 31. Several of the substrates used showed a slight decrease in the percentage cellulose digested after cobalt had been fed at high levels. The cellulose of Solka-Floc showed the highest percentage digestion and the greatest drop in digestibility. In the control experiment, before the feeding of cobalt sulfate had started, 86.3% of the cellulose of the Solka-Floc was digested. In the experiment started July 19, 81.8% was digested and in the experiment started July 31 only 69.9% was digested. When unfertilized hay No. 6535 was used, the corresponding values for percentage cellulose digested were 29.1, 22.6 and 21.5%. Before the feeding of cobalt sulfate, 35.3% of the cellulose in the pith from bagasse was digested but only 28.7% after cobalt feeding. The percentage

TABLE 32

THE EFFECT OF FEEDING HIGH LEVELS OF COBALT ON THE IN VITRO DIGESTION OF CELLULOSE[1]

Substrate	Cellulose digested					
	6-3-54		7-19-54		7-31-54	
	Replicates	Average	Replicates	Average	Replicates	Average
	%	%	%	%	%	%
Solka-Floc	85.1		81.8		71.6	
Solka-Floc	86.7		81.2		69.7	
Solka-Floc	87.1	86.3	82.3	81.8	68.4	69.9
Untreated cotton linters	37.6					
Untreated cotton linters	40.2					
Untreated cotton linters	35.4	37.7				
Extracted cotton linters	53.0		44.1			
Extracted cotton linters	55.2		41.7			
Extracted cotton linters	51.2	53.1	39.8	41.9		
Alfalfa leaf meal	49.8		51.7		50.3	
Alfalfa leaf meal	48.4		51.9		48.7	
Alfalfa leaf meal	52.0	50.1	50.0	51.2	54.2	51.1
Unfertilized hay #6535	28.4		30.6		23.0	
Unfertilized hay #6535	35.2		20.3		23.0	
Unfertilized hay #6535	23.8	29.1	16.9	22.6	18.5	21.5
Pith from bagasse	36.0		26.0			
Pith from bagasse	35.2		32.1			
Pith from bagasse	34.8	35.3	28.1	28.7		

TABLE 32 (CONCLUDED)

Substrate	Cellulose digested					
	6-3-54		7-19-54		7-31-54	
	Replicates	Average	Replicates	Average	Replicates	Average
	%	%	%	%	%	%
Unfertilized hay #2543	27.6		34.7		28.1	
Unfertilized hay #2543	30.0		38.4		30.7	
Unfertilized hay #2543	25.7	27.8	33.2	35.4	34.1	31.0
Fertilized hay #2543	35.6		35.9		36.8	
Fertilized hay #2543	36.1		37.6		30.1	
Fertilized #2543	37.2	36.3	30.3	34.6	29.4	32.1

[1] Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from steer No. 741
Mineral solution - Complex No. 3
Non-protein nitrogen - 0.1% urea added to the inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 24 hours

of the cellulose of extracted cotton linters which was digested dropped from 53.1 in the case of the inoculum taken June 3 to 41.9 in the case of the inoculum taken July 19.

On the other hand, when alfalfa leaf meal was used as the substrate, the cellulose digested was maintained at approximately the same level, the values obtained for the samples taken June 3, July 19 and July 31 being 50.1, 51.2 and 51.1%, respectively.

Table 33 shows the amounts of cellulose digested when inocula from the animal receiving the high levels of cobalt were used and when urea was not added to either the fermentation mixture or the outer liquid. It can be seen that with the sample taken July 14 the percentage cellulose digested was usually equal to or more than that obtained with the sample taken July 19 shown in Table 33. However, the percentage cellulose digested when Solka-Floc was the substrate was 81.8% in the sample taken July 19 and only 57.3% in the sample taken July 14. When the results from the two inocula shown in Table 33 are compared, it can be seen that the decrease in the ability to ferment cellulose shown by the sample taken July 31 was greater than in the fermentations to which urea was added.

Rate of cellulose digestion. One disadvantage of the use of a 24-hour fermentation period is the possibility that the substrate might exert a limiting effect on cellulose digestion. This in itself would give rise to useful information, since it would be of interest to know the percentage of the cellulose in different feeds that is accessible to the rumen microorganisms. On the other hand, the rate of cellulose digestion is also of great importance. Accordingly, experiments were

TABLE 33

THE EFFECT OF FEEDING HIGH LEVELS OF COBALT ON THE IN VITRO DIGESTION OF CELLULOSE[1]

Substrate	Cellulose digested			
	7-14-54		7-31-54	
	Replicates	Average	Replicates	Average
	%	%	%	%
Solka-Floc	58.3		47.2	
Solka-Floc	58.2		45.7	
Solka-Floc	55.3	57.3	47.6	46.8
Untreated cotton linters	27.7			
Untreated cotton linters	30.7			
Untreated cotton linters	27.8	28.7		
Extracted cotton linters	47.1			
Extracted cotton linters	48.7			
Extracted cotton linters	41.5	45.8		
Alfalfa leaf meal	53.7		46.9	
Alfalfa leaf meal	54.2		48.0	
Alfalfa leaf meal	54.0	54.0	38.1	44.3
Unfertilized hay #6535	28.3		15.6	
Unfertilized hay #6535	27.9		15.9	
Unfertilized hay #6535	23.0	26.4	12.9	14.8
Pith from bagasse	30.8			
Pith from bagasse	34.3			
Pith from bagasse	31.2	32.1		
Unfertilized hay #2543	33.1			
Unfertilized hay #2543	31.0			
Unfertilized hay #2543	26.7	30.3		
Fertilized hay #2543	33.5			
Fertilized hay #2543	36.6			
Fertilized hay #2543	33.6	34.6		

[1] Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from steer No. 741
Mineral solution - Complex No. 3
Non-protein nitrogen - not added
Readily available carbohydrate - not added
Incubation - 39°C for 24 hours

conducted to determine the amounts of Solka-Floc cellulose and of alfalfa leaf meal cellulose that were digested after incubation periods of different lengths. The results obtained are shown in Table 34 and 35 in Figure I.

The percentage cellulose digested in previous experiments was generally higher when Solka-Floc was used as the substrate than when other substrates were used. It can be seen from Table 34 that in the 24-hour fermentation period the higher percentage cellulose digested was again obtained when Solka-Floc was used as the substrate. However, after 6 hours of incubation, only 24% of the cellulose digested in 24 hours had been digested when Solka-Floc was the substrate. When alfalfa leaf meal was the substrate (Table 35), more than 50% of the 24-hour cellulose digestion was accomplished in 6 hours. Examination of the values for percentage of initial cellulose digested shows that this difference was not due to the lower cellulose digestion in 24 hours shown by the alfalfa leaf meal. For incubation periods up to 12 hours, a higher percentage of the initial cellulose was digested when alfalfa leaf meal was the substrate than when Solka-Floc was used. After 16 hours, the percentage cellulose digested, based on the cellulose initially present, was approximately the same for both substrates. At the beginning of the experiments, the concentrations of cellulose present when Solka-Floc was the substrate and when alfalfa leaf meal was the substrate were 10.9 and 9.7 mg. per ml., respectively.

Table 36 shows the results of two experiments to determine whether appreciable cellulose digestion could be obtained when a cell inhibitor was added to the fermentation mixture. It can be seen that the addition

TABLE 34

RATE OF DIGESTION OF CELLULOSE IN SOLKA-FLOC[1]

Experiment	Fermentation period	Cellulose digested: Based on initial[2] — Replicates	Based on initial[2] — Average	Based on final[3] — Average
no.	hr.	%	%	%
S-25-1	3	5.8		
S-25-2	3	6.8		
S-25-3	3	5.7	6.1	9.0
S-25-4	6	9.9		
S-25-5	6	10.1		
S-25-6	6	7.6	9.2	13.6
S-25-7	9	14.9		
S-25-8	9	16.7		
S-25-9	9	16.9	16.2	24.0
S-25-10	12	30.0		
S-25-11	12	29.5		
S-25-12	12	26.0	28.5	42.2
S-25-13	16	41.0		
S-25-14	16	39.8		
S-25-15	16	39.1	40.0	59.3
S-25-16	24	65.0		
S-25-17	24	68.5		
S-25-18	24	68.9	67.5	100.0

1 Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea added to the inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 3 to 24 hours

2 Based on the amount of cellulose present at start

3 Expressed as percentage of the cellulose digestion obtained in 24 hours

TABLE 35

THE RATE OF DIGESTION OF CELLULOSE IN ALFALFA LEAF MEAL[1]

Experiment	Fermentation period	Cellulose digested		
		Based on initial[2]		Based on final[3]
		Replicates	Average	Average
no.	hr.	%	%	%
S-30-1	3	14.9		
S-30-2	3	14.0		
S-30-3	3	15.8	14.9	32.2
S-30-4	6	25.0		
S-30-5	6	25.3		
S-30-6	6	20.3	23.5	50.9
S-30-7	9	33.2		
S-30-8	9	32.5		
S-30-9	9	31.0	32.2	69.7
S-30-10	12	38.7		
S-30-11	12	37.1		
S-30-12	12	36.2	37.4	80.9
S-30-13	16	40.3		
S-30-14	16	41.1		
S-30-15	16	39.9	40.4	87.4
S-30-16	24	45.0		
S-30-17	24	46.8		
S-30-18	24	46.9	46.2	100.0

1 Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Substrate - 4% alfalf leaf meal
Non-protein nitrogen - 0.1% urea added to inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 3 to 24 hours

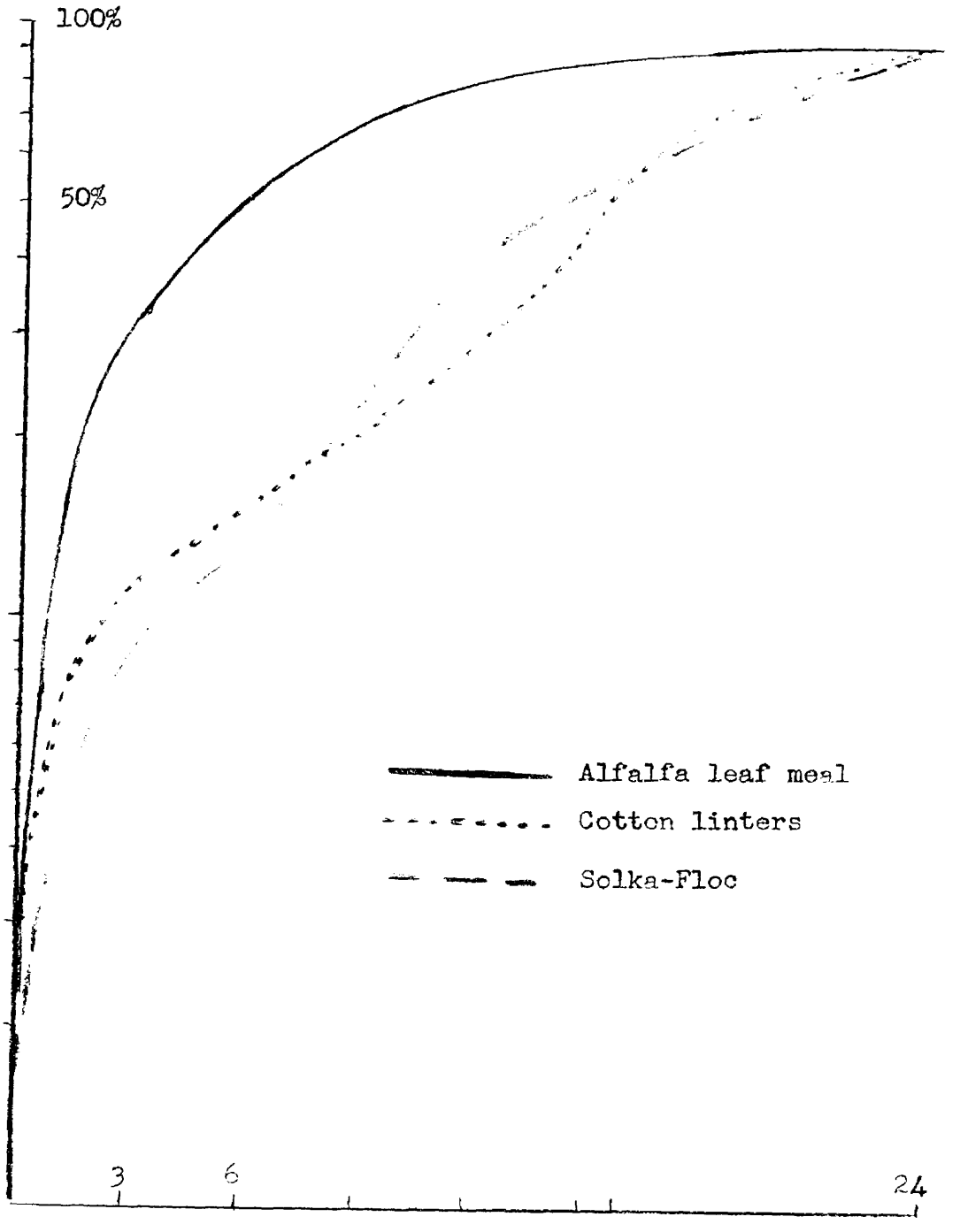

FIGURE I. Rates of digestion of cellulose in alfalfa leaf meal, cotton linters and Solka-Floc. Ordinate - incubation time in hours. Abscissa - cellulose digested, as percentage of the amount digested in 24 hours (logarithmic scale).

of thymol at the start of the fermentation period causes a very great reduction in the percentage cellulose digested, probably by preventing the growth and multiplication of the microorganisms.

Cellulase activity. It would appear from the results shown in Table 36 that thymol could be used to prevent the growth and multiplication of microorganisms in the fermentation mixture. The small amount of cellulose digestion obtained in the presence of thymol might then be interpreted as a measure of the cellulase activity of the fermentation mixture. On this assumption, an experiment was conducted to determine whether this cellulase activity changed during the course of in vitro incubation. The results are shown in Table 37 (Appendix). All of the fermentation mixtures were incubated for 24 hours. Thymol was added to different sets of replicates at 0, 3, 6, 9, 12, and 16 hours. In order to estimate the cellulase activity, the data from experiment No. S-25 and S-32 were used to calculate the percentage cellulose digested in each fermentation mixture while it was exposed to the thymol. The results of these calculations are shown in Table 38 (Appendix) and in Figure II.

In experiment S-25, 67.5% of the cellulose was digested in 24 hours and in experiment S-32, 74.0% was digested in 24 hours when thymol was not added. In both experiments, therefore, the percentage cellulose digested was calculated as percentage of the cellulose digested in 24 hours without thymol. This put the two experiments on a more comparable basis, since the digestion for each incubation period was expressed as percentage of the final digestion for that experiment. When thymol was added at the start of the fermentation, the average rate of cellulose digestion was 0.30% per hour. When the thymol was not added until

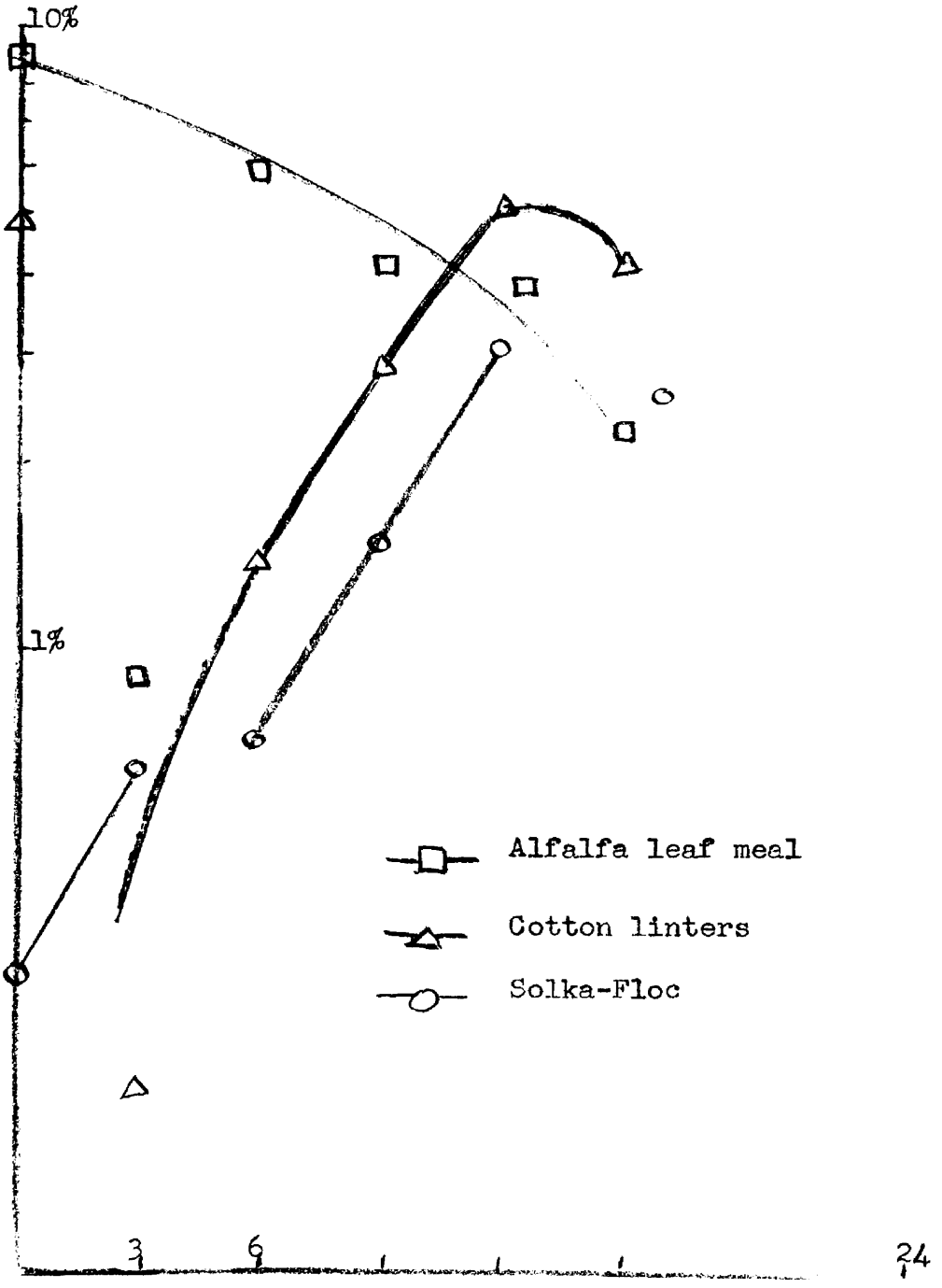

FIGURE II. Average hourly rates of cellulose digestion in the presence of thymol. Ordinate - time of incubation before addition of thymol (hours). Abscissa - cellulose digested per hour in the presence of thymol, as percentage of the amount digested in 24 hours without thymol (logarithmic scale).

after 3 hours of normal incubation, the average rate of cellulose digestion during exposure to thymol increased to 0.647% per hour. When thymol was added after 6, 9, 12, and 16 hours of normal incubation, the corresponding values for average rate of cellulose digestion during exposure to thymol were 0.722, 1.45, 3.12 and 2.63% per hour, respectively. Thus, if the average rate of cellulose digestion in the presence of thymol can be taken as a measure of the cellulase activity at the time the thymol was added, then the cellulase activity increased from a low value at 0 hour to a high value at 12 hours and then began to decrease. It is possible, however, that the lower hourly rate when the addition of thymol was made at 16 hours was due to the limiting effect of the substrate, since approximately 40% of the initial cellulose had been digested when the thymol was added.

Tables 39, 40 and 41 (Appendix) show the results of experiments to determine the ability of the rumen fluid inocula to digest cellulose in the presence of thymol when alfalfa leaf meal was used as the substrate. The results of similar experiments in which cotton linters were used as the substrate are shown in Tables 42, 43 and 44. In Figure II, these results are compared with those obtained using Solka-Floc as the substrate. It can be seen that Solka-Floc and cotton linters behaved similarly when thymol was added at different times during the incubation period but that with alfalfa leaf meal the cellulose digestion in the presence of thymol decreased as the thymol was added later in the incubation period. From Figure I it can be seen that alfalfa leaf meal gave a different type of rate curve than was obtained with the other two substrates in the absence of thymol also.

Comparison of in vitro with in vivo cellulose digestion. Table 45 (Appendix) shows the results of experiments in which samples of roughages which had been used in digestion trials were used as substrates and the in vitro digestion of cellulose determined. The in vivo digestibilities are given as 80% of the fecal digestibilities determined by digestion trials because of the finding by Hale et al. (1940), using alfalfa hay, that the average digestibility coefficient of cellulose in the rumen was approximately 80% of the average fecal digestibility coefficient.

DISCUSSION

Determination of cellulose. In view of the uncertainty as to the structure of "native" cellulose the evaluation of analytical methods is rather difficult. Moreover, cellulose in the plant exists in close association with hemicelluloses and lignin and it is doubtful whether these can be completely removed without degrading some of the cellulose. Methods involving the hydrolysis of cellulose followed by the determination of reducing sugars are not applicable to plant materials where hydrolytic products of other substances may contribute to the reducing power of the hydrolyzed material. These methods appear to be even less applicable to material from the rumen in the view of the finding of Heald (1951) that non-fermentable reducing substances were present in large quantities in the hydrolysates of rumen microorganisms. The reducing power of hydrolysates would consequently be affected by reducing substances of non-cellulosic origin to a degree dependent upon undetermined differences in the composition of the original material.

The methods involving the removal of non-cellulosic materials by various means are more applicable to plant materials and rumen contents. Some investigators have attempted in this way to obtain a residue which consists almost entirely of anhydroglucose residues. Druce and Wilcox (1949) have used this approach but even under the conditions of their method they obtained a residue containing only 90% hexosan. These authors used an oxidation in an acid medium followed by another oxidation in an alkaline medium. The low values for percentage cellulose obtained when this

method was used (Table 12) may have been at least partially the result of unfamiliarity with the method. However, Hägglund (1951) stated that when oxidizing agents are used, part of the cellulose may be converted into oxycellulose or other degradation products which will then dissolve on subsequent treatment with alkali.

Norman (1937) favored the use of a method that will determine both cellulose and cellulosans, the hemicelluloses closely associated with structural cellulose. There is much to be said for this point of view, since even strong chemical treatment does not remove all of the hemicellulose from "native" cellulose and it may be that the biological unit with which the nutritionist is concerned is a close association of cellulose and hemicelluloses rather than cellulose alone. According to Crampton and Maynard (1938), however, there is some evidence that hemicelluloses are utilized to a greater extent than is cellulose. If this were true, the determination of cellulose and hemicelluloses as one unit would preclude the observation of independent variations in the utilization of the two components. In spite of this, the method of Norman and Jenkins (1933) may determine a fraction which more closely approaches the biological unit than that determined by some other methods, particularly if both "true" and "crude" cellulose are determined. The procedure is time consuming, however, and for this reason the method of Crampton and Maynard (1938) has been used here. As shown by Ferguson (1942) the method of Kürschner and Hanak (1930), which is essentially the same as the Crampton and Maynard (1938) method, gives values for "crude" cellulose somewhat below those obtained with the Norman and Jenkins (1933) method. The values obtained for "true" cellulose were similar for both methods. The modification of

the Norman and Jenkins method reported by Matrone et al. (1946) permits a greater number of determinations per day than does the original method but not as many as the method of Crampton and Maynard as used in this study.

It can be seen from Table 13 that the method of determining cellulose used in the present work gave satisfactory reproducibility even though the results shown include the error involved in pipetting from a suspension of plant material. It is difficult to pipette suspensions of pith or cotton linters, but with these materials also, satisfactory agreement among replicates was obtained.

The fermentation methods. The environmental conditions associated with an in vitro fermentation method inevitably differ from those present when rumen studies are performed in vivo. Cellulose digestion in the rumen may be affected by factors which are modified or eliminated by the in vitro method used and new factors may be introduced. These changes in environmental conditions may or may not be desirable from the investigator's point of view, but in any case they should always be considered when interpreting data obtained in vitro.

The intermittent loss of portions of the fermentation mixture by passage from the rumen, a factor which complicates in vivo work, is conveniently eliminated in laboratory fermentations. The loss of volatile fatty acids and other materials through the rumen wall is another variable involved in animal work. In those fermentation methods in which no semipermeable membrane is used, this loss is prevented. When a semipermeable bag is used there is a loss from the fermentation mixture but in this case by dialysis, while it is quite possible that the loss of some materials from the rumen is by selective absorption. This possibility is supported by the work of a

number of investigators with acetic, propionic and butyric acids. There is little agreement as to the relative rates of absorption of these three acids and some of the work is discredited by the finding of Masson and Phillipson (1951) that the rate of the disappearance of these acids from the rumen does not parallel their concentration in the blood draining the rumen. However, it seems likely that the acids are absorbed at different rates. Moreover, Pennington (1951, 1952) found that rumen epithelium metabolized butyrate in vitro to a much greater extent than acetate.

There are other departures from rumen conditions that are evident in laboratory fermentation procedures. The inoculum is small by comparison with the massive inoculum present in the rumen when feed is introduced, and usually strained rumen fluid is used rather than the whole rumen material. Either of these conditions might lead to the development of a microbial population that is different than the one normally occurring in the rumen. In the method of Burroughs et al. (1950), CO_2 is bubbled through the fermentation mixture during incubation. It is possible that this technique may decrease the partial pressures of volatile constituents of the milieu sufficiently to affect some of the reactions taking place within it.

In the laboratory fermentations, investigators have frequently used simple substrates such as glucose or cellulose which differ considerably from the feed which the animal normally receives. That this induces an atypical fermentation is indicated by the results obtained by a number of workers. Elsden (1945) observed a greater production of propionic than acetic acid when glucose, lactic acid, or cellulose was used as the substrate. Marston (1948), using cellulose as the substrate, also found that more propionic acid than acetic acid was produced. McNaught (1951) found

that when maltose or xylose was used as the substrate more acetic acid than propionic acid was produced but that the proportion of propionic acid was greater than that produced *in vivo*. Belasco (1954b), using wood cellulose as the substrate, found that a much greater proportion of propionic acid was produced *in vitro* than occurred *in vivo* and that frequently more propionic acid than acetic acid was produced.

On the other hand, the amount of propionic acid produced *in vitro* when forages were used as substrates, though greater than that produced *in vivo*, has generally been found to be less than the amount of acetic acid produced. Elsden (1945), using dehydrated grass, Gray and Pilgrim (1950), using alfalfa hay, and Gray and Pilgrim (1950) and Gray *et al*. (1951), using wheaten hay, found a somewhat higher proportion of propionic acid *in vitro* than is normally found *in vivo*. Balasco (1954b) found that the molar ratio of propionic to acetic acid was higher when a protein meal was used as the nitrogen source *in vitro* than when urea was used.

Another factor which may lead to departure from rumen conditions is the use of an extended incubation period or of serial fermentations. It seems likely that the effect of atypical conditions would be more pronounced in a longer period of incubation than in a shorter one. The use of serial fermentations results in considerable dilution of the original inoculum and the continued subculturing may lead to more rapid growth with a possible increase in the proportion of fast-growing microorganisms.

Examination of the fermentation methods used. In the experiments described in Experimental, Section I, exceptionally low values were obtained for percentage cellulose digested when the method of Huhtanen *et al*. (1954) was used. With the technique of Wasserman *et al*. (1952) much higher values

had been obtained, although these too were considerably lower than many of the values given in the literature. Since the most obvious difference between these two methods was that in the method of Wasserman et al. (1952) CO_2 was continuously passed through the fermentation mixture, the possibility existed that the poor digestion of cellulose might be due to failure to obtain anaerobic conditions. Quastel and Stephenson (1926a) have reported that cysteine and other -SH compounds can be used to lower the oxidation-reduction potential of a medium in order to make it more suitable for the growth of anaerobic organisms. Accordingly, cysteine hydrochloride and sodium thioglycollate were used with the fermentation of Huhtanen et al. (1954). As shown in Table 14 and Table 15 this treatment did not effect an increase in cellulose digestion.

The percentage cellulose digestion obtained by Burroughs et al. (1950) was higher than had been obtained in the preliminary experiments with either of the methods used. In order to have a basis for further examination of the methods, an experiment was performed using the fermentation procedure described by these authors. If comparable cellulose digestion could be obtained the implication would be that the low results heretofore obtained were not due to manipulation of the sample before the in vitro fermentation, and further work could be done to determine the difference in fermentation conditions which resulted in the low percentage cellulose digestion. A number of different mineral solutions were used in order to determine their effect upon the extent of cellulose digestion. A much higher percentage cellulose digestion was obtained using this method. When the average values for three successive 24-hour fermentation periods are considered (Table 17), there appears to be little difference due to the mineral

solution used. Although Complex No. 2 gave the most uniform results for the three periods, the best results for the initial 24-hour fermentation were obtained with the various modifications of the artificial saliva of McDougall (1948). In agreement with results obtained by Burroughs et al. (1950) and Arias et al. (1951), higher percentage cellulose digestion was obtained in the second fermentation period when the mineral solutions described by these authors were used. This may indicate an adaptation on the part of the microorganisms. No similar increase was noted with the other mineral solutions used. As shown by experiment No. B-1-9 to E-1-12 (Table 17) this increase was greater when a centrifuged inoculum was used, but this may merely reflect the low first-period digestion obtained with centrifuged inocula.

The experiments using the fermentation procedure of Burroughs et al. (1950) indicated that the low results previously obtained were due to the conditions of the in vitro fermentation. The most obvious inference is that the introduction of CO_2 during fermentation is required. However, Table 18 shows that the percentage cellulose digestion increased with decreased rate of passage of CO_2 through the fermentation mixture and was greatest when no CO_2 was used except for mixing when the zero-hour samples were taken. In these experiments a modification of the artificial saliva of McDougall (1948) was used, and when this mineral solution was used in the fermentation procedure of Huhtanen et al. (1954) an even higher percentage of cellulose digestion was obtained (experiment No. S-15, Table 21). It would appear that although a satisfactory digestion of cellulose could be obtained when Complex No. 2 was used in the fermentation procedure of Burroughs et al. (1950), a different mineral solution was required to

obtain appreciable cellulose digestion when the procedure of Huhtanen et al. (1954) was used.

The results shown in Table 20 indicate that the unsuitability of Complex No. 2 was not due to its low calcium content. It can be seen that the percentage cellulose digested when Complex No. 2 was used, although considerably lower than that obtained with the modification of McDougall's (1948) artificial saliva, is much higher than had been obtained in the preliminary experiments. A possible explanation is that, in experiment No. S-19, Complex No. 2 was gassed with CO_2 for a longer period than in the previous experiments. This explanation would be in agreement with the fact that values obtained for the percentage cellulose digestion when Complex No. 2 was used in the fermentation procedure of Burroughs et al. (1950) (Table 18) were essentially the same as those shown in Table 20.

The results shown in Table 19 indicate that neither the use of Complex No. 2 nor the continuous passage of CO_2 into the fermentation mixture was responsible for the rather low values for percentage cellulose digested when the procedure of Wasserman et al. (1952) was used. In contrast with the results obtained using the procedure of Burroughs et al. (1950) the greatest percentage cellulose digestion was obtained when CO_2 was passed through the fermentation mixture. No explanation can be offered for this, except that the increments in percentage cellulose digestion were so small as to suggest that they were apparent rather than real.

The results shown in Table 22 suggest that one factor responsible for the lower value for percentage cellulose digested when the procedure of Wasserman et al. (1952) was used is the low ratio of the volume of the inoculum to the volume of the mineral solution. Possibly the concentration

of an essential nutrient is lowered by dialysis into the outer liquid. One compound which might behave in this manner is valeric acid. Bentley et al. (1954a) found that n-valeric acid stimulated the digestion of cellulose by rumen microorganisms, and Bryant and Doetsch (1954) found that a combination of n-valeric and isovaleric acids added to a basal medium permitted the growth of Bacteroides succinogenes without the addition of rumen fluid.

The results shown in Table 21 indicate that the semipermeable sac is necessary for maximum cellulose digestion under the conditions used. The greater alkalinity and higher salt concentration of the modification of McDougall's (1948) artificial saliva used may have contributed to the lack of cellulose digestion when the residue from 25 ml. was used. The anomalous effect of added glucose, stimulation of cellulose digestion in experiment No. S-16 and depression in experiment No. S-28, may be due to difference in feed consumption by the donor animal. This animal was on hay alone but occasionally received the corn silage left by other animals. The sample for experiment No. S-28 was taken about one month after the sample for experiment No. S-16 and about 1½ hours earlier in the day.

Under the conditions used it would appear that the maximum cellulose digestion and most reproducible results were obtained when a semipermeable sac was used, the inoculum:mineral-solution ratio was small, CO_2 was not passed through the fermentation mixture during incubation, and a modification of McDougall's (1948) artificial saliva (Complex No. 3) was used outside the sac.

Factors Affecting Cellulose Digestion In Vitro

The effect of 2,4-D. Under the conditions used, 2,4-D had no appreciable effect on cellulose digestion as shown in Table 24. In view of the

results of a later experiment (Table 35) it is unlikely that a 24-hour incubation period would reveal differences in rate of cellulose digestion when alfalfa leaf meal is used as the substrate, particularly if these differences existed early in the incubation period. The highest concentration used here probably approached the levels attained in the rumen by Mitchell et al. (1946) when they fed 5.5 g. of 2,4-D per day.

The effect of alkali treatment of corn fiber. The results shown in Table 25 indicate that treatment of corn fiber with lime, followed by extraction with water, appreciably increases the availability of the cellulose to rumen microorganisms. This is in accordance with the work of Ferguson (1942) on wheat straw and wheat straw pulp. He found that the alkali-treated wheat straw pulp gave an average digestibility coefficient for "crude" cellulose of 87.7 and for "true" cellulose of 86.7. Each of these digestibility coefficients was approximately 40% higher than the corresponding value for wheat straw.

The effect of urea concentration. Most in vitro investigations have involved the use of relatively small amounts of non-protein nitrogen. Burroughs et al. (1950) and Arias et al. (1951) have used a 0.1% concentration of urea in their in vitro work. Belasco (1954, 1954b) used a non-protein nitrogen level equivalent to slightly less than 0.04% urea. Agrawala et al. (1953), using a calf on a natural ration, found a concentration of non-protein nitrogen in the rumen of 0.135% of the dry matter just before feeding and 0.154% 6 hours after feeding. This was equivalent to 0.037% and 0.043% urea, respectively, on the wet basis.

The effect of glucose concentration. The results shown in Table 28 indicate that concentrations of glucose up to 0.2% have no appreciable

effect on the percentage of cellulose digested under the conditions used. Arias et al. (1951) have shown that addition of readily available carbohydrate at low concentration stimulates cellulose digestion. However, these workers used serial fermentations according to the method of Burroughs et al. (1950) and averaged the results. Their greatest apparent stimulation when readily available carbohydrate was added occurred in the first and third periods. In these periods the percentage of the cellulose digested in the absence of added starch or sugar was relatively low. This suggests the possibility that the stimulating effect was actually the overcoming of depressing forces such as sub-optimal conditions or nutritional deficiencies. The method used by Hoflund et al. (1948), by which small amounts of glucose were shown to stimulate cellulose digestion, was too empirical to permit the estimation of the percentage cellulose digested.

The effect of added cobalt or vitamin B_{12}. As can be seen from Tables 29 and 30, increasing the concentration of cobalt brought about a decrease in the percentage cellulose digestion. However, the decrease obtained when the method of Burroughs et al. (1950) was used with added urea and glucose (Table 29) was much less than that obtained with the method of Huhtanen et al. (1954). This may have been an artifact due to differences in the conditions associated with the two methods or possibly the presence of glucose and/or the higher level of urea when the Burroughs method was used was responsible for a greater ability to tolerate high levels of cobalt. Another, though remote, possibility is that a critical level of added cobalt existed between 91 ppm. and 100 ppm.

As shown in Table 31, addition of vitamin B_{12} up to 50 micrograms per ml. did not have any appreciable effect on cellulose digestion. This does

not mean that vitamin B_{12} is not required for cellulose digestion, since to show this it would first be necessary to rigidly exclude vitamin B_{12} from the inoculum. On the other hand, it is possible that some other form of vitamin B_{12} serves as a growth factor for rumen microorganisms. Ford and Porter (1952) isolated a compound closely related to vitamin B_{12} from the rumen contents and feces of calves and Pfiffner et al. (1951) isolated a closely related compound from the culture of a rumen anaerobe.

Examination of the cellulose digesting ability of rumen fluid obtained from a steer receiving high doses of cobalt sulfate daily also indicated that the addition of urea to the in vitro fermentation increased cellulose digestion (Tables 32 and 33). With the sample taken July 19 to which urea was added the percentage digestion of the cellulose in Solka-Floc was higher (81.8%) than with the sample taken July 14 to which no urea was added (57.3%). With the sample taken July 31, the percentage digestion of the cellulose in Solka-Floc, alfalfa leaf meal and unfertilized hay was higher (69.9%, 51.1%, and 21.5%, respectively) when urea was added than when urea was not added (46.8%, 44.3%, and 14.8%, respectively). The reason for this difference is not apparent from the results. It is possible, however, that the maintenance of relatively high levels of cobalt in the rumen results in the chelation of nitrogen compounds which are thus made unavailable to the microorganisms. This could bring about a nitrogen deficiency which would be counteracted by the addition of urea to the in vitro fermentations.

Rates of Cellulose Digestion

Tables 34 and 35 and Figure I show the rate of cellulose digestion in Solka-Floc and alfalfa leaf meal as determined in vitro. A comparison of

the results obtained with these two substrates indicates a difference in their rates of digestion. It can be seen that in 6 hours 50% of the amount of the cellulose of the alfalfa leaf meal that was digested in 24 hours had already been digested. With the Solka-Floc only 14% of the 24-hour digestion had been accomplished within this period. On the other hand, with Solka-Floc almost 60% of the 24-hour digestion was accomplished in the last 12 hours of incubation while with alfalfa leaf meal only 20% of the 24-hour digestion was accomplished within this period. Thus, the rate of digestion of the Solka-Floc was slower in the first part of the incubation and faster in the last part, while the reverse was true of the alfalfa leaf meal. These observations suggest at least two possible explanations. First, the alfalfa leaf meal may provide nutrients not furnished by the Solka-Floc. Since the Crampton and Maynard (1938) method of cellulose determination also includes some hemicellulose, the faster initial rate with alfalfa leaf meal may reflect a more rapid digestion of hemicelluloses. On the other hand, the differences in the rate of digestion may indicate a difference between "native" cellulose as represented by alfalfa leaf meal and treated cellulose as represented by Solka-Floc and the slower initial rate shown by the Solka-Floc may be due to a period of adaptation required by the change in substrate. In this connection, it is interesting to note that McBee (1953) found a lag period of several hours in the digestion of a shredded wood pulp <u>in vitro</u>, measured by gas production, by rumen fluid from sheep on an alfalfa hay ration. Addition of the wood pulp to the ration reduced the lag period to about 15 minutes.

However, the curves obtained when Solka-Floc and cotton linters (Figure I) were used as substrates were very similar in appearance. This

suggests that the difference between the curves obtained with alfalfa leaf meal and Solka-Floc was due to some factor other than a structural difference between "native" and treated cellulose. The decrease in the slope after about 3 hours incubation may indicate that some required factor(s) is present in the inoculum but is quickly utilized so that its concentration falls below an optimum level. Similarly, the increase in slope which occurs later may indicate that the optimum level has been restored by the activity of the microorganisms, that the organisms responsible for cellulose digestion have adapted to the new conditions, or that certain less fastidious strains have multiplied sufficiently to increase the rate of cellulose digestion.

On the assumption that the cellulose digestion in the presence of thymol might be an indication of the cellulase activity at the time the thymol was added, experiments were performed with Solka-Floc and alfalfa leaf meal as substrates and with thymol added after different periods of incubation. As can be seen from Tables 38 and 41 (Appendix) and Figure II, a different pattern was obtained when Solka-Floc was the substrate than when alfalfa leaf meal was used. When Solka-Floc was used the cellulose digested per hour in the presence of thymol increased with the time of incubation without thymol up to 12 hours. The slight decrease in the hourly rate when thymol was added at 16 hours may have been due to the limiting effect of the substrate since 40% of the initial amount had been digested at this time, and the hourly rate was calculated from the digestion over the last 8 hours in the presence of thymol. When alfalfa leaf meal was used as the substrate the highest hourly rate of cellulose digestion in the presence of thymol was obtained when thymol was added before incubation. Thereafter

the rate of cellulose digestion in the presence of thymol decreased with the time of incubation without thymol. Another striking difference between the behavior of Solka-Floc and that of alfalfa leaf meal was the percentage of cellulose digested in 24 hours incubation with thymol. With Solka-Floc, only 7% of the amount digested in 24 hours in the absence of thymol was digested in 24 hours in the presence of thymol. With the alfalfa leaf meal, 41% of the amount digested in 24 hours in the absence of thymol was digested in 24 hours in the presence of thymol.

Table 44 (Appendix) and Figure II show that the pattern of cellulose digestion in the presence of thymol with cotton linters as the substrate followed that of Solka-Floc rather than that of alfalfa leaf meal. The reason for this difference in behavior is not clear. However, there is no positive assurance that the addition of thymol completely inhibits the action of the microorganisms and it may be, because of the more complex array of organic matter in the alfalfa leaf meal, that the microorganisms are not inhibited to the same extent when alfalfa leaf meal is used as when the simpler substrates are used. This would be in keeping with the greater percentage cellulose digestion with alfalfa leaf meal when incubated with thymol for 24 hours (41% of the 24-hour digestion) as compared to the value obtained for Solka-Floc (7% of the 24-hour digestion) or cotton linters (10% of the 24-hour digestion).

Consequently, it should be emphasized that the results obtained when cellulose was digested in the presence of thymol are merely suggestive and further work is required before any definite interpretations can be made. The lack of assurance that the thymol completely prevented microbiological activity indicates the advisability of treating the results with caution.

Moreover, additional data should be accumulated to eliminate the possibility that the apparent difference between the behavior of alfalfa leaf meal and that of Solka-Floc and cotton linters was an artifact.

Comparison of *In Vitro* with *In Vivo* Cellulose Digestion

The results shown in Table 45 (Appendix) do not indicate any close parallel between the digestion of the cellulose in different roughages as determined *in vitro* and their digestion as calculated from digestion trials. This is not surprising in view of the difficulty experienced in estimating digestion in the rumen on the basis of values obtained for fecal digestibility. Moreover, the *in vivo* results are average digestibilities over a period of days or weeks and must to some extent be a function of the rate and manner of passage of the ingesta through the animal.

SUMMARY

Four methods of incubating rumen fluid for the purpose of studying cellulose digestion *in vitro* were examined. Under the conditions used, it was found that the maximum cellulose digestion and most reproducible results were obtained when a semipermeable sac was used and the inoculum: mineral-solution ratio was small. When CO_2 was passed through the fermentation mixture during incubation, the percentage cellulose digested was slightly lower than when CO_2 was used very briefly at the start of the fermentation period. A modification of the "artificial saliva" of McDougall (1948) was adopted as a source of mineral nutrients.

Cellulose digestion was increased by addition of urea up to 0.1% when no readily available carbohydrate was added but decreased by additions of 0.15 or 0.20%. Addition of glucose up to 0.2% did not appreciably affect the amount of cellulose digested.

Addition of large amounts of cobalt (up to 200 ppm.) depressed cellulose digestion, the amount of depression being greater as the concentration of the cobalt increased. Addition of vitamin B_{12} (up to 50 micrograms per liter) had no appreciable effect on cellulose digestion.

Inocula obtained from a steer receiving high levels of cobalt had a decreased ability to digest the cellulose in a wood cellulose, alfalfa leaf meal and timothy hay when incubated without the addition of urea. When urea was added before incubation the inocula had a decreased ability to digest the cellulose in the wood cellulose and timothy hay but the digestion of

the cellulose in alfalfa leaf meal was essentially unchanged.

A comparison of rates of cellulose digestion *in vitro* showed that the cellulose in alfalfa leaf meal was digested at a relatively greater rate than the wood cellulose or the cellulose of cotton linters when compared on the basis of percentage of 24-hour digestion.

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APPENDIX

TABLE 36

THE EFFECT OF THE ADDITION OF AN INHIBITOR AT THE START OF THE FERMENTATION PERIOD ON CELLULOSE DIGESTION

Experiment no.	Inhibitor	Treatment of inoculum	Cellulose digested %
Fermentation procedure - Burroughs et al. (1950)[1]			
B-14-1	None	Uncentrifuged	31.0
B-14-2	Thymol	Uncentrifuged	3.6
B-14-3	None	Centrifuged	29.8
B-14-4	Thymol	Centrifuged	12.5
Fermentation procedure - Huhtanen et al. (1954)[2]			
S-14-1	None	Uncentrifuged	81.7
S-14-2	None	Uncentrifuged	83.7
S-14-3	None	Uncentrifuged	83.7
S-14-4	Thymol	Uncentrifuged	6.0
S-14-5	Thymol	Uncentrifuged	9.1
S-14-6	Thymol	Uncentrifuged	10.4

1 Inoculum - 200 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea
Readily available carbohydrate - 0.1% glucose
Incubation - 40° for 24 hours

2 Inoculum - 25 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea added to inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 24 hours

TABLE 37

THE AMOUNTS OF CELLULOSE DIGESTED WHEN THYMOL WAS ADDED AT DIFFERENT TIMES DURING THE FERMENTATION PERIOD[1]

Experiment	Thymol added at	Cellulose digested		
		Based on initial[2]		Based on final[3]
		Replicates	Average	Average
no.	hr.	%	%	%
S-32-1	0	4.8		
S-32-2	0	5.6		
S-32-3	0	5.9	5.4	7.3
S-32-4	3	14.4		
S-32-5	3	21.0		
S-32-6	3	14.6	16.7	22.6
S-32-7	6	16.2		
S-32-8	6	20.4		
S-32-9	6	22.4	19.7	26.6
S-32-10	9	35.1		
S-32-11	9	33.2		
S-32-12	9	33.4	33.9	45.8
S-32-13	12	61.2		
S-32-14	12	62.3		
S-32-15	12	53.2	58.9	79.6
S-32-16	16	61.4		
S-32-17	16	56.5		
S-32-18	16	60.4	59.4	80.3
S-32-19	Not added	73.2		
S-32-20	Not added	74.0		
S-32-21	Not added	74.9	74.0	100.0

1 Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Substrate - 1% Solka-Floc
Non-protein nitrogen - 0.1% urea added to inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 3 to 24 hours

2 Based on the amount of cellulose present at 0 hr.

3 Expressed as the percentage of the cellulose digestion obtained in 24 hours

TABLE 38

THE CALCULATION OF THE AVERAGE HOURLY RATES OF CELLULOSE DIGESTION IN THE PRESENCE OF THYMOL FROM DATA IN TABLES 34 AND 37

Cellulose digestion					
Without inhibitor[1]		With inhibitor[2]			
Time incubated	Cellulose digested[3] (A)	Thymol added at	Cellulose digested[4] (B)	B - A	Cellulose digested per hour[5]
hr.	%	hr.	%	%	%
		0	7.3	7.3	0.30
3	9.0	3	22.6	13.6	0.65
3	13.6	6	26.6	13.0	0.72
9	24.0	9	45.8	21.8	1.45
12	42.2	12	79.6	37.4	3.12
16	59.3	16	80.3	21.0	2.63

1 Calculated from the data of experiment No. S-25 (Table 34)

2 Calculated from the data of experiment No. S-32 (Table 37)

3 Expressed as percentage of the cellulose digestion obtained in 24 hours

4 Expressed as percentage of the cellulose digested in 24 hours in the absence of an inhibitor

5 During the exposure to thymol

TABLE 39

THE RATE OF DIGESTION OF CELLULOSE IN ALFALFA LEAF MEAL[1]

Experiment	Fermentation period	Cellulose digested Based on initial[2]		Based on final[3]
		Replicates	Average	Average
no.	hr.	%	%	%
S-44-1	3	10.3		
S-44-2	3	9.6		
S-44-3	3	10.0	10.0	22.2
S-44-4	6	17.7		
S-44-5	6	17.6		
S-44-6	6	18.8	18.0	40.0
S-44-7	9	26.5		
S-44-8	9	26.6		
S-44-9	9	26.6	26.4	58.7
S-44-10	12	32.9		
S-44-11	12	33.8		
S-44-12	12	33.7	33.5	74.4
S-44-13	15	36.6		
S-44-14	15	34.1		
S-44-15	15	35.1	35.3	78.4
S-44-16	24	43.9		
S-44-17	24	46.3		
S-44-18	24	44.9	45.0	100.0

1 Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from steer No. 741
Mineral solution - Complex No. 3
Substrate - 4% alfalfa leaf meal
Non-protein nitrogen - 0.1% urea added to inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 3 to 24 hours

2 Based on the amount of cellulose present at start

3 Expressed as percentage of the cellulose digestion obtained in 24 hours

TABLE 40

THE AMOUNTS OF CELLULOSE DIGESTED WHEN THYMOL WAS ADDED AT DIFFERENT TIMES DURING THE FERMENTATION PERIOD[1]

Experiment	Thymol added at	Cellulose digested		
		Based on initial[2]		Based on final
		Replicates	Average	Average
no.	hr.	%	%	%
S-43-1	0	14.0		
S-43-2	0	12.1		
S-43-3	0	9.8	12.0	26.7
S-43-4	3	13.0		
S-43-5	3	13.3		
S-43-6	3	7.2	11.2	24.8
S-43-7	6	26.5		
S-43-8	6	23.8		
S-43-9	6	28.0	26.1	58.0
S-43-10	9	33.6		
S-43-11	9	32.2		
S-43-12	9	30.6	32.1	71.3
S-43-13	12	40.4		
S-43-14	12	37.2		
S-43-15	12	38.4	38.7	86.0
S-43-16	15	43.8		
S-43-17	15	45.1		
S-43-18	15	44.6	44.5	99.0

1 Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from steer No. 741
Mineral solution - Complex No. 3
Substrate - 4% alfalfa leaf meal
Non-protein nitrogen - 0.1% urea added to inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 3 to 15 hours without thymol, 3 hours with thymol (S-43-16, S-43-17, and S-43-18 incubated 9 hours with thymol)

2 Based on the amount of cellulose present at start

3 Expressed as the percentage of the cellulose digestion obtained in 24 hours in experiment No. S-44

TABLE 41

THE CALCULATION OF THE AVERAGE HOURLY RATES OF CELLULOSE DIGESTION IN THE PRESENCE OF THYMOL FROM DATA IN TABLES 39 AND 40

Cellulose digestion					
Without inhibitor[1]		With inhibitor[2]			
Time incubated	Cellulose digested[3] (A)	Thymol added at	Cellulose digested[4] (B)	B - A	Cellulose digested per hour[5]
hr.	%	hr.	%	%	%
		0	26.7	26.7	8.9
3	22.2	3	24.8	2.6	0.9
6	40.0	6	58.0	18.0	6.0
9	58.7	9	71.3	12.6	4.2
12	74.4	12	86.0	11.6	3.9
15	78.4	15	99.0	20.6	2.3

1 Calculated from the data of experiment No. S-44 (Table 39)

2 Calculated from the data of experiment No. S-43 (Table 40)

3 Expressed as percentage of the cellulose digestion obtained in 24 hours

4 Expressed as percentage of the cellulose digested in 24 hours in the absence of an inhibitor

5 During the exposure to thymol

TABLE 42

THE RATE OF DIGESTION OF CELLULOSE IN COTTON LINTERS[1]

Experiment	Fermentation period	Cellulose digested		
		Based on initial[2]		Based on final[3]
		Replicates	Average	Average
no.	hr.	%	%	%
S-47-1	3	5.3		
S-47-2	3	6.2		
S-47-3	3	5.1	5.5	11.4
S-47-4	6	6.6		
S-47-5	6	8.3		
S-47-6	6	7.6	7.5	15.6
S-47-7	9	10.0		
S-47-8	9	10.6		
S-47-9	9	9.9	10.2	21.2
S-47-10	12	14.8		
S-47-11	12	15.2		
S-47-12	12	13.1	14.4	29.9
S-47-13	15	23.2		
S-47-14	15	24.0		
S-47-15	15	24.6	23.9	49.7
S-47-16	24	47.5		
S-47-17	24	49.4		
S-47-18	24	47.5	48.1	100.0

1 Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from steer No. 741
Mineral solution - Complex No. 3
Substrate - 1% cotton linters
Non-protein nitrogen - 0.1% urea added to inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 3 to 24 hours

2 Based on the amount of cellulose present at start

3 Expressed as percentage of the cellulose digestion obtained in 24 hours

TABLE 43

THE AMOUNTS OF CELLULOSE DIGESTED WHEN THYMOL WAS ADDED AT DIFFERENT TIMES DURING THE FERMENTATION PERIOD[1]

Experiment	Thymol added at	Cellulose digested		
		Based on initial[2]		Based on final[3]
		Replicates	Average	Average
no.	hr.	%	%	%
S-46-1	0	2.0		
S-46-2	0	1.6		
S-46-3	0	4.4	2.7	4.9
S-46-4	3	6.3		
S-46-5	3	6.9		
S-46-6	3	6.7	6.6	12.0
S-46-7	6	11.0		
S-46-8	6	9.7		
S-46-9	6	12.1	10.9	19.8
S-46-10	9	14.8		
S-46-11	9	18.7		
S-46-12	9	15.7	16.4	30.0
S-46-13	12	23.0		
S-46-14	12	25.8		
S-46-15	12	26.3	25.0	45.4
S-46-16	15	32.9		
S-46-18	15	34.0		
S-46-18	15	36.3	34.4	62.4
S-46-19	24	54.0		
S-46-20	24	55.7		
S-46-21	24	56.7	55.5	100.7
S-46-22	Not added	53.7		
S-46-23	Not added	53.6		
S-46-24	Not added	57.9	55.1	100.0

1 Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from steer No. 741
Mineral solution - Complex No. 3
Substrate - 1% cotton linters
Non-protein nitrogen - 0.1% urea added to inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 3 to 27 hours

2 Based on the amount of cellulose present at start

3 Expressed as percentage of the cellulose digestion obtained in 24 hours

TABLE 44

THE CALCULATION OF THE AVERAGE HOURLY RATES OF CELLULOSE DIGESTION IN THE PRESENCE OF THYMOL FROM DATA IN TABLES 42 AND 43

Cellulose digestion					
Without inhibitor[1]		With inhibitor[2]			
Time incubated	Cellulose digested[3] (A)	Thymol added at	Cellulose digested[4] (B)	B - A	Cellulose digested per hour[5]
hr.	%	hr.	%	%	%
		0	4.9	4.9	1.6
3	11.4	3	12.0	0.6	0.2
6	15.6	6	19.8	4.8	1.6
9	21.2	9	30.0	8.8	2.9
12	29.9	12	45.4	15.5	5.2
15	49.7	15	62.4	12.7	4.2
24	100.0	24	100.7	0.7	0.2

1 Calculated from the data of experiment No. S-47 (Table 42)

2 Calculated from the data of experiment No. S-46 (Table 43)

3 Expressed as percentage of the cellulose digestion obtained in 24 hours

4 Expressed as percentage of the cellulose digested in 24 hours in the absence of an inhibitor

5 During the exposure to thymol

TABLE 45

A COMPARISON OF THE CELLULOSE DIGESTION OBTAINED IN VITRO WITH THAT OBTAINED IN DIGESTION TRIALS

Substrate	Cellulose digested				
	In vitro[1]		In vitro[2]		In vivo[3]
	Replicates	Average	Replicates	Average	
	%	%	%	%	%
2nd. cut alfalfa hay	51.6		41.8		
2nd. cut alfalfa hay	50.9	51.3	37.6	41.6	51.7
2nd. cut alfalfa hay			45.3		
Alfalfa-brome hay	42.3		38.9		
Alfalfa-brome hay	39.9	41.1	32.5	37.4	52.5
Alfalfa-brome hay			40.8		
Corn silage	38.6		38.7		
Corn silage	47.3	43.0	35.5	34.9	49.0
Solka-Floc	81.9		82.2		
Solka-Floc	83.0	82.5	82.0	82.2	
Solka-Floc			82.3		

1 Fermentation procedure - Burroughs et al. (1950)
Inoculum - 250 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Substrates - Solka-Floc; hay and silage samples from digestion trials
Non-protein nitrogen - 0.1% urea
Readily available carbohydrate - not added
Incubation - 40°C for 24 hours

2 Fermentation procedure - Huhtanen et al. (1954)
Inoculum - 25 ml. of strained rumen fluid from cow No. A-55
Mineral solution - Complex No. 3
Substrates - Solka-Floc; hay and silage samples from digestion trials
Non-protein nitrogen - 0.1% urea added to the inoculum bottle
Readily available carbohydrate - not added
Incubation - 39°C for 24 hours

3 80% of the fecal digestibility, determined by digestion trials