STUDIES ON EFFECT OF CITRIC ACID ON *IN VITRO* CELLULOSE DIGESTION BY RUMEN MICROORGANISMS FROM NORMAL AND TRAUMATIC GASTRITIS ANIMALS

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ABSTRACT

STUDIES ON EFFECT OF CITRIC ACID ON IN VITRO CELLULOSE DIGESTION BY RUMEN MICROORGANISMS FROM NORMAL AND TRAUMATIC GASTRITIS ANIMALS

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A study was made of in vitro cellulose digestion in order to determine the effects of citric acid when inocula were obtained from normal fistulated animals. Alfalfa leaf meal, Solka-Floc and timothy hay were used as sources of cellulose. It was demonstrated that there was no statistically significant difference between the percentages of cellulose digested with 0.2 percent, 0.1 percent, 0.05 percent and 0.01 percent concentrations of citric acid added in the test fermentations as compared to equivalent amounts of dextrose added in the control fermentations.

A study of in vitro fermentation of cellulose by rumen microorganisms drawn from traumatic gastritis animals was made to determine the effect of 0.1 percent citric acid on cellulose digestion and volatile fatty acid production. The fermentation mixture consisted of alfalfa leaf meal or Solka-Floc as the source of cellulose, rumen liquor drawn from traumatic gastritis animals, and 0.1 percent citric acid or 0.1 percent dextrose. No significant difference was found in percentages of cellulose digestion and volatile fatty acid production by adding 0.1 percent citric acid in the test fermentation. A wider variability in the results was encountered with the percentage of cellulose digestion and volatile fatty acid production by rumen microorganisms drawn from traumatic gastritis animals than by inocula from normal animals.

Determination of citric acid disappearance from the fermenters showed that most of the citric acid was metabolized by rumen micro-organisms after 16 hours of fermentation.

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INTRODUCTION

The utilization of cellulose has long been recognized as a primary factor in the nutrition of ruminants. Since ruminants are an expensive experimental subject for use in preliminary investigations several types of artificial rumens have been devised for *in vitro* studies of fiber breakdown and other problems relating to ruminant digestion. It is believed that the rate of cellulose digestion *in vitro* by the microorganisms of the rumen may be used as a measure of cellulose digestion *in vivo*.

Since unusual types of bacteria digest the food in the rumen, it is necessary to ascertain that the same bacteria are functioning in digestion in the artificial rumen. The most popular criterion of activity of microorganism in the artificial rumen is the percentage of breakdown of pure cellulose (Burroughs, Headley, Bethke and Gerlaugh, 1950; Stranks, 1956; Davey, Cheeseman and Briggs, 1960). The end products of fermentation, volatile fatty acids (VFA), have also been used as criteria of activity (Gray, Pilgrim and Weller, 1951; Warner 1956; Barnett and Reid, 1957). Studies have also been made using similar systems on the effects of various substances on *in vitro* ruminant fermentation. Burroughs *et al.* (1950) studied the effects of cereal grain and high protein feeds and Burroughs, Latona, DePaul, Gerlaugh and Bethke (1951) studied mineral influences. Wasserman, Duncan, Churchill and Huffman (1952) investigated the effects of antibiotics.

The purpose of this study was to test the effects of citric

acid on the *in vitro* activity of the rumen microorganisms from normal animals and from animals suffering from traumatic gastritis. Citric acid was selected as a test material because of its well known role as an energy source and easy availability at low cost.

The objective was attained by determining the percentage of *in vitro* cellulose digestion and VFA production. The disappearance of citric acid was observed to insure the validity of the approach.

REVIEW OF LITERATURE

In vitro fermentation methods

Tappiener (1888) was the first to investigate the in vitro fermentation of cellulose by rumen microorganisms. He found that paper pulp and cotton wool, when incubated under anaerobic conditions with the fluid contents of the rumen dissolved slowly with the evolution of methane and carbon dioxide and the formation of fatty acids. Analyzing the distillate of the medium from prolonged in vitro fermentation of cellulose by rumen contents he stated that acetic acid and butyric acids were the main products of the fermentation. Markoff (1911, 1913) using the same technique as described by Tappiener (1888) stated that the average molecular weight of the fatty acid products of the fermentations of cellulose was the same as that of butyric acid. Further work on in vitro study of rumen fermentation was mainly on VFA production by cellulose digestion. Barcroft, McAnally and Phillipson (1944) used fistulated animals from which samples were drawn for VFA determination. Similarly, Daniell, Hitchcock, Marshall and Phillipson (1945), and Gray (1947) analyzed the rumen contents drawn frequently from fistulated animal.

The early workers did not compare the conditions in vitro to those which occur in vivo until Marston (1948) developed the technique to simulate more closely the natural environment in the rumen. He considered the low pressure of oxygen, the high carbon dioxide 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. 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 nitrogen over the fermentation mixture.

Marston (1948) did not remove the nongaseous fermentation end-products and as they accumulated they might have been expected to slow and eventually inhibit the rate of digestion. To test the influence of such an accumulation of break-down products, Louw, Williams and Maynard (1949) compared cellulose digestion after incubation with fresh rumen fluid (700 ml) in a closed vessel and in a semi-permeable bag suspended in a large volume of aqueous growth medium. They showed that their semi-permeable system was capable of maintaining good rumen population during 24 hours of fermentation.

Burroughs et al. (1950) devised an artificial rumen which consisted of 500 ml glass fermentation flasks incubated in a waterbath at 40 C. A three hole stopper was inserted in the flasks for carbon dioxide inlet and outlet and for regulating the pH of the contents. The fermentation was carried out continuously in a 36 hour period and subsequent inocula consisted of one-half the residue of the preceding fermentation.

Gall and Glaws (1951) compared an all glass impermeable apparatus of Burroughs et al. (1950) and a permeable type as described by Louw et al. (1949). Rumen fluid was added to cellulose or grass in a salt

solution in these systems and neutralized frequently. The fermentation was carried out for 24-28 hours under anaerobic conditions. They found that in the impermeable system, accumulation of cellulase may account for complete cellulose digestion rather than viable bacteria. Poor culturability, altered-Gram reactions and lack of anaerobic organisms were found in seven trials with the impermeable system. They concluded that the process in the permeable system was more nearly like rumen digestion.

Huhtanen and Gall (1952) modified the artificial rumen of Louw et al. (1949), using a small cellophane sac suspended in a 4 ounce screw cap jar which contained a solution similar in mineral composition to sheep saliva. The sac containing the substrate and rumen fluid was held in place by screwing the cap onto the sac. The whole artificial rumen was sufficiently small to be placed in an incubator. The sac consisted of dialysis tubing, about 6 inches in length and tied at one end with a firm knot to form a sac with a volume of at least 10 ml. They used 10 ml of rumen fluid which had been filtered through two layers of cheese cloth as inoculum in the sac. The miniature artificial rumen used by Huhtanen et al. (1952) is particularly useful in routine work where large numbers of determinations of fiber digestion are desired.

McBee (1953) used the manometric procedure for evaluating the total activity of rumen microorganisms. The basis of his method is the assumption that the rate of fermentation of any substrate by the microorganisms in the rumen fluid is proportional to the total activity of all organisms in the rumen capable of attacking that substrate and the compounds derived from it. The total numbers and morphological types of organisms were ignored in his study. The results obtained

by McBee were influenced by the method used for obtaining the rumen content, the fraction of rumen fluid used, the buffer system in the Warburg vessel and the time of collection of the sample relative to the time of feeding. Johns (1951) carried out fermentations, using washed suspension (resting cells) of rumen organisms in a flask attached to a mercury filled manometer connected to an adjustable reservior which was adjusted to keep the gas at atmospheric pressure. It was so arranged that after inoculation under sterile conditions the apparatus could be filled with sterile nitrogen. The volume of gas produced by fermentation included that indicated by the mercury manometer and the dissolved gas in the liquid (mainly CO_2) which was determined in the VanSlyke manometric gas analysis apparatus. The gas in the manometer was analyzed by means of a Haldane gas analysis apparatus.

Doetsch and Robinson (1953) used the same technique as described by McBee (1953) except that the inoculum used was a suspension of washed cells. A number of substrates were used and the gas production was measured manometrically using the Warburg apparatus. Volatile fatty acid were also determined. Henderson, Horvat and Block (1954) used a simplified method for the study of cellulose digesting activity of rumen microorganism in vitro. They measured the cellulose activity by the loss of weight of parchment paper strips incubated with the frozen culture of the rumen microorganisms blended with a warmed nutrient solution containing a number of salts in standard bacteriological test tubes. The loss in dry weight of the parchment strips was considered to represent cellulose digestion. Bentley, Johnson, Hershberger, Cline and Moxon (1955) used the sediment of the strained centrifuged rumen juice, for the inoculum in their study on cellulolytic

factor activity of short chain fatty acids by rumen microorganisms *in vitro*. The sediment from one liter of juice was suspended in 200 ml of phosphate buffer (pH 7) which contained 0.01 percent cystine hydrochloride. Eight ml of this suspension were used in fermentation flasks containing cellulose, urea, glucose and mineral solutions. The pH was adjusted to 6.7 to 6.9 by saturated sodium carbonate solution. Carbon dioxide bubbled through the flask contents, served to establish anaerobiosis and to agitate the suspensions. At the end of 30 hours of fermentation the volume was adjusted to 100 ml and the disappearance of cellulose was determined on 10 ml of the reaction mixture by the method of Crampton and Maynard (1938) modified by Hersberger, Engle, Bentley and Moxon (1955).

Dehority, Bentley, Johnson and Moxon (1957) used an *in vitro* rumen fermentation technique essentially the same as described by Bentley *et al.* (1955) except that the urea concentration was changed from 0.168 g to 0.126 g per 100 ml. Also an improvement in the procedure for the collection of the rumen microorganism was made by drawing a line one inch from the bottle of the celluloid liner placed inside the Sharples centrifuge. Only the sediment above this line was used for inoculation of the *in vitro* fermentation flasks, by these workers. Warner (1956) has classified *in vitro* rumen procedures as follows; (a) Undiluted or slightly diluted rumen liquor incubated with the substrate in an all glass impermeable system, this technique was applied by Pearson and Smith (1943), Quin (1943), and Gray *et al.* (1951); (b) Whole rumen liquor diluted to half strength with mineral solution incubated with substrate in an impermeable system, this system was used by Burroughs *et al.* (1950), and McBee (1953); (c) Various fractions of rumen liquor

used in an impermeable system, e.g. centrifuged cells and washed cell suspension, this was used by McNaught (1951), Cheng, Hall and Burroughs (1955), and Bentley, Johnson, Vanecko and Hunt (1954); (d) Rumen liquor usually whole or undiluted incubated with a substrate in a semipermeable container and dialysing against a mineral solution; this system was used by Louw et al. (1949) Huhtanen and Gall (1952), and Wasserman et al. (1952).

Johnson (1958) improved the method of inoculum preparation of Bentley et al. (1954). The basal medium was the same as described by the latter investigators, except that the cellulose level was raised to 2 g per 100 ml of medium. Quicke, Bentley, Scott and Moxon (1959) applied the in vitro technique of Bentley et al. (1955) modified by Dehority et al. (1957) and Johnson et al. (1958) in their evaluation of cellulose digestion with forages, with some further modifications.

Davey et al. (1960) designed an improved artificial rumen for continuous control during prolonged operation. The important feature of their apparatus is the high degree of control which can be exercised over the progress of in vitro ruminal fermentation, permitting experiments of considerable duration. The criteria used for the validity of their fermentation apparatus include quantitative and qualitative bacteriological data, volatile fatty acid production, pH levels, and cellulose digestion rates. These were examined by them with a variety of diets using cows on similar diets as control.

Stewart, Daryl, Warner and Seeley (1961) have recently reported preliminary tests of reliability of continuous culture for studying the activities of mixed microbial population of the rumen in vitro. They designed an apparatus for continuous culture (5.5 liter) maintained in an 8 liter glass vessel at a constant temperature of 39 C. The culture

was stirred with Lucite (methyl methacrylate) paddles, and the sampling device consisted of a suction flask connected to half-inch glass tubing which extended down into half the depth of the culture. They tried to simulate the conditions of the normal rumen by their continuous culture apparatus and suggested that the normal rumen fermentation can be reproduced at a biochemical and cellular level.

Determination of cellulose

There are two common ways by which determination of cellulose can be accomplished. In the first of these, the cellulose is hydrolyzed and the increase in reducing power due to release of glucose units is determined. This method is not applicable in the present study because of the heterogenous nature of rumen fluid and the probability of the development of interfering substances during hydrolysis. In the second method, non-cellulosic materials are removed and the residual cellulose can be determined by volumetric or gravimetric methods. None of the methods of cellulose determinations are entirely satisfactory (Salsbury 1955). However, there are some methods in the second group which give reproducible results and which are applicable to the type of study presented here.

Kurschner and Hanak (1930) used a method for the quantitative estimation of cellulose, in which the sample was freed of non-cellulose organic constituents by digestion with an acetic acid-nitric acid reagent. The treatment involved boiling the samples with the reagent for twenty minutes.

Kurschner and Hoffer (1931) substituted alcohol-nitric acid as the digestion reagent and the sample was boiled with the reagent for

two and sometimes three successive hour periods.

Crampton and Maynard (1938) modified the method of Kurschner and Hanak (1930) by using alcohol instead of water for the first washing to free the cellulose from the digesting reagent. They used 15 ml of 80 percent acetic acid and 1.5 ml of concentrated nitric acid and boiled this gently for 20 minutes. The sample and liquid were transferred to a 50 ml centrifuge tube with about 20 ml alcohol. This was centrifuged for 10 minutes and then the residual fiber was transferred to a crucible and, by using suction this was washed with hot benezene, hot alcohol, and ether.

Viles (1948) developed the anthrone procedure used in the determination of the undigested fiber and for colorimetric analysis of starch and cellulose at a wavelength of 625 mμ. In this method sulfuric acid (60 percent by volume) is used to digest the material prior to analysis. It is then treated with water and anthrone reagent. He found that 60 percent sulfuric acid give optimum results for rapid disolution of cellulose. Cotton is the only form of cellulose referred to in his work.

Huhtanen, Saunders and Gall (1954) used the method described by Viles (1948) for cellulose determination, with modifications. They used a preliminary extraction of the water soluble carbohydrates present in the sample followed by a conversion of the remaining carbohydrate material into a form that will react with the anthrone to give a colored product. This colored product could be read in a Beckman spectrophotometer at a wavelength of 625 mμ.

Henderson et al. (1954) used a simplified method of cellulose determination. They incubated weighed strips of parchment paper before fermentation. At the end of fermentation the paper strips

were removed, washed in running water, air dried and reweighed. They found as much as 40 percent of the weight of the strips was lost by digestion and that the strips had retained sufficient strength to allow washing, drying and weighing. Hershberger et al. (1955) described a rapid volumetric method for the determination of cellulose with rumen microorganisms in vitro. In this method cellulose was suspended in water, centrifuged and the supernatant fluid decanted. The cellulose in the tube was suspended in 6.5 ml of glacial acetic acid and 1.5 ml of concentrated nitric acid and placed in a boiling water bath for 20 minutes. After cooling the tubes were centrifuged for 10 minutes, the top of cellulose leveled and after that recentrifuged for 5 minutes. The volume of cellulose in the tube was determined and compared to the control tubes. The authors claimed the advantage of rapidity of their volumetric method over the gravimetric method of Crampton and Maynard (1938).

Volatile fatty acid determination and citric acid determination

Volatile fatty acid determination. An early investigation on quantitative analytical methods for the determination of individual volatile fatty acid (VFA) in a mixture has been based upon such differences as rate of distillation or partition between immiscible solvents. Ramsey and Patterson (1945) were the first to devise a method for chromatographic separation of saturated VFA (C1 to C4). They used silica gel previously saturated with water containing a suitable indicator in its alkaline form in a column as in the ordinary chromatography. The chloroform solution of the volatile acid was added to the column which was then developed by washing with chloroform containing suitable amounts of n-butanol. The position of each

acid in the column was indicated by a color change in the indicator. The different bands containing one acid (or a mixture of isomeric acids in case of butyric) were then eluted from the column and collected separately. Elsden (1946a) introduced a technique based on partition chromatography for the separation and estimation of lower members of the saturated series of fatty acids. He used wet silica gel impregnated with bromocresol green. A small sample of the mixture of acids dissolved in chloroform was introduced at the top of the column and after its penetration into the gel, a suitable mixture of butanol and chloroform was allowed to percolate through the columns. Elsden was able to estimate propionic and butyric acids in samples subjected to previous distillation to quantitatively remove the acetic acid. Acetic acid could not be estimated by the column. This method has been used by Elsden (1946b) and modified by Elsden, Hitchcock, Marshall and Phillipson (1946) with excellent results in the analysis of rumen contents.

Paper chromatography has also been applied to the separation and quantitative determination of relatively non-volatile organic acids. The volatility of lower fatty acids however limits the direct application of paper chromatography techniques for their separation and identification. Fink and Fink (1949) overcame this difficulty by chromatographing the acids as their hydroxamate derivatives and spraying the developed chromatograms with ferric chloride. The derivatives were then visible as purple spots on a yellow background but accurate quantitative determination was not possible. Brown and Hall (1950) and Reid and Lederer (1951) devised a similar procedure by which lower fatty acids from C_2-C_7 could be separated, identified and estimated. They

employed the ascending development for 20-24 hour as described by William and Kirby (1948). The solvent used was butanol saturated with aqueous 1.5 N ammonia. The acids migrated on the chromatogram in the form of ammonium salts. Bromocresol purple in ethanol containing formaldehyde was used as a spraying agent. Using this technique lactic acid and formic acids could not be separated from acetic acid when lactic acid was present in significant amounts and it had to be removed by a preliminary distillation.

Keeney (1955) used a direct method for the chromatic determination of C1-C6 fatty acids in rumen fluid, as described by Harper (1953) for cheese samples. Keeney (1955) used silicic acid which had been mixed with an ethylene glycol solvent and then slurried with butanol-hexane to prepare the columns. Two ml of rumen fluid acidified with one drop of 50 percent sulfuric acid were mixed with 2 g of dry silicic acid and this mixture was transferred to the column. The solvents used in Keeney's method were a series of n-butanol concentrations in n-Hexane.

Emery, Smith, and Huffman (1956) used the method as described by Harper (1953), for volatile fatty acid determination in rumen fluid, with some modification. They used 13 g of dry silicic acid and 7.5 ml of 1.6 M potassium phosphate (pH 6.3) buffer for the buffered section. The sulfuric acid section of Harper (1953) was omitted by these workers and they found no effect on the results of their experiment. Furthermore, they prepared the sample by weighing 6 to 7 g of rumen liquid directly on to 10 g of dry silicic acid on a watch glass and thoroughly mixed this in a mortar. Fifty ml of chloroform were used to form a slurry and this was used as a vehicle to transfer to the column.

The use of celite in preference to silica gel columns has been suggested by Bueding and Yale (1951) and by Gray (1947), because of unpredictable chromatographic properties encountered with various preparations of the silica gel. Gray (1947) reported losses when passing the first sample of acids through celite columns. These results were rejected by Wiseman and Irvin (1957). They found that this loss could be prevented by an addition to the stationary phase of either 3 g of ammonium sulfate or 0.5 ml of 0.1 N H_2SO_4. Bueding (1951) used this phosphate buffer with celite column and quantitative separations of VFA were accomplished by the pH variables of the buffer present in the non-mobile aqueous phase and the butanol concentration of the eluent. Wiseman and Irwin (1957) designed a method for determination of organic acids in silage. They used celite columns with an internal indicator for the quantitative separation of silage acids including butyric and succinic acid. They found that the aqueous internal phase employed minimal amounts of sulfuric acid to prevent retention of organic acids, and sugar was added to increase elution resistance. The eluting solvents used were mixtures of acetone with Skellysolve B. The method was designed to eliminate steam distillation or ether extraction of acids and mechanical equipment for the collection of fractions. They found single zone collections made possible a reduced number of titrations with increased accuracy. Hungate, Mah, and Simensen-Mogens (1961) applied the method of Wiseman and Irwin (1957) in the determination of butyric, propionic and acetic acid in the rumen of lactating cows. Two ml of acidified liquid were mixed with the cap material on the top of a Wiseman-Irwin column, and the acids eluted with solvents were titrated with alcoholic sodium hydroxide. They found that butyric acid was

eluted with 1 percent acetone in mixed hexanes, propionic acid with 5 percent acetone-hexane mixture and acetic acid with 10 percent and then 20 percent acetone in hexanes.

Citric acid determination. Several methods for estimation of citric acid utilize the Furth and Herrman (1935) reaction in which color is formed in the presence of pyridine and acetic anhydride. Saffaran and Denstedt (1948) applied this reaction to the colorimetric determination of citric acid in animal fluids and tissues. Babad and Shtrikman (1951) modified the procedure to estimate citric acid in milk serum. In both procedures the sample was held for 10 minutes at 60 C in the presence of an excess of acetic anhydride, pyridine was added and the mixture held for further 40 minutes period at 60 C and then cooled in an ice bath. The two heating periods were accurately timed to minimize variability. Reducing the amount of water added with the sample was found to lower the intensity of the yellow color but enhanced the reproducibility (Babad et al., 1951).

Saffaran and Denstedt (1948) found that temperatures higher than 60 C decreased color intensity and stability while lower temperatures gave greater sensitivity but required longer holding periods for full color developments. Addition of pyridine to the hot solution caused a vigorous reaction that made it difficult to keep the tubes stoppered during the subsequent holding period in the procedure of Babad et al. (1951). Reinart and Nisbett (1957) modified the method to reduce the violence of the reaction at 60 C whereas Murthy and Whitney (1957) recommended the use of glass stoppers instead of rubber stoppers to prevent possible contamination of the sample.

Marrier and Boult (1958) improved the method of color development using lower reaction temperatures and this led to a procedure at a reaction temperature of 32 C for direct determination of citric acid in milk. They found that the reaction was strongly exothermic, and that a constant reaction temperature was essential to obtain reproducible results. They also found that rapid color formation at temperatures below 60 C could be induced by introducing more water with the sample; however, the water and acetic anhydride concentrations were difficult to control if they were allowed to interact before the addition of pyridine. On the basis of these observations pyridine was added to the sample prior to acetic anhydride and as color development was not affected the need for timing the pyridine step was obviated. All reagents were added at room temperature and the tubes were placed in water bath at 32 C immediately after adding the anhydride. In this manner they controlled the vigorous nature of the reaction and even at the reaction temperature of 60 C it was no longer necessary to stopper the tubes.

Chromatographic separation of citric acid in plant and animal tissues was attempted by a number of workers. The reader is referred to papers by Bulen, Varner and Burrel (1952), Phares (1952), Varner (1957), Jones, Dowling and Skroba (1953), Bush, Hurlbert and Potter (1952), Owens, Smith and Wright (1943), and Bryant and Overall (1953). None of the chromatographic techniques for the estimation of citric acid were applicable in the present study due to the lactic and succinic acid in the rumen samples. Citric acid could also be determined by the method of Natelson, Pincus and Lugovoy (1948) modified by Elliott (1957) in which colorimetric determination of citric acid is based on the oxidation

of citric acid to pentabromoacetone and conversion of the later to a colored complex. The reader is also referred to the procedure of Weil-Malherbe and Bone (1949) modified by Taylor (1953).

Rumen Microflora and their activity in in vitro fermentation

Rumen microflora. The ruminant differs from other mammals in that its food is subjected to microbial fermentation in the rumen before it passes into the true stomach and intestinal tract where most mammalian digestion occurs. The environment of the rumen is well adapted for the maintenance of a large and diverse microbial population. The temperature is held relatively constant at about 39 C. The pH of the ingesta is usually slightly acid and is held relatively constant by the influx of food, water and heavily buffered saliva (Masson and Phillipson, 1951). The Eh of the ingesta is held at a low level due to intense microbial activity and the low oxygen tension of the gaseous phase (Huhtanen et al., 1952 and Bryant, 1959). Many different groups of microorganisms have been isolated from the rumen, however it is now generally believed that the organisms of functional significance in the rumen are protozoa and bacteria, capable of growth under the anaerobic conditions prevailing in the rumen. Although a large number of bacteria of diverse morphological characteristics can be demonstrated readily in the rumen contents by microscopic examinations only a small number of these bacteria have been grown in pure culture and described as to their morphological and physiological characteristics. Hastings (1944) recognized that many of the bacteria in the rumen can not be cultivated by the more common bacteriological techniques, because of their fastidious growth requirements.

Isolation and culture media. As early as 1928 Bechdel isolated an organism from the rumen which was identified as Flavobacterium vita rumen. This bacterium was grown on nutrient agar under anaerobic conditions. Since then pure cultures of other species have been isolated from the rumen many of these having been cellulose decomposing bacteria. Hungate (1947) described an anaerobic technique and an agar medium containing minerals, carbonic acid bicarbonate buffer, and sterile rumen fluid for the isolation of cellulolytic bacteria. He showed that large numbers of non-cellulolytic bacteria also could be grown on this medium. Gall, Stark and Loosli (1947) isolated rumen microorganisms from rumen contents diluted as much as 100 billion times.

Sijpesteijn (1949) published anaerobic techniques for isolating cellulose decomposing bacteria from the rumen of cattle. The cultural procedure described by Gall et al. (1947) is satisfactory for the isolation of many rumen organisms but this technique had several weaknesses which made the isolation of several more delicate rumen organisms rather difficult. Gall, Hendrickson, Thomas and Loosli (1949) used a rich organic broth medium and emphasized the necessity of continued maintenance of anaerobic conditions and speed in preparing cultures of bacteria from high dilutions of rumen contents. Sijpesteijn (1951) used a similar medium as described by Hungate (1947) that contained yeast autolysate in place of rumen fluid to cultivate cellulolytic cocci.

Huhtanen et al. (1952) devised an improved technique for the isolation of rumen bacteria from high dilutions of rumen fluid. They designed a procedure to create a suitable pH and Eh in both the dilution blanks and broth and to control the production of acid by the more

rapidly growing organisms. They found the ability to culture rumen bacteria increased and growth of several delicate anaerobics typical of the adult roughage fed ruminant is encouraged by this method.

Microbial species in the rumen. Huhtanen and Gall (1953) isolated and purified nine types of curved rods, and related rod-types from the rumen. They described the rods and divided them into three main groups based on morphology; large curved rods, small curved rods and straight rods. All the organisms were nonspore-forming obligate anaerobes, which attacked fiber and produced short chain fatty acids, carbon dioxide and methane.

Bryant and Burkey (1953) using culturing techniques similar to Hungate (1950) found that on rumen fluid glucose cellobiose Agar (RGCA) medium there were higher colony counts and larger colonies were obtained, than from the agar medium described by Gall et al. (1947) or a tomato juice yeast extract trypticase agar with the carbondioxide-bicarbonate buffer system. Counts of 1,100 million, 180 million and 380 million per ml of rumen fluid were obtained from a single sample of rumen contents cultured on three media respectively. They also found that higher colony counts were obtained from the solid portions of rumen contents obtained by squeezing the liquid off through cheese cloth than from the liquid fraction. Furthermore, they found that out of 20.2 billion bacteria per ml seen under the microscope, only 1.7 million bacteria per ml were viable. They suggested that many of the bacteria either were not viable when cultured or were not capable of growing under the cultural conditions used. It was of interest that none of the common groups of bacteria such as members of the family

Enterobacteriaceae, enterococci or spore forming anaerobes were found in the rumen under the conditions applied by Bryant and Burkey (1953).

Hungate (1944, 1950, 1952) isolated Clostridium cellobioparus, Bacteriodes succinogenes and cocci. Sijpesteijn (1949, 1951) isolated Ruminococcus flavifaciens. Johns (1951) isolated Veilonella gazogenes an anaerobic, propionic acid producing coccus from the rumen of sheep. Gall et al. (1951) have described pure cultures of lactic acid bacteria but refrained from assigning names.

Hungate (1952) isolated 25 strains of cellulolytic bacteria and divided these into 4 groups: Bacteriodes; butyric rods, cocci and spore formers. He suggested that the cellulolytic flora are not a constant association of organisms but that any strain might predominate depending upon the particular animal and the particular time.

Mackay and Oxford (1959) and Mann, Oxford and Masson (1954) and Mann and Oxford (1955) have characterized several different kinds of bacteria isolated from a young calf and a goat. The organisms identified included, species of coliforms, lactobacilli, streptococci and Gram-negative, facultative anaerobic, urease positive rods. Hungate (1957) isolated Ruminococcus albus from the rumen of a cow.

An extensive study on the bacterial species of the rumen by Bryant (1959) produced evidence that the rumen contains a great variety of bacterial species, many of which are present in large numbers in one animal held under one set of conditions. Bryant concluded that the groups of bacteria corresponding to definite species are sometimes easily identified, e.g. the genera Butyrivibrio and Ruminococcus and the succinic acid producing Bacteriodes, but the great variability in characteristics among strains in these groups suggests that species specific patterns

will be very difficult to find. Furthermore, he stated that based on their function in making the energy of the ration available to the ruminant, the cellulolytic bacteria are often the most important ruminal microorganisms. Based on numbers present and the rate of cellulose digestion the genus Ruminococcus is one of the most important cellulolytic groups.

Physiology of rumen microflora. A symbiotic relation exists between the ruminant and bacteria found in the rumen in that the host supplies the bacteria with materials to be utilized and the bacteria in turn breaks down these substrates to products which may be absorbed into the blood stream (Thorton, Howie, Baker, Phillipson, Synge and Elsden, 1952). Notable among the functions of the bacterial flora are the digestion of cellulose, production of VFA and synthesis of protein and vitamins of the B-complex. A number of reviews on the functions of rumen bacteria are available and the reader is referred to the papers by McElory and Goss (1940), Wegner, Booth, Bohstedt and Hart (1941), Pearson and Smith (1943), Owen et al. (1943) Baker and Harris (1947), Conrad, Hibbs, Pounden, and Sutton (1950), Quin and Clark (1951) McNaught (1951), Arias, Burroughs, Gerlaugh, and Huffman (1951), Wasserman et al. (1952), Dehority, El-Shazly and Johnson (1960), Huffman (1953), Salsbury, Hoffer and Luecke (1961), Hinders and Ward (1961) and Bryant and Robinson (1961), for more detailed information on the subject.

In vitro activity. Biological and chemical criteria have been used in attempts to establish the validity of the results obtained with the artificial rumen systems. Biologically, attention has been

paid to motility of the protozoa and predominant morphological or cultural types of bacteria. Chemically most attention has been paid to the proportion of cellulose digested; though other relative values have been used, little attention has been given to the actual rates of digestion of the total substrate. It is essential to ensure that the microbial multiplication which takes place should proceed in a fashion similar to that found in the rumen of the living animal. When the environment or substrate is not that found in the rumen of the animal from which the rumen liquor inoculum was taken, then the results of this metabolism may bear little or no relation to events in the rumen *in vivo*.

Pearson and Smith (1943) used undiluted or slightly diluted rumen fluid incubated with substrate in an all glass impermeable system as used by Quin (1943) and Gray *et al.* (1951). On the basis of their findings they claimed that their system remained normal for 2-4 hours of fermentation and that, numbers and types of mainly the iodophilic bacteria showed no significant changes during that time. With the same technique McNaught and Owen (1949) showed that the concentration of O-phenanthroline which suppressed urea utilization *in vitro* corresponded to a concentration of ferrous ion of the same order as that found *in vivo*. With a similar system with rumen liquor free of protozoa McNaught (1951) found that the CO_2/CH_4 ratio in the evolved gases had an average value similar to that found *in vivo* though the range of values was larger.

Quin (1943) and McAnally (1944) showed that the rates of gas output following addition of glucose was similar *in vivo* and *in vitro* but the incubation periods observed were very short.

Burroughs *et al.* (1950) claimed that there were no marked changes

in numbers, size or predominant types of bacteria throughout, but, that some of the types of protozoa failed to survive. However the main criterion of normal rumen function relied on by those workers was the high degree of cellulose digestion obtained. They used 3 g per liter of cellulose per artificial rumen contents in early experiments. Gray et al. (1951) successfully applied a number of criteria of normal rumen function to their in vitro system. They microscopically observed the activity of the protozoa, the rate of methane produced to fodder supplied, and the digestibility of cellulose and pentosans. These total digestions were found to be similar in vivo and in vitro but the authors noted that the rates of digestion of these substrates and of methane production were only one-half of those found in vitro. They used as buffer ammonium-carbonate equivalent to 103 mg N/ 100 ml, a concentration of NH_3 very rarely found in the rumen of healthy animals even when fed a diet rich in a readily attacked protein.

Bentley et al. (1955) found that rumen microorganism required the presence of certain VFA, particularly valeric acid, for digestion of cellulose in vitro. However, Bryant and Doetsch (1955) working with cultures of Bacterioides succinogenes, a cellulose digesting organism isolated from the rumen, reported that this organism required a combination of a branched chain fatty acids, isobutyric, iso-valeric or DL-α - methyl-n-butyric acid, and a straight chain fatty acid, valeric or caproic acid for growth on a purified medium. Allison, Bryant and Doetsch (1958) investigated the volatile fatty acid requirement of several strains of Ruminococcus flavifaciens and Ruminococcus albus, both anaerobic cellulolytic cocci isolated from the rumen. They found that a mixture of acetate, isobutyrate and valeric acid

isomers and also a mixture of acetate and isovalerate greatly stimulated the growth of these organisms.

Warner (1956) using an artificial rumen system of Louw et al. (1949) showed that in the presence, though not in absence, of substrate the numbers of bacteria and protozoa were maintained approximately constant in the artificial rumen for 7 hours. For the microbial population to remain normal in numbers and activity, it was shown to be necessary to use as test substrate in vitro, only substances similar to diet fed to animal from which the rumen liquor inoculum was taken.

Dehority, et al. (1960) studied the cellulolytic fractions of bacteria by differential centrifugation. They found that the fraction of bacteria still in suspension at 15 x G but sedimented at 3000 x G contained the majority of the cellulolytic rumen bacteria. Fermentation studies with the 3000 x G sediment showed that after incubation with cellulose as the source of carbohydrate a predominance of Gram-negative micrococci were present along with a lesser number of very small Gram-negative rods. These observations indicated that the Gram-negative cocci are the principal cellulose digesting organisms which proliferate in vitro under these conditions. These same kind of organisms were also found in large numbers in the inoculum obtained from the rumen. They also reported that the microorganisms showed a strict requirement for both valeric acid and biotin.

Stewart et al. (1961) using a continuous culture method for in vitro fermentation found that the values for pH, rates of total VFA production, proportions of VFA, and numbers of coliform and amylolytic organisms were similar to values which have been reported by Balch and Rowland (1957) and Reid, Hogan and Briggs (1957). The rates of

growth of oligotrich protozoa in the in vitro experiments using hay-concentrate substrate were equal to the fastest rates of growth reported in vitro by other workers (Hungate, 1942, 1943 and Coleman, 1958). They found the apparent growth of oligotrichs in their experiments with an all hay substrate consistent with that reported by Oxford and Sugden (1953). Both holotrichs and oligotrichs showed good motility in their experiments.

Mechanism of cellulose breakdown

Ruminants do not possess a salivary enzyme for degradation of cellulose nor is there any such enzyme secreted into the rumen. Thus the degradation of cellulose is clearly a function performed by the flora found in the rumen.

Henneburg (1919) first reported detailed microscopic studies of disintegration of plant tissues in the rumen. An important finding was that the areas of erosion on the surfaces of plant materials were usually occupied by cocci. This method of approach was extended by the studies of Baker (1939) who, using polarized light, examined microscopically cellulosic material undergoing digestion by rumen bacteria. The most characteristic result of the attack on plant tissues was found to be the excision of clear cut cavaties or lacunae in which iodiphilic organisms were enclosed. An important finding by Baker (1939) was that the morphological characteristics of the rumen micro-organisms were correlated with their functional activities.

Norman and Fuller (1940) stated

> "Since cellulose is completely insoluble in water and obviously therefore to accomplish decomposition any organism must possess an extra cellular enzyme system capable of producing some soluble product that can pass into the cell. It seems to be

> some indication that two types of hydrolytic system may be concerned, one accomplishing the primary attack, perhaps breaking cross linkages or accomplishing substantial chain shortening and the other bringing about subsequent hydrolysis of these fragments."

They concluded that it seems likely that exoenzyme production is only stimulated by direct contact with the fiber in the case of a wholly insoluble substrate. Baker and Harris (1947) found that the rumen microorganisms reach the susceptible plant structure by invading the middle lamella of the 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 microorganism with the result that cavaties are formed. At first these cavaties generally follow the form of microorganisms but as digestion proceeds the cavaties enlarge and form corrosion figures which lack the birifrigence shown by the neighboring plant material.

Hungate (1944) has suggested that the apparent requirement for 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.

Considering the available observations Siu (1951) gave a tentative scheme for the enzymic breakdown of cellulose. He postulated a separate enzyme C_x. The postulation of C_x enzyme was based on the observation made by Reese (1950) that the carboxy-methyl cellulose was attacked not only by filtrates from cellulolytic microorganisms but also by those from non-cellulolytic fungi. According to Siu (1951) the linear polysaccharide is degraded by C_x enzyme directly into glucose without formation of cellobiose as an intermediate. Siu (1951)

extended his scheme for breakdown of cellulose step wise. He stated that the first step was the rupture of Vanderwall's forces in the amorphous region of the cellulose and of the hydrogen bonds in the crystalline region. Second step would be the rupture of the cross linkage which are assumed to join the linear chains of the anhydrous glucose units. For the third step, the breakdown by the C_x enzyme of linear polysaccharide into units which can diffuse into the cell. He reserved the term 'Cellulase' for the enzyme bringing about the initial change in the native cellulose molecule.

Levinson, Mandles and Reese (1951) used glucose oxidase for the determination of glucose and beta-glucosidase followed by the determination of glucose formed with glucose dehydrogenase for the determination of cellobiase and found that cellobiose was the principal produce of cellulose degradation. The cellulolytic organisms studied produced large amounts of C_x enzyme which diffused into the medium. Only small amounts of β-glucosidase were found outside the cell. They found that the cellobiose resulting from C_x activity can enter the cells as rapidly as can glucose. They hypothesized that the principal final product of C_x activity is cellobiose and the presence of cellobiose in the medium is not a prerequisite to utilization of cellobiose by the organism. They stated that this hypothesis is a correction of the hypothesis previously made in the same laboratory by Reese (1950) that glucose appeared to be the final product of C_x activity.

Reese and Gilligan (1953) separated the C_x enzyme into three components by differential adsorption onto cellulose and Kaolin. The number of components varied depending upon the species of micro-organisms used and the conditions of growth. They could not reach

the conclusion whether these components represented individual enzymes or were due to interaction of a single enzyme with other elements of the system.

Kitts and Underkofler (1954) using rumen organisms in an anaerobic fermentation found that unlike aerobic fungi these rumen organisms do not liberate cellulase into the medium. Using undiluted rumen fluid to which an inhibitor was added the yields of glucose obtained were of a magnitude of 2-3 mg/ml in 24 hour when a substrate concentration of 16 mg/ml of Solka-Floc was used.

Whitaker (1953, 1954) has concluded that Myrothecium verrucaria cellulase is a single enzyme capable of hydrolyzing native cellulose to glucose. Kooiman, Roelofsen and Sweeris (1953) agree with Whitaker except that they involve a separate enzyme (Cellobiase) in the hydrolysis of cellobiose to glucose. Jermyn (1952) demonstrated the presence of several β-glucosidases some of which he believes act on comparatively long chains as found in carboxy-methyl cellulose (CMC).

The recent developments on mechanism of cellulose hydrolysis were summarized diagramatically by Reese (1956) as follows:

$$\text{Native cellulose} \xrightarrow{C_1} \underset{\text{chains}}{\text{Linear cellulose}} \xrightarrow[A,B,C\ \text{etc}]{C_x} \text{Cellobiose} \xrightarrow{\beta\text{ gluc.}} \text{glucose}$$

β-glucosidase A,B,C etc (= transferases).

He explained that the components of cellulolytic systems may differ largely in their preference for chains of a particular length. If this is true then there may be components bridging the gap between the cellulases and β-glucosidases. Such components would have maximum activity on the short chains (6 to 10 units long) and lesser activity as the chains become longer or shorter. Not enough is known about

C_1 to characterize it. He proposed the existence of C_1 enzyme when C_x was considered to be a single enzyme. He deduced the presence of C_1 enzyme from the fact that some organisms unable to attack native cellulose did produce enzyme capable of hydrolyzing degraded cellulose (e.g. CMC). Reese (1956) stated that it is uncertain whether C_1 is that form of C_x acting primarily on the longest chains or whether it may indeed act on some minor linkage or minor component, in native cellulose.

Reese (1956) concluded that glucose is certainly the end product of the complete hydrolysis of cellulose by the enzymes when β-glucosidase is present in the cellulolytic complex. Much of the glucose results from β-glucosidase action on cellobiose, but it is still uncertain whether glucose may be produced in the absence of β-glucosidase.

Factors affecting in vitro cellulose digestion

Several factors could be of significant importance in in vitro cellulose fermentation. These factors include: Lignification and stage of maturity of substrate, amount of cellulose, particle size, rumen liquor source, amount of rumen liquor, pH adjustments, addition of trace minerals, length of fermentation, various techniques used and the nitrogen source.

Kamstra, Moxon and Bentley (1958) reported the effect of lignification and stage of maturity of plant in in vitro cellulose digestion. They observed that the amount of cellulose in the flasks affected the amount of cellulose digested by the microflora in the fermentation flasks because of the amount of cellulose available to them. Furthermore, the maturity of the forage affects the in vitro digestion of

cellulose in plant material. With first stage alfalfa, an average of 83.6 percent of cellulose was digested in 30 hours whereas only 46.7 percent of the cellulose was digested in alfalfa in the third stage of growth. Lignin *per se* does not appear to influence the activity of rumen organisms *in vitro*; however, the lignin inhibits cellulose digestion by an encrusting action based upon the deposition of lignin around the cellulose during the growth of the plant. These observations were similar to those reported by Norman (1935), Crampton and Forshaw (1939), Dyer, Weaver and Comfort (1954), Ely, Kane, Jacobson and Moore (1953) and Trimberger, Kennedy, Turk, Loosli, Reid and Slack (1955) on the maturity of forages and their feeding values and digestibility. The effect of lignin on digestion of fiberous plant material *in vitro* has also been reported to be similar by Baker and Harris (1947) and Howie and Baker (1952).

Perlin, Michaelis, and Mcfarlane (1947) and Norkans (1950) using cellulolytic microorganisms showed that the amount of cellulose breakdown was inversely proportional to the degree of crystallinity of the cellulose fraction. Furthermore, they showed that by changing the crystalline cellulose to amorphous cellulose they increased the rate of microbial decomposition of the cellulose. Ranby and Immurgot (1955) have demonstrated that the physical constants are not identical for cellulose from different sources.

Stewart and Schultz (1958), Salsbury, Vanderkolk, Baltzer and Luecke (1958) and Quicke, Bentley, Scott and Moxon (1959) found little difference in cellulose digestion by various rumen liquor sources. However, Huhtanen *et al*. (1952) and Asplund, Berg, McElory and Pigden (1958) have observed qualitative and quantitative differences in

in vitro digestion for rumen liquors from steers fed different diets.

McLeod and Brumwell (1954) using purified substrate and washed cell suspensions as described by Cheng *et al.* (1955) reported that the amount of rumen liquor may have an appreciable influence on the extent of *in vitro* digestion of cellulose or dry matter. This information was confirmed by Church and Peterson (1960) who applied the same procedures as described by Barnett and Reid (1957).

In many of the *in vitro* experiments reported, pH adjustments have been made in the range of 6.4 to 6.9. Wegner, Booth, Bohstedt and Hart (1940) suggested adjustment between 6.0 to 7.5 whereas Meites, Burrel, and Sutton (1951) have reported an optimum pH between 4.53 and 7.35 for cellulose digestion. Kitts and Underkofler (1954) and Stanley and Kesler (1959) working with isolated rumen cellulose preparations found optimum cellulase activities at pH 5.5 to 6.0. Reis and Reid (1959) observed that optimum pH for ammonia accumulation varied between 6.0 to 6.7 on different diets and appeared to be correlated with the range of pH normally encountered in the rumen on each diet. Virtanen (1946) found that mesophilic bacteria fermented finely ground wood dust more completely than coarsely ground dust, possibly due to less interference by lignin.

Church and Peterson (1960) confirming the results of Reis and Reid (1959) and Baker, Quicke, Bentley, Johnson and Moxon (1959) indicated little difference in the digestion of purified wood cellulose when separated into different particle sizes. However they reported that the fermentation of some substrates (e.g. alfalfa hay) may be altered appreciable by fine grinding. They suggested that fine grinding may have altered solubilities of the other carbohydrate

constituents with the resultant change in the cellulose digesting flora. Cheng et al. (1955) and Dehority et al. (1957) reported that increasing levels of dried grass in the fermentation flasks, depressed the percentage of digestibility of dry matter and cellulose. Church and Peterson (1960) also found the same effect of dried grass added to their cellulose fermentation. Huhtanen and Elliott (1956) found that cellulose digestion was not influenced by the quantity of alfalfa added within the ranges studied and with the technique used.

Baumgardt, Cason, and Markley (1958) found that the relation between in vitro and in vivo cellulose digestion was not significant when all forages were considered, but the relationship was highly significant for the grass hays.

The mineral supply to rumen microorganisms is vital to efficient cellulose digestion in vitro. Phosphorous and iron have been named as two minerals most likely to affect the cellulose splitting organisms. Little, Cheng and Burroughs (1958) found that a balanced mineral nutrient solution is needed by the rumen microorganisms for optimum cellulose digestion and the increase reached a maximum at a concentration of 12.5 ppm. The addition of 7.5 ppm of zinc or manganese, or 15 ppm of magnesium also increased cellulose digestion but to a lesser extent than could be obtained with the addition of iron. Cobalt and copper failed to stimulate cellulose digestion at any concentration tested and they were found to be inhibitory to cellulose digestion at levels of 2.5 ppm and above. Selenium was found to decrease cellulose digestion when added to the basal medium by these workers. Church and Peterson (1960) demonstrated that changes in mineral medium concentration within the ranges used have only negligible effect on

in vitro digestion of dry matter and cellulose, or VFA production.

Cardon (1953) reported that a high intake of salt adversely affects cellulose digestion.

Using alfalfa leaf meal as a substrate, Huhtanen and Elliott (1956) showed the following rate of cellulose digestion: at 6 hours 17 percent, at 12 hours 34 percent; at 18 hours 40 percent, and at 24 hours 49 percent. The 24 hour period was recommended as suitable for the *in vitro* fermentation by those workers. Lefevere and Kamastra (1958) compared cellulose digestion *in vitro* and *in vivo* with 22 different rations and found the 48 hour *in vitro* fermentation period as most nearly simulating *in vivo* cellulose digestion.

LeFevere and Kamastra (1960) using the technique of Bentley, Lehumkuhl, Johnson and Hershberger (1954) in the *in vitro* digestion of cellulose found that 24 hour fermentation was below that obtained *in vivo*. Cellulose digestion coeficients obtained *in vitro* with sheep and cattle rumen fluid as inoculum were lower for rations containing 25% roughage than for those containing 75 percent roughage.

The factors affecting *in vitro* cellulose digestion may be variable due to the different techniques employed by the several workers. Hungate (1947), Sijpesteijn (1951) and Bryant and Berkey (1953) used pure cultures for some studies on cellulose digestion. Cheng *et al.* (1955) modified this technique by centrifuging and washing the rumen microorganisms. Bentley *et al.* (1954) employed centrifuged unwashed bacterial cells from rumen fluids while Wasserman *et al.* (1952) and Huhtanen *et al.* (1954) worked with whole unaltered rumen fluid. Some authors have pooled the rumen liquor samples from several animals (Barnett, 1957) or washed suspensions of microorganisms (Doetsch and

Robinson, 1953) as a means of reducing variability between inoculums.

Arias et al. (1951) found that the degree to which energy requirements of the rumen microorganisms were fulfilled depended upon their ability to utilize urea or other ammonia supplying compounds. They suggested that the probability exists that there is specific need for small amounts of readily available energy, such as that from dextrose, sucrose or starch when the principal energy source is cellulose, to bring about the most efficient utilization of urea in the fermentation. Huhtanen and Elliott (1956) found that the addition of urea or glucose alone markedly inhibited the in vitro breakdown of cellulose in timothy hay substrate but, when they were added together, normal activity was restored. The effect was less marked or absent with alfalfa leaf meal. Bloomfield, Muhrer and Pfander (1958) studied the relation of the source of energy to urea utilization by rumen microorganisms and found that lactic acid, pyruvic acid and alanine stimulated urea utilization indicating a possible necessity for a functional group on the alpha carbon. Starch was found to be more effective than the simple sugars, fructose was found to be superior to other hexoses, and equal amounts of glucose and fructose were superior to similar amounts of sucrose as sources of energy.

Salsbury, Hoffer and Luecke (1961) found that a ration of cellulose, cornstarch and urea was the simplest combination capable of maintaining the cellulose digesting ability of the rumen microflora for three days. Cellulose plus soyabean protein was found more effective than cellulose plus urea. Furthermore, when the ration consisted of only cellulose, starch and urea, a source of amino acids was required to maintain cellulose digestion for more than 3 days. Determination

of molar concentration of VFA indicated that feeding ground cellulose in addition to hay and supplement lowered the ratio of acetic to propionic acid in the rumen.

Barnett (1957) reported that by the use of chemically untreated feed-stuffs and whole filtered rumen liquor the operative microflora may be kept sufficiently active *in vitro* for a suitable length of time. He concluded that the microbial population in an artificial rumen bears no relationship at the end of run to the original population. He also stated that different animals on the same diet have approximately the same microbial population but different animals on different diets show considerable variation. LeFevere and Kamastra (1958) found that there was no significant difference in cellulose digestion *in vitro* and *in vivo* when the animals used were on the same ration. However when the animals used as sources of animal fluid were on different rations, the cellulose digestion values *in vitro* with the same basal medium were of different magnitudes. Greater cellulose digestion was indicated for rations high in roughage both *in vitro* and *in vivo*.

Donefer, Lloyd and Crampton (1961) reported that the digestion of Solka-Floc cellulose was much more affected by source and type of inoculum than were any of the forage substrates. Timothy hay digestion was stimulated by casein hydrolysate supplementation only when re-suspended cell inoculum was used.

Burroughs *et al*. (1950), Ruf, Hale, and Burroughs (1953) and Bentley *et al*. (1955) demonstrated cellulolytic factor activity in hot water extracts of alfalfa, timothy hay, ladino clover, cow manure, autolysed yeast, dried brewers yeast and distilled dried fermentation

soluble molasses. Hall, Cheng, Hale and Burroughs (1954) found that the partial hydrolysate (acid, alkali and enzymatic) of casein or soya-bean protein, chicken feathers, hair and blood-meal also stimulated cellulose digestion. Huhtanen, Saunders and Gall (1954) noted that the addition of 50 mg Difco yeast extract or enzyme hydrolysed casein to their in vitro system increased the digestion of Solka-Floc by as much as 100 percent over the control.

Wasserman et al. (1952) showed that antibiotics interfered with in vitro cellulose digestion. The effect of the antibiotic chlorotetracycline hydrochloride on cellulose digestion was further investigated by Huhtanen and Elliott (1956) who reported that this form of antibiotic when fed had no correlative inhibitory action on cellulose digestion over the test period. Gas production by rumen microorganisms increased greatly after a few days on the antibiotic regime. Lodge, Miles, Jacobson and Quinn (1956) showed that the tetracyclines tend to depress cellulose digestion over a period of time and that resistant strains of organisms appear in the rumen.

Contradictory evidence has also been forthcoming on the influence of added fat and steroids on cellulose digestion. Brooks, Garner, Muhrer and Pfander (1954a) reported that steroids are generally shown to stimulate cellulose digestion while Brooks, Garner, Muhrer and Pfander (1954b) reported that the position of fat is still in dispute.

Citric acid utilization

There are two major stages in the process by which energy becomes available to ruminants; a preliminary conversion of the protein, or metabolism of carbohydrates and fats of the diet via acetyl Co A or an

intermediate of the tricarboxylic acid cycle and the subsequent oxidation of these relatively simple compounds.

The direct utilization of citric acid by rumen microorganism is not known, however citric acid utilization by other microorganisms has been reported by several workers. Early recognition of citrate utilization in milk and in glucose-citrate-broth by Hammer (1920), Hucker and Pederson (1930) and Van Beynum and Pette (1939) led to the appreciation of the role of Streptococcus lactis, Streptococcus citrovorous and Streptococcus paracitrovorous in better flavor development by the production of acetoin and diacetyl. In most cases, however, a fermentable sugar also was required to obtain citrate fermentation. Brewer and Werkman (1938) found pyruvate in citrate-glucose fermentation of S. paracitrovorous, but were unable to find citrate as its precursor. Citrate fermentation in Aerobacter aerogenes was formulated by Deffner and Franke (1938, 1939) as follows:

4 citrate -----> 7 Acetate + 5 CO_2 + Formate + Succinate.

Carbon dioxide and acetic acid were the major products determined. Brewer and Werkman (1939, 1940) obtained evidence that oxaloacetate and pyruvate are intermediate in citrate fermentation by S. paracitrovorous. Campbell and Gunsalus (1944) showed that energy can be derived from citrate in the absence of carbohydrate fermentation and lactate production by homofermentative lactic acid bacteria. They found that at pH 7.0 lactic acid is not produced, but acetate, carbon dioxide and formic acid were the main products. At an acid pH, acetylmethylcarbinol and lactate become major products. The citrate cleaving enzyme has been shown to be present in Streptococcus faecalis, A. aerogenes and Escherichia coli by Dagly and Dawes (1953) and Grunberg-Monago and

Gunsalus (1953). The cleavage of citrate in microbial fermentation is catalyzed by a distinct enzyme which does not involve coenzyme A as shown by Wheat, Wong and Ajil (1954), Wheat and Ajil (1954, 1955) and Smith, Stamer and Gunsalus (1956).

In vivo studies on feeding trials with sodium citrate in the ration of lambs was reported by Packett, Butcher and Fordham (1960). They stated that addition of 1.63 percent equivalent citric acid to the diet, produced 47 percent increase in gain. The gain enhancement was greatest during the initial period but gains were continuously greater throughout the experiment. Increase in feed consumption, feeding efficiency, salt intake and water consumption were greater according to these workers.

Heany, Pritchard, and Sylvestre (1961) used sodium citrate buffer with feeding roughage rations (pelleted) in steer feeding trials. They expected sodium citrate would provide a readily fermentable anion and a residual cation. They used citric acid also to provide only the readily fermentable anions. They found that sodium citrate increased the intake of pellets in all periods but citric acid had no effect. Furthermore, they reported that there was a tendency for the consumption of pellets to increase during the period of fermentation. When compared with consumption of chopped hay the consumption of pellets was greater in all groups, the sodium citrate groups showing the greatest increase.

Gray and Anthony (1961) reported the effect of citric acid and citric acid plus yeast extract fed with Bermuda Hay in vivo digestion trials among eight steers. They found that there was no statistically significant difference among the treatments in either phase. However,

steers receiving citric acid supplement did have an increased average daily gain that approached significance.

EXPERIMENTAL AND RESULTS

Section I

Experimental

Effect of citric acid on in vitro cellulose digestion by rumen inocula from normal animals

The experiments were carried out using normal cows in an attempt to determine the in vitro cellulose digestion using different substrates and to estimate a suitable concentration of citric acid to be used in the following studies.

Animals used. Inocula were obtained from three nine-year-old Holstein cows (A-130, T-26, and K-139) maintained on a ration of seven pounds of alfalfa hay and four pounds of grain mixture. All three animals had been fistulated and fitted with screw capped lucite fistula plugs similar in construction to one described by Hentschl, Berry and Huffman (1954).

Collection of samples. Inocula were obtained 2-3 hr after the animals had been fed. The upper portion of the rumen contents was removed through the fistula to approach the area of the ventral sac of the rumen. The rumen fluid was then squeezed into a polyethylene bottle through two thickness of cheese cloth. The bottle with the rumen sample was kept under tepid water and was brought to the laboratory as soon as possible. The sample was then transferred to a glass bottle, and aerated with CO_2 while immersed in a 39 C waterbath.

Substrates used. Substrates used in these studies were air dried alfalfa leaf meal, Solka-Floc and timothy hay. (See Table 1.)

TABLE 1. Percent of cellulose in substrates

Substrate	Cellulose percentage	G used to obtain 0.2 g of cellulose
Alfalfa leaf meal	22.5	0.80
Solka-Floc	95.2	0.20
Timothy hay	31.6	0.60

Alfalfa leaf meal and Solka-Floc were used throughout these studies, while timothy hay was used only in the preliminary experiments.

The concentrations of citric acid tested were 0.2 percent, 0.1 percent, 0.05 percent and 0.01 percent (w/v) of citric acid in distilled water. These concentrations were made from stock solutions stored at 4 C and were brought to a temperature 39 C in a water bath. Dextrose was used as the standard at the same concentrations as citric acid in each run. Urea was used as the source of nitrogen. The concentration of urea used throughout the studies was 0.1 percent as recommended by Salsbury (1955).

Fermentation procedure. The fermentation procedure used in these studies was similar to that described by Huhtanen et al. (1954). As inocula 20 ml of rumen fluid were placed in Visking cellophane tubing,[1] 6-1/2 inches in length and 1 inch in diameter; these sacs were placed in 4 oz screw-capped bottles. Twelve fermentation vessels of each substrate were set with the test concentrations of citric acid and 12 with the same concentrations of dextrose as a standard. Each experiment was performed in duplicate. Composition of the mineral mixture

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

used was one-half strength artifical saliva as described by McDougall (1948). Eighty ml of the mineral mixture plus citric acid or dextrose were used outside the Visking tubing in each individual fermentation vessel. The fermentation jar containing the substrate, rumen fluid and citric acid or glucose in the Cellophane bag was sealed and placed in a water bath at 39 C. Fermentations were stopped at 0, 4, 12, 16, 20 and 24 hours by freezing the fermentation vessel at -10 C.

Determination of cellulose. Cellulose was determined by the modified method of Crampton and Maynard (1938). In these experiments the mixture in the cellophane bag was transferred to a 150 ml Berzelieus beaker, the bag was rinsed with distilled water and the solution evaporated to dryness at 105 C. The dried sample was treated with 15 ml of 80 percent acetic acid and 1.5 ml of concentrated nitric acid. A glass, round bottom flask, filled with cold water was placed on the beaker and the contents refluxed on a hot plate for 20 minutes. The beaker was removed from the hot plate, the sides of the beaker and bottom of the flask were rinsed with 95 percent ethyl alcohol, and the beaker was set to cool. The contents of the beaker were then filtered through a Selas 40XF sintered porcelain crucible and the crucible was dried 18 hours at 105 C. It was then cooled in a desiccator over calcium sulfate and weighed to determine the weight of the crucible with cellulose. The cellulose in the weighed crucible was then muffled for 2 hr at 700 C, cooled again in a desiccator and weighed to determine the weight of the crucible and ash. The percent cellulose digestion was calculated from comparison with the residual cellulose in the zero hour samples.

Results

The percentages of cellulose digested in 8 hr and 24 hr fermentation periods are shown in Table 2. When alfalfa leaf meal, Solka-Floc and timothy hay were used as substrates, the concentrations of dextrose and citric acid used were 0.2 percent, 0.1 percent, 0.05 percent and 0.01 percent. There was a decrease in the percent of cellulose digested when 0.2 percent citric acid or 0.2 percent dextrose was used in all the substrates. Citric acid gave higher percentages of cellulose digestion in 24 hours of fermentation when compared to equivalent amounts of dextrose, although there was only slight change observed with added citric acid.

The change in the percent of cellulose digestion with these concentrations of citric acid with alfalfa leaf meal, Solka-Floc and timothy hay was not statistically significant at the 5 percent level as tested by the t-test (Stearman, 1955).

Figure 1 shows the average percent of cellulose digestion of Solka-Floc, alfalfa leaf meal, and timothy hay using 0.2 percent added citric acid or dextrose when the inocula were taken from cow K-139. The percentage of digestion obtained in 24 hours of fermentation was lower using Solka-Floc as substrate. An increase in the percent of cellulose digestion with all three substrates was observed when citric acid was added, but this increase was not significantly different from that of the standard.

Figure 2 shows the percent cellulose digestion with the same substrates, after adding 0.1 percent citric acid or dextrose. The percent of cellulose digestion in 24 hours was in agreement with that of previous workers (Huhtanen et al., 1954, Huhtanen and Elliott, 1956)

TABLE 2. Effect of various concentrations of dextrose and citric acid on the *in vitro* digestion of cellulose

Cellulose Digestion*									
		Dextrose concentration**				Citric acid concentration**			
Substrate	Time of fermentation	0.2	0.1	0.05	0.01	0.2	0.1	0.05	0.01
Alfalfa leaf meal	8 hr	10.5	20.1	25.4	25.4	16.0	21.4	18.8	23.8
	24 hr	34.7	46.5	49.9	50.9	45.9	47.5	46.9	52.1
Solka-Floc	8 hr	0	8.6	10.8	6.1	3.0	0.3	7.8	5.5
	24 hr	50.5	67.2	55.3	55.8	49.7	67.8	57.9	60.0
Timothy hay	8 hr	1.8	5.96	8.86	10.5	5.8	5.75	7.6	7.6
	24 hr	34.8	25.9	44.2	35.3	33.3	29.5	35.7	33.3
Average of all substrates	8 hr	4.1	11.6	15.0	14.0	8.2	12.5	11.4	12.3
	24 hr	40.0	46.5	49.8	47.3	42.9	48.3	46.8	48.5

*Average values of experiments expressed in percent.

**Concentration of citric acid and dextrose expressed in percent.

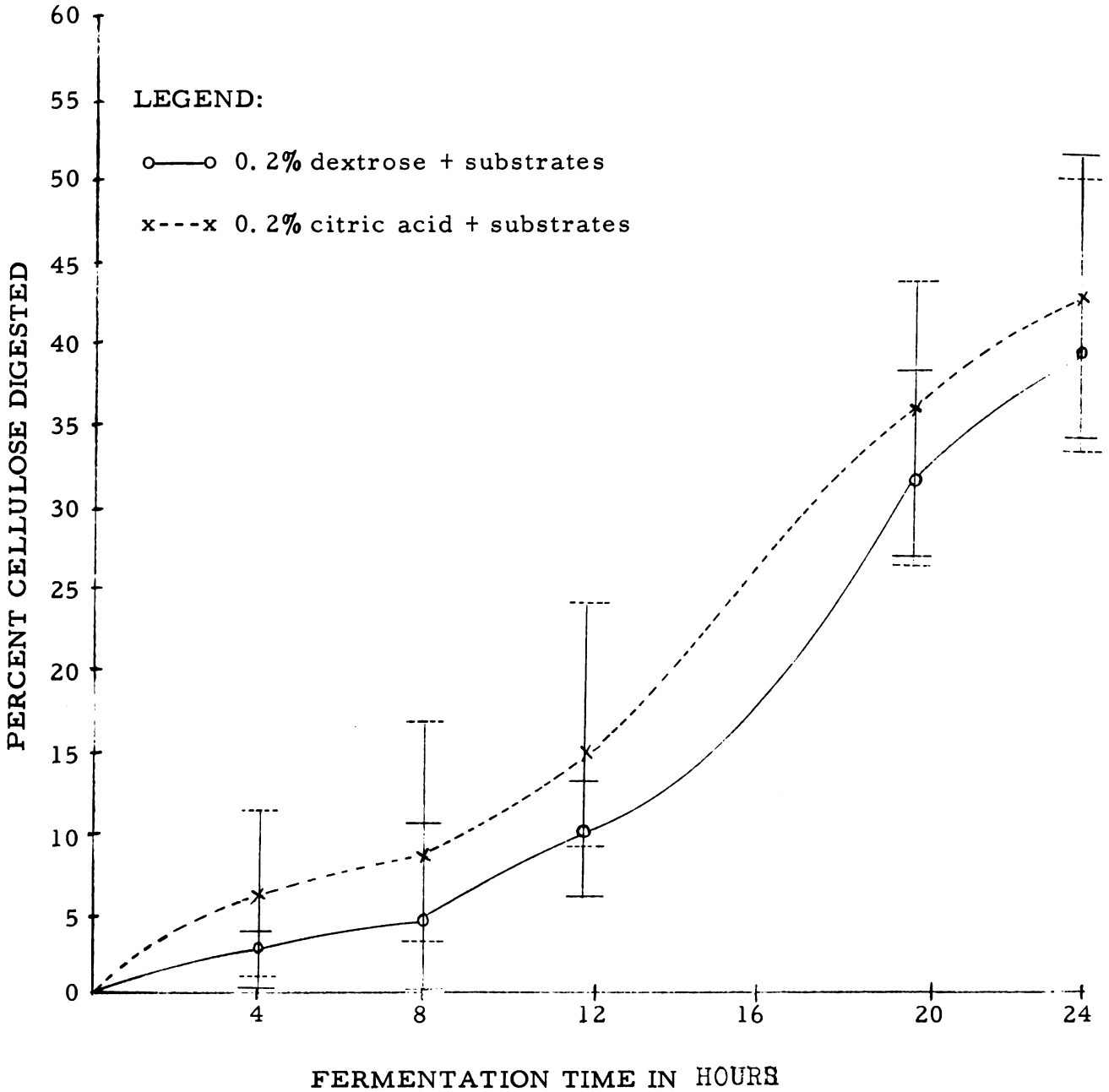

Fig. 1. Effect of 0. 2 percent citric acid on the percent of the cellulose digested by inocula from cow K-139 with alfalfa leaf meal, Solka-Floc and timothy hay as substrates.

Each point represents an average value of three duplicate experiments. The perpendicular lines represent the range of variation with the substrates with dextrose and citric acid.

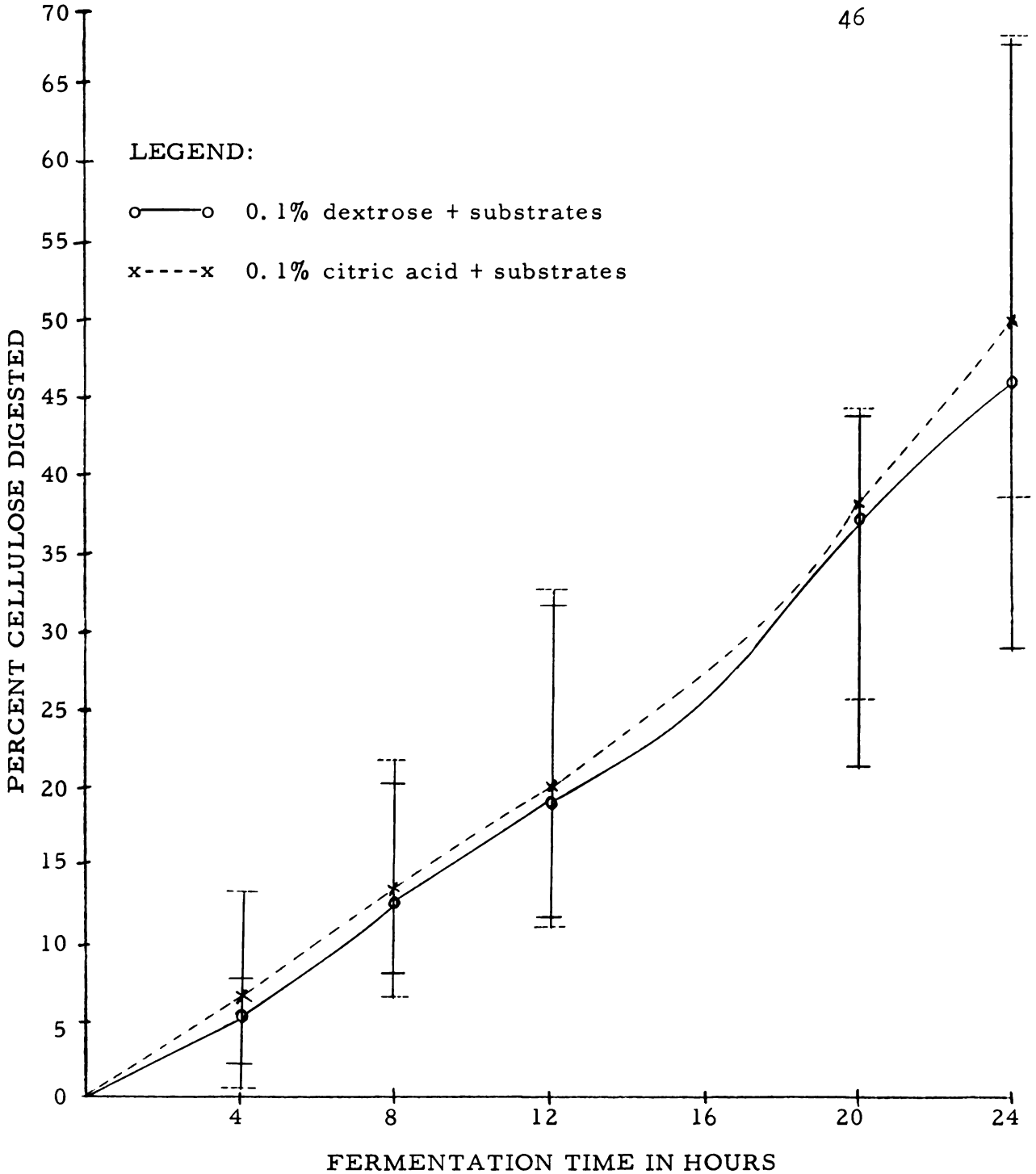

Fig. 2. Effect of 0.1 percent citric acid on the percent of the cellulose digested by inocula from normal animals with alfalfa leaf meal, Solka-Floc and timothy hay as substrates.

Each point represents an average value of six duplicate experiments. The perpendicular lines represent the range of variation with the substrates used with dextrose and citric acid.

in this field. When Solka-Floc was used as the substrate the increase in cellulose digestion with added citric acid was observed up to 8 hours of fermentation and with timothy hay as a substrate this increase was noted after 8 hours. The increase in percent cellulose digestion with 0.1 percent citric acid is not statistically significant at the 5 percent level with all the substrates used.

Figure 3 shows the average percent cellulose digestion with alfalfa leaf meal, Solka-Floc and timothy hay using 0.05 percent citric acid or dextrose. Solka-Floc and timothy hay gave a slight increase in percent cellulose digestion with added citric acid. Using Solka-Floc as a substrate the percent of cellulose digested was lower in the 24 hours fermentation period when citric acid was added as compared to added dextrose.

Figure 4 shows the average percent of the cellulose digested with the same substrates using 0.01 percent added citric acid or dextrose. Citric acid increased the percent of cellulose digestion with alfalfa leaf meal as compared to added dextrose. The percent of cellulose digested with added citric acid was lower in 24 hours of fermentation when Solka-Floc was used as a substrate.

Table 3 shows the percent of the cellulose digested in Solka-Floc and alfalfa leaf meal in 8 hour and 24 hour fermentation periods using 0.1 percent citric acid or dextrose with inocula from different animals. The difference in percent of the cellulose digested with citric acid or dextrose added was not statistically significant after 8 hours of fermentation. A wide variation was observed.

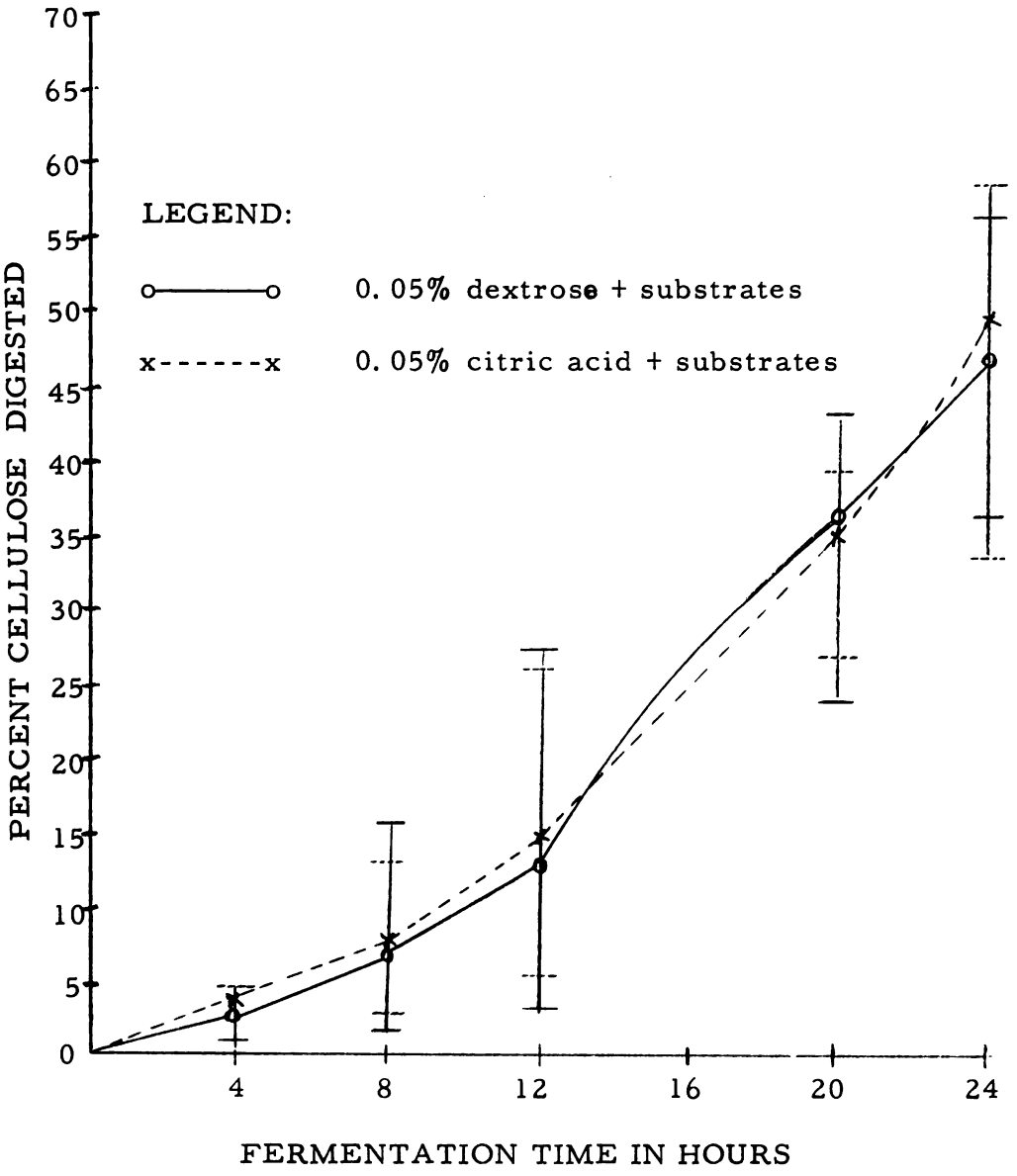

Fig. 3. Effect of 0.05 percent citric acid on the percent of the cellulose digested by inocula from cow T-26 with alfalfa leaf meal, Solka-Floc and timothy hay as substrates.

Each point represents the average value of three duplicate experiments. The perpendicular lines represent the range of variation with the substrates used with dextrose and citric acid.

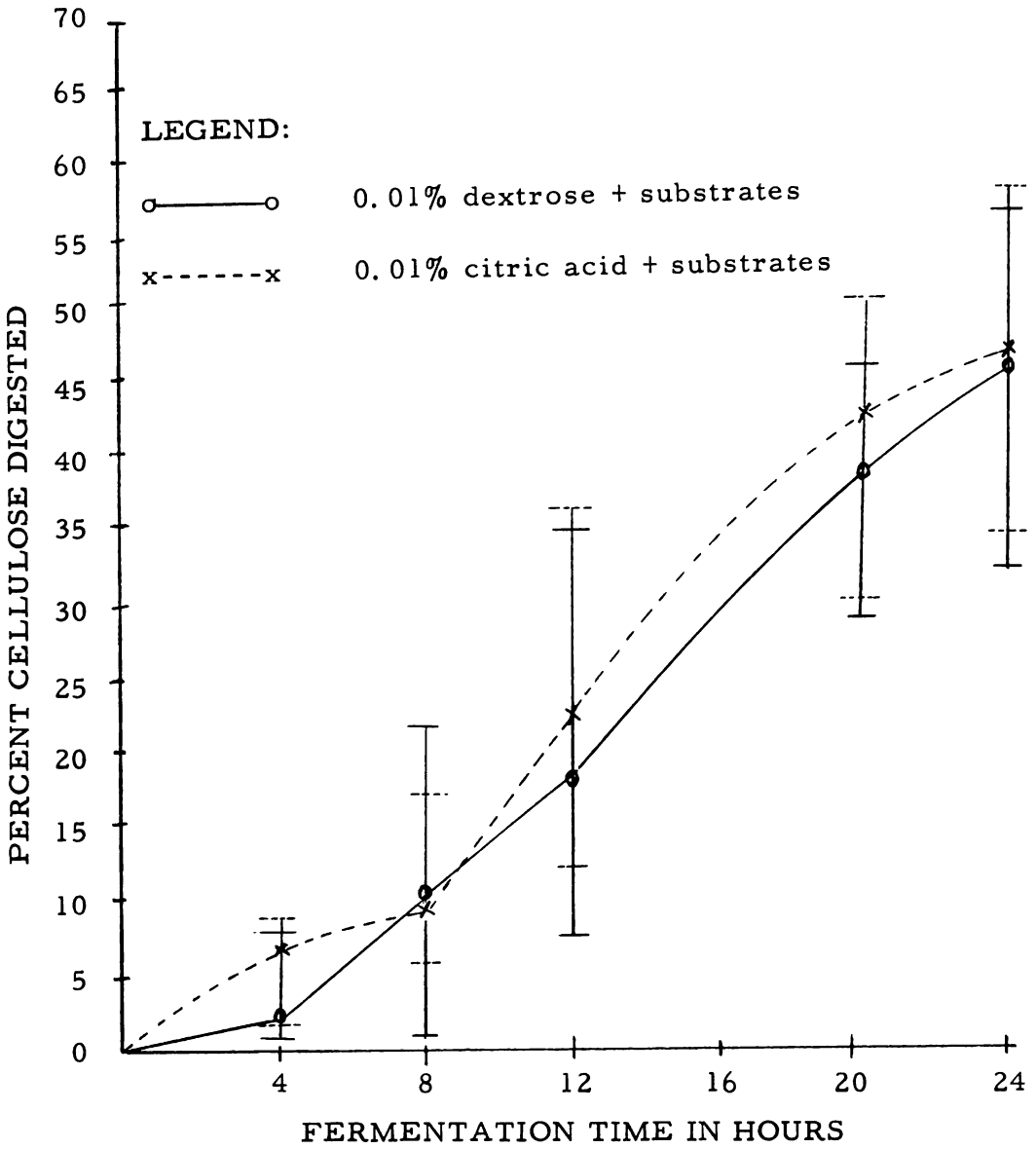

Fig. 4. Effect of 0.01 percent citric acid on the percent of the cellulose digested by inocula from cow T-26 with alfalfa leaf meal, Solka-Floc and timothy hay as substrates.

Each point represents the average value of three duplicate experiments. The perpendicular lines represent the range of variation with the substrates used with dextrose and citric acid.

TABLE 3. Percent of cellulose digested *in vitro* by inocula from normal animals in 8 hr and 24 hr fermentation periods

Animal	Alfalfa leaf meal				Solka-Floc			
	8 hr		24 hr		8 hr		24 hr	
	Dextrose 0.1 percent	Citric acid 0.1 percent	Dextrose 0.1 percent	Citric acid 0.1 percent	Dextrose 0.1 percent	Citric acid 0.1 percent	Dextrose 0.1 percent	Citric acid 0.1 percent
Cow Holstein (A-130)	12.9	25.0	42.4	48.6	9.6	8.0	67.0	66.8
Cow Holstein (T-26)	16.7	18.4	46.1	50.6	--	--	--	--
Cow Holstein (T-26)	16.1	13.5	47.4	44.4	9.7	11.4	59.9	67.8
Cow Holstein (K-139)	26.9	25.3	46.0	45.6	0	0	65.8	64.7
Cow Holstein (K-139)	16.2	12.5	46.3	44.2	6.5	11.4	76.1	71.8
Steer Holstein	32.0	33.7	50.9	51.6	--	--	--	--
Average	20.1	21.4	46.5	47.5	6.4	7.8	67.2	67.8

Section II

Experimental

Effect of citric acid on cellulose digestion by cattle with clinical traumatic gastritis

Traumatic gastritis, a widely occurring disease in ruminants, is caused by metallic objects swallowed by the animal incidently or with the feed. The "hardware" is usually lodged in the ventral part of the reticulum, where it penetrates or causes continuous irritation to the animal. This may slow the rumen contractions and may cause death of the animal if there is penetration through the diaphragm and puncturing of the heart. The animal displays anorexia, rumen contractions are slower and weakened, there is rise in body temperature and tenderness in the region of the xiphoid cartilage. The treatment is removal of the foreign body by rumenotomy.

Rumen samples from nine cases of traumatic gastritis from the Michigan State University cattle clinic, were obtained during rumenotomy. They were treated with 0.1 percent citric acid or dextrose as were the regular rumen samples. The percent of cellulose digestion, VFA production and citric acid disappearance were studied. The animals used were eight Holstein cows from 3 yr-12 yr of age and a yearling steer of the Holstein breed. The history of all the animals in these studies was similar. The animals were anorexic with a body temperature above 102.5 F and they had slow rumen movements. Traumatic gastritis was diagnosed in all cases. Rumenotomy was performed in the clinic and the foreign bodies were removed.

Cellulose fermentations were carried out using the methods described above. Substrates tested in these studies were alfalfa leaf meal and Solka-Floc. The experiments were designed so that both

substrates could be used at the same time and treated with inocula from the same animal. The concentration of citric acid or glucose tested was 0.1 percent in all the experiments. The urea concentration of 0.1 percent was kept constant.

The fermentations were stopped at 0, 4, 8, 16, 20 and 24 hours, the material within the sac and the artificial saliva were mixed and centrifuged at 800 X G after the fermentation period. A twenty ml sample of supernatant fluid was stored at -10 C for later chromatographic analysis of volatile fatty acids and colorimetric determination of citric acid. The sediment of the fermenter was treated for residual cellulose analysis as described previously.

Results

Figure 5 shows the average values of percent of the cellulose digested with 0.1 percent citric acid, using alfalfa leaf meal and Solka-Floc as substrates at different periods of fermentation. The difference observed in both substrates due to 0.1 percent added citric acid was not significant; however, with alfalfa leaf meal a slight increase in the percent cellulose digested was noted until 16 hours of fermentation.

Table 4 shows the percent cellulose digested in 8 and 24 hour fermentation periods using alfalfa leaf meal and Solka-Floc as substrates when the inocula were taken from nine clinical cases of traumatic gastritis. The average difference in percent of the cellulose digested in 8 hour fermentation with 0.1 percent citric acid or dextrose added was tested using the t-test and was found not to be significant at the 5 percent level. A wide variation in percent cellulose digestion

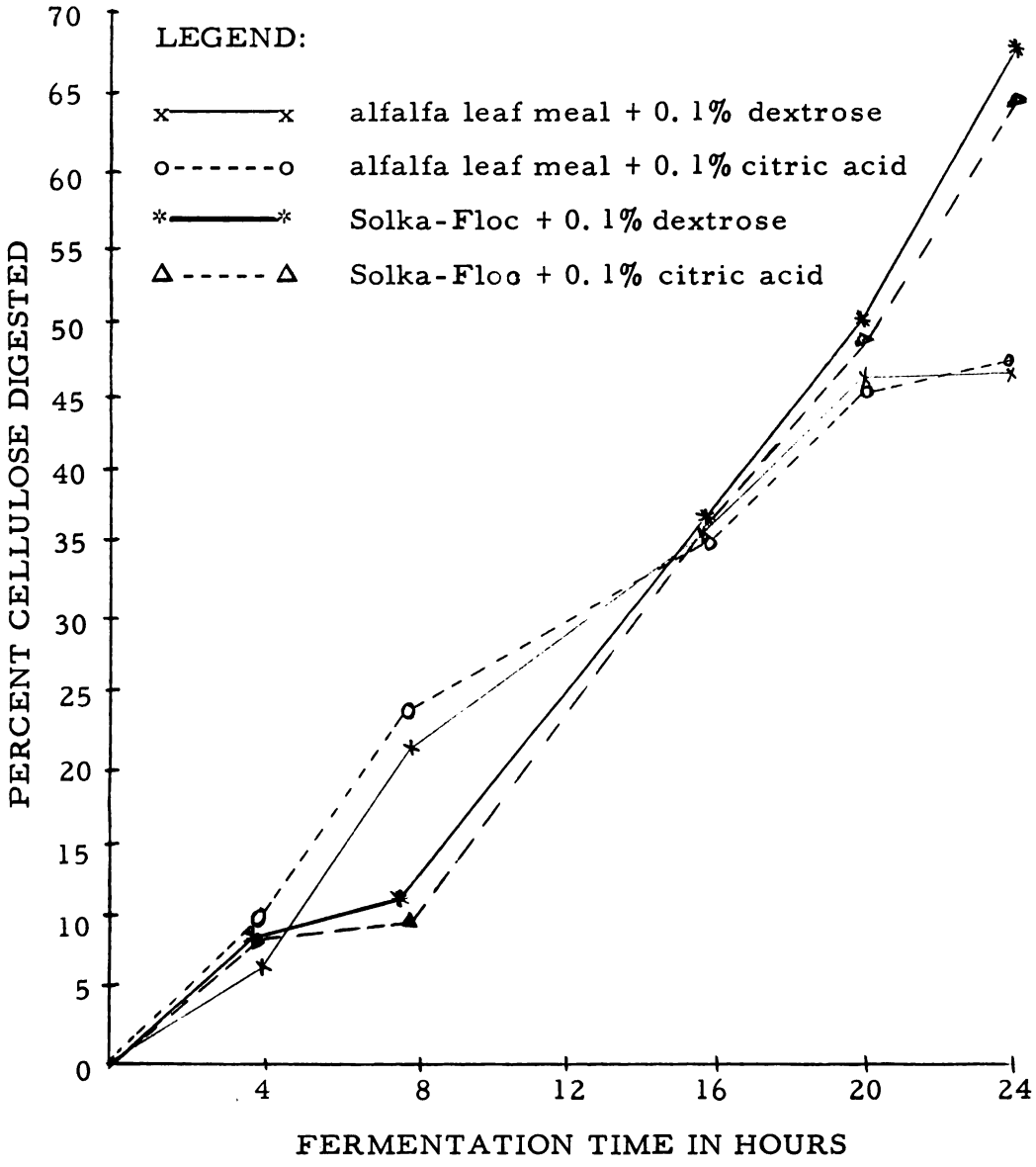

Fig. 5. Percent of cellulose digested with 0. 1 percent dextrose or 0. 1 percent citric acid by inocula taken from traumatic gastritis animals.

Each point represents the average value of nine duplicate experiments.

TABLE 4. Percent cellulose digested by inocula taken from 9 clinical cases of traumatic gastritis in 8 hour and 24 hour fermentation periods

Substrate	Alfalfa leaf meal				Solka-Floc			
	8 hr		24 hr		8 hr		24 hr	
Case No.	Dextrose 0.1 percent	Citric acid 0.1 percent	Dextrose 0.1 percent	Citric acid 0.1 percent	Dextrose 0.1 percent	Citric acid 0.1 percent	Dextrose 0.1 percent	Citric acid 0.1 percent
1	12.0	23.3	46.9	46.7	5.2	3.2	61.8	47.2
2	2.97	5.2	44.2	46.8	--	--	--	--
3	23.5	21.5	43.1	44.9	6.1	4.7	71.4	68.6
4	23.0	27.5	50.3	48.8	13.1	11.3	60.5	59.9
5	35.6	35.1	51.7	51.4	13.1	7.0	69.8	65.4
6	22.7	19.4	46.6	47.8	8.3	5.5	70.8	67.8
7	25.98	28.8	46.3	48.8	9.8	16.2	73.8	73.6
8	23.8	26.4	40.8	34.4	21.2	17.3	61.9	57.1
9	25.2	26.7	44.7	51.0	10.9	9.4	73.1	77.4
Average	21.6	23.7	46.0	46.7	11.0	9.3	67.8	64.6

in both alfalfa leaf meal and Solka-Floc at 8 hour fermentation was observed. This variation may possibly be due to the difference in activity of rumen microorganisms obtained from different cases of traumatic gastritis.

In Table 5 the average percent cellulose digestion is compared in normal and traumatic gastritis animals with alfalfa leaf meal as substrate. There was not a significant difference at 8 hr of fermentation. At 16 hr of fermentation, the percent cellulose digestion in traumatic gastritis animal was lower than that in normal animals. In 24 hr there was no difference in percent cellulose digestion between normal and traumatic gastritis animals.

When Solka-Floc was used as a substrate there was a considerable difference in percent cellulose digestion between normal and traumatic gastritis animals. In normal animals it was lower than in traumatic gastritis animals at 4 hr and 8 hr of fermentation; while at 16 and 20 hr of fermentation, there was no difference in cellulose digestion between the two groups. In both normal and traumatic gastritis animals there was no statistically significant difference in the cellulose digestion with both alfalfa leaf meal and Solka-Floc.

TABLE 5. Percent *in vitro* cellulose digestion by normal and traumatic gastritis animals with 0.1 percent dextrose or 0.1 percent citric acid added

	Alfalfa leaf meal				Solka-Floc			
	Normal animals		Traumatic gastritis animals		Normal animals		Traumatic gastritis animals	
Fermentation	Dextrose	Citric acid	Dextrose	Citric acid	Dextrose	Citric acid	Dextrose	Citric acid
4 hour	7.5	13.1	6.6	8.6	3.1	2.3	8.7	8.8
8 hour	20.1	21.4	21.6	23.7	8.6	10.3	11.0	9.3
12 hour	31.7	32.2	--	--	12.6	10.9	--	--
16 hour	44.1	43.1	36.8	34.9	42.8	41.5	36.2	36.6
20 hour	44.2	44.0	46.0	45.4	43.0	43.0	49.4	48.5
24 hour	46.5	47.5	46.0	46.7	67.2	68.1	67.8	64.6

Section III

Experimental

Volatile Fatty Acid (VFA) Determinations

Volatile fatty acids were determined by column chromatography, based on the method described by Bueding (1951), with modifications.

Samples prepared from the fermentation mixtures of 0, 4, 16 and 24 hr with 0.1 percent citric acid or dextrose added, were applied to a column of celite and sulfuric acid. Acetic, propionic and butyric acids were separated by development with hexane-butanol solvent. A standard column was run with known amounts of acetic, propionic and butyric acids and a recovery of 98.4 percent was obtained. VFA were determined in each sample with Solka-Floc and alfalfa leaf meal as substrates and fermented by inocula from normal and traumatic gastritis animals.

Celite[1] was washed twice by decantation with two volumes of purified diethyl ether. It was air dried overnight and then dried at 110 C for 4 hr. Six grams of this celite were used to prepare a column.

Sulfuric acid solutions. 0.2 N H_2SO_4 and 10 N H_2SO_4 solutions were made from Analytical Reagent grade H_2SO_4 of 35.5 N strength.

Ethanolic potassium hydroxide. 1 N KOH was made by dissolving (w/v) potassium hydroxide pellets (Reagent grade) in 95 percent ethanol. 0.01 N KOH was made by diluting 1 N KOH with 95 percent ethanol and standardized against 0.01 N potassium biphthalate. 0.01 N standardized ethanolic KOH was prepared fresh for each experiment.

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[1] Johns-Manville No. 535.

Indicator. 0.1 percent bromothymol blue in ethanol was used as an indicator.

Solvent. 1, 3, 5 and 25 percent butanol concentrations were prepared (v/v) in n-Hexane (Skelly solve B). Each solution was equillibriated with 0.2 N H_2SO_4 three times for 6 hours and the H_2SO_4 was separated by means of a separatory funnel.

Columns. Chromatographic tubes, 20 mm (inside diameter) x 400 mm, sealed with a sintered glass disc at the bottom were used in these analyses. The columns were prepared using 6 grams of celite. The celite was weighed in a beaker and 2.6 ml of 0.2 N H_2SO_4 were added and mixed until completely homogenous. Sufficient hexane (Skelly solve B) was added to wet the celite. Hexane was added to the column until the column was approximately one-half full. The celite was then added and the column was packed lightly with a solid glass rod. Fifty ml of hexane were passed through the column using 3 pounds of nitrogen pressure. One inch of hexane was left on the column above the celite. Ten ml of the sample of the supernatent fluid preparation of the fermentation described previously were pipetted into a 100 ml beaker and was adjusted to a pH of 10-12 by 1N KOH (Asplund et al., 1950) and dried on a steam bath. The dried sample was dissolved in 2 ml of 12 percent H_2SO_4 and 1 ml of this solution, acidified with 3 drops of 10 N H_2SO_4, was used for the chromatographic analysis.

Chromatographic procedure

One ml of the sample was mixed with 2 grams of dried celite until completely homogenous. This was then transferred quantitatively to the column and packed evenly. Traces of celite were transferred by

wiping the beaker, spatula and inside walls of the column with the glass wool plug. The flow rate was controlled to approximately 5 ml per minute and five ml fractions of effluent were collected in individual 20 mm x 150 mm test tubes. The effluent was titrated with 0.01 N KOH using two drops of 0.1 percent bromothymol blue as an indicator. Butyric, propionic and acetic acids were eluted with 1 percent, 3 percent and 25 percent butanol in hexane respectively. The butyric acid was eluted using 100 ml of 1 percent butanol in hexane, propionic acid was eluted with 125 ml of 3 percent butanol in hexane and acetic acid was eluted using approximately 100 ml of 25 percent butanol in hexane. After an acid was completely eluted the next solvent was added. The blank value was subtracted from the titrations for each tube to obtain the amount of VFA eluted.

Results

The titration values for the individual VFA were added and calculated as mM percent of the acid per fermenter. Each experiment was performed in duplicate. The blank determinations were made with each lot of reagents and appropriate corrections made.

Figure 6 shows production of mM of VFA per fermenter with alfalfa leaf meal as substrate in 0, 4, 16 and 24 hour of fermentation when inocula were used from Cow K-139. It was found that butyric acid was increased three-fold in 4 hours of fermentation over the initial concentration in the fermentation containing dextrose, while with citric acid the increase was two-fold as great as the initial concentration. During the 4-16 hour period of fermentation there was little difference in the butyric acid production between the fermenters with and without added citric acid. The propionic acid produced in 4 hours

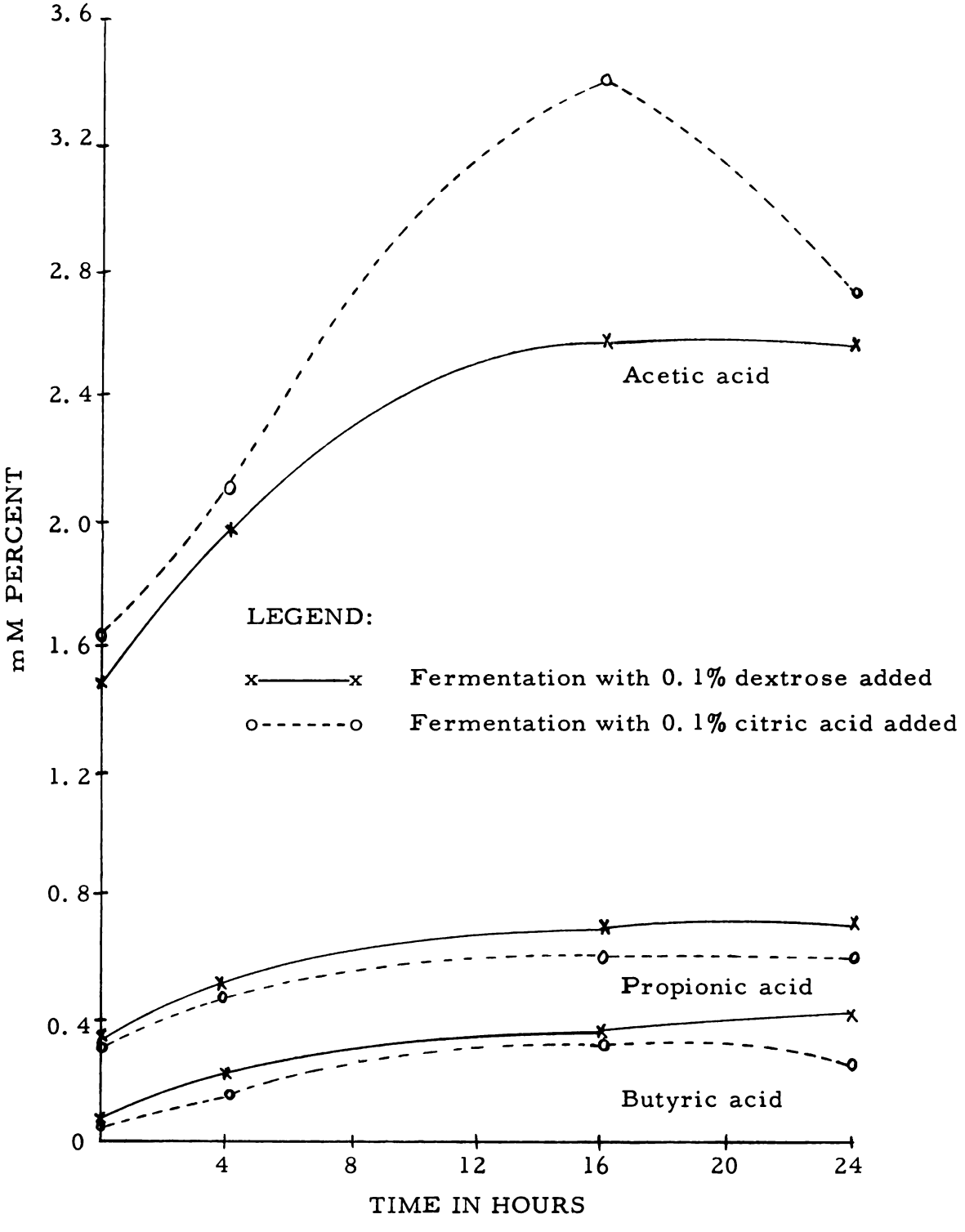

Fig. 6. Volatile fatty acid production during cellulose fermentation of alfalfa leaf meal substrate when the inocula were used from cow K-139.

of fermentation was 50 percent of the total propionic acid produced in the 24 hour period in both dextrose and citric acid fermentation. There was no significant difference in the propionic acid production between the dextrose and the citric acid fermentations.

Acetic acid production with alfalfa leaf meal as substrate was markedly higher in the fermentation with 0.1 percent citric acid than in the 0.1 percent dextrose. The difference in acid production between the two fermentations was greatest at 16 hours of fermentation. The fermentation with 0.1 percent citric acid contained 3.43 or 3.4 mM percent while the control with 0.1 percent dextrose contained 2.59 or 2.6 mM percent. Figure 7 shows VFA production in 0, 4, 16 and 24 hours by inocula from Cow K-139 using Solka-Floc as substrate, with 0.1 percent dextrose or 0.1 citric acid. Butyric acid production was similar until 16 hours of fermentation in both cases. During the 16-24 hour period of fermentation the production of butyric acid was higher in the fermentation with 0.1 percent dextrose.

Propionic acid produced was not significantly different in the two fermenters in the 0-16 hour period of fermentation. During the 16-24 hour period there was increased production of propionic acid in the 0.1 percent dextrose fermentation.

There was an increase in acetic acid production during the 0-16 hour period with citric acid fermentation as compared to the 0.1 percent dextrose fermentation but they were similar at 24 hours.

Figure 8 shows VFA production by inocula from traumatic gastritis animals with alfalfa leaf meal as substrate. Here butyric acid increased until 16 hours of fermentation in both 0.1 percent dextrose and 0.1 percent citric acid samples. There was an apparent utilization of butyric acid in both fermentations during the 16-24 hour period. There were no

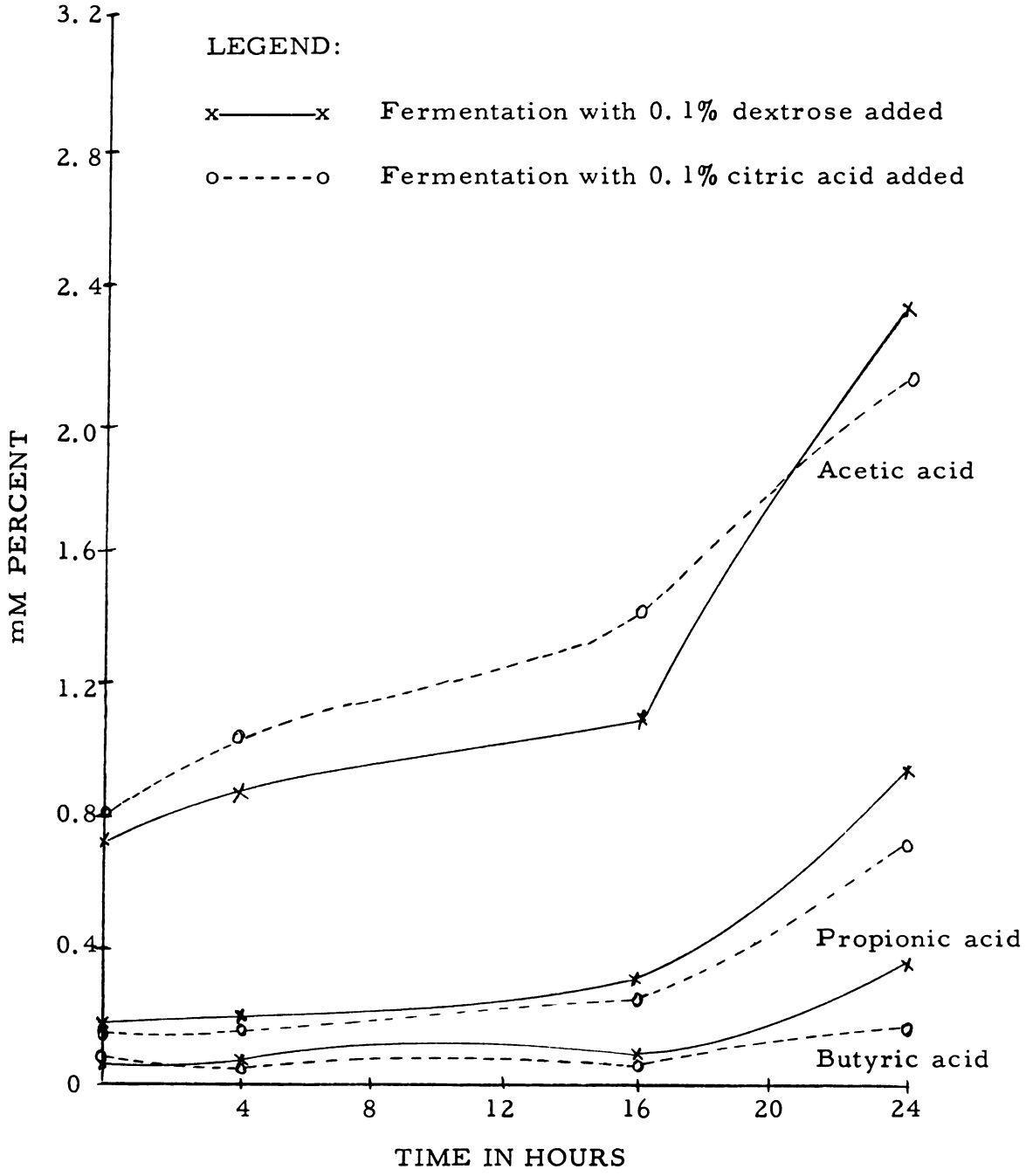

Fig. 7. Volatile fatty acid production during cellulose fermentation of Solks-Floc substrate when the inocula were used from cow K-139.

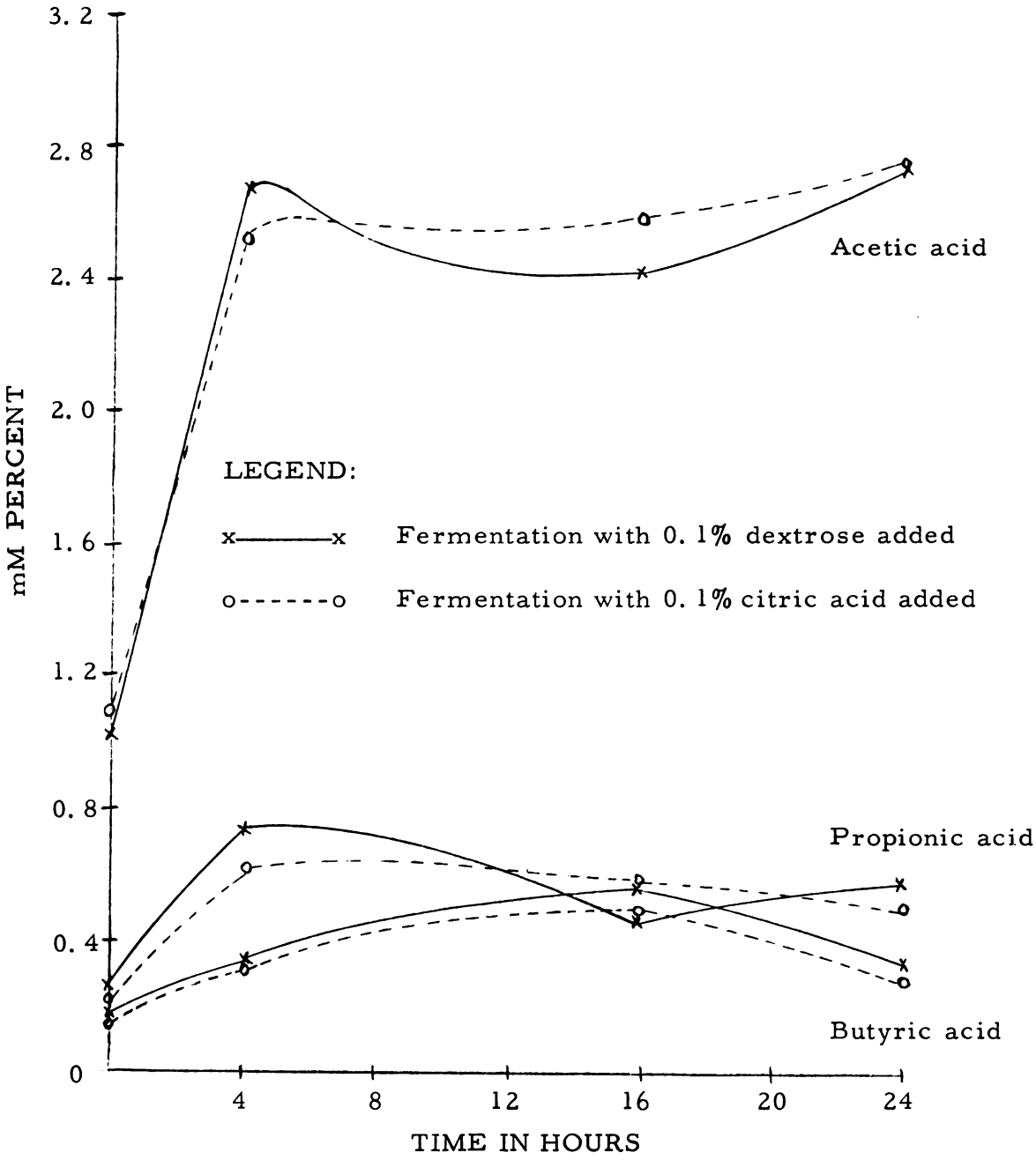

Fig. 8. Volatile fatty acid production during cellulose fermentation of alfalfa leaf meal substrate when the inocula were used from traumatic gastritis animals.

significant differences observed between the two fermentations at 0, 4, 16 and 24 hours.

The propionic acid produced was maximum in 4 hours of fermentation in both dextrose and citric acid fermentations and then demonstrated apparent utilization. While the acetic acid produced was higher at the 4 hour period in dextrose fermentation than in citric acid fermentation, during the 4-24 hour period of fermentation acetic acid production did not change in either fermentation. Most of the acetic acid production in these fermentations was completed during the 0-4 hour period. Figure 9 shows VFA production in traumatic gastritis animals with Solka-Floc as substrate at 0, 4, 16 and 24 hours of fermentation. There was no difference in butyric acid production with 0.1 percent citric acid as compared to 0.1 percent dextrose in the 0-16 hour period of fermentation; however, lower amounts of butyric acid were noted at 24 hours with the citric acid sample.

Propionic acid production was increased slightly in both cases during the 0-16 hour fermentation period; however, during the 16-24 hour period a marked increase in propionic acid production in the dextrose fermentation over that in the citric acid fermentation was observed. Acetic acid production was found higher in citric fermentation in the 0-16 hour period; however, there was no measurable amount of acetic acid produced in the 0-4 hour fermentations in either the dextrose or citric acid samples.

The total amount of VFA produced in 0, 4, 16 and 24 hours of fermentation with alfalfa leaf meal and Solka-Floc is presented in Table 6. It shows that the 0.1 percent citric acid fermentation increased the total VFA production during the 4-16 hour period as

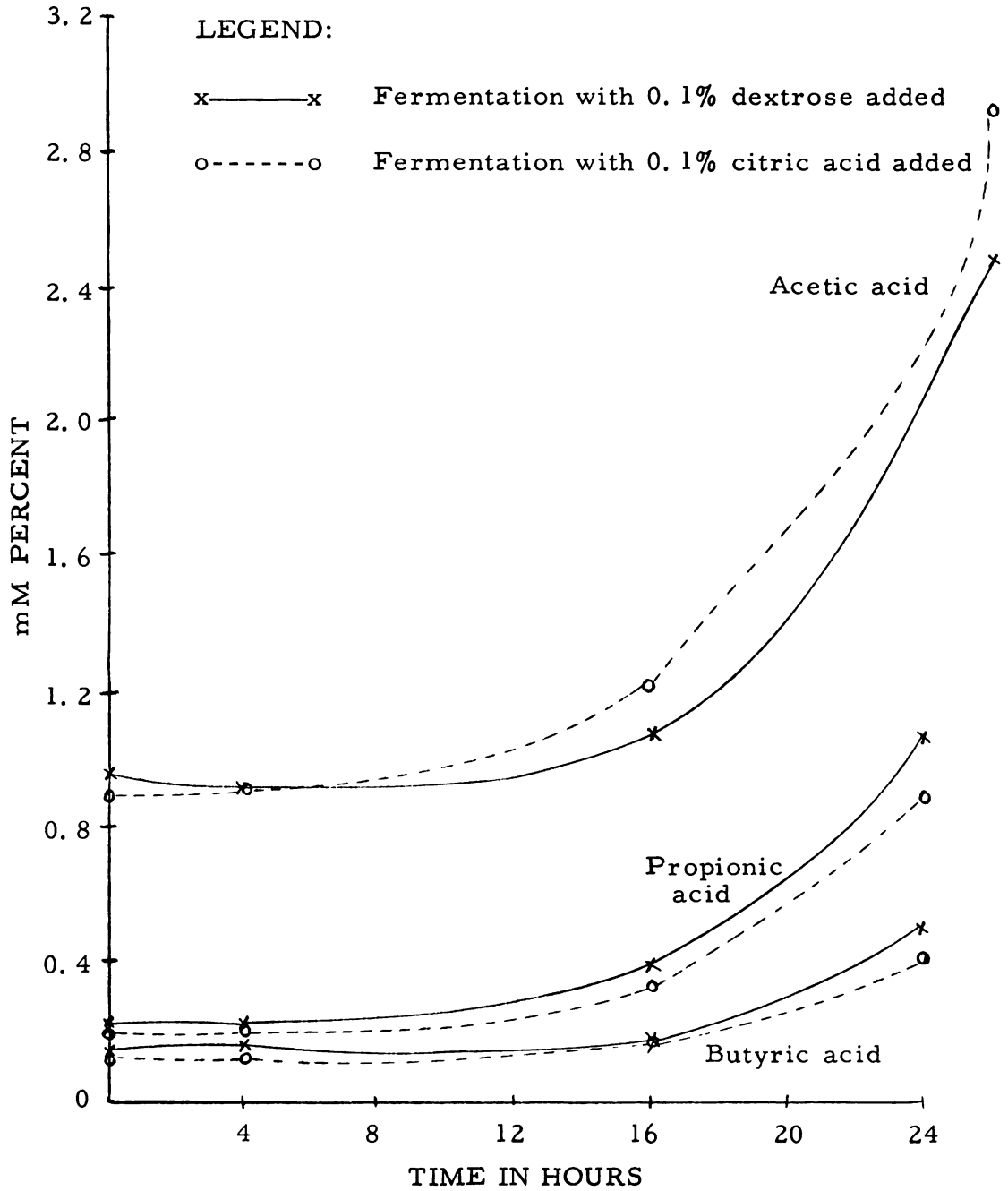

Fig. 9. Volatile fatty acid production during cellulose fermentation of Solka-Floc substrate when the inocula were used from traumatic gastritis animals.

compared to 0.1 percent dextrose when alfalfa leaf meal was used as substrate. There was no significant difference in the amounts of VFA at 24 hours of fermentation.

TABLE 6. Total VFA produced during in vitro cellulose fermentation by inocula taken from cow K-139

Fermentation time in hours	Alfalfa leaf meal		Solka-Floc	
	Dextrose	Citric acid	Dextrose	Citric acid
0	1.885*	2.010	0.946	0.915
4	2.720	2.730	1.090	1.125
16	3.635	4.335	1.395	1.650
24	3.660	3.605	3.655	3.045

*Acids expressed in mM percent.

When Solka-Floc was used as a substrate the total VFA production during 16 hours of fermentation was similar in both groups; however, at the end of the 24 hour period the total VFA production was lower with added citric acid than with dextrose.

Table 7 shows the total amount of VFA production at 0, 4, 16 and 24 hours of fermentation with alfalfa leaf meal and Solka-Floc as substrates when the inocula used were taken from traumatic gastritis animals.

VFA production was lower during the 0-4 hour period due to the citric acid addition when alfalfa leaf meal was used as a substrate. During the 4-16 hour fermentation period VFA production of the citric acid fermentation was increased over that of the dextrose fermentation. After 24 hours of fermentation there was no significant

difference in the amount of VFA in the two samples. The difference in VFA production between dextrose and citric acid fermentations was not measurable until after 4 hours of fermentation when Solka-Floc was used as a substrate. During the 4-16 hour period the citric acid fermentation produced more VFA and from 16-24 hours the VFA production in both dextrose and citric acid fermenters was marked but essentially the same.

TABLE 7. Total VFA produced during in vitro cellulose fermentation by inocula from traumatic gastritis animals

	Alfalfa leaf meal		Solka-Floc	
Fermentation time in hours	0.1 percent Dextrose	0.1 percent Citric acid	0.1 percent Dextrose	0.1 percent Citric acid
0	1.490*	1.460	1.343	1.233
4	3.809	3.495	1.305	1.275
16	3.465	3.750	1.635	1.745
24	3.685	3.575	4.360	4.260

*Acids expressed in mM percent.

Section IV

Experimental

Colorimetric determination of citric acid

Citric acid in the fermentation mixtures at 0, 4, 16 and 24 hours was determined colorimetrically based on the Furth and Herrman reaction (1935) modified by Hartford.[1]

Equipment

a) A colorimeter equipped to measure color intensity at 420 mu

[1]Personal communication, 1961.

b) A water-bath set at a temperature of 32 ± 0.25 C.

c) Self-filling 10 ml microburette from both acetic anhydride and pyridine.

Reagents

a) Reagent grade acetic anhydride and Baker analysed reagent-pyridine solution.

b) Citric acid stock standard solution of 69.9069 grams of trisodium citrate dehydrate (Reagent grade) made up to one liter with distilled water. This solution is equivalent to 50,000 µg citrate per ml. Dilute standards were made from this stock solution and stored at 4 C.

Preparation of test samples

The supernatant fluids of the centrifuged fermentation mixtures at 0, 4, 16 and 24 hours, stored at -10 C were brought to room temperature and recentrifuged at 800 x G for 10 minutes. One ml of the supernatent fluid was used for the colormetric determination of citric acid.

Preparation of standard samples

Dilutions were made from the stock solutions containing 50,000 µg citric acid per ml. Dilutions containing 5, 10, 25, 50, 75, 100, 125, 150, 200, 250 and 350 µg citrate per ml were used for standard curve, plotting optical density versus µg equivalent of citric acid monohydrate of the above concentrations.

Procedure

One ml of test sample, standard or blank was added to a colorimeter tube. Then 1.30 ml of pyridine were added and the tube was swirled

briskly. After similar additions had been made to all tubes in a series, 5.70 ml of acetic anhydride were added and the tube swirled again and immediately placed in the constant temperature (32 C) water bath for 30 minutes. Color intensity at 420 mμ was read with the blank set at 0.000 optical density. Blank determinations were run with each experiment. The standard curve was made by taking the average of five repeated trials at different times with each concentration of citrate as described above. The citric acid content of the fermentation mixture was estimated with reference to the standard curve.

Results

One thousand μg citric acid per ml was added to the 0 hr fermenter and the colorimetric reading gave the initial concentration of citric acid for the test fermentations. The μg of citric acid recovered at 4, 16 and 24 hours of fermentation was subtracted from the 0 hr reading to obtain the citric acid disappearance during these periods of fermentation. It is shown (Fig. 10 and Fig. 11) that the 0 and 24 hour fermentation samples, without citric acid and dextrose added, had essentially the same colorimetric reading. The calculated amounts of citric acid with reference to the standard curve are shown in Table 8. This also shows the percent of citric acid disappearance in 0, 4, 16 and 24 hours of fermentation with Solka-Floc and alfalfa leaf meal when inocula were used from normal animals. Seventy-four percent of the total citric acid disappeared in 4 hours of fermentation with alfalfa leaf meal and Solka-Floc with inocula from normal animals. At 16 and 24 hours of fermentation the remaining citric acid disappeared. The rate of percent disappearance was slightly lower with Solka-Floc than with alfalfa leaf meal.

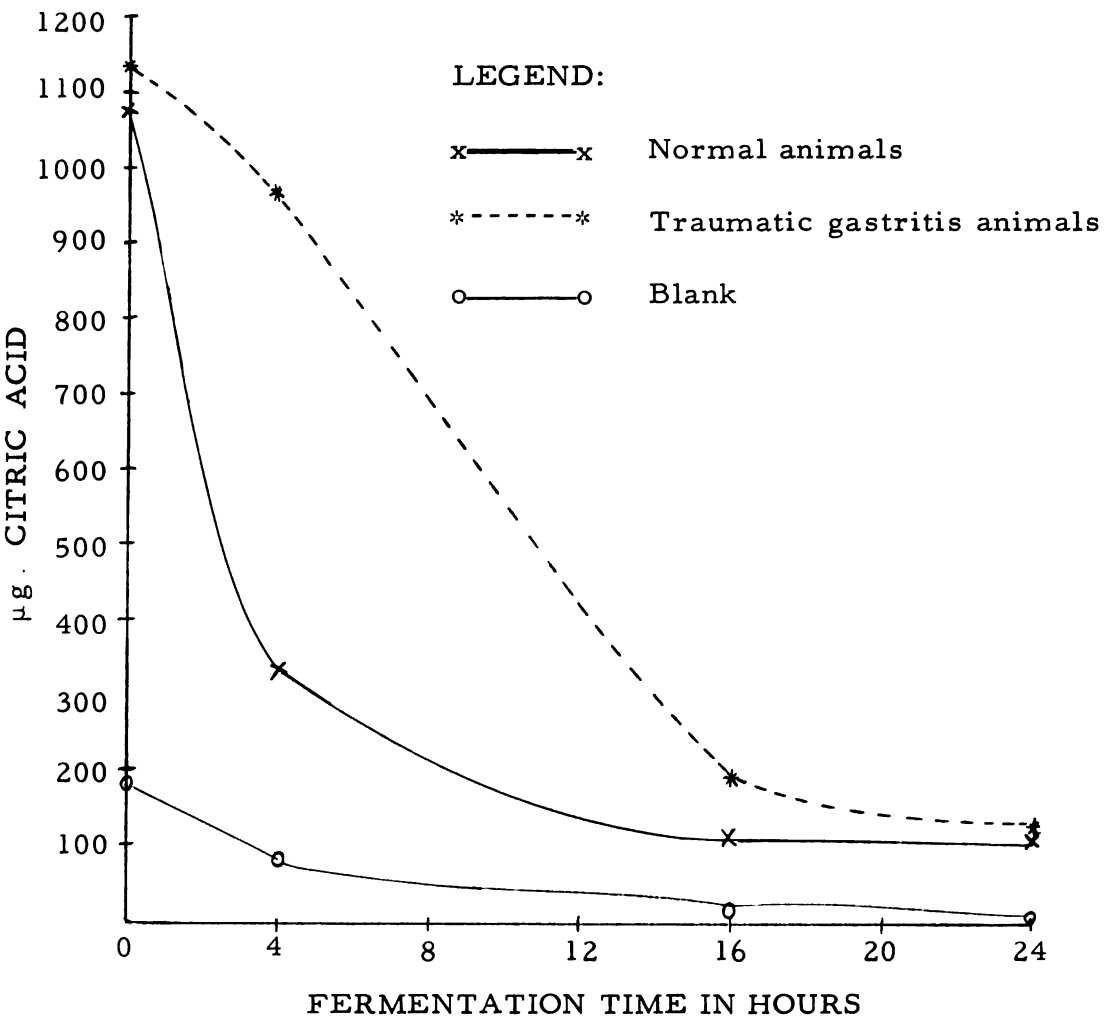

Fig. 10. Citric acid disappearance during cellulose fermentation of alfalfa leaf meal substrate by inocula taken from normal and traumatic gastritis animals.

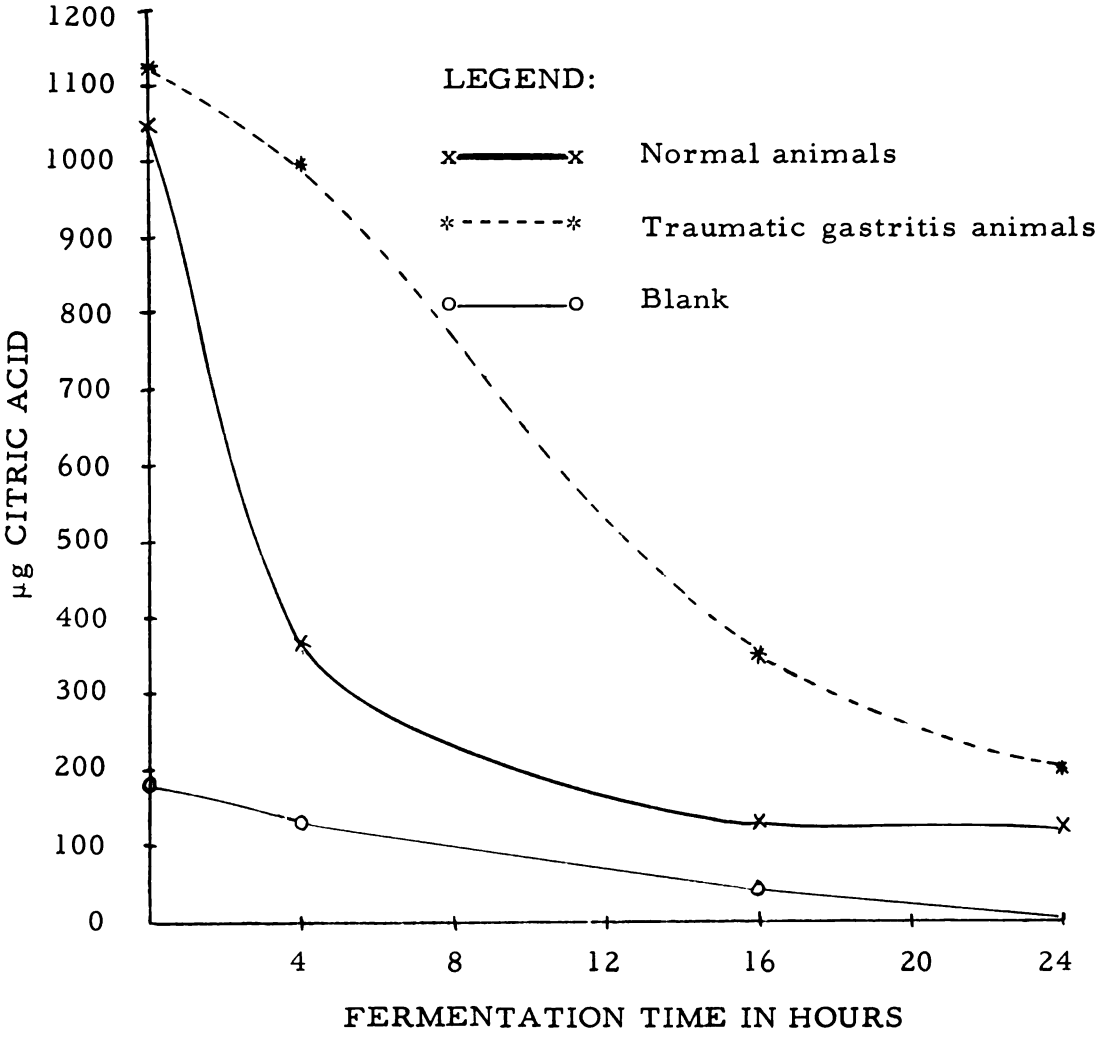

Fig. 11. Citric acid disappearance during cellulose fermentation of Solka-Floc substrate by inocula from normal and traumatic gastritis animals.

TABLE 8. Percent of citric acid disappearance *in vitro* with inocula taken from normal animals

	Alfalfa leaf meal			Solka-Floc		
Fermentation time in hours	µg of citric acid per ml	µg of citric acid disappeared	Percent disappeared	µg of citric acid per ml	µg of citric acid disappeared	Percent disappeared
0	1077.5	0	0	1050.0	0	0
4	335.0	742.5	74.25	367.5	683.5	68.35
16	118.0	959.5	95.95	135.0	915.0	91.5
24	115.0	962.5	96.25	125.0	925.0	92.5

Table 9 shows the percent of disappearance of citric acid in inocula from traumatic gastritis animals. In both alfalfa leaf meal and Solka-Floc substrates only 16.2 percent and 13.2 percent respectively of citric acid had disappeared at 4 hours of fermentation, while at 16 hours 93.9 percent of the citric acid had disappeared with alfalfa leaf meal as substrate as compared to 68.2 percent with Solka-Floc. After 24 hours all of the citric acid had disappeared from the fermentation samples with alfalfa leaf meal as substrate while 93.2 percent of the citric acid had disappeared with Solka-Floc as substrate.

Figure 10 shows a comparison of the citric acid disappearance with alfalfa leaf meal as substrate in normal and traumatic gastritis animals. In normal animals 75 percent of the total citric acid disappeared in 4 hours of fermentation as compared to traumatic gastritis animals where only 16 percent of citric acid disappeared from the fermentation mixture.

In Figure 11 citric acid disappearance is shown with Solka-Floc substrate in normal and traumatic gastritis animals. Two-thirds of the total citric acid disappeared during 4 hours of fermentation in normal animals while in traumatic gastritis animals the rate of disappearance was slow and most of the citric acid did not disappear until the 4-16 hour period of fermentation. After 24 hours of fermentation 93.2 percent and 92.5 percent of the citric acid disappeared in traumatic gastritis and normal animals respectively.

TABLE 9. Percent of citric acid disappearance in vitro with inocula taken from traumatic gastritis animals

	Alfalfa leaf meal			Solka-Floc		
Fermentation time in hours	µg of citric acid per ml	µg of citric acid disappeared	Percent disappeared	µg of citric acid per ml	µg of citric acid disappeared	Percent disappeared
0	1132.0	0	0	1132.5	0	0
4	970.0	162.0	16.2	1000.0	132.5	13.25
16	192.5	939.5	93.95	350.0	682.5	68.25
24	125.0	1007	100.7	200.0	932.5	93.25

DISCUSSION

Percent cellulose digestion by inocula from normal and traumatic gastritis animals with dextrose and citric acids added

The data of percent cellulose digestion by both normal and traumatic gastritis animals show that no significant difference is effected by the addition of 0.1 percent citric acid. (Tables 2, 3 and 4). It is interesting to note the decrease in the percent of cellulose digested in all substrates when 0.2 percent citric acid was used in normal cows. The percent of cellulose digestion in all three substrates was considerably lower than that reported by previous workers for in vitro cellulose digestion studies (Huhtanen and Elliott 1956, Salsbury 1955). One-tenth percent dextrose maintained the normal percent of cellulose digestion in 24 hours. This is in agreement with the findings of Salsbury (1955), Burrough et al. (1950), and Huhtanen et al. (1954).

Among the three substrates used, (alfalfa leaf meal, Solka-Floc and timothy hay) the cellulose of alfalfa leaf meal was most rapidly attacked, timothy hay was intermediate and Solka-Floc was most resistent. This is in excellent agreement with the results obtained by Huhtanen and Elliott (1956).

The difference in percent cellulose digestion by the addition of 0.1 percent citric acid was not significant as compared to that with 0.1 percent dextrose. Dextrose or citric acid in 0.05 percent concentration lowered the percent of cellulose digestion in 24 hours of fermentation when Solka-Floc was used as a substrate. The 0.01 percent

concentration of citric acid tended to increase the percent of cellulose digested as compared to the 0.01 percent dextrose; however these values were below those of the 0.1 percent level. Salsbury (1955) reported that the percent cellulose digested increased gradually as the glucose concentration was increased from 0.005 to 0.15 percent, but the differences obtained in his experiments were small. He found agreement among replicates best when no glucose was added and when 0.2 percent glucose was added.

Hoflund, Quin and Clark (1948) have shown that small amounts of glucose stimulate cellulose digestion, but the methods used were too emperical to permit the estimation of the percent cellulose digested. Arias et al. (1951) reported that the addition of readily available carbohydrate at low concentrations stimulates cellulose digestion. These workers used serial transfer of the fermentations according to the method of Burroughs et al. (1950) and averaged the results. They suggested the possibility that the stimulating effect was actually the overcoming of depressing forces such as suboptimal conditions or nutritional deficiences.

The small amount of citric acid added in the fermentation mixtures shows greater though not significant increases in cellulose digestion, than that with dextrose. This indicates that citric acid can be degraded by the rumen microorganisms as a readily metabolizable energy source. As can be seen in Table 2 the effect of citric acid noted was only evident at 8 hours of fermentation, but, after 24 hours the change was slight or none. This is possibly because citric acid was utilized in the 8 hour period of fermentation.

There were wide variations in the percent of cellulose digestion

as can be seen in Table 3 when the inocula were taken from different animals. The variation was marked when inocula were used from cow A-130 with alfalfa leaf meal as substrate and from cow K-139 with Solka-Floc as substrate. It seems likely that the microbial population of the rumen might vary according to the type of forage used as substrate. Variations were less in the fermentation trials with the other experimental animals and different forages with 0.1 percent citric acid, while the variations were rather larger when 0.05 percent and 0.01 percent citric acid concentrations were used.

When the inocula were used from traumatic gastritis cases and the activity of rumen microorganisms was tested with alfalfa leaf meal and Solka-Floc, variations in the percent of cellulose digested in the Solka-Floc substrate were greater at 8 hour of fermentation as compared to that with alfalfa leaf meal. This could be explained on the basis of the finding by Donefer et al. (1961) that digestion of Solka-Floc was much more affected by the source and type of inoculum than were any of the other forages tested.

The average percent cellulose digestion in the 8 hour fermentation period with alfalfa leaf meal as a substrate was higher than that with Solka-Floc; however, in the 24 hour period the average percent cellulose digestion with Solka-Floc as substrate was greater. These results are in agreement with those reported by Salsbury (1955) in that the rate of digestion of Solka-Floc was slower in the first part of the incubation and faster in the latter part, while the reverse was true with alfalfa leaf meal. This may be due partially to the fact that the alfalfa leaf meal provide nutrients not furnished by Solka-Floc and the lack of crystallinity of the alfalfa leaf meal cullulose. The

addition of 0.1 percent citric acid as compared to the Solka-Floc fermentation increases in the percent of cellulose digestion and it more closely approximates that observed at 8 hour of fermentation with alfalfa leaf meal. It is possible that the rumen microorganisms were not adapted to the Solka-Floc substrate and the lower activity and retarded rumen movements due to traumatic gastritis. Solka-Floc is not the usual feed of cattle and Warner (1956) has reported that wide variation of cellulose digestion can be seen with feeds other than the normal feed of the animal which provide the inocula for *in vitro* studies. Furthermore he suggested the use of a substrate similar to the feed of the animal that provides the inocula for *in vitro* fermentation to achieve proper activity of the rumen microorganisms. With alfalfa leaf meal as substrate the addition of 0.1 percent citric acid increased the percent cellulose digestion over that of dextrose in the 8 hour fermentation period. Since the Crampton and Maynard (1938) method of the determination of cellulose digestion also includes some hemicellulose, the faster initial rate with alfalfa leaf meal may reflect a more rapid digestion of hemicellulose.

In the 24 hour fermentation period the average percent cellulose digestion by 0.1 percent citric acid was essentially the same in both. This may be explained by the fact that citric acid had disappeared by 24 hour of fermentation. In any event the change due to 0.1 percent citric acid was not significant as calculated by the t-test as can be seen from Fig. 5.

Table 5 shows that, after 4 hours of fermentation there was a considerable increase in cellulose digestion due to 0.1 percent citric acid with alfalfa leaf meal with inocula from normal animals while there was little change in the percent of cellulose digestion observed

in traumatic gastritis animals. This would suggest the activities of the rumen microorganisms from traumatic gastritis animals were lower than those from the normal animals. There is no other evidence available to confirm this. After 8 hour of fermentation the percent of cellulose digested in 0.1 percent citric acid or dextrose fermentation was similar in both normal and traumatic gastritis animals when alfalfa leaf meal was used as substrate. This indicates that the microorganisms of the traumatic gastritis animals were equally as active as those of the normal animals after 8 hours of fermentation. After 24 hours the percent cellulose digestion in both 0.1 percent citric acid and dextrose fermentations by rumen inocula from normal and traumatic gastritis animals were essentially the same. This indicates that the 0.1 percent citric acid was effective only in the early phase of cellulose digestion by rumen microorganisms.

When Solka-Floc was used as a substrate the percent of cellulose digested after addition of citric acid was not affected in the traumatic gastritis animals at the 4 hour fermentation period. Since pure cellulose is attacked at a slower rate, according to the report by Huhtanen and Elliott (1956), it is possible that the citric acid was attacked instead of cellulose by the rumen microorganisms during the 0-4 hour period. By the 8 hour period the increment in cellulose digestion in the 0.1 percent citric acid sample shows that the rumen microorganisms were adapted to the Solka-Floc substrate. The cellulolytic activity of the rumen microorganisms in traumatic gastritis cases was found to be less.

During the 16-24 hour period of fermentation the percent of the cellulose digested with 0.1 percent citric acid or dextrose added was

essentially the same with inocula from normal and traumatic gastritis animals when alfalfa leaf meal was used as a substrate.

Although the increase in the percent of cellulose digestion in both substrates with 0.1 percent citric acid was demonstrated in certain cases further work is required to claim the definite superiority of citric acid over dextrose as an adjuvant in cellulose digestion by rumen microorganisms. It could be fruitful in the improvement of cellulose digestion by traumatic gastritis animals which exhibit anorexic conditions and it may aid the recovery of traumatic gastritis animals after the foreign body has been removed.

VFA production as a criterion of in vitro cellulose digestion

Gray, Pilgrim and Weller (1951), Warner (1956) and Barnett and Reid (1957) have reported volatile fatty acids as the end products of cellulose fermentation and VFA production as an index to measure cellulose digestion to their in vitro studies.

The precise role of microflora and the importance of VFA production is controversial. One school of thought stresses the nutritional importance of VFA found in the rumen. This view is typified by the work of Phillipson and McAnally (1942), and Barcoft et al. (1944). The opposing view is that the VFA are of little nutritional value to the animal and that it is the microflora itself which is important, serving as a source of carbohydrate, protein and B-complex vitamins. This later point is favored by Baker (1942) who stated that it is the substances synthesized such as microbial protein and polysaccharides, rather than the initial products of decomposition such as organic acids that are utilized by the host animals. It is assumed by

Baker (1942) that the organisms convert the starch and fiber of the diet into bacterial starch. These organisms laden with starch pass through the forestomachs into the small intestine where the starch is hydrolysed to glucose by the pancreatic and intestinal amylases.

Pearson and Smith (1943) have shown that because of the increased amount of material available for digestion, a higher percent of VFA was produced in _in vitro_ fermentation experiments and absorption of VFA was absent. Therefore one may expect a slightly higher proportion of acid to be present _in vitro_ than _in vivo_ (Elsden 1946, and Davey _et al._, 1960).

Gray and Pilgrim (1952) reported that as the concentration of total VFA in the rumen increased there was a decrease in the ratio of acetic to propionic acid. A similar difference was shown in the experiments of Elshazly (1952) between samples taken before and after feeding but not in the experiments of Shambye and Phillipson (1949) where high concentrations of lactic acid were observed and the ratio of acetic to propionic acid was unusually low. Gray _et al._ (1951) have reported that fatty acids produced could be taken to represent those actually found in the rumen. They stated that the fatty acids are removed continuously by absorption through the rumen wall and failure to reproduce this phenomenon _in vitro_ may have some influence on the rate of fermentation and the proportions in which the acid products are found.

Barnett and Reid (1957), Sijpestejn (1951), Sijpestejn and Elsden (1952) and Hungate (1950) suggest that certain microorganisms, for example _Ruminococcus flavifaciens_ and _Bacteriodes succinogenes_, breakdown cellulose to succinic acid which in turn is rapidly converted to propionic acid presumably by decarboxylation. These findings have been

confirmed by *in vivo* studies and it is significant that succinic acid does not accumulate in the rumen. Elsden (1946) has shown that lactic acid is an intermediate in short chain VFA production in the rumen. He found that propionic acid is the acid produced in the greatest amount; however, Gray and Pilgrim (1952), Gutierrez (1953) and Davey *et al*. (1960) found acetic acid in the greatest amount. Elsden, Volcani, Gilchrist and Lewis (1956) found that cellulose was rapidly fermented with the production of VFA and considerable quantities of gas. They found that glucose used in the fermentation was exhausted within 24 hours, and acetic acid predominated *in vivo* fermentation whereas propionic was the main product *in vitro*.

The results obtained in these studies agree in part with the results of Elsden *et al*. (1956) but in contrast to his results acetic acid predominated as can be seen from Figure 6.

The production of acetic acid with 0.1 percent citric acid added was observed to be considerably higher after 16 hours of fermentation when alfalfa leaf meal was used as a substrate. This increase could be due to the acetic acid produced from the citric acid, or to the utilization of butyric acid and propionic acid. Acetate accumulation was reported by Doestch, Robinson, and Shaw (1952) as a result of rapid oxidation of maltose and glucose in *in vitro* fermentation. After 24 hours of fermentation the production of VFA was in close agreement with the percent cellulose digested in both 0.1 percent dextrose and 0.1 percent citric acid.

When Solka-Floc was used as a substrate similar results were obtained as with alfalfa leaf meal as substrate, with 0.1 percent added

dextrose or citric acid. The production of acetic acid was not markedly higher at 16 hours of fermentation but the increase was evident during the 0-16 hour period of fermentation as can be seen from Figure 7. The amount of each volatile fatty acid produced was similar in both substrates. These results are in agreement with the report by Church and Peterson (1960) that VFA production was dependent upon the amount of cellulose in the fermentation.

Figure 8 shows the production of VFA by inocula taken from animals with traumatic gastritis. A great deal of variation was encountered in the fermentation at different time intervals. The total VFA produced with alfalfa leaf meal as substrate in 0.1 percent dextrose or citric acid was produced in 4 hours. At 16 hours the total VFA produced was higher in the fermentation containing citric acid. At 24 hours no difference in total VFA was observed.

When Solka-Floc was used the results were similar until 16 hours of fermentation. After 24 hours the total VFA was lower with added citric acid but the difference was not statistically significant.

It can be seen from Table 7 that in the traumatic gastritis cases the total VFA produced with alfalfa leaf meal and Solka-Floc as substrates was lower when 0.1 percent citric acid was added during the 0-4 hour period of fermentation while at the 16 hour period of total VFA was slightly higher in citric acid fermentation. This suggests that the activity of microorganism was greater during 4-16 hours of fermentation due to a delayed utilization of citric acid. However, the difference in total VFA production with 0.1 percent dextrose and 0.1 percent citric acid was not significant with both Solka-Floc and alfalfa leaf meal substrates. This is in agreement with the results

obtained for the percent cellulose digestion with the two substrates.

This is also in agreement with the result obtained by Jacobson (1958) who reported that animals showing anorexia had an essentially normal fatty acid ratios in the rumen; however, the corresponding fatty acid production *in vitro* was quite unusual. He suggested that even though total fatty acid production in the rumen was altered, the molar percentage of the fatty acids present in the rumen may remain within the normal range.

The total VFA fluctuation during the course of the experiments with inocula from traumatic gastritis animals may be due to variable diets, the degree of sickness of the animals and the interval of feeding time.

Citric acid disappearance

The disappearance of citric acid from the *in vitro* rumen fermentation was followed in order to gain knowledge whether citric acid was metabolized by the rumen microorganisms. The time and magnitude of disappearance was thought to be in accordance with the cellulose digestion and VFA production. The utilization of citric acid by rumen microorganism has not been demonstrated. However, dissimilation of compounds of the citric acid cycle, such as succinate degradation, has been studied by Sijpestejn and Elsden (1952), Johns (1948) and Doetsch, Robinson, Brown, and Shaw (1953). Pyruvate, fumarate, malate, and oxaloacetate are degraded by rumen microorganisms with the production of gases and volatile fatty acids (Doetsch *et al.*, 1953). They established that lactate is not attacked with the production of detectable VFA and fumarate yields solely acetate.

The results obtained in the experiments in which the disappearance of citric acid from the fermentation mixture was followed showed that 75 percent of the citric acid disappeared during the 0-4 hour period of fermentation when the inocula were obtained from normal animals and alfalfa leaf meal was the substrate. When Solka-Floc was used as a substrate 68 percent of citric acid disappeared from the fermentation mixture (Table 8). This shows the relatively slow activity of rumen microorganisms with Solka-Floc as compared to alfalfa leaf meal. This is in agreement with the work reported by Salsbury (1955) and Huhtanen and Elliott (1956) who reported that with Solka-Floc as substrate the *in vitro* activity of rumen microorganisms was slower during the first period of fermentation.

Doestch *et al.* (1953) using pyruvate as substrate found that pyruvate was always vigorously attacked and was not detectable in the cell substrate mixture after 5 hours of fermentation. They indicated that pyruvate must be utilized as rapidly as it is formed and that the anaerobic mechanism of the rumen process is one involving low energy yielding anaerobic dissimilations.

When inocula were from traumatic gastritis animals the citric acid disappearance at 4 hours of fermentation with both substrates was very slow. This supports the idea that the activity of rumen microorganisms from animals suffering from traumatic gastritis is lower as compared to normal animals. Jacobson *et al.* (1958) reported that animals off-feed may have an abnormal bacterial metabolism though it is not clear as to whether the change would proceed or follow the development of inappetance.

After 16 hours of fermentation using inocula from traumatic

gastritis animals 93 percent of the citric acid disappeared when alfalfa leaf meal was used as a substrate and 68 percent disappearance was observed with Solka-Floc. This again shows the relative slow activity of rumen microorganisms with Solka-Floc as a substrate. At 24 hour all of the citric had disappeared with alfalfa leaf meal as a substrate and 93 percent of the citric acid disappeared with Solka-Floc as a substrate.

SUMMARY

In vitro cellulose digestion studies were undertaken to test the effect of citric acid concentrations of 0.2 percent, 0.1 percent, 0.05 percent and 0.01 percent, on cellulolytic activity of rumen microorganisms obtained from normal fistulated animals. Alfalfa leaf meal, Solka-Floc and timothy hay were used as sources of cellulose. All control fermentations contained equivalent amounts of dextrose as well as the cellulose source. The percent of cellulose digestion was determined at 0, 4, 8, 12, 16 and 24 hours of fermentation. The difference in percent cellulose digestion between the dextrose and citric acid fermentations was found not to be statistically significant at the 5 percent level (t .975, $>$ 0.05 percent). The 0.1 percent citric acid concentration was found suitable to work with during further experiments.

Rumen flora obtained from nine animals with traumatic gastritis were used as sources of inocula to observe the affect of 0.1 percent citric acid when added to the Solka-Floc and alfalfa leaf meal. No significant difference was found in percent cellulose digestion by adding 0.1 percent citric acid in the test fermentations. Wide variability in the results was postulated to be due to the condition of the inocula obtained from the different animals.

Volatile fatty acids were determined on Celite 535 columns at 0, 4, 16 and 24 hours of fermentations when inocula from normal and traumatic gastritis animals were used. The kinds of VFA produced

were acetic acid, propionic acid, and butyric acid. No significant differences in the VFA production between the fermentations with 0.1 percent dextrose and 0.1 percent citric acid were found in normal and traumatic gastritis animals. Variability in VFA production with inocula from traumatic gastritis animals was observed.

Citric acid disappearance was determined from the fermenters at 0, 4, 16 and 24 hour periods. It was found that about two-thirds of the total citric acid disappeared in the initial 4 hour fermentation period when alfalfa leaf meal and Solka-Floc were used as substrates and the inocula were obtained from normal animals. Only about one-sixth of the total citric acid disappeared when the source of inocula was traumatic gastritis animals. After 16 hours of fermentation most of the citric acid had disappeared in both situations. Factors causing variability in the percent cellulose digestion and VFA production are discussed.

It is concluded that under the experimental conditions used in these studies there was no statistically significant effect of adding 0.1 percent citric acid on the percent cellulose digestion and VFA production when inocula were obtained from normal and traumatic gastritis animals.

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