1953

Metabolism of cellulose by microorganisms of the rumen

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NOTE TO USERS

METABOLISM OF CELLULOSE BY MICROORGANISMS OF THE RUMEN

by

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

Major Subject: Biochemistry

Approved:

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Iowa State College
1953

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I. INTRODUCTION

The chemistry of ruminant digestion is unique in that bacteria elaborate various enzymes which play an essential part in the well-being of ruminant animals. The mammalia do not have the capacity to secrete cellulolytic enzymes, but in the rumen of the herbivore large numbers of microorganisms are present which digest the cellulosic plant material, through the agency of their enzyme systems, and produce end-products that may be utilized by the host. A symbiotic relationship therefore exists between the rumen microorganisms and the animal in question.

The knowledge of the enzyme system effecting the primary attack on cellulose by rumen microorganisms is very meager. The conventional view is that exoenzymes bring about the hydrolysis of this large polysaccharide with consequent liberation of glucose. The latter then is subsequently further dissimilated within the bacterial cell to form the various end-products.

It is not known whether cellulose dextrins are produced in the enzymic attack of cellulose by rumen microorganisms. Reese, Siu and Levinson (102) and Levinson, Mandels and Reese (67), using a number of cellulolytic mold filtrates, indicated that two types of hydrolytic systems may be concerned in the degradation of cellulose to the soluble sugars. The primary attack on the cellulose molecule would be the rupturing of the cross-linkages in the molecule of the polysaccharide thus resulting in the production of linear, polyanhydroglucose chains. The second attack would then be the subsequent hydrolysis of these linear chains to produce soluble, smaller molecules capable of diffusion into the bacterial cell.

The present investigation was undertaken to further the knowledge of the chemistry of cellulose digestion by the rumen microorganisms with special reference to the carbohydrate intermediates formed. In order to elucidate the series of biochemical transformations undergone by a substrate molecule, the breakdown products must be identified.

In this work, the use of bacterial and/or enzyme inhibitors and filter paper chromatography have made possible the study of the action of rumen microorganisms

on cellulose, such as filter paper and alphacel, and cellulose derivatives, namely the water soluble carboxymethylcelluloses. Rumen fluid, washed rumen microorganisms, rumen isolates, and cellulolytic cell-free bacterial extracts prepared from the rumen microorganisms and from a culture isolated from the rumen have all been studied as to their mode of action on the cellulose molecule.

II. REVIEW OF THE LITERATURE

A. Introduction

The subject of rumen microbiology covers many pages in the scientific literature and has been reviewed from time to time by a number of investigators (7,36,42,44,53,75,80,91). The topic in general covers a vast field of microbiological activities including cellulose, starch and protein digestion as well as vitamin synthesis. The literature on all these subjects will not be reviewed here. However, pertinent material on the metabolism of cellulose by the rumen bacteria including related information will be discussed.

There are no cellulases in the digestive juices of mammals and the breakdown of cellulose, whenever it occurs, is brought about by microorganisms, which obtain their energy for growth by the fermentation of this and related materials. Ruminants have in addition to an enlarged caecum and colon, a modified and capacious gastric system. This forms the main fermentation chamber and is well adapted to maintain a large and active population of microorganisms.

The early work on the breakdown of fiber in the ruminant has been comprehensively reviewed by Mangold. A condensation of this review is given below: (74 p. 648)

> The process of digestion of fibre . . . was first observed by Sprengel in 1832, described by Haubner and Sussdorf in 1855, and subjected to exact chemical study by Henneberg and Stohman over the period from 1860 to 1864 . . . .
>
> The bacterial nature of fibre digestion in the rumen and intestines of ruminants was demonstrated by Scheunert in 1909 and by Ellenberger in 1915. The nature of the flora was carefully studied by Ankerschmidt, 1905; Hopffe, 1919; Henneberg, 1922, 1926; and by Schieblich in 1929 and 1932. It has been shown by Tappeiner in 1882, 1885, and by Zantz with Markoff and other collaborators, in 1913, that the end products of bacterial fermentation of fibre are gases (carbon dioxide, methane and hydrogen) and acids (acetic, lactic, butyric, isobutyric, valerianic, formic, propionic and succinic).

The presence of the gases and organic acids as end-products resulting from the fermentation of cellulose in the rumen has been confirmed many times by other workers (32,90, 97,144).

B. Nature of the Cellulolytic Microorganisms

Little work has been conducted in order to determine whether particular microorganisms can be said to be functional members of the rumen population. Baker (3,6) classified the normal rumen microflora and microfauna of cattle firstly on their starch iodine reaction, secondly on their size, and lastly on their morphological type. Later, Elsden and Phillipson (36) stated two requirements that must be fulfilled before an organism can be classed as an active rumen bacterium:

(a) the organism must perform a chemical reaction known to occur in the rumen; and (b) the organism must be present in the rumen in numbers sufficient to perform this reaction. Recently Gall and Huhtanen (40) described a number of criteria for judging whether a bacterium isolated from the rumen is a true rumen organism. These criteria are: (a) anaerobiosis; (b) presence in numbers of at least one million per gram of fresh rumen contents; (c) at least ten isolations of a similar type bacterium from two or more animals; (d) isolation of similar type bacteria in at least two geographical locations; and (e) production by the organism of end-products found in the rumen from substrates found in the rumen.

The isolation in pure culture of anaerobic cellulose bacteria has only been achieved with success in recent years. In the ruminant the breakdown of cellulose seemed to be associated with iodophile bacteria which were found in large numbers on the surface and within the enzymic cavities of the plant tissue and also on pure cellulose (4,5).

Hungate, in his studies on cellulose fermentation (51, 52,54,55), has outlined a technique for isolating anaerobic bacteria. He was successful in obtaining a pure culture of Clostridium cellobioparus from the rumen of cattle, and stated that it differed from other bacteria in this group in being easily isolated and in growing well on both glucose and

cellobiose (51). A number of monographs have stated that cellulose-digesting bacteria gradually lose their fermentation properties when grown in an isolated state. Hungate (51) suggested that this gradual loss of cellulose-fermenting ability may be due to the appearance of mutations in the particular strain. However this investigator observed that his isolated culture of Clostridium cellobioparus did not show any tendency toward loss of cellulose-digesting capacity during four years of pure culture. The fact that cellulose bacteria appeared to retain their cellulose-digesting capacity to a greater degree when in mixed cultures (54) may be explained in that coexisting forms were present and played a symbiotic role in the stated fermentation.

The success in using mixtures has usually been interpreted as indicating that the accompanying forms in some way aid the cellulose digestion. Vartiovaara and his collaborators (128,129), using caecal and rumen contents as inocula, noted that anaerobic decomposition of cellulose was always associated with the presence of cocci which could be seen in chains or pairs embedded in cellulose particles.

For purposes of clarity the different agents that are capable of digesting cellulose can be discussed under three main headings: (1) aerobic cellulose fermenters; (2) anaerobic cellulose fermenters; and (3) other agents of

cellulose digestion.

1. Aerobic cellulose fermenters

Waksman (132) in his discussion of the microbiology of cellulose decomposition, reported that the aerobic cellulose-digesting bacteria can be definitely separated into at least four distinct groups: (a) long sinuous rods, belonging to the genus Cytophaga; (b) short bent rods of the vibrion type, under Cellvibrio; (c) short rods designated as Celfalcicula; and (d) myxobacteria and myxococci. These four groups of bacteria are relatively unimportant in ruminal digestion.

Recently investigators at the Tôhoku University in Japan (121) isolated aerobically 102 bacterial strains from the rumen of the bovine and found that they did not decompose cellulose. The ratio of aerobic bacteria against the total number of bacteria in the rumen was estimated at 1:4300. Sijpesteijn (107) stated that in order to obtain an insight into the nature of the microbial agents active in the process of cellulose breakdown in the rumen, the physiological conditions prevailing in that organ are the determining factors and thus she concluded that the microorganisms predominating in the rumen depend on an anaerobic metabolism.

2. Anaerobic cellulose fermenters

The anaerobic cellulolytic bacteria, which have been isolated, fall into five categories: actinomycetes, thermophilic sporeformers, nonsporeforming rods and cocci, and mesophilic sporeformers. The attempted isolation of pure cultures of anaerobic cellulose bacteria has attracted the attention of microbiologists for a very long time; however, it is only in recent years that unqualified success has been achieved. Many workers have reported the isolation of cellulose decomposing microorganisms from rumen contents. The reviews of Hungate (55) and Sijpesteijn (107) covered extensively the early work done on this problem up to 1950 and outlined the bacteriological techniques involved in order to isolate a pure culture of a cellulose-digesting microorganism from the rumen. Since that time a number of investigations have been published reporting on improved techniques employed to isolate these cellulolytic bacteria from the rumen.

In April 1951, Gall and Huhtanen (40) reported that about 5,000 isolations of bacteria from the rumen of cattle and sheep had been studied. A description of some of the physiological characteristics of five rumen bacteria was included. In November of the same year, Sijpesteijn (108) reported that two strains of Ruminococcus flavefaciens, important cellulose-

decomposing bacteria, were isolated. In the same month Bryant (15) discussed some characterists of the different bacteria present in the rumen of cattle. Huhtanen, Rogers and Gall (48), in December of 1951, reported on an improved technique for the isolation of rumen bacteria from high dilutions of rumen contents.

During the following year Doetsch, Robinson and Shaw (30) presented data resulting from a critical examination of some of the cultural techniques published earlier on studies of rumen contents. They found that this investigation was necessary since difficulty had been encountered with these methods.

During the latter half of 1952, Bryant (16) reported the isolation and characterization of an anaerobic spirochete from the bovine rumen. It was found to grow rapidly at 39°C. on media containing rumen fluid, fermentable sugar, and with an initial pH of 6.5 to 7.0. Recently Bryant and Burkey (17) discussed cultural methods and characteristics of some of the more numerous groups of bacteria in the bovine rumen.

3. Other agents of cellulose digestion

The possibility of plant cytases hydrolysing the walls of plant cells under favorable conditions in the rumen must not be overlooked. Excised barley embryos have been shown by

Boswell (14) to attack slices of potato tubers and the fibers of filter paper with which they were in contact.

The function of the ruminal infusoria has often been discussed. It is now recognized that protozoa play no vital role and can be dispensed with without any loss to the host, (11,126). Van der Wath and Myburgh (126) suggested that the cellulose-digesting power of protozoa is attributed to cellulose-splitting bacteria ingested by the infuzoria. Hungate (49) demonstrated that a ciliate, Eudiplodinium neglectum, had a cellulose-digesting capacity, secreted a cellobiase and produced glucose as an end-product. A year later, in 1943, Hungate (50) cultured a number of species of Diplodinia. They were all strict anaerobes and required cellulose and dried grass in the medium. The generation time was about 24 hours, and all contained a cellulase which could be obtained cell-free. It was not decided whether this cellulase was produced by the protozoa or by the symbiotic bacteria. In a recent review Hungate (56) stated that, since protozoa are so numerous in the rumen, it must be realized that in addition to the action of some protozoa in digesting starch and cellulose, they play a significant role in the food chain leading to the host. Sijpsteijn (107) summarized the present state of the knowledge of the cellulose-decomposing activity of protozoa in the rumen of cattle by saying that some species may contribute to cellulose

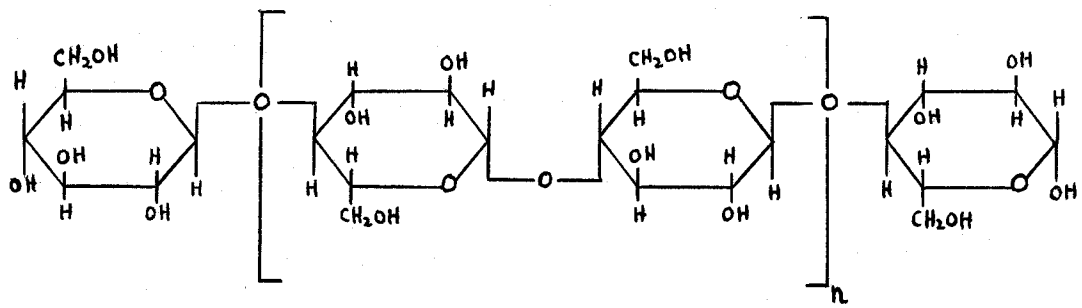
digestion, but that on the other hand this cooperation is not at all essential to the normal functioning of cellulose digestion and utilization.

Actinomyces and molds have been shown by enzyme preparations (14) and by polarized light and microchemical tests (2) to be agents of cellulose digestion. Hungate (52) isolated a cellulose-digesting actinomycete from the alimentary tract of a termite and from cultures of rumen protozoa (49) and identified it as an anaerobic strain of Micromonospora. The slow growth of this group revealed that it would be of limited utility in the symbiotic digestion of cellulose.

The appearance of lactic acid as an intermediate compound in the decomposition of glucose by rumen microorganisms suggested on the one hand the presence of lactic acid bacteria and on the other the presence of lactic acid decomposing organisms. This claim has been suggested by several investigators (32,96,97). Strains of propionic acid bacteria were isolated by Elsden (32) and Johns (57) from cellulose and glucose enrichment cultures; however, there is no knowledge of the numbers of each type present.

C. Chemistry of the Cellulose Molecule

To elucidate the mechanism by which cellulose is degraded by the microorganisms of the rumen there must be first a clear understanding of the chemical and physical nature of the material involved. In the light of the present knowledge the predominating portion of the cellulose molecule can be assigned the following conventional form, where n is a very large integer:

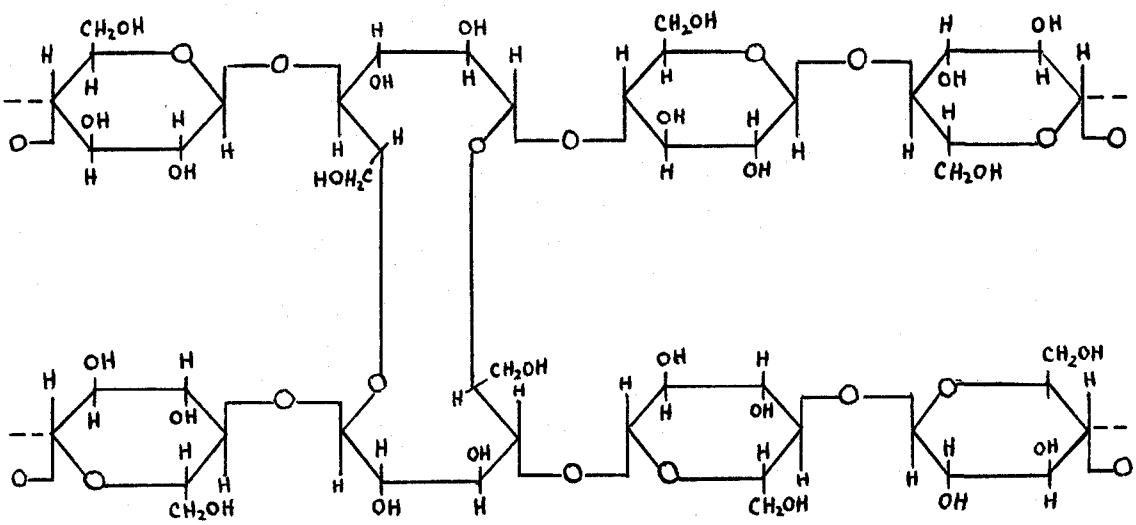


It is to be noted that the two end units differ from the glucose residues in the middle of the chain in two respects. Firstly the terminal members contain four instead of three hydroxyl groups. Secondly, the glucose unit to the extreme right of the structure, shown above, is aldehydic in nature and therefore possesses reducing properties. Carothers (21) pointed out that the picture of this linear molecular chain of anhydrous units accounts for all of the properties exhibited by cellulose as satisfactorily as formulas of simpler organic compounds explain their reactions.

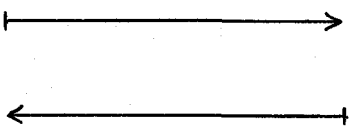
This long chain structure involves 3,000 or more anhydrous units joined together by β-glycosidic linkages and is at least 99 per cent correct (111). No exact information is available as to the chemical nature, number and location of the remaining 1 per cent of the bonds.

Several theories have been advanced on the presence of linkages which are more susceptible to hydrolysis than the normal glycosidic bonds. The cleavage of these labile cross-linkages are considered primarily responsible for the initial degradation of cellulose.

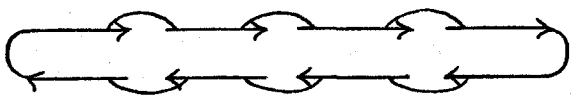
Hess and Steurer (46) suggested that adjacent pyranose rings of parallel aligned chains open up and furnish inter-meshing oxygen bridges. Cross linkages would then involve 12 membered rings which would be formed by the union of two open anhydroglucose units. Such a structure is given below:



Haworth (45) accepted the possibility of cross-linkages between adjacent cellulosic chains and proposed chemical bonds joining adjacent polysaccharide chains at definite intervals. The approximate intervals were estimated at 25 to 30 anhydroglucose units. He assumed that on the average an equal number of chains would be aligned so that the potential reducing group of the chains point in opposite directions as shown:

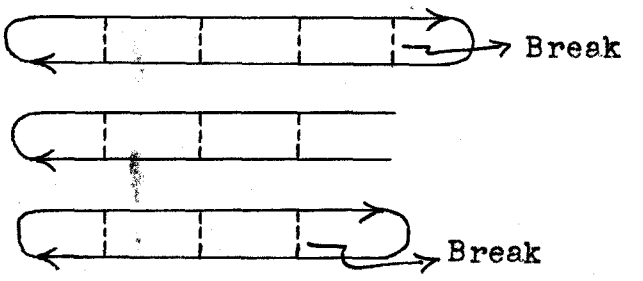


He further stated that cellulose exists in chains of limited minimum length linked together by a different kind of bond from that of the general chain and this would give a loop by the closure of the extremities:



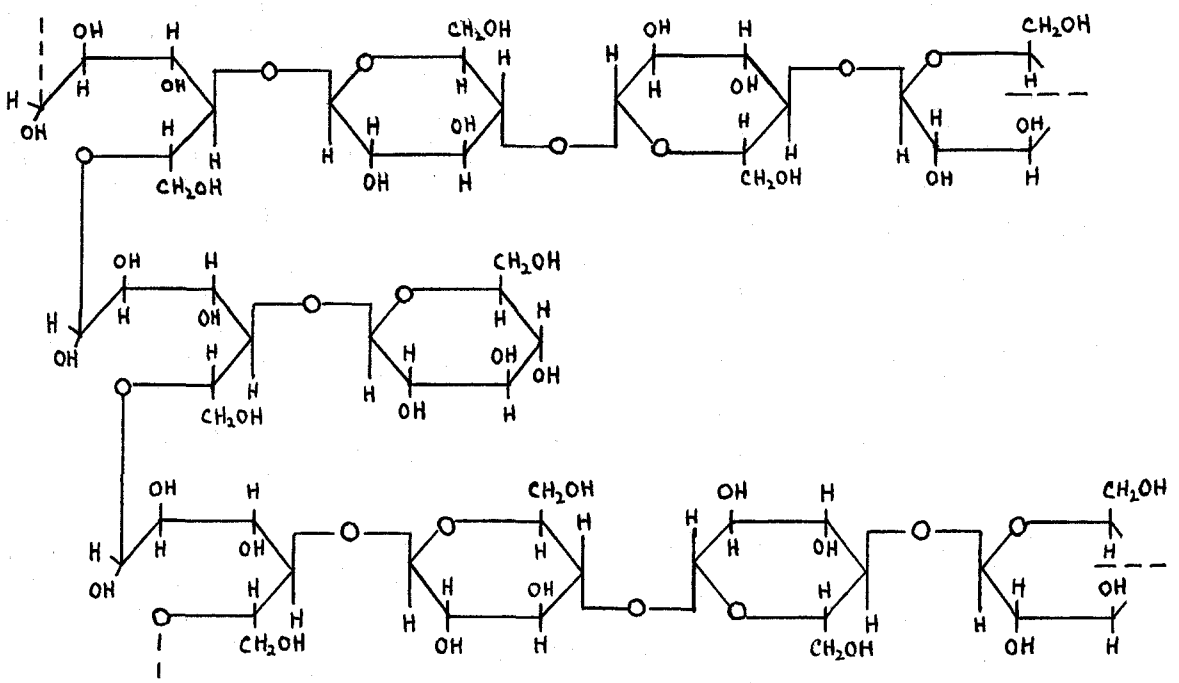
The polymeric or interconnecting bonds were thought to be of a weaker nature than that in the main chain. Therefore during hydrolysis these would represent the potential breakdown points in the chain and the free ends would immediately reunite to form a looped structure of smaller size. This concept can

be diagrammed as follows:



Still another concept has been advanced on the presence of linkages between the long polysaccharide chains. Pascu (92), in 1947, found that mild acid treatment decreased the reducing power of cellulose. This, he indicated, would suggest that cellulose contains another type of covalent bond in addition to the normal 1,4-glycosidic linkages. The acid-sensitive bonds must somehow be correlated with reducing units of the cellulose molecule. Pascu (92) considered the acid-sensitive linkages in the cellulose molecule as hemiacetal bonds of an open glucose unit. Such a grouping can be located either at the reducing end of the chain or as cross links between adjacent cellulose chains. On the rupture of these

linkages, linear molecules would result. A diagram showing the essential features of this concept is shown below:



The original reducing power of the cellulose molecule would correspond to the total number of hemiacetal linkages. The gradual decrease in the reducing power would be caused by the loss of the small molecule during acid treatment. Since these small compounds on leaving the fiber after hydrolysis of their acid-sensitive hemiacetal linkages, do not create new reducing groups, the reducing power of the hydrocellulose would become less than that of the original material (92).

Despite disagreements over the precise nature of the cross-linkages, these workers all seem to agree that in native cellulose small amounts of chemical bonds other than the 1,4 β-glycosidic type are present which serve to join the linear chains together.

D. Biochemistry of Cellulose Decomposition

Pasteur (93), in 1885, was of the opinion that the normal development of any animal depends upon the presence and activities of its intestinal microorganisms. Since cellulose is insoluble in water and therefore cannot diffuse into the microorganism, it is logical to expect that the organism itself must secrete chemical substances, i.e. enzymes, which solubilize the cellulose. The soluble product or products can then diffuse into the cells and be further metabolized. The insolubility of cellulose alone is sufficient reason why in pure culture-pure substrate studies decomposition is often slow in starting unless a heavy inoculum is employed. Mammals have not the ability to elaborate cellulases in the digestive juices and thus the breakdown of the large cellulose molecule, whenever it occurs, is brought about by various microorganisms which obtain their energy for growth by the fermentation of this and related materials.

Considering the extent to which cellulose is ingested by animals, both vertebrate and invertebrate, it is surprising that digestive secretions with cellulases are relatively uncommon. Among invertebrates such secretions are found in some woodboring beetle larvae and in some herbivorous mollusca, for example certain snails (79). Borradaile, Eastham, Potts and Saunders (13 p. 435) stated:

> . . . the great majority of insects do not possess a cellulase and as all plant-cell contents occur within cellulose envelopes it is clear that digestion can only follow when either protoplasm is released by mechanical injury of the cell wall or when the enzymes are able to penetrate the cell wall . . . .

Among vertebrates no record exists (79) of the presence of cellulase. The main site of breakdown of cellulose in ruminants is in the rumen, and in non-ruminants in the large intestine and caecum.

Although cellulose is by far the most important carbohydrate in ruminant nutrition, there is little knowledge as to the method of its actual breakdown in the rumen. Norman and Fuller (90) stated that the primary attack on cellulose during anaerobic fermentation is as follows:

$$\text{cellulose} + \text{water} \longrightarrow \text{cellobiose}$$
$$\text{cellobiose} + \text{water} \longrightarrow \text{glucose}$$

These workers were able to isolate the degradation products of cellulose fermentation, namely cellobiose and glucose. Hungate (51) while studying the microorganism, Clostridium cellobioparus, which he isolated from the rumen, observed that cellobiose did not accumulate in the medium until after the fermentation ceased. The rate of formation of cellulose digestion products was apparently the factor limiting the rate of growth of the microorganisms. It was

shown by Hungate (51) that liquid media containing cellulose showed a somewhat slower development of the bacteria than the same media with glucose or cellobiose.

The closeness with which the cellulose bacteria adhere to the cellulose particles is explained (51) upon the hypothesis that an extracellular cellulase is formed. The closer the bacterium approaches the substrate the greater benefit it derives from its extracellular enzymes. This follows from a consideration of the several steps by which the cellulose is hydrolyzed and the products become useful to the cell. These are: (a) the cellulase must be secreted and diffuse to the substrate; (b) it must be adsorbed on the cellulose and liberate soluble sugars; and (c) the sugar must diffuse back into the cell.

It is evident that the farther the bacterium is from the cellulose fiber the less is the chance the hydrolytic enzymes will be able to react on that material. The extracellular nature of the cellulase formed by Clostridium cellobioparus was clearly demonstrated by Hungate (51). Around each colony on cellulose agar tubes a clear area developed in which the cellulose had been digested.

For the past 65-70 years various attempts have been made to show that microorganisms elaborate enzymes that have the capacity to hydrolyze cellulose. Tappeiner (116), in 1884,

appeared to be the first to show experimentally that the disappearance of cellulose in the digestive tract is effected by a fermentation brought about by microorganisms inhabiting the alimentary canal. In 1886 De Bary (26) was the first to study the dissolution of cell walls of plants by the action of fungi. A few years passed till von Euler, in 1912, (37) published his work "Zur Kenntnis der Cellulase" in which he claimed to have obtained an enzyme preparation from the wood destroying fungus Merulius lacrymans which acted not upon cellulose itself but upon its hydrolytic products (73). In the same year, Pringsheim (101) demonstrated the production of cellobiose from the hydrolysis of cellulose. He postulated the existence of two hydrolytic enzyme systems namely cellulase and cellobiase, the former producing cellobiose from cellulose and the latter hydrolyzing the disaccharide to glucose. By the use of various antiseptics such as toluene and iodoform, he was able to inhibit the growth of the microorganisms in a vigorous thermophilic fermentation. This resulted in an accumulation of reducing substances identified as cellobiose and glucose.

Woodman (142) and Woodman and Stewart (145) using practically the same technique as Pringsheim, identified an accumulated reducing substance in a thermophilic fermentation as glucose. If the culture was incubated at 37°C. instead of 65°C. no cellobiose could be detected. Kalninš (59) found that

in the soil, no cellobiose was formed during the fermentation of cellulose.

Simola (110), in 1931, obtained results while studying the fermentation habits of two aerobic cellulose bacteria, Cellulobacillus myxogenes and Cellulobacillus mucosus, that supported Pringsheim's theory of the existence of two distinct steps in the breakdown of cellulose: cellulose ⟶ cellobiose, and cellobiose ⟶ glucose. Simola (110) also found that the enzymes involved in this hydrolytic reaction possessed the greatest activity in phosphate buffers between pH 5.0 and 6.0. Complete inhibition took place at pH 8.5, and the optimum temperature was in the neighborhood of 37°C.

In 1935 Pochon (99) similarily found that an accumulation of reducing bodies occurred when toluene was added to a healthy culture of Plectridium cellulolyticum. Glucose was partially identified and evidence for the presence of cellobiose was given. Vartiovaara (127) found that reducing sugars accumulated in the decomposition of cellulose by fungi when conditions were unfavorable for growth. These unfavorable conditions for growth of the fungi were brought about by preventing access of oxygen or by the addition of toluene or iodoform to well established cultures.

To date cellulolytic enzymes are still far from being isolated in pure form. According to Siu (112) a number of

investigators have demonstrated cellulolytic activity of crude enzymic preparations from various sources such as the higher plants (wheat, barley etc.), animal life (shipworm, snail, termite, etc.), protozoa, fungi and bacteria. Siu appeared skeptical that many of these preparations were cell-free or sterile.

In 1948, Saunders, Siu and Genest (105) were successful in obtaining a cellulolytic enzyme preparation from the mold, Myrothecium verrucaria and showed that the main breakdown product from cellulose was glucose. A few years later, Whitaker (135,136) outlined a method of purifying the cellulase of this mold and, with a colleague, performed various inhibition and stimulation studies on the purified enzyme preparation (10). Recently Whistler and Smart (134) noted that a crude cellulolytic enzyme preparation of Aspergillus niger hydrolyzed swollen cellulose to glucose as the only degradative product. However, when the preparation was purified by adsorption on and elution from powdered cellulose, both cellobiose and glucose were isolated from the enzymic hydrolyzate of cellulose.

Early investigations (81) indicated that cellulose undergoes a direct gaseous fermentation to which a very large percentage of the carbon is converted into carbon dioxide and methane. Such a direct gaseous fermentation, where a large part or all of the cellulose carbon forms waste gases, is

incompatible with the energy values obtained from cellulose in the ruminant. The fate of the cellulose in the rumen must be explained in another way.

Woodman (143) discounted the theory of bacterial digestion of cellulose in the rumen to give organic acids and gases. He stated this because at that time the organic acids were believed to be inferior to glucose for energy and the only other products would be the waste gases, methane, hydrogen, and carbon dioxide. The explanation Woodman could find was that glucose (or some other sugar capable of hydrolysis to glucose) is formed as a primary product of the bacterial action on cellulose.

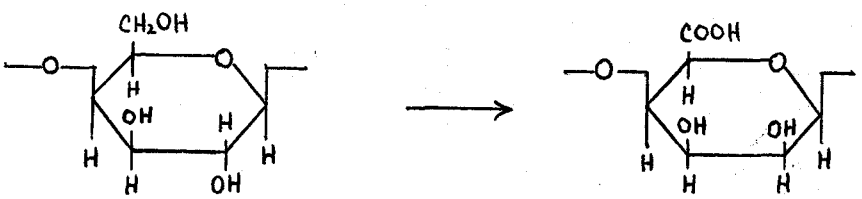
Woodman and Evans (144), as a result of further work, concluded that although glucose is undoubtedly an intermediate product in cellulose fermentation, the quick breakdown which it undergoes precludes the possibility of its being a main end-product of cellulose digestion in the animal. If cellulose does reach the blood stream in the form of glucose at all, this can only happen to a very limited degree.

It was concluded from the investigations of Woodman and Evans (144) that: (a) cellobiose, glucose and lactic acid are intermediate products of cellulose fermentations; (b) the hypothesis that the fat forming power of cellulose may be attributed to glucose or to lactic acid appear untenable; and (c) the principal products of cellulose decomposition in

addition to waste gases are the volatile fatty acids.

With the structure of cellulose in mind it is possible to see what the end-products of bacterial digestion could be. Up to now a number of theories have been proposed to explain the biochemical transformation undergone by the cellulose molecule.

Winogradsky (140), in 1929, outlined a theory to explain the biochemical breakdown of cellulose to various products. He stipulated that the first step involved the oxidation of the primary hydroxyl group to give 6-carboxycellulose.



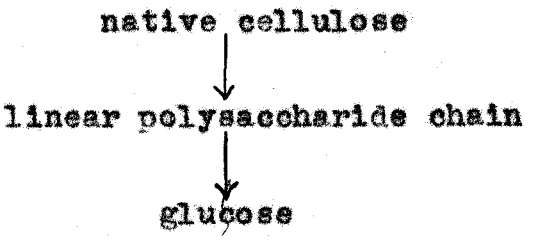
This carboxy compound was then, presumably, hydrolyzed to soluble compounds. The evidence against this explanation is so great that it must now be discarded (112 p. 286). Siu (111) stated that although glucose has been isolated from the breakdown products of cellulose decomposition, the 6-carboxy derivative has not been reported. Furthermore, he continued, preliminary studies showed that 6-carboxycellulose was resistant to cellulose-decomposing microorganisms. From these observations it was, therefore, difficult to expect that the first step in the fermentation of cellulose by microorganisms

resulted in the formation of such a resistant compound.

Pringsheim (101) proposed an additional theory. He stated that the enzyme cellulase hydrolyzes cellulose to cellobiose and then the latter is split further to glucose by the enzyme cellobiase. The general pattern in which the glucose is metabolized by the cell seems to be well established by the work of Lipmann (68) and Meyerhof (85,86) and is included in many biochemical text-books.

The theory of Pringsheim (101) has been critized recently by Siu (111). Siu stated first that cellobiose is not a necessary intermediate product arising from the breakdown of cellulose. He based this on the fact that he had obtained enzymic preparations which were able to convert cellulose to glucose but not cellobiose to glucose. These preparations were obtained from microorganisms growing on cellulose. With the above statement Siu attempted to explain the transformation of the insoluble native cellulose to the soluble sugar, glucose, without passing through cellobiose. He stated that evidence showed that there were at least two separate steps concerned in the solubilization of native cellulose. The two enzymic steps involved in the microbiological breakdown of

native cellulose would then be as follows:



At the present time there is nothing known about the mechanism of the first step (above) which is responsible for converting the native cellulose to the linear chain. In regard to the second step, Clayson (23) believed that the organism proceeds at one end of the polysaccharide chain and lops off one anhydroglucose unit after another. In contrast to this opinion Siu (112 p. 293) claimed that glycosidic linkages in the internal as well as terminal unsubstituted anhydroglucose units in the cellulose molecules are susceptible to attack.

Siu obtained these results by inoculating substrates of partially acetylated cellulose of different degrees of polymerization or chain lengths with the cellulose-digesting mold, Myrothecium verrucaria. The samples were prepared in phosphoric acid solution so that acetylation occurred more or less at random throughout the anhydroglucose units in the cellulose chain. The high degree of breakdown indicated that the internal glycosidic linkages were susceptible to micro-organisms. Siu, Darby, Burkholder and Barghoorn (113) reported

that it was not certain whether or not the terminal or the internal linkages were attacked at greater rates, however it was speculated that they were equally susceptible.

In regard to cellulose derivatives, complete resistance to microorganisms was exhibited only when there was one or more substituents on every anhydroglucose unit (113). The glycosidic linkages adjacent to any substituted anhydroglucose unit seemed to be readily attacked. Siu et al. (113) continued that the degree of resistance did not appear to be affected by the nature of the substituent. The cellulose derivatives that they tested included methyl, ethyl, tosyl, iodotosyl, carboxy, carboxymethyl and acetyl compounds.

Langwell and Lymn (65) and Neuberg and Cohen (89), in 1923, studied independently the hydrolysis of cellulose by microorganisms and in the case of anaerobic bacteria elucidated the characteristic products of the fermentation as follows:

$(C_6H_{10}O_5)_n + (n-1)H_2O \longrightarrow n(C_{12}H_{22}O_{11})$
cellulose — disaccharide conjugate

$C_{12}H_{22}O_{11} + H_2O \longrightarrow 2C_6H_{12}O_6$
glucose

$2C_6H_{12}O_6 + H_2O \longrightarrow 2CH_3CHOHCOOH + CH_3COOH + CH_3CH_2OH + 2CO_2 + 2H_2$

$2CH_3CH_2OH \longrightarrow CH_3COOH + 2CH_4$

$2CH_3CHOHCOOH \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$

$CH_3CHOHCOOH + 2H \longrightarrow CH_3CH_2COOH + H_2O$ (63)

$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$

Although these reactions are largely hypothetical, they serve, nevertheless, to explain the processes involved in the formation of the various acids, alcohols and gases in anaerobic decomposition of cellulose.

Much work has been reported on the study of the end-products arising from the fermentation of cellulose in the rumen. Barker (8), in 1941, demonstrated that methane was not a primary product of cellulose on decomposition but resulted from a transfer of hydrogen from one of the primary fermentation products to carbon dioxide by organisms other than cellulose fermenters. Sijpesteijn (107) remarked that the presence of methane in a cellulose digesting culture indicates that the bacterial population is not pure.

Elsden (32,33) and Elsden, Hitchcock, Marshall and Phillipson (35) showed that the mixture of lower fatty acids formed from cellulose or other carbohydrate fermentation in the rumen was made up of acetic, propionic and butyric acids with acetic acid always predominating. The identity of the acids present in the rumen has been made possible by the development of partition chromatography by Elsden (34), Peterson and Johnson (94) and Gray, Pilgrim and Weller (43).

Acetic acid, the main component of the volatile acid mixture, has been shown to be both a source of energy and an

important anabolic intermediary (72,83).

It is thus evident that the ruminant produces and absorbs the acetic acid, a compound which is usually considered to be a metabolite rather than a product of digestion. Propionic acid is known also to be converted into carbohydrate. Several investigators (18,27,31) have shown that this volatile fatty acid is converted into liver glycogen.

The role of butyric acid formed in the rumen is controversial. Eckstein (31) and Deuel, Butts, Hallman and Cutler (27) could not demonstrate that butyric acid gave significant glycogen deposition in the liver of rats. Later on Buchanan, Hastings and Nesbett (18) found butyric acid was converted into liver glycogen.

E. Number of Microorganisms in the Rumen

Rupel, Bohstedt and Hart (104) and Hastings (44) reported that the number of protozoa in the rumen material ranged from 5×10^8 to 1×10^9 per ml. of rumen fluid. More important than the protozoa are the many morphological types of bacteria present in the rumen fluid (6,44,104). It has been estimated that 10 per cent or more of the total rumen contents consists of bacteria and protozoa (104).

Köhler (62) studied numbers of rumen bacteria by several techniques. By direct count he found about 13×10^9 per gram;

however by indirect procedures he calculated that there should be over 1×10^{11} per gram. Gall, Stark and Loosli (41), using a direct slide count, found bacteria in numbers ranging from 5×10^{10} to 1×10^{11} per gram of fresh rumen contents. Van der Wath (124,125) also made direct counts of rumen microorganisms and found an average of 1×10^{9} to 2×10^{9} per ml. of rumen contents. Recently Gall, Burroughs, Gerlaugh and Edgington (39), while studying rumen bacteria in cattle and sheep on practical farm rations, reported an average count of 5×10^{10} to 9.61×10^{10} per gram of fresh rumen contents depending on type of ration.

Both Köhler (62) and van der Wath (124) stated in their publications that their direct slide counts were too low. Johnson, Hamilton, Robinson and Garey (58) counted rumen organisms by plating out on a simple medium and incubating them aerobically. They obtained a very low figure of 6.5×10^{6} organisms per ml. This figure shows only the number of microorganisms which can grow under the prevailing conditions. Reliable viable counts of rumen population can only be obtained by the use of specific cultural conditions, and it is certain that no one medium will be adequate for all bacteria present in the ruminal mass.

F. Fate of the Ruminal Microorganisms

The rumen is an exceedingly complex microcosm and the cellulose-digesting microorganisms are only one of many groups performing significant functions. The role of the ruminant microflora is that of synthesis. This is based on the fact that bacteria as well as the host utilize the products of degradation.

Rumen microorganisms are eliminated by various routes. A considerable number of bacteria are ingested by the protozoa. The bacteria are then digested and the bacterial proteins and polysaccharides are thus transformed into protozoan protein and paraglycogen respectively. Baker (4) demonstrated that protozoa are more readily accessible to the action of the digestive enzymes of the abomasum than are the iodophilic microorganisms.

The existing bacteria in the rumen may also be eliminated by autolytic disintegration during their passage through the alimentary tract.

A great portion of the rumen microflora is probably digested by the host. Baker (6) observed that there was an absence of the iodophilic bacteria in the faeces and the proven utilization of the synthesized materials was evidence that the bacteria are eliminated and utilized, in part at least.

III. MATERIALS

A. Special Materials

1. Cellulose

As a source of cellulose, Whatman No. 12 filter paper, Alphacel and carboxymethylcellulose derivatives were used. The filter paper was prepared for fermentation purposes by grinding it through the fine mesh screen in a Wiley Mill. Alphacel, the trade name for powdered cellulose, was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. The various carboxymethylcelluloses were obtained from the Hercules Powder Company, Wilmington 99, Delaware.

2. Inocula

The source of the inocula used in the experiments reported here originated from the liquid portion of rumen contents removed from a fistulated Holstein cow maintained on a regular dairy farm ration and kept at the Iowa State College Dairy Farm. The material was strained through four thicknesses of No. 50 grade cheesecloth and placed in warm thermos bottles. The rumen liquid was then taken to the laboratory and used immediately for experimentation purposes. The rumen contents were removed from the animal between eight and eight-thirty in the morning on the days the various

experiments were initiated.

3. Mineral solution

The mineral solution employed in the fermentation media was the one given by Burroughs, Headley, Bethke and Gerlaugh (20) and had the following composition:

Sodium phosphate, monobasic	52.50 gm.
Sodium bicarbonate	52.50 gm.
Potassium chloride	7.50 gm.
Sodium chloride	7.50 gm.
Magnesium sulfate	2.25 gm.
Calcium chloride	0.75 gm.
Ferrous sulfate	0.15 gm.
Manganous sulfate	0.08 gm.
Zinc sulfate	0.08 gm.
Copper sulfate	0.04 gm.
Cobalt chloride	0.02 gm.
Distilled water	2000 ml.

4. Urea solution

A urea solution containing 84 gm. reagent grade urea per liter of distilled water was used as a nutrient for the rumen microorganisms.

5. Phosphorylated sugars

The phosphorylated sugars, glucose-1-phosphate (potassium salt), glucose-6-phosphate (barium salt) and fructose-1,6-diphosphate (barium salt), employed in this investigation were obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio.

6. Eugon agar (B.B.L.)

Eugon Agar was obtained from the Baltimore Biological Laboratories, Baltimore 18, Maryland.

7. Phytone

The powdered phytone, a papaic digest of soya meal, was obtained from the Baltimore Biological Laboratories, Inc., Baltimore 18, Maryland.

8. Trypticase

Trypticase, a peptone derived from casein by pancreatic digestion, was obtained from the Baltimore Biological Laboratories, Inc., Baltimore 18, Maryland.

9. Trypticase-phytone medium

This medium had the following composition per liter:

Trypticase (B.B.L.)	15.0 gm.
Phytone (B.B.L.)	5.0 gm.
Sodium chloride	4.0 gm.
Sodium citrate	1.0 gm.
Sodium sulfite	0.2 gm.
L-Cystine	0.2 gm.
Glucose	5.0 gm.

10. Yeast extract

The yeast extract used in this investigation was the dehydrated powder form of Difco Bacto Yeast Extract manufactured by the Difco Laboratories, Detroit 1, Michigan.

B. Chemicals

All chemicals used during this investigation were of the C.P. grade and were obtained through ordinary commercial sources.

IV. METHODS

A. Fermentation Apparatus

The study of the metabolism of cellulose by the rumen bacterial population can be done adequately in the laboratory. This *in vitro* study, used to similate the *in vivo* processes, has been made possible by the use of the laboratory rumen, now more commonly termed the artificial rumen (19,47,70,76).

The artificial rumen consisted of a number of Erlenmeyer flasks or test tubes of various sizes depending on the type of experiment performed and the amount of cellulose-digesting culture required. The culture vessels were placed in a thermostatically controlled water bath at 40°C. for a prescribed length of time; the physical arrangement, similar to that described by Burroughs, Frank, Gerlaugh and Bethke (19), is shown in Figure 1. A manifold was fixed above the water bath having an adjustable needle valve for each fermentation culture. The carbon dioxide was introduced into the cultural medium by extending a glass tube, which was connected to rubber tubing leading to the needle valve, through the appropriate two-hole rubber stopper to within one-half inch of the bottom of the vessel. A small glass aeration tube was placed through the second hole of the rubber stopper to serve as a vent for the

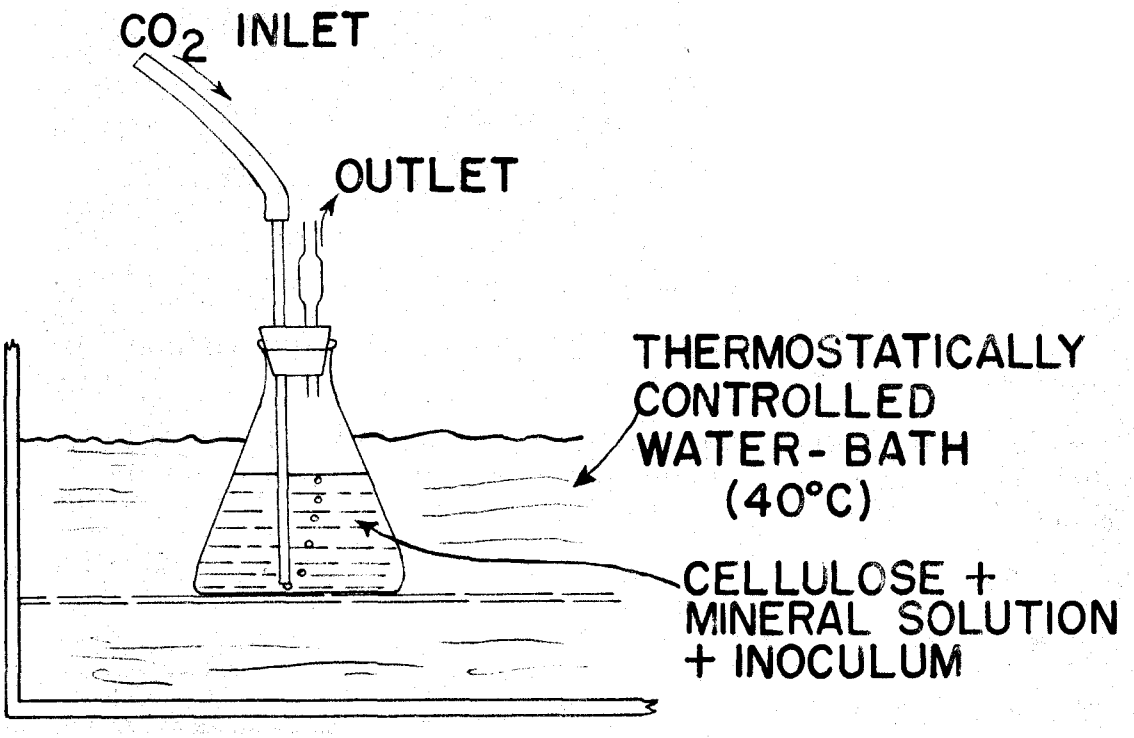


Figure 1. The artificial rumen

carbon dioxide and water vapor. The carbon dioxide gas was bubbled through the medium at a slow rate in order to maintain anaerobic conditions in the fermentation culture.

B. Fermentation Procedure

In a large percentage of the experiments that were conducted during this investigation of cellulose digestion by the microorganisms of the rumen, the fermentation cultures were prepared in 500 ml. wide-mouth Erlenmeyer flasks. The materials placed initially in the flasks consisted of 4 gm. cellulose or a derivative of cellulose, 32.1 ml. mineral solution described under Materials, 4.5 ml. urea solution, and enough warm distilled water to make a volume of 200 ml. To this, 200 ml. of inoculum was added. The pH of the medium at this stage was usually at the desired pH 6.5 - 7.0 and thus did not require any alteration. Immediately following the preparation of the cultures for experimental purposes an aliquot was withdrawn from each flask for subsequent analyses. The flasks were then placed in the thermostatically controlled water bath. The rubber stopper assembly for each flask was then inserted and the stream of carbon dioxide directed through the media. The pH of the cultures were checked periodically throughout the experiment by inserting into the media long lead electrodes from a McBeth pH meter, and when found to be below 6.2 they were adjusted to 6.8 with a solution of saturated

sodium carbonate. During various time intervals aliquots were withdrawn from the fermentation cultures for subsequent analyses.

C. Analytical Procedures

1. Clarification of fermentation solutions

Samples of the fermentation media were deproteinized by the procedure of Doak (29) using cadmium hydroxide with slight modification. Instead of filtering the solution during the clarification procedure, the deproteinized sample was centrifuged and the clear supernatant decanted from the residue for further use.

2. Determination of reducing sugars

The method of Underkofler, Guymon, Rayman and Fulmer (122) was followed without modification for the quantitative determination of reducing sugars. The total reducing substances in each sample was calculated in terms of glucose per 100 ml. fermentation medium.

3. Determination of total volatile acids

The distillation of the volatile fatty acids was conducted as outlined by Neish (88). The distilled acids were titrated with standard sodium hydroxide to the phenol red end-point. The total volatile acids was calculated in terms of acetic

acid per 100 ml. fermentation medium.

4. Qualitative determination of reducing sugars by filter paper chromatography

The method to detect reducing sugars by filter paper chromatography was essentially the same as that used by Wild (139)

The chromatograms were made on sheets of Eton and Dikeman 613 filter paper cut to 8 5/8 in. by 8 in. This particular size was chosen because it could be used nicely in the containers that were available.

The samples from the fermentation media were first deproteinized by the method described and evaporated to dryness by a stream of warm air. The residue was then dissolved with a small amount of distilled water. These samples of carbohydrates were spotted equidistant from each other along a line 2 cm. from the bottom edge. In most cases they were spaced 3 cm. apart, however spots could be placed as close as 2 cm. and still give satisfactory results. Small quantities (3-4 µl.) of the samples and of control sugars (1 per cent) were applied with a platinum loop. In some cases to obtain a high enough concentration of material, successive loops of solution were applied to the same spot after the preceding application had dried.

Ascending type chromatography was used in all experiments. The containers used were wide mouthed gallon jars with screw lids obtained from the Owens-Illinois Glass Company, Bridgeton, New Jersey.

After the spots were applied to the filter paper and dried, the paper was formed into a cylinder by bringing the edges together and loosely joining them with metal staples, care being taken not to allow the edges to touch. The paper cylinder was then placed in a gallon jar which contained approximately 70 ml. of developing solvent and the bottle capped. The chromatogram was allowed to stand in the solvent until the solvent had reached the top of the paper by capillary action. This required from 6 to 8 hours. The chromatogram was then removed from the jar, dried at room temperature and returned to the jar for another ascent if desired.

The developing solvent consisted of three parts of water, four parts of pyridine and six parts of n-butyl alcohol by volume (22). The solvent, known as 3-4-6, was used for four or five ascents and then discarded as its effectiveness in moving and resolving materials on the chromatograms was significantly reduced.

The reagent used to detect the reducing carbohydrates was the alkaline copper reagent. It was prepared by dissolving 7.5 gm. of copper sulfate in 100 ml. of water and adding this,

with stirring, to a solution of 25 gm. of Rochelle salt and 40 gm. of anhydrous sodium carbonate in 300 ml. of water. To this mixture was added 500 ml. of methyl alcohol and the resulting solution diluted to 1 liter with distilled water. This solution was sprayed on the chromatogram until uniformly moistened employing a DeVilbiss No. 31 atomizer with a continuous air stream. The chromatogram was then heated in an oven at 110°C. for 5 min. After this period the chromatogram was sprayed with a phosphomolybdic acid solution (117), which brought out as blue spots the areas where reduction of the copper by the sugar had occurred.

The phosphomolybdic acid solution was prepared by adding approximately 500 ml. of distilled water in small amounts to 150 gm. ammonia-free molybdic acid and 75 gm. anhydrous sodium carbonate. The mixture was then heated to boiling or until all of the molybdic acid had dissolved. After the solution had been filtered, 300 ml. of 85 per cent orthophosphoric acid was added. The solution was then cooled and diluted to 1 liter with distilled water.

This combination of reagents employed in this procedure can be used to detect as little as 1 microgram of glucose (139).

V. EXPERIMENTAL RESULTS

In a recent review on the subject of rumen digestion, Elsden and Phillipson (36) commented that an increasing number of physiologists, biochemists, microbiologists and nutritionists realize that the ruminant offers not only an intrinsically interesting field of investigation, but also one which will yield results of primary importance to man. With the continued use of the conventional laboratory animal many aspects of intermediary metabolism and digestion could very well have been overlooked if it was not for the emphasis that has been placed on the use of the ruminant for biochemical investigations.

The physiology of digestion of ruminants is unique in that bacteria elaborate various enzymes which play an intregal part in the well-being of those animals. The microbiological hydrolysis of cellulose may be studied <u>in vitro</u> in at least two ways (84), either directly by determining the amount of cellulose digested (25) or indirectly by the quantitative determination of reducing substances which accumulate during the digestion of cellulose in the presence of bacterial growth or enzyme inhibitors. Pringsheim (101) was the first to employ compounds, such as toluene or iodoform, to arrest the growth of cellulose-digesting bacteria, and as a result reducing substances accumulated in the culture media.

A. The Effect of Toluene on the Cellulolytic Activities of Rumen Microorganisms

Woodman and Evans (144) were the first to show that antiseptics such as toluene favored the production of reducing sugars in rumen liquid. This work was followed a few years later by Meites, Burrell and Sutton (84) who stated that toluene was the most effective antiseptic to prevent further changes of reducing substances that were produced during the fermentation of cellulose by rumen bacteria. A number of experiments were conducted in order to confirm the results given by Meites et al. (84). Cultures were prepared in 250 ml. Erlenmeyer flasks by adding a definite amount of rumen liquid, as inoculum, to a medium consisting of buffer (mixture of 0.067 M Na_2HPO_4 and KH_2PO_4, pH 6.98), cellulose (ground filter paper) and toluene. The cultures were then incubated in a thermostatically controlled water bath at 40°C. for 72 hours. A typical culture had the following composition:

Rumen liquid	25 ml.
Cellulose	200 mgm.
Buffer	95 ml.
Toluene	10 ml.

At various intervals an aliquot was withdrawn from each flask and analyzed in duplicate for the accumulation of reducing substances. For convenience the reducing substances were calculated as gm. glucose per 100 ml. fermentation culture.

From the results of this experiment, as given in Table I, it was found that toluene arrested the growth of the cellulolytic bacteria and brought about an accumulation of reducing substances. It was noted however, that after 48 hours, the accumulation of reducing substances decreased markedly in culture number 4. This may be due to the fact that the toluene was removed by vaporization to a level that allowed the bacteria to use the available sugars for their nutrition. The work of Meites *et al.* (84) did not mention this behavior, and therefore in future experiments the volume of toluene was increased. Nevertheless these results do confirm previously reported work which showed that the presence of toluene in active cellulose-digesting cultures resulted in the accumulation of reducing substances in the growth menstruum.

Ruf (103), in his studies concerning cellulose utilization by ruminal microorganisms, employed a mineral solution (see Materials) patterned after the composition of sheep saliva. It was felt that it would be interesting to compare this complete salt solution with the buffer solution used by Meites *et al.* (84), as given previously, for their capacity to maintain the activities of the rumen microorganisms and/or their enzymes in *in vitro* fermentations. In addition to this, the concentration of cellulose, as ground filter paper, and the volume of inoculum were varied in the media. The composition of the cultures is given in Table II.

Table I. Fermentation of cellulose by rumen microorganisms in the presence of toluene

Culture	Cellulose	Toluene	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				
			0	12	24	48	72
1	+	-	0.000	0.000	0.000	0.001	0.001
2	-	-	0.000	0.000	0.000	0.001	0.001
3	+	-	0.000	0.001	0.001	0.002	0.002
4	+	+	0.000	0.011	0.071	0.080	0.028
5	-	+	0.000	0.005	0.059	0.068	0.019

Table II. Composition of the cellulose-digesting cultures.

Culture	Cellulose gm.	Buffer sol. ml.	Mineral sol. ml.	Urea sol. ml.	Toluene ml.	Distilled water ml.	Rumen liquid ml.
1	0.6	285					75
2	0.6	285			90		75
A	0.6	285					75
B	0.6	285			90		75
I	0.6		28	4		253	75
II	0.6		28	4	90	253	75
III	3.6		28	4		148	180
IV	3.6		28	4	90	148	180

The results of this experiment as given in Table III showed that either one of the salt solutions contained adequate nutrients for the microorganisms and/or their enzymes. It was also noted that the increased level of the antiseptic prevented the accumulated reducing substances to be utilized by the bacteria as was experienced in the previous experiment.

Several investigators (32,90,97,144) showed that volatile fatty acids such as formic, acetic and butyric, were produced as end-products of cellulose digestion by ruminal microorganisms. An experiment was designed to show the relationship of the production of short-chain fatty acids to the accumulation of reducing substances during cellulose digestion in the presence and absence of toluene. The composition of the culture media employed for this and future experiments was designed after that employed by Ruf (103) and described under Methods.

During the fermentation, aliquots were withdrawn from the cultures and deproteinized. The clarified solutions were then used for the determination of total reducing substances and total volatile acids. The results of this experiment are given in Table IV and Figure 2. It is evident that the volatile acids remained constant and the reducing substances accumulated in the cultures having toluene added during the fermentation. The breakdown of the cellulose by the microorganisms to produce

Table III. The accumulation of reducing substances in cellulose-digesting cultures in the presence of toluene

Culture	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours			
	0	24	48	72
1	0.000	0.002	0.002	0.000
2	0.000	0.085	0.098	0.098
A	0.000	0.000	0.003	0.002
B	0.000	0.028	0.090	0.090
I	0.000	0.000	0.000	0.000
II	0.000	0.059	0.072	0.074
III	0.000	0.000	0.000	0.000
IV	0.000	0.198	0.196	0.190

Table IV. Fermentation of cellulose by rumen microorganism in the presence and absence of toluene

Culture	Cellulose	Toluene	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours			Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours		
			0	24	48	0	24	48
1	+	-	0.000	0.000	0.001	0.000	0.387	0.620
2	+	+	0.000	0.107	0.120	0.000	0.013	0.023
3	-	+	0.000	0.059	0.066	0.000	0.075	0.121

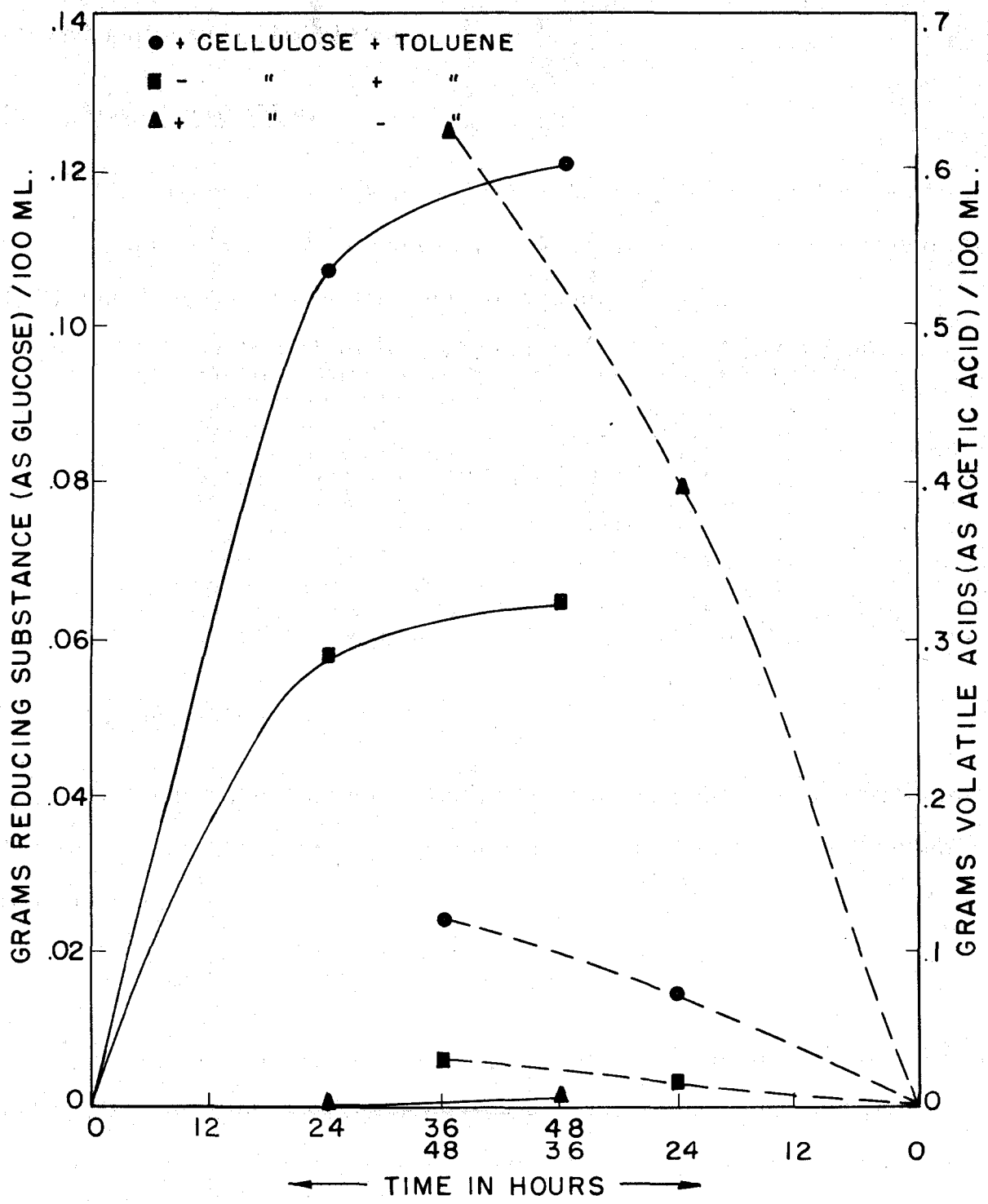


Figure 2. Fermentation of cellulose by rumen microorganisms in the presence and absence of toluene

volatile fatty acids as end-products was not hampered in the absence of the antiseptic. It appeared that toluene had the ability to inhibit the growth of the microorganisms, but not the activity of the enzyme system which catalyzed the hydrolysis of the cellulose to smaller reducing compounds.

An additional experiment was conducted in order to supplement these results. A vigorous fermentation of cellulose (as ground filter paper) by rumen microorganisms was prepared, which required a period of 24 hours in a thermostatically controlled water-bath. At this time toluene was added and the fermentation allowed to continue. At various intervals the media were analyzed for reducing substances and volatile acids.

The results from this experiment (Table V) added additional evidence that toluene inhibited part of the organic metabolism of the rumen microorganisms. It is clearly shown by culture number 2 that digestion of cellulose continues to the point at which volatile acids as well as other gaseous products are produced at a fast rate, i.e., 24 hours of fermentation. At this peak toluene and 2 gm. cellulose were added to the culture. The production of short-chain fatty acids stopped abruptly while the reducing substances accumulated rapidly in the culture medium. The other three cultures were used as controls in this test.

Table V. Fermentation of cellulose by rumen microorganisms in the presence and absence of toluene

Culture	Cellulose added at: hours		Toluene added at: hours		Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
	0	24	0	24	0	24	48	72	0	24	48	72
1	+	-	-	-	0.000	0.000	0.000	0.000	0.000	0.428	0.595	0.757
2	+	2 gms. +	-	+	0.000	0.000	0.070	0.074	0.000	0.445	0.474	0.485
3	-	-	-	+	0.000	0.000	0.001	0.006	0.000	0.198	0.196	0.205
4	-	-	+	-	0.000	0.025	0.016	0.022	0.000	0.000	0.044	0.060

B. Fermentation of the Carboxymethylcelluloses by Rumen Microorganisms

The previous experimental work on this subject has shown that the rumen microorganisms are able to digest cellulose, in the form of finely ground filter paper, to produce the various end-products. Since this form of cellulose is a solid, insoluble material, contact of the rumen bacteria with the cellulose fibers cannot be very uniform. Experiments were therefore conducted employing soluble cellulose derivatives, namely the carboxymethylcelluloses, as the source of the cellulosic material in the various fermentation media. These compounds were supplied by the Hercules Powder Company, Wilmington, Delaware, under the industrial designation of CMC.

CMC, technically, is the sodium salt of carboxymethylcellulose having a closely controlled number of sodium carboxymethyl groups introduced into the cellulose molecule to bring about solubility in water. The product is manufactured by a process which involves cellulose of high purity to be treated first with alkali and then reacted with sodium monochloracetate as follows:

$$RONa + ClCH_2COONa \longrightarrow ROCH_2COONa + NaCl$$

where "R" represents the cellulose structure.

Since each anhydroglucose unit in the cellulose structure contains three reactive hydroxyl groups with which the sodium monochloroacetate can react, theoretically complete reaction would mean the introduction of three sodium carboxymethyl groups per anhydroglucose unit. Such a completely reacted product would have a substitution of 3.0. The Hercules Powder Company manufactures these cellulose derivatives having a substitution range of 0.3 to 1.2 (0.7 being the regular type). For example CMC-70 has a degree of substitution of 0.7 or 7 out of each 10 anhydrous units have one substituent carboxymethyl group.

The fermentation of CMC-70H by rumen microorganisms was carried out utilizing the artificial rumen technique as described previously. The CMC compound was put into solution by vigorous agitation using the Waring Blendor. Toluene was added to the various cultures to act as the antiseptic or inhibitor.

The results of this experiment, as given in Table VI, and Figure 3, show clearly that the rumen bacteria had the capacity to attack the soluble cellulose product, and degraded the large molecule to smaller, reducing constituents. As was expected a larger concentration of CMC increased the extent of fermentation which resulted in a larger accumulation of reducing substances.

Table VI. Fermentation of CMC-70H by rumen microorganisms in the presence or absence of toluene

Flask	CMC	Toluene	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
			0	24	48	72	0	24	48	72
1	+ 0.6%	-	0.000	0.010	0.010	0.005	0.000	0.138	0.374	0.399
2	-	+	0.000	0.105	0.118	0.111	0.000	0.030	0.042	0.079
3	+ 0.6%	+	0.000	0.171	0.178	0.191	0.000	0.005	0.024	0.024
4	+ 1.0%	+	0.000	0.192	0.217	0.229	0.000	0.011	0.011	0.050

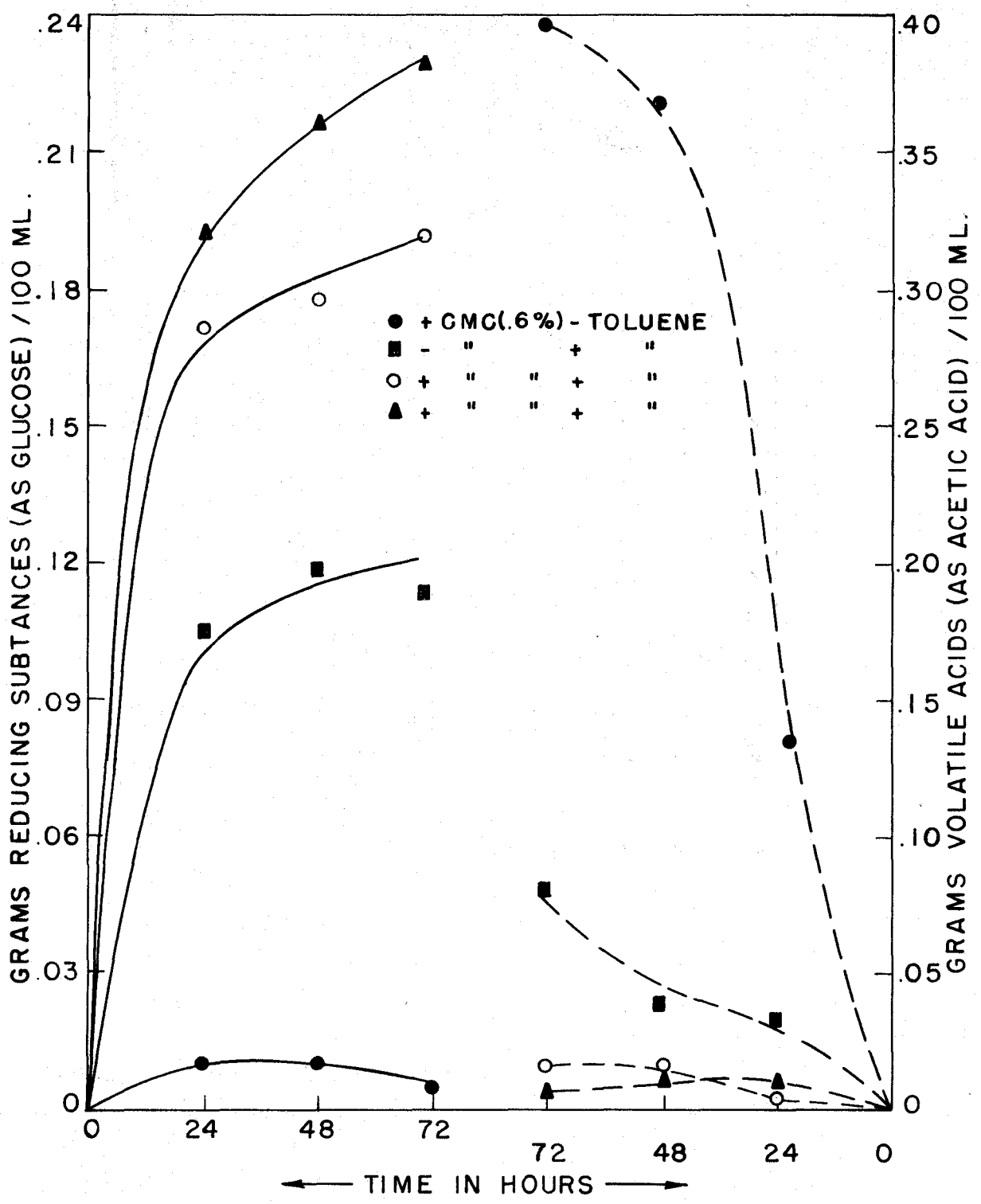


Figure 3. Fermentation of CMC-70H by rumen microorganisms in the presence and absence of toluene

1. Effect of various chemical compounds on the fermentation of CMC by rumen microorganisms

Upon request, the Hercules Powder Company supplied a number of carboxymethylcellulose products for experimental use. An experiment was designed to observe which soluble cellulose compound was most susceptible to the action of the rumen microorganisms. The results of this study (see Table VII) showed that all the CMC samples used were digested rapidly by the microbial action. The finely ground filter paper and Alphacel were degraded to a much slower or lesser degree. In all cases toluene was found to prevent the conversion of the reducing substances to volatile fatty acids.

As a result of these observations a number of chemical compounds were tested in order to find other inhibitory substances that would prevent the conversion of formed reducing sugars to short-chain fatty acids during the digestion of cellulose by rumen microorganisms.

The fermentation of CMC-70H by the rumen bacteria was carried out utilizing the artificial rumen as described under Methods. Eight chemical compounds were tested in a preliminary experiment in order to note which compound would bring about the greatest accumulation of reducing substances in the fermentation medium.

Table VII. Fermentation of cellulose and soluble cellulose derivatives by rumen microorganisms in the presence of toluene

Flask	Cellulose	Toluene	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours			
			0	12	24	48
1	-	+	0.000	0.037	0.046	0.053
2	FP	+	0.000	0.157	0.163	0.192
3	A	-	0.000	0.001	0.000	0.000
4	A	+	0.000	0.168	0.179	0.180
5	CMC-120M	-	0.000	0.000	0.000	0.000
6	CMC-120M	+	0.000	0.188	0.216	0.227
7	CMC-70L	-	0.000	0.021	0.028	0.029
8	CMC-70L	+	0.000	0.255	0.268	0.318
9	CMC-70M	-	0.000	0.023	0.033	0.040
10	CMC-70M	+	0.000	0.253	0.274	0.296
11	CMC-70H	-	0.000	0.014	0.020	0.025
12	CMC-70H	+	0.000	0.256	0.279	0.282

FP - Finely ground filter paper
A - Alphacel
L,M,H - Designates degree of viscosity (Low, Medium, High)

The results of this experiment, tabulated in Table VIII, indicate that the different compounds added to a cellulose fermentation varied in their inhibitory action. It was learned from this investigation that each compound should be tested separately in different concentrations so that an optimum concentration could be found that would result in the greatest accumulation of reducing substances.

The following compounds were tested for their inhibitory action during the fermentation of cellulose by ruminal microorganisms: (a) sodium cyanide; (b) iodoform; (c) sodium fluoride; (d) malonic acid; (e) thymol; (f) iodoacetic acid; (g) toluene; (h) formaldehyde; (i) phenol; (j) chloroform; (k) m-xylene; and (l) benzoic acid.

During the fermentation of CMC-70L by the rumen microorganisms in the presence and absence of the various chemical compounds, aliquots were removed from the culture media and analyzed for reducing substances and volatile acids. The results obtained from these experiments, Tables IX to XV and Figures 4 to 9, show that, as the concentration of the various inhibitors was increased in the fermentation media, a greater accumulation of reducing substances was obtained with little to no production of volatile fatty acids. As the reducing substances increased in the culture media the production of short-chain fatty acids decreased thus showing

Table VIII. Fermentation of CMC-70H by rumen microorganisms in the presence or absence of various chemical compounds.

Flask	Cellulose	Antiseptic and molar concentration		Inoculum	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours			
					0	12	24	48
1	1%	Toluene	35 ml.	+	0.000	0.192	0.202	0.211
2	1%	Sodium cyanide	0.01M	+	0.000	0.001	0.008	0.011
3	1%	Iodoform	0.01M	+	0.000	0.001	0.008	0.008
4	1%	Sodium fluoride	0.01M	+	0.000	0.004	0.007	0.006
5	1%	Iodoacetic acid	0.01M	+	0.000	0.010	0.013	0.013
6	1%	Form-aldehyde	20 ml.	+	0.000	0.000	0.000	0.000
7	1%	Thymol	0.01M	+	0.000	0.092	0.111	0.117
8	1%	Chloro-form	71 ml.	+	0.000	0.130	0.131	0.046
9	-	Toluene	35 ml.	+	0.000	0.047	0.049	0.014
10	1%	-		+	0.000	0.002	0.004	0.003
11	1%	Toluene	35 ml.	-	0.000	0.003	0.003	0.000

Table IX. Fermentation of CMC-70L by rumen microorganisms in the presence or absence of sodium cyanide

Flask	CMC %	Molar concentration of sodium cyanide	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
			0	12	24	48	0	12	24	48
1	1	1	0.000	0.000	0.001	0.001	0.000	0.001	0.000	0.069
2	1	0.1	0.000	0.044	0.050	0.062	0.000	0.004	0.010	0.092
3	1	0.01	0.000	0.025	0.035	0.037	0.000	0.025	0.075	0.085
4	1	0.001	0.000	0.019	0.022	0.024	0.000	0.132	0.237	0.354
5	1	0.000	0.000	0.012	0.012	0.015	0.000	0.214	0.405	0.616
6	1	0.5 (no inoculum)	0.000	0.000	0.000	0.000	0.000	0.003	0.003	0.000

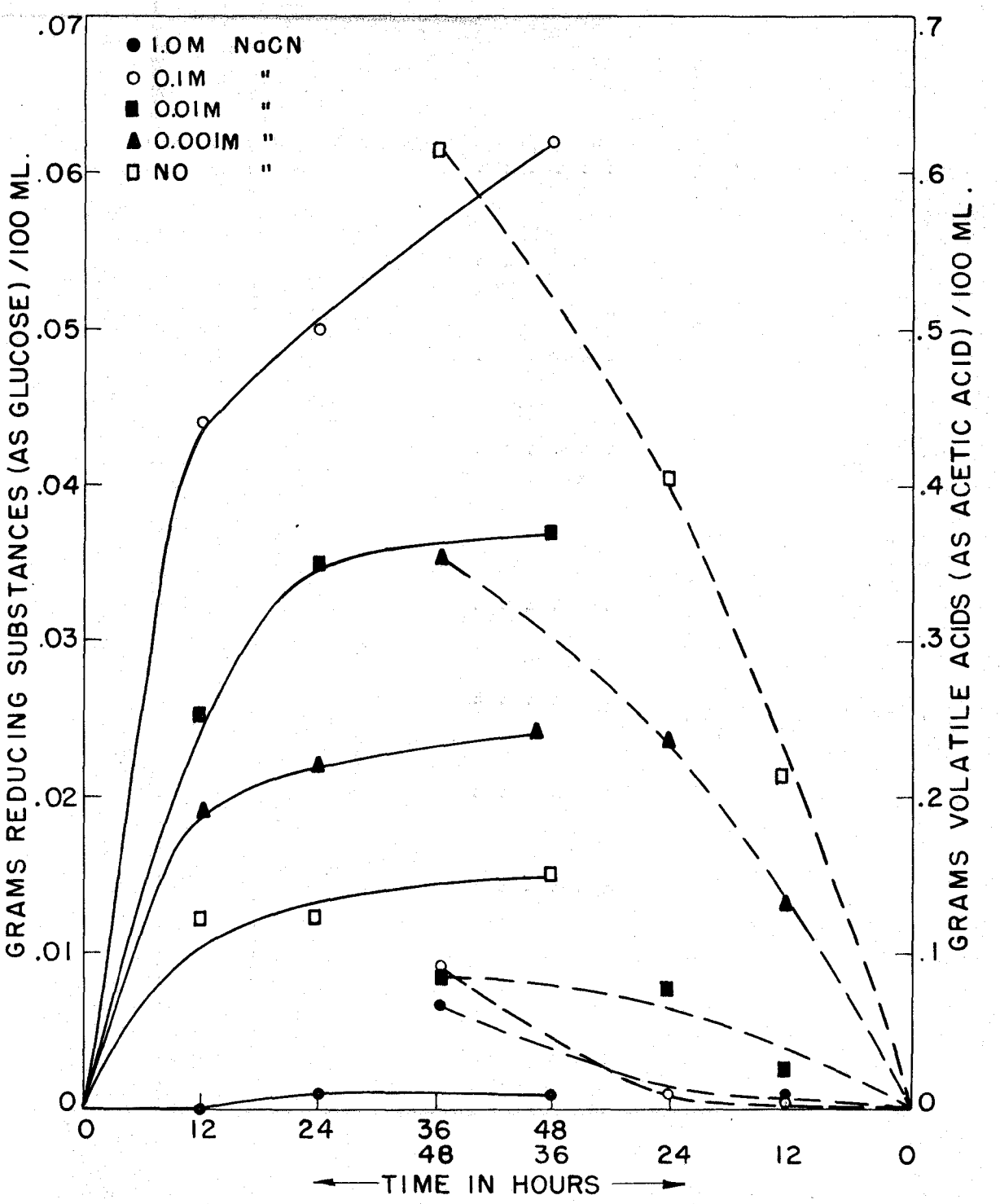


Figure 4. Fermentation of CMC-70L by rumen microorganisms in the presence and absence of sodium cyanide

Table X. Fermentation of CMC-70L by rumen microorganisms in the presence or absence of iodoform

Flask	CMC %	Molar concentration of iodoform	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
			0	12	24	48	0	12	24	48
1	1	0.1	0.000	0.033	0.055	0.069	0.000	0.060	0.088	0.088
2	1	0.05	0.000	0.027	0.048	0.056	0.000	0.041	0.081	0.066
3	1	0.01	0.000	0.035	0.044	0.050	0.000	0.058	0.077	0.076
4	1	0.001	0.000	0.024	0.033	0.033	0.000	0.068	0.178	0.250
5	1	0.000	0.000	0.023	0.022	0.023	0.000	0.141	0.276	0.347
6	1	0.05 (no inoculum)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

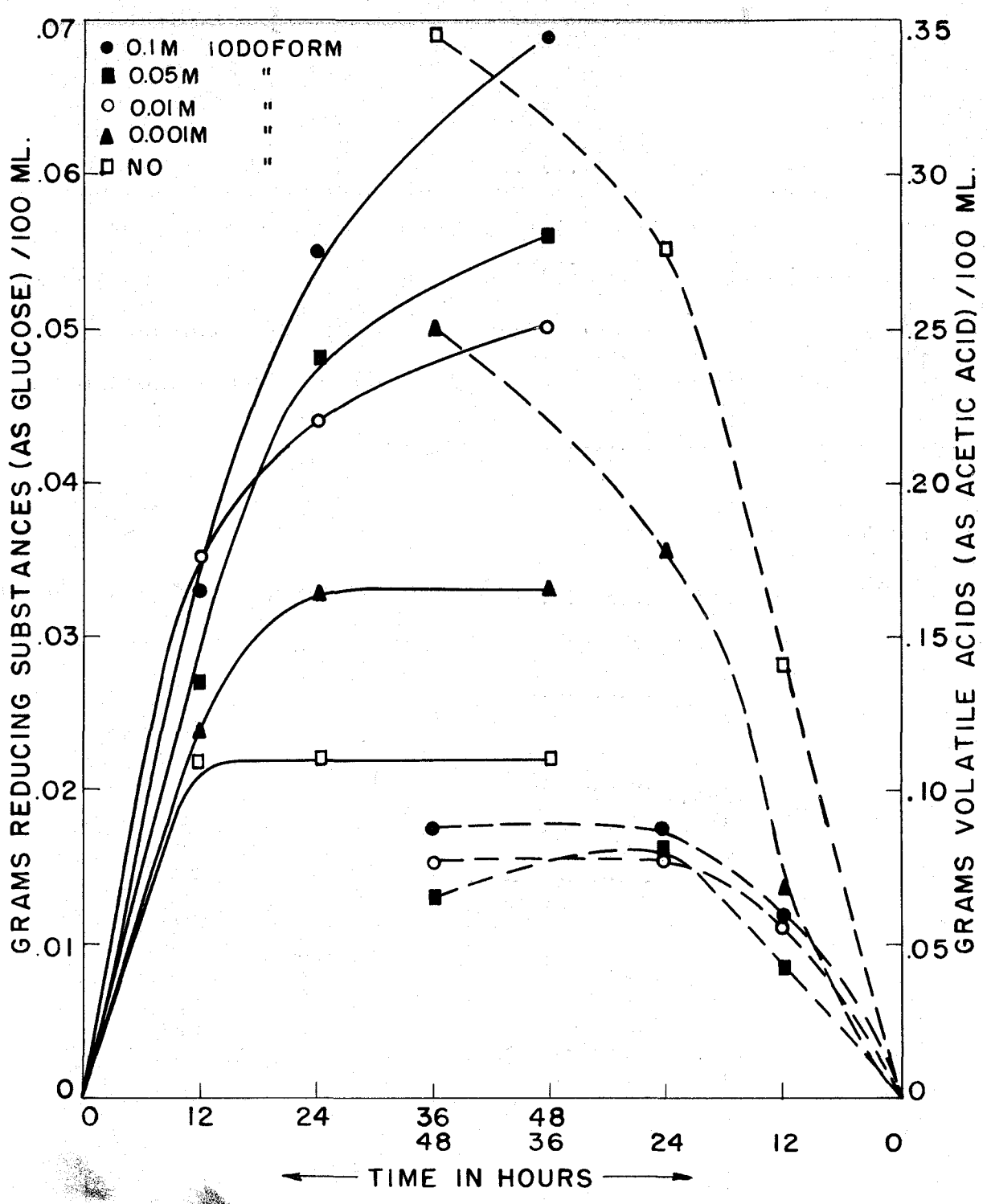


Figure 5. Fermentation of CMC-70L by rumen microorganisms in the presence and absence of iodoform

Table XI. Fermentation of CMC-70L by rumen microorganisms in the presence and absence of sodium fluoride

Flask	CMC %	Molar concentration of sodium fluoride	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
			0	12	24	48	0	12	24	48
1	1	0.5	0.000	0.197	0.268	0.328	0.000	0.071	0.082	0.088
2	1	0.1	0.000	0.059	0.069	0.084	0.000	0.008	0.076	0.087
3	1	0.01	0.000	0.037	0.042	0.055	0.000	0.128	0.210	0.285
4	1	0.001	0.000	0.021	0.020	0.020	0.000	0.124	0.207	0.326
5	1	0.000	0.000	0.021	0.021	0.021	0.000	0.132	0.224	0.316
6	1	0.5 (no inoculum)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

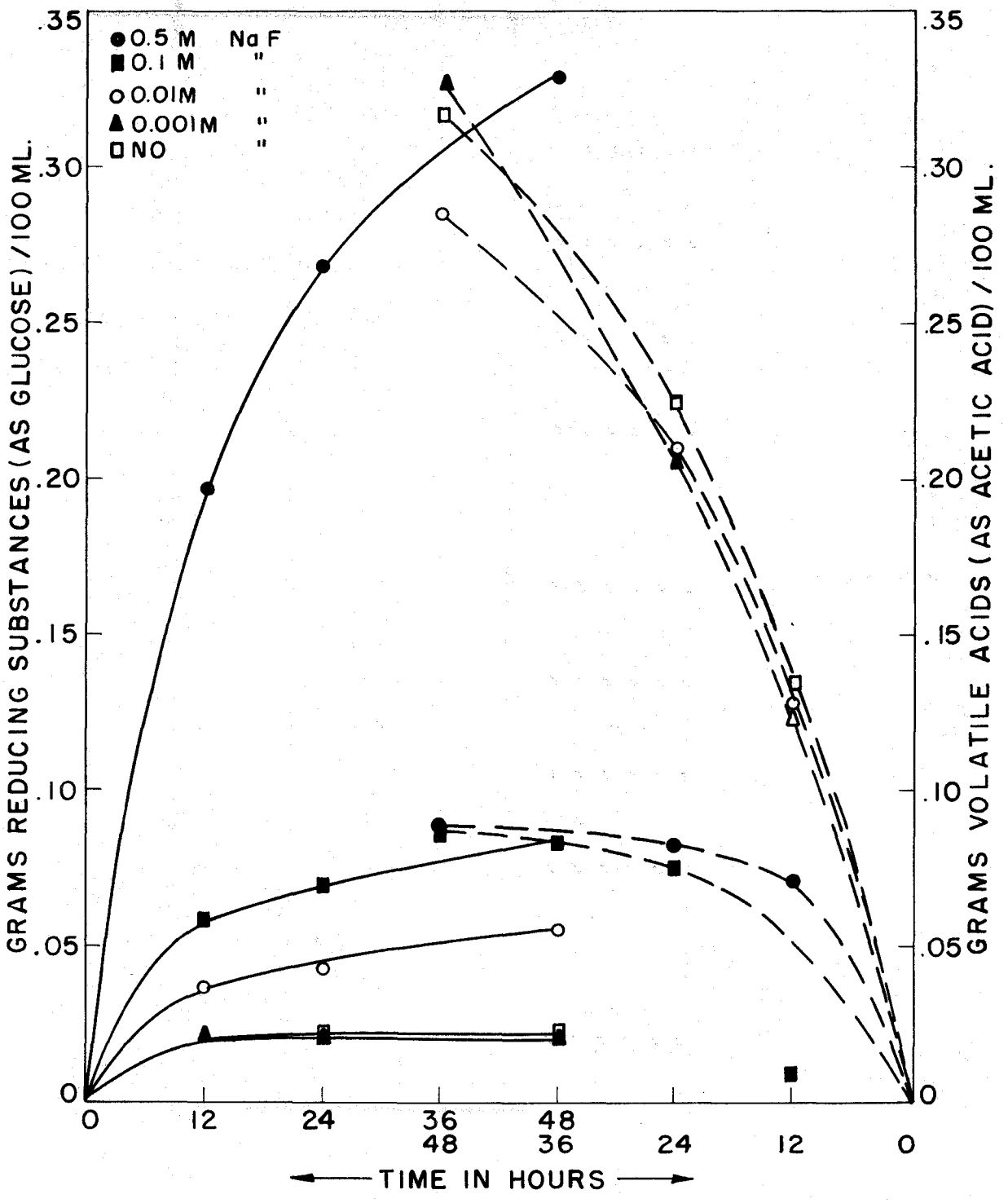


Figure 6. Fermentation of CMC-70L by rumen microorganisms in the presence and absence of sodium fluoride

Table XII. Fermentation of CMC-70L by rumen microorganisms in the presence or absence of malonic acid

Flask	CMC %	Molar concentration of malonic acid	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
			0	12	24	48	0	12	24	48
1	1	0.5	0.000	0.031	0.031	0.031	0.000	0.000	0.000	0.000
2	1	0.1	0.000	0.031	0.033	0.040	0.000	0.115	0.362	0.673
3	1	0.01	0.000	0.036	0.046	0.046	0.000	0.201	0.288	0.352
4	1	0.001	0.000	0.037	0.045	0.060	0.000	0.027	0.231	0.267
5	1	0.000	0.000	0.005	0.019	0.018	0.000	0.181	0.328	0.404
6	1	0.5 (no inoculum)	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001

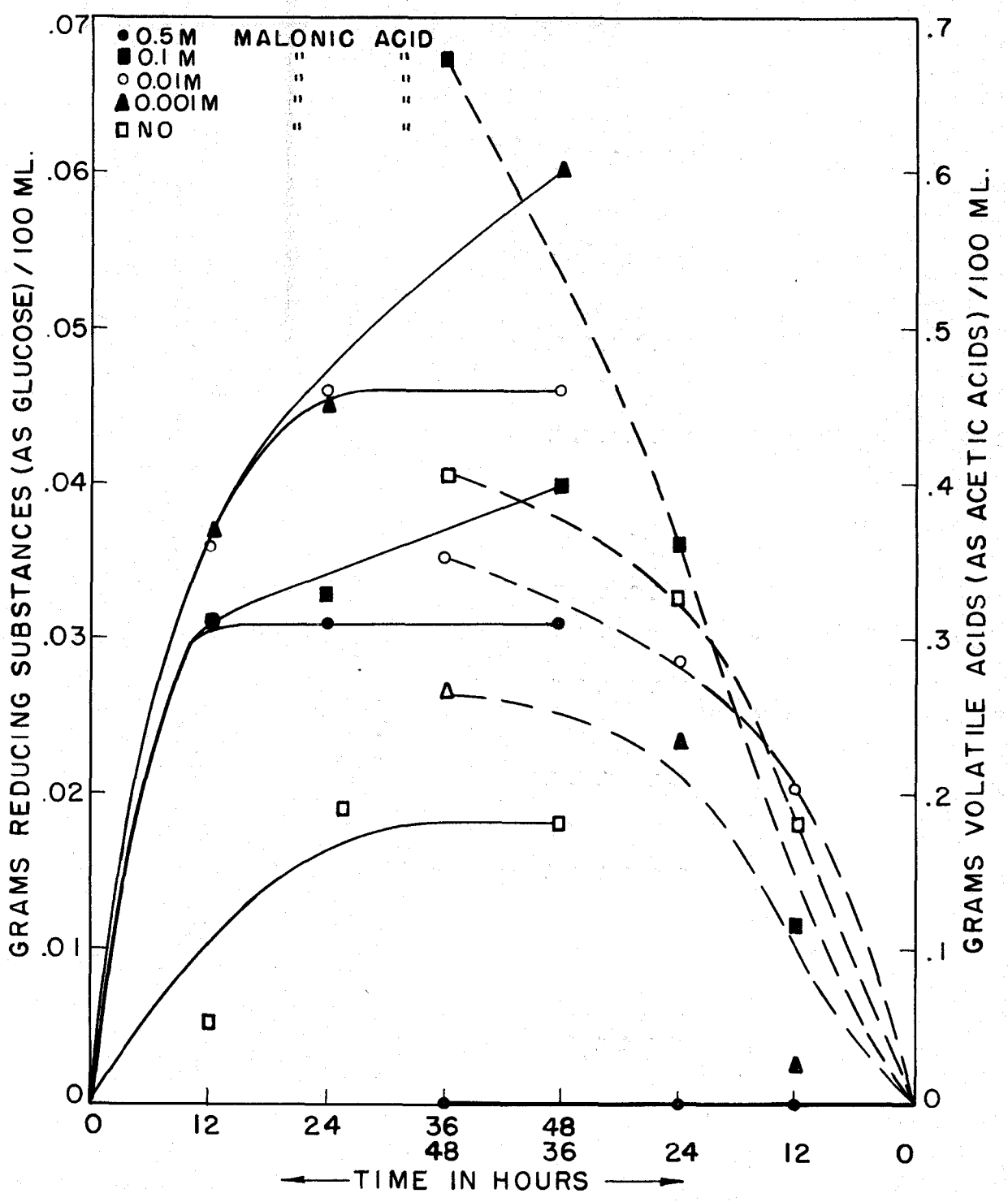


Figure 7. Fermentation of CMC-70L by rumen microorganisms in the presence and absence of malonic acid

Table XIII. Fermentation of CMC-70L by rumen microorganisms in the presence or absence of thymol

Flask	CMC %	Molar concentration of thymol	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
			0	12	24	48	0	12	24	48
1	1	0.5	0.000	0.315	0.351	0.472	0.000	0.000	0.000	0.006
2	1	0.1	0.000	0.283	0.456	0.474	0.000	0.008	0.000	0.000
3	1	0.01	0.000	0.272	0.455	0.424	0.000	0.045	0.046	0.039
4	1	0.001	0.000	0.040	0.067	0.078	0.000	0.191	0.240	0.253
5	1	0.000	0.000	0.004	0.019	0.018	0.000	0.182	0.329	0.404
6	1	0.5 (no inoculum)	0.000	0.002	0.002	0.002	0.000	0.000	0.000	0.000

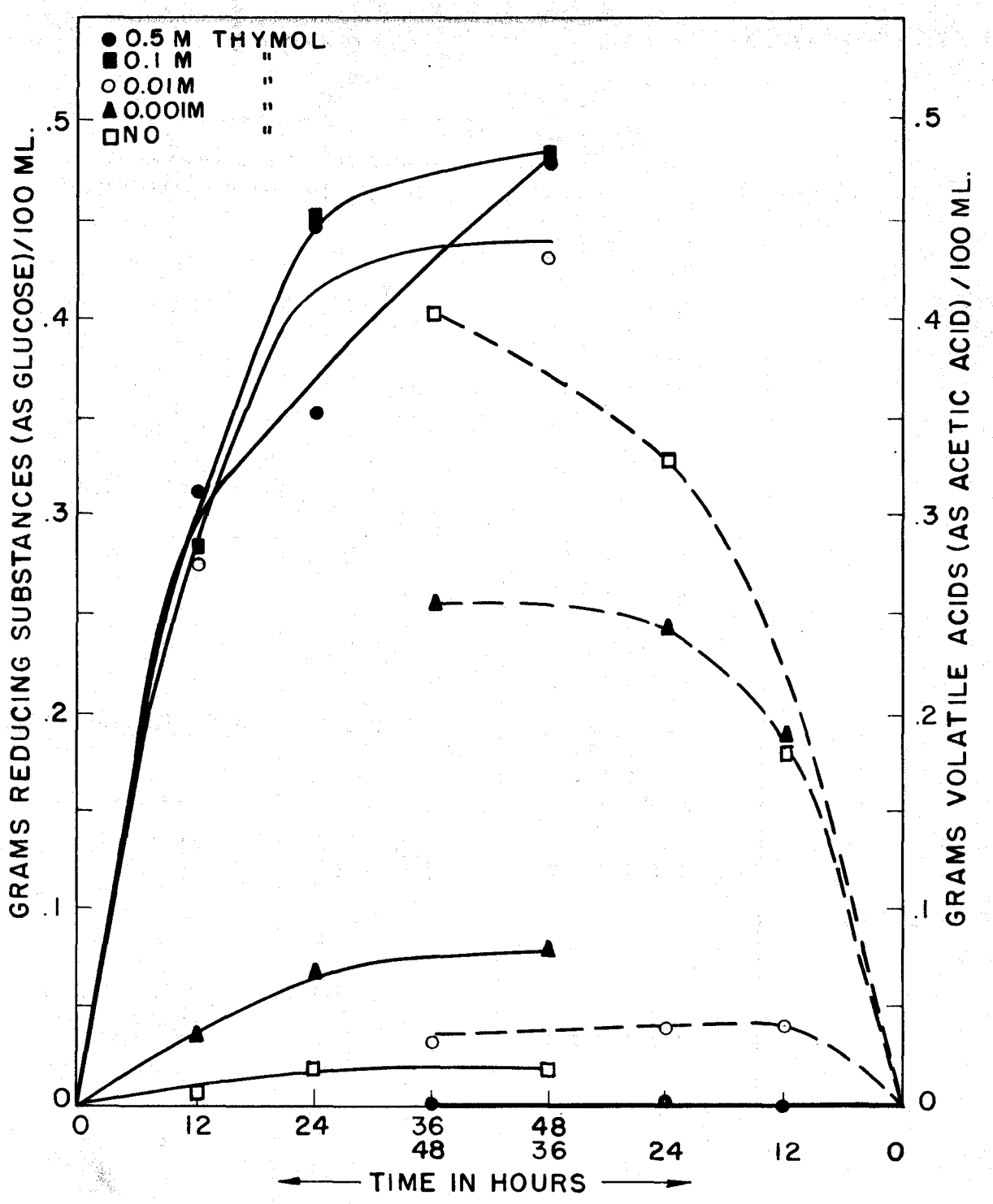


Figure 8. Fermentation of CMC-70L by rumen micro-organisms in the presence and absence of thymol

Table XIV. Fermentation of CMC-70L by rumen microorganisms in the presence or absence of iodoacetic acid

Flask	CMC %	Molar concentration of iodoacetic acid	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
			0	12	24	48	0	12	24	48
1	1	0.5	0.000	0.149	0.154	0.103	0.000	0.002	0.021	0.062
2	1	0.1	0.000	0.071	0.084	0.086	0.000	0.066	0.117	0.162
3	1	0.01	0.000	0.038	0.044	0.045	0.000	0.095	0.115	0.150
4	1	0.001	0.000	0.018	0.038	0.038	0.000	0.123	0.153	0.186
5	1	0.000	0.000	0.020	0.016	0.016	0.000	0.091	0.163	0.225
6	1	0.5 (no inoculum)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

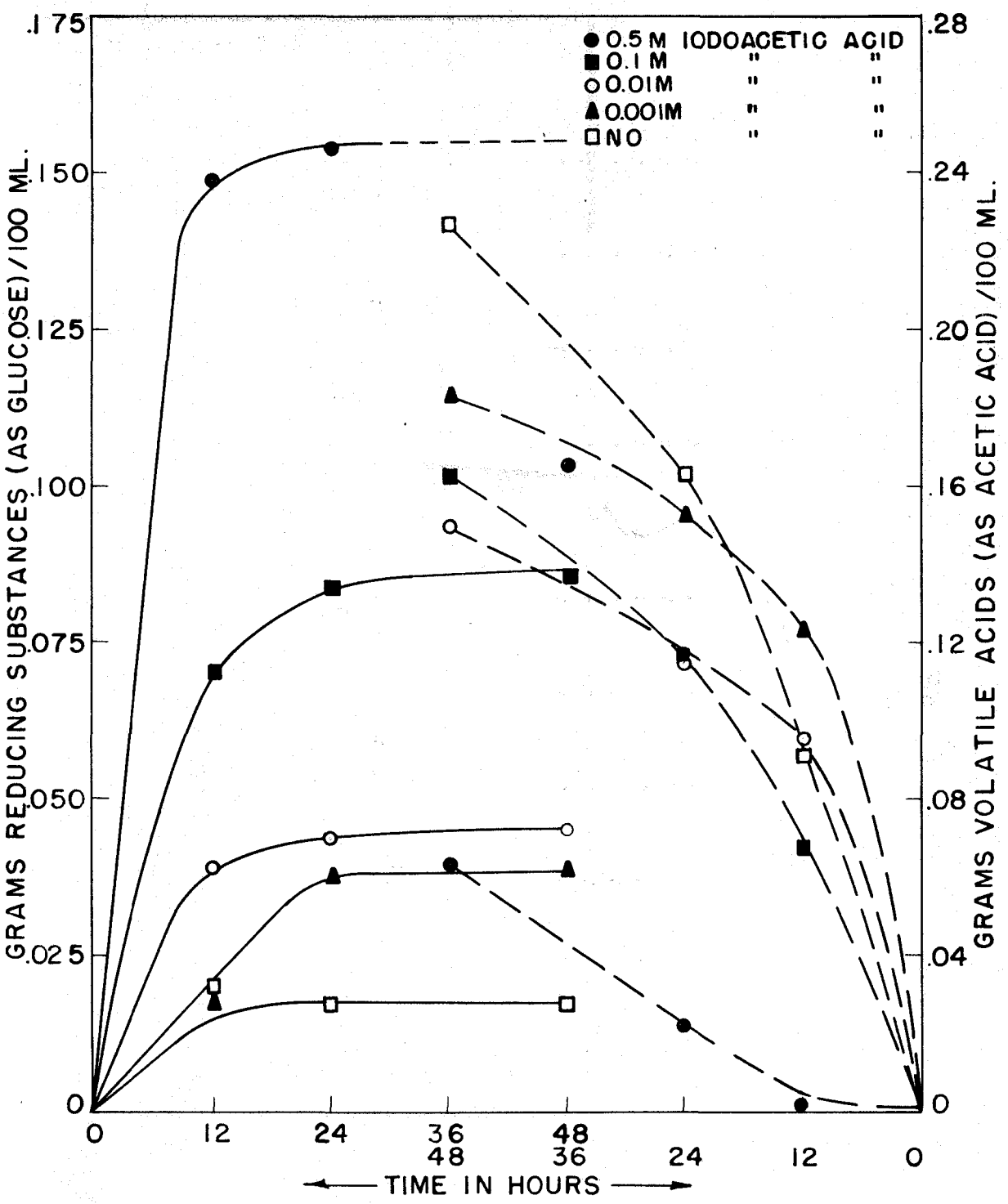


Figure 9. Fermentation of CMC-70L by rumen microorganisms in the presence and absence of iodoacetic acid

Table XV. Fermentation of CMC-70L by rumen microorganisms in the presence or absence of various organic compounds

Flask	CMC %	Organic compound	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
			0	12	24	48	0	12	24	48
1	1	Toluene 50 ml.	0.000	0.155	0.222	0.222	0.000	0.009	0.010	0.012
2	1	Form-aldehyde 15 ml.	0.000	0.011	0.024	0.025	0.000	0.020	0.042	0.050
3	1	Phenol 19 gm.	0.000	0.000	0.019	0.026	0.000	0.005	0.008	0.014
4	1	Chloro-form 50 ml.	0.000	0.212	0.324	0.372	0.000	0.000	0.000	0.027
5	1	m-Xylene 50 ml.	0.000	0.166	0.213	0.167	0.000	0.012	0.031	0.031
6	1	Benzoic acid 5 gm.	0.000	0.043	0.054	0.054	0.000	0.213	0.263	0.278
7	1	Control (no inhibitor)	0.000	0.027	0.027	0.024	0.000	0.124	0.189	0.203

that the added inhibitory agents prevented the complete conversion of simple sugars to the volatile acids as end-products.

It is interesting to note, at this point, the action of malonate during the cellulose fermentation process. The results obtained using this compound as inhibitor appeared to be the reverse to those obtained from the other compounds. In this case as the concentration of malonate increased, the accumulation of reducing substances decreased and the production of volatile acids increased. High concentration of this organic compound in the fermentation medium had a bacteriocidal effect on the rumen microorganisms.

The absolute mechanism of the inhibitory action of these chemical compounds is unknown, however, it is felt that these compounds either prevent the growth of the bacteria or interfere with the phosphorylation of the formed glucose and/or other simple sugars before they can be metabolized to the usual end-products. The phosphorylation of these soluble sugars may be required before they can gain entrance into the interior of the bacterial cell. The enzymes responsible for the hydrolysis of the cellulose molecule to smaller soluble constituents were not affected by these inhibitory compounds as demonstrated by the foregoing experiments.

Verzár and Süllman (130) and Verzár and Wirz (131) believed that iodoacetic acid inhibited the process of phosphorylation in fermentation and glycolysis. Later Laszt (66) reported that phosphorylation of sugars can be controlled by oral administration of monoiodacetic acid and sodium sulfide in diabetic animals. In the same year Beevers (12) concluded from his experiments that iodoacetic acid in concentrations greater than 10^{-3} M inhibited yeast zymase fermentation, suggesting that the phosphorylation mechanism of the sugars was impaired.

With these conclusions in mind it is noted from the experiments reported here that as the concentration of iodoacetic acid is increased in the culture media, the accumulation of reducing substances increased markedly and the production of volatile acids decreased. At a concentration of 0.05 M iodoacetic acid, practically no volatile acids appeared in the fermentation medium.

2. Effect of phosphorylation inhibitors on the fermentation of CMC by rumen microorganisms

Selective poisons have been used for many years in the study of enzyme systems. Most of this work has been confined to acellular preparations and enzyme extracts although a limited number of studies have also been made in which the respiration of intact cells was investigated in the presence

of various poisons. Despite the fact that the intact cell possesses a mosaic of enzyme systems, this approach appears promising in attempts to further elucidate the synthetic and degradative processes in the living cell (98).

Barron (9) stated that since the oxidative enzymes are systems made up of a number of components, inhibitors can produce their effect either on the enzyme protein, or on the oxidative-reduction systems. The activity of the protein may be altered in a number of ways (9): (a) the inhibitor may combine with the initial prosthetic group (inhibition by fluoride or phosphate of Mg^{++} proteins); (b) the inhibitor may destroy or combine with the essential side chain of the protein (e.g. -SH, -OH); (c) the protein may have its spatial configuration altered (e.g. denaturation); and (d) the active centers of the enzyme may be blocked by combination with substances of close chemical configuration to the substrates, the prosthetic groups or to the oxidation-reduction systems.

All enzyme reactions do not necessarily occur on the surface of the cell (9). Enzymes are distributed inside the cytoplasm as well as at the surface of the cell in the cell membrane. It seems, therefore, that hexokinase is distributed at the surface of the cell and that glucose, before penetrating into the cell's interior, has to be

phosphorylated. The latter reaction was shown to occur in the living cell by Wiggert and Werkman (137,138). It appears now that phosphorylation is a mechanism by which energy is made available to the organism. There is evidence that a biological oxidation makes its energy available to the organism through phosphorylation reactions (133).

Dixon (28) in his study of multi-enzyme systems, stated that the phosphokinases apparently required Mg^{++} and -SH in their molecules for activity. There is some reason for believing that the enzyme-substrate combination involves the formation of an -SH-Mg-PO_4 complex. The results of a number of experiments previously described here showed that the presence of sodium fluoride in an active cellulose digesting culture brought about a large accumulation of reducing sugars in the growth menstruum. An insoluble protein-magnesium-fluorophosphate could very well have been formed which would prevent the normal phosphorylation of the formed glucose from the enzymic hydrolysis of the cellulose molecule and therefore an accumulation of reducing sugars in the fermentation medium resulted. Munoz and Leloir (87) found that the specific inhibitor of phosphorylation, the fluoride ion, inhibited the oxidation of butyrate by liver enzymes.

Ajl and Werkman (1), while studying the oxidative decarboxylation of α-ketoglutaric acid, noticed that fluoride inhibited the reaction and thus suggested the participation of a phosphorylated intermediate in the above oxidative reaction.

Pett and Wynn (95), Massart and Dufait (78) and Utter and Werkman (123) showed conclusively that magnesium or manganese were required in phosphorylation reactions. The latter workers (123) observed that sodium fluoride seemed to inhibit the unstimulated enzyme and the magnesium enzyme completely, but merely decreased the activity of the manganese enzyme.

Thus when manganese was used in place of magnesium, fluoride was ineffective in inhibiting the enolase. Manganese does not form a sufficiently insoluble compound, with the fluoride, and enough remains available to activate the enolase system (123).

With these published results in mind an experiment was designed in order to show whether the addition of manganese to a cellulose fermentation culture in the presence of sodium fluoride would reverse the inhibiting action of this compound. The results are tabulated in Table XVI. It is noticed that the addition of the manganese ions to the CMC-fluoride culture media resulted in an increased accumulation of reducing sugars,

Table XVI. The effect of added manganese to the fermentation of CMC-70L by rumen microorganisms in the presence of sodium fluoride

Flask	CMC %	Molar concentration of NaF	Molar concentration of $MnSO_4$	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
				0	12	24	48	0	12	24	48
1	1	0	0	0.000	0.008	0.008	0.009	0.000	0.236	0.371	0.449
2	1	0.5	0	0.000	0.211	0.234	0.240	0.000	0.065	0.070	0.082
3	1	0.5	0.25	0.000	0.237	0.312	0.364	0.000	0.048	0.048	0.048
4	1	0.5	0.175	0.000	0.208	0.253	0.297	0.000	0.047	0.047	0.050

and a substantial decrease in the production of volatile acids. In this case manganese did not reverse the action of the fluoride ion, however it was pointed out by Utter and Werkman (123) that manganese ions in high concentration, had an inhibitory action, such was the case in this investigation.

Other phosphorylation inhibitors have been studied in this investigation, and in each case these enzyme inhibitory compounds have been shown to result in an accumulation of reducing substances and a decrease in the production of volatile acids in in vitro cellulose fermentations using rumen microorganisms.

A number of investigators (61,69,118,119,120) showed conclusively that 2-4 dinitrophenol inhibited phosphorylation reactions. A cellulose fermentation experiment was conducted having various concentrations of this organic compound added to the culture medium. The results are given in Table XVII and Figure 10. Again it was noted that, as the concentration of the inhibitor was increased in the fermentation media, a greater accumulation of reducing substances appeared suggesting an impairment of the phosphorylating mechanism of glucose or other simple sugars before they could be oxidized to short-chain fatty acids. It was also noticed from these results that the inhibitor required approximately 12 hours before it became effective.

Table XVII. Fermentation of CMC-70L by rumen microorganisms in the presence or absence of 2-4 dinitrophenol

Flask	CMC %	Molar concentration of 2-4 dinitrophenol	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
			0	12	24	48	0	12	24	48
1	1	0.1	0.000	0.018	0.130	0.134	0.000	0.272	0.393	0.399
2	1	0.01	0.000	0.013	0.094	0.105	0.000	0.210	0.280	0.291
3	1	0.001	0.000	0.018	0.077	0.080	0.000	0.142	0.206	0.214
4	1	0.000	0.000	0.011	0.021	0.026	0.000	0.088	0.209	0.272
5	1	0.1 (no inoculum)	0.000	0.011	0.012	0.011	0.000	0.003	0.000	0.003

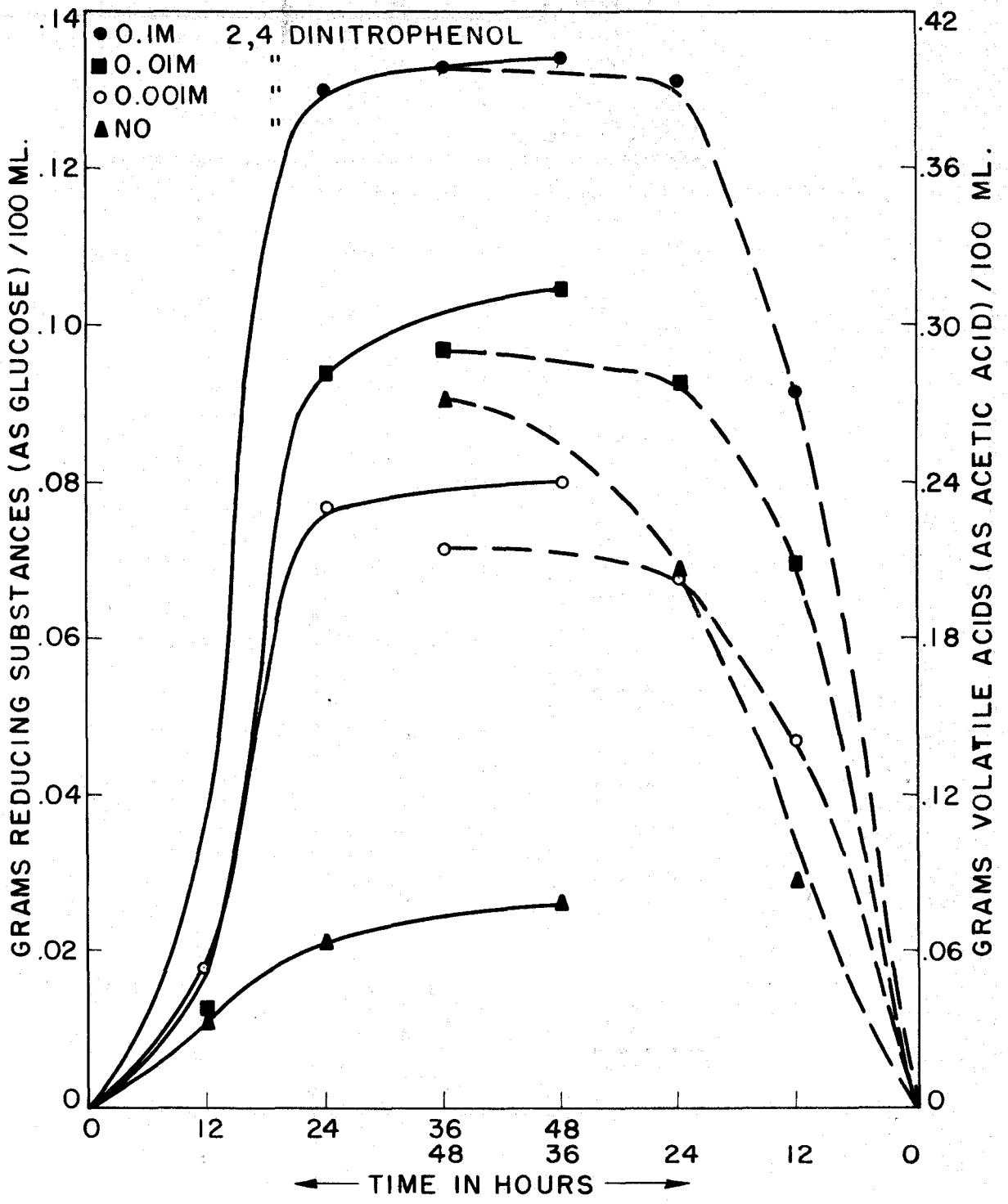


Figure 10. Fermentation of CMC-70L by rumen microorganisms in the presence and absence of 2-4 dinitrophenol

Two other compounds, phloridzin and sodium azide have been recognized as phosphorylating inhibitors by a number of workers. Lundsgaard (71) found that phloridzin inhibited glucose phosphorylation in tissue extracts. Later several workers (60,106) gave exacting evidence that this glucoside possessed the ability to specifically inactivate phosphorylation or phosphatase action.

A number of publications have appeared lately concluding that sodium azide also acted as an inhibitor to this phosphorylase action. Winzler (141) showed that azide inhibited anaerobic assimilation of glucose by yeast. Spiegelman (114) and Spiegelman and Kaman (115) discussed the site of uncoupling of phosphorylation from carbohydrate metabolism in the presence of sodium azide. They showed that coupling reaction between oxidative processes and the formation of high energy phosphate bonds are inhibited by azides.

Since there is evidence that phloridzin and sodium azide can act as bacterial enzyme inhibitors two experiments were conducted to show the effect of various concentrations of these compounds on <u>in vitro</u> cellulose digestion by ruminal microorganisms.

The results of these experiments are tabulated in Table XVIII and XIX. It is noticed that phloridzin brought about an accumulation of reducing sugars and prevented the production of short-chain fatty acids in the cultural medium at a concentration of 0.03 M. At smaller concentrations, though, there was a slight accumulation of reducing substances, with a normal or increased production of volatile fatty acids. This is an interesting point as these results may be interpreted in the sense that phloridzin may bring about a stimulatory effect in low concentrations, rather than an inhibitory effect (77 p. 2)

Sodium azide (Table XIX) also appeared to prevent the glucose or other reducing sugars to be utilized by the cellulose-digesting microorganisms to produce fatty acids. At 0.1 M and 0.5 M concentrations this active agent seemed to decrease markedly the production of acids by the bacteria and thus an accumulation of reducing compounds appeared in the cultural medium.

In reference to phosphorylation inhibition by various compounds it is generally noticed that glucose tends to inhibit the growth of isolated cellulose-digesting microorganisms, however no explanation of this inhibitory mechanism has been suggested. A possible explanation for this reaction

Table XVIII. The effect of various concentrations of phloridzin on the fermentation of CMC-70L by rumen microorganisms

Flask	CMC %	Molar concentration of phloridzin	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at hours			
			0	12	24	48	0	12	24	48
1	1	0.0025	0.000	0.030	0.040	0.040	0.000	0.285	0.355	0.420
2	1	0.005	0.000	0.043	0.043	0.046	0.000	0.280	0.351	0.458
3	1	0.01	0.000	0.073	0.077	0.077	0.000	0.225	0.285	0.474
4	1	0.03	0.000	0.213	0.267	0.277	0.000	0.000	0.012	0.000
5	1	0.00	0.000	0.008	0.008	0.009	0.000	0.236	0.361	0.449

Table XIX. The effect of various concentrations of sodium azide on the fermentation of CMC-70L by rumen microorganisms

Flask	CMC %	Molar concentration of sodium azide	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
			0	12	24	48	0	12	24	48
1	1	0.000	0.000	0.014	0.022	0.022	0.000	0.048	0.257	0.593
2	0	0.5	0.000	0.000	0.000	0.000	0.000	0.141	0.153	0.177
3	1	0.5	0.000	0.392	0.467	0.466	0.000	0.005	0.112	0.115
4	1	0.1	0.000	0.178	0.200	0.207	0.000	0.112	0.222	0.207
5	1	0.01	0.000	0.054	0.095	0.095	0.000	0.094	0.100	0.142
6	1	0.5 (no inoculum)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

in these isolated cases may be the inhibitory action of glucose on the phosphorylase system. Cori and Cori (24) observed that glucose had the ability to inhibit α-glucosan phosphorylase, an enzyme that transforms glycogen into glucose-1-phosphate. A similar enzyme may be elaborated by various cellulose-digesting microorganisms and thus could be inhibited by an increased concentration of glucose in the cultural medium.

Since a number of chemical compounds were tested for their inhibitory effect during the fermentation of CMC-70L by rumen microorganisms, data are tabulated in Table XX in order to compare the effectiveness of one compound with another. The values for the exact accumulation of reducing substances and volatile fatty acids produced in the various fermentation experiments were determined by taking the amounts that were formed in the particular medium at 48 hours fermentation. From these figures, thymol, sodium azide, chloroform, sodium fluoride, phloridzin, toluene, m-xylene, 2-4 dinitrophenol and iodoacetic acid, in decreasing order, appear to be the most effective in preventing the conversion of simple soluble sugars to short-chain fatty acids. As a result reducing substances accumulate in the cellulose fermentation media. In subsequent experiments thymol or sodium fluoride were used to stop the fermentation of cellulose by

Table XX. The accumulation of reducing substances and the production of volatile acids in poisoned and non-poisoned fermentation media

Inhibitor	Reducing substances (as glucose formed, grams per 100 ml.		Total volatile acids (as acetic acid) formed, grams per 100 ml.	
	Medium with inhibitor	Medium without inhibitor	Medium with inhibitor	Medium without inhibitor
Sodium cyanide	0.062	0.015	0.092	0.616
Iodoform	0.069	0.023	0.088	0.347
Sodium fluoride	0.328	0.021	0.088	0.316
Malonic acid	0.060	0.018	0.267	0.404
Thymol	0.474	0.018	0.000	0.404
Iodoacetic acid	0.103	0.016	0.062	0.225
Toluene	0.222	0.024	0.012	0.203
Formaldehyde	0.025	0.024	0.050	0.203
Phenol	0.026	0.024	0.014	0.203
Chloroform	0.372	0.024	0.027	0.203
m-Xylene	0.167	0.024	0.031	0.203
Benzoic acid	0.054	0.024	0.278	0.203
2-4 Dinitrophenol	0.134	0.026	0.399	0.272
Phloridzin	0.277	0.09	0.000	0.449
Sodium azide	0.466	0.022	0.115	0.593

the rumen microorganisms at the glucose stage. By this means the pathway of cellulose degradation by rumen bacteria with special reference to the carbohydrate intermediates formed could be studied.

C. Filter Paper Chromatographic Studies

A number of preliminary experiments were conducted in order to obtain information as to the identity of the reducing sugars that accumulated in a cellulose-digesting culture having an antiseptic added. The fermentation media containing ground filter paper or CMC-70L, as the cellulosic substrate, and toluene as the inhibitor, were inoculated with fresh strained rumen liquid and incubated under carbon dioxide at 40°C. for 48 hours. After this period of time the media were tested qualitatively for reducing sugars employing the filter paper chromatographic technique.

It was found in all cases, using xylose, glucose and cellobiose for controls, that only glucose and a trace of xylose were present in the cellulose digestion media. There was, however, no trace of the disaccharide cellobiose in any of the samples tested. However, after 48 hours incubation, the height of the cellulose fermentation had passed, and if cellobiose was formed during the degradation of the cellulosic substrate by the microorganisms of the rumen, it could very

easily have been hydrolyzed to glucose during that period of time. With this probability in mind a more comprehensive investigation was conducted to study the carbohydrate intermediates formed during the rumen microbial breakdown of cellulose.

Before studying the pathway of cellulose digestion by the rumen microorganism, an experiment was conducted to show the effect of various concentrations of CMC-70L on the digesting ability of the rumen bacteria. In order to follow the fermentation of CMC-70L by these microorganisms it was felt that a greater initial concentration of CMC-70L in the fermentation medium might result in larger yields of degradative cellulose products and thus a greater concentration of these smaller sugar molecules could be more easily detected by chromatographic means. The media were prepared, as described before, using thymol as the active inhibitor. Each flask was inoculated with fresh rumen liquid and incubated anaerobically at 40°C. The results of this experiment are tabulated in Table XXI.

It is noticed from these results that the accumulation of reducing substances in the culture media increased with increased concentration of CMC-70L as the cellulosic substrate. In preparing the media prior to inoculation the larger concentrations of the CMC-70L made the final culture media very

Table XXI. The fermentation of various concentrations of CMC-70L by rumen microorganisms in the presence of thymol (0.1 M)

Flask	CMC %	Inhibitor	Inoculum	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours			
				0	12	24	48
1	0.0	+	+	0.000	0.159	0.164	0.214
2	1.0	-	+	0.000	0.026	0.026	0.026
3	1.0 Charged 1% every 12 hr.	+	+	0.000	0.285	0.403	0.539
4	0.5	+	+	0.000	0.222	0.236	0.252
5	1.0	+	+	0.000	0.228	0.261	0.278
6	1.5	+	+	0.000	0.224	0.292	0.331
7	2.0	+	+	0.000	0.247	0.334	0.338
8	3.0	+	+	0.000	0.302	0.366	0.422
9	4.0	+	+	0.000	0.338	0.447	0.538
10	5.0	+	+	0.000	0.368	0.464	0.580
11	1.0	+	-	0.000	0.002	0.002	0.002

viscous. Since this hindered the securing of a homogeneous sample at various time intervals for analytical purposes, the 2 per cent concentration was chosen as the most suitable level to use in the following study.

In the previous experiment an aliquot from a 48 hour cellulose-digesting culture, under the influence of an inhibitor such as toluene, was tested for reducing sugars by chromatographic means. The results showed that glucose was present as well as a smaller molecular size reducing sugar. Since it was felt that at the 48 hour point, most of the cellulolytic action had ceased, this result was not considered to be the true picture of the pathway of cellulose digestion by rumen microorganisms.

A number of cellulose fermentation cultures having CMC-70L or Alphacel as the cellulosic substrate and sodium fluoride or thymol as the inhibitor were prepared. At hourly intervals for 16 hours an aliquot was withdrawn from each culture and prepared to be analyzed quantitatively and qualitatively for reducing sugars. It was found that the usual method for clarifying the media was inadequate and thus the samples to be analyzed chromatographically were deproteinized by using methyl alcohol. The sample was first brought to a boil and hot methyl alcohol was added until its concentration reached 75 per cent in the mixture. The sample was

then heated for a few minutes, cooled and centrifuged. The clear supernatant was decanted carefully and evaporated to one-third of its original volume. After the sample was cooled, it was qualitatively analyzed for reducing sugars by means of filter paper chromatography.

The results of this experiment, as seen in Table XXII and Figures 11 and 12, indicate that during the fermentation of Alphacel and CMC-70L three reducing sugars were formed, as evidenced by their appearance on the chromatograms. Of the three sugars, glucose appeared in the greatest concentration, while cellobiose and xylose were noticed to be present in very slight traces. There was no evidence of any reducing molecule larger than cellobiose on the chromatograms.

Since xylose was detected in all these samples it appeared that traces of xylan must be present in the cellulosic substrate as a contaminant, therefore acid hydrolysis of CMC-70L was performed using 1 M and 2 M sulfuric acid. A 5 per cent solution of CMC-70L was prepared and brought to a boil. An equal volume of boiling sulfuric acid was added and the solution allowed to boil for 30 min. After this time half of the solution was poured over calcium carbonate and mixed until a yellow methyl red end-point was obtained. The remaining acid mixture was allowed to boil for an additional 30 min. to make the total hydrolyzing time 60 min. This

Table XXII. Fermentation of CMC-70L and alphacel by rumen microorganisms in the presence of 0.5 M sodium fluoride or 0.1 M thymol

Flask	CMC %	Alphacel %	Inhibitor	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours								
				0	1	2	3	4	5	6	7	8
1	2	-	Sodium fluoride	0.000	0.053	0.077	0.160	0.208	0.220	0.236	0.244	0.253
2	-	1	Sodium fluoride	0.000	0.022	0.039	0.057	0.062	0.072	0.078	0.089	0.089
3	2	-	Thymol	0.000	0.107	0.134	0.164	0.224	0.233	0.254	0.260	0.270
4	-	1	Thymol	0.000	0.048	0.079	0.090	0.111	0.120	0.130	0.137	0.137

Table XXII. (Continued)

Flask	CMC %	Alphacel %	Inhibitor	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours							
				9	10	11	12	13	14	15	16
1	2	-	Sodium fluoride	0.259	0.270	0.280	0.286	0.287	0.294	0.300	0.309
2	-	1	Sodium fluoride	0.098	0.103	0.106	0.106	0.107	0.107	0.109	0.110
3	2	-	Thymol	0.274	0.282	0.291	0.298	0.306	0.316	0.327	0.340
4	-	1	Thymol	0.157	0.158	0.159	0.164	0.164	0.167	0.168	0.170

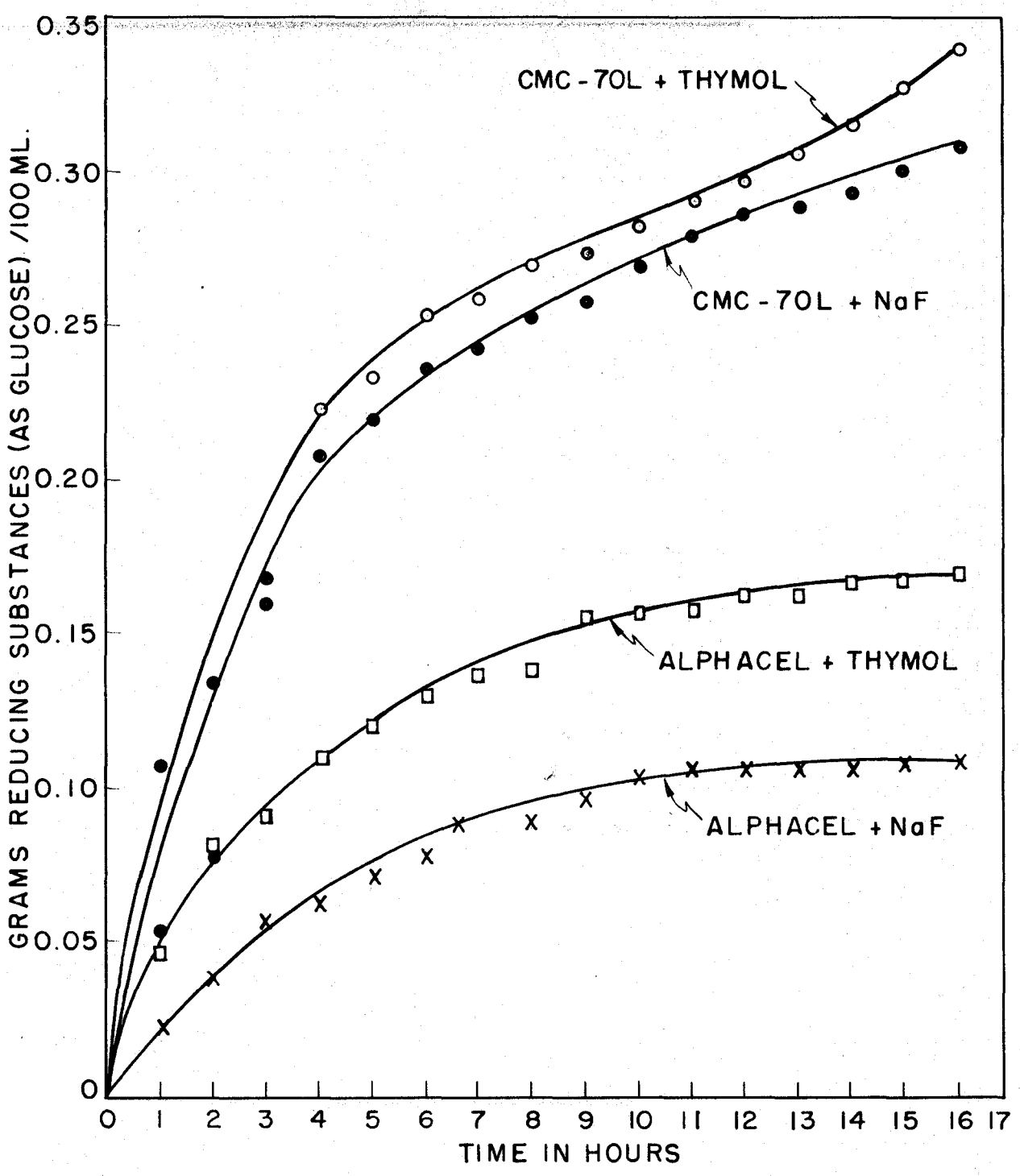
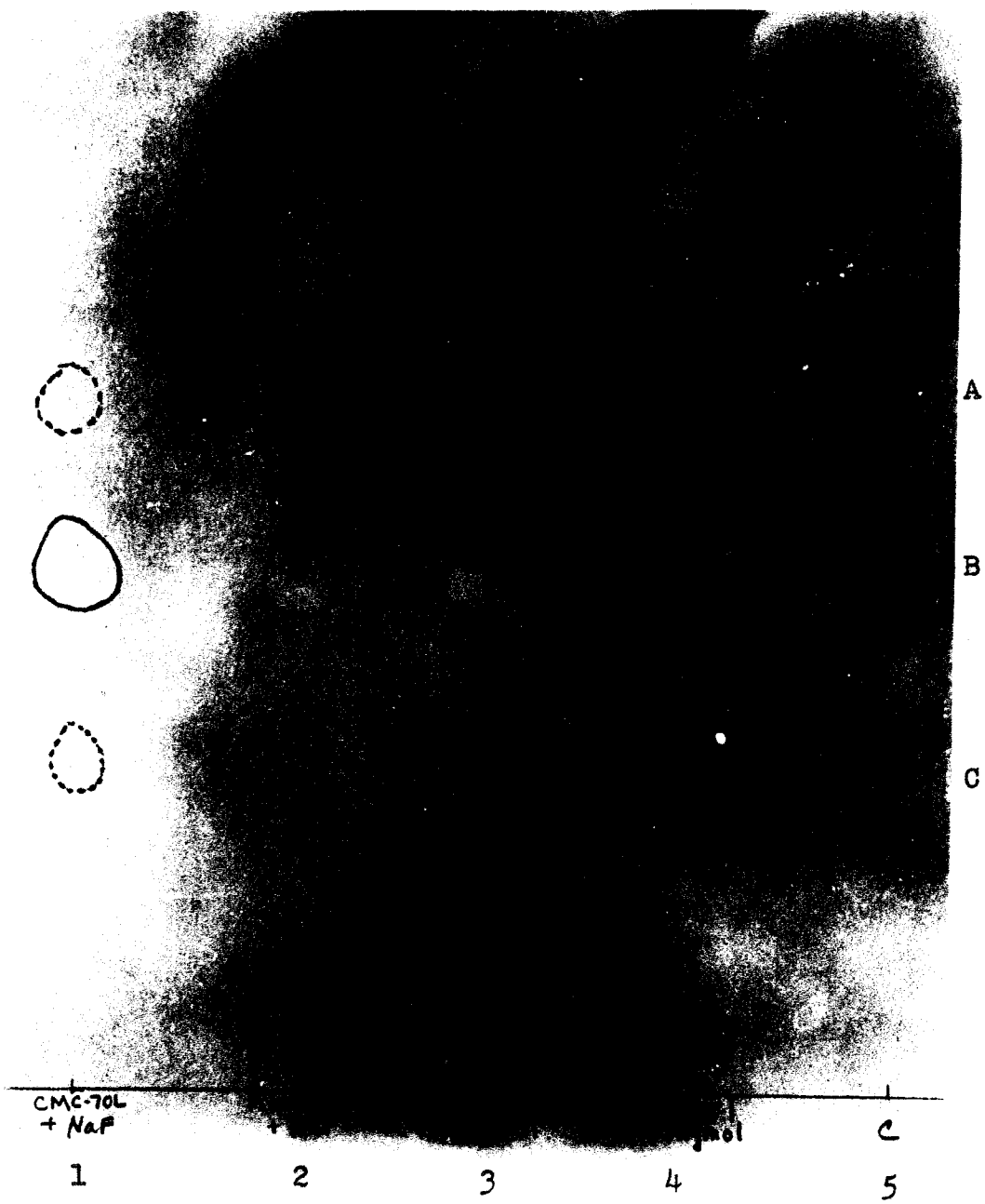


Figure 11. Fermentation of CMC-70L and alphacel by rumen microorganisms in the presence of sodium fluoride or thymol

Figure 12. Degradation of CMC-70L and Alphacel by rumen microorganisms in the presence of sodium fluoride and thymol

(1) CMC-70L + sodium fluoride
(2) Alphacel + sodium fluoride
(3) CMC-70L + thymol
(4) Alphacel + thymol
(5) Control
    (A) Xylose
    (B) Glucose
    (C) Cellobiose


A
B
C
CMC-70L
+ NaF
1
2
3
4
5

solution was also neutralized with calcium carbonate as described above.

Each neutralized solution was filtered and the clear liquid analyzed for degradative sugars employing the chromatographic method. The results showed that several reducing sugars were formed during the acid hydrolyzing action. A definite compound appeared above glucose on the chromatogram. At first this was thought to be glucuronic acid because during the preparation and purification of cellulose (as filter paper or Alphacel) or cellulose derivatives (as CMC-70L), a number of primary alcohol groups present in the glucose residues are oxidized to the carboxyl group (112 p. 31). Therefore when the refined cellulose product was degraded by the acid, it was thought that glucuronic acid would be freed and thus would be detected on the chromatogram. The unknown compound above glucose was proven not to be glucuronic acid as the latter* was tested chromatographically and was found to have a lower Rf value than glucose (Figure 13). By using as controls known xylose, glucose and cellobiose, the unknown compound was found to be the pentose, xylose.

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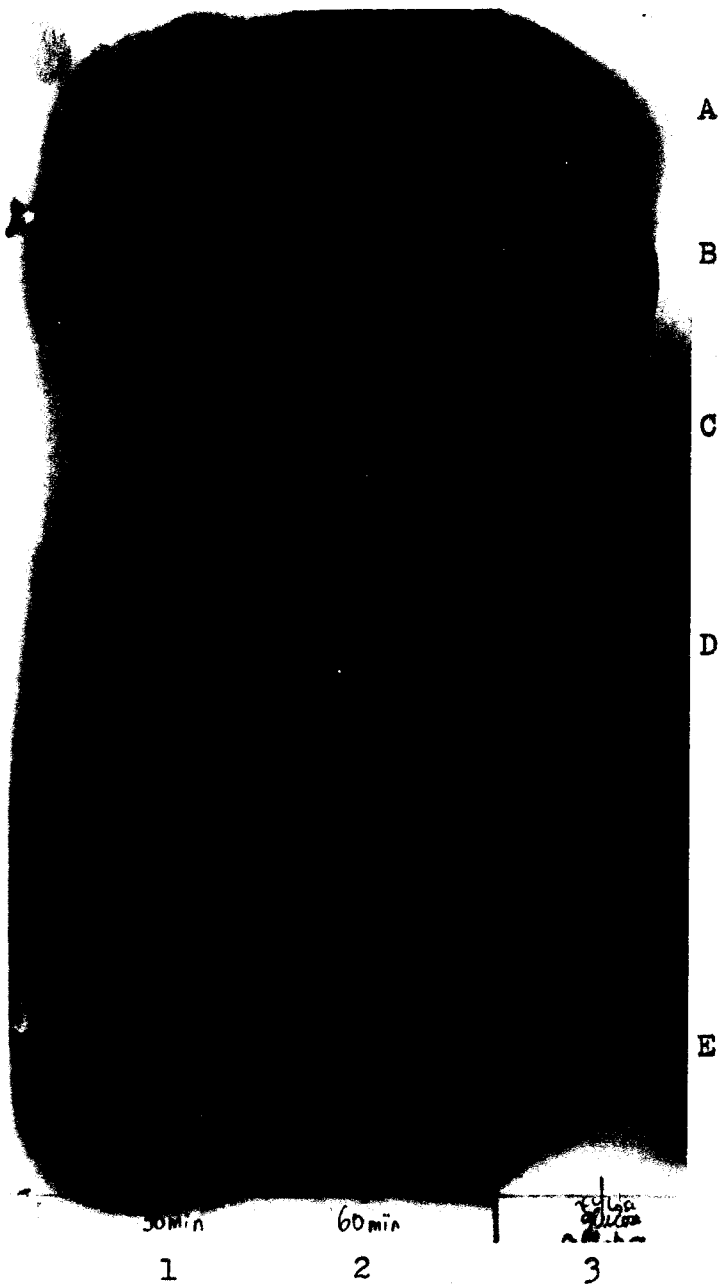
*A sample of glucuronic acid was obtained from Dr. G.N. Bollenback of Corn Products Refining Company, Argo, Illinois. A subsequent letter from Dr. Bollenback stated that the glucuronic acid sample was contaminated slightly with glucuronic acid lactone.

Figure 13. Hydrolysis of CMC-70L by 2 M sulfuric acid

(1) Hydrolyzed for 30 min.
(2) Hydrolyzed for 60 min.
(3) Control
    (A) Glucuronic acid lactone
    (B) Xylose
    (C) Glucose
    (D) Cellobiose
    (E) Glucuronic acid


A
B
C
D
E
30min
60min
1
2
3

The compound above xylose in Figure 13 is glucuronic acid lactone. This compound was present as a contaminant in the control sample of glucuronic acid used.

D. Factors Affecting the Activities of Rumen Microorganisms

From previous experiments it has been shown that a number of known phosphorylating inhibiting compounds arrested the fermentation of cellulose by rumen microorganisms at the glucose stage. It is the opinion of the writer that phosphorylating enzymes such as hexokinases are distributed on the surface of the bacterial cell and that glucose before passing into the cell's interior must be phosphorylated. An experiment was designed to study the effect of phosphorylated sugars on the activities of rumen bacteria in the presence and absence of sodium fluoride. Glucose-1-phosphate, glucose-6-phosphate and fructose-1,6-diphosphate were added to active cellulose-digesting cultures. The inoculum used in this experiment was fresh rumen fluid.

The results of this experiment, tabulated in Table XXIII, show that a greater amount of reducing sugars accumulated in the cellulose-digesting cultures having the phosphorylated sugars and inhibitor present. The active cultures with added phosphorylated sugars with no inhibitor showed greater production of volatile acids as compared with the control

Table XXIII. The effect of various phosphorylated sugars on the fermentation of CMC-70L by rumen microorganisms in the presence and absence of sodium fluoride

Flask	CMC 1%	NaF 0.5M	Inoculum	Sugar	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
					0	12	24	48	0	12	24	48
1	+	+	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	-	+	+	-	0.000	0.004	0.138	0.157	0.000	0.000	0.000	0.000
3	+	-	+	-	0.000	0.017	0.017	0.018	0.000	0.093	0.187	0.197
4	+	+	+	-	0.000	0.203	0.249	0.278	0.000	0.000	0.000	0.000
5	+	+	+	G1P	0.000	0.303	0.362	0.415	0.000	0.000	0.017	0.039
6	+	+	+	G6P	0.000	0.260	0.295	0.360	0.000	0.000	0.000	0.004
7	+	+	+	FDP	0.000	0.298	0.338	0.393	0.000	0.000	0.021	0.016
8	+	-	+	G1P	0.000	0.012	0.014	0.014	0.000	0.193	0.248	0.328
9	+	-	+	G6P	0.000	0.007	0.007	0.007	0.000	0.182	0.244	0.333
10	+	-	+	FDP	0.000	0.007	0.009	0.009	0.000	0.205	0.276	0.359

G1P - Glucose-1-phosphate
G6P - Glucose-6-phosphate
FDP - Fructose-1,6-diphosphate

cultures. This information showed that the rumen microorganisms appeared to utilize the phosphorylated sugars in their metabolic processes. The three phosphorylated sugars appeared to stimulate the activities of the bacteria approximately to the same degree. Since this was the case, fructose-1,6-diphosphate was used subsequently as the representative phosphorylated sugar.

It has been known for many years that yeast products contain known and unknown nutrients that are beneficial for bacterial growth. Various investigators studying aspects of rumen fermentation and cellulose digestion utilized yeast extract in their cultural studies (55,100,103,107). An experiment was designed to observe the effect of yeast extract on cellulose utilization by rumen bacteria. Yeast extract, in a concentration of 1 per cent, was added to the active cellulolytic cultures in the presence and absence of sodium fluoride. At various intervals an aliquot was withdrawn from each culture and analyzed for reducing sugars and short-chain, volatile fatty acids. From the results of this investigation, (see Table XXIV and Figure 14) it is clearly seen that yeast extract increased the utilization of cellulose as evidenced by a greater accumulation of reducing sugars in the inhibited cultures and a greater production of volatile fatty acids in the non-inhibited cultures as compared with the control cultures.

Table XXIV. The effect of yeast extract on the fermentation of CMC-70L by rumen microorganisms in the presence and absence of sodium fluoride

Flask	CMC 1%	NaF 0.5M	Inoculum	Yeast extract 1%	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
					0	12	24	48	0	12	24	48
1	+	+	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	-	+	+	-	0.000	0.004	0.138	0.157	0.000	0.000	0.000	0.000
3	+	-	+	-	0.000	0.017	0.017	0.018	0.000	0.093	0.187	0.197
4	+	+	+	-	0.000	0.203	0.249	0.278	0.000	0.000	0.000	0.000
5	+	+	+	+	0.000	0.233	0.278	0.321	0.000	0.005	0.026	0.039
6	+	-	+	+	0.000	0.000	0.000	0.004	0.000	0.425	0.484	0.601

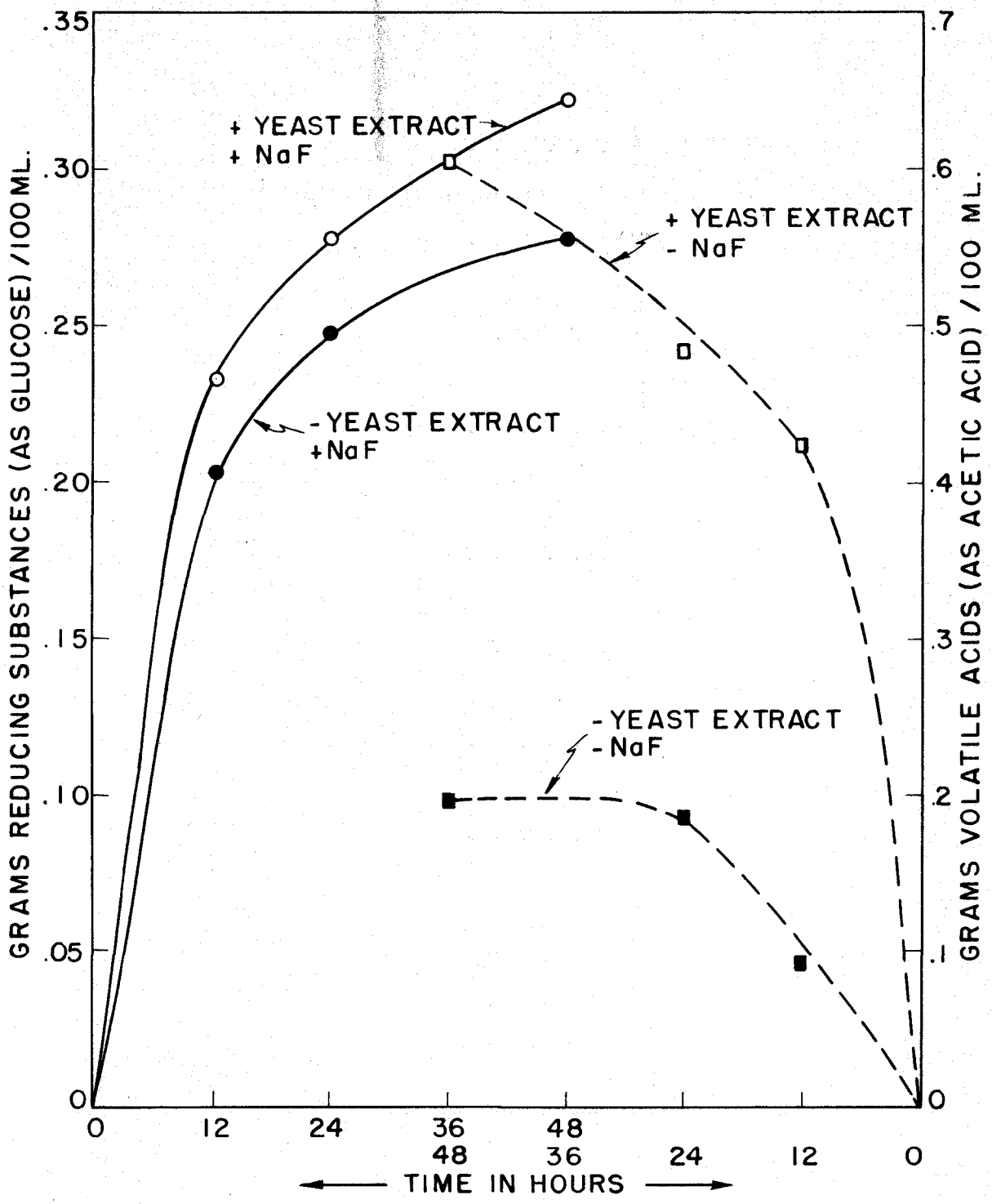


Figure 14. Effect of yeast extract on the fermentation of CMC-70L by rumen microorganisms in the presence and absence of sodium fluoride

In the experiments reported above the inoculum used was rumen liquid which had been strained through several thicknesses of cheesecloth. By using this liquid to inoculate the laboratory artificial rumen a certain amount of cellulose present in the liquid would be introduced into the fermentation media. Since this was the case as seen from the results of the previous experiments (Table XXIII and XXIV) washed suspensions of rumen microorganisms were employed in subsequent experiments. These washed microorganisms were prepared as follow:

A fresh sample of ruminal liquid, obtained from the fistulated cow, was strained through four thicknesses of No. 50 cheesecloth. The sample was then centrifuged at 1,000 r.p.m. for 5 min. to remove the course material. The resulting solution was decanted and centrifuged at 10,000 r.p.m. for 20 min. The centrifugate was then decanted and the bacterial cells were washed two times with sterile 0.067 M phosphate buffer solution. After the final centrifugation the wash solution was discarded and the cells resuspended in sterile buffer solution. Each cultural medium under test was inoculated with the amount of bacterial cells that would be present in 75 ml. of fresh rumen liquid.

An experiment was designed to show the effect of the presence and absence of various nutrients such as fructose-

1,6-diphosphate, sterile rumen liquor and yeast extract on the cellulolytic action of the washed rumen bacteria. The results of this experiment, tabulated in Table XXV, showed that sterile rumen liquid and yeast extract contained nutrients that were required by the cellulose-digesting bacteria. A combination of both the sterile rumen liquid and yeast extract added to the fermentation cultures brought about the best bacterial activity. The addition of fructose-1,6-diphosphate to the various cultures resulted in a greater accumulation of reducing sugars in the inhibited cultures and a greater production of volatile fatty acids in the non-inhibited cultures.

E. Isolation of the Cellulose-Digesting Microorganisms of the Rumen

A number of isolates were cultivated from the rumen of the bovine employing principally the techniques of Hungate (55) and Doetsch et al. (30). A description of the technique employed to isolate these cultures includes preparation of the dilution blanks and culture media, source of sample and processing procedure.

1. Preparation of the dilution blanks

In order to incubate rumen samples under controlled anaerobic conditions, dilution blanks of the composition given

Table XXV. The effect of sterile rumen liquid (SRL), yeast extract (YE) and fructose-1,6-diphosphate (FDP), on the fermentation of CMC-70L by washed rumen microorganisms

No.	CMC 1%	NaF .5M	Nutrient	YE 1%	FDP .3%	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours					Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours				
						0	12	24	48	72	0	12	24	48	72
1	-	+	SRL	-	-	0.000	0.038	0.042	0.042	0.042	0.000	0.011	0.011	0.022	0.022
2	+	-	SRL	-	-	0.000	0.008	0.012	0.020	0.020	0.000	0.010	0.016	0.151	0.184
3	+	+	SRL	-	-	0.000	0.047	0.070	0.084	0.108	0.000	0.005	0.033	0.034	0.034
4	+	+	SRL	-	+	0.000	0.050	0.078	0.103	0.115	0.000	0.000	0.005	0.000	0.000
5	+	-	SRL	-	+	0.000	0.009	0.012	0.026	0.026	0.000	0.011	0.032	0.090	0.128
6	+	+	SRL	+	-	0.000	0.024	0.051	0.102	0.122	0.000	0.000	0.000	0.000	0.000
7	+	-	SRL	+	-	0.000	0.000	0.000	0.000	0.000	0.000	0.079	0.258	0.326	0.354
8	-	+	-	-	-	0.000	0.000	0.000	0.003	0.003	0.000	0.005	0.000	0.000	0.000
9	+	-	-	-	-	0.000	0.005	0.013	0.019	0.020	0.000	0.000	0.059	0.071	0.073
10	+	+	-	-	-	0.000	0.023	0.040	0.058	0.064	0.000	0.000	0.005	0.023	0.023
11	+	+	-	-	+	0.000	0.039	0.064	0.107	0.111	0.000	0.000	0.006	0.000	0.000
12	+	-	-	-	+	0.000	0.002	0.010	0.016	0.016	0.000	0.001	0.112	0.129	0.135
13	-	+	YE	-	-	0.000	0.014	0.027	0.033	0.033	0.000	0.006	0.011	0.023	0.011
14	+	-	YE	-	-	0.000	0.011	0.011	0.011	0.011	0.000	0.017	0.104	0.164	0.231
15	+	+	YE	-	-	0.000	0.045	0.074	0.117	0.120	0.000	0.001	0.000	0.000	0.000
16	+	+	YE	-	+	0.000	0.046	0.076	0.135	0.137	0.000	0.012	0.006	0.000	0.006
17	+	-	YE	-	+	0.000	0.000	0.000	0.003	0.002	0.000	0.000	0.122	0.214	0.395

below were prepared which were light orange when anaerobic and light pink when exposed to trace amounts of oxygen. In addition Tween 80 was added to disperse the microorganisms from the large cellulose fibers. The blanks were prepared on the same day they were used and sterilized at 15 p.s.i. for 15 min. in 100 ml. square, milk dilution bottles. Short glass rods were inserted in the one-hole rubber stoppers of the dilution bottles so that during the autoclaving procedure, the stoppers could be placed on the lip of the bottle. Immediately after sterilization these stoppers were placed in position to insure anaerobic condition in the dilution blanks. The latter were gassed with carbon dioxide for 2 min. prior to use.

The composition of the blanks was as follows (30):

Ammonium sulfate	0.05	gm.
Potassium phosphate, monobasic	0.02	gm.
Potassium phosphate, dibasic	0.05	gm.
Calcium chloride	0.005	gm.
Magnesium sulfate	0.005	gm.
Sodium chloride	0.1	gm.
Sodium bicarbonate	0.4	gm.
L-Cystine	0.075	gm.
Sodium thioglycolate	0.05	gm.
Glucose	0.05	gm.
Resazurin	0.001	gm.
Tween 80	0.01	gm.
Distilled water to	100	ml.

2. Preparation of culture media

Three media were employed in this investigation.

(a) Medium A

This medium, proposed by Hungate (55), was used with good results. It consisted of the following:

Inorganic salt solution A*	20.0	ml.
Filtered rumen liquid	15.0	ml.
Resazurin	0.0001	gm.
CMC-70L	1.0	gm.

This mixture was sterilized at 15 p.s.i. for 15 min. After it was cooled, 50 ml. of Solution B**, which

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*Solution A:

Ammonium sulfate	0.05	gm.
Potassium phosphate, monobasic	0.05	gm.
Potassium phosphate, dibasic	0.02	gm.
Calcium chloride	0.005	gm.
Magnesium sulfate	0.005	gm.
Sodium chloride	0.1	gm.
Distilled water to	100	ml.

**Solution B:

Sodium thioglycolate	0.1	gm.
L-Cystine	0.155	gm.
Glucose	0.4	gm.
Cellobiose	0.4	gm.
Cystiene hydrochloride	0.04	gm.
Sodium carbonate	0.6	gm.
Sodium bicarbonate	0.5	gm.
Distilled water to	100	ml.

was previously sterilized by filtration through a Seitz filter, was added. The total mixture was made up to a volume of 100 ml. with sterile distilled water.

(b) Medium B.

This medium, proposed by Doetsch et al. (30) was used primarily to avoid the necessity of using rumen liquid. It consisted of the following:

Eugon agar (B.B.L.)	4.54 gm.
Inorganic salt solution A	20.0 ml.
Solution B	50.0 ml.
Distilled water to	100 ml.

The method used to prepare this medium was the same as that used to prepare Medium A.

(c) Medium C.

This medium was essentially the same as Medium B, however it differed in that 3.05 gm. powdered Trypticase-phytone medium (see Materials) was used in place of the Eugon Agar (B.B.L.)

3. Source of sample

Fresh rumen contents were obtained from a fistulated bovine and strained through four thicknesses of No. 50 cheesecloth.

4. Processing procedure

Ten grams of the well-mixed sample was added from a sterile graduate to a gassed 90 ml. dilution blank. The sample was shaken vigorously for 2 min. Serial 1 ml. transfers were made in the same fashion using gassed 9 ml. and 99 ml. blanks to a dilution of 10^{-10} of the original sample.

One milliliter of dilutions 10^{-7}, 10^{-8} and 10^{-9} were added to 6 ml. of melted, cooled Media A and B contained in rubber stoppered (125 mm. x 16 mm.) test tubes. The samples were mixed in the agar under carbon dioxide and then the agar was solidified in a thin film on the walls of the test tube by rotating under cold water (55). In a number of cases the rubber stoppers of the culture tubes were covered with molten paraffin, however in later experiments this step was omitted as anaerobic conditions were maintained in the culture media without this precaution. The tubes were incubated at 39°C. for 48 hours. After this time interval or after well defined microbial colonies had developed in the culture media, a number of well isolated colonies were transferred to the broth Medium C. When good growth had occurred as evidenced by turbidity in the broth medium, the relatively pure cultures were once again cultured into Medium A. From these cultures 32 well defined and well isolated colonies were

transferred to Medium C and incubated at 39°C. for 48 hours. The pure cultures were stored in the refrigerator as control cultures and transferred semi-monthly (30).

The fermentation habits of each of these isolated cultures were investigated and it was noticed that all 32 cultures fermented starch, dextrin, maltose, cellobiose and glucose, however only two cultures (designated in future experiments as 31 and 32) were found to ferment cellulose and CMC-70L at an active rate.

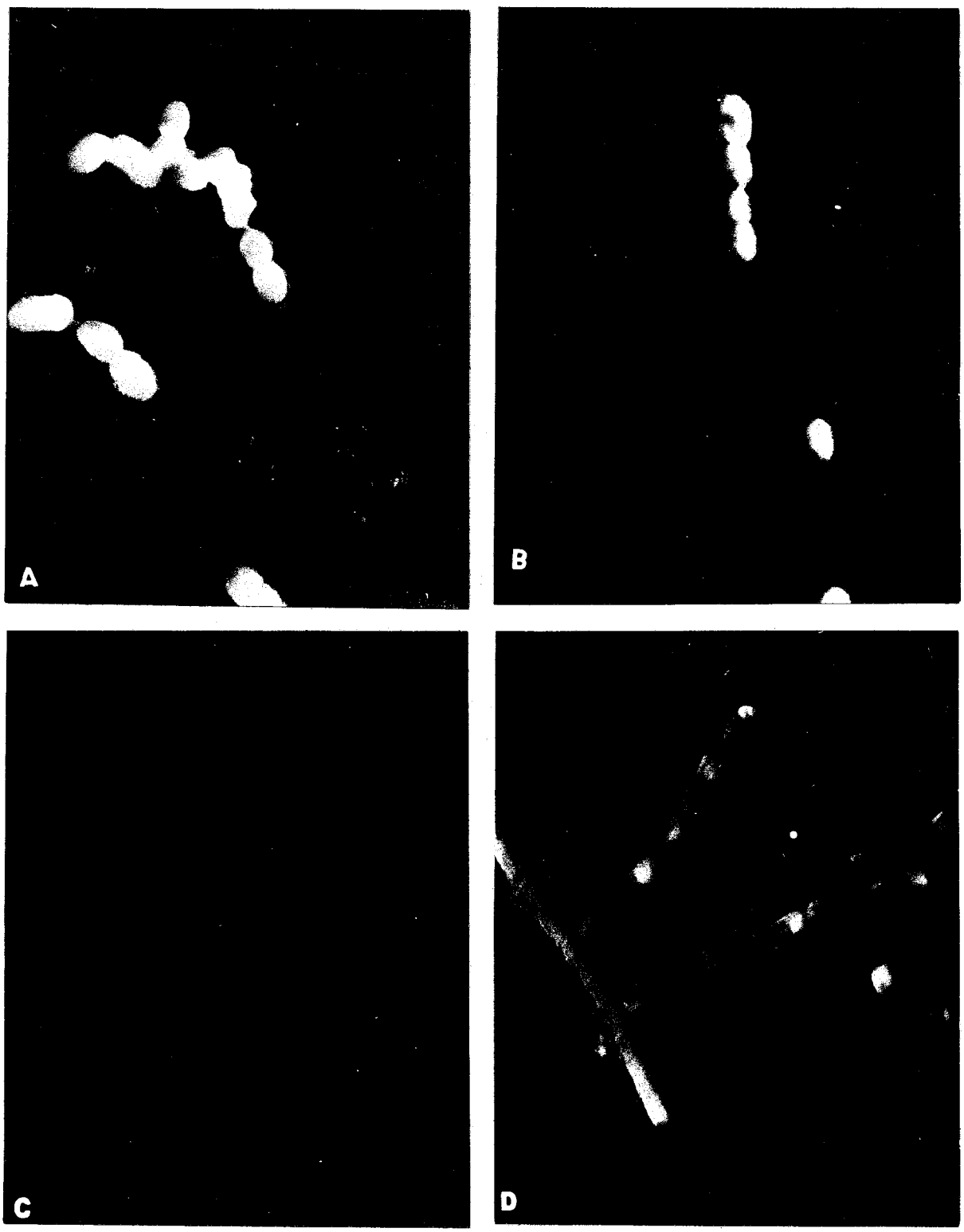
Microscopic studies revealed that the cells of Culture 31 were spherical and non-motile occurring singly, in pairs and in short chains. In cultures of all ages the cells invariably stained Gram positive. Culture 32 was a rod form of varying length and motile. Its staining characteristics were variable; in young cultures the bacteria were Gram negative, however in older cultures they were Gram positive. Both cultures were strict anaerobes.

Electron microphotographs of 24 hour cultures of Culture 31 and 32 were prepared and are shown in Figure 15. The cellulolytic activities of these two isolates were studied further and are reported in the following section.

Figure 15. Electron microphotographs of 24-hour cultures of rumen isolates

A and B - Culture 31 (magnification 10,000 X)

C and D - Culture 32 (magnification 10,000 X)


A
B
C
D

F. Fermentation of Carboxymethylcellulose-70L by Bacterial Cultures Isolated from the Rumen

The effects of various inhibitory compounds and nutrients on the fermentation of CMC-70L by rumen microorganisms have been studied. Since two pure rumen cultures were isolated and found to digest cellulose in preliminary experiments it was felt desirable to conduct a number of investigations to show the effects of inhibitory compounds and nutrients on the fermentation of CMC-70L by these isolates.

Inhibitors such as sodium fluoride, thymol and toluene, and the nutrient, fructose-1,6-diphosphate were added to the various cellulose fermentations by Cultures 31 and 32. The inocula were prepared by transferring Cultures 31 and 32 into Medium C in which 1 per cent CMC-70L was added in the place of the glucose. After 24 hours incubation at 39°C. the cells were harvested by centrifugation, and washed with sterile distilled water. After two washings the cells were resuspended in sterile distilled water. The composition of the culture medium was as follows:

Bacto yeast extract	1.5 gm.
Mineral solution	11.7 ml.
Urea solution	1.7 ml.
Inoculum (Culture 31 or 32)	20.0 ml.
Distilled water to	150 ml.

From the results of this investigation, as seen in Table XXVI and XXVII, the action of these chemical compounds

Table XXVI. Fermentation of CMC-70L, by cultures 31 and 32 in the presence and absence of sodium fluoride, thymol and toluene

No.	CMC 1%	Inhibitor	Isolate	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
				0	24	48	72	0	24	48	72
1	+	-	31	0.000	0.000	0.000	0.000	0.000	0.319	0.440	0.526
2	-	Sodium fluoride	31	0.000	0.024	0.024	0.025	0.000	0.000	0.000	0.000
3	+	Sodium fluoride	31	0.000	0.066	0.072	0.072	0.000	0.000	0.041	0.041
4	+	Thymol	31	0.000	0.061	0.062	0.062	0.000	0.032	0.032	0.032
5	+	Toluene	31	0.000	0.063	0.066	0.066	0.000	0.000	0.000	0.000
6	+	-	32	0.000	0.000	0.000	0.000	0.000	0.307	0.466	0.557
7	-	Thymol	32	0.000	0.022	0.026	0.027	0.000	0.000	0.000	0.000
8	+	Sodium fluoride	32	0.000	0.099	0.112	0.128	0.000	0.047	0.092	0.126
9	+	Thymol	32	0.000	0.051	0.052	0.055	0.000	0.000	0.000	0.000
10	+	Toluene	32	0.000	0.049	0.052	0.055	0.000	0.012	0.012	0.012

Table XXVII. Fermentation of CMC-70L by culture 32 in the presence and absence of thymol and fructose 1,6 diphosphate (FDP)

Flask	CMC 1%	Thymol	FDP	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
				0	24	48	72	0	24	48	72
1	-	+	-	0.000	0.022	0.026	0.027	0.000	0.000	0.000	0.000
2	+	-	-	0.000	0.000	0.000	0.000	0.000	0.204	0.362	0.403
3	+	+	-	0.000	0.043	0.049	0.049	0.000	0.067	0.013	0.013
4	+	+	+	0.000	0.064	0.073	0.089	0.000	0.000	0.000	0.000
5	+	-	+	0.000	0.000	0.000	0.000	0.000	0.251	0.410	0.476

was the same as occurred when rumen liquor and washed rumen microorganisms were used as inocula. In the presence of an inhibitor, an accumulation of reducing substances occurred, while in the absence of such an inhibitor the digestion of the soluble cellulose derivative by the rumen isolates was not arrested at the glucose stage and thus the familiar end-products of such a fermentation were found in the culture medium. Fructose-1,6-diphosphate appeared to stimulate the activities of Culture 32 (Table XXVII) and thus it seemed that this particular microorganism could utilize phosphorylated sugars in its metabolic processes.

By the use of paper chromatography, the reducing substances which accumulated during the fermentation of CMC-70L by Cultures 31 and 32 were found to consist of two reducing sugars, glucose and xylose. There was no evidence of cellobiose or cellulose dextrins as the breakdown products being present in the culture.

G. Preparation and Study of a Cell-Free Cellulolytic Extract from Rumen Microorganisms

In the microbiological degradation of cellulose specific enzymes are elaborated which are able to split the large polysaccharide molecule into smaller water-soluble compounds. These latter compounds can then diffuse into the interior of the organism for further metabolism. It is not

certain but it seems probable that these smaller sugar identities must be phosphorylated before entrance into the cell. There have been several observations that the cellulolytic microorganisms act upon the insoluble cellulose fiber only by direct contact with that substance.

Various experimental attempts were made to show whether the cellulose-splitting enzymes were exocellular and present in the rumen liquid. Fresh rumen contents were first strained through four thicknesses of cheesecloth and then centrifuged at 10,000 r.p.m. for 20 min. Part of the centrifugate was tested for its cellulose-digesting activity. The remaining portion was then filtered through a Berkefeld filter to remove any bacteria that were present in the previous clear liquid. This filtered sample was also tested for its cellulose-digesting activity.

The results of this investigation, as shown in Table XXVIII, indicated that the filtered rumen liquid had no cellulolytic activity while the centrifuged, non-filtered sample possessed slight activity. This slight enzyme activity could have been the result of a small number of cellulolytic microorganisms present in the non-filtered sample.

It can be postulated from the above experiment that the cellulose-splitting enzymes were strictly endoenzymes. This

Table XXVIII. Fermentation of CMC-70L by centrifugate (C) and filtrate (F) from rumen liquid in the presence and absence of sodium fluoride

Flask	CMC 1%	NaF .5M	Inoculum	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				Total volatile acids (as acetic acid) formed, grams per 100 ml. Determined at: hours			
				0	24	48	72	0	24	48	72
1	-	+	C	0.000	0.061	0.064	0.064	0.000	0.000	0.011	0.011
2	+	-	C	0.000	0.012	0.013	0.013	0.000	0.242	0.218	0.383
3	+	+	C	0.000	0.055	0.055	0.055	0.000	0.113	0.142	0.169
4	-	+	F	0.000	0.004	0.003	0.003	0.000	0.000	0.028	0.011
5	+	-	F	0.000	0.000	0.000	0.000	0.000	0.000	0.136	0.142
6	+	+	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011

hypothesis cannot be readily reconciled because it is difficult to envision that the insoluble molecules enter into the interior of the bacterial cell for the initial degradation. The specific enzymes in question may be tightly adsorbed on the outer surface of the cell membrane and not liberated as such in the external medium.

The next step to locate the site of the cellulolytic enzymes was to endeavor to prepare a cellulolytic cell-free bacterial extract. Enzyme extracts from rumen microorganisms and from Culture 32 isolated from rumen contents were prepared by the use of the conventional 9 kc. Model 5-102 Magnetostriction Oscillator*. However, the preparation was shown, experimentally, to be of low cellulolytic activity. As this was the case the method outlined by McIlwain (82) was used to prepare an enzyme extract from rumen microorganisms.

Four quarts of a fresh sample of rumen liquid was obtained from the fistulated animal and passed through four thicknesses of No. 50 cheesecloth. The resulting solution was centrifuged at 1,000 r.p.m. for 2 min. to remove plant material and protozoa (109). The bacterial cells in the supernatant liquid were then harvested by high speed centrifugation, employing the Sharples Centrifuge. After the

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*Manufactured by the Raytheon Manufacturing Company, Waltham, Massachusetts.

cells had been washed three times with a pH 6.98 phosphate buffer, they were repacked in the centrifuge, weighed and placed in a previously cooled mortar. Polishing Alumina A-303* was added in the amount of 2½ to 3 times the weight of the cells. The mixture was then thoroughly ground with a cold pestle for 5 to 10 min. After this time sterile physiological saline solution was added, the amount being two times by volume of the weight of the cells. The mixture was thoroughly stirred until a uniform suspension was obtained. This suspension was then centrifuged under refrigeration for 1 hour at 12,000 to 15,000 r.p.m. The resulting clear liquid was decanted carefully and used as the cell-free cellulolytic bacterial extract.

An index of cellulolytic activity of the different preparations was obtained by the amount of reducing substances formed from CMC-70L under anaerobic conditions with carbon dioxide and incubated at 40°C. In the test, 5 ml. of the preparation was added to 5 ml. of a phosphate buffer (136), pH 5.5, and 100 mgm. of CMC-70L. Sodium fluoride (0.21 gm.) or thymol (0.20 gm.) was added as an antiseptic to the mixture in the reaction vessel. At various times during the period of incubation, aliquots were withdrawn, filtered and analyzed for reducing substances.

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*Obtained from the Aluminum Ore Company, East St. Louis, Illinois.

A number of experiments were conducted in order to determine the activity of the prepared cell-free extract. The extract was added to media containing 1 per cent CMC-70L in the presence and absence of sodium fluoride and thymol. It was observed (Table XXIX) that this extract possessed good cellulolytic activity. The addition of sodium fluoride or thymol prevented the reaction mixture from becoming contaminated. Each culture was analyzed at 0 and 24 hours for total volatile acids, however it was shown that no short-chain fatty acids were produced.

Paper strip chromatographic analysis of the degradation of CMC-70L by the cellulolytic extract was also done at various time intervals during the above experiment. In the early hours of the investigation only glucose was detected in the media, however at 20 and 24 hours of incubation glucose and xylose appeared on the chromatograms. The presence of xylose suggested that xylan was present in the cellulose substrate and that the cellulolytic preparation contained a number of enzymes one of which was capable of degrading the xylan to xylose.

Cellobiose was not detected in any of the media as a degradative product of CMC-70L by the extract. It was possible that cellobiose was not formed or that if it was, it could have been rapidly hydrolyzed to glucose during the fermentation.

Table XXIX. Enzymic breakdown of CMC-70L by a cell-free extract of rumen microorganisms in the presence and absence of thymol and sodium fluoride

Tube	CMC 1%	Inhibitor	Extract	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours							
				0	0.5	0.75	1.5	3.0	6.0	20	24
1	+	-	+	0.000	0.011	0.053	0.071	0.084	0.113	0.057	0.062
2	-	Thymol	+	0.000	0.000	0.001	0.002	0.003	0.006	0.006	0.006
3	+	Thymol	-	0.000	0.000	0.000	0.000	0.001	0.003	0.003	0.003
4	+	Thymol	+	0.000	0.017	0.067	0.085	0.087	0.102	0.104	0.112
5	+	Sodium fluoride 0.5M	+	0.000	0.017	0.044	0.062	0.076	0.106	0.131	0.140

In view of the latter possibility, the action of the cellulolytic preparation on CMC-70L, Alphacel and cellobiose was studied. The reaction mixtures were prepared as described above.

From Table XXX it may be observed that the extract had the ability to hydrolyze Alphacel as well as CMC-70L. Cellobiose was degraded likewise to glucose (as shown by chromatographic analysis), its rate of hydrolysis was greater than that of CMC-70L or Alphacel (Figure 16). It is probably true from these results that the detection of cellobiose in the reaction mixtures during the fermentation of CMC-70L or Alphacel would be difficult because of the rate in which the cellobiose would be hydrolyzed to glucose.

In order to ensure that the action of the cellulolytic preparation was not due to contaminating microorganisms, samples of the enzyme solution were tested with and without sterilization by bacteriological sintered glass filtration. The results given in Table XXXI clearly show that the conversion of CMC-70L into reducing sugars was not brought about by contaminating microorganisms.

A number of investigations were conducted to study the properties of the prepared cellulolytic bacterial extract. Phosphate buffer solutions (64) were employed in this and subsequent experiments. A series of enzyme-buffer CMC-70L

Table XXX. Fermentation of CMC-70L, alphacel and cellobiose by the cellulolytic cell-free extract in the presence of sodium fluoride

Tube	CHO Source	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours					
		0	3	6	20	24	48
1	CMC 1%	0.000	0.057	0.072	0.102	0.106	0.113
2	Cello-biose 0.5%	0.000	0.081	0.109	0.203	0.242	0.253
3	Alphacel 1%	0.000	0.015	0.016	0.021	0.022	0.033
4	-	0.000	0.001	0.001	0.002	0.002	0.002

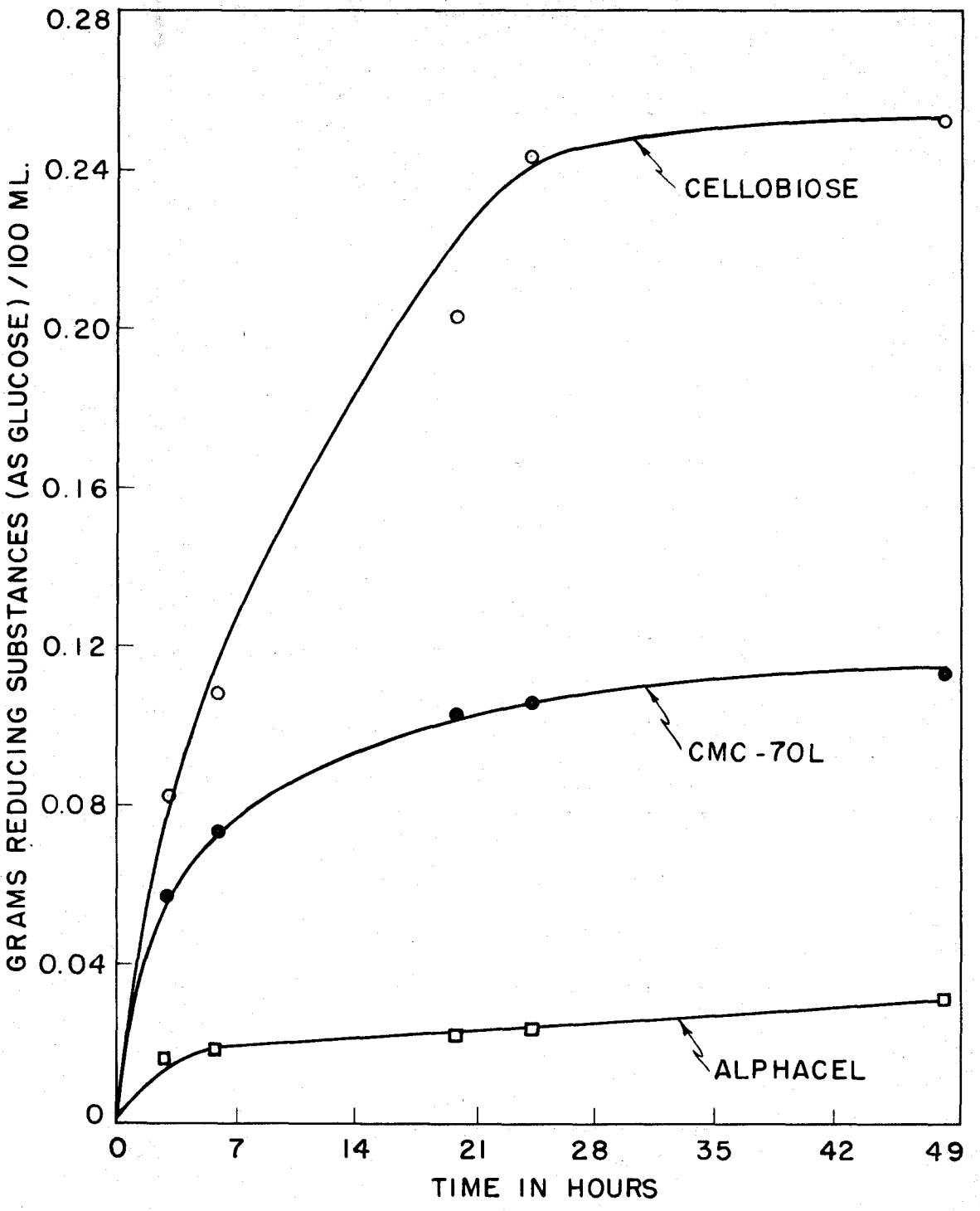

Figure 16. Fermentation of CMC-70L, alphacel and cellobiose by the cellulolytic cell-free extract

Table XXXI. Effect of filtration on the activity of the cell-free cellulolytic preparation

Treatment of preparation	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours		
	0	6	20
Not filtered	0.000	0.090	0.094
Sintered glass filtered	0.000	0.094	0.099
Control	0.000	0.006	0.015

mixtures of pH 4.0 to pH 8.0 were prepared and incubated anaerobically at 40°C. The results in Table XXXII and Figure 17 indicated that the optimum pH for the cellulolytic activity of the extract occurred at 5.5.

In testing the stability of this extract, portions of the same preparation were kept at -15°C., 10°C., and 25°C. for 144 hours. During this time cellulolytic activity of the samples were tested using CMC-70L as described before. Results showed, as given in Table XXXIII, that after 144 hours of storage the frozen and chilled samples showed no loss of activity. However, the sample kept at room temperature lost 34 per cent of its enzymic activity.

The optimum temperature for enzymic action was determined by preparing a number of enzyme-buffer-CMC-70L mixtures (pH 5.5) and incubating them at temperatures from 10°C. to 80°C. under carbon dioxide. The results of this study, as given in Table XXXIV and Figure 18, showed that the optimum temperature for maximum cellulolytic activity of the extract was between 40° to 50°C. The curves in Figure 18 showed that the incubation temperature of 50°C. increased the activity of the extract during the 6 hour reaction period. However after a longer period of incubation, the extract showed an optimum temperature of 40°C.

Table XXXII. The effect of pH on the activity of the cell-free cellulolytic preparation

pH	CMC-70L	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours		
		0	6	20
4.0	+	0.000	0.021	0.031
4.5	+	0.000	0.057	0.084
5.0	+	0.000	0.090	0.110
5.5	+	0.000	0.094	0.116
6.0	+	0.000	0.075	0.096
6.5	+	0.000	0.069	0.084
7.0	+	0.000	0.053	0.066
7.5	+	0.000	0.030	0.036
8.0	+	0.000	0.024	0.031
7.0	-	0.000	0.010	0.016

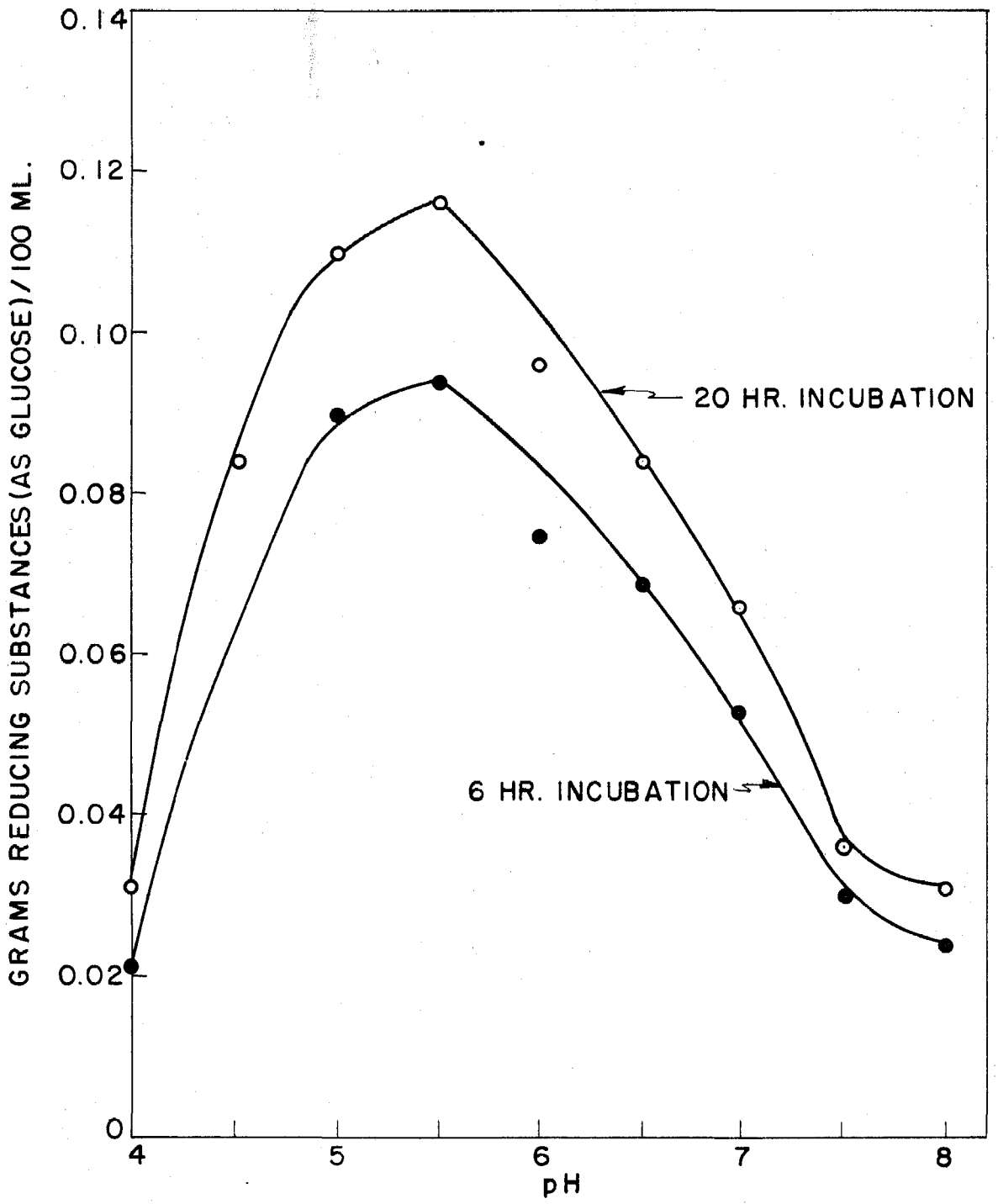

Figure 17. Effect of pH on the activity of the cellulolytic cell-free preparation

Table XXXIII. Effect of storage at different temperatures on the activity of the cell-free cellulolytic preparation

Storage time of sample hr.	Storage temperature °C.	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours incubation		
		0	6	20
0	-	0.000	0.088	0.108
24	25	0.000	0.079	0.091
	10	0.000	0.087	0.102
	-15	0.000	0.088	0.106
48	25	0.000	0.074	0.086
	10	0.000	0.087	0.100
	-15	0.000	0.087	0.108
144	25	0.000	0.058	0.061
	10	0.000	0.087	0.099
	-15	0.000	0.087	0.106

Table XXXIV. The effect of temperature on the activity of the cell-free cellulolytic preparation

Temperature °C	CMC-70L	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours		
		0	6	20
10	+	0.000	0.041	0.078
20	+	0.000	0.070	0.113
30	+	0.000	0.104	0.143
40	+	0.000	0.125	0.166
50	+	0.000	0.132	0.158
60	+	0.000	0.097	0.115
70	+	0.000	0.037	0.047
80	+	0.000	0.032	0.040
40	-	0.000	0.000	0.000

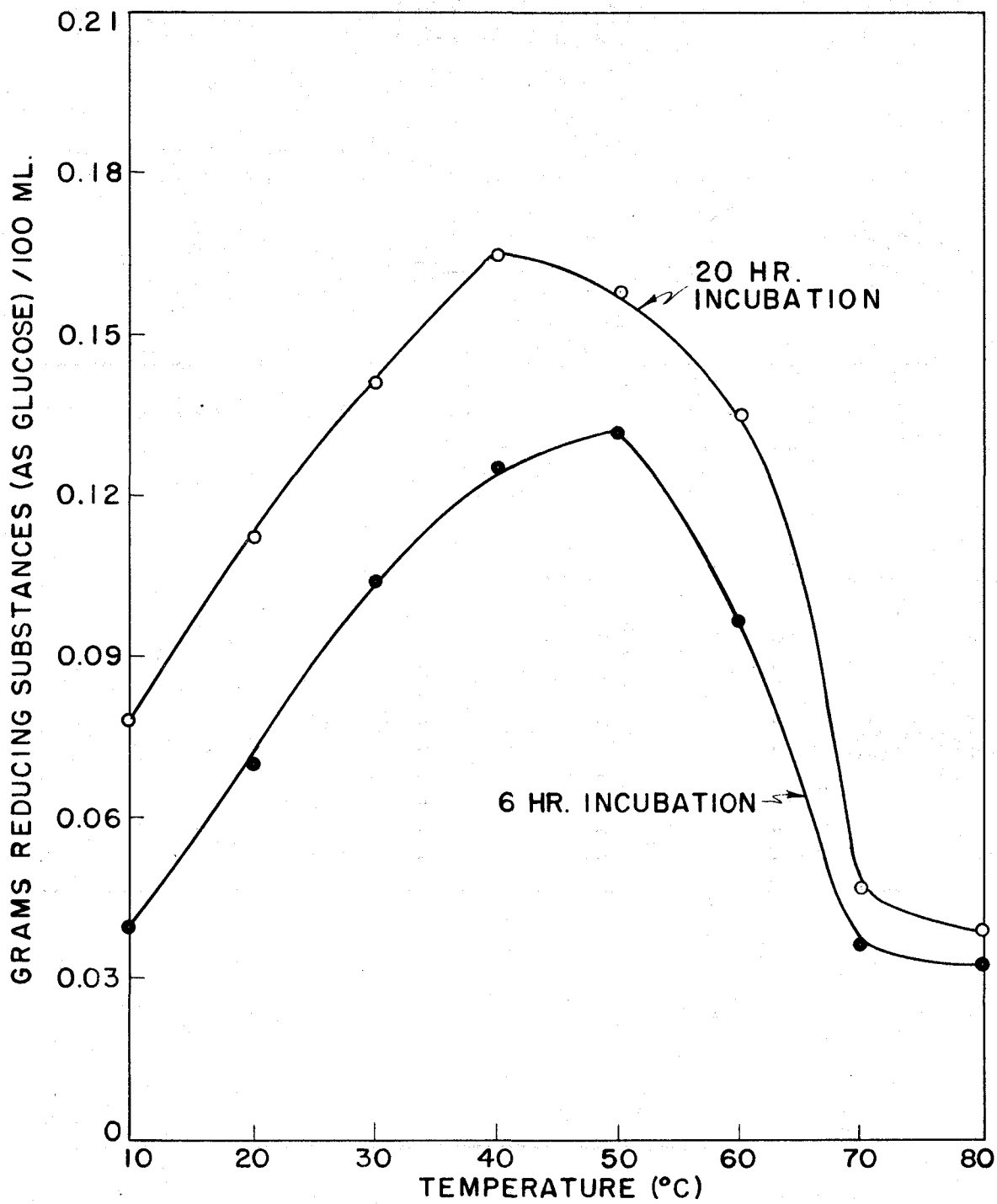

Figure 18. Effect of temperature on the activity of the cellulolytic cell-free preparation

It was indicated previously that yeast extract increased the utilization of cellulose by the rumen microorganisms. Since this was the case a number of experiments were conducted to study the effect of yeast extract on the action of the cellulolytic preparation. It was noticed, as shown in Table XXXV, that the addition of yeast extract stimulated the action of the enzymic solution on the hydrolysis of Alphacel, however little or no increase of degradation of CMC-70L appeared.

McIlwain's procedure (82) for obtaining a cell-free bacterial extract, was found to be useful in preparing an enzyme solution from rumen microorganisms. As a result a similar extract was prepared from rumen Culture 32. The extract obtained was tested for its cellulolytic activity (Table XXXVI) and it was shown to have the ability to hydrolyze CMC-70L and Alphacel. Glucose was detected by filter paper chromatographic means, as the only degradative product of the cellulosic substrates in the reaction media.

Table XXXV. The effect of yeast-extract on the fermentation of CMC-70L and alphacel by the cellulolytic cell-free rumen bacterial extract

Tube	Cellulose source 1%	Yeast extract 0.5%	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours				
			0	3	6	20	24
1	CMC	-	0.000	0.057	0.072	0.102	0.106
2	CMC	+	0.000	0.059	0.081	0.107	0.123
3	Alphacel	-	0.000	0.015	0.016	0.021	0.022
4	Alphacel	+	0.000	0.045	0.050	0.057	0.060
5	-	-	0.000	0.001	0.001	0.002	0.002
6	-	+	0.000	0.005	0.010	0.011	0.011
A	CMC	-	0.000	0.015	0.038	0.046	0.049
B	CMC	+	0.000	0.020	0.034	0.048	0.051
C	Alphacel	-	0.000	0.019	0.027	0.035	0.035
D	Alphacel	+	0.000	0.035	0.038	0.054	0.057
E	-	+	0.000	0.002	0.011	0.011	0.011

Table XXXVI. Enzymic breakdown of CMC-70L and alphacel by a cell-free extract of culture 32

Tube	Cellulose %	Extract	Reducing substances (as glucose) formed, grams per 100 ml. Determined at: hours	
			0	16
1	CMC-70L	+	0.000	0.026
2	Alphacel	+	0.000	0.017
3	-	+	0.000	0.007

VI. GENERAL DISCUSSION AND CONCLUSIONS

The chemistry of cellulose digestion by rumen micro-organisms has been investigated with special reference to the identification of the carbohydrate intermediates formed. The end-products formed during a normal fermentation of cellulose by the rumen bacteria are principally the short-chain fatty acids, such as formic, acetic, propionic and butyric acids. Therefore in order to study the pathway of cellulose degradation during this type of fermentation, the process must be arrested at an appropriate stage so that the various compounds formed during the hydrolysis of cellulose can be identified.

The use of the laboratory rumen of Burroughs *et al.* (19), now more commonly termed the artificial rumen, has made possible such an investigation. Strained rumen contents, obtained from a fistulated bovine, was used as the initial inoculum in early experiments. However, in later studies washed rumen microorganisms, rumen isolates and cellulolytic cell-free bacterial extracts were also employed as inocula. The flask cultures of nutrient solution containing cellulose, were incubated in a thermostatically controlled water-bath at 40°C. with a slow stream of carbon dioxide bubbled through to maintain anaerobic conditions.

Preliminary work showed that when the toluene was added to the cellulose-digesting cultures, reducing sugars accumulated in considerable amounts in the media, but in the absence of toluene very little reducing sugars could be detected. The yields of volatile acids were markedly decreased in those cultures having toluene added as compared with the yields of end-products in the absence of toluene. In a 48 hour fermentation culture containing 1 per cent original cellulose, as finely ground filter paper, in the absence of toluene, 0.620 gm. of volatile acids (as acetic acid) and only 0.001 gm. reducing sugars (as glucose) were produced, while in the presence of toluene, 0.023 gm. of volatile acids and 0.120 gm. of reducing sugars were formed.

It appeared that toluene arrested the growth of the microorganisms thus affecting the action of the enzyme systems which catalyze the conversion of the soluble sugars to the volatile fatty acids. However the inhibitor had little or no effect on the enzymes that catalyze the conversion of cellulose to the soluble reducing sugars.

Since cellulose is, even in the form of finely ground filter paper as used in these experiments, a solid, insoluble material, contact of the organisms with the cellulose cannot be very uniform. Experiments were therefore conducted employing the soluble cellulose derivatives, namely the

carboxymethylcelluloses (CMC) as the cellulosic substrate. In comparing the fermentation of ground filter paper, Alphacel (a commercial powdered cellulose product) and several grades of CMC by rumen microorganisms, it was found that in all cases the extent of digestion was greater with the soluble CMC samples than with the insoluble celluloses. Thus the results of these experiments indicated that CMC-70L was a suitable cellulosic substrate for the cellulolytic rumen bacteria.

It was felt that in order to identify the carbohydrate intermediates formed during the degradation of the cellulose molecule, an efficient inhibitor would be required so that a large accumulation of reducing sugars would occur in the culture. Previous experiments showed that toluene had the ability to inhibit cellulose fermentation by rumen microorganisms at the glucose stage. Therefore eleven other compounds were tested as to their effectiveness in blocking the metabolic pathway of cellulose digestion by the same bacteria. Of all compounds tested thymol, chloroform, sodium fluoride, toluene, m-xylene and iodoacetic acid, in the order of decreasing effectiveness, were found to prevent the further utilization of the reducing sugars by the rumen bacteria to form the volatile fatty acids as end-products. Of these compounds listed, thymol and sodium fluoride were chosen to

be used in future experiments to study the identity of the carbohydrate intermediates formed during the degradation of cellulose.

The hydrolysis of the cellulose molecule by the rumen bacterial enzymic systems appeared to be a hydroclastic reaction not affected by the various inhibitory compounds. It was felt that the action of these substances affected the enzymic processes that phosphorylate the soluble sugars before they entered the bacterial cell. It seemed probable that before the sugars can gain entrance into the cell's interior for further metabolism to the various end-products, they must be phosphorylated. With this hypothesis in mind, general phosphorylation inhibitors, such as 2-4 dinitrophenol, phloridzin and sodium azide, were tested to observe if they would inhibit the rumen fermentation of cellulose at the glucose stage. Results indicated that they had such inhibitory capacity, and thus reducing sugars accumulated in the culture media.

Phosphorylated sugars such as glucose-1-phosphate, glucose-6-phosphate and fructose-1,6-diphosphate, were added to cellulose-digesting cultures and it was noted that increased production of end-products resulted as compared with the control cultures having no added phosphorylated sugars. These results indicated that the rumen microorganisms

can utilize phosphorylated sugars and that the various inhibitory compounds may have arrested the growth and reproduction of the microorganisms by affecting the hexokinases that are distributed on the surface of the cell in the cell membrane.

Preliminary experiments that were conducted in order to elucidate the pathway of cellulose digestion by rumen microorganisms suggested that glucose was the only product formed. However, these investigations were done on inhibited cultures that had been incubated for 48 hours. It is reasonable to assume that the height of the cellulose fermentation had passed, and if cellobiose, cellotriose, cellotetrose, etc., were formed during the degradation of the cellulosic substrate, they could have been easily hydrolyzed to glucose by microbial action during that period of time. With this assumption in mind a more comprehensive study was done to follow the rumen bacterial breakdown of cellulose.

Cellulose-digesting cultures, inhibited with sodium fluoride or thymol, were prepared and the media were analyzed qualitatively at hourly intervals for carbohydrate intermediates. During this study it was found, by filter paper chromatography, that three reducing sugars appeared as the intermediate compounds of cellulose degradation. Of the three, glucose was in the greatest concentration, while

xylose and cellobiose were detected only in traces.

In various enzymic starch degradation studies, especially with Clostridium acetobutylicum (38), an amyloglucosidase has been found, the principal catalytic activity of which was the hydrolytic removal of individual glucose units from the non-reducing terminus of a starch chain. When a branch point in the structure was encountered the enzymic action was slowed down. From the results of the study of cellulose degradation described previously, it appeared that the cellulose-splitting enzyme elaborated by rumen microorganisms may be a "celloglucosidase", one which hydrolyzes individual glucose units from the ends of the cellulose chains. This postulation is similar to the one proposed by Clayson (23) in 1943. He believed that the cellulolytic microorganisms began at the end of the cellulose chain and lopped off one anhydroglucose unit after the other.

The presence of a small amount of cellobiose may be, therefore, due to the action of a small number of microorganisms such as Clostridium cellobioparus. Hungate (51), who isolated the latter from the rumen, found that a pure culture of this bacterium degraded cellulose to cellobiose but not to the glucose stage.

The presence of the xylose molecule in the cellulose-digesting media may be explained in that the cellulosic

substrates were contaminated with xylan. This polysaccharide is very susceptible to enzymic attack and thus would be hydrolyzed easily to the pentose, xylose.

An important consideration in the digestion of cellulose by rumen microorganisms is whether the cellulolytic enzymes are extracellular, intracellular or perhaps held on the surface of the bacteria. In the microbiological degradation of cellulose specific enzymes are elaborated which are able to split the large, polysaccharide molecules into smaller, water-soluble compounds. These latter compounds can then diffuse into the interior of the organisms for further metabolism. It is not certain, but it seems probable, that these smaller sugar identities must be phosphorylated before entrance into the cell. There have been several observations (51) that the attacking microorganisms act upon the insoluble fiber only by direct contact with the cellulose.

A number of experiments were conducted using centrifuged and filtered rumen fluid as inocula. The results showed that the filtered liquid had no cellulolytic activity, while the centrifuged, non-filtered sample possessed slight activity. This slight activity could have been due to the presence of a small number of microorganisms in the non-filtered sample. It could be postulated from these results that cellulose-splitting enzymes were strictly endoenzymes.

However, this hypothesis is difficult to believe because the insoluble cellulose molecule could not pass into the bacterial cell for the initial degradation. It can be concluded, however, that the cellulolytic enzymes of the rumen microorganisms are not present as such in the rumen fluid, but are associated with the bacterial cells.

The preparation of a cellulolytic cell-free extract from washed rumen microorganisms and from a culture isolated from the rumen added additional evidence that the cellulolytic enzymes are oriented and tightly adsorbed on the outer surface of the cell membrane and are not liberated far into the external medium.

A number of experiments were conducted to study the effect of yeast extract on the action of the cellulolytic cell-free preparation on Alphacel and CMC-70L. It was noticed that the addition of yeast extract seemed to stimulate the action of the enzyme extract on the hydrolysis of Alphacel. However, little or no increase of hydrolysis of CMC-70L compound appeared. In addition to the enzyme and the substrate many enzymes require or are activated by a third substance called a "coenzyme" or an "activator". In many cases certain inorganic ions are necessary, but there are a number of coenzymes that are organic in nature. In the experiment described briefly above it appeared that yeast

extract contained a "co-factor" that activated the cellulolytic action of the cellulose-splitting enzyme on insoluble cellulose while the degradation of CMC-70L was not influenced by the presence of yeast extract. From this information and the study of the pathway of cellulose degradation with reference to the identity of the carbohydrate intermediates formed, the digestion of cellulose by rumen microorganisms can be explained on the basis of at least a three-step breakdown as follows: (a) transformation of the insoluble cellulose molecule into linear polyanhydroglucose chains; (b) the breakdown of the linear hydrocellulose structure directly to cellobiose or glucose; and (c) hydrolysis of cellobiose (if formed) to glucose.

If a "celloglucosidase" is elaborated by the rumen microorganisms, its action would take place during the second stage after the insoluble cellulose molecule had become solubilized by another enzyme system.

VII. SUMMARY

1. The chemistry of cellulose digestion by rumen microorganisms has been investigated with special reference to the identification of the carbohydrate intermediates formed. Flask cultures containing cellulose and mineral solution were inoculated with strained rumen fluid, obtained from a fistulated cow and incubated under carbon dioxide at 40°C.

2. Preliminary experimental results showed that when toluene was added to active cellulose-digesting cultures, reducing sugars accumulated in the medium in considerable amounts with little or no production of volatile fatty acids. In the absence of such an inhibitor little or no reducing sugars accumulated in the culture medium and the production of the various end-products (volatile acids) was considerable.

3. Soluble derivatives of cellulose, namely the carboxymethylcelluloses (CMC) were found to be actively digested by rumen microorganisms. As a result experiments were conducted employing CMC-70L as the source of the cellulosic substrate in the fermentation media.

4. Twelve chemical compounds were tested as to their effectiveness in blocking the metabolic pathway of cellulose digestion by rumen microorganisms. Of all the compounds

tested, thymol, chloroform, sodium fluoride, toluene, m-xylene and iodoacetic acid, in the order of decreasing effectiveness, were found to prevent further utilization of the reducing sugars by the rumen bacteria to form the various end-products.

5. Three general phosphorylating inhibitors, 2-4 dinitrophenol, phloridzin and sodium azide, were found to inhibit the cellulose digestion by rumen bacteria at the glucose stage.

6. The addition of phosphorylated sugars to the cellulose fermentation media resulted in a greater production of volatile fatty acids by the rumen microorganisms thus indicating that these bacteria can utilize phosphorylated sugars.

7. The pathway of cellulose digestion by rumen microorganisms was studied with special reference to the carbohydrate intermediates formed. Aliquots were withdrawn at hourly intervals from cellulose fermentation cultures having CMC-70L or Alphacel as the cellulosic substrate with sodium fluoride or thymol added as the inhibitor. Glucose and a slight amount of xylose and cellobiose were detected by filter paper chromatography in the fermentation media. Other possible carbohydrate degradation products from the cellulosic substrate, such as cellotriose, cellotetrose, etc. were not detected.

8. A number of bacterial isolates were cultivated from the rumen of the bovine and tested for their fermentation habits on several carbohydrates. Of the 32 cultures isolated, two (Cultures 31 and 32) were found that would ferment CMC-70L and Alphacel at an active rate. Investigations revealed that Culture 31 was anaerobic, non-motile, spherical in shape and invariably stained Gram positive. Culture 32 was a rod form of varying length, strictly anaerobic, motile and stained Gram negative or Gram positive depending on the age of the culture. Electron microphotographs were prepared of each culture.

9. The inhibitors such as sodium fluoride, thymol and toluene, and the nutrient, fructose-1,6-diphosphate had the same effect on the action of the two isolates on CMC-70L as reported for the mixed cultures above. Only glucose was detected as the degradative product of CMC-70L by the cellulolytic action of Cultures 31 and 32.

10. Experiments showed that the cellulolytic enzymes of the rumen microorganisms are not present as such in the rumen fluid, but are associated with the bacterial cells.

11. A method was described for the preparation of a cell-free enzymic extract from rumen bacteria capable of degrading cellulose and CMC-70L.

12. The activity of the cell-free cellulolytic enzyme (or enzymes) was maximum at an optimum temperature of 40°C. and an optimum pH of 5.5. It was partially inactivated when allowed to stand at room temperature for 144 hours. The main hydrolytic product resulting from action of the enzyme cellulose was shown to be glucose.

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IX. ACKNOWLEDGEMENTS

The writer wishes to take this opportunity to express his sincere appreciation to Dr. L.A. Underkofler for suggesting this problem and also for his helpful advice and criticism during the course of this study.

Thanks are extended to Dr. P.H. Carr for making the electron microphotgraphs used in this investigation and to Mr. C.C. Claridge for his technical assistance.

The writer also wishes to express his thanks to his wife, Eileen, for preparing the preliminary and final forms of this report and for her patience and cooperation throughout this study.