INTRODUCTION:

In the October of 1930 I sent to the Rockefeller Foundation a short report of the work carried out up to the end of September 1930. On the November 20, 1930 I was awarded an extension of the Fellowship from the Rockefeller Foundation for an additional six months. After finishing this term I received a Fellowship from the Wisconsin Alumni Research Association for the Summer-school of 1931, and first Semester of the Academic Year 1931/32.

As a fellow of the University of Wisconsin I have continued my study of the problem of the isolation and biochemical investigation of the cellulose fermenting organisms. Therefore I am giving in this report an account of work which has been completed after the expiration of my fellowship from the Rockefeller Foundation. In my last report I gave the reason why I altered to a certain extend the plans submitted originally to the Board of the Rockefeller Foundation. Now I can state with pleasure that it was advantageous to my scientific ~~training~~ and the broadening of my education to have done so.

In general my activities were carried out along two main lines:

I. Experimental work

II. General Studies and Activities.

I.

A. Studies on mesophilic cellulose fermenting bacteria.
B. Studies on anaerobic ~~cellulose~~ mesophilic cellulose fermenting bacteria.
C. Studies on anaerobic thermophilic cellulose fermenting bacteria.

D. Studies on filterable viruses of mosaic diseases of tobacco.
E. The use of viscose sacks for ultrafiltration.
F. The growth of anaerobic bacteria in Petri dish cultures.
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A.

Studies on aerobic, mesophilic cellulose fermenting bacteria:

The bacteria were isolated from the slime of Lake Mendota at Madison, Wisconsin. At first enrichments cultures were secured, and from these I started the isolation of the cellulose organisms on solid media. For this purpose, cellulose agar and starch agar are most suitable. The usual culture media are unsuitable, since the contaminating forms, especially B. proteus, outgrow the cellulose dissolving bacteria. In some respects starch agar plates are better than cellulose agar, because the colonies are visible in two days, while on the cellulose agar plates, the dissolved zones are seldom present before a month or two. The characteristics of these bacteria were investigated in accordance with the Descriptive Chart of the Society of American Bacteriologists. The important points of all these characteristics will be presented later.

Some of more important findings are presented below:

a. The amount of cellulose decomposed. In optimal conditions is decomposed 40 per cent of cellulose.

b. Products. Small quantities of volatile and non volatile acids are produced. No alcohol or reducing substances were recovered.

c. Reaction. The optimum is between PH 7.0 and 8.0. The growth and the dissolving of cellulose occurs in reactions between pH 6.3 and 8.5. These bacteria change the hydrogen ion

concentration slightly towards acidity.

d. The role of dyes.- Gentian violet for example, in concentration of 1:80.000 is the limit in which the bacteria will grow and dissolve the cellulose. The use of indicators for isolation purposes was tried, but the amount of acid formed was not sufficient to distinguish the bacteria which dissolve the cellulose from other forms.

e. The carbohydrate fermentation.- The ability of these bacteria to ferment different carbohydrates was tested. Arabinose, Xylose, Sucrose, Glucose, Galactose, Fructose, Maltose, Lactose, Mannitol and Glycerol were added to the medium with or without cellulose, and after fermentation the residual sugar was determined. Fructose, Mannitol and Glycerol are not fermented. In the tubes where both a carbohydrate such as Mannitol, Glycerol or Fructose, with cellulose were added, only Cellulose was fermented. Where any of other above mentioned carbohydrates were added to the cellulose culture, the cellulose was not dissolved, but instead the carbohydrate was fermented.

f. Oxidizing and reducing agents.- Oxidizing substances did not influence the growth of fermenting power of those organisms. Reducing substances as cysteine-hydrochloride had an inhibitory influence on growth and fermenting power of these bacteria.

This results will be published shortly.

During the study of these bacteria it was observed that in some Petri dish cultures an starch agar, there appeared frequently zones of dissolved bacteria. These zones were much like those appearing in bacterial cultures infected with bacteriophage. This phenomenon was observed only in cultures on solid medium. The zones spread rapidly over the whole dish. If transfered from

phaged cultures, growth was obtained only if large inoculum was used. The bacteria from cleared zones did not stain with the exception of small granules. This phenomenon could not be observed on other than starch medium, because of the scantiness of the growth. On solid media with cellulose, observations could not be made because on this substrate only zones were formed and not typical colonies.

B.

Studies on mesophilic anaerobic cellulose fermenting bacteria:

As a result of my investigations of the aerobic cellulose fermenting bacteria, I came to the conclusion that these organisms play a minor role in the bacterial decomposition of cellulose. Therefore to aid in solving this very complicated problem the work wit anaerobic cellulose fermentation was begun. The range of the temperature at which these bacteria grow is very wide.

This group of bacteria is of special interest because until recently nobody has obtained a pure culture of these organisms. My experiments were made with material from soil, manure and slime from the lake. At the beginning, the fermentation of cellulose proceeded very slowly; after a long period of cultivation of these bacteria on selective media, the process of purification was started. Many different kinds of media were used, and most suitable was a medium containing an extract from the slime of the bottom of the lake. Many strains of enrichment cultures of these bacteria developed on this medium and dissolved cellulose. In purified cultures they gew very well at a temperature of 30°C and were seen in microscopic preparations as thin long rods with terminal spores, and thick short rods with oval spores. Many preliminary attempts to get pure culture on cellulose agar plates and other solid media failed. Some new media with cellulose, and low concentrations of agar were tried, using the anaerobic chamber described in the re-

print attached. Using this method some of the bacteria were grown in the Petri dish cultures. In microscopic preparations the culture look pure; the bacteria are slender rods with terminal rohnd spores. The cultures obtained in this way were grown for one year. The growth and the dissolving of cellulose are very slow. This series of experiments will be completed at the earliest opportunity.

C.

Studies on the thermophilic anaerobic cellulose fermenting bacteria:

From all groups of cellulose fermenting bavteria, the thermophilic are the most interesting. They are very active. If the process of the fermentation of the cellulose by means of these bacteria could be effectively controlled, it could be applied succesfully in fermentation industries. Also from a purely scientific point of view these bacteria represent one of the most interesting group of microorganisms. A number of bacteriologists have attempted to isolate pure culture of this organism. However from the evidence given in their publications it seems to be doubtful whether one of them was succesful in this respect. If a pure culture of these organisms has been obtained by anybody on non-cellulosic bacteriological media, this culture when transferred to media containing cellulose failed to ferment it. This has been explained by the suggestion that these bacteria if grown on media without cellulose have lost the power of fermentig cellulose . If the cellulose was decomposed, always a number of forms was observed in microscopic preparations.

My study of this problem was carried out in two directions:

A. Fermentation studies with highly purified enrichment cultures,

B. Bacteriological study of the organisms.

For the fermentation experiments the best stock, and newly isolated cultures were selected. The results of these experiments are given in the table attached.

The bacteriological experiments were made only on newly isolated cultures. During many month of inntensive work on the part of myself nad Miss N. Kimball as my assistant, the majority of general bacteriological methods have been applied but we could not succeed in obtaining a pure culture of these organisms. If the cellulose was fermented always three forms could be demonstrated. Two of these forms grew well on ordinary bacteriological media in pure cultures, but if transferred separately or together to the cellulose media they did not attack the cellulose. The third form, seen only in microscopic preparations from the cellulose fermenting culture, never grew on other than cellulose media, amd only then in the presence of the above mentioned contaminants. Both contaminants have been studied in their characteristics. From the results of this experiment we came to the conclusion that cotrary to the generally accepted explanation, these bacteria hardly could be responsible for for the removing of the intermediate products in the cellulose fermentation, which were considered as toxic for the cellulose fermentig organisms. We obtained the evidence that the contaminating organisms should be considered as resposible for rendering the medium more suitable for the development of the specific cellulose organisms.

After obtaining this evidence further experiments were made alongtwo lines.

Firstly, to change in the culture the ratio between the contaminants

and the specific organism. The previous observations indicated clearly that the contaminants are in much larger number than the cellulose fermenting organisms. Secondly, by creating in the medium the conditions in which the pure culture of the cellulose arganisms could find suitable conditions for growth.

Recently after a long series of experiments both problems seem to be solved.

The ratio between the contaminants and the specific organisms was changed by applying an original method based on following facts. During the experimenting some observations indicated that in a medium without cellulose, suitable for the growth of the contaminating organisms, the cellulose arganisms do not develope and probably do not germinate. Based on this the folloving experiments were arranged. From the culture in which the fermentation of the cellulose was well advanced, samples were taken and dilutions made in tubes of nutrient broth. These tubes were inoculated for several days. During this time the broth cultures were heated every few hours for 10 to 15 minutes in boiling water. Immediately after the heating, secondary dillutions from these tubes were made in sterile water blanks and used for inoculation of tubes with cellulose medium. The first experiments proved unsuccesful. The failure couls be explained either by accepting that the cellulose organisms were killed during the heatings or that the conditions in the medium were not suitable for growth in the absence of associated organims. The first possible explanation could hardly be accepted because of the fact that 17 hours heating at 100 C was necessary to kill the cellulose fermenting organism in purified culture. The second assumption, that the conditions of the medium were not satisfactory was therefore investigated.

For the purpose of the improvement of the conditions of the medium, yeasts were considerdd from the following reasons:
1. Yeasts could replace in the medium some labile substances which were destryed by very long sterilisation. The medium must be sterilised many hours in order to be free from the living spores of thermophilic organisms. 2. They might replace the living contaminant by creating the proper oxydation-reduction potential in the medium. 3. By the presence of specific enzymes they could remove the small quantities of sugar, which as a possible intermediate product in frementation of cellulose may be harmful to the cellulose decomposing organisms.

In the last series of experiments pure suspension of yeasts grown on glucose agar were added to a series of tubes with cellulose medium. After few days of incubation, in several tubes, cellulose was slowly digested. In microscopic preparations made from this tubes only one type of organisms was present. No growth occured if transferred on usual bacteriologidal media. In consecutive transfers to the same medium with cellulose and yeasts, cellulose was always fermented. This pure culture is much less active in cellulose decomposition than the crude. As a nitrogen source gelatine was found better than peptone.

Further studies on this problem are in progress. The results presented in this report on this problem will be read at the December Meeting of the Society of Americam Bacteriologists in Baltimore.

D.

Studies on the filterable virusxes of the mosaic diseases of tobacco:

After I obtained the extension of my fellowship from the Rockefeller Foundation I devoted a part of my time to the study of mosaic diseases of plants. Dr. L.R. Jones and Dr. James Johnson suggested that I could acquire experience in this field by studying the problem of the ultrafiltration of the virusxes. The viruses of tobacco mosaic, yellow tobacco mosaic and spot necrosis were selected for my experiments.

For the ultrafiltration I used collodion and viscose sacs. After a preliminary work on the preparation of graded collodion sacs a slight modification of the method described by Olitzky and Gates was followed. The method of Eggerth was proved not satisfactory, and the sacs prepared according to this method were very fragile. Also Cellulose sacs prepared from viscose have also been tried. From the results of my experiments carried through a period of one year as a minor problem, may be drawn the following conclusions:

1. The virusxes if dialised do not pass collodion or viscose sacs.

2. Filtered under the slight pressure of the liquid itself present in the sacs the virus ~~stems~~ also to be retained.

3. If higher pressure of two to three lbs per square inch of the sac be applied the virusxes seem to penetrate the membranes but only to a small degree.

These experiments are nearing completion.

E.

The use of viscose sacs for ultrafiltration:

In the preparation of the collodion sacs for ultrafiltration, difficulties have arisen in connection with their uniformity. The mixture of alcohol and ether used as solvent for celloidine is very volatile. This is is the cause of the difficulties in obtaining a sac of uniform permeability. In the search for a substitite it has been found that the viscose, which is widely used in industry may be employed succesfuly for this purpose. It has been found that viscose sacs are very uniform and strong. They can stand easily the pressure of 3 to 5 lbs per square inch. The permeability differs very little from the permeability of medium graded collodion sacs. The proteins and enzymes pass viscose membranes. The viscose sacs a are more resistant against bacterial deterioration. The viscose sacs were tried in several series of experiments wit satisfactory results. They proved be superior to collodion sacs in many respects.

Mr.M. Johnson, Assistant in the Department collaborated with me in these experiments.

The details will be published shortly.

F.

The growth of anaerobic bacteria in Petri dishes.

The results of these experiments were published in the Zentrbl.f. Bakt. etc. II. Abt. Bd. 82. p. 109-110. 1930.

The reprint of this paper is attached to this report.