STUDIES ON THE DECOMPOSITION OF CELLULOSE BY MICROORGANISMS

by

Arthur Saul Perlin

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GENERAL INTRODUCTION

Cellulose is attacked and decomposed to simpler chemical compounds by a host of microorganisms in a variety of natural processes. In this manner, a very large part of the carbon of decaying vegetation is reconverted into forms utilizable by other living organisms, and a proper balance of the carbon cycle is thus maintained.

A large number of different organisms are associated in the breakdown, and the cellulose itself is in intimate contact with other plant materials, notably hemicelluloses and lignin. The majority of the studies on cellulose decomposition, however, have been confined to the use of pure cultures of microorganisms, with pure cellulose as the substrate, so that experimental conditions are more easily controlled.

However, even with isolated systems much of the information obtained has been of little value, so that the existing knowledge concerning the microorganisms involved and the mechanisms by which the decomposition is accomplished, is relatively scanty.

The increasing economic importance being attributed to cellulosic residues - the largest by-product of the agricultural and lumbering industries - as well as the desirability of fully understanding the decomposition of plant materials in the soil and in the rumen of herbivorous animals, accentuates the need for further research into the action of microorganisms on cellulose.

This study was undertaken as a contribution to the knowledge of cellulose decomposition.

HISTORICAL INTRODUCTION

A factor of primary importance in cellulose decomposition by microorganisms is the relative insolubility and inert nature of cellulose itself. It would appear, therefore, that soluble products which can be absorbed and assimilated must first be produced from the cellulose, before energy is derived by the organisms attacking it. The maximum number of polymerized glucose units which can be absorbed across the cell-membrane is not known, but it is probably of a relatively small order. Most of the breakdown of the cellulose molecule must therefore occur in the environment of the organism, through the action of a powerful extra-cellular enzyme system.

The existence of such an enzyme system was first indicated by Debary (8), in 1886, who observed that the plant cell-wall is dissolved by certain species of fungi. A number of workers have illustrated the diffusible nature of this exo-enzyme in the case of bacteria, by the clear zone surrounding colonies growing on cellulose-agar.

In 1912, Pringsheim (26) sought to explain the mechanism of the decomposition. Working with thermophilic bacteria he demonstrated the formation of glucose and cellobiose in fermenting cellulose cultures. This was effected by interrupting the normal activity of the organisms through the addition of

antiseptics, such as toluene and iodoform, or by raising the incubation temperature at the height of the fermentation. "It is thus possible," stated Pringsheim, "to demonstrate the separate nature of the vital and digestive functions of the thermophilic bacteria." On the basis of these results he postulated the existence of two hydrolytic enzymes - one, which produces cellobiose from cellulose, now called "cellulase", and another, which breaks cellobiose down to glucose, now called "cellobiase".

Woodman et al (37) (38), using mixed cultures of rumen bacteria, and Kalnins (18), working with the aerobic Bacterium protozoides and some Vibrio species, employed the techniques of Pringsheim (26) and isolated glucose from cellulose cultures. However, cellobiose was not found. Scott et al (29) demonstrated the production of glucose in the thermophilic fermentation of cellulose by raising and lowering the incubation temperature during active fermentation, but they also were unable to identify cellobiose.

More recently, Fahraeus (10) showed that some members of the Cytophaga genus produce glucose from cellulose when the cultures are sisted. Furthermore, by using dried preparations of bacteria he was able to obtain a quantitative relationship between cellulose decomposed and glucose formation. On the other hand, Hungate (15) found that the anaerobe, Clostridium

cellobioparus, brings about the accumulation of considerable amounts of cellobiose when an active cellulose culture of the organism is incubated for long periods at a hydrogen-ion concentration too low for normal growth.

The production of glucose or cellobiose has thus been demonstrated in a number of instances through the use of antiseptics and by using abnormal conditions in culturing. These techniques have been employed on the assumption that the products of the decomposition of cellulose under normal conditions are rapidly oxidized and, therefore, difficult to detect. The validity of such an assumption was strengthened by the experiments of Imshenetskii (17) who cultured C. vulgaris and C. hutchinsoni in a limited oxygen supply and obtained reducing sugars which, however, could not be detected when normal air was used.

Grassman et al (13) studied the action of enzyme extracts from various molds. They were able to effect a separation of two distinct enzymes from A. niger - one corresponding to cellulase and the other to cellobiase. The cellulase was shown to convert cellulose to dextrins approximating the cellohexoase stage, while the cellobiase carried the hydrolysis further to form glucose.

In 1930, Tetrault (32), working with unidentified thermo-

philic bacteria, claimed to have detected reducing sugars as a product of cellulose decomposition, without artificial treatment of the cultures. More recently, Alarie (2) reported the presence of reducing substances in normal cultures of two aerobic soil-bacteria, V. perimastix and B. effluens, when ammonium sulphate was used as the source of nitrogen.

On the basis of findings such as the aforementioned, a hydrolytic theory of cellulose decomposition has been developed and strengthened. The decomposition is pictured as resulting from hydrolysis by an extra-cellular enzyme system in which glucose or cellobiose, or both, are split off from the long cellulose chains. The soluble, absorbable products thus formed are thought to be utilized by the organisms in the maintenance of their life processes.

For a number of years this theory had been questioned because, in numerous instances, glucose was not readily utilized by cellulose-decomposing bacteria. This objection has now been removed, at least in the case of Cytophaga, as Stanier (31) has found that growth was inhibited by poisonous products formed from glucose on autoclaving in salt solution.

In contrast to the hydrolysis theory of cellulose decomposition an "oxidative" mechanism has been proposed

by Winogradsky (36). It had its origin in the observation that residual cellulose, isolated from aerobic cultures, resembles chemically-prepared oxycelluloses. Loicjanskaya (21) and Walker and Warren (34) supported the oxidative theory. They isolated a mucilaginous material from cellulose cultures of Cytophaga which contained uronic acid groups and which was described by the latter workers as an oxycellulose of the acidic, non-reducing type, and regarded by them as an intermediate in cellulose decomposition. Accordingly, they postulated that the free primary alcohol groups in the cellulose molecule were oxidized through aldehyde to carboxyl groups, with the resulting instability permitting easy fragmentation of the chain. Loicjanskaya (21) suggested that the polyuronic acids are hydrolysed to glucuronic acids which, presumably, could then be assimilated.

The oxidative theory has been criticized by Norman and Fuller (11) (24) on the grounds that it presupposes an oxidative exo-enzyme which is without precedent. At the same time, it was pointed out that the oxycellulose produced was still insoluble and hence unavailable to the organism. Norman and Bartholomew (23) made a study

of a number of aerobic mesophilic bacteria and presented data to indicate that the uronide-containing substances of aerobic cultures were in reality synthesized polysaccharides. They concluded that there was no evidence to support the oxidative theory of cellulose decomposition.

More recently, Fahraeus (10) studied the mucilaginous material of Cytophaga cultures described by Loicjanskaya (21) and Walker and Warren (34) - the material which was an important factor in the development of the oxidative theory. He showed that this mucilage could be obtained from glucose as a substrate as well as from cellulose. Accordingly, it was considered to be a synthesized cell product rather than an intermediate of cellulose degradation, and the validity of the oxidative theory was thereby questioned.

Referring to the work of Cori and Cori (7) with starch and glycogen, Stanier (31) suggested that a phosphorolytic, rather than a hydrolytic fission, could also take place. However, no experimental evidence was presented in support of this suggestion.

The opposition to the oxidative theory and the absence of any other acceptable explanation of the mechanism of cellulose decomposition, has resulted in general acceptance

of the hydrolytic theory. However, the hydrolytic theory may be criticized as offering an inadequate explanation, in its present form, of the reactions which convert the complex cellulose molecule to the simpler end - products of dissimilation, such as carbon dioxide.

In only a few instances has the presence of hydrolytic enzymes been established, and where reducing sugars have been caused to accumulate a quantitative relationship to cellulose decomposition has rarely been demonstrated. Theoretically, hydrolysis should result in a progressive shortening of the cellulose chains, but products corresponding to cellodextrins have not been isolated from actively-decomposing cultures. Moreover the mode of utilization of glucose or cellobiose, should they be present, has not been taken into account in the hydrolytic theory.

A number of products have been isolated from cellulose cultures but their identification has contributed very little to elucidating the nature of the decomposition reactions. This has been due in part to the lack of accurate balance sheets. In the case of the aerobic mesophilic bacteria, moreover, the situation has been further obscured by the fact that the only products of consequence appear to be carbon dioxide and synthesized cell-materials. For example,

Walker and Warren (34) recovered two-thirds of the decomposed cellulose-carbon as carbon dioxide, and the remaining one-third as a polyuronide mucilage.

On the other hand, the thermophilic and anaerobic bacteria produce appreciable quantities of alcohol, and acids such as acetic, butyric and lactic, the amounts and proportions varying considerably with different bacteria involved. The sequence of reactions giving rise to these products is unknown. It has been assumed in some cases that they are products of the dissimilation of glucose, by mechanisms similar to those already understood for other bacteria.

The number of studies which have been conducted on cellulose decomposition by fungi is small and very little is known of the mechanisms involved.

It may be concluded, therefore, that the decomposition of cellulose by microorganisms is far from being clearly understood. The greatest difficulties heretofore have been concerned with bacteriological methods, since the isolation and culturing of cellulose-decomposing organisms has presented a number of unique problems. Furthermore, the application to cellulose decomposition of the modern concepts and techniques employed in the study of carbohydrate metabolism has been extremely slow. It is conceivable, however, that the metabolism

of microorganisms growing on cellulose differs considerably from that of microorganisms utilizing other carbohydrate substrates. Moreover, there is as yet no reason to believe that the mechanism of the decomposition of cellulose is similar for all groups or species of organisms attacking it.

EXPERIMENTAL

I. Media

Salt Solution: The standard salt solution used was similar to that of Kalnins (18) and consisted of

Na_2HPO_4	- 1.0 gm.	NaCl	- 0.1 gm.
$MgSO_4.7H_2O$	- 0.2 gm.	$FeCl_3$	- 0.02 gm.
$CaCl_2$	- 0.1 gm.	$NaNO_3$	- 1.0 gm.

Distilled water - 1000 ml.

The hydrogen-ion concentration was approximately pH 7.2, and during the course of the study, KH_2PO_4 was used in addition to adjust the pH, and to buffer the solution. However, the total phosphate was always maintained at 0.1 percent.

Cellulose Broth: Tubes containing 7 ml. of salt solution with a long narrow strip of filter paper in each constituted cellulose broth.

Cellulose Agar: Cellulose agar suitable for plates was prepared with 7.5 gm. precipitated cellulose, 15 gm. washed agar, and one litre of salt solution. The precipitated cellulose was prepared according to the method of Scales (28) by treating with 60 percent sulphuric acid at about 65°C. for 20 to 30 seconds. This treatment almost dissolved the cellulose and it was immediately reprecipitated by pouring into a large volume of cold water. It was found that the cellulose was most easily recovered by filtering on several folds of cheese-cloth, which also facilitated rapid washing

to remove acid. By incorporating the precipitated cellulose into the medium with the aid of a Waring blendor, an agar was obtained which was homogeneous and almost colorless.

Starch and Glucose Broth: These consisted of one percent starch or glucose in salt solution.

Starch-Peptone Agar: Prepared by adding 1.5 percent washed agar and one percent peptone to starch broth.

Cellulose: Whatman No. 41 filter paper was used throughout as the cellulose substrate.

II. Nature of the Organism

An impure culture of Vibrio perimastix, an aerobic cellulose-decomposing organism isolated from the soil (2) was employed in these studies. V. perimastix has been isolated but was not as active in decomposing cellulose as the impure culture.

Only one other organism, also a Vibrio, appears to be associated with the V. perimastix. It has not yet been successfully cultured alone. Reproducible results were usually obtained with the impure culture.

Active decomposition of cellulose took place over a range of 20° C. to 40° C. and from pH 5.0 to 7.5. There was no growth under anaerobic conditions.

Starch-peptone agar was used to maintain cultures of the organisms, with transfers being made periodically from cellulose medium. It has been reported that cellulose-decomposing organisms grown for a long period on carbon sources other than cellulose may lose their activity. However, inoculations from cultures maintained on starch-peptone agar were found to be as effective in attacking the cellulose as those from cellulose medium.

III. Physiological Characteristics

The pure and impure cultures differ markedly in their physiological characteristics. The former decomposes cellulose more readily with peptone or ammonium sulphate as the nitrogen source, whereas the latter utilizes nitrate most efficiently. Growth on starch-peptone agar is restricted and appears almost transparent in the case of the pure culture, whereas the mixed culture produces a heavy, slimy growth and a greenish-yellow pigment.

Thirty-three different media, including a number of sugars and enriched media, were tested, but good growth was obtained only with cellulose, starch, glucose, and cellobiose. Activity was rapid in cellulose broth with nitrate as the

nitrogen source, the filter paper strips being consistently cut at the air-liquid interface within three days. Using peptone, however, the time was extended to about ten days, and with ammonium chloride to about twenty days. Rupture of the paper was not confined to the interface, for considerable activity was also evident throughout the length of the paper immersed in the solution. The decomposition was accompanied by the production of a yellow pigment and the reduction of nitrate to nitrite, the latter being detected with Tromsdorff's Reagent.

Activity was limited on cellulose agar, the extent of the clear zonations around the colonies being very small. Growth was good on starch agar, and the starch was partially hydrolysed (Iodine test). Heavy growth was obtained with starch and glucose broth in cultures aerated with sterile air. Suspensions of the bacteria were obtained by this method of culturing, and these were used in some of the experiments to be described below.

It is of interest to note that considerably better growth was obtained with glucose broth sterilized by Seitz-filtration or autoclaved in distilled water, than with glucose broth autoclaved with the salt solution. Similar results were obtained by Stanier (31) using bacteria of the Cytophaga group.

Small amounts of starch and glucose (0.01-0.02 percent) added to the cellulose medium were found to considerably retard the rate of decomposition.

IV. Decomposition Studies

A series of decomposition studies was undertaken in order to investigate the metabolism of the bacteria on cellulose medium.

A. Method of Culture:

The organisms were cultured by the submerged culture method: sliced filter paper was suspended in salt solution contained in a round-bottom pyrex flask. The flask was provided with an air inlet, an air outlet, and an additional opening through which samples could be withdrawn. A vigorous stream of sterile air was obtained through suction and served to agitate the cellulose suspension and provide aeration.

This method of culturing was later replaced by mechanical shaking, which had a number of advantages, including more uniform aeration, more efficient agitation, and less danger of contamination. Agitation is especially important because the cellulose, with adhering bacteria, tends to settle to the bottom of the flask, so that it becomes difficult to provide an adequate oxygen supply.

Because of the uniformity of treatment afforded each culture by shaking, an attempt was made to determine the rate of decomposition of cellulose. For comparison, the rate was also determined with V. perimastix:

Two sets of 250 Erlenmeyer flasks were set up, each flask containing 0.25 gm. of filter paper and 50 ml. of salt solution. The rate of decomposition by the impure culture was considerably greater than that of V. perimastix. The results are illustrated in Figure I.

Cellulose cultures of large volume (750 ml. to 1000 ml.) were constantly maintained by the mechanical shaking method for use in many of the experiments to be described.

B. Analysis of the Products:

Minor variations in procedure were employed to identify the culture products and all of the tests were not applied to every culture. The general scheme of analysis was as follows:-

At the end of the incubation period the contents of the flask were filtered through filter paper or bolting silk, to remove the residual cellulose, and the pH of the filtrate was determined.

a) Cellulose: The cellulose was washed several times with hot water, the washings constituting the "hot-water extract".

This was followed by extraction with 95 percent alcohol, the "alcohol extract", and with ether, the "ether extract". The cellulose was then dried to constant weight at low temperature (about 80°C.) and weighed.

b) Filtrate: The filtrate was immediately tested for reducing sugars using Benedict's or Fehling's solution and a portion was hydrolysed with dilute sulphuric acid, neutralized, and reducing substances (calculated as reducing sugars) determined by the Shaffer method (30). Tests were also made for pentose sugars or uronide groups using Tollen's phloroglucinol reaction (14).

A portion of the culture liquid was treated, according to the procedure of Walker and Warren (34), with about three volumes of 98 percent ethyl alcohol containing a few drops of concentrated hydrochloric acid, in order to precipitate any mucilaginous materials such as have been reported as occuring in various aerobic decompositions (16) (34). Precipitation was also effected by adjusting the hydrogen-ion concentration of the filtrate to about pH 3 with dilute hydrochloric acid (Congo Red), as was done by Fahraeus (10). The suspension was allowed to stand in the cold for several hours, when the precipitate settled and was easily separated by centrifuging.

Another portion of the culture solution was centrifuged to separate excessive bacterial growth and then filtered with suction through a Chamberland bacteria-filter. The bacteria-free solution was placed in sterile test tubes containing filter paper strips in order to detect enzyme activity. An attempt was made to concentrate the filtrate by dialysing against a concentrated dextrin paste (39), but this was not satisfactory. The ninhydrin test was applied to the bacteria-free filtrate to detect soluble proteins or amino acids.

The culture solution was tested for volatile acids, bases, or alcohol, by steam-distilling an acid or alkaline solution under atmospheric pressure. Later, ether-extraction was substituted for steam-distillation in the detection of acids. The procedure was as follows:

The culture was acidified to Congo Red with hydrochloric acid. The cellulose was filtered off and extracted with redistilled ether in a Soxhlet apparatus. The moisture carried over into the distilling-flask was separated from the ether layer with the aid of a separatory-funnel. The filtrate was extracted with redistilled ether in a liquid-liquid extractor. A measured volume of distilled water was added to each ether-extract, the ether distilled off, and the residual liquid titrated with standard alkali.

c) Carbon Dioxide: Duplicate apparatus were set up for the determination of small amounts of carbon dioxide. They were arranged so that the cultures were oxygenated with a stream of air freed from carbon dioxide by passage first through concentrated potassium hydroxide and then soda lime. The efficiency with which carbon dioxide was removed, was determined by inserting, in the gas chain, an indicator tube containing dilute barium hydroxide and thymolphthalein. The moist air leaving the cultures was dried with calcium chloride and Anhydrone (anhydrous magnesium perchlorate), and the carbon dioxide contained therein was absorbed in Pregl micro-absorption tubes containing Ascarite, and determined gravimetrically. A standardized weighing technique was adopted in which three pairs of wiping-cloths were employed.

Trial runs were made with the apparatus, of several hours duration and using sodium carbonate and dilute sulphuric acid as the source of carbon dioxide, and gave satisfactory results.

C. Results:

a) General Characteristics of the Decomposition:

The changes in physical appearance of the cultures followed practically the same course in every case. In three to four days the filter paper strips were considerably frayed, a yellow pigment appeared, and considerable foaming occured at the surface of the culture which probably indicates protein synthesis. The yellow coloration and the degree of foaming increased for a period of a few days and then remained fairly constant. In the case of the bubbler-aeration technique, the paper was reduced to a very fine pulp after about five days when it tended to settle to the bottom of the vessel despite the agitation by the incoming air, and necessitated periodic shaking of the flasks.

Quantitative results obtained in four experiments using the bubbling method of aeration are given in Table I.

TABLE I. DECOMPOSITION OF CELLULOSE

Experiment	Time (days)	Temp. (°C)	Decomposition (%)	Initial pH	Final pH
I	21	22°	5.6	7.2	8.3
II	19	31°	14.9	7.4	8.9
III	9	33°	10.0	7.6	8.6
IV	9	34°	19.6	6.8#	8.3

(# adjusted with KH_2PO_4)

The decided shift to a basic reaction during the course of the decomposition suggested the formation of ammonia. Accordingly, air which had passed through a culture was bubbled into Nessler's Reagent (14). Compared with a blank carried out concurrently a positive test was obtained within 30 minutes with one six-day culture.

To ascertain if the appearance of the yellow coloration was associated with the observed rise in pH, samples were examined before and after the appearance of the pigment. However, no change in pH could be detected until several days after the pigment had appeared.

Figure I illustrates the marked difference in the rates of decomposition of cellulose by the pure and impure cultures of V. perimastix.

Reducing sugars were not found in any of a large number of cultures examined either during the period of active decomposition or in the filtrates at the end. To detect traces of reducing sugar, the residual cellulose from one culture was boiled several times in distilled water; the water-extract, together with some of the culture fluid, was concentrated in-vacuo to a small volume and clarified with charcoal. The concentrate failed to give a positive test for reducing sugar.

Hydrolysis of the culture liquid with acid produced small amounts of reducing substances, the highest yield being 0.03 percent. This may have been produced from the colloidal material which gave the filtrate an opalescent appearance and which could only be removed by precipitation with alcohol or acid. This material is discussed below.

A positive test was always obtained for pentoses even in small test tube cultures. Acid-distillation into phloroglucinol and hydrochloric acid gave a positive test for furfuraldehyde, which suggested the presence of uronic acid complexes; the amount was very small. The original filter paper gave a negative test.

Distillation of volatile products yielded basic substances, largely ammonia, but no acids or alcohol. Traces of acid were detected by the ether-extraction method, but the amount was too small to permit identification even when a litre of culture fluid was taken for the test. Qualitative tests for acetylmethylcarbinol and lactic acid gave negative results.

Bacteria-free filtrates failed to attack cellulose and contained no proteinaceous material.

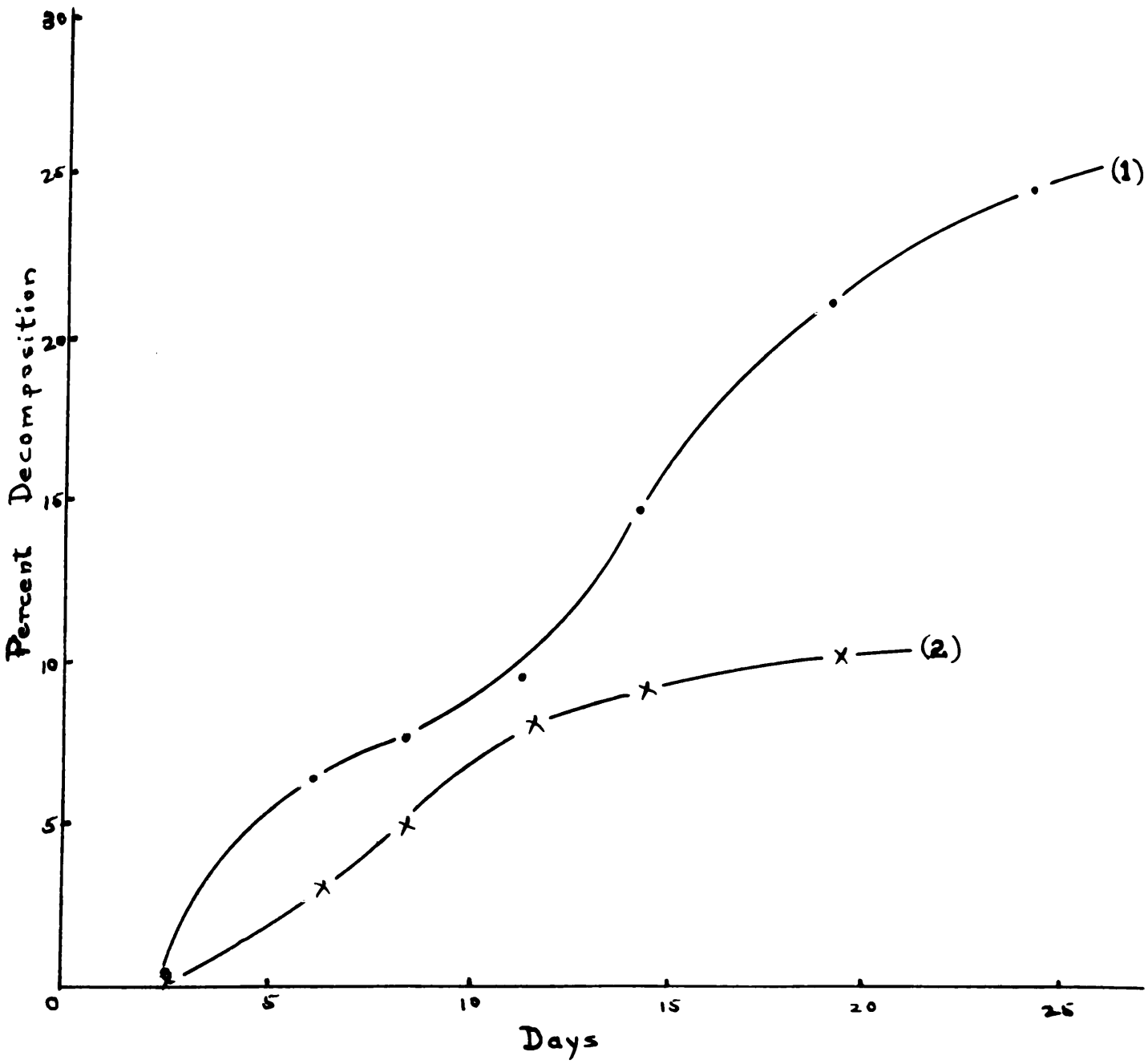


Figure I. Decomposition of cellulose by a pure and an impure culture of V. perimastix.

(1) Impure culture (2) Pure culture

b) Acid-Alcohol Precipitate: Small amounts of a white, bulky precipitate, which resembled the "mucilaginous material" of Walker and Warren (34), were separated from the filtrate by alcohol and by acid precipitation. However, the material gave a negative test for uronic acids and therefore was not identical with the precipitate which they obtained.

The material was insoluble in hot water, dilute acid and alkali. It dissolved slowly in concentrated sulphuric acid and was reprecipitated by diluting the solution with water, thus resembling cellulose.

A culture solution which had been filtered through a Chamberland filter was also treated with acidified alcohol, but the coagulated material did not settle out for several days. This indicated that only a portion of the precipitable material was of chain-length of the order of bacterial dimensions.

Because of the similarity to cellulose the rates of acid hydrolysis of the precipitated material and of very fine shreds of residual cellulose were compared. The precipitate was first dried and ground to a fine powder. It was then suspended in a small amount of acetone in order to lyse adhering bacterial cells, and the acetone was removed by centrifuging. This was followed by extraction in the cold (3^{o}C.) with three 20 ml. portions of trichloroacetic acid.

Finally, it was washed with acetone and dried in vacuo. The residual cellulose was similarly treated with trichloroacetic acid.

The samples were refluxed on a steam-bath with 2 N hydrochloric acid and aliquots were removed periodically for reducing sugar determinations. Reducing values were determined by the Shaffer-Somogyi method (30), after neutralizing the hydrolysates. Results are given in Table II. The rate of hydrolysis of the precipitated material was considerably more rapid than that of the residual cellulose.

TABLE II. RATES OF HYDROLYSIS OF ACID-PRECIPITATE AND RESIDUAL CELLULOSE

(mg. glucose per ml.)

Time (min.)	Precipitate	Cellulose
15	0.44	trace
30	0.54	0.28
60	0.56	-
120	0.63	0.45
180	0.73	0.45

The nitrogen content of the purified material as determined by the micro-Kjeldahl method (6) was 7.24 percent.

c) Alcohol Extract: The alcohol extract was usually a deep yellow-green color, and when evaporated to dryness in-vacuo it yielded a dark, oily residue. This residue was very slightly soluble in cold and hot water and a fraction of the suspension could be extracted with ether. This fraction was separable into acetone-soluble and acetone-insoluble portions, the former having a particularly pungent odor. These fractions were too small for characterization. Their appearance and solubilities, however, indicate that they were lipoid in nature. The substances were only found in cultures which had undergone extensive autolysis, and hence they were probably bacterial cell-materials rather than direct products of the decomposition of cellulose.

d) Pigment: In addition to the pigment in the alcohol extract there was present in all cultures a greenish-yellow, fluorescing, water-soluble pigment resembling riboflavin. This pigment could not be extracted with ethyl ether, petrol ether, chloroform, or ethyl acetate. It was extracted, however, by benzyl alcohol in the presence of barium chloride (5) and by butyl alcohol saturated with sodium sulphate (22) - both, methods for extraction of riboflavin - and these extracts exhibited fluoreseence which could be measured by the Coleman Fluorometer using B_2 and PC-2 filters. For example, a benzyl alcohol extract of 3 ml. of one

bacterial culture gave a reading of 13, compared with a blank reading of 6. The pigment could also be adsorbed on freshly-prepared lead sulphide from which it could be eluted with a mixture of 70 parts water, 2 parts glacial acetic acid and 30 parts pyridine.

The intensity of the fluorescence was diminished rapidly by ultra-violet radiations and by reduction with sodium hypo-sulphite. Oxidation by permanganate (22) destroyed a part of the fluorescence; for example, a butyl alcohol extract of one culture gave fluorometer readings of 75 and 66 before and after treatment with permanganate. The pigment was stable to con-centrated sulphuric acid.

The absorption spectrum of the pigment was in part investigated with the use of a Coleman spectrophotometer. No areas of maximum absorption were found in the visible range, but the absorption curve of the pigment in butyl alcohol rose sharply in the range 4300A to 4000A. On the other hand, a butyl alcohol extract of riboflavin exhibited one absorption band at about 4400A.

A quartz spectrograph (Littrow-mounting) was also used, in order to examine the near ultra-violet, from about 2000 to 3500A, since riboflavin shows maximum absorption in this range. However, all of the solvents which extract the pigment also

absorb strongly in this region of the spectrum.

An attempt was made to clarify a sample of culture fluid directly, so as to avoid the use of immiscible solvents. Seitz filtration removed the pigment along with the bacteria. When the liquid was dialysed to remove salts and other soluble substances which might have been present, the yellowish fluorescence changed to a bluish tinge.

e) Carbon Dioxide: While attempting to determine the amount of carbon dioxide produced in the decomposition of cellulose, it was found that newly-inoculated cultures failed to show activity, no growth being apparent after five to six days. The apparatus for the determination of carbon dioxide had been designed so as to duplicate as nearly as possible the most favourable conditions for culturing the bacteria. As previously pointed out, the oxygen tension in the system was lower than was obtained with the shaking method. However, this was not believed seriously detrimental to the bacteria. A more striking difference lay in the fact that carbon dioxide was absent from the air entering the culture-flasks. Experiments were, therefore, undertaken to ascertain if the organisms required carbon dioxide.

To obtain some measure of the carbon dioxide evolved in the decomposition of cellulose, an experiment was carried out

using 100 ml. aliquots removed aseptically from an actively-growing culture. The duration of the experiment was 8 days, and the amount of decomposed cellulose appearing as carbon dioxide was found to be about 30 percent.

D. The Role of Carbon Dioxide:

a) Essentiality of Carbon Dioxide: The carbon dioxide requirement of the bacteria was examined in the following manner:

Four flasks, fitted with Folin bubblers, and containing cellulose media were inoculated in the usual manner. The air entering two of the flasks passed through potassium hydroxide and soda lime, thus removing carbon dioxide, while the other two flasks received air containing the normal complement of carbon dioxide. Aeration was vigorous in order to prevent the possible accumulation of respired carbon dioxide in the carbon dioxide-depleted flasks. The percent decomposition of cellulose was determined and the hydrogen-ion concentration of the cultures was recorded at the beginning and end of the experiments.

At first the flasks were shaken and the air from which carbon dioxide was to be removed was passed through two

bubbler-bottles containing 50 percent potassium hydroxide and two U-tubes containing soda lime. Under these conditions cellulose decomposition was always considerably reduced in cultures deprived of carbon dioxide, but never completely inhibited. In some experiments it was found, by using barium hydroxide and thymolphthalein, that carbon dioxide had passed through the train.

To more effectively remove carbon dioxide, a tube of soda lime (2 feet long and 1½ inches diameter) similar to that used by Gladstone et al (12) was substituted for the U-tubes, and because of the large amount of rubber tubing necessary, shaking was eliminated. Flasks of different design were tested. A type which was fairly satisfactory and permitted vigorous aeration of cellulose is illustrated in the diagram of the apparatus (Plate I). A needle-point inoculum was used so as to minimize the amount of carbon dioxide formed in the preliminary stages through bacterial respiration per se. The importance of a small inoculum was emphasized by Rahn (27).

Under these conditions there was no growth or cellulose decomposition in the cultures from which carbon dioxide was excluded. The results of a typical experiment are given in Table III.

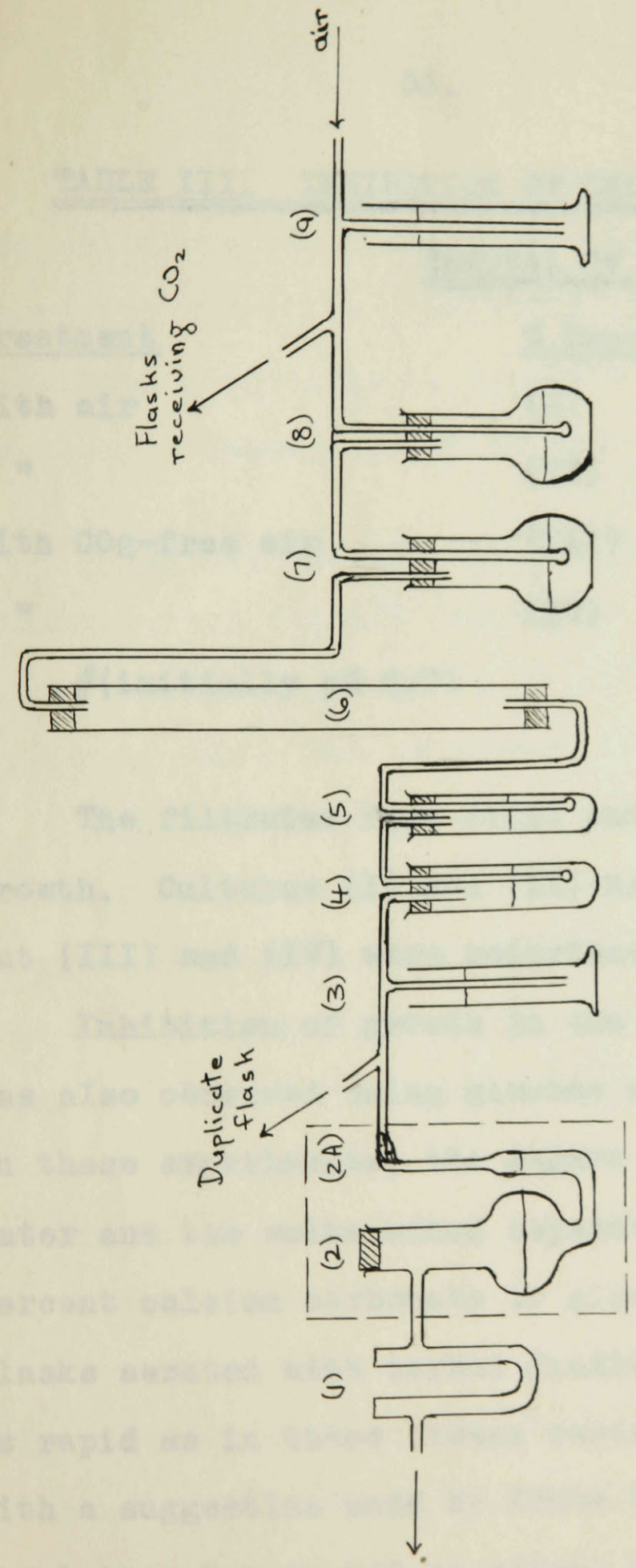


Plate I. Diagram of apparatus for studying the effect of carbon dioxide.

TABLE III. INHIBITION OF CELLULOSE DECOMPOSITION BY THE REMOVAL OF CARBON DIOXIDE

Treatment		% Decomposition	Days	pH#
With air	(I)	5.2	8	7.0
"	(II)	6.0	8	7.0
With CO_2-free air	(III)	0	8	6.6
"	(IV)	0	8	6.6

#(initially pH 6.7)

The filtrates from (III) and (IV) showed no bacterial growth. Cultures (I) and (II) had an intense yellow color but (III) and (IV) were colorless.

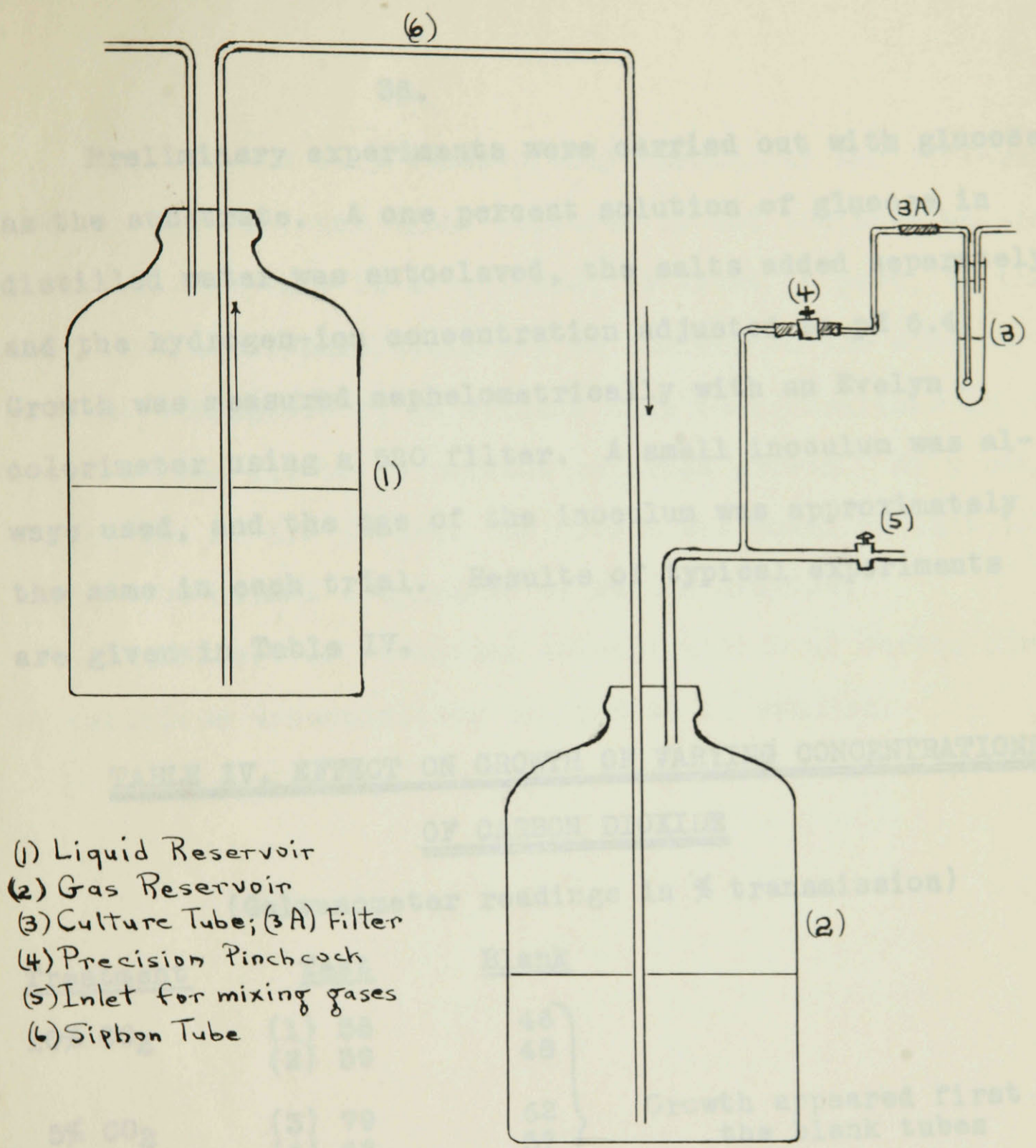
Inhibition of growth in the absence of carbon dioxide was also observed using glucose and cellobiose as substrates. In these experiments, the sugars were autoclaved in distilled water and the salts added separately. The addition of one percent calcium carbonate to glucose permitted growth in the flasks aerated with carbon dioxide-free air, but it was not as rapid as in those flasks receiving normal air. In connection with a suggestion made by Krebs (20), sodium citrate was added to glucose but it had no effect in replacing carbon dioxide.

On the other hand, the addition of calcium carbonate to cellulose was found unsatisfactory for growth and decomposition under the conditions employed. Hence, it has thus far been impossible to obtain a carbon balance-sheet of cellulose decomposition by the organisms.

b) Effect of Varying the Concentration of Carbon Dioxide: Since carbon dioxide was found to be essential for growth of the bacteria, the effect of using concentrations of carbon dioxide above that of normal air was investigated.

An aspirator was used to prepare the gas mixtures. Its design is illustrated in Plate II. The enrichment of air with carbon dioxide was carried out in the lower flask, which served as the gas reservoir. The water used for displacement of the gas was adjusted to below pH 3 so as to prevent absorption of carbon dioxide from the gas mixtures and also to prevent microbial growth in the reservoir. The system was completely air-tight.

A blank determination, using normal air, was always included in each trial. Unfortunately, only one aspirator was available, so in the case of the blank, air was simply drawn through by suction. The rate of bubbling in the test and blank tubes was kept relatively constant by adjustment with precision pinchcocks.



(1) Liquid Reservoir
(2) Gas Reservoir
(3) Culture Tube; (3A) Filter
(4) Precision Pinchcock
(5) Inlet for mixing gases
(6) Siphon Tube

Plate II. Diagram of apparatus for studying the effect of varying the concentration of carbon dioxide.

Preliminary experiments were carried out with glucose as the substrate. A one percent solution of glucose in distilled water was autoclaved, the salts added separately and the hydrogen-ion concentration adjusted to pH 6.4. Growth was measured nephelometrically with an Evelyn colorimeter using a 520 filter. A small inoculum was always used, and the age of the inoculum was approximately the same in each trial. Results of typical experiments are given in Table IV.

TABLE IV. EFFECT ON GROWTH OF VARYING CONCENTRATIONS OF CARBON DIOXIDE

(Galvanometer readings in % transmission)

Treatment	Test	Blank	
10% CO_2	(1) 56	46	Growth appeared first in the blank tubes
	(2) 59	48	
5% CO_2	(3) 79	62	
	(4) 62	37	
2.5% CO_2	(5) 60	54	
	(6) 46	35	
1.2% CO_2	(7) 36	37	Growth appeared in both tubes at approx. the same time
	(8) 36	38	
Normal air	32	31	

The results vary considerably throughout and differences between the test and blank cultures do not indicate an orderly progression. This is attributed, at least in part, to the fact that all trials could not be limited to the same period of time. However, it is evident that concentrations of carbon dioxide from 2.5 to 10 percent retarded growth. At a concentration of 1.2 percent the growth was equal to that in air having the normal complement of carbon dioxide.

The effect of increased concentrations of carbon dioxide on cellulose decomposition has yet to be studied.

V. Experiments on the Mode of Attack on Cellulose

A. Respiration Studies:

a) Methods:

1. Thunberg Method: This technique detects and measures dehydrogenase activity. It is based on the reduction of methylene blue, a reducible dye which may be conveniently used as the oxidizer in biological systems in place of molecular oxygen. Hence, in the enzymic oxidation of a substance, it acts as the hydrogen-acceptor whilst the substrate acts as the hydrogen-donor.

By adding methylene blue to suspensions of bacteria in neutral buffer solution it is thus possible to determine the effect of various substances on the time required to reduce the dye. However, the reduced methylene blue is readily reoxidized by atmospheric oxygen so that the reactions must be carried out in special Thunberg tubes, which can be evacuated. A good description of the apparatus and its use is given by Ahlgren (1).

2. Direct Warburg Technique: Cell respiration is measured by a manometric method employing a constant volume type of respirometer. The reaction takes place in a special type of vessel having an outlet which leads directly to a U-type of manometer, open to atmospheric pressure. The gas evolved, or absorbed, can be readily measured by the differences in the readings of each arm of the manometer. A description of the

apparatus and the techniques involved is given by Dixon (9).

3. Bacterial Suspensions: These were prepared by growing the organisms in starch-peptone or glucose-peptone broth or on cellulose medium, from which they could be readily separated by centrifuging, but after filtration in the case of cellulose. The bacteria were washed with, and suspended in, M/5 Na_2HPO_4-KH_2PO_4 buffer of pH 7.0.

b) Application:

1. Thunberg Experiments: A large number of experiments were carried out, many of which proved valueless. Considerable difficulty was encountered in obtaining the organisms in a sufficiently impoverished state so that they would attack the substrates instead of using their own metabolites.

To impoverish the organisms they were vigorously oxygenated in phosphate buffer, then recentrifuged and resuspended. It was difficult to determine the proper length of time of oxygenation, because in some cases the decolorations required several hours. Typical results are given in Table V.

TABLE V. - DEHYDROGENASE ACTIVITY

Substrate	Time (min.)	Conditions
(1) 1% cellobiose (in PO_4 buffer)	46	pH - 7.0
(2) 1% glucose " " "	43	Temp. - 35° C.
(3) 1% cellulose " " "	#	MeB - 0.5 ml.
(4) Blank " " "	62	Bacterial suspension - 1.0 ml.
(5) Blank " " "	58	Total volume - 6.5 ml.

(Larger than the blank determinations)

As can be seen, dehydrogenase activity was found for both glucose and cellobiose but none with cellulose. The dehydrogenases for cellobiose and glucose could be demonstrated to better advantage by reoxidizing the contents of the tubes (thus oxidizing the leuco-methylene blue) by shaking for about a minute while the tubes were open to the air, and then allowing the reaction to again take place. The results of one such experiment, where the original times for the test solutions and the blanks were about equal, is given in Table VI.

TABLE VI. DEHYDROGENASE ACTIVITY

Substrate	Time (min.) I	II	III	Conditions
(1) 1% cellobiose (in PO_4 buffer)	100	77	70	pH - 7.0
(2) 1% glucose " " "	97	74	60	Temp. - 35°C.
(3) Blank " " "	100	131	214	MeB - 0.5 ml.
(4) Blank " " "	104	118	260	Bacterial suspension-1.0 ml.
				Total volume - 6.5 ml.

The prolongation of decoloration time in tubes containing cellulose may have been due to a changed oxidation-reduction potential brought about by the presence of interfaces. This possibility is suggested by Oppenheimer and Stern (25) when referring to the finding of L. Michaelis that methylene blue when adsorbed on filter paper is not reduced by a suspension of platinized asbestos saturated with hydrogen.

It appeared desirable, therefore, to investigate the problem from another standpoint by carrying out a Warburg experiment.

2. Warburg Experiments:

(a) Utilization of Postulated Intermediates: The results of two experiments which verify those obtained by the Thunberg technique, are illustrated in Figure II. Bacterial suspensions from two sources are represented - one, grown on starch-peptone, the

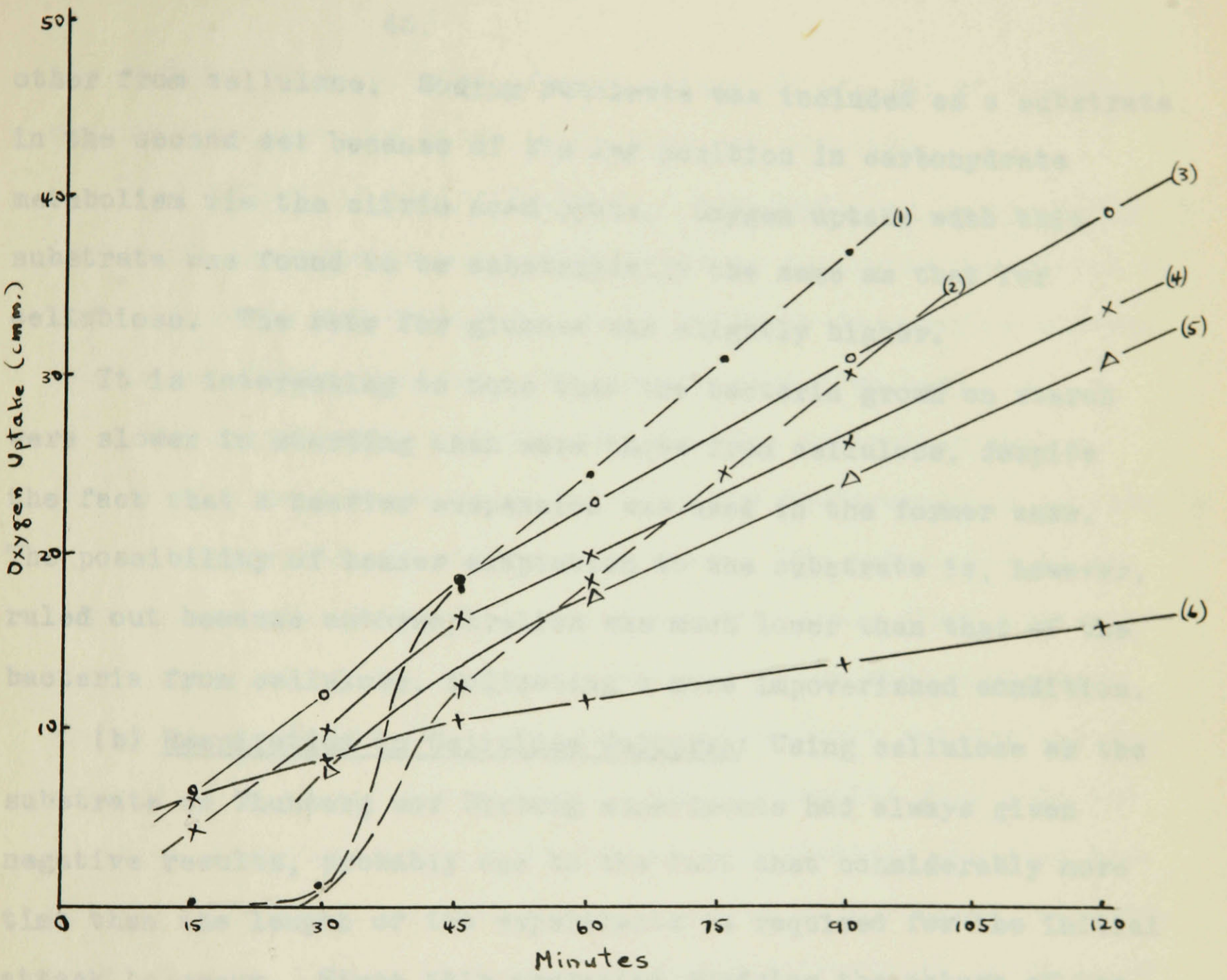


Figure II. Utilization of postulated intermediates - Warburg Direct Method.

(1) Glucose (bacteria from starch)
(2) Cellobiose " " "
Autorespiration " " " - (zero)
(3) Glucose (bacteria from cellulose)
(4) Cellobiose " " "
(5) Succinate " " "
(6) Autorespiration " " "

other from cellulose. Sodium succinate was included as a substrate in the second set because of its key position in carbohydrate metabolism via the citric acid cycle. Oxygen uptake with this substrate was found to be substantially the same as that for cellobiose. The rate for glucose was slightly higher.

It is interesting to note that the bacteria grown on starch were slower in starting than were those from cellulose, despite the fact that a heavier suspension was used in the former case. The possibility of lesser adaptation to the substrate is, however, ruled out because autorespiration was much lower than that of the bacteria from cellulose, indicating a more impoverished condition.

(b) Respiration in Cellulose Cultures: Using cellulose as the substrate in Thunberg and Warburg experiments had always given negative results, probably due to the fact that considerably more time than the length of the experiments is required for the initial attack to occur. Since this prevented studying the nature of the decomposition with respect to such things as inhibitors and stimulants, the possibility of using suspensions of active cellulose cultures in the Warburg vessels was investigated. It was found that cultures in which decomposition had progressed to a considerable degree furnished satisfactory suspensions: the culture was well dispersed, samples of the cellulose together with adhering bacteria were taken with wide-mouth pipettes and used directly in

the Warburg vessels. The rate of oxygen uptake was of a readily-measurable order and good duplication was obtained.

(c) Effect of Adding Postulated Intermediates to Cellulose: A number of experiments were carried out to determine the effect of adding glucose and cellobiose to an actively-decomposing culture. Sodium succinate, maltose, and lactose were also used.

The oxygen uptake of the cultures was determined over a period of 2 to 3 hours so as to allow for differences in uniformity of the suspensions in different vessels. The substrates to be tested were then added from the side-arms, their final concentrations being about one percent. Results of one such experiment are given in Figure III. The cellulose-bacteria suspensions were taken from a 17-day old culture. Similar results were obtained on three occasions using cultures of approximately the same age. However, identical experiments carried out with younger cultures, about 8 days, failed to show appreciable differences between the substrates added to cellulose.

(d) Influence of Carbon Dioxide: The above-described Warburg experiments were carried out before the essentiality of carbon dioxide had been established. Since the Warburg "direct" method involves exclusion of carbon dioxide, the "indirect" method of Warburg was employed to investigate the effect of the presence of carbon dioxide. In the latter method oxygen uptake is measured

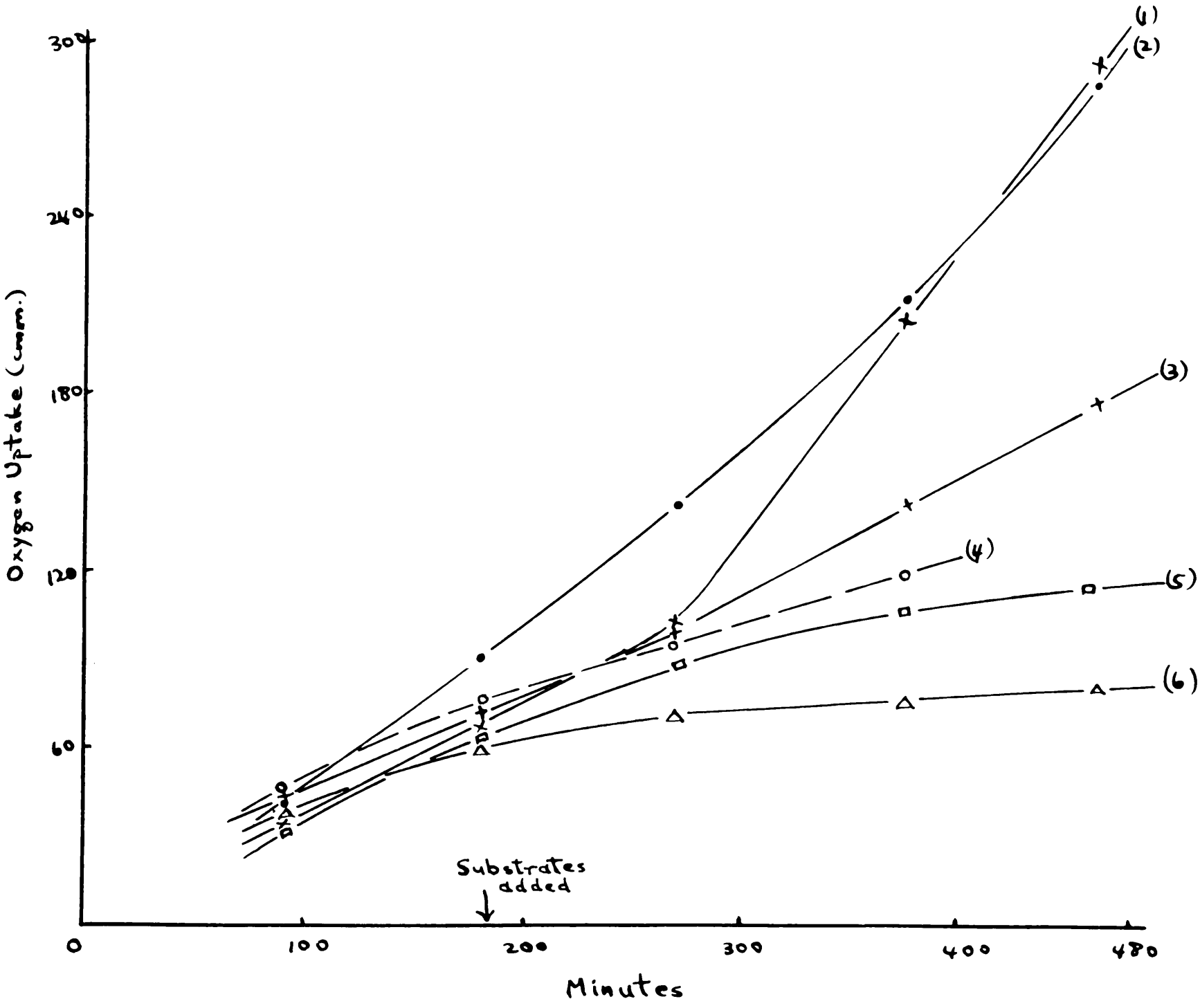


Figure III. Effect on oxygen uptake of adding postulated intermediates to cellulose - Warburg Direct Method.

(1) with added Cellobiose
(2) " " Glucose
(3) " " Maltose
(4) Cellulose (blank)
(5) with added Lactose
(6) " " Succinate

in the presence of carbon dioxide, and the carbon dioxide output may also be determined. Detailed descriptions of the method are given in (9) and (33).

(i) Respiration in cellulose cultures: The oxygen uptake of two different cultures on cellulose medium was measured by the direct and indirect methods, that is, in the presence and in the absence of carbon dioxide. The results are illustrated in Figure IV. Numbers I and II were taken from one culture (final pH 7.4) which was 17 days old, while III and IV, from another culture (final pH 7.6), were 20 days old. The overall oxygen uptake in both cultures was greater in the presence of carbon dioxide than when carbon dioxide was removed from the vessels.

(ii) Effect of adding compounds presumed to be intermediates: The effect of adding glucose and cellobiose to actively-growing cultures was re-investigated by the indirect Warburg method, using 20-day old cultures (final pH 7.6). The results were essentially the same as those obtained by the direct method, but the stimulation of oxygen uptake by cellobiose and glucose was more evident. The rates of carbon dioxide evolution followed trends similar to the rates of oxygen uptake, as illustrated in Figure V.

These experiments were repeated at 27°C. with essentially the same results as at 37°C.

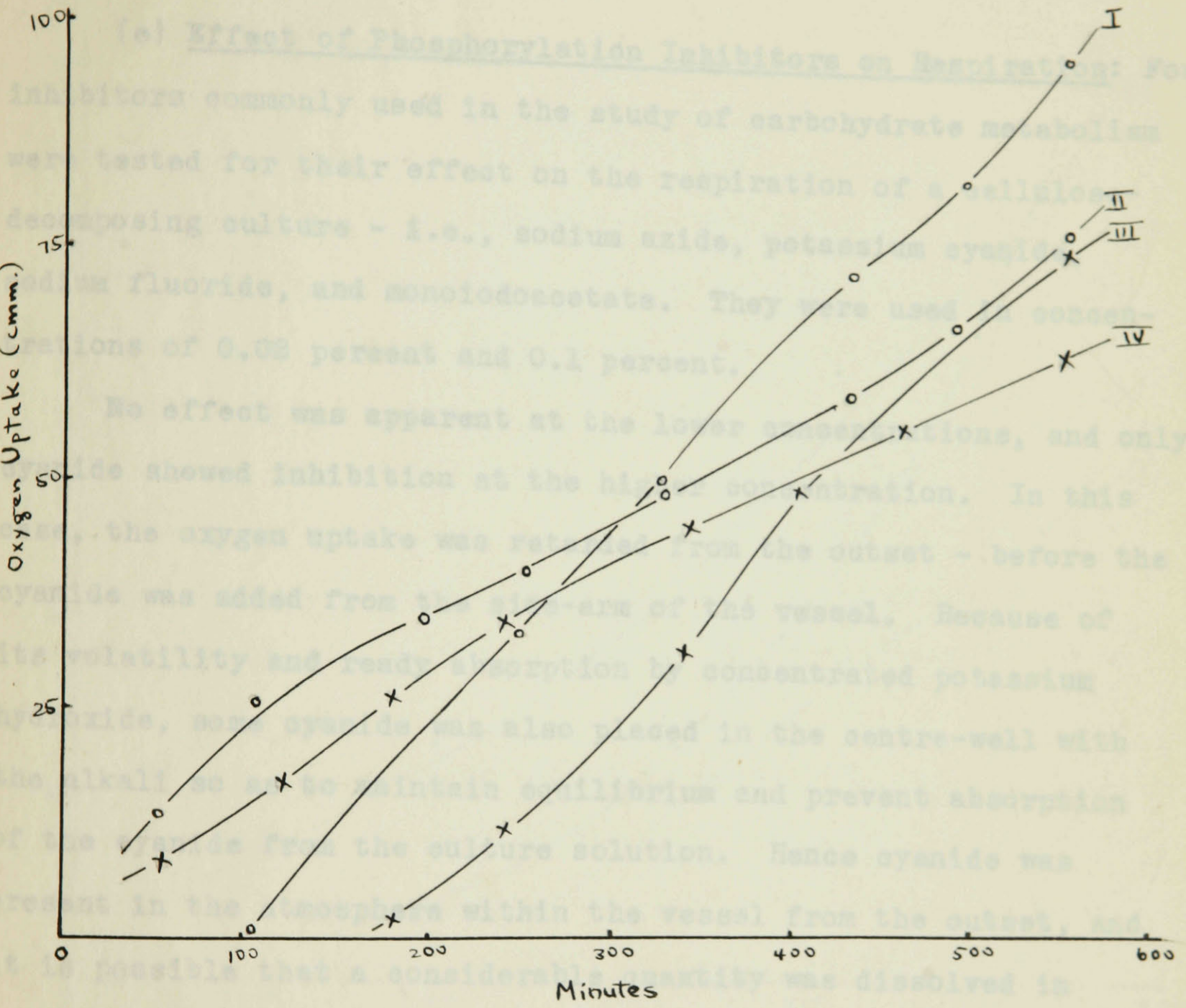


Figure IV. Influence of carbon dioxide on oxygen uptake of a cellulose culture.

Culture A: I - with CO_2 Culture B: III - with CO_2

II - without CO_2 IV - without CO_2

(e) Effect of Phosphorylation Inhibitors on Respiration: Four inhibitors commonly used in the study of carbohydrate metabolism were tested for their effect on the respiration of a cellulose-decomposing culture - i.e., sodium azide, potassium cyanide, sodium fluoride, and monoiodoacetate. They were used in concentrations of 0.02 percent and 0.1 percent.

No effect was apparent at the lower concentrations, and only cyanide showed inhibition at the higher concentration. In this case, the oxygen uptake was retarded from the outset - before the cyanide was added from the side-arm of the vessel. Because of its volatility and ready absorption by concentrated potassium hydroxide, some cyanide was also placed in the centre-well with the alkali so as to maintain equilibrium and prevent absorption of the cyanide from the culture solution. Hence cyanide was present in the atmosphere within the vessel from the outset, and it is possible that a considerable quantity was dissolved in the culture solution.

To allow for the possibility of slow diffusion of the inhibitors into the cellulose fibres, an experiment was done in which oxygen uptake was measured twelve hours after adding the inhibitors. A concentration of one percent of the inhibitors was used and phlorhidzin was also tested. Results are given in Table VII.

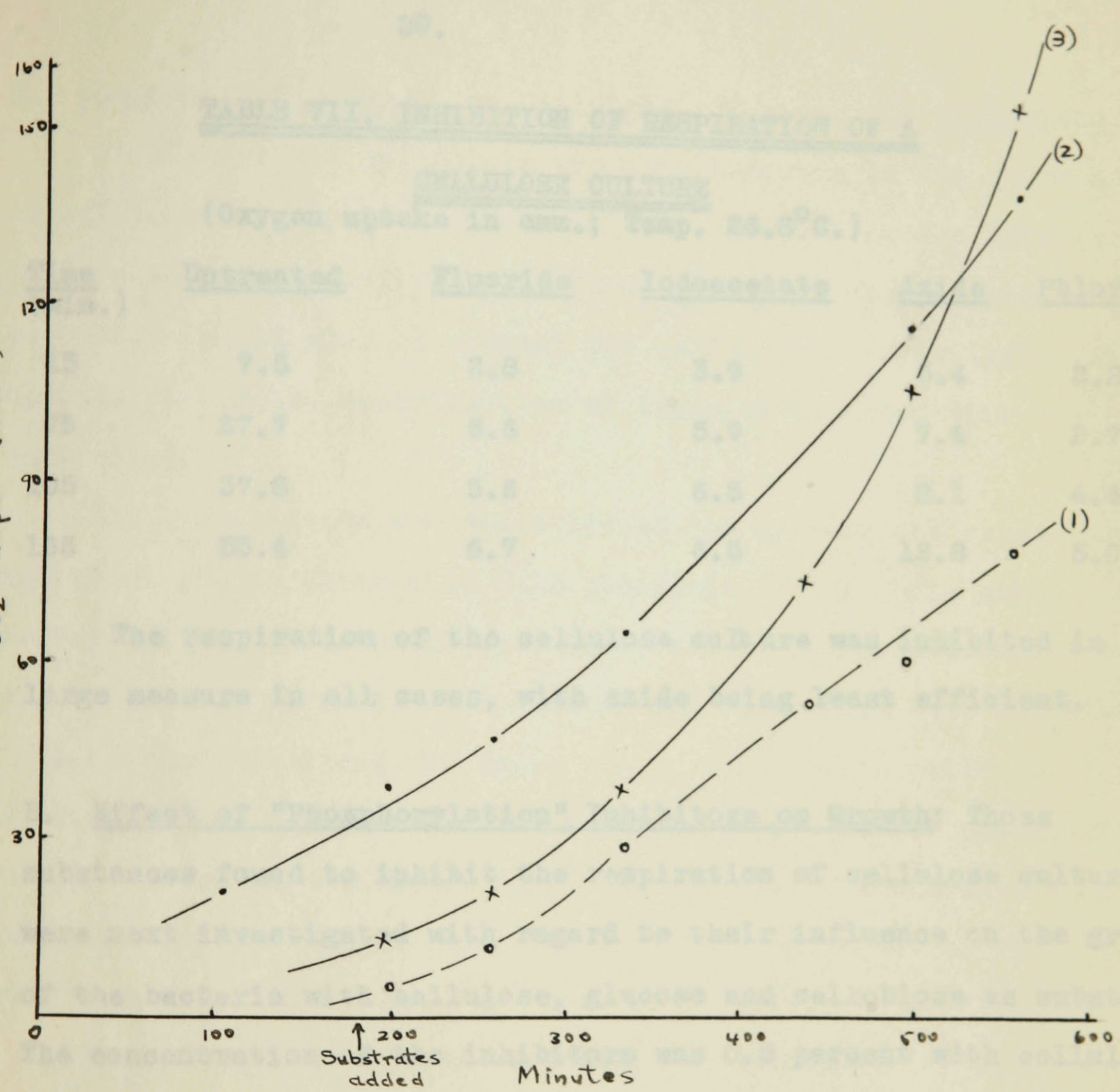


Figure V. Effect on carbon dioxide output of adding postulated intermediates to cellulose - Warburg Indirect Method.
(1) Cellulose (blank) (2) with added Glucose
(3) with added Cellobiose

TABLE VII. INHIBITION OF RESPIRATION OF A CELLULOSE CULTURE

(Oxygen uptake in cmm.; Temp. 26.8°C.)

Time (min.)	Untreated	Fluoride	Iodoacetate	Azide	Phlorhidzin
15	7.5	2.8	3.9	3.4	2.2
75	27.7	5.6	5.9	7.4	2.7
105	37.8	5.6	6.5	8.1	4.4
165	55.4	6.7	6.5	12.8	5.0

The respiration of the cellulose culture was inhibited in large measure in all cases, with azide being least efficient.

B. Effect of "Phosphorylation" Inhibitors on Growth: Those substances found to inhibit the respiration of cellulose cultures, were next investigated with regard to their influence on the growth of the bacteria with cellulose, glucose and cellobiose as substrates. The concentration of the inhibitors was 0.5 percent with cellulose and 0.25 percent with glucose and cellobiose.

In each case, with the exception of fluoride, complete inhibition resulted; in the presence of fluoride there was a very slight growth in glucose and cellobiose broth. The untreated cultures all showed normal growth.

C. Optimum pH for Growth on Cellulose, Glucose, and Cellobiose:

If glucose and cellobiose are intermediates in the decomposition of cellulose, it would be reasonable to expect that the hydrogen-ion concentration most favourable for growth on glucose and cellobiose would also be optimal for cellulose. Accordingly, the optimum pH for the decomposition of these three substrates was determined.

The hydrogen-ion concentration of the glucose and cellobiose solutions was adjusted with M/15 phosphate buffer. The usual amounts of the other salts were used, and the concentration of the sugars was one percent. The glucose and cellobiose were autoclaved in distilled water and the salts added later. With cellulose, growth was not obtained with M/15 buffer, hence it was necessary to use the normal concentration of phosphate. The concentration of cellulose was one percent. Equal amounts of inoculum were used and the cultures were prepared in duplicate.

With sugars, growth was measured nephelometrically with an Evelyn colorimeter, using a 520 filter, and with cellulose the percent decomposition was measured.

Results of this experiment are illustrated in Figure VI.

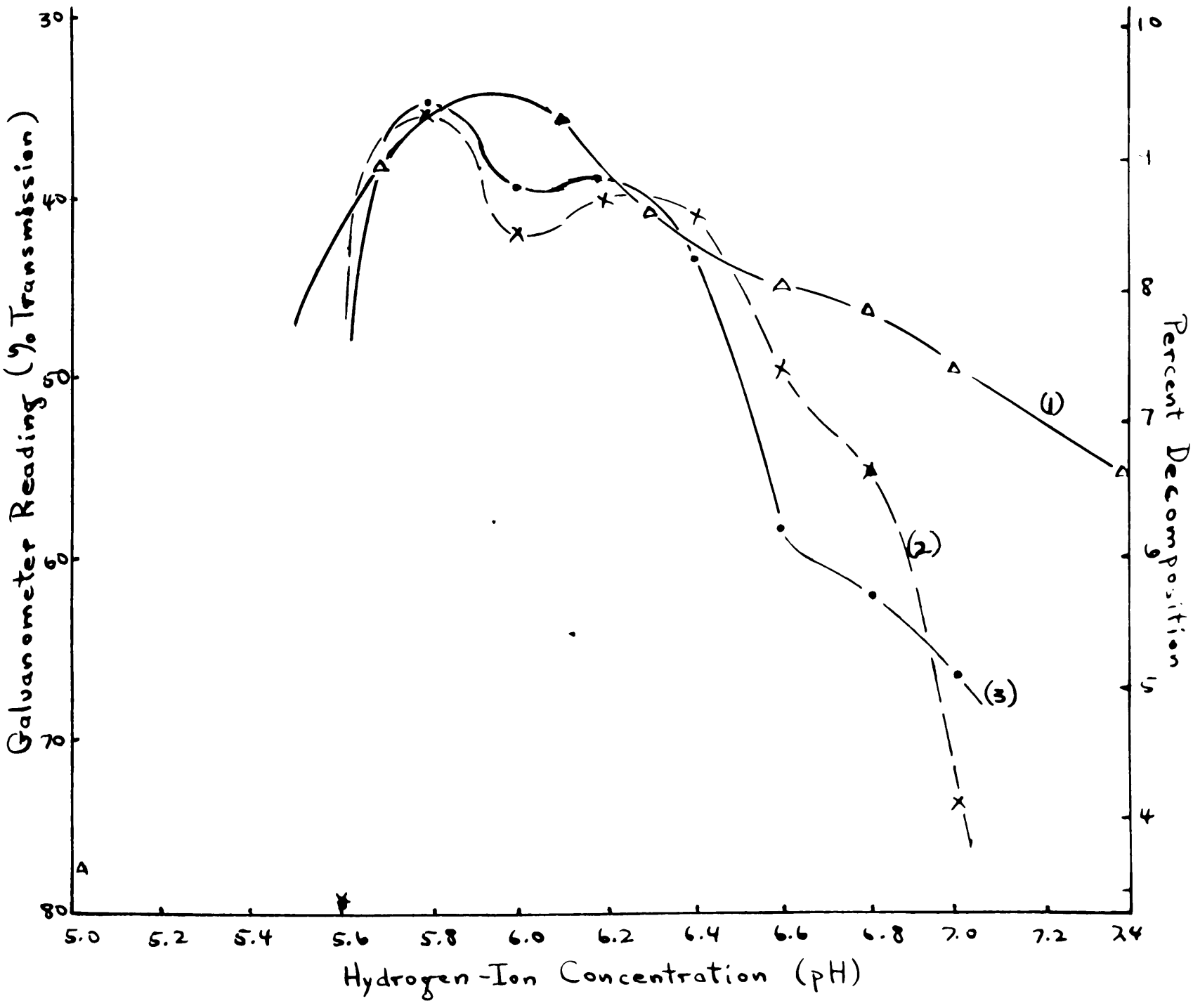


Figure VI. Optimum pH for growth on cellulose, glucose and cellobiose.

(1) Cellulose (2) Glucose

(3) Cellobiose

D. Production of Reducing Sugars:

A number of workers have demonstrated the formation of reducing sugars in cellulose cultures by employing various treatments designed to disrupt the normal activity of the organisms. In some cases, however, these methods have failed to cause an accumulation of reducing sugars. The ability of the bacteria, used in this study, to produce reducing substances from cellulose was therefore examined.

Aliquots of cellulose cultures showing normal activity were adjusted to about pH 6.5; 5 ml./per 50 ml. of culture fluid were of toluene added, and the cultures were incubated at 26°C. Aliquots were removed from these periodically and reducing sugars determined by the Shaffer-Somogyi method (30).

The amount of reducing sugars produced varied considerably with the age and final pH of the cultures. In some cases the rate of formation was rapid and the final concentration of sugar was of a high order. For example, one culture showed an accumulation of 56.3 mg. reducing sugars per 50 ml. after 4 days incubation.

a) Identification of Reducing Sugar:

About 500 ml. of an active cellulose culture was incubated with toluene for 5 days, the cellulose filtered off and the culture

liquid concentrated in vacuo to a small volume. To precipitate inorganic salts, 95 percent ethyl alcohol was added, and finally the alcohol was distilled-off. The remaining liquid was clarified with charcoal and diluted to a known volume. The concentration of sugar was too small to permit identification by measuring optical rotation.

With phenylhydrazine hydrochloride and sodium acetate, an osazone formed in about 45 minutes. Glucosazone and cellobiosazone were also prepared, the latter forming only on cooling the solution after boiling for over an hour, while glucosazone formed in ten minutes.

Examined microscopically, the crystals of the unknown had the characteristic appearance of glucosazone; only one type of crystal was present.

Melting-point determinations were carried out with the unknown osazone and glucosazone. Both gave melting points of 203° to 205°C. corrected (theoretical 204° to 205°C.). A mixed melting-point carried out with equal portions of the unknown and glucosazone, gave a reading of 205°C., corrected.

The reducing sugar produced in the cellulose cultures was therefore, glucose.

b) Effect of Inhibitors on the Formation of Glucose:

The effect of iodoacetate and fluoride on the production of glucose was examined. Azide and phlorhidzin could not be used in these experiments because they interfered with the determination of reducing sugars.

The compounds were added in concentrations of one percent to aliquots of cultures treated with toluene in the usual manner, and reducing sugars determined periodically. Results are given in Table VIII. Iodoacetate completely inhibited the formation of glucose, while fluoride partially inhibited glucose production.

TABLE VIII. INHIBITION OF GLUCOSE FORMATION
(mg. glucose per 100 ml.)

Time (hrs.)	Untreated	Fluoride	Iodoacetate
13	12	trace	0
19	25	12	0
38	43	18	0

c) Effect of Dialysis on the Formation of Glucose:

The rate of glucose formation was generally found to decline gradually after a few days, and in some cases, dropped off sharply.

The possibility was considered that accumulation of glucose might retard the additional formation of glucose by a mass-action effect. Dialysing the sugar as rapidly as it is formed should, in such a case, increase the total sugar produced over a given period.

Accordingly, aliquots of active cellulose cultures which were treated in the usual manner with toluene, were dialysed in collodion sacs against a salt solution of equal salt concentration and the same pH; toluene was also added to the dialysate.

Dialysing 50 ml. aliquots of cellulose culture against 100 ml. and 200 ml. portions of salt solution did not show a significant increase in the total glucose produced in 4.5 days. The values obtained were 56.4 mg. and 56.3 mg., respectively, as compared with 53.6 mg. produced by 50 ml. of the same culture incubated with toluene in an Erlenmeyer flask.

When dialysis was carried out in like manner against a larger volume of salt solution - 400 ml. or 500 ml. - no production of reducing sugars occured in the same period of time. To investigate this point, 50 ml. aliquots of a culture treated with toluene were dialysed against 100 ml., 300 ml., and 500 ml. portions of salt solution, respectively. Another aliquot was incubated in an Erlenmeyer flask for comparison. The rates of glucose formation were determined with the results given in Table IX.

TABLE IX. EFFECT OF DIALYSIS ON THE FORMATION OF GLUCOSE

(Total mg. glucose, including dialysate)

Time (days)	Untreated	Dialysed against 100 ml.	300 ml.	500 ml.
1	11	0	0	0
2	20	21	trace	0
4	29	27	33	0
5	-	43	40	-

These results suggested that some substance(s) produced by the bacteria, and necessary for glucose production was being removed by dialysis; also, that in order for glucose to appear, a sufficient concentration of the dialysable factor would have to be produced in order to counteract the effect of dialysis.

The hydrogen-ion concentration inside the collodion membranes did not change appreciably and hence a Donnan equilibrium-effect was considered unlikely.

Should a dialysable factor be involved, then by discontinuing the dialysis the concentration of such a factor should be increased in the culture sufficient to permit glucose formation.

To investigate this possibility, the contents of a membrane, which had been dialysed against 500 ml. of salt solution and in which no glucose had been formed, was divided into two test tubes. These were incubated with toluene and reducing values determined periodically. Reducing sugars were found to be present in 12 hours, the concentration being 0.2 mg. per ml.

Further evidence that a dialysable factor is involved in glucose formation was obtained as follows:-

Two 50 ml. aliquots of a toluene-treated culture were dialysed against 100 ml. and 300 ml. of salt solution, respectively. When glucose was detected in the 100 ml. dialysate, equal portions of the culture which was being dialysed against 300 ml. of salt solution and which contained no glucose, were transferred to two test tubes. To Tube I was added 10 ml. of the 100 ml. dialysate, which should have contained an appreciable concentration of the dialysable factor, and to Tube II was added 10 ml. of a freshly-prepared salt solution.

After 8 hours incubation, Tube I contained 0.10 mg. glucose per ml., glucose was not present in Tube II. At 22 hours the concentration in Tube I had increased to 0.14 mg. per ml., and only a trace appeared in Tube II.

E. Influence of the Physical Nature of Cellulose on Decomposition:

The chemical inertness of cellulose and its relative insolubility in water, requiring a powerful exo-enzyme system, are generally considered primary factors affecting the extent of its utilization by bacteria. In woody materials, however, where interpenetrating layers of lignin encrust the cellulose fibre, the physico-chemical structure also becomes of considerable importance in respect to the availability of cellulose to microbial attack.

In the light of the modern concept of the structure of cellulose, the closely-packed nature of the macromolecules of the crystalline portion renders difficult the penetration of chemical and physical agents between the micelles. On the other hand, the less orderly amorphous portion would be more readily affected by these same agents. Therefore, it seems reasonable to suggest that the amorphous type of cellulose should be more readily decomposed than crystalline cellulose, being more accessible to bacteria and permitting diffusion of the large enzyme molecules.

To test this hypothesis an experiment was carried out in which cellulose was treated to increase its amorphous content, and the rate of its decomposition compared with untreated cellulose.

The amorphous portion of cellulose was increased by treating according to Assaf et al (4), i.e., about 0.25 gm. of filter paper was immersed in 15 ml. of 13 percent sodium hydroxide at 3.5°C. for 3 hours, then diluted with 2 percent sodium hydroxide and allowed to stand for half-an hour. (Note: The method of Assaf et al employs ten percent sodium hydroxide with cotton linters. It has been found that with No. 41 Whatman filter paper 13 percent sodium hydroxide is the minimum concentration which will give satisfactory swelling of the fibres. This concentration may vary with the grade of filter paper.)

The filter paper was recovered by filtration and was thoroughly washed with water, 1 percent acetic acid, distilled water, and salt solution, in that order. It was then suspended in 50 ml. of salt solution in a 250 ml. Erlenmeyer flask. In this way the cellulose was not allowed to dry.

A set of such flasks was inoculated and cultured by mechanical shaking together with a set containing untreated filter paper. Flasks were removed from the shaker at various intervals and the loss in weight determined. Results are illustrated in Figure VII.

The rate of decomposition of cellulose having a higher amorphous content was markedly greater than that for ordinary cellulose. Cultures containing treated cellulose were considerably more yellow in color and the cellulose-free solutions had much heavier bacterial suspensions than cultures containing untreated cellulose. The treated cellulose was not so readily pulped by agitation as was the untreated; the pieces of filter paper appeared to remain more or less intact for the first few days, after which disintegration was very rapid.

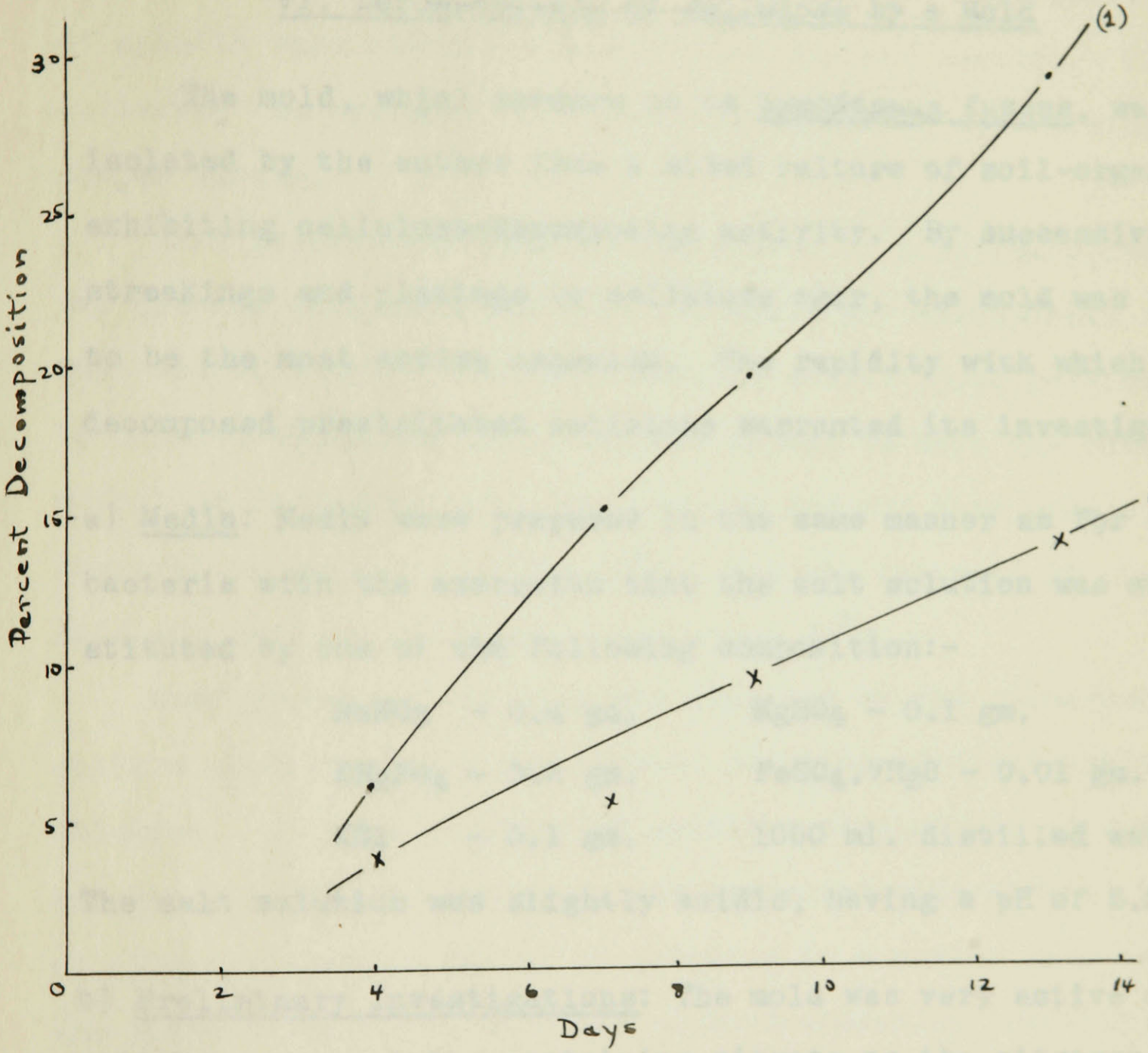


Figure VII. Decomposition of cellulose treated with alkali, and of untreated cellulose.
(1) Treated (2) Untreated

VI. Decomposition of Cellulose by a Mold

The mold, which appears to be Zygodesmus fuscus, was isolated by the author from a mixed culture of soil-organisms exhibiting cellulose-decomposing activity. By successive streakings and platings on cellulose agar, the mold was found to be the most active organism. The rapidity with which it decomposed precipitated cellulose warranted its investigation.

a) Media: Media were prepared in the same manner as for the bacteria with the exception that the salt solution was substituted by one of the following composition:-

$NaNO_3$ - 0.4 gm.	$MgSO_4$ - 0.1 gm.
KH_2PO_4 - 0.2 gm.	$FeSO_4.7H_2O$ - 0.01 gm.
KCl - 0.1 gm.	1000 ml. distilled water

The salt solution was slightly acidic, having a pH of 5.2.

b) Preliminary Investigations: The mold was very active on cellulose agar plates containing nitrate as the nitrogen source. From a stab inoculation on such plates a zonation of 4-5 inches in diameter was usually produced in three days. Microscopic examination revealed the complete absence of cellulose fibres in the cleared zones in the agar. The

amount of the mold growth was very sparse, but fine threads of mycelia were evident wherever clearing had occured. This indicated the presence of a powerful cellulose-decomposing enzyme system.

When peptone was used as the source of nitrogen growth was greatly increased but less cellulose was decomposed. The addition of a small amount of soluble starch (0.02 percent) to nitrate-cellulose agar, did not appear to have any retarding effect on decomposition. The results did not vary over a temperature range of 22° to 30°C., but the activity of the mold was reduced at temperatures above 30°C.

Good growth of the mold was obtained by the submerged culture method using a one percent solution of distiller's solubles. A large quantity of mycelium obtained in this way was dried in the frozen state and extracted with a small volume of phosphate buffer (M/15) by grinding in a mortar with sand. The extract failed to show cellulose-decomposing activity on cellulose agar. Intact mold from the same culture, however, was as active in decomposing cellulose as when transferred from cellulose medium.

Cellulose in submerged culture was rapidly decomposed, the material remaining in the flask after 13 days being recognizable only as mold. Residual cellulose from the bacterial

cultures was even more readily attacked, apparently being completely converted to mold mycelium in nine days. From 9.0 gm. of residual cellulose 7.4 gm. were recovered as mold (dry weight) which contained 7.0 percent protein, 5.4 percent lipid, and 2.1 percent ash. Carbon dioxide is the only product of the decomposition identified.

DISCUSSION

The rate of decomposition of cellulose in pure and impure cultures follows a course resembling a typical growth curve, which is to be expected in a biological process. The stimulation produced in the impure culture is of interest. It may be due either to competition between V. perimastix and the associated bacteria for assimilable breakdown products, or to the production of a substance(s) which catalyzes the cellulose-decomposing enzymes. This example of symbiosis illustrates how it is possible for cellulose to be so rapidly destroyed under natural conditions.

On the other hand, the physical state of the cellulose probably has an important bearing on the rate of decomposition in Nature. The fact that amorphous cellulose is more susceptible to attack by microorganisms indicates that the physical structure facilitates permeation by the enzymes or the bacteria, for it is believed that crystalline and amorphous cellulose are chemically the same. The physical and mechanical conditions which exist in the soil favour the increase in amorphous cellulose, and thus may facilitate the action of microorganisms.

Failure to detect cellulose-decomposing activity in the bacteria-free solutions is not unexpected in view of the fact that the isolation of cellulose-decomposing enzymes has proven to be particularly difficult. Furthermore, the concentration

of free enzyme in the filtrate is likely to be very small in view of the highly inert nature of the cellulose fibre and the manner in which the bacteria adhere to it. The tenacious attachment of the organisms to the substrate is illustrated by the fact that when filter paper, which had been attacked by the organisms, is boiled in water and washed with alcohol and acetone, bacteria are still adhering to it. This supports the suggestion that the enzyme system is of a sparingly-diffusible nature.

The different rates of hydrolysis of the alcohol-acid precipitate and residual cellulose, makes it unlikely that the former is composed of short cellulose chains, but is possibly a synthesized bacterial polysaccharide. However, on hydrolysis the initial production of reducing substances from the precipitated material was rapid and then declined sharply, indicating that the material was not pure.

There is a striking similarity between the chemical and physical properties of the fluorescent pigment and riboflavin, so that the former is conceivably part of a flavin respiratory enzyme. If this is correct, the pigment would be expected to be present in large amounts in cultures showing rapid decomposition. The highest concentration of the pigment is obtained with alkali-treated cellulose, when the rate of decomposition is also most rapid.

Carbon dioxide has been found to be an essential growth factor for the bacteria and it is suggested that it plays some fundamental role in metabolic processes. Heterotrophic assimilation of carbon dioxide has been recognized as an important process in the metabolism of several bacteria (35), and the list of organisms which require carbon dioxide continues to grow. This, however, appears to be the first instance to be reported of the essentiality of carbon dioxide in the case of aerobic cellulose-decomposing bacteria.

It is not known whether carbon dioxide is required for the breakdown of cellulose itself, or for the utilization of intermediate products. The carboxylation of pyruvic acid is the only known reaction which involves carbon dioxide assimilation (20), but this does not preclude other possibilities. Considering this reaction in the case of cellulose decomposition, the metabolic pathway of the polysaccharide would then, in part, coincide with that involved in the metabolism of many other carbohydrates.

The decomposition of cellulose is not measurable by the methods at present in use until a period of three or four days has elapsed. During this initial period the bacteria multiply rapidly and, therefore, must be rapidly synthesizing proteins and lipids. This would necessitate a readily-available supply

of carbon and nitrogen. The bacteria are able to reduce nitrate to nitrite, which is a preliminary step in protein synthesis. The carbon could be supplied by the decomposition of cellulose with the subsequent formation of pyruvic acid, at which stage fixation of carbon dioxide could occur. The reaction could then join the nitrate reduction chain with the eventual formation of amino acids. On the other hand, it has been pointed out that reduction of carbon dioxide might yield organic acids (3), so that carbon dioxide could then be used as a carbon substrate during the preliminary growth phase.

The retarded growth observed in the presence of concentrations of carbon dioxide above that normally present in air, may be due to the bacteria being adapted to lower carbon dioxide concentrations. In penicillin fermentations it has been found that varying the concentration of carbon dioxide affects the activity when the gas mixtures are bubbled through the cultures, but not when the cultures are merely shaken in an atmosphere containing the same gas mixtures (19). No explanation is given, but this observation indicates that the results may depend on the type of treatment.

There is a marked increase in the oxygen uptake with cellulose in the presence of carbon dioxide over that in the absence of carbon dioxide, which emphasizes the importance of carbon dioxide in the metabolism of the bacteria. The greater initial respiration rate in the absence of carbon dioxide, which is evident in both cultures, is not explained.

The demonstration of the presence of appropriate dehydrogenases and the more rapid oxygen uptake with glucose, would be in accord with the postulated role of glucose and cellobiose, respectively, as intermediates. Furthermore, the pH activity curves on glucose, cellobiose and cellulose are similar, and glucose is also produced in fairly large amounts when the decomposition takes place in the presence of toluene. The increase in the respiration of an active cellulose-decomposing culture when cellobiose or glucose are added may also be significant. If the extra oxygen uptake is merely due to decomposition of the added glucose or cellobiose, then the respiration would be expected to follow a course similar to that with the glucose or cellobiose alone. This is not the case, however, for the oxygen uptake in the absence of cellulose is greater with glucose than with cellobiose, and in the presence of cellulose the results are reversed.

These observations do not necessarily prove that glucose or cellobiose are normal intermediates in the decomposition of cellulose. Nevertheless, they strongly suggest a similarity in the metabolism of the bacteria on the three substrates. It is also possible that phosphorylation mechanisms are involved, since phosphorylation inhibitors prevent growth on these carbohydrates, inhibit respiration of cellulose cultures, and retard the production of reducing sugars. In such a case, glucose, cellobiose and cellulose could have common pathways of dissimilation, and glucose and cellobiose could accumulate if the normal decomposition is disturbed, since they would be in equilibrium with other intermediates of cellulose decomposition.

Glucose is produced in relatively large amounts when an active cellulose culture is treated with toluene. It is likely that a dialysable factor, possibly a coenzyme, which is produced by the bacteria is involved in the production of glucose under these conditions. The formation of reducing sugars when toluene is present has been demonstrated with a number of other bacteria, but it has not previously been indicated that a dialysable factor is involved. When the nature of this factor is known it may be possible to elucidate the mechanism of glucose formation, and possibly

obtain an insight into the nature of the enzymic processes involved in cellulose decomposition about which little is known at the present time.

SUMMARY and CONCLUSIONS

1. The metabolism of an impure culture of an aerobic cellulose-decomposing bacterium, *V. perimastix,* was studied. The products of cellulose decomposition included carbon dioxide, which was produced in large amounts, traces of acidic substances, a fluorescent pigment closely resembling riboflavin, a polysaccharide which did not appear to be cellulose, and other unidentified substances.

2. The bacteria required carbon dioxide, without which cellulose decomposition and growth on glucose and cellobiose was inhibited. Calcium carbonate partially replaced carbon dioxide of the air, but was not satisfactory for use with cellulose. Heterotrophic assimilation of carbon dioxide is suggested.

Increasing the content of carbon dioxide of the air above 1.5 percent retarded growth on a glucose broth medium, but there was no appreciable difference in growth in normal air and air enriched with 1.5 percent carbon dioxide.

3. Respiration studies were made by the Thunberg and Warburg techniques, using cellulose, glucose, and cellobiose as substrates. The rate of oxygen uptake was higher with glucose than with cellobiose when cellulose was absent, but

the reverse held when cellulose was present. The rate of oxygen uptake of cellulose cultures in the presence of carbon dioxide was higher than when carbon dioxide was removed.

4. Glucose was produced when toluene was added to cellulose cultures during active decomposition. Evidence is presented that a dialysable factor essential for glucose formation was produced by the bacteria. The pH activity curves of the bacteria were similar with cellulose, glucose or cellobiose as substrate.

5. Phosphorylation inhibitors prevented growth of the bacteria on cellulose, glucose and cellobiose, inhibited respiration of active cellulose cultures, and retarded the production of glucose by toluene-treated cellulose cultures.

6. Cellulose, treated with alkali to increase the proportion of amorphous to crystalline cellulose, was more rapidly decomposed than untreated cellulose.

7. A mold grown in submerged culture on cellulose medium was found to actively decompose the cellulose. The decomposition of filter paper was apparently complete in nine days, since none could be found in the residue, which contained about seven percent protein and five percent lipid.

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