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## Doctor's Dissertation

The Hypothesized Carbonic Acid Ester Linkages  
in Cellulose Oxidized by Aqueous Chlorine  
at pH 4.5

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THE HYPOTHESIZED CARBONIC ACID ESTER LINKAGES  
IN CELLULOSE OXIDIZED BY AQUEOUS CHLORINE AT pH 4.5

A thesis submitted by

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## PRESENTATION OF PROBLEM

Aqueous chlorine systems have long been used in the textile and paper industries for bleaching. The action of these oxidative systems on cellulose is very important to product stability and end use. Care must be taken to maintain controlled bleaching conditions, for certain deviations may rapidly destroy the cellulose. On the other hand, there are also industrial processes for which change in the chemical and physical properties of the cellulose is a required effect of the bleaching operation. Thus, to understand how certain changes in cellulose properties are effected by the oxidative action of aqueous chlorine systems is very important.

The action of aqueous chlorine on cellulose has been widely investigated. The general conclusion from this work is that chlorine, hypochlorous acid, and hypochlorite ion are nonspecific oxidizing agents, reacting with the cellulose macromolecule at different points and leading either to the "reducing" type of cellulose or to the "acidic" type, as defined by Birtwell, Clibbens, and Ridge (1). The most recent extensive study dealing with the chemical changes produced in cellulose by chlorine, hypochlorous acid, and hypochlorite ion was made by the Russian workers, Kaverzneva, Salova, and Ivanov (2-5). This work was an outstanding contribution, for it represents one of the most detailed chemical (functional group) analyses made on a series of oxidized celluloses. On the basis of the data obtained, numerous statements were made regarding the nature of these oxidized celluloses. Further study of one of their conclusions, the proposal that carbonic acid ester linkages are present in cellulose oxidized by aqueous chlorine at pH 4 to 5 (predominantly aqueous hypochlorous acid), is the subject of this thesis.

The possibility of the formation of carbonic acid ester linkages in cellulose, involving carbon 1 of the anhydroglucose molecule, was first suggested by Staudinger (6, 7) in connection with his hypothesis on defective celluloses. Kaverzneva, et al., revived this hypothesis on the basis of indirect evidence which is suggestive but not conclusive. Therefore, the primary aim of this research is to test the validity of the carbonic acid ester hypothesis. The merit of this undertaking stems from the important part such a structure may play in the alkali sensitivity of acid chlorine-oxidized cellulose.

## LITERATURE RELATED TO PROBLEM ANALYSIS

### CELLULOSE OXIDATION

The effect of pH on the aqueous chlorine oxidation of cellulose has been observed by many (1, 8-10); their observations may be summarized as follows:

1. The oxidation rate is greatest at pH 7.
2. The oxidized cellulose from acidic oxidations contains more carbonyl groups than carboxyl groups.
3. The oxidized cellulose from alkaline oxidations contains more carboxyl than carbonyl groups.

The foregoing statements were basically derived from oxidant consumption rates and empirical tests such as the methylene blue and copper number determinations.

Kaverzneva, Salova, and Ivanov (2-5) have recently applied new techniques to the examination of celluloses oxidized by aqueous chlorine and have obtained data from which a better estimate of the oxidation products and the mechanism of their formation can be made. They obtained data supporting the presence of aldehydic and ketonic carbonyls, lactones, acids, and carbonate esters in the oxidized celluloses examined.

In a review of the field of cellulose oxidation, Meller (11) has discussed some possible reactions that may occur during the oxidation of cellulose. These include attack at carbon 6 to give an aldehydic carbonyl group which can further be oxidized to a carboxyl group. An attack at carbons 2 and 3 may form ketonic carbonyl groups or upon cleavage of the carbon-carbon

bond may yield a dialdehyde. The dialdehyde could be oxidized to a diacid. Another postulated reaction involves the cleavage of the bond between carbons 1 and 2, leaving carbon 1 as a carbonate ester group and carbon 2 as an aldehyde group. Also, the pyran ring may be opened between carbon 1 and carbon 5 with further oxidation resulting in a carboxylic ester grouping at carbon 1. With Meller's comments in mind, it must be recalled that for cellulose oxidized by aqueous chlorine at pH 4.5<sup>a</sup> there is no evidence of a quantitatively significant bias with respect to the oxidation of carbons 1, 2, 3, and 6 of the anhydroglucose unit. Thus, all of the above reactions may be involved in this type of oxidation.

#### CARBONIC ACID ESTER HYPOTHESIS

The oxidative cleavage of carbon 1 and carbon 2 of the glucopyranose unit by an acid chlorine oxidation was first suggested by Staudinger (6, 7). Although no direct evidence was presented for the support of the proposed mechanism for the formation of this "ester cellulose" or for its chemical constitution, the alkali sensitivity of acid chlorine-oxidized cellulose was consistent with easily saponifiable ester groups. No further development of the carbonic acid ester linkage hypothesis was made until Kaverzneva, Salova, and Ivanov (2-5) obtained experimental evidence that indirectly supported such a structure (see Figure 1). Their data suggested that the number of such linkages present in oxidized cellulose was extremely small but was detectable by Salova's method (13) for the microdetermination of carbon dioxide. The carbon dioxide that was determined by these workers was evolved

<sup>a</sup> According to the calculations of Whitney (12), 96 to 99% of the available chlorine is present as hypochlorous acid over the pH range of 4 to 5. Aqueous chlorine at pH 4.3 ± 0.3 is the only oxidative system to be used in this research. Oxidized celluloses will henceforth be denoted either acid chlorine-oxidized cellulose or simply oxidized cellulose.

by treating the cellulose with a dilute sodium hydroxide solution and subsequently acidifying this alkaline mixture (see Figure 1 for the proposed mechanism).<sup>a</sup>

Kaverzneva, et al., oxidized cotton with aqueous chlorine (buffered at about pH 4.5 with an acetate buffer system), then washed, dried, and saponified it. The saponification was effected with a 0.025 normal solution of sodium hydroxide. After an interval of time, the saponification mixture was acidified and carbon dioxide<sup>b</sup> was liberated. This carbon dioxide was ascribed to the saponification of carbonic acid ester linkages. Unoxidized cellulose and cellulose oxidized in an alkaline medium gave off almost no carbon dioxide. Data presented by these workers also showed that the uronic-acid carboxyl content of these oxidized celluloses was greater than the total carboxyl. They stated that this was due to the presence of lactones and carbonic acid ester groupings in the cellulose.

Meller (11), in his review of the literature in the field of cellulose oxidation, discussed the data presented by Kaverzneva, et al., and concluded that the hypothesized carbonic acid ester linkage was consistent with the existing information. However, he noted that further evidence was needed in order to verify such a hypothesis, for it may not represent an ultimately satisfactory interpretation of the experimental results. [For example, the saponifiable carbon dioxide in the oxidized cellulose may be the result of carbon dioxide adsorbed on the cellulose during its oxidation or in part or

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<sup>a</sup> The cellulose ester of methyl carbonic acid is unstable in the presence of alkali (14).

<sup>b</sup> Carbon dioxide derived from oxidized cellulose by this procedure will subsequently be called saponifiable carbon dioxide.

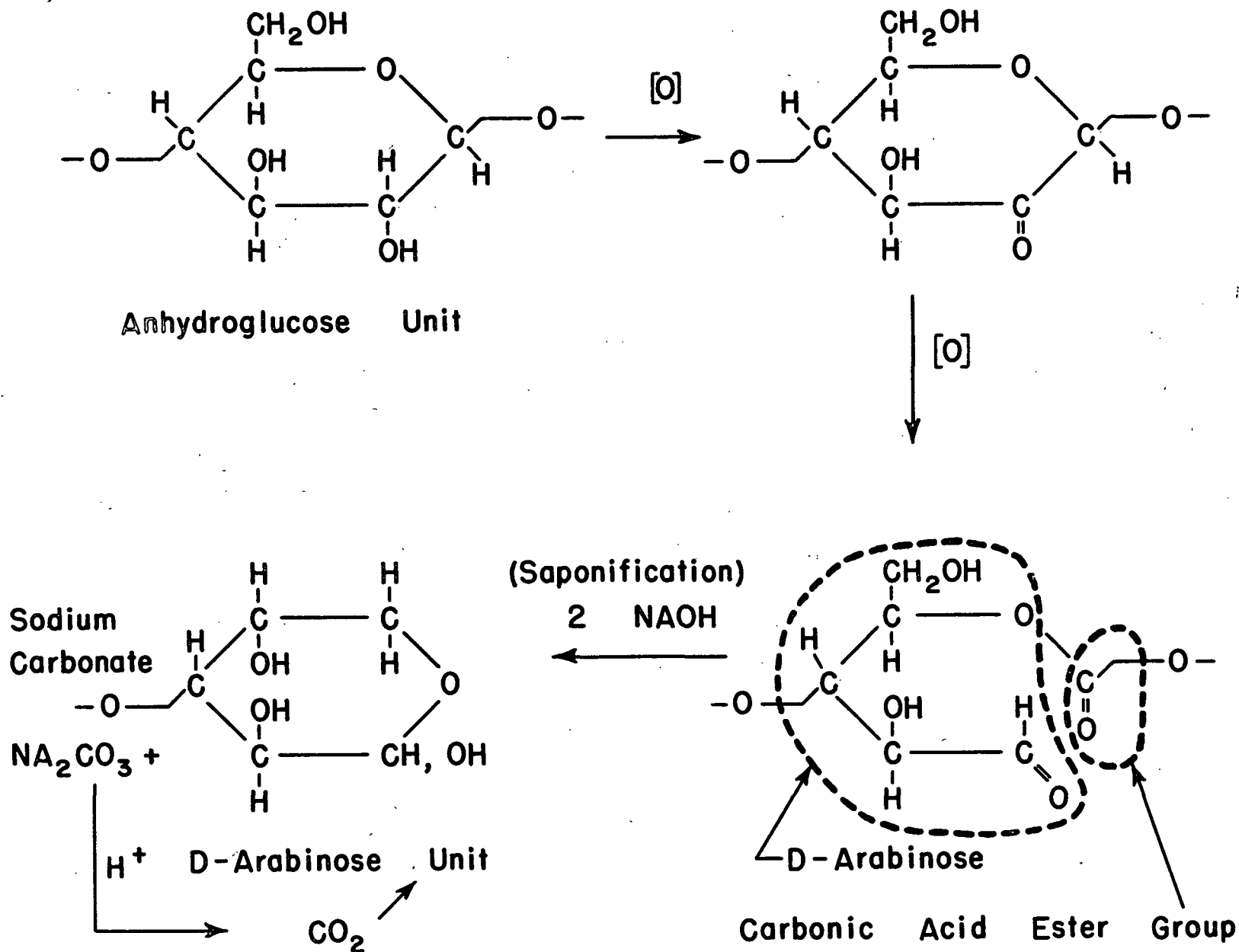


Figure 1. Formation of Hypothesized Carbonic Acid Ester Linkage, According to Kaverzneva (2).

in toto due to some other structure or structures not presently associated with oxidized cellulose.]

Henderson (15) conducted experiments of a preliminary nature on cellulose oxidized by aqueous chlorine at pH 4.5. He reported the isolation and characterization of D-arabinose from the oxidized cellulose and stated that the D-arabinose was derived from an oxidative cleavage of carbons 1 and 2 of the glucopyranose unit. The carbon 2 was left as a carbonyl group, and this became the carbon 1 of the arabinose unit. The original carbon 1 of the glucopyranose unit was left as a carbonic acid ester linkage, which may be easily hydrolyzed.

#### CARBON-14 LABELED CELLULOSE

Two approaches are reported for the preparation of specifically labeled cellulose. One is the biosynthesis of carbon-14 labeled cotton from D-glucose-1-C<sup>14</sup> according to Greathouse (16) or Shafizadeh and Wolfrom (17). The other is the growth of carbon-14 labeled bacterial cellulose from D-glucose-1-C<sup>14</sup> or D-glucose-6-C<sup>14</sup> using the bacterium Acetobacter xylinum according to Greathouse, et al., (18, 19). The first method has not shown promise due to its long growth cycle and the uncertainty of yield and label distribution. Consequently, the second method, the bacterial cellulose preparation, has more potential because of the rapidity of cellulose synthesis and reproducibility of yield and specificity of label.

Specifically labeled bacterial cellulose had not been prepared in the quantity necessary to sustain a comprehensive research program dealing with cellulose oxidation. However, Hestrin (20) and Greathouse (21) have

indicated the belief that published methods can be scaled up to meet such a demand. The method of Greathouse (19) should prove adequate for such an operation; he has prepared both carbon 1 and carbon 6 labeled bacterial celluloses by this procedure.

This method (19) has produced yields, based on the D-glucose supplied, ranging from 40 to 45% and has given activity yields of 20 to 30%, based on the carbon-14 supplied in the form of D-glucose-1-C<sup>14</sup> or D-glucose-6-C<sup>14</sup>. On complete analysis of these labeled bacterial celluloses, the label distributions were found to be:

<u>Cellulose-1-C<sup>14</sup></u> (Cellulose prepared from D-glucose-1-C <sup>14</sup> )		<u>Cellulose-6-C<sup>14</sup></u> (Cellulose prepared from D-glucose-1-C <sup>14</sup> )	
C(1)	- 79.1%	C(1)	- 9.3%
C(2)	- 0.4%	C(2)	- 1.7%
C(3)	- 11.0%	C(3)	- 3.9%
C(4)	- 8.0%	C(4) and C(5)	- 3.5%
C(5)	- 0.4%	C(6)	- 81.6%
C(6)	- 1.1%		

The labeling was not specific; nevertheless, for many studies it would be adequate to support significant quantitative conclusions of a high degree of statistical certainty.

An obvious objection to the use of bacterial cellulose in cellulose research relating specifically to cotton arises with respect to possible differences between bacterial and cotton celluloses. This problem has been studied by many, including Hibbert and Barsha (22), Greathouse and Shirk (23), Schramm and Hestrin (24), and others. The findings of these workers support the conclusion that the chemical and internal physical properties of purified bacterial cellulose are essentially identical with those of cotton cellulose.

Therefore, it is reasonable to assume that bacterial cellulose is directly comparable to cotton with respect to the qualitative manner in which the two celluloses would respond to a given treatment.

#### THE ISOTOPE EFFECT

The isotope effect must be considered when reactions involving carbon-14 labeled compounds are studied, for it is well known that the mass of a molecule has an effect on the reaction rate and equilibrium constants of any reaction in which it takes part. This effect has been found to be small for the systems that have been studied, i.e., up to an 8% decrease in rate and equilibrium constants for carbon-14 with respect to carbon-12 (25). Two studies that consider the isotope effect in homogeneous oxidative reactions, similar to the type of reactions that may take place in the oxidation of cellulose, are:

1. The oxidation of a secondary carbinol to an acid by Bonner and Collins (25) for which they found an isotope effect ranging from 2 to 8%, depending on the degree of oxidation, 90 and 20% completion, respectively, and
2. The oxidation of acetic acid by Evans and Huston (26) for which they found that the rate of oxidation for the carbon-14 labeled acid was 5% less than that for the carbon-12 acid.

No information is available in the literature relating to the magnitude of the isotope effect for heterogeneous reactions such as is represented by the oxidation of cellulose. Consequently, it is necessary to assume that the isotope effect for the oxidation of carbon-14 labeled cellulose does not exceed the maximum found for homogeneous oxidations. On the basis of the above examples, an isotope effect that may be operative in the oxidation of carbon-14 labeled cellulose would not be expected to exceed 10%.

Other assumptions that must be made in order to qualify the suitability of carbon-14 labeled cellulose as a research tool for cellulose oxidation studies are:

1. Intrapolymer distribution of labeled anhydroglucose units is random.
2. The portions of the polymer that are accessible to oxidation are randomly oxidized.

#### LEFÈVRE AND TOLLENS URONIC ACID METHOD

When uronic acids, either free or as lactones, or combined as polyuronides, are boiled with 12% hydrochloric acid, the carboxyl groups are quantitatively decomposed to yield the theoretical amount of carbon dioxide. Since Lefèvre and Tollens (27) developed this method, it has undergone modifications and has become widely used for the determination of the uronic acid content of celluloses. Some investigators, e.g., Kaverzneva, et al., (3), have even used this method in the interpretation of very minor differences in the nature of oxidized celluloses. These interpretations have been made in the light of evidence which prompted Browning (28) to state that amounts of uronic acid carbon dioxide in the range of 0.2 to 0.4%, based on the cellulose, cannot be considered definite evidence of the presence of uronic acid groups.

Browning's statement is supported by the experimental evidence of many investigators, e.g., Nevell's (29) determination of uronic acids on a periodate-oxidized cellulose containing negligible uronic acid (either as free-acid or lactone) which gave a value corresponding to 1.2% uronic anhydride. This observation emphasizes the inadequacy of interpretations made on the

basis of small changes or differences in the "uronic acid" content of oxidized celluloses.

Currently, it is futile to attempt detailed analysis of discrepancies that may be noted when the Lefèvre and Tollens uronic acid method is applied to a given oxidized cellulose. This dilemma is due to the limited knowledge of the structures (sugars, nonuronic acids, etc.) present in oxidized celluloses and of the mechanisms involved in the evolution of carbon dioxide from these by the action of boiling 12% hydrochloric acid.

## MATERIALS

### PURIFIED COTTON

The cotton used in this experimental program was obtained from the Southern Regional Laboratories of the Department of Agriculture at New Orleans, Louisiana. When received, this cotton had been ginned and subsequently cleaned by passing it through a Shirley Analyzer.

The cotton was Wiley-milled through one-quarter inch holes in order to break up the long fibers and to give a more uniform sample. One hundred and five grams of this cotton were extracted for three hours with 1500 ml. of absolute ethyl alcohol under reflux; it was then washed with 1500 ml. of acetone and air dried. The air-dry cotton was next extracted with 3500 ml. of 1% potassium hydroxide under reflux. After refluxing for two hours, the cotton was filtered onto a fritted-glass filter funnel, washed with a liter of 1% acetic acid, and then washed with water until the washings were neutral. The water-washed cotton was next washed with two liters of absolute ethyl alcohol and two liters of acetone and allowed to dry. The air-dry, purified cellulose was stored in Mason jars.

### LABELED BACTERIAL CELLULOSE

The method used for the preparation of bacterial cellulose was patterned after the method reported by Greathouse (19). This procedure was scaled up for the biosynthesis of about 30 grams of cellulose per run. The increased scale of preparation consisted primarily of using larger vessels for the growth of the cellulose. The above-mentioned yield was effected by the use

of about one hundred 3000-ml. Erlenmeyer flasks for each run; each flask had approximately 1.7 sq. cm. of solution surface per cc. of nutrient solution. It was found that the surface-to-volume ratio of the nutrient media was important to the yield of cellulose based on the glucose supplied in the media. The 1:7 ratio appeared to be about optimum for the conditions used in this experimental work.

Acetobacter xylinum, A.T.C.C. strain no. 12733, was the organism used for the growth of the bacterial cellulose. The nutrient media used consisted of an aqueous solution of 0.5% glucose, 2% Difco yeast extract, and 0.1%  $\text{KH}_2\text{PO}_4$  (weight-to-volume percentages, w/v). The initial pH of the media was 6.0. The inoculation was made by the transfer of a three-day-old colony of the bacterium from an agar<sup>a</sup> slant to a flask of the nutrient media. This was done by suspending the colony in about 5 ml. of a one-day-old population of the bacterium in nutrient media; this suspension was added directly to the flask. The cellulose pellicles were collected from the flasks seven days after inoculation.

For the preparation of the carbon-14 labeled cellulose, 60% of the total glucose addition, including all of the labeled D-glucose, was added about 24 hours after the inoculation. This was done so that physiological activity of the bacterial population had passed its peak before the radioactive glucose was added; this delay in the addition of the labeled glucose gives a marked increase in the activity yield and the specificity<sup>b</sup> of label.

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<sup>a</sup> Agar slant composition (w/v): 2% glucose, 0.5% bacto-peptone, 0.5% yeast extract, 2.5% agar.

<sup>b</sup> Specificity denotes the percentage of the total radioactivity present in a molecule that is present in a specific position.

After harvesting, the bacterial cellulose pellicles were washed with hot tap water ( 50°C.) for about 8 hours before they were autoclaved for 2 hours at 15 lb./sq. in. gage in a 1% sodium hydroxide solution. Fifteen hundred milliliters of 1% sodium hydroxide solution were used for about 6 grams of cellulose. The caustic-extracted cellulose was washed with water, then washed with a 0.5% acetic acid solution and again washed with water until the washings were neutral. The cellulose was next dried by the solvent-replacement technique, using absolute ethyl alcohol, acetone, and ethyl ether, in that order. The purified, solvent-dried bacterial cellulose was next Wiley-milled past a 40-mesh screen and stored.

#### SPECIAL SOLUTIONS

In many cases, experiments required carbon dioxide-free water and carbonate-free sodium hydroxide solutions.

In the preparation of carbon dioxide-free water, triple distilled water was purged of any dissolved carbon dioxide by heating it to 95°C. and then bubbling nitrogen through it for 30 minutes. When the nitrogen flow and heat were discontinued, the bottle containing the water was tightly stoppered, and as the water cooled, the pressure was allowed to equilibrate through an Ascarite (asbestos and sodium hydroxide) guard tube. The carbon dioxide-free water was siphoned off as needed.

Carbonate-free sodium hydroxide solutions were prepared from Acculutes<sup>a</sup> of concentrated sodium hydroxide; carbon dioxide-free water was used to make

<sup>a</sup> Acculute is the trademark of standard volumetric solutions prepared by Anachemia Chemicals Limited.

up the desired concentrations. The preparation of the solutions was conducted in a carbon dioxide-free atmosphere; a constant-atmosphere box with neoprene arm tubes was used for this operation. The constant-atmosphere box was kept under a positive pressure with compressed air. This air was passed through a calcium chloride column, an Ascarite column, and a soda-lime solution before it entered the box. The top of the box was fitted with plate glass so that work conducted inside the box could be viewed easily without allowing carbon dioxide contamination. Polyethylene bottles were used for the storage of the carbonate-free sodium hydroxide solutions.

## APPARATUS AND METHODS

### CELLULOSE OXIDATION

#### OXIDANT PREPARATION

All oxidations were performed with an aqueous chlorine system buffered at pH 4.5 with either an acetate or a phosphate buffer system. The stock oxidant solution was a normal solution of sodium hypochlorite. The preparation of the oxidant involved the bubbling of chlorine through a normal solution of sodium hydroxide until the pH dropped to 9.5. The hypochlorite solution was then stored in a stoppered amber bottle to cool. After cooling and just prior to using, the solution was standardized according to White (30) with respect to chloride and available chlorine. The chlorate content of the solution was found to be negligible. The pH of the hypochlorite solution was adjusted to 4.5 with either acetic or phosphoric acid (depending on the buffer system) immediately before its addition to the reaction vessel containing the buffered cellulose slurry.

#### CELLULOSE OXIDATION APPARATUS

Before the method requiring the apparatus shown in Figure 2 was adopted, two different procedures were used for the oxidation of cellulose. They were different only in the manner in which the carbon dioxide that was evolved during the oxidation was treated, i.e., the carbon dioxide was in one case neglected, whereas in the other it was absorbed on Ascarite.<sup>a</sup> All of the

<sup>a</sup> When Ascarite was used for collecting the carbon dioxide, it was necessary to remove any chlorine in the gas stream; this was done by passing it through a strongly acidic ferrous sulfate solution. The gas was then dried before it reached the Ascarite tube; calcium chloride was utilized for the drying.

more important oxidations were conducted utilizing the final method given below.

The oxidation apparatus, Figure 2, was arranged as a closed system, i.e., once the oxidation was initiated, the reaction vessel did not need to be opened until the termination of the oxidation. The glass reaction vessel was about 400 ml. in volume and was covered with aluminum foil. On initiation of an oxidation, the reaction vessel was sealed with a rubber stopper which was fitted with a glass electrode, a calomel electrode, a gas exit tube, and a gas inlet tube. The gas inlet tube extended to near the bottom of the reaction vessel, and the gas exit tube extended into the vessel but was always above the surface of the reaction mixture. The electrodes were connected to a Beckman Model M pH meter so that the pH of the reaction mixture could be checked intermittently. A magnetic stirrer was used for occasional agitation of the reaction mixture.

The only gas that entered the reaction vessel after it was closed was purified nitrogen which was passed into the reaction mixture near the end of the oxidation in order to purge it of dissolved carbon dioxide. The remainder of the time, the gas inlet tube was closed.

The carbon dioxide that was evolved during the oxidation of the cellulose passed out of the reaction vessel through the gas outlet tube and then bubbled into a carbonate-free sodium hydroxide-absorbing solution. The bubbles were ejected into the solution from a capillary 2 mm. in diameter. The capillary opening was at the bottom of the gas washing bottle; therefore the bubbles had to pass up through the 16 ml. of 2N caustic solution. The total distance the bubbles traveled in the solution was about 45 mm. The 2N caustic gave an adequate excess of caustic for all oxidations that were conducted.

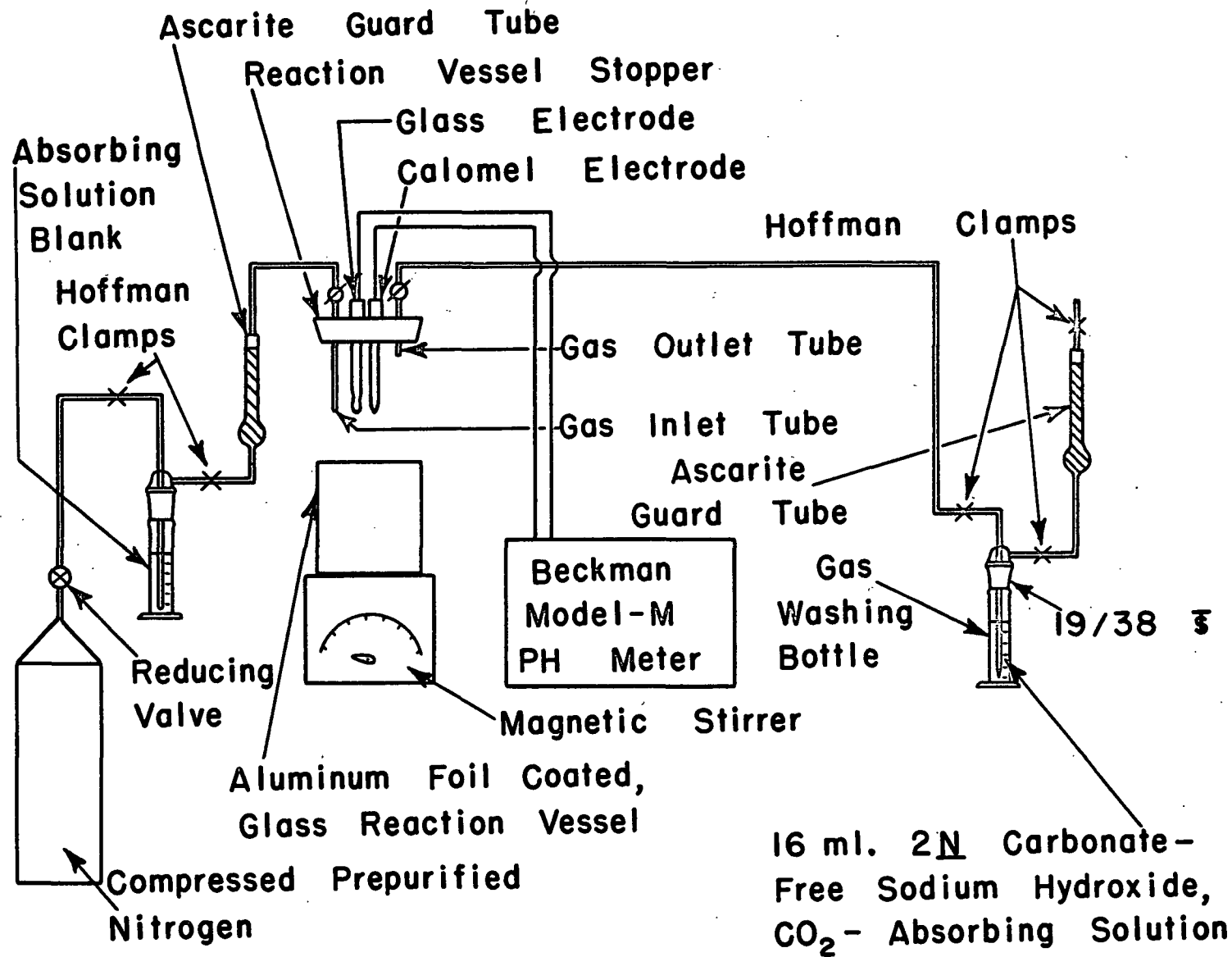


Figure 2. Schematic of Cellulose Oxidation Apparatus

Ascarite guard tubes were so placed in the apparatus that they protected the carbonate-free sodium hydroxide solution, the blank and absorbing solutions, from contamination by atmospheric carbon dioxide.

#### OXIDATION PROCEDURE

For each oxidation, about 12 grams of cellulose were suspended in either an acetate or a phosphate buffer system. The buffer system used for a given oxidation had sufficient buffering capacity to hold the pH at  $4.3 \pm 0.3$  throughout the oxidation. The consistency of the cellulose suspension was adjusted to 5% by the addition of the oxidant and make-up water.

Immediately after the addition of the oxidant, the reaction mixture was made up to the proper consistency. The vessel was then sealed with the reaction vessel stopper; the stopper was secured with rubber bands. The gas outlet tube was then opened so that gas evolved during the oxidation could pass out of the vessel into the absorbing solution; the outlet tube remained open for the duration of the oxidation. The oxidations were allowed to proceed for 40 hours; at this point all or nearly all of the oxidant had been consumed. For four hours preceding the termination of the oxidation, purified nitrogen was bubbled into the reaction mixture in order to remove the carbon dioxide dissolved in the solution.

On completion of the oxidation, the gas washing bottles, containing the carbon dioxide-absorbing solution and the absorbing solution blank, were closed and set aside for analysis. The spent oxidizing solution was then separated from the oxidized cellulose and analyzed for chloride, available chlorine, and chlorate (30). The chlorate content was again found to be negligible. From these analyses and the initial concentration of chloride and

available chlorine, the oxidant consumption was calculated. The oxidized cellulose was washed with water, 1.5%  $\text{NaHSO}_3$  solution, water, 0.5% acetic acid, water, absolute ethyl alcohol, acetone, and ether, in that order. The cellulose was washed by centrifugation and decantation. The oxidized cellulose was then vacuum-dried to constant weight (at 753 mm. Hg vacuum and  $50^\circ\text{C}.$ ), and its yield was determined. The cellulose was then stored in a tightly sealed weighing bottle.

### SAPONIFICATION OF CELLULOSE

Two saponification methods were employed for the determination of the saponifiable carbon dioxide in celluloses. Method (a) was used in conjunction with the titrimetric method for the determination of carbon dioxide (see page 21), and method (b) was used in conjunction with the manometric method for the determination of carbon dioxide (see page 23).

#### METHOD A

About 2 grams of cellulose were suspended in 100 ml. of carbon dioxide-free water. This mixture was then made 0.025N in sodium hydroxide by the addition of 21 ml. of 0.15N carbonate-free sodium hydroxide.<sup>a</sup> This alkaline mixture was allowed to stand for 12 hours before it was acidified with 4 ml. of normal sulfuric acid. The carbon dioxide liberated by this treatment was collected and quantitatively determined.

<sup>a</sup> The constant-atmosphere box (see page 15) was utilized for all transfers of carbonate-free sodium hydroxide solutions. This was done because of the necessity of maintaining a carbon dioxide-free atmosphere in order to insure against contamination of these solutions.

## METHOD B

About 2 grams of cellulose were suspended in 30 ml. of carbon dioxide-free water. The mixture was then made 0.025N in sodium hydroxide by the addition of 6.2 ml. of 0.15N carbonate-free sodium hydroxide. This alkaline mixture was allowed to stand for 12 hours before it was acidified with 2 ml. of normal sulfuric acid. The carbon dioxide liberated by this treatment was collected and quantitatively determined.

The foregoing saponification techniques in conjunction with their respective methods for the determination of the saponifiable carbon dioxide gave comparable results for a given cellulose.

## TITRIMETRIC MICROMETHOD FOR THE DETERMINATION OF CARBON DIOXIDE

A titrimetric method for the determination of minute quantities of inorganic carbonates (0.5 to 3.0 mg. as carbon dioxide) in aqueous solution was developed. This method was tested with known sodium carbonate solutions and was found to have an average inherent positive error of 0.15 mg. of carbon dioxide. This error is believed to originate in the handling of the barium hydroxide absorbing solution. However, inasmuch as the absolute value of the quantity of carbonate present in a sample was obtained by correcting the sample for a blank, the inherent positive error was nullified. The confidence limits (95%) for data obtained by this method were  $\pm 0.04$  mg. of carbon dioxide; the sample size utilized for this analysis was approximately 0.8 mg., as carbon dioxide (see Appendix I, page 91).

## APPARATUS

The apparatus used in this procedure is shown in Figure 3. A brief description of the component parts of the apparatus follows:

1. Prepurified nitrogen supply;
2. Reaction flask with gas inlet tube extending to the bottom of the flask;
3. Acid reservoir above the reaction flask, 5 ml. volume;
4. Hoffman clamp;
5. Gas inlet tip, 2-mm. capillary tube with 1-mm. opening into absorption tube;
6. Absorption tube, 8-mm. glass tubing, 50 cm. in length;
7. Stoppered absorption flask with a side arm with a 19mm. stopcock;
8. Stopcock, absorption flask side arm;
9. Ascarite guard tube, and
10. Water manometer.

Tygon tubing was used exclusively where tubing connections were made in the apparatus.

## PROCEDURE

The carbonate sample was placed in the reaction flask, 2, and the flask was connected to the apparatus. Prepurified nitrogen, 1, was passed through the system for 5 minutes to remove the air containing carbon dioxide. The nitrogen was stopped, and then the absorption system was closed at points 4 and 8. Ten milliliters of 0.02N barium hydroxide (titrated with 0.01N hydrochloric acid before each run) were then added to the absorption system through the top of the absorption flask, 7; the flask was quickly closed

after the addition of the absorbent. Nitrogen flow through the apparatus was resumed at a flow rate of about 1500 ml. per hour. At time zero, adequate normal sulfuric acid was added to the reaction flask from the acid reservoir, 3, to acidify the alkaline carbonate solution to about pH 3. This acidification decomposed the carbonate and liberated carbon dioxide. The flow of nitrogen through the system was continued for 3 hours. After completion of the run, the absorption system was again closed at points 4 and 8 and was severed from the apparatus. The barium hydroxide absorbing solution was washed from the absorption system with carbon dioxide-free water under nitrogen pressure. The washings were removed through the side arm of the absorption flask and were collected in a 125-ml. flask covered with dental dam; the total washings amounted to 50 ml. Phenolphthalein was added to the solution, and it was then titrated through dental dam with 0.01N hydrochloric acid. During the titration, agitation was continuously effected with a magnetic stirrer. The amount of carbon dioxide absorbed by the barium hydroxide solution was then calculated. This value was corrected for a blank in order to obtain an absolute value for the amount of carbonate in the sample.

#### MANOMETRIC MICROMETHOD FOR THE DETERMINATION OF CARBON DIOXIDE

For the analysis of a given carbonate solution, the result obtained by the manometric method was comparable to that obtained by the titrimetric method.

#### APPARATUS

The apparatus utilized for the manometric determination of carbon

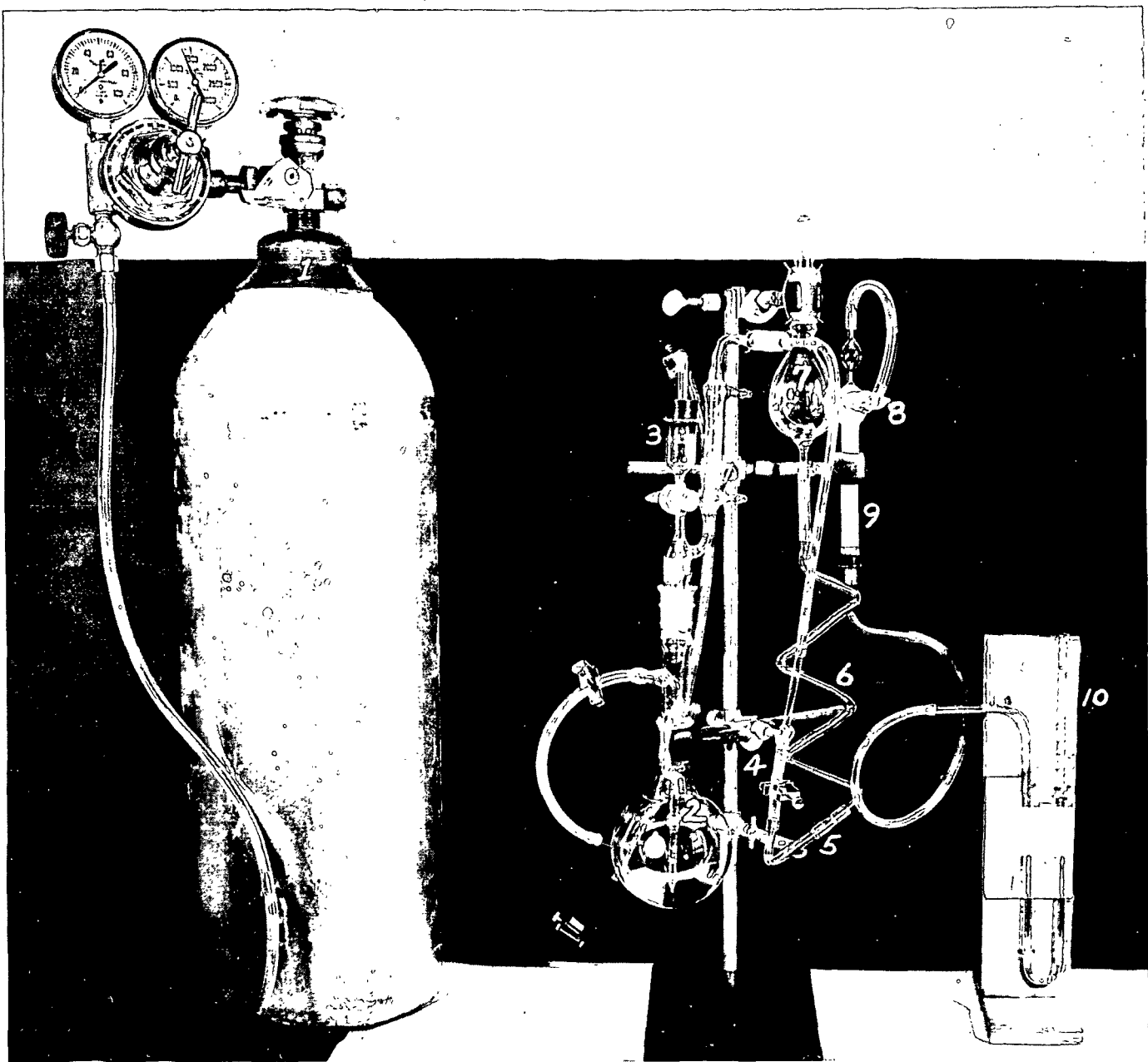


Figure 3. Apparatus for the Titrimetric Determination of Carbon Dioxide

dioxide was an adaptation of the Van Slyke-Neill (31) manometric apparatus. The modified apparatus was identical with the modification used by Van Slyke and Folch (32); it is pictured in Figure 4. A brief description of the major components of the apparatus follows:

1. Reaction vessel for carbonate determination, either a 100-ml. round-bottomed flask (female, 19/38,  $\text{F}$ ) or a 25-ml. test tube (female, 19/38,  $\text{F}$ );
2. Acid reservoir;
3. Delivery tube, connecting reaction flask to the gas buret;
4. Calibrated gas buret, marked at 50, 10, and 2-ml. volumes;
5. Mercury manometer, and
6. Aspirator line.

This apparatus was used for the quantitative determination of carbon dioxide derived from total wet combustion of potassium gluconate, the periodate oxidation of potassium gluconate, the mercurous chloride decomposition of formic acid, and the decomposition of sodium carbonate by acidification (pH 3).

#### PROCEDURE

The Van Slyke-Folch method (32) was adhered to for the determination of the carbon dioxide derived from all the sources mentioned above, i.e., once the carbon dioxide was absorbed in the alkaline-hydrazine solution in the gas buret, the Van Slyke-Folch method was used for its quantitative analysis. Published techniques will be mentioned in a subsequent section for all the above reactions involving the evolution of carbon dioxide with the exception of the methods for the acidification of carbonate solutions described below.

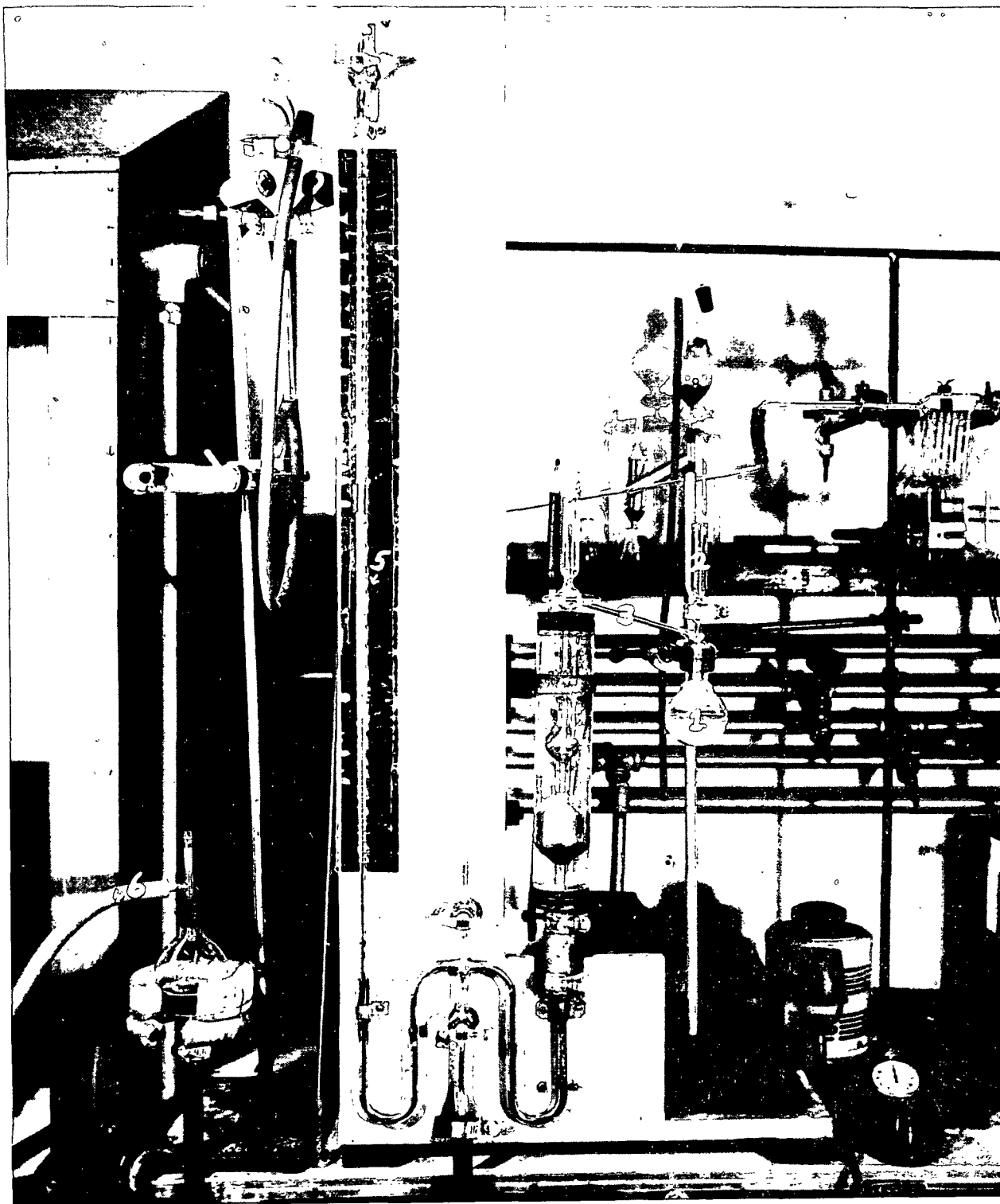


Figure 4. Apparatus for the Manometric Determination of Carbon Dioxide

Carbonate solutions with and without cellulose suspended in them were analyzed; method (c) was used for solutions containing cellulose and method (d) for others.

#### METHOD C

The reaction vessel, 1, was connected to the gas buret, 4, with the connecting tube, 3; it was then evacuated in the normal manner (32). During the evacuation, the sample was heated to about 40°C.; at this temperature and with the reduced pressure, the solution began to boil, thus helping to rid the sample of dissolved gases. Two milliliters of alkaline-hydrazine solution were then added to the buret to absorb the carbon dioxide liberated on acidification of the carbonate solution. Next, the carbonate sample was acidified with normal sulfuric acid and the evolved carbon dioxide was absorbed by the alkaline solution. The carbon dioxide so liberated was completely transferred from the reaction flask to the absorbing solution by sweeping the gases back and forth between the gas buret and the flask for 8 minutes (about 50 sweeps). This was done by alternately lowering and raising the mercury level in the buret. During the sweeping operation, the solution in the reaction vessel was maintained at a temperature of about 40°C.; this caused the solution to boil intermittently as the pressure was raised and lowered, thus aiding in the expulsion of carbon dioxide from the solution.

#### METHOD D

This procedure was identical with method (c) with the exception that the sample was agitated with a magnetic stirrer rather than heated in order

to assist in the expulsion of dissolved carbon dioxide from the acidified carbonate solution.

The manometric method was used for the determination of carbon dioxide over the range of 0.15 to 3.0 mg. carbon. The submicro technique (32) was used for samples with 0.15 to 0.7 mg. of carbon, and the micromethod (32) was used for samples with 0.7 to 3.0 mg. of carbon. (See Appendix III, page 96, for a more complete description of the manometric determination of carbon.)

#### URONIC ACID DETERMINATION

Institute Method 25 (1952) with two modifications was used for analyzing celluloses for their uronic acid content; the modifications were:

1. A sodium hydroxide solution, instead of Ascarite, was used for the absorption of carbon dioxide. The carbon dioxide absorbed was determined by the manometric method.
2. The nitrogen flow rate through the apparatus was changed from 8 to 6 cc. per minute.

The gas washing bottle used for the absorption of the carbon dioxide contained 16 ml. of 0.15N carbonate-free sodium hydroxide. This absorption vessel was identical with that used in the determination of the carbon dioxide evolved during cellulose oxidation.

This uronic acid method was checked with known glucurone; it gave results that were about 7% below theory for samples yielding approximately 10 mg. of carbon dioxide. The low results were undoubtedly due to the inefficiency of the method used for the carbon dioxide absorption.

## CHARACTERIZATION OF LABELED CELLULOSE - POSITIONS 1 AND 6

In order to determine the distribution of label in a labeled cellulose, it was first necessary to hydrolyze the cellulose to glucose, for the anhydroglucose polymer is not amenable to such analysis. The glucose was subsequently oxidized to potassium gluconate which was the compound basic to the analyses that were made.

The cellulose was hydrolyzed to glucose by first treating it with 72% sulfuric acid (0.5 gram per 9 ml.) for one hour at room temperature and then, after diluting the solution to 3%, by refluxing it for three hours. The hydrolyzate was neutralized to pH 6.0 with barium hydroxide and then filtered through a Celite-actuated carbon bed. The hydrolyzate was next put through an Amberlite IR-120 ion-exchange column to remove the barium ions. This hydrolyzate was concentrated to a syrup; the syrup was then oxidized with potassium hypiodite, according to Moore and Link (33), to potassium gluconate. The potassium gluconate was recrystallized from aqueous methyl alcohol and melted at 178-180°C. with decomposition.

The specific activity<sup>a</sup> of the carbon in the potassium gluconate was determined according to the method of Van Slyke, Steele, and Plazin (34) for the wet combustion and manometric determination of total carbon as carbon dioxide and the subsequent measurement of its radioactivity.

Carbons 1 and 6 of the potassium gluconate were liberated as carbon dioxide according to the method of Eisenberg (35) (see Appendix IV, page 101).

<sup>a</sup> The specific activity of a substance is the radioactivity per unit weight of the substance, e.g., m $\mu$ c/mg. or c.p.m./mg., etc. (m $\mu$ c = millimicrocurie; c.p.m. = counts per minute).

The carbon dioxide from each position was collected and determined manometrically by using the Van Slyke-Folch (32) apparatus (see Figure 3). The specific activity of the carbon dioxide was determined by the gas phase proportional counting method of Van Slyke, Steele, and Plazin (34).

From the specific activities determined above, the specificity of label in positions 1 and 6 of the labeled cellulose was calculated.

#### DETERMINATION OF THE RADIOACTIVITY OF CARBON DIOXIDE

The activity of radioactive carbon dioxide (carbon-14) was determined by the gas phase counting technique of Van Slyke, Steele, and Plazin (34). The gas phase proportional counter of Bernstein and Ballentine (36) was utilized for the activity measurement.

The carbon dioxide was quantitatively determined just prior to its transfer to the counting tube; therefore, its specific activity could be calculated once the sample activity was known. The detailed procedure followed in the preceding analysis is given in Appendix III, page 96.

#### CHROMATOGRAPHIC TECHNIQUES

Descending paper chromatography was used for the qualitative analysis of the neutral sugars in the hydrolyzates of oxidized and unoxidized celluloses. Chromatographic methods were also applied in other investigations, viz., (1) the quantitative determination of arabinose in acid chlorine-oxidized cellulose, (2) the quantitative determination of the yield of glucose from the total hydrolysis of a purified bacterial cellulose, and (3) the analysis of the hydrolyzate of a purified bacterial cellulose for amino acids.

## PROCEDURES

Well-known methods were used for qualitative chromatographic analyses, and the spectrophotometric method of Piper and Bernardin (37) was used for the quantitative determination of pentose and hexose sugars. To facilitate better adaptation of these methods to the analysis of cellulose hydrolyzates containing trace quantities of various sugars, a fractionation procedure was developed for the purification and concentration of certain fractions of the hydrolyzates.

Whatman 3MM paper was used for fractionations; elsewhere, Whatman no. 1 filter paper was utilized. A number of developers and spray reagents were used in the course of the experimental work; the most useful of these were:

### Developers

- A. Butanol, pyridine, water (10:3:3)
- B. Ethyl acetate, acetic acid, formic acid, water (18:3:1:4)
- C. Methyl ethyl ketone saturated with water

### Spray Reagents

- A. 2-Amino biphenyl (sugars)(38)
- B. p-Anisidine hydrochloride (sugars)(39)
- C. 1% Aqueous methyl orange (acids)
- D. Periodate-permanganate (general)(40)
- E. Ninhydrin (amino-acids)(41)
- F. Hydroxylamine (lactones)(42)
- G. Anthrone (ketoses)(43)

## Fractionation of Cellulose Hydrolyzates

### Cellulose Hydrolysis

Nine milliliters of 72% sulfuric acid were added to 0.5 gram of cellulose, and the mixture was held at room temperature (about 25°C.) for one hour. The mixture was then diluted with 207 ml. of distilled water and refluxed for 3 hours. After cooling, the solution was neutralized to pH 6.0 with barium hydroxide, filtered through a Celite-activated carbon bed, passed through an Amberlite IR-120 ion-exchange column, and concentrated to dryness in vacuo. The syrup was redissolved in a small quantity of water and filtered through glass wool. The solution was then made up to the desired volume.

### Fractionation

The cellulose hydrolyzate was heavily spotted along a line on a chromatogram (20 cm. in width) prepared from 3MM paper. Pilot strips were prepared to each side of the material to be fractionated; these were spotted with either the cellulose hydrolyzate or a known sugar mixture. The chromatogram was developed in the butanol: pyridine: water system (10:3:3) for 60 hours at about 25°C.; this developing time gave the best separation of hexose, pentose, and tetrose sugars. The chromatogram was then removed from the developer and dried. The pilot strips were cut from the chromatogram and sprayed with either p-anisidine or 2-amino-biphenyl spray reagent. The strips were then heated at 105°C. for 5 minutes in order to develop the colors. The regions of interest, using the pilot strips as a guide, were

then cut from the unsprayed portion of the chromatogram. These strips were eluted utilizing the apparatus shown in Figure 5.

For elution, the strip, 4, was placed between the ends of a pair of lantern slides, 1, which extended from an elevated Petri dish, 2, containing distilled water. About 0.5 cm. of the strip was held by the slides, the remainder was bent down, as shown in Figure 5. A battery jar, 3, was placed over the apparatus to decrease evaporation. The water moved by capillary action down the strip, maintaining a uniform front perpendicular to the length of the strip. When the solvent front reached the tip of the paper, the flow of the solution was directed into a receiver, 5, by a glass rod, 6, that was attached to the strip near its tip. This glass rod also served to keep the strip in a downward position. About 2 ml. of solution were collected before the eluting was discontinued; this quantity of solvent gave complete removal of the sugar from the strip.

The solution was concentrated to dryness in the receiver at 50°C.; this was done using a rotary vacuum concentrator. The residue was redissolved in a few drops of water and filtered through glass wool. It was then made up to the desired volume.

The isolated fractions were rechromatographed in order to obtain chromatograms that were not masked by glucose; comparison of these chromatograms with knowns gave a clearer interpretation of the identification of the trace sugars. The arabinose fractionated from one of the oxidized cellulose hydrolyzates was quantitatively determined by the spectrometric method of Piper and Bernardin (37). Application of the fractionation procedure to a

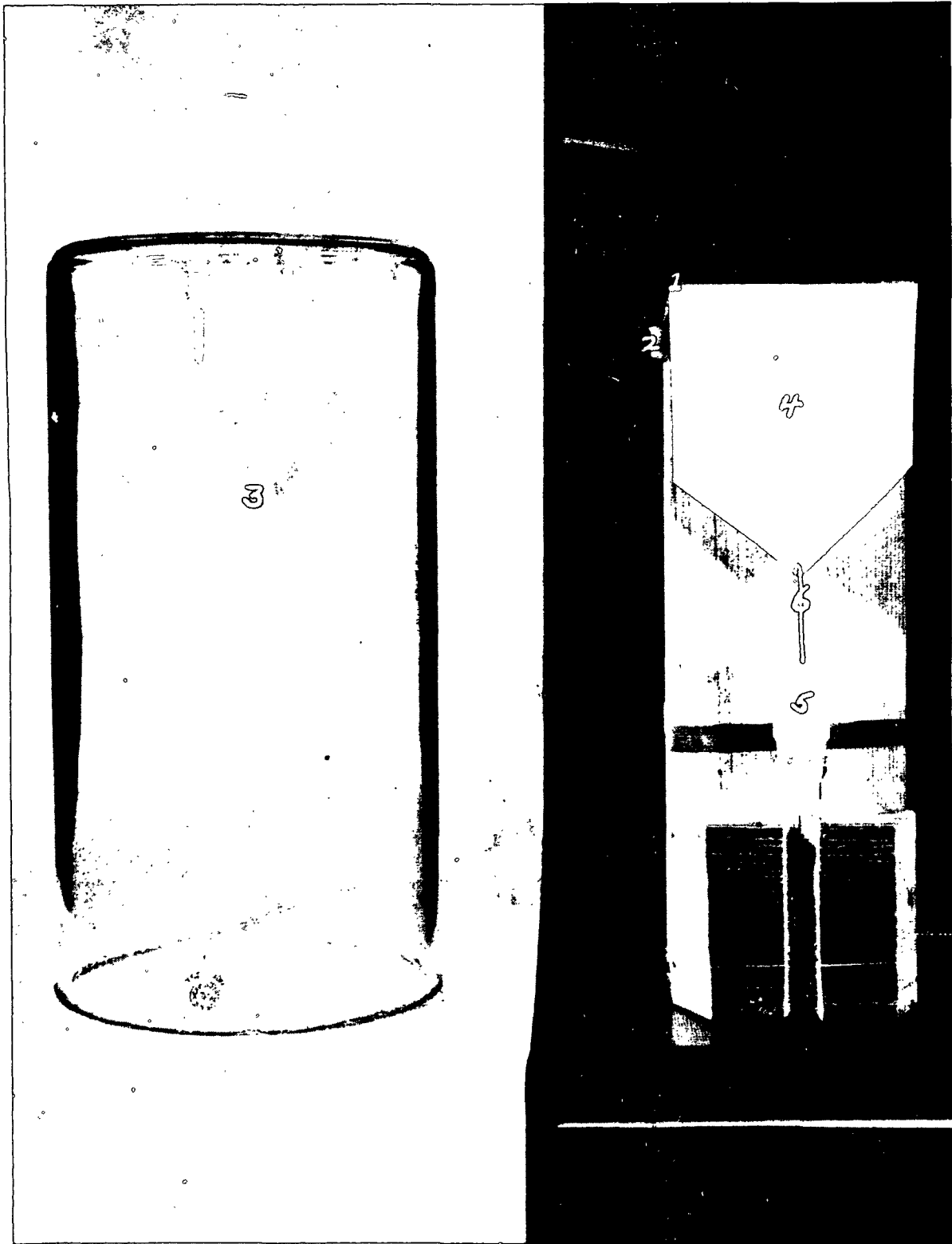


Figure 5. Eluting Apparatus

99 to 1 glucose-arabinose mixture gave a separation of more than 93% of the arabinose; the arabinose fraction contained only a small quantity of glucose.

#### X-RAY DIFFRACTION PATTERNS AND THE DEGREE OF CRYSTALLINITY

In preparation for X-ray analysis, a cellulose sample was Wiley-milled past a 40-mesh screen; 200 mg. of the cellulose were then pressed into a 1.3-cm. diameter pellet under a pressure of about 10,000 lb. per sq. in.; the pressure was applied for one minute. All pellets had a density of 1.40  $\pm$  0.02 g. per cc. The sample was trimmed to fit the sample holder.

A North American Philips Co., Inc., instrument with a copper target and a nickel filter was used to obtain the X-ray diffraction patterns. The instrument was set at 35 kilovolts and 20 milliamperes. The scanning angle ( $2\theta$ ) was 4 to 32 degrees, and the scanning rate was one-quarter degree per minute. The following slit settings were used: divergence slit—one-sixth degree, receiving slit—0.006 in., and scattering slit, one-sixth degree. The rate meter settings were: scale factor—4, multiplier—1.0, and time constant—4 seconds. The chart speed was one inch per minute.

The degree of crystallinity was calculated from the X-ray diffraction patterns according to the methods of Ant-Wuorinen (44) and Hermans and Weidinger as interpreted by Ant-Wuorinen (44).

#### INFRARED ANALYSIS

Infrared analyses were run by L. O. Sell of the Institute Analytical Department. A Perkin-Elmer Model 21 Recording Infrared Spectrophotometer, in conjunction with the potassium bromide pellet technique, was used for the

analyses. Cellulose samples were Wiley-milled past an 80-mesh screen before they were mixed with potassium bromide for pellet preparation.

#### DETERMINATION OF THE DEGREE OF POLYMERIZATION

The degree of polymerization (D.P.) of oxidized and unoxidized celluloses was determined by viscosity measurements. Two different cellulose solutions were used for the determinations; these were: (1) cellulose as cellulose trinitrate (CTN) in acetone solution (non-alkaline) and (2) cellulose in cupriethylenediamine (CED) solution (alkaline). The intrinsic viscosity  $[\eta]$  for these solutions was determined, and the D.P. of the celluloses was calculated according to the Staudinger equation, as given below:

$$[\eta] = K_w M_w^a$$

$M_w$  = weight average molecular weight

$K_w, a$  = constants

The constants used for CTN in acetone were  $K_w$  equals  $1.7 \times 10^{-5}$  and  $a$  equals 1.00; these were based on light-scattering measurements of Benoit, and Doty (45). The constants for CED solutions were obtained by the direct comparison of intrinsic viscosity values for CED solutions of unoxidized celluloses and the acetone solutions of the trinitrates of these celluloses. This was done on the assumption that no alkali-labile linkages were present in the unoxidized cellulose; therefore, both methods would give rise to identical D.P. values. The CED constants were  $K_w$  equals  $3.0 \times 10^{-5}$  and  $a$  equals 1.00. This comparison of the two viscometric methods was not ideal, but it did give a defined basis for comparison.

NONALKALINE D.P. (CELLULOSE NITRATE IN ACETONE SOLUTION)

Cellulose nitrate was prepared according to the method of Bennett and Timell (46). By this method, a nitrogen content of about 14% was easily obtained. The degree of nitration of the cellulose nitrates was determined by the semimicro Kjeldahl method of Timell and Purves (47).

The viscosity of the cellulose nitrates was determined in acetone solution. Solutions were accurately prepared at concentrations of about 0.025, 0.050, 0.100, and 0.200 gram of the nitrated celluloses per 100 ml. of acetone. The viscosities of the solutions were measured in Cannon-Fenske viscometers at 20°C.

The intrinsic viscosity of each sample was obtained by the extrapolation of zero concentration of a plot of the logarithm of reduced viscosity versus concentration. The D.P. was calculated from the Staudinger equation, as discussed above.

ALKALINE D.P. (CELLULOSE IN CUPRIETHYLENEDIAMINE SOLUTION)

The technique of Browning, Sell,<sup>m</sup> and Abel (48) was used for the preparation of solutions containing known quantities of cellulose of approximately 0.2, 0.4, and 0.6 gram per 100 ml. of 0.5M cupriethylenediamine. The precautions of removing residual oxygen from prepurified nitrogen, protecting the serum bottles from light, and flushing the viscometers with nitrogen were not observed. The viscosities of the solutions were measured in Cannon-Fenske viscometers at 20°C. The intrinsic viscosity of the solutions and D.P. of the cellulose were calculated as discussed above.

NITROGEN DETERMINATION ON CELLULOSE--NISS COLORIMETRIC METHOD

The Niss colorimetric method (49) for the determination of trace quantities of nitrogen in organic materials was used for the analysis of nitrogen in purified oxidized and unoxidized celluloses. A detailed description of the method is given in Appendix V, page 103.

## EXPERIMENTAL RESULTS AND DISCUSSION

### CHEMICAL STUDIES

#### SAPONIFIABLE CARBON DIOXIDE IN OXIDIZED CELLULOSES

The initial studies conducted in this research program were designed to check the findings of Kaverzneva, *et al.*, (2-5) with respect to the presence of saponifiable carbon dioxide (SCD) in acid chlorine-oxidized cellulose. Oxidations I and II (see Table I) were made with the foregoing in mind; they proved successful, for both the oxidized celluloses contained SCD, about 0.37 mole of carbon dioxide per 100 moles of anhydroglucose for each. Blanks were run on the unoxidized cotton; the results obtained ranged from 0.01 to 0.03 mole of SCD for 100 moles of anhydroglucose. Treatment of the unoxidized cotton with the buffer solutions had no effect on its SCD content. Therefore, it was apparent that the oxidative treatment had effected the formation of saponifiable structures<sup>a</sup> in the purified cotton celluloses. These results support the data reported by the Russian workers.

In the course of this research, SCD was determined on eleven different oxidized celluloses (Table I). The degree of oxidation of these celluloses varied from 0.3 to 4.3 equiv. of hypochlorous acid per mole of anhydroglucose. These oxidations were made on cotton and bacterial celluloses. The bacterial cellulose was used in connection with the radiochemical studies; these studies will be discussed later. The oxidized bacterial cellulose was found to contain SCD; however, for the samples examined the quantity was

<sup>a</sup> Saponifiable structures should be construed to mean either chemical or physical structures that liberate carbon dioxide when subjected to either of the saponification treatments (page 20).

less than that in oxidized cotton cellulose at comparable levels of oxidation (see Figure 6).

The duration of the saponification treatment was observed to have an effect on the apparent SCD content of a given cellulose. It was found that a one-hour saponification gave rise to less SCD than did a 12-hour treatment; after 12 hours, no additional increase was noted (see items 12a and 12b in Table I). It is believed that this is due to the time aspect of the penetration of the 0.025N sodium hydroxide solution into the cellulose and the subsequent breakdown of the carbon dioxide-yielding structures by the dilute caustic. The results reported by Kaverzneva (2) were derived from one-hour saponifications, whereas, unless otherwise noted, the results listed herein were obtained from 12-hour saponifications. It will be shown later (page 55) that the SCD from a one-hour saponification apparently has the same origin as that from a 12-hour treatment.

The titrimetric and manometric methods that were used for the determination of SCD gave comparable results for a given sample (see items 2a and 2b in Table I). The manometric results were, however, consistently lower by a few per cent. In spite of this, the results obtained by the manometric method were not corrected, for the general precision of the two methods was not good enough to merit the development of a correction factor. Both methods are considered adequate for the analysis of oxidized celluloses for SCD.

The acetate and phosphate buffer systems that were utilized for the control of the pH in the oxidation mixtures were not found to have an effect

TABLE I

## CELLULOSE OXIDATION DATA AND SCD RESULTS

Number	Sample Identification (Oxidation Number, etc.)	Material	Buffer System (pH 4.3±0.3)	Oxidant Consumption <sup>d</sup> , equiv. HCl/mole <sup>a</sup> anhydroglucose	Initial D.P. of Cellulose	Yield <sup>b</sup> , %	Method of SCD <sup>c</sup> Deter- mination	SCD <sup>d</sup> , mole CO <sub>2</sub> /100 moles anhydroglucose
1	Oxidation I (Sample 1)	Cotton	Acetate	4.34	1750	74.3	Titrimetric	0.37
2a	Oxidation II (Sample 2)	Cotton	Phosphate	2.59	1750	80.3	Titrimetric	0.38
2b	Oxidation II (Sample 2)	Cotton	Phosphate	2.59	1750	80.3	Manometric	0.35
3	Oxidation II (Sample 3)	Cotton	Phosphate	2.66	1750	77.8	Titrimetric	0.37
4	Oxidation X	Cotton	Acetate	1.54	1750	82.0	Manometric	0.25
5	Oxidation VI	Bacterial cellulose	Acetate	0.29	3370	96.2	Manometric	0.10
6	Oxidation VII	Bacterial cellulose	Acetate	0.82	3370	89.7	Manometric	0.15
7	Oxidation XII	Bacterial cellulose	Phosphate	0.91	3370	89.8	Manometric	0.15
8	Oxidation VIII	Bacterial cellulose	Acetate	1.37	3370	86.9	Manometric	0.17
9	Oxidation IX	Bacterial cellulose	Acetate	1.38	2750	86.1	Manometric	0.18
10	Oxidation XI	Bacterial cellulose	Acetate	1.39	2800	85.9	Manometric	0.19
11	Oxidation IV	Bacterial cellulose	Phosphate	2.31	1230	77.8	Titrimetric	0.24
12a	Oxidation V	Bacterial cellulose	Acetate	2.65	--	84.1	Manometric	0.25
12b	Oxidation V	Bacterial cellulose	Acetate	2.65	--	84.1	Manometric (one hour)	0.11
13	Unoxidized purified cotton	--	--	--	--	--	Titrimetric	0.02
14	Unoxidized purified bacterial cellulose	--	--	--	--	--	Titrimetric	0.01

<sup>a</sup> Purified cotton and bacterial celluloses are assumed to be 100% anhydroglucose, i.e., for calculations of this type.

<sup>b</sup> Yields are calculated on o.d. basis; o.d. is defined in this thesis as a sample that has been dried over P<sub>2</sub>O<sub>5</sub> at 30°C. and 735 mm. Hg vacuum for 12 hours.

<sup>c</sup> SCD = saponifiable carbon dioxide.

<sup>d</sup> Averages of duplicate determinations.

on the quantity of SCD in oxidized cellulose at a given level of oxidation. Items 6 and 7 in Table I support the foregoing statement; they also serve to illustrate the reproducible character of the oxidized cellulose. In the light of these observations, it appears unlikely that the buffer systems actively enter into the cellulose oxidations.

The SCD values reported by Kaverzneva, et al., covered a range of 0.07 to 0.22 mole of carbon dioxide per 100 moles of anhydroglucose. These values corresponded to oxidant consumptions of 0.1 to 0.9 equiv. of hypochlorous acid per mole of anhydroglucose. The above data compare favorably with those obtained in this research, i.e., over the same range of oxidant consumptions. From their results, it appears that the SCD in an oxidized cellulose is related to the oxidant consumption; this is also substantiated by results reported herein (see Figure 6). For eight oxidized bacterial celluloses, the SCD increased for each significant increase in oxidant consumption; no definite leveling off point had been reached at the highest level of oxidation used. However, for samples of oxidized cotton, the SCD content leveled off at about 0.37 mole of carbon dioxide per 100 moles of anhydroglucose; this leveling off point corresponded to an oxidant consumption of about 2.5 equiv. of hypochlorous acid per mole of anhydroglucose. Kaverzneva, et al. (3) suggested that the leveling off effect noted for cotton may be due to a balance between the formation and the destruction of the saponifiable groupings.

The stability of the chemical or physical structures giving rise to the SCD has not been thoroughly investigated. Kaverzneva (2) stated that her hypothesized carbonic acid ester linkage was rather unstable, but she did not

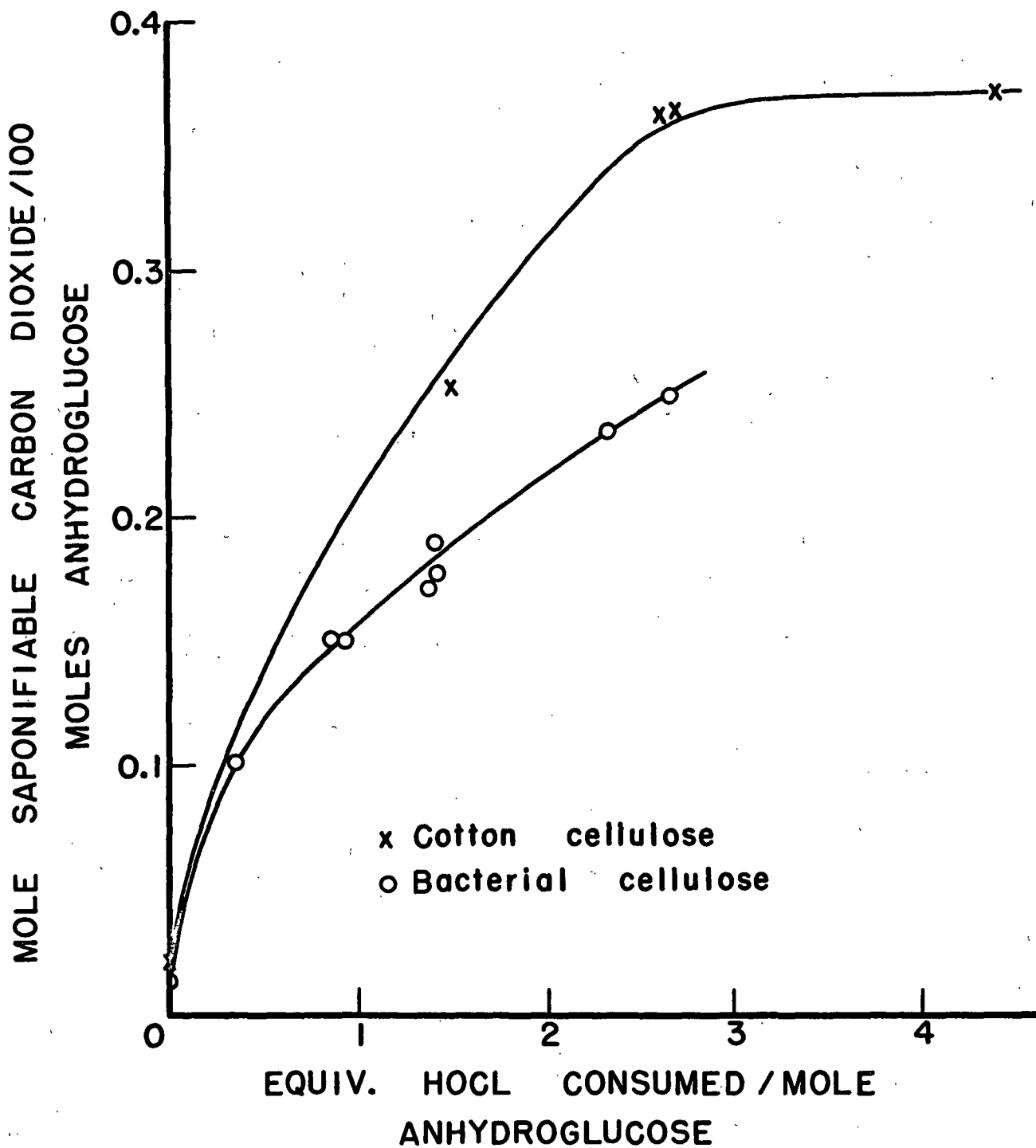


Figure 6. Effect of Oxidant Consumption on Saponifiable Carbon Dioxide Content of Oxidized Cellulose

give supporting evidence, i.e., other than the action of the saponification treatment. It was found in this research that the SCD in an oxidized cotton cellulose (0.37 mole  $\text{CO}_2$ /100 moles anhydroglucose) was not affected significantly by either an acidity of pH 3 (0.36) or a short treatment with distilled water at 45°C. (0.35). The effect of higher temperatures and higher acidities is not known.

#### NEUTRAL OXIDATION PRODUCTS IN OXIDIZED CELLULOSE

The isolation of D-arabinose from acid chlorine-oxidized cellulose by Henderson (15) gave Kaverzneva's carbonic acid ester hypothesis additional experimental support (see Figure 1). Henderson concluded that the arabinose found was a product of the cleavage of the anhydroglucose unit between carbons 1 and 2 to form the hypothesized carbonic acid ester linkage and D-arabinose. This suggests the possibility of a relationship between SCD and the arabinose in an oxidized cellulose. If the relationship were consistently found to be 1:1 on a molar basis, it would give additional support to the ester hypothesis. It was with a view toward the evaluation of this ratio that oxidized celluloses were hydrolyzed and analyzed for arabinose. This work also served as an attempt to substantiate Henderson's isolation of D-arabinose as well as to detect any other neutral sugars that are present in the oxidized cellulose as oxidation products.

Two samples of oxidized cotton, oxidation I (sample 1) and oxidation II (sample 2), and a sample of purified unoxidized cotton (0.5 gram each) were hydrolyzed and quantitatively prepared for paper chromatographic analysis. The hydrolyzates were qualitatively chromatographed using both a basic

developer (developer A - butanol, pyridine, water; 10:3:3) and an acidic developer (developer B - ethyl acetate, acetic acid, formic acid, water; 18:3:1:4). For the study of neutral products, the basic developer showed more promise, for it moved acids very slowly and gave good resolution of neutral sugars. Even without the acidic fraction, satisfactory analysis of the neutral oxidation products in the hydrolyzates was still impossible, for the large concentration of glucose in the hydrolyzates tended to mask trace components. Therefore, it was necessary to fractionate and concentrate these trace materials before they could be accurately determined. When the unfractionated oxidized cellulose hydrolyzates were chromatographed using developer A and sprayed with *p*-anisidine spray reagent, a pinkish streak in the pentose region and a faint yellow spot in the tetrose region were observed. Evaluation of these chromatograms was uncertain, for both of these areas were heavily streaked with glucose.

A 99:1 glucose-arabinose mixture and D-threose<sup>a</sup> were used on the pilot strips of the chromatograms utilized for fractionation; they defined the pentose and the tetrose regions, respectively. The pentose and tetrose fractions were separated by cutting the proper sections from the developed chromatograms; the pilot strips were used as guides. The fractionated materials were quantitatively separated (eluted, concentrated, etc.) and rechromatographed with known reference compounds in order to ascertain the sugars present.

On chromatographing and spraying with *p*-anisidine spray reagent, the pentose fraction from the oxidized cotton cellulose hydrolyzates gave a

<sup>a</sup> D-threose was prepared and purified according to the method of Perlin and Brice (50).

discrete pink spot that corresponds to arabinose ( $R_g - 1.32^a$ ) and the tetrose fraction gave a discrete yellow spot ( $R_g - 3.14$ ) that moved slightly slower than D-threose ( $R_g - 3.28$ ). This second compound was also noted by Henderson (15). Neither of the foregoing compounds was detected by the fractionation and analysis of the unoxidized cellulose hydrolyzate; therefore, these materials are obviously products of the acid chlorine oxidation of the cellulose.

The tetrose fraction from the oxidized cellulose hydrolyzates was also chromatographed in developers B and C (methyl ethyl ketone saturated with water). A variety of spray reagents was utilized in the analysis of the chromatographed material. A summary of the information obtained from these tests follows:

- (1) It is a reducing sugar that moves slightly slower than D-threose in all the developers used.
- (2) It gives the same color reactions with p-anisidine and 2-aminobiphenyl spray reagents as does D-threose.
- (3) It is neither a ketose, a lactone, nor an acid.

From this chromatographic evidence and the published information of Perlin and Brice (50) with regard to the chromatographic comparison of D-erythrose and D-threose, it is apparent that D-erythrose is a product of the acid chlorine oxidation of cellulose. As suggested by Staudinger (6, 7), the formation of D-erythrose during cellulose oxidation may result from the cleavage of carbons 2 and 3 of the anhydroglucose unit; subsequent oxidation

<sup>a</sup> All  $R_g$  values (R value with respect to glucose) are for developer A, unless otherwise noted.

may lead to a carbonic acid ester. Also, erythrose may be derived by further oxidation of arabinose formed by the cleavage of carbons 1 and 2.

The tetrose fraction of the oxidized bacterial cellulose hydrolyzate, believed to be erythrose, was freeze-dried together with potassium bromide, according to the method of Schiedt and Reinwein (52). The resulting powder was then pressed into a pellet, and the infrared spectra of the material was obtained. This spectrum did not compare favorably with that of an authentic sample of D-erythrose.<sup>a</sup> The discrepancies between the two infrared spectra are felt to be due to impurities in the tetrose fraction; a small quantity of glucose was later found to be present. Also, the elution of the fraction from unwashed 3MM chromatographic paper undoubtedly gave rise to other impurities. Purification of the fraction for further infrared study was not tried.

Further chromatographic analysis of the pentose fraction of the oxidized cellulose hydrolyzates was conducted using developer B; this study confirmed previous work relating the presence of arabinose ( $R_g - 1.6$ , developer B) in the oxidized cellulose hydrolyzates. The arabinose fractions from the different hydrolyzates were quantitatively compared with each other and with quantitative arabinose knowns. The hydrolyzate representing sample 2 of oxidation II was found to contain more arabinose than did other hydrolyzates examined. It was determined by the method of Bernardin and Piper (37) that the arabinose in this sample represented 0.2% by weight of the original cellulose.

<sup>a</sup> Infrared spectrum of authentic D-erythrose obtained through the courtesy of E. S. Perlin, National Research Council of Canada.

In the oxidized celluloses examined, the quantity of arabinose did not exceed about 0.2% by weight; this value is considerably lower than the 1% found by Henderson (15). The reason for this difference may stem from the conditions of the respective oxidations, for it was impossible to duplicate the conditions of Henderson's oxidation. He reported that in his oxidation the purified cotton consumed 4.4 equiv. of hypochlorous acid per mole on anhydroglucose with a yield of 95%, whereas oxidation II, sample 2 (see Table I), consumed about 2.6 equiv. per mole but gave a yield of only 80%. The methods of hydrolysis used were also slightly different in the secondary hydrolysis stage. However, neither of these hydrolysis techniques was found to effect significant change in the ratio of glucose to arabinose in a known 99:1 mixture. In short, the reason for the quantitative difference in the results reported by Henderson and those obtained in this research is not clearly defined.

Unoxidized and oxidized (oxidation VI, Table I) bacterial celluloses were also hydrolyzed and analyzed as outlined above. The oxidized sample was found to contain arabinose, xylose ( $R_g - 1.67$ ), and erythrose as oxidation products. These materials appeared to be present in the following order with respect to descending magnitude: xylose, erythrose, and arabinose. No attempt was made to estimate these compounds quantitatively.

The xylose in the oxidized bacterial cellulose hydrolyzate was found by taking two fractions from the pentose region of developed fractionation chromatograms; one corresponded to an arabinose known and the other to a xylose known. In the fractionation of the oxidized cotton hydrolyzates, only the fraction corresponding to arabinose was taken for analysis; thus, xylose

may have been present although it was not detected. The xylose found was probably derived from decarboxylation of glucuronic acid groups in the oxidized cellulose. This may have occurred either during the oxidation or during the hydrolysis of the cellulose (51).

RATIO OF SAPONIFIABLE CARBON DIOXIDE TO  
ARABINOSE IN OXIDIZED CELLULOSE

As suggested earlier, the ratio of SCD to arabinose in oxidized cellulose may give support to the carbonic acid ester hypothesis. This indirect approach involves the quantitative determination of both the SCD and the arabinose in oxidized celluloses. The methods used for the analysis of oxidized cellulose for SCD were found to be adequate; however, the determination of arabinose proved difficult and uncertain due to the small quantity that was present.

The ratio of SCD to arabinose in sample 2 of oxidation II was found to be about 1.7:1. This result has two possible interpretations: (1) All of the SCD in this oxidized cellulose is not entirely associated with the carbon 1 position and the carbonic acid ester linkage, or (2) the arabinose formed by the oxidative cleavage of carbons 1 and 2 of the anhydroglucose unit is subsequently oxidatively degraded, while the carbonic acid ester grouping, formed by the oxidation, either remained intact or was hydrolyzed at a slower rate than the arabinose was degraded.

Due to the lack of a satisfactory method for quantitating small quantities of arabinose in oxidized cellulose and to the uncertainty of the meaning of the SCD-arabinose ratio obtained for an oxidized cellulose, this

approach to the study of the carbonic acid ester hypothesis showed little promise and was abandoned.

#### SUMMARY OF CHEMICAL STUDIES

Saponifiable carbon dioxide was shown to be present in acid chlorine-oxidized cotton and bacterial celluloses.

Neutral oxidation products identified in the hydrolyzates of oxidized cotton and bacterial celluloses were arabinose and erythrose. In addition, xylose was detected in the hydrolyzate of an oxidized bacterial cellulose. The chromatographic characterization of arabinose as an oxidation product qualitatively supports the finding of Henderson (15); however, the maximum quantity of arabinose found was 0.2% by weight, whereas Henderson reported 1%. This discrepancy remains unexplained.

As a means of obtaining evidence relating to the carbonic acid ester hypothesis, the relationship between the SCD and the arabinose in an oxidized cellulose was determined. The ratio obtained was amenable to several different interpretations, none of which clearly added to the question of the validity of the hypothesis. Therefore, this indirect approach to the study of the hypothesis was abandoned due to its lack of promise.

#### RADIOCHEMICAL STUDIES

##### INTRODUCTION

The isolation of the SCD from acid chlorine-oxidized cellulose and the characterization of this carbon dioxide as to its origin, carbon 1, 2, etc.

of the anhydroglucose molecule, constitute a direct test of the validity of Kaverzneva's carbonic acid ester linkage (see Figure 1). If the SCD were shown to consist of carbon dioxide derived exclusively from carbon 1, the hypothesis would be greatly strengthened. However, if carbon 1 were shown to represent little or none of the SCD, the probability of the existence of the postulated stable ester linkage would be reduced; such a negative finding would also serve to open the way for consideration of the true source of the SCD in acid chlorine-oxidized cellulose. Data capable of being interpreted in the foregoing terms can only be obtained through the use of isotopically labeled cellulose.

#### LABELED BACTERIAL CELLULOSE PREPARATION

The radioactive isotope of carbon most conveniently used is carbon-14; therefore, it was utilized in the preparation of the specifically labeled celluloses. The labeled cellulose was grown using the bacterium Acetobacter xylinum with the labeling being introduced in the form of specifically labeled D-glucose. The preparation and use of labeled bacterial cellulose were contingent upon the successful preparation of an unlabeled bacterial cellulose, its oxidation, and the analysis of this oxidized cellulose for SCD. The success of these operations (see page 39), showed the feasibility of the use of labeled bacterial cellulose for the proposed investigations.

$C^{14}$ -labeled cellulose was biosynthesized by Acetobacter xylinum from both D-glucose-1- $C^{14}$  and D-glucose-6- $C^{14}$ . These labeled bacterial celluloses were prepared in yields of 35-40% on the basis of the glucose supplied and 16-21% on the basis of the carbon-14 supplied (see Table II). The labeled

celluloses were not entirely specific despite the use of specifically labeled glucose; this was due to the entry of part of the labeled glucose into the metabolic process of the organism (53). The label in the cellulose-1- $C^{14}$ <sup>a</sup> was 80% specific at carbon 1 and 2% specific at carbon 6; these values compared favorably with those reported by Greathouse (19). For the cellulose-6- $C^{14}$ <sup>b</sup> the label was 24% specific at carbon 1 and 59% specific at carbon 6; whereas Greathouse (19) reported an 82% specificity in position 6 of a similarly prepared labeled cellulose. This low result was probably due to one or both of the following:

- (1) The nutrient media for this preparation was contaminated with unknown species of bacteria, as evidenced by abnormal turbidity and color of the medium.
- (2) Greathouse's method (19) of preparation was not adhered to exactly; the alcohol specified for the nutrient media was not used.

Although the specificity of the labeled celluloses was less than 100%, it is felt that both preparations were adequate for the support of the conclusions drawn from the studies described below.

#### ANALYSIS OF SAPONIFIABLE CARBON DIOXIDE IN LABELED BACTERIAL CELLULOSE

Cellulose-1- $C^{14}$  was oxidized to five different levels of oxidation; the oxidant consumption for these oxidations ranged from 0.29 to 2.65 equiv. hypochlorous acid per mole of anhydroglucose. The oxidized celluloses were washed and solvent dried before they were analyzed for SCD. These celluloses represented oxidations V, VI, VII, VIII, and XII. Oxidation V utilized batch 1 of the labeled cellulose; the other oxidations were made on batch 3 (mixture of batches 1 and 2) of the labeled cellulose (see Table II). These

<sup>a</sup> Denoted cellulose-1- $C^{14}$  because of its biosynthesis from D-glucose-1- $C^{14}$ .

<sup>b</sup> Denoted cellulose-6- $C^{14}$  because of its biosynthesis from D-glucose-6- $C^{14}$ .

TABLE II

## PURIFIED LABELED BACTERIAL CELLULOSE PREPARATIONS

Purified Labeled Bacterial Cellulose	Yield <sup>a</sup> , g.	Yield <sup>b</sup> , %	Yield of <sup>14</sup> C- Carbon, %	Nitrogen in Cellulose, %	D.P. of Cellulose	Sp. Act. of Anhydro-glucose Unit, c.p.m./mg. C <sup>c</sup>	Sp. Act. in C(1)-Position, c.p.m./mg. C	Activity <sup>d</sup> in C(1)-Position, %	Sp. Act. in C(6)-Position, c.p.m./mg. C	Activity <sup>e</sup> in C(6)-Position, %	Estimates <sup>f</sup> of Sp. Act. in C(2)-, C(3)-, C(4)-, and C(5)-Positions, c.p.m./mg. C			
											C(2)	C(3)	C(4)	C(5)
Batch 1: Cellulose-1-C <sup>14</sup> , used in oxidation V	36.3	40.2	16.1	--	--	2774 <sup>g</sup>	13,313	80.2	--	--	--	--	--	--
Batch 2: Cellulose-1-C <sup>14</sup>	36.0	40.0	17.8	--	--	3092	--	--	--	--	--	--	--	--
Batch 3: Mixture of batches 1 and 2, used in oxidations VI, VII, VIII and XII	--	--	--	0.20	3370	2914	13,953	79.8	362	2.1	110	1950	1050	110
Batch 4: Cellulose-6-C <sup>14</sup> , used in oxidation IX	31.7	35.2	21.3	0.07	2750	1920	2,773	24.1	6807	59.1	450	1050	400	350

<sup>a</sup> For all calculations, cellulose assumed to be 100% anhydroglucose.

<sup>b</sup> Yield based on glucose supplied.

<sup>c</sup> Specific activity, counts per minute per milligram of carbon.

<sup>d</sup> Specificity at carbon 1.

<sup>e</sup> Specificity at carbon 6.

<sup>f</sup> Estimates calculated on basis of distributions of label in bacterial celluloses, as presented by Greathouse (19)(see page 8), and total activity of anhydroglucose units.

<sup>g</sup> All activity values represent average of two determinations, counting error less than 3% for all counts made in this research.

labeled celluloses had a specific activity in position 1 of about 13,300 and 13,900 c.p.m./mg., respectively; these activities represented specificities of approximately 80%.

The SCD content of each of the oxidized celluloses was determined by the manometric method; the specific activity of this carbon dioxide was determined by the gas phase counting procedure of Van Slyke and Folch (32) (see Appendix III, page 96). The results obtained are listed in Table III.

The average specific activity of the SCD from samples representing oxidations V, VI, VII, VIII, and XII were 2774, 2015, 2382, 2283, and 2052 c.p.m./mg. C, respectively. On the basis of the specific activity of the carbon in position 1 of the cellulose-1-C<sup>14</sup> that was oxidized, the maximum percentage of the SCD from the respective samples that could have originated at position 1 was 20.8, 14.5, 17.1, 16.4, and 14.7%. The quantity and specific activity of the SCD from a given oxidized cellulose were reproducible.

The effect of saponification time was checked on samples from oxidation VI. The quantity of SCD liberated by a one-hour treatment was less than that liberated by a 12-hour treatment, 0.07 as opposed to 0.10 mole carbon dioxide per 100 moles of anhydroglucose. The specific activities of the SCD liberated by these different saponifications were comparable, 1915 and 2015 c.p.m./mg. C, respectively. Therefore, it seems likely that the source of the SCD was in both cases about the same. This study was conducted in order to put the results of Kaverzneva, et al., (one-hour saponification time) and the results obtained in this research (12-hour saponification time) on a common basis.

TABLE III

## SUMMARY OF RESULTS—SAPONIFIABLE CARBON DIOXIDE FOUND IN ACID CHLORINE-OXIDIZED LABELED BACTERIAL CELLULOSE

Item Number	Sample Identification	Buffer System (pH 4.3±0.3)	Oxidant Consumption, equiv. HOCl/mole anhydroglucose	Carbon Dioxide Liberated During Oxidation, % <sup>a</sup>	Sp. Act. of CO <sub>2</sub> Liberated During Oxidation, c.p.m./mg. C	Yield of Oxidized Cellulose, %	SCD, mole CO <sub>2</sub> /100 moles anhydroglucose	Specific Activity of SCD, c.p.m./mg. C	Maximum of Saponifiable Carbon Dioxide (SCD) Originating at the C(1) <sup>b</sup> -Position, %
1	Oxidation VI Batch 3	Acetate	0.29	0.61	1039	96.2	0.069 (one-hour saponification)	1915	13.7
							0.102 (12-hour saponification)	2015	14.5
2	Oxidation VII Batch 3	Acetate	0.82	2.09	1352	89.7	0.154	2420	17.1
							0.15	2382	
3	Oxidation XII Batch 3 (free of included solvents)	Phosphate	0.91	1.99	2841	89.8	0.143	2041	14.7
							0.15	2052	
4	Oxidation VIII Batch 3	Acetate	1.37	4.65	1348	86.9	0.173	2262	16.4
							0.17	2283	
5	Oxidation V Batch 1	Acetate	2.65	—	—	84.1	0.261	2736	20.8
							0.25	2774	
6	Oxidation IX Batch 4	Acetate	1.38	5.10	728	86.1	0.173	1582	16.0 <sup>c</sup>
							0.18	1604	
							0.181	1626	

<sup>a</sup> Based on original cellulose.

<sup>b</sup> Limitation valid provided assumptions made with respect to isotope effect and label distribution hold.

<sup>c</sup> For this sample the maximum SCD originating at position 6 is 17%.

Interpretation of the results that were obtained from the radio-chemical analysis of the SCD from oxidized C(1)-labeled celluloses gives rise to the conclusion that the SCD in acid chlorine-oxidized cellulose is not derived primarily from position 1 of the anhydroglucose molecule. Of the five samples prepared and analyzed, the maximum contribution of the SCD that could have been made by position 1 ranged from 14 to 21%. In reality, the contribution of position 1 could never have reacted the maximum, for all the other positions of the anhydroglucose molecule had some label.

A sample of cellulose-6-C<sup>14</sup> was oxidized (oxidation IX) to a level of oxidation comparable to that of oxidation VIII; this was done in an attempt to ascertain the contribution made by position 6 to the SCD in a cellulose oxidized to this level of oxidation. The specific activity of the SCD from the oxidized cellulose-6-C<sup>14</sup> was 1604 c.p.m./mg. C. By assuming that the SCD from the two oxidized celluloses, oxidations VIII and IX, had the same origin and that any carbon dioxide derived from positions other than 1 and 6 was of zero activity, the maximum contributions of position 1 and 6 were calculated as 16 and 17%, respectively. Of course, one or both of these values are high, for the second assumption made above is known to be erroneous. Only one oxidation was made using cellulose-6-C<sup>14</sup>, so the analysis of the part played by position 6 may be considered preliminary.

#### SUMMARY OF RADIOCHEMICAL STUDIES

Labeled bacterial celluloses were satisfactorily biosynthesized from D-glucose-1-C<sup>14</sup> and D-glucose-6-C<sup>14</sup> with Acetobacter xylinum. These labeled celluloses were prepared in yields of 35 to 40% based on the glucose supplied and 16 to 21%

based on the carbon- $^{14}$  supplied. The specificity of label in position 1 for the cellulose- $1-C^{14}$  was 80%, and the specificity of label in position 6 for the cellulose- $6-C^{14}$  was 59%. The specificity of position 6 of cellulose- $6-C^{14}$  was not as high as published results but was adequate for the satisfactory interpretation of the experiments utilizing this cellulose.

From the analysis of the specific activity of the SCD from oxidized cellulose- $1-C^{14}$  and cellulose- $6-C^{14}$ , it was clearly indicated that the SCD does not represent a single position in the anhydroglucose molecule and position 1 can be the source of not more than 21% of the SCD, in fact, probably much less. Therefore, the probability of the existence of the hypothesized carbonic acid ester linkage is reduced.

#### POSTULATED SOURCES OF SAPONIFIABLE CARBON DIOXIDE

There may be some carbonic acid ester linkages in acid chlorine-oxidized cellulose, but the origin of most of the SCD is not known. There are two broad explanations for this SCD; these are presented below.

1. Carbon dioxide liberated during the oxidation of cellulose may be physically absorbed or included in the cellulose, with these physical bonds or obstructions being labile to dilute alkali.
2. Some structure or structures, not involving position 1, that liberate carbon dioxide when subjected to the saponification treatment are present in acid chlorine-oxidized cellulose.

(Both of these may occur simultaneously.)

#### ABSORBED OR INCLUDED CARBON DIOXIDE

From the specific activity of the carbon dioxide liberated during the oxidations of the labeled celluloses, Table III, it was apparent that the

SCD was not due to only adsorption or inclusion of part of this carbon dioxide in the cellulose. Specifically, this conclusion stemmed from the fact that the specific activity of the carbon dioxide liberated during the oxidations was not directly related to the specific activity of the SCD. Nevertheless, it was felt that this possibility could not be completely discounted without additional experimental evidence. The experiment conducted in analysis of possible adsorption or inclusion of carbon dioxide in cellulose during acid chlorine oxidations follows:

Radioactive carbon dioxide with a specific activity of about 12,000 c.p.m./mg. C was continuously cycled through the oxidation mixture throughout the oxidation of an unlabeled bacterial cellulose (oxidation XI)(see Appendix II, page 93). When the bulk of the oxidant was consumed, cycling of the radioactive carbon dioxide was discontinued, and the oxidized cellulose was washed and solvent dried. The specific activity of the SCD in the oxidized cellulose was then determined; it was found to be approximately zero. Therefore, it was concluded that neither physical adsorption nor inclusion of carbon dioxide is likely to be responsible for any of the SCD in oxidized cellulose.

#### CHEMICAL STRUCTURES

Inasmuch as the carbonic acid ester (position 1) can make only a minor contribution (<21%) to the SCD in acid chlorine-oxidized cellulose, it is necessary for other structures, not associated with position 1, to make up the major part of the SCD, i.e., if chemical structures are solely responsible for the SCD. Hypotheses for the formation of monocarbonate and formate esters

are presented below. It must be pointed out that these proposed structures and the mechanism for their formation is for the most part strictly speculation. Also, these structures are by no means felt to be the only ones possible; they are presented as a guide to further thinking with respect to the explanation of the experimental results obtained in this research.

The postulated monocarbonate ester grouping may be formed from carbon 3 or 6. The formation in both cases involves a  $\gamma$ -lactone intermediate of the C(3)- or C(6)-acid. The mechanism depicted in Figure 7 is for the formation of the monocarbonate ester from carbon 3. The supporting evidence for the steps in the formation of this structure are listed below:

- (1) The presence of erythrose in acid chlorine-oxidized cellulose has been chromatographically verified in this research.
- (2) Jayme and Maris (54) have suggested that the aldehyde group at position 3 reacts much faster than does that at position 2.
- (3) Erythronic acid has not been identified in acid chlorine-oxidized cellulose, but it has been identified by Whistler and Schweiger (55) in acid chlorine-oxidized amylopectin.
- (4) Kaverzneva, et al., (3) showed the presence of lactones in acid chlorine-oxidized cellulose.
- (5)  $\gamma$ -lactones are known to be very stable in acidic media; however, it is not known whether an oxidative carbon-carbon cleavage could take place leaving the lactone group intact. One theory that may support such a cleavage is that pertaining to the oxidative cleavage of a carbon-carbon bond with lead tetraacetate leaving a lactol linkage intact; this theory was advanced by Perlin and Brice

(50) for the lead tetraacetate oxidation of sugars.

- (6) The stability and reactivity (ability to cross link) of a monocarbonate ester of the type shown are not known. It is known that monocarbonate esters are normally rather unstable (56).

[The above supporting evidence, where applicable, may be construed to cover monocarbonate formation via the C(6)-carbon.]

The formation of formate esters via lactol intermediates (carbons 2, 3, and/or 6) is hypothesized. These formate esters would be saponifiable in the presence of dilute alkali, thus forming sodium formate which on acidification would be converted to formic acid. The formic acid may be readily decomposed to carbon dioxide in the presence of the reducible groupings in the oxidized cellulose. Metal ions present in the acidic cellulose suspension could catalyze such a reaction.

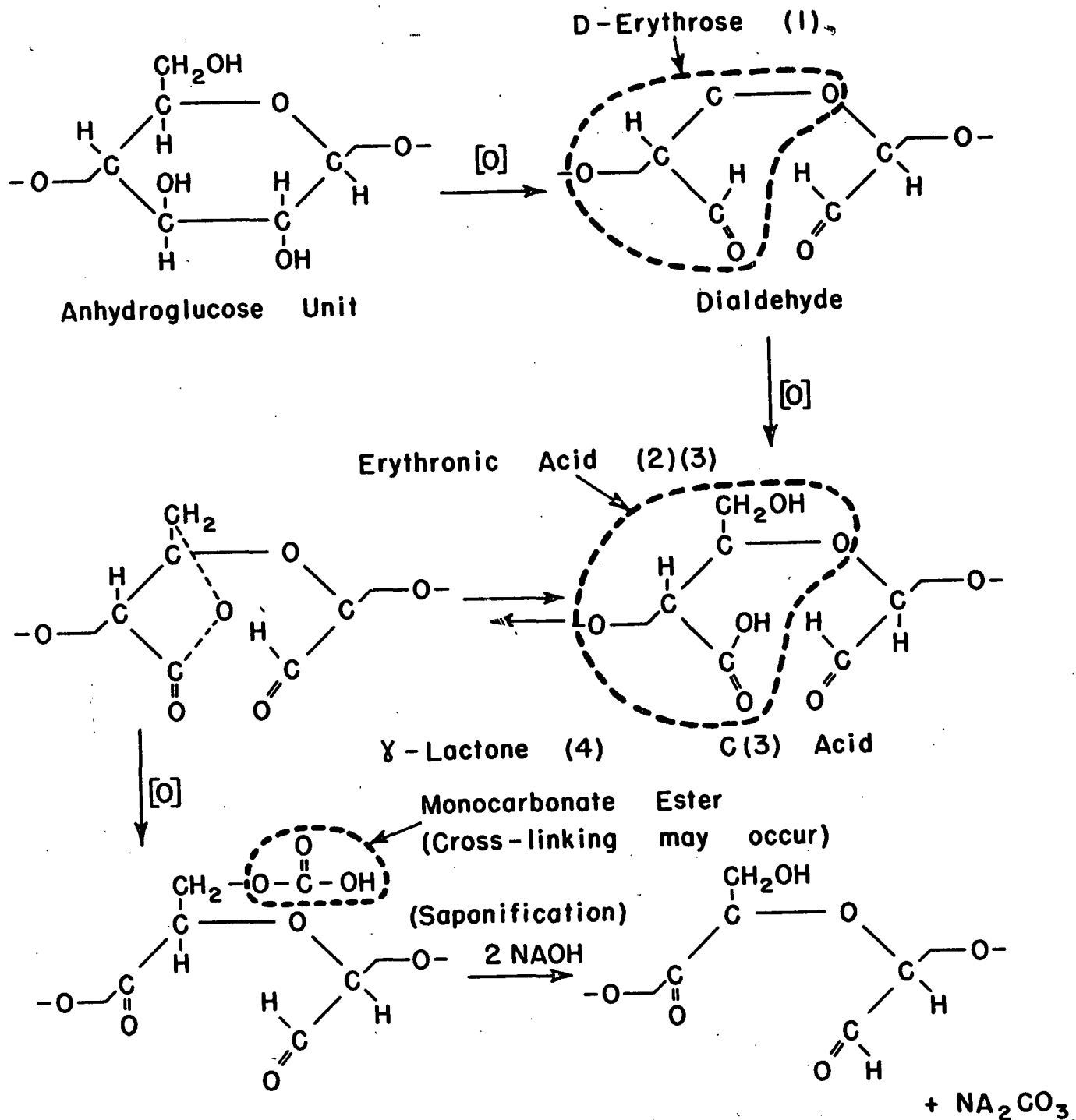
(The specific activity of the SCD found in the acid chlorine-oxidized celluloses may be easily explained on the basis of various combinations of the structures hypothesized above--see Table II.)

#### RELATED MISCELLANEOUS STUDIES

##### COMPARISON OF UNOXIDIZED AND OXIDIZED COTTON AND BACTERIAL CELLULOSES

As has been noted earlier, bacterial cellulose responds to acid chlorine oxidation in about the same manner as does cotton; differences are listed below.

- (1) The yield of oxidized bacterial cellulose is higher than that of oxidized cotton at comparable levels of oxidation, e.g., 85.9% as



NOTE: Monocarbonate ester may be formed via C(3)- or C(6)-carboxyl; consider above as via C(3)-carboxyl.

Figure 7. Monocarbonate Ester Hypothesis ( $\gamma$ -Lactone Intermediate)

opposed to 82.0% at an oxidation level of 1.45 equiv. hypochlorous acid per mole of anhydroglucose.

- (2) The content of SCD in oxidized bacterial cellulose is lower than that in cotton oxidized to a comparable level of oxidation, e.g., 0.19 as opposed to 0.25 mole carbon dioxide per 100 moles of anhydroglucose at an oxidation level of 1.45 equiv. HOCl/mole of anhydroglucose.
- (3) The neutral sugar oxidation products were found to be the same in the two celluloses with the exception that xylose, as well as arabinose and erythrose, was noted in the hydrolyzate of oxidized bacterial cellulose. [This finding may have been due to a slightly different technique used for the analysis of the hydrolyzate of the oxidized bacterial cellulose (see page 48).]

It cannot be concluded on the basis of these results whether cotton and bacterial celluloses are markedly different in their physical and/or chemical character. Therefore, several physical and chemical comparisons were made in order to ascertain the soundness of extending to both celluloses interpretations made on the basis of results obtained for only one type. These comparisons were patterned after those made by Hibbert and Barsha (22), Greathouse and Shirk (23), and Schramm and Hestrin (24). The findings of these men support the conclusion that the chemical properties of bacterial and cotton celluloses are essentially identical.

### Physical Properties

#### X-Ray Studies

X-ray analyses were made on both oxidized and unoxidized cotton and

bacterial celluloses (see Appendix VI, page 105, for diffraction patterns). In each case, the major reinforcement angles were found at  $2\theta$  values of  $14.5^\circ$ ,  $16.8^\circ$ , and  $22.5^\circ$ . Crystallinity, according to Ant-Wuorinen (44) and Hermans and Weidinger as interpreted by Ant-Wuorinen (44) was calculated from the X-ray data for each sample studied.

The degree of crystallinity (DCr) according to Hermans and Weidinger is interpreted as an index of the per cent of crystallinity of cellulose. The crystalline index (CrI) of Ant-Wuorinen is interpreted as an index of the degree of order of the crystalline region of cellulose (see Table IV for data).

The cotton and bacterial celluloses were found to have about the same per cent crystallinity and crystalline order; the bacterial cellulose was slightly higher for both. A minor increase in the per cent crystallinity and crystalline order was noted for each sample after oxidation; this was probably due to a preferential removal of the amorphous and less ordered crystalline regions of the cellulose by the oxidation.

#### Infrared Studies

Infrared spectra were obtained for oxidized and unoxidized cotton and bacterial celluloses. On comparison, these spectra were found to be greatly similar, with the exception that at 13.3 microns ( $750 \text{ cm.}^{-1}$ ), the bacterial celluloses had an absorption band not observed in the spectrum for cotton. This band may result from absorption caused by structural configurations (57) that are found in many amino acids (58) (see Appendix VII, page 106). Variations in absorption intensity and definition were attributed to differences

in the preparation of the various samples. The infrared spectra of oxidized cotton and bacterial celluloses had an absorption band at 5.75 microns ( $1740 \text{ cm.}^{-1}$ ). The new band found in the oxidized celluloses may be attributed to either aldehydic, ketonic, lactone, or ester carbonyl stretch vibrations (57).

TABLE IV

## CELLULOSE CRYSTALLINITIES

Sample Identification	Sample Density, g./cc.	Measure of Crystallinity	
		Degree of Crystallinity (DCh) <sup>a</sup>	Crystalline Index (CrI) <sup>b</sup>
(1) Purified unoxidized cotton	1.40 ± 0.02	0.84	0.78
(2) Oxidized cotton sample, oxidation II	1.40 ± 0.02	0.85	0.81
(3) Unoxidized cellulose-1-C <sup>14</sup> , batch 3	1.40 ± 0.02	0.86	0.79
(4) Oxidized cellulose-1-C <sup>14</sup> , oxidation VIII	1.40 ± 0.02	0.87	0.82
(5) Unoxidized cellulose-6-C <sup>14</sup> , batch 4	1.40 ± 0.02	0.87	0.81
(6) Oxidized cellulose-6-C <sup>14</sup> , oxidation IX	1.40 ± 0.02	0.87	0.82

<sup>a</sup> Degree of crystallinity according to Hermans and Weidinger as interpreted by Ant-Wuorinen (44).

<sup>b</sup> Ant-Wuorinen's crystalline index (44).

## Chemical Properties

### Glucose Content

The hydrolyzates of purified, unoxidized cotton and bacterial celluloses were found to contain largely glucose with some oligosaccharides when chromatographed in butanol, pyridine, and water developer (10:3:3) and sprayed with p-anisidine spray reagent. The bacterial cellulose hydrolyzate was also found to contain a trace of an amino acid (unidentified); this was chromatographically detected using the above developer and ninhydrin spray reagent. The cotton did not contain detectable amino acids.

The glucose in the bacterial cellulose hydrolyzate was determined according to the quantitative method of Piper and Bernardin (37); the amount found represented 87% by weight of the original purified cellulose. In the light of the quantity of oligosaccharides remaining in the hydrolyzate, this value compares favorably with values previously reported for purified cotton, e.g., the 93% reported by Schramm and Hestrin (24); a similar method was used for their glucose determination. From this result, it is apparent that bacterial cellulose is composed primarily of glucose linked by  $\beta$  1-4 linkages. The  $\beta$ -linkage has been checked by hydrolysis with emulsin.

### Cellulose Nitration

Bacterial celluloses were nitrated according to the procedure of Bennett and Timell (46), and the degree of nitration was determined by the method of Timell and Purves (47). The yields for these cellulose nitrates were compared with yield and degree of nitration data obtained for cotton using the same experimental procedures (see Table V).

TABLE V

## CELLULOSE NITRATION

Sample Identification	Cellulose Nitrate Yield, %	Nitrogen in Cellulose Nitrate, %
(1) Purified cotton linters, nitrated by Major (59)	98-99	13.9
(2) Unoxidized, purified cellulose-1-C <sup>14</sup> (batch 3)	95.8	13.89
(3) Unoxidized, purified cellulose-6-C <sup>14</sup> (batch 4)	95.1	13.94

The results show that the bacterial celluloses gave lower yields of cellulose nitrate than are normally obtained with cotton. The lower yields of nitrated bacterial cellulose probably stemmed from high mechanical loss. In spite of the fact that the bacterial cellulose nitrate yields were 5% below theoretical, the results obtained, in view of the high degree of nitration, are quite valuable, for they show rather conclusively that the bacterial cellulose is at least 95% cellulosic.

## Alkali Sensitivity

Alkali sensitivity of acid chlorine-oxidized cotton cellulose has long been recognized (60). Thus, as another means of noting the similarity of cotton and bacterial celluloses, a study was made to determine the alkali sensitivity of acid chlorine-oxidized bacterial cellulose. Alkali sensitivity was evaluated by making a comparison of D.P. values (viscometric, weight average D.P.) obtained from cellulose nitrate in acetone and those obtained from the raw cellulose in cupriethylenediamine.

The unoxidized bacterial cellulose was assumed to be free of alkali-sensitive linkages and, consequently, to have the same D.P., according to both methods. This assumption was made in order to arrive at defined constants that were adequate for the comparison of the two methods used (see page 30). It was on the basis of the constants defined above and the assumption that alkali-labile linkages are not degraded by nitration that the alkaline and nonalkaline D.P. values were compared. These comparisons showed that oxidized bacterial cellulose is alkali sensitive and, consequently, is similar to cotton in this respect (see Table VI).

TABLE VI

## ACID CHLORINE-OXIDIZED BACTERIAL CELLULOSE ALKALI SENSITIVITY STUDY

Sample Identification	Oxidant Consumption, equiv. HOCl/mole anhydroglucose	D.P. (cellulose nitrate in acetone)	D.P. (cellulose in CED)	D.P.	Alkali-Labile Linkages/1000 Anhydroglucose Units	SCD, mole CO <sub>2</sub> /1000 moles anhydroglucose
(1) Unoxidized cellulose-1-C <sup>14</sup> (Batch 3)	--	3370	3370	--	--	--
(2) Unoxidized cellulose-6-C <sup>14</sup> (Batch 4)	--	2750	2750	--	--	--
(3) Oxidized cellulose-1-C <sup>14</sup> (Oxidation VI)	0.29	1090	622	468	0.85	1.0
(4) Oxidized cellulose-1-C <sup>14</sup> (Oxidation VIII)	0.82	813	422	391	0.95	1.5

In this study of the alkali lability of acid chlorine-oxidized bacterial cellulose, the primary function was the comparison of oxidized cotton and

bacterial celluloses. However, in the course of this work it became apparent, as discussed below, that the findings made could be combined with previously obtained results to shed light on the relationship between SCD, carbonic acid ester linkages (carbon-1), and alkali sensitivity in oxidized cellulose.

The sample from oxidation VI had about 0.85 alkali-labile linkages per 1000 anhydroglucose molecules and the sample from oxidation VII had about 0.95. These oxidized celluloses had 1.0 and 1.5 mole of SCD per 1000 moles of anhydroglucose, respectively; therefore, the structures in acid chlorine-oxidized cellulose that liberate SCD are not exclusively associated with alkali-sensitive linkages, i.e., groupings capable of cellulose chain cleavage, for example, the hypothesized carbonic acid ester linkage. It can be further stated on the basis of data already presented that the carbonic acid ester grouping could be responsible for only a small percentage of the alkali-labile structures causing chain cleavage in acid chlorine-oxidized cellulose. The most likely major causes of alkali lability in this type of oxidized cellulose are: (1) the instability of aldehydic and ketonic structures (61) and (2) the saponification of gluconic acid esters, with the former being considerably more important than the latter. (Henderson (15) reported the chromatographic identification of 2-keto-gluconic acid in the hydrolyzate of acid chlorine-oxidized cotton.)

#### Nitrogen in Cellulose

The nitrogen content of oxidized and unoxidized cotton and bacterial celluloses was determined according to the colorimetric method of Niss (49) (see Appendix V, page 108). The results are listed in Table VII.

TABLE VII

NITROGEN CONTENT OF PURIFIED, OXIDIZED AND UNOXIDIZED  
COTTON AND BACTERIAL CELLULOSES

Sample Identification	Nitrogen in Cellulose, %
(1) Purified cotton	0.03
(2) Oxidized cotton (oxidation X)	0.02
(3) Purified bacterial cellulose [cellulose-1-C <sup>14</sup> (batch 3)]	0.20
(4) Oxidized bacterial cellulose [cellulose-1-C <sup>14</sup> (oxidation VI)]	0.14
(5) Oxidized bacterial cellulose [cellulose-1-C <sup>14</sup> (oxidation VII)]	0.07
(6) Oxidized bacterial cellulose [cellulose-1-C <sup>14</sup> (oxidation VIII)]	0.07
(7) Purified bacterial cellulose [cellulose-6-C <sup>14</sup> (batch 4)]	0.07
(8) Oxidized bacterial cellulose [cellulose-6-C <sup>14</sup> (oxidation IX)]	0.04

The per cent of nitrogen in the bacterial celluloses was found to be greater than that in cotton. This is easy to understand when the origin of the bacterial cellulose is considered. Proteinaceous material probably accounts for the bulk of the nitrogen in both types of cellulose. The nitrogen and its source in the purified bacterial cellulose may be responsible for as much as 1.4% noncellulosic material. This is more than six times the maximum amount of proteinaceous material in the purified cotton; however, for the purpose of this study, the difference is not felt to constitute a significant dissimilarity in the celluloses.

THE QUANTITY AND RADIOACTIVITY OF "URONIC ACID" CARBON DIOXIDE IN  
LABELED OXIDIZED AND UNOXIDIZED BACTERIAL CELLULOSES

Kaverzneva, et al., (2, 3) have stated that the reason that the uronic acid content of acid chlorine-oxidized cellulose, as determined by a modification of the Lefèvre and Tollens method, is higher than the total carboxyl (calcium acetate method) is partly due to the SCD in the oxidized cellulose. These workers have also drawn various conclusions about acid chlorine-oxidized cellulose (3) based on very small changes in the uronic acid content as determined above. It was the purpose of the following investigation to: (1) evaluate the contribution made to the "uronic acid" carbon dioxide<sup>a</sup> by the SCD in oxidized cellulose and (2) to determine just how meaningful the uronic acid analysis is when applied to celluloses containing small amounts of "uronic acid" carbon dioxide. Labeled bacterial celluloses were used in these studies.

The uronic acid content was determined for unoxidized and oxidized C(1)- and C(6)-labeled bacterial celluloses. The specific activity of the carbon dioxide evolved was determined for each sample. Conclusions drawn from these data were made on the assumption that if only glucuronic acid contributes to the "uronic acid" carbon dioxide liberated from the labeled celluloses, the specific activity of this carbon dioxide would be the same as that of the C(6)-position in the cellulose. In other words, the isotope effect is small. (See Table VIII for summary of data.)

The "uronic acid" carbon dioxide was found to be about the same in

<sup>a</sup> Carbon dioxide liberated from cellulose by application of any adaptation of Lefèvre and Tollens' uronic acid method.

unoxidized cotton and bacterial celluloses (items 1, 2, and 3, Table VIII) as well as in cotton and bacterial celluloses oxidized to corresponding levels of oxidation (items 7 and 10, Table VIII). This finding gives a basis for interpreting results obtained for bacterial cellulose in terms of cotton cellulose treated in a similar manner.

The saponification of an oxidized cellulose does not affect a change in its "uronic acid" carbon dioxide, item 4 in Table VIII. This is true for low levels of oxidation; however, for overly degraded celluloses, the saponification treatment (0.025N sodium hydroxide solution) apparently causes a selective removal of highly oxidized regions of the cellulose, item 5 in Table VIII. This selective removal of part of the cellulose by the alkali treatment was noted in two ways: (1) Some oxidized celluloses underwent a loss in weight of up to 5% due to the saponification treatment; the alkaline extraction was also evidenced, without checking the weight loss, by the yellow color of the caustic solution used for saponification; (2) The quantity and specific activity of the "uronic acid" carbon dioxide was lowered in the highly oxidized celluloses. If the SCD were responsible for this drop in "uronic acid" carbon dioxide content, the specific activity of that remaining would have increased rather than decreased, this being due to their respective specific activities.

The specific activity values of the "uronic acid" carbon dioxide from cellulose-1-C<sup>14</sup> at various levels of oxidation ranged from about 5000 down to 1700 c.p.m./mg. C. Therefore, the "uronic acid" carbon dioxide did not have the C(6)-position as its only point of origin, because the specific activity

TABLE VIII

## URONIC ACID DETERMINATIONS

Sample Identification	"Uronic Acid" Carbon Dioxide Evolved, %	"Uronic Anhydride", %	Specific Activity of "Uronic Acid" Carbon Dioxide, c.p.m./mg. C
(1) Unoxidized bacterial cellulose: Cellulose-1-C <sup>14</sup> (Batch 3)	0.10	0.40	4968
(2) Unoxidized bacterial cellulose Cellulose-6-C <sup>14</sup> (Batch 4)	0.13	0.52	1109
(3) Unoxidized purified cotton <sup>a</sup>	0.07	0.27	--
(4) Oxidized bacterial cellulose Cellulose-1-C <sup>14</sup> (Oxidation VI, 0.29 equiv. HOCl/ mole anhydroglucose)	Before saponification 0.20 After saponification 0.20	0.80 0.80	3698 3609
(5) Oxidized bacterial cellulose Cellulose-1-C <sup>14</sup> (Oxidation VII, 0.82 equiv. HOCl/ mole anhydroglucose)	Before saponification 0.41 After saponification 0.38	1.64 1.52	3199 2925
(6) Oxidized bacterial cellulose Cellulose-1-C <sup>14</sup> (Oxidation XII, 0.91 equiv. HOCl/ mole anhydroglucose)	0.43	1.72	2825

<sup>a</sup> Data reported by Kaverzneva, *et al.* (3).

TABLE VIII (Continued)

Sample Identification	"Uronic Acid" Carbon Dioxide Evolved, %	"Uronic Anhydride", %	Specific Activity of "Uronic Acid" Carbon Dioxide, c.p.m./mg. C
(7) Oxidized bacterial cellulose  Cellulose-1-C <sup>14</sup> (Oxidation VIII, 1.4 equiv. HOCl/ mole anhydroglucose)	0.62	2.48	1698
(8) Oxidized bacterial cellulose  Cellulose-6-C <sup>14</sup> (Oxidation IX, 1.4 equiv. HOCl/ mole anhydroglucose)	0.57	2.28	1790
(9) Oxidized cotton <sup>a</sup>  (0.26 equiv. HOCl/ mole anhydroglucose)	0.29	1.14	--
(10) Oxidized cotton <sup>a</sup>  (0.89 equiv. HOCl/ mole anhydroglucose)	0.52	2.08	--

<sup>a</sup> Data reported by Kaverzneva, et al. (3).

Note: (1) All the values reported above that were obtained in this current research are based on duplicate determinations with a deviation from the average ranging from  $\pm 1$  to 5%.

(2) "Uronic acid" carbon dioxide values are low by as much as 7% due to error in the modified Institute Method 25 that was used.

in position 6 of the cellulose-1-C<sup>14</sup> used was only 362 c.p.m./mg. C. The specific activity decreased as the level of oxidation and the per cent of "uronic acid" carbon dioxide in the cellulose increased (see Figure 8). This shows a change in the fraction of the carbon dioxide coming from the various positions in the cellulose as a function of oxidation level.

About 30% of the "uronic acid" carbon dioxide from the unoxidized celluloses (items 1 and 2, Table VIII) was derived from position 1 and as much as 4% may have come from position 6. The basis for these estimates was the specific activity of positions 1, 2-5 (as a composite), and 6 in the labeled celluloses (see Table II). The foregoing suggests some decomposition of the glucose hydrolyzed from the cellulose, by the 12% hydrochloric acid, with the liberation of the C(1)-position as carbon dioxide. On analysis of items 7 and 8 in Table VIII, it is apparent that no more than about 22% of the "uronic acid" carbon dioxide from these oxidized celluloses came from the C(6)-position and no more than 12% came from the C(1)-position.<sup>a</sup> Thus, positions 2 and 3 of the cellulose undoubtedly contribute substantially to the "uronic acid" carbon dioxide from this type of oxidized cellulose at this level of oxidation. The carbon dioxide originating at positions 2 and 3 may be derived from erythronic or glyoxylic acids or from some type of ketonic or aldehydic structure that is unstable under the drastic conditions of the uronic acid determination.

Neither erythronic nor glyoxylic acid has been shown to be present in acid chlorine-oxidized cellulose. These materials have been found in acid chlorine-oxidized amylopectin (55) and, therefore, may be present in oxidized

<sup>a</sup> The limitation of the contribution of the C(6)-position was based on the analysis of only one sample of oxidized cellulose-6-C<sup>14</sup>; thus, it must be considered preliminary.

cellulose. The glyoxylic acid is known to give off some carbon dioxide (1.86%) on treatment with 12% hydrochloric acid at 137°C. for 23 hours (62), but the amount liberated by erythronic acid has not been determined. The "uronic acid" carbon dioxide from erythronic acid presumably would not greatly exceed the 8% given off by gluconic acid (62).

It is improbable that the amount of gluconic, erythronic, or glyoxylic acid units present in this type of oxidized cellulose could account for all of the "uronic acid" carbon dioxide not derived from glucuronic acid. More studies of the type initiated in this work will be required in order to obtain a clear picture of the pseudouronic acid carbon dioxide liberated from acid chlorine-oxidized cellulose by the uronic acid determination.

From the preceding data, the following conclusions may be drawn:

- (1) The saponifiable carbon dioxide in acid chlorine-oxidized celluloses is not responsible for a significant amount of pseudouronic acid carbon dioxide in the oxidized celluloses.
- (2) The application of the uronic acid determination to either unoxidized or acid chlorine-oxidized celluloses (over the range examined) is meaningless when interpreted in terms of C(6)-carboxyl (uronic anhydride), for a large percentage of the "uronic acid" carbon dioxide does not originate at the C(6)-position. Therefore, in light of this and other findings made in this research, it is obvious that small changes in the uronic acid content of acid chlorine-oxidized celluloses (according to any adaptation of the Lefèvre and Tollens method) defy precise interpretation.

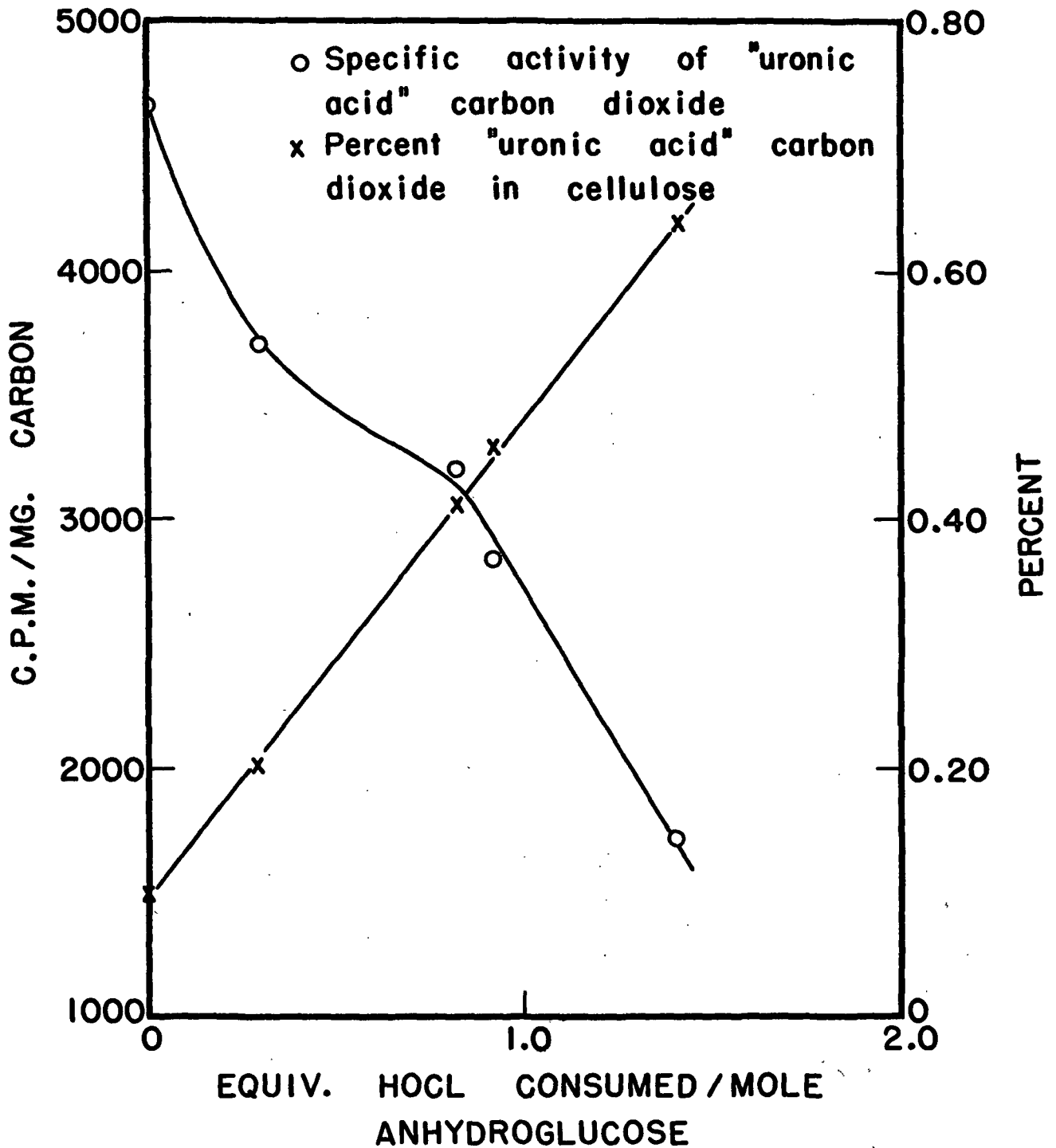


Figure 8. "Uronic Acid" Carbon Dioxide from Cellulose-1-C<sup>14</sup> (Batch 3) at Various Oxidation Levels.

EXAMINATION OF CARBON DIOXIDE EVOLVED DURING THE  
OXIDATION OF LABELED BACTERIAL CELLULOSE

The specific activity of the carbon dioxide evolved during oxidations VI through IX (about 1350 c.p.m./mg. C for the oxidations of cellulose-1-C<sup>14</sup>) was impossible to explain by either random oxidation of the cellulose or preferential attack at positions 1 and/or 6 (see Tables II and III). Although preferential attack of the intermediate positions presented a possibility, it was felt that such an explanation was not well supported, for there were no three-carbon fragments detected in the oxidized celluloses, based on the chromatographic analysis of the cellulose hydrolyzates. Therefore, other alternatives were explored; these studies accomplished the following:

- (1) The presence of inert organic material (about 4%) in the labeled bacterial celluloses was established. This was done by making a comparison of the specific activity of the cellulose with that of potassium gluconate prepared from the cellulose. The cellulose was found to have a lower specific activity (see Table IX). This inert organic material was concluded to be primarily solvent that was included during the solvent drying of the purified cellulose (63). The included solvent was principally diethyl ether with perhaps some acetone and ethyl alcohol. These solvents were found to liberate some carbon dioxide due to the oxidative action of aqueous chlorine at pH 4.5.
- (2) The acetate buffer system used in the cellulose oxidations was found to be oxidized by aqueous chlorine at pH 4.5; some carbon dioxide was evolved.

TABLE IX

INERT ( $C^{14}$ -FREE) NONCELLULOSIC MATERIAL  
IN LABELED BACTERIAL CELLULOSE

	Specific Activity of Cellulose, c.p.m./mg. C	Specific Activity Potassium Gluconate, c.p.m./mg. C	Inert Carbon in Cellulose % <sup>a</sup>
Run 1	1841	1920	4.0
Run 2	1857	1920	3.3
Run 3	1853	1920	<u>3.4</u>
Average			3.6

<sup>a</sup> Calculated from difference in the two specific activities.

The solvent was removed from a sample of solvent-dried, purified cellulose- $l$ - $C^{14}$  (batch 3) by boiling it in water for three hours. The cellulose was filtered from the water and was washed with boiling water. This cellulose was oxidized in a phosphate buffer system (oxidation XII). Carbon dioxide with a specific activity of 2841 c.p.m./mg. C was evolved from the oxidation mixture. This result showed the effect that the included solvent and acetate buffer system had had in previous oxidations. The specific activity of the carbon dioxide evolved during this oxidation was very near that of the cellulose- $l$ - $C^{14}$  (2914 c.p.m./mg. C) that was oxidized.

It is believed that the bulk of the carbon dioxide evolved during this apparently random oxidation was derived from subsequent oxidation of solubilized fragments of the cellulose.

SUMMARY

(1) In confirmation of previous work (2), SCD was shown to be present in acid chlorine-oxidized cellulose. Over a limited range of oxidation, the amount of SCD in an oxidized cellulose was found to be dependent on the level of oxidation. The SCD content of oxidized cotton leveled off at 0.37 mole carbon dioxide per 100 moles of anhydroglucose; this corresponded to an oxidation level of about 2.5 equiv. hypochlorous acid per mole of anhydroglucose. No leveling-off point was reached for bacterial cellulose; the highest oxidation level studied was about 2.7 equiv. hypochlorous acid per mole of anhydroglucose.

(2) Arabinose and erythrose were chromatographically identified as oxidation products in the hydrolyzates of oxidized cotton and bacterial celluloses. In addition, xylose was detected in the hydrolyzate of an oxidized bacterial cellulose. The identification of arabinose qualitatively confirmed the finding of Henderson (15). However, quantitative confirmation could not be made, for no more than 0.2% arabinose by weight could be found in the oxidized celluloses examined; Henderson reported that 1% was present in his oxidized cellulose. This discrepancy remains unexplained.

The origins of the chromatographically identified neutral oxidation products are hypothesized as follows.

- (a) Arabinose is derived from end group oxidation and/or the formation and breakdown of carbonic acid ester groupings at the C(1)-position.
- (b) Erythrose is formed by the oxidative cleavage of the C(2)-C(3) glycol grouping of the anhydroglucose unit.

(c) Xylose is formed by the decarboxylation of glucuronic acid groups; this decarboxylation may occur either during the oxidation or during the hydrolysis of the oxidized cellulose.

(3) A relationship between SCD and arabinose was obtained (1.7:1; SCD: arabinose, on a molar basis) for an oxidized cotton cellulose. Interpretation of this ratio did not clearly add to the question of the validity of the carbonic acid ester hypothesis. Hence, this indirect approach to the study of the hypothesis was dropped due to its lack of promise.

(4) Gram-lot quantities (about 35 grams per batch) of purified, specifically labeled bacterial celluloses were successfully prepared by scaling up published methods. Cellulose-1-C<sup>14</sup> was prepared with 80% of the carbon-14 in position 1; however, the preparation of cellulose-6-C<sup>14</sup> was not as successful, for it was only 59% specific in position 6. The low specificity of label in cellulose-6-C<sup>14</sup> was not caused by the method but by the technique of preparation; the nutrient media was contaminated with unknown bacteria. The labeled bacterial celluloses were prepared in yields of 35 to 40% based on the glucose supplied and 16 to 21% based on the carbon-14 supplied.

(5) Comparison of physical and chemical properties of unoxidized and acid chlorine-oxidized cotton and bacterial celluloses was made. From these comparisons, it was evident that qualitative interchangeable usage of the two celluloses may be made. Therefore, the usefulness of labeled bacterial cellulose as a tool for cellulose oxidation research would not be impaired, i.e., so long as the following assumption held:

(a) the isotope effect is negligible,

- (b) intrapolymer distribution of labeled anhydroglucose units is random, and
- (c) the portions of the polymer that are accessible to oxidation are randomly oxidized.

(6) The per cent of crystallinity and degree of order of the crystalline regions of a cellulose are slightly increased by acid chlorine oxidation. This may be interpreted as a slightly preferential oxidative removal of the amorphous and less ordered crystalline regions of the cellulose.

(7) The infrared spectra of acid chlorine-oxidized cotton and bacterial celluloses showed an absorption band at  $1740 \text{ cm.}^{-1}$  (5.75 microns); this band was not present in the spectra of unoxidized cellulose. The new band in the oxidized cellulose may be attributed to either aldehydic, ketonic, lactone, or ester carbonyl stretch vibrations.

(8) Acid chlorine-oxidized bacterial cellulose was found to contain alkali-sensitive linkages. This was determined by the comparison of the alkaline D.P. (cellulose in cupriethylenediamine) and the nonalkaline D.P. (cellulose nitrate in acetone) of the oxidized celluloses. On a molar basis, the quantity of SCD in these oxidized celluloses was greater than the number of alkali-labile linkages. Therefore, the structures that permit liberation of SCD are obviously not all incorporated into the cellulose as groupings that are capable of cellulose chain cleavage, e.g., the hypothesized carbonic acid ester linkage.

(9) Analysis of the results that were obtained from the determination of the specific activity of the SCD from oxidized cellulose- $1\text{-C}^{14}$  and

cellulose-6-C<sup>14</sup> showed conclusively that the SCD in acid chlorine-oxidized cellulose is not derived primarily from position 1 of the anhydroglucose molecule. For the five oxidized cellulose-1-C<sup>14</sup> samples analyzed, the maximum contribution to the SCD resulting from position 1 ranged from 14 to 21%. In reality, the contribution of position 1 could never have reached the maximum, for all the other positions of the anhydroglucose molecule had some label.

(10) The physical adsorption or inclusion of carbon dioxide in cellulose during its oxidation was considered as a possible source of SCD in acid chlorine-oxidized cellulose. These possibilities were experimentally investigated, and on the basis of the evidence obtained, it was clear that neither adsorption nor inclusion of carbon dioxide is responsible for any of the SCD in oxidized cellulose.

(11) The saponification of mildly oxidized cellulose (up to 0.3 equiv. hypochlorous acid per mole of anhydroglucose) did not effect a change in its "uronic acid" carbon dioxide. However, for overly degraded cellulose, the saponification treatment caused a selective removal of highly oxidized regions of the cellulose. This caused a loss in weight of the cellulose and a lowering of the per cent and specific activity of the "uronic acid" carbon dioxide in the residue. If the SCD were responsible for this drop in "uronic acid" carbon dioxide content, the specific activity of that remaining would have increased rather than decreased, this being due to their respective specific activities.

(12) By the determination of the specific activity of the "uronic acid"

carbon dioxide from oxidized and unoxidized cellulose-1-C<sup>14</sup> and cellulose-6-C<sup>14</sup>, it was found that a large portion of the carbon dioxide so evolved did not stem from position 6. The specific activity of the "uronic acid" carbon dioxide from cellulose-1-C<sup>14</sup> decreased as the level of oxidation and the per cent of "uronic acid" carbon dioxide increased. This indicates a change in the fraction of the carbon dioxide coming from the various positions in the cellulose as a function of oxidation level.

(13) The quantity and specific activity of the carbon dioxide evolved by the oxidative degradation of labeled bacterial celluloses were determined in an attempt to gain an insight into any preferential oxidative attack of the oxidant on cellulose. All results except that for oxidation XII were in error due to inert carbon dioxide derived from the oxidation of solvent that was included in the cellulose during solvent drying and from the oxidation of the acetate buffer system that was used to buffer these oxidations. Oxidation XII was carried out with solvent-free cellulose in a phosphate buffer; this combination gave rise to a correct result. The specific activity of the carbon dioxide evolved during this oxidation (2841 c.p.m./mg. C) closely approaches that of the cellulose-1-C<sup>14</sup> that was oxidized (2914 c.p.m./mg. C). This result supports a random oxidation.

## CONCLUSIONS

(1) The SCD in acid chlorine-oxidized cellulose is not derived from carbon in a single position in the anhydroglucose molecule. Position 1 can be the source of not more, and probably less than 21% of the SCD. Therefore, the probability of the existence of the hypothesized carbonic acid ester linkage is greatly reduced.

(2) Some chemical structure or structures, not involving position 1, are responsible for the SCD that cannot be accounted for by the carbonic acid ester linkage.

(3) The carbonic acid ester linkage does not play a significant role in the alkali sensitivity of acid chlorine-oxidized cellulose.

(4) The application of the uronic acid determination to unoxidized or to acid chlorine-oxidized cellulose is meaningless when interpreted in terms of C(6)-carboxyl, for much of the "uronic acid" carbon dioxide does not originate at the C(6)-position. The fraction of the "uronic acid" carbon dioxide coming from the various positions in a cellulose changes as a function of the oxidation level of the cellulose. Hence, small changes in the uronic acid content of acid chlorine-oxidized celluloses defy precise interpretation.

(5) The SCD in acid chlorine-oxidized cellulose is not responsible for a significant amount of the pseudouronic acid carbon dioxide that is present in these celluloses.

(6) The net interpretation of all the present results is that the acid

chlorine oxidation of cellulose is nonspecific, i.e., the structure of acid chlorine-oxidized cellulose cannot be precisely formulated on the basis of a single primary point of oxidation.

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LITERATURE CITED

1. Birtwell, C., Clibbens, D. A., and Ridge, B. P., J. Textile Inst. 16:T13(1925).
2. Kaverzneva, E. D., Izvest. Akad. Nauk S.S.S.R. 6:791(1951); Communication to the XIIIth International Congress for Pure and Applied Chemistry, Stockholm, 1953.
3. Kaverzneva, E. D., Ivanov, V. I., and Salova, A. S., Izvest. Akad. Nauk S.S.S.R., Otdel. Khim. Nauk, no. 4:751-62(1952).
4. Kaverzneva, E. D., Ivanov, V. I., and Salova, A. S., Izvest. Akad. Nauk S.S.S.R., Otdel. Khim. Nauk, no. 1:185-9(1952); C.A. 46:7323.
5. Kaverzneva, E. D., Ivanov, V. I., and Salova, A. S., Bumazh. Prom. 28, no. 7:6-11(1953); C.A. 47:12799.
6. Staudinger, H., Ber. 72:1709(1939).
7. Staudinger, H., and Sohn, A. W., J. prakt. Chem. 155:177-84(1940).
8. Clibbens, D. A., and Ridge, B. P., J. Textile Inst. 18:T135(1927).
9. Hisey, W. O., and Koon, C. M., Paper Trade J. 103, no. 6:36(1936).
10. Weber, O. H., and Husemann, E., J. prakt. Chem. 161:1-30(1942); C.A. 37:4893.
11. Meller, A., Revs. Pure and Appl. Chem. (Australia) 6, no. 1:40-60 (1956); C.A. 50:10397.
12. Whitney, R. P. Composition of aqueous chlorine systems. In Wise and Jahn's Wood Chemistry. Vol. 2, p. 1038, New York, Reinhold, 1952.
13. Salova, A. S., Zhur. Anal. Khim. 4:354(1949); C.A. 44:4249.
14. Rogovin, Z. A., Kozlova, Yu. S., and Derevitskaya, V. A., Khim. Nauka i Prom. 2:264(1957); C.A. 51:18580.
15. Henderson, J. T., J. Am. Chem. Soc. 79:5304-8(1957).
16. Greathouse, G. A., Science 117, no. 3047:553-4(1953).
17. Shafizadah, F., and Wolfrom, M. L., J. Am. Chem. Soc. 77:5182-3(1955).
18. Minor, F. W., Greathouse, G. A., Shirk, H. G., Schwartz, A. M., and Harris, M., J. Am. Chem. Soc. 76:1658-61(1954).

19. Greathouse, G. A., J. Am. Chem. Soc. 79:4505-7(1957).
20. Hestrin, S. Personal communication, 1957.
21. Greathouse, G. A. Personal communication, 1957.
22. Barsha, J., and Hibbert, H., Can. J. Research 10:170-9(1934).
23. Greathouse, G. A., and Shirk, H. G., Anal. Chem. 24:1774-5(1952).
24. Schramm, M., and Hestrin, S., Biochem. J. 56:163-6(1954).
25. Bonner, W. A., and Collins, C. J., J. Am. Chem. Soc. 75:2308-11(1953).
26. Evans, E. A., and Huston, J. L., J. Chem. Phys. 19:1214(1951).
27. Lefèvre, K. U., and Tollens, B., Ber. 40:4513(1907).
28. Browning, B. L. Determination of polyuronide carboxyl groups. In Wise and Jahn's Wood Chemistry. Vol. 2. p. 1206, New York, Reinhold, 1952.
29. Nevell, T. P., J. Textile Inst. 39:T118-30(1948).
30. White, J. F., Am. Dyestuff Repr. 31:485(1942).
31. Van Slyke, D. D., and Neill, J. M., J. Biol. Chem. 61:523(1924).
32. Van Slyke, D. D., and Folch, J. J., J. Biol. Chem. 136, no. 1:509-41(1940).
33. Moore, S., and Link, K. P., J. Biol. Chem. 133:293(1940).
34. Van Slyke, D. D., Steele, R., and Plazin, J., J. Biol. Chem. 192, no. 2: 769-805(1951).
35. Eisenberg, F., Jr., J. Am. Chem. Soc. 76:5152(1954).
36. Bernstein, W., and Ballentine, R., Rev. Sci. Instr. 21:158-62(1950).
37. Piper, C., and Bernardin, L. J., Tappi 41, no. 1:16-18(1958).
38. Timell, T. E., Gloudemans, C. P., Jr., and Currie, A. L., Anal. Chem. 28, no. 12:1916-20(1956).
39. Hough, L., Jones, J. K. N., and Wadman, W. H., J. Chem. Soc., 1950:1702-6.
40. Lemieux, R. V., and Bauer, H. F., Anal. Chem. 26:920(1954).
41. Hirs, C. H. W., Moors, S., and Stein, W. H., J. Biol. Chem. 195:669-83 (1951).

42. Abdel-Akher, M., and Smith, F., J. Am. Chem. Soc. 73:5859-60(1951).
43. Johanson, R., Nature 172:956-7(1953).
44. Ant-Wuorinen, O., Paperi ja Puu 37, no. 8:335-69(1955).
45. Holtzer, A. M., Benoit, H., and Doty, P., J. Phys. Chem. 58:624(1954).
46. Bennett, C. F., and Timell, T. E., Svensk Papperstidn. 55, no. 8:281-5 (1955).
47. Timell, T. E., and Purves, C. B., Svensk Papperstidn. 54, no. 9:303-32 (1951).
48. Browning, B. L., Sell, L. O., and Abel, W., Tappi 37, no. 7:273(1954).
49. Niss, H. F. Unpublished work, Purdue University, 1953.
50. Perlin, A. S., and Brice, C., Can. J. Chem. 34:541-53(1956).
51. Franken, H., Biochem. Z. 250:53(1932).
52. Schiedt, U., and Reinwein, H., Z. Naturforsch. 7b:270-7(1952); C.A. 46:8522.
53. Schramm, M., Gromet, Z., and Hestrin, S., Nature 179:28-9(1957).
54. Jayme, G., and Maris, S., Ber. 77:383(1944).
55. Whistler, R. L., and Schweiger, R., J. Am. Chem. Soc. 79:6460-4(1957).
56. Ivanov, D., Compt. rend. 189:51-3(1929); C.A. 23:4925.
57. Bellamy, L. J. The infrared spectra of complex molecules. p. 13, 55, 114, and 153. New York, John Wiley and Sons, 1956.
58. Karrer, P. Organic chemistry. p. 285. New York, Elsevier Publishing Company, 1950.
59. Major, W. D. Personal communication, 1957.
60. Davidson, G. F., J. Textile Inst. 29:T195(1938).
61. Ott, E., Spurlin, H. M., and Grafflin, M. W. Cellulose and cellulose derivatives. Vol. 1. p. 147. New York, Interscience, 1954.
62. Taylor, E. W., Fowler, W. F., Jr., McGee, P. A., and Kenyon, W. O., J. Am. Chem. Soc. 69:342(1947).

63. Staudinger, H., and Dohle, W., J. prakt. Chem. 161, no. 8-10:219-40 (1943).
64. Most, D. S. The sorption of certain slash pine hemicellulose fractions by cellulose fibers. Doctor's Dissertation. p. 108. Appleton, Wis., The Institute of Paper Chemistry, 1957.
65. Sakami, W., J. Biol. Chem. 187:369(1950).

APPENDIX I

TEST OF THE TITRIMETRIC MICROMETHOD FOR THE DETERMINATION OF CARBON DIOXIDE

The reproducibility of the titrimetric micromethod for the determination of carbon dioxide was tested by analyzing a series of identical carbonate solutions. From these data, the 95% confidence limits for the method were calculated. The results obtained showed the method to be reproducible within limits of  $\pm 3$  to 5% (95% of the time) over the range of greatest interest in this work, 0.8 to 1.2 mg. of carbon dioxide. (See calculations below,)

$$\sigma = \sqrt{\frac{\sum x^2 - (\sum x)^2/N}{N - 1}} \quad (1)$$

$$\text{C.L. (95\%)} = \frac{\pm \sigma (t_{0.05})}{\sqrt{N}} \quad (2)$$

where

$\underline{x}$  = individual result,

$\underline{N}$  = number of results,

$\sigma$  = standard deviation,

C.L. (95%) = 95% confidence limits, and

$\underline{t}$  = "Student's  $\underline{t}$ ", 5% probability level.

Trial Number	Result ( $\underline{x}$ ), mg. carbon dioxide	$\underline{x}^2$
(1)	0.816	0.6659
(2)	0.755	0.5700
(3)	0.826	0.6823
(4)	0.782	0.6115
(5)	0.740	0.5476
$\underline{N} = 5$	$\sum \underline{x} = 3.919$	$\sum \underline{x}^2 = 3.0773$

$$\bar{x} = 0.784$$

$$t_{0.05} \text{ (for } \underline{N} = 5) = 2.78$$

$$\frac{(\sum x)^2}{\underline{N}} = 3.0714$$

$$\sigma = 0.0385$$

C.L. (95%) =  $\pm 0.043$  mg. carbon dioxide

$\bar{x} \pm \text{C.L. (95\%)} = 0.784 \pm 0.043$  mg. carbon dioxide

APPENDIX II

STUDY OF CARBON DIOXIDE ADSORPTION ON OR INCLUSION IN  
CELLULOSE DURING ITS OXIDATION

The apparatus used in this experiment is schematically shown in Figure 9. Reference will be made to this labeled sketch throughout the description of the experimental procedure.

To the constant atmosphere bottle were added a liter of acetate buffer solution at pH 4.5 (500 ml. 2N acetic acid and 500 ml. sodium acetate solution, 16.4 g./100 ml.) and 500 ml. of normal hypochlorous acid solution. The apparatus was then closed and purged of air with compressed carbon dioxide. Next, a sodium carbonate solution containing a 0.1 millicurie of carbon-14 was quantitatively added through the funnel into the constant atmosphere bottle. The pump was started and the gas in the apparatus was cycled at a rate of about 60 cc./min. for 18 hours in order to bring the system to equilibrium. After 18 hours, a sample of carbon dioxide was taken and its specific activity was determined (12,200 c.p.m./mg. C).

At this point, the reaction flask was closed off and removed from the system. To the flask were added 12 grams of purified unlabeled bacterial cellulose (batch 5), 150 ml. of buffer solution (pH 4.5), and 95 ml. of 1.2N hypochlorous acid. The flask was then replaced in the apparatus and the cycling of the radioactive carbon dioxide through the reaction mixture was begun. The carbon dioxide was cycled at a rate of 60 cc./min. for 24 hours; at this time the bulk of the oxidant had been consumed and the experiment was terminated. Immediately prior to the completion of the experiment, another

sample of the carbon dioxide was taken. The specific activity of this sample was found to be 12,150 c.p.m./mg. C; thus, the specific activity of the carbon dioxide cycled was constant at about 12,000 c.p.m./mg. C.

The oxidized unlabeled bacterial cellulose was washed, solvent dried, and analyzed (the determination of the specific activity of its SCD, etc.) in the usual manner.

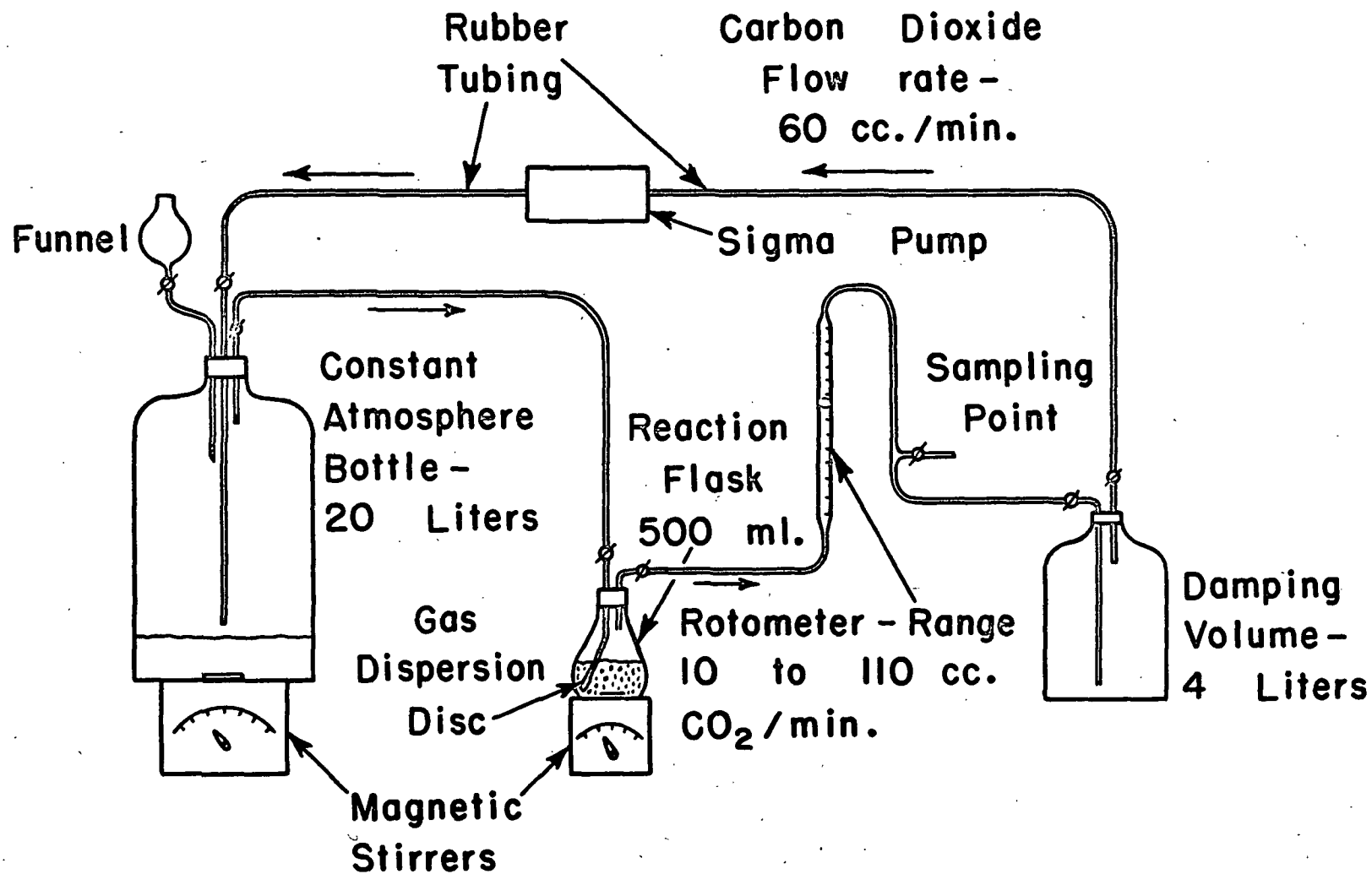


Figure 9. Apparatus for Adsorption Experiment

### APPENDIX III

#### ANALYSIS OF RADIOACTIVE CARBON DIOXIDE

Figure 10 shows a sketch of the manometric apparatus pictured in Figure 4: this apparatus in conjunction with the accessories shown was used for the determination of the specific activity of carbon in the gas phase, as carbon dioxide. This labeled sketch will be referred to throughout the description of the method.

#### MANOMETRIC DETERMINATION OF CARBON

The reaction vessel was connected to the calibrated gas buret by means of a snug fitting rubber coupling. After the air was removed from the vessel, 2 ml. of sodium hydroxide-hydrazine solution were added to the buret to absorb the carbon dioxide generated by either the wet combustion of an organic sample (32) or the acidification of a carbonate solution. The concentration of the absorbing solution was preadjusted according to the amount of carbon dioxide anticipated.

The remainder of this procedure for the determination of the specific activity of carbon was conducted in the manner described by Most (64), as follows:

"Upon completion of the combustion [or acidification], the gases are swept back and forth between the buret and the combustion tube. This is accomplished by alternately raising and lowering the level of the mercury in the buret. Van Slyke and Folch [(32)] present data showing that 20 of these excursions (requiring about 2 min.) produce 100% absorption of the liberated carbon dioxide in the presence of the inert gases.

"After the carbon dioxide has been absorbed, . . . the inert gases present above the solution in the buret are carefully ejected through

the cup above the stopcock. The solution is then extracted under vacuum for two minutes by pulling the mercury level down to the 50-ml. mark of the buret. The buret assembly is shaken, during this time, by a motor-driven assembly attached to the water jacket around the buret. The inert gases removed from the solution during this extraction are also ejected through the cup above the buret.

"A measured volume (2 ml.) of 2N lactic acid is then added to the absorbing solution to liberate the carbon dioxide. Lactic acid is strong enough to liberate carbon dioxide from carbonates, but not strong enough to liberate sulfur dioxide from sulfites. If any sulfur dioxide were generated at this point, the analysis would be useless. Sulfur dioxide could be formed during the combustion. The reaction of the acid with the carbonate solution is allowed to proceed under vacuum for two minutes while the buret is shaken. At this stage of the procedure, the gas mixture is solely carbon dioxide and water vapor. The volume of the gases is then carefully set at either of the calibration points on the buret (50, 10, or 2 ml.) and the pressure is read from the attached manometer and recorded as  $p_1$ . The temperature of the water bath and the volume of the gas are also noted."

The carbon dioxide is then transferred to a counting tube; this operation will be discussed in detail later.

"...The gas space above the absorbing solution is set at the same volume as before and the corresponding pressure is noted and recorded as  $p_2$ . Therefore,  $p_1 - p_2$  is the total carbon dioxide pressure corresponding to the temperature and volume of the measurement. The carbon dioxide due to the reagents alone is obtained by running a blank analysis. This value is subtracted from the measured total...pressure. The final corrected value of carbon dioxide pressure is then multiplied by the appropriate factor, as listed by Van Slyke and Folch [(32)]...[to obtain the weight of carbon]."

#### CARBON-14 DETERMINATION BY PROPORTIONAL COUNTING

"The carbon dioxide...[to be analyzed]...contains both carbon-12 and carbon-14 dioxide. To transfer this carbon dioxide mixture to a proportional counting tube, the water trap is connected to the cup above the buret while the counting tube is connected to the stopcock fitting on the water trap and the whole space thus enclosed is evacuated to a pressure of 0.3 mm. or less (checked by a tilting McLeod gauge in the line). The pump is then removed from the system by closing the cock on the water trap and the buret is opened to the system. The pressure of gases in the buret causes a mixture of water vapor and carbon dioxide to distill over

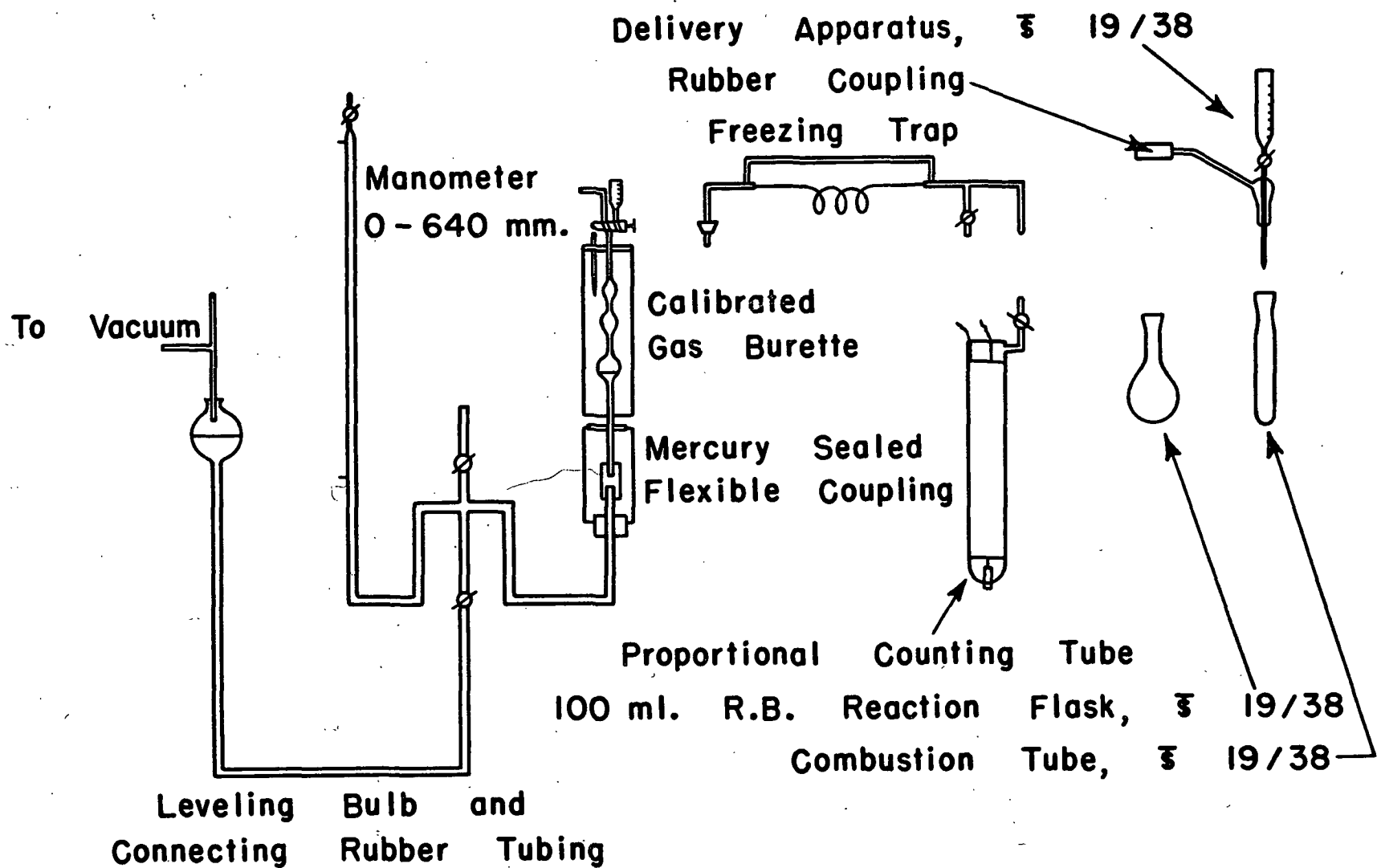


Figure 10. Schematic of Apparatus Used in the Determination of Total Carbon and Its Radioactivity

towards the counting tube, the bottom end of which is immersed in liquid nitrogen. The water is removed from the gas stream in the coils of the freezing trap which are immersed in a dry ice-alcohol bath. The carbon dioxide passes into the counting tube and is condensed at the bottom. When all the gas has been transferred, the buret and counting tube are closed. The tube is removed from the liquid nitrogen and allowed to warm to room temperature. The pressure above the absorbing solution, now stripped of carbon dioxide, is measured as described above...

"When the carbon dioxide in the counting tube has come to room temperature, it is simultaneously diluted with methane and brought to atmospheric pressure by means of the leveling bottle shown in [Figure 10]. The detailed procedure for this operation is described by Van Slyke, Steele, and Plazin [(34)].

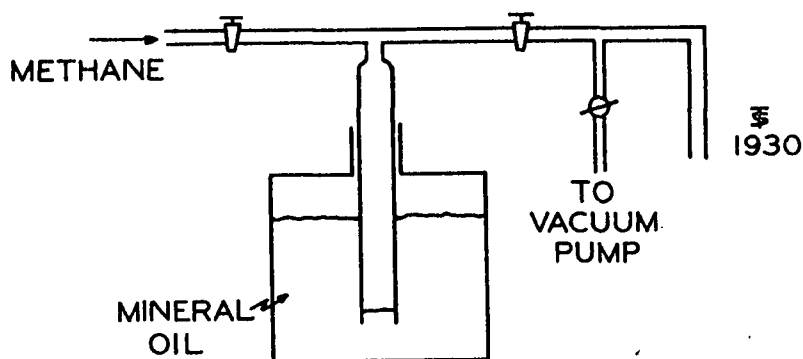


Figure 11. Methane Leveling Bottle

"The sample thus prepared is ready for counting. In the present work, a Nuclear Instrument and Chemical Corp. scaler, model 182X, was used. This instrument meets the specifications outlined by Bernstein and Ballentine [(36)] for proportional counting of low energy beta-ray emitters.

"The counting tube is then placed in a special shielded holder and attached to the scaler. The voltage is set at the proper value for the particular tube<sup>a</sup> being used, and the counting rate of the gas is measured."

From the observed counting rate, corrected for background activity, and the quantity of carbon transferred to the counting tube, the specific activity

<sup>a</sup> The Bernstein and Ballentine counting tubes were calibrated according to the method of Van Slyke, Steele, and Plazin (34).

of the carbon in the carbon dioxide was calculated. The equation relating sample counting rate, background counting rate, standard deviation, and counting time is discussed below.

Based on Equation (1) below, as given by Van Slyke, Steele, and Plazin (34), an estimate was made of the standard error which could be expected for a measured counting rate under the conditions which prevailed for these experiments. The counting error did not exceed the 3% level for any count made in this research.

$$N_s = \frac{1 + \frac{4N_oTs^2}{T} + 1}{Ts^2} \quad (1)$$

where

$\frac{N_s}{s}$  = sample counting rate (500 c.p.m.).

$\frac{N_o}{o}$  = background counting rate (170 c.p.m.).

$\underline{T}$  = counting time (10 minutes), and

$\underline{s}$  = standard error (?).

The calculation showed that  $\underline{s} = \pm 2.6\%$  could be expected.

APPENDIX IV

CHARACTERIZATION OF LABELED BACTERIAL CELLULOSE:  
DETERMINATION OF THE SPECIFIC ACTIVITY OF THE  
CARBON IN POSITIONS 1 AND 6 (35)

Sixty milligrams of potassium gluconate prepared from labeled bacterial cellulose were dissolved in 10 ml. of 0.5M sodium phosphate buffer at pH 5.8. The flask containing the foregoing solution was connected to the Van Slyke-Folch apparatus (see Figure 4)(manometric method D used), the system was evacuated, and then 3.1 ml. of freshly prepared 0.5M sodium periodate were added. The oxidation of the gluconate with periodate liberated carbon dioxide from position 1, formic acid from positions 2, 3, 4, 5, and formaldehyde from position 6. The carbon dioxide evolved was collected in 2 ml. of alkaline-hydrazine solution by sweeping the gases back and forth between the reaction flask and the gas buret (32); this was terminated after 45 minutes (~95% of theoretical evolved). The specific activity of the carbon dioxide (the carbon from position 1) was then determined (34).

An hour after the periodate oxidation was initiated, the mixture was chilled and the oxidation stopped by the addition of 1 ml. each of 6N sulfuric acid and 20% potassium iodide solution. Sufficient 2M sodium arsenite was then added to destroy the iodine. Four milliliters of 3N sodium hydroxide were added, and the alkaline solution was distilled to near dryness several times after repeated additions of water. The distillate, containing formaldehyde, was collected in a receiver chilled in a salt-ice-bath. The partially frozen distillate was oxidized to formic acid by treatment with 20 ml. each of normal sodium hydroxide and 0.1N iodine for 1 hour at 0 to 5°C. At the end of

the oxidation period, the solution was acidified and the iodine destroyed with sodium arsenite. The solution, now containing formic acid, was added to a 100-ml. round-bottomed flask, neutralized, evaporated to 10 ml. and acidified with glacial acetic acid. A large wad of glass wool was added, and the flask was connected to the Van Slyke-Folch apparatus. The mixture was boiled with sweeping to remove preformed carbon dioxide; the gases evolved were then ejected. To the boiling solution were added 30 ml. of Sakami's mercurous chloride reagent (65). By trapping the newly precipitated mercury salts, the glass wool served to overcome the tendency to bump. The evolved carbon dioxide was absorbed in 2 ml. of alkaline-hydrazine solution as before. The specific activity of the carbon dioxide (the carbon from position 6) collected was then determined (34).

APPENDIX V

NISS COLORIMETRIC METHOD FOR THE DETERMINATION OF NITROGEN  
IN ORGANIC MATERIALS (49)

PROCEDURE

Reagents

- (1) 50% sulfuric acid solution
- (2) Buffer - equal volume mixture of 0.1M sodium citrate and 1.0% sodium hydroxide (w/v)
- (3) 5% phenol solution (w/v) - (solution must be colorless)
- (4) borate-hypochlorite solution - prepared by dissolving 2 g. of boric acid and 2 g. of sodium hydroxide in 50 ml. of water, cooling, then adding 15 ml. of Chlorox and diluting to 100 ml.
- (5) 30% hydrogen peroxide solution

Digestion

A sample (about 0.5 g. cellulose) containing 80 to 2500 micrograms of nitrogen was added to a 100-ml. Kjeldahl flask. Two milliliters of 50% sulfuric acid were added to the flask, and it was heated on a digestion rack until the solution had fumed for 5 minutes. The flask was then removed from the rack and allowed to cool for about a minute. Three or four drops of 30% hydrogen peroxide were added to the cooled solution, and it was heated for 2 minutes. This process was repeated until the solution became clear; this normally required about five treatments. After the last treatment, the solution was allowed to cool before 100 ml. of buffer solution were added. The flask was then stoppered with a rubber stopper, and the contents of the flask were thoroughly mixed.

### Color Development

Two milliliters of the sample, 2 ml. of 5% phenol solution, and 2 ml. of the borate-hypochlorite solution were added to a spectro cuvette; the cuvette was then heated for 5 minutes in boiling water. The solution was cooled, and the per cent transmission of the solution with reference to a blank, was determined at 640 millimicrons.

The range for the method as described above is 80 to 800 micrograms of nitrogen. If the per cent transmission for a sample drops below 15%, 10 ml. of water are added to the cuvettes (sample and blank), and the transmission values are read again. This increases the range to 250 to 2500 micrograms of nitrogen. The standard curve for this method was prepared from known ammonium chloride solutions.

APPENDIX VI

X-RAY DIFFRACTION PATTERNS

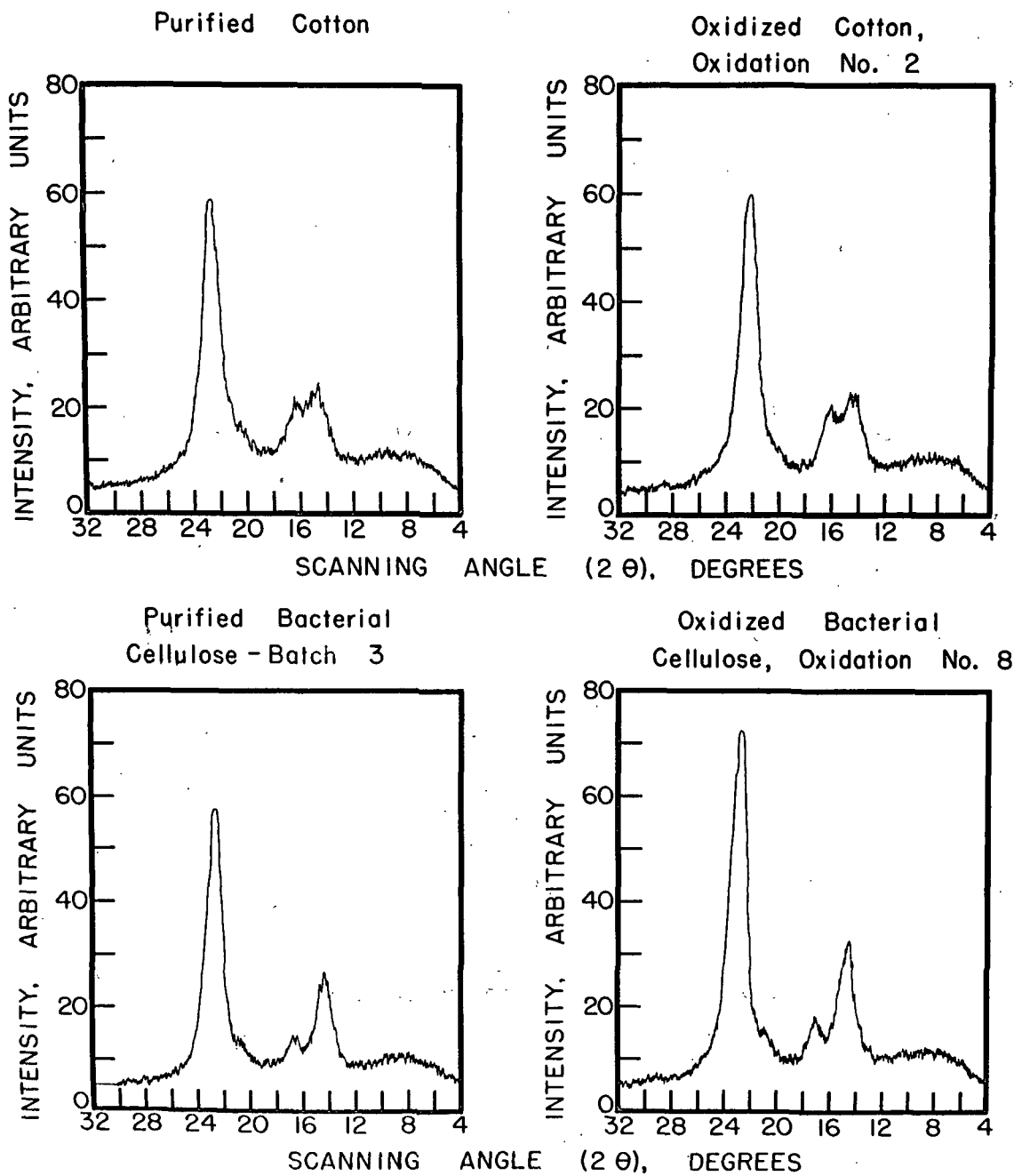


Figure 12. X-ray Diffraction Patterns of Unoxidized and Oxidized Cotton and Bacterial Celluloses

APPENDIX VII

INFRARED SPECTRA

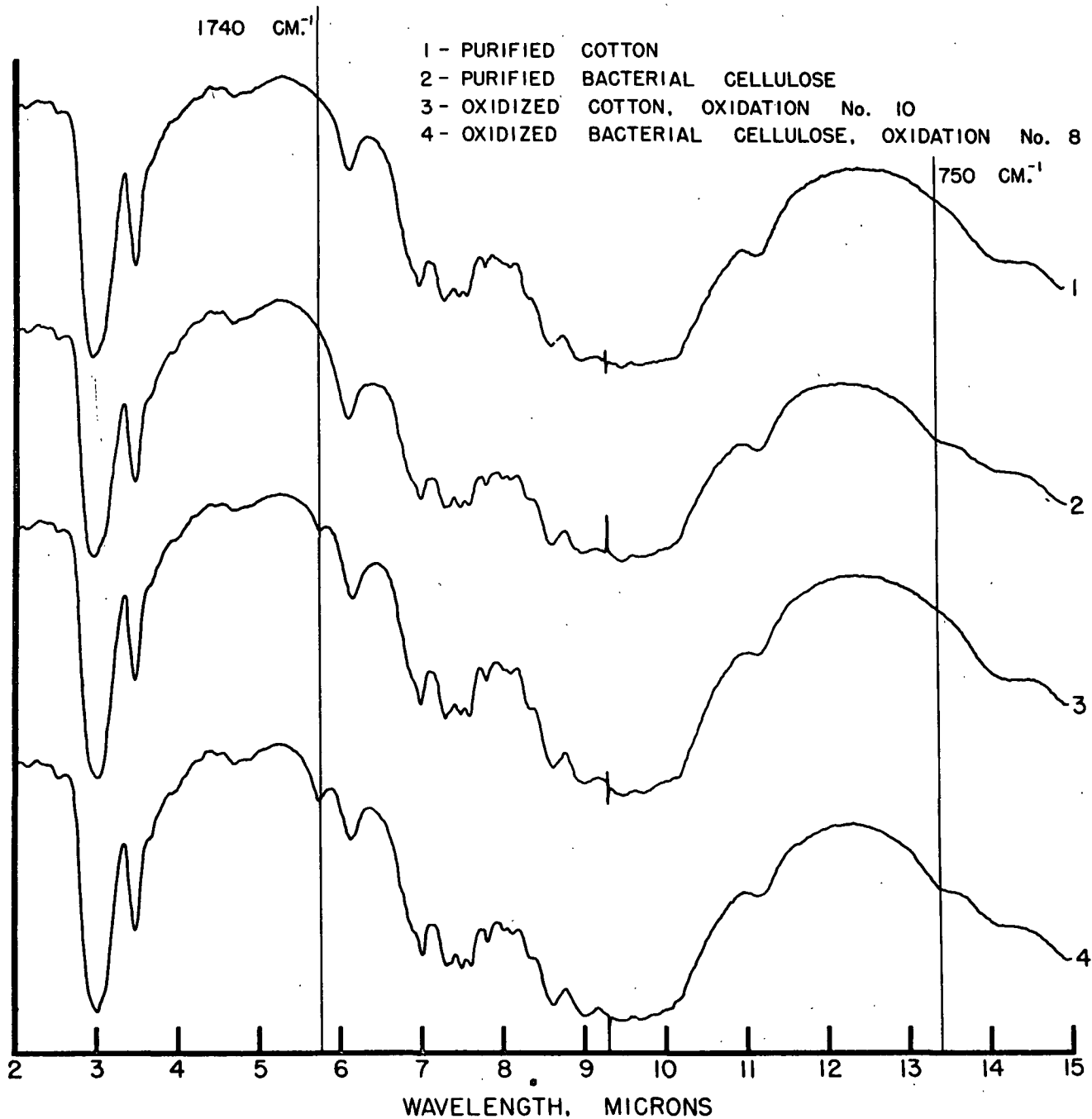


Figure 13. Infrared Absorption Spectra of Unoxidized and Oxidized Cotton and Bacterial Celluloses