

The Institute of Paper Chemistry

Appleton, Wisconsin

Doctor's Dissertation

Linkages between Glucose and Mannose
in Slash Pine Alpha-Cellulose

Austin F. Anthis

June, 1956

LINKAGES BETWEEN GLUCOSE AND MANNOSE
IN SLASH PINE ALPHA-CELLULOSE

A thesis submitted by

Austin F. Anthis

B.S. 1950, The Rice Institute
M.S. 1952, Lawrence College

in partial fulfillment of the requirements
of The Institute of Paper Chemistry
for the degree of Doctor of Philosophy
from Lawrence College,
Appleton, Wisconsin

June, 1956

TABLE OF CONTENTS

PRESENTATION OF THE PROBLEM	1
HISTORICAL REVIEW	3
Mannan Determination	3
Mannan as a Homopolymer in Wood	5
Resistant Mannans in Wood	9
EXPERIMENTAL PROCEDURES	13
Acetolysis	13
Deacetylation	14
Carbon Column	15
Preparation	15
Operation	16
Concentration of Fractions	17
Paper Chromatography	18
For Qualitative Identification	18
Procedure	18
Differences in Solvents	18
For Separation in Quantity	20
Determination of Constituents of Disaccharides	21
Hydrolysis	21
Oxidation and Hydrolysis	22
PRESENTATION AND DISCUSSION OF DATA	24
Obtaining the Disaccharides	24
Acetolyses	24
Recovery of Acetolyzates	24
Acetolyzate I	24

Acetolyzate II	26
Discussion	26
Deacetylation	28
Carbon Column	29
Preliminary Trials	29
First Development	30
Operation	30
Difficulties	31
Second Development	33
Discussion	34
Separation on Paper Chromatograms	35
Characterization of Disaccharides	37
Disaccharide 1 (4-O- β -D-glucopyranosyl-D-mannose)	37
Acetylation	38
Properties of the Acetate	39
Melting Point and Specific Rotation	39
X-ray Powder Diagram	39
Carbon and Hydrogen Analyses	40
Conclusions	40
Disaccharide 3 (possibly 4-O- β -D-mannopyranosyl-D-glucose)	41
Acetates	41
Attempt to Crystallize the Free Sugar	42
Deacetylation and Crystallization	43
Melting Point and Specific Rotation	45
Carbon and Hydrogen Analyses	45
X-ray Powder Diagram	46

Conclusions	47
Disaccharides 2-A, 2-B, and 4	47
Disaccharides 2-A and 2-B	47
Disaccharide 4	50
Tests for Presence of Artifacts	51
Subjecting Simple Sugars to Acetolysis Conditions	51
Test of Deacetylation Procedure	52
Significance of the Disaccharides	53
POSSIBILITIES OF FUTURE WORK	55
SUMMARY OF RESULTS AND CONCLUSIONS	56
LITERATURE CITED	58

PRESENTATION OF THE PROBLEM¹

When wood chemists classify the various polysaccharides in the cell walls of wood fibers, they usually do so along either academic or analytical lines. In either case, the polysaccharides can be divided into two groups, the actual point of division being somewhat different in each case. Using the academic view, the different polysaccharides are included either in the term "true" cellulose (long chains of glucose units linked β -glycosidically through positions 1 and 4) or in the term hemicellulose. This latter term would then include all xylose, mannose, uronic acid, galactose, and arabinose units, as well as possibly some glucose or other sugar units.

The analytical classification places the polysaccharides under the terms of either alpha-cellulose or hemicellulose. The alpha-cellulose is defined as the alkali-insoluble residue obtained under controlled (but somewhat arbitrary) conditions from the delignified, extractive-free cell walls of the wood; the hemicelluloses are the soluble portion. The insoluble residue contains sugar units other than glucose, such as mannose and xylose. In the academic view these latter sugar units would be classified as resistant hemicelluloses.

In the case of the softwoods the resistant hemicelluloses are predominantly composed of mannans², which are found not only in alpha-celluloses but in purified pulps and in other cellulose preparations from softwoods as well. These mannans have never been conclusively shown to

¹The experimental evidences alluded to in this section are more fully discussed in the historical review.

²The term mannan as used here is the same as is used by most wood chemists and signifies only that mannose is obtained upon hydrolysis of the material.

exist as a homopolymer in wood, and some evidence has been obtained (also inconclusive) indicating that the anhydromannose units might be combined chemically with anhydroglucose units in the wood. If such chemical linkages could be definitely established it would increase our knowledge of the structure of these mannans and might help in explaining some of the difficulties and reactions involved in purifying wood pulps and utilizing such pulps for chemical conversion.

It was the specific purpose of this work to establish, if possible, the presence of linkages between glucose and mannose units in an alpha-cellulose of slash pine (Pinus caribaea). This was to be accomplished by the isolation and identification, from an alpha-cellulose acetolyzate, of disaccharide degradation products containing both glucose and mannose.

In the course of this work disaccharides other than cellobiose and those containing both glucose and mannose were isolated, and were investigated in order to obtain further knowledge of the structure of the carbohydrates that constitute the alpha-cellulose.

HISTORICAL REVIEW

MANNAN DETERMINATION

Mannans are present to the extent of 10% or more in softwoods, but as yet it is not definitely known whether chains consisting of only anhydromannose units are present, or whether mannose and other sugars are combined into chains.

Many of the early wood chemists assumed that the mannan in wood was a homopolymer, for such polymers had been obtained from other materials such as vegetable ivory, tubers of various orchids, and certain yeasts (1). They were also aided in their assumption by the fact that the mannan determinations used in those times did not always reveal the presence of the "resistant" mannans in wood or pulp preparations. Some of the methods which led to possibly false conclusions are discussed below.

Schorger (2) in 1917 presented a method for the determination of mannan in wood by refluxing the wood meal for two successive 3.5-hour periods with fresh 5% hydrochloric acid. The filtrate and washings were neutralized, acidified slightly with acetic acid, and concentrated. The mannose in the solution was then precipitated as the phenylhydrazone. He assumed that this was a quantitative removal of mannan, because a third acid treatment did not produce an appreciable increase in mannose phenylhydrazone.

One of the reasons why Schorger's assumption was not valid was brought out by Nowotnowna (3), when she showed that the precipitation of mannose

phenylhydrazone was not quantitative below a mannose concentration of about 1%. Also, Hägglund and Klingstedt (4) showed that the residue left after a Schorger mannan determination on sprucewood (assaying 8.6% mannan) still retained about 3% mannan, as shown by completely dissolving the residue with 72% sulfuric acid and then determining the mannose in the solution after complete hydrolysis.

Nishida, Hamashima, and Fukai (5) also claimed that Schorger's method gave low results, and recommended heating the wood meal in 5% sulfuric acid for 44-45 hours on a water bath.

In 1921 Lenze, Pleus, and Müller (6) attempted to determine the mannan content of delignified wood by extracting it twice with 17% caustic soda and determining the mannan in the extracts. They assumed that all the mannans had been removed by the extraction, since they did not detect mannan in the third extract obtained with 17% caustic soda. However, Heuser and Dammel (7) showed that the residue from the alkaline extractions still contained mannan.

In order to include these resistant mannans, Hägglund and Bratt (8) hydrolyzed the entire carbohydrate portion of the wood after first dissolving it in 72% sulfuric acid. Wise, Ratliff, and Browning (9) slightly modified and improved their procedure, and it now seems to be the most reliable method for determining mannans in wood or pulps, providing the mannan content is not too low.

Because of these inadequate methods available to the early workers, their results and conclusions as to the possible chemical constitution of wood mannan should be interpreted with caution.

MANNAN AS A HOMOPOLYMER IN WOOD

One of the earliest reported attempts at isolation of a pure mannan polysaccharide from wood was that of Sherrard and Blanco (10). They used a method given by Browne (11) for obtaining mannan from other plant materials. Sprucewood was refluxed for 2 hours in 2.8% sodium hydroxide and then filtered. The filtrate was diluted to 2-1/2 times its original volume, and a portion of it had about a 1/4 volume of Fehling solution added to it. A small quantity of copper oxide was deposited, but no insoluble copper-mannan compound was precipitated. They then hydrolyzed another portion of the diluted filtrate with dilute acid, and it did not give a test for mannose. In view of the dilute conditions of the test some mannose may have been present, however. Since the mannan in the wood was not obtained by this method they concluded that mannose units might be linked to the glucose.

Hess and Lüdtke (12) described the isolation from spruce sulfite pulp of what they considered to be a pure mannan. They extracted the pulp with eight per cent sodium hydroxide and recovered the hemicelluloses thus removed. This material was dissolved in Schweizer reagent and the "mannan" was selectively precipitated by making the solution 0.2 N in sodium hydroxide. The free polysaccharide was regenerated with acetic acid. The solution, precipitation, and regeneration was repeated twice, and a product was obtained whose specific rotation was not changed by further purification.

They found that this material was very much like ivory nut mannan "A"

(which is essentially a pure mannan) in the following respects: (1) their specific rotations in cuprammonium checked (no data given); (2) their powder x-ray diagrams were very similar; and (3) their specific rotations in 1 N sodium hydroxide were nearly identical, (See Table I). In addition, they stated that only mannose was detected in the hydrolyzate of the material, but they gave no yield figures or experimental details.

There has been no published confirmation of this work on the isolation of a pure mannan from wood, and in view of the results of Huseman and Yundt, as discussed below, it is doubtful that they obtained a mannan homopolymer.

Yundt (13), in attempting to isolate a mannan by Hess and Lüdtke's general procedures, used as his starting material a mildly hydrolyzed hemicellulose obtained by extracting a chlorite holocellulose with 8% sodium hydroxide and then autoclaving the extracted material for 3 hours at 120°C. and a pH of 4. The autoclaving supposedly selectively hydrolyzed xylans, so that they could be at least partially separated from the mannan-containing chains. This mannan-enriched portion was then dissolved in cupriethylenediamine, which was then made N in sodium hydroxide in order to precipitate the mannan-containing chains. He found that the cupriethylenediamine was easier to work with than the cuprammonium, as used by Hess and Lüdtke. The carbohydrate material that was then regenerated with acetic acid was dissolved in sodium hydroxide, which was then very slightly acidified with acetic acid and heated at 65-70°C. Crystals were thereby obtained which agreed in appearance and characteristics with those obtained from ivory nut mannan "A." The recrystallization procedure was repeated five times, and the crystals assayed 50 and 47% mannan after the

third and fifth times, respectively. The remaining carbohydrate material was not determined.

The following table shows the comparative values of specific rotation for various "mannans," as given by Yundt.

TABLE I
SPECIFIC ROTATIONS OF "MANNANS"

Material	Concentration, g./l.	Solvent	$[\alpha]_D$	$[\alpha]_{436}$
Hess's ivory nut mannan "A"	6	4% NaOH	-44.6°	-----
Yundt's ivory nut mannan "A"	47	6% NaOH	-46°	-84°
Slash pine hemicellulose crystals containing 50% mannan	54	6% NaOH	-41°	-----
Hess and Lüdtke's spruce "mannan"	--	4% NaOH	-44.6°	-----
Ivory nut mannan "A"	5.2	TAPPI Cuprammonium	-----	+303°
Slash pine hemicellulose crystals containing 50% mannan	4.9	TAPPI Cuprammonium	-----	+ 12°

From the above, it can be seen that a hemicellulose containing 50% mannan had essentially the same specific rotation in alkali as ivory nut mannan, as well as the fact that it produced the same type of crystals, and therefore these characteristics cannot be used alone as a basis of judgment as to whether or not a material is a pure mannan. It is unfortunate that Hess and Lüdtke did not give their data on the specific rotation of their spruce "mannan" in cuprammonium for comparison.

Huseman (14), as well as Hess and Lüdtk^ue, placed considerable weight upon the fact that their "mannan" had the same specific rotation as ivory nut mannan "A." She obtained her material by treating sprucewood for a prolonged period with a dilute solution of chlorine dioxide containing pyridine and then extracting the residue with 8% sodium hydroxide. After giving the extract another treatment with chlorine dioxide, she fractionally precipitated the product from a sodium hydroxide solution by stepwise addition of methanol. All of the fractions thus obtained had essentially the same specific viscosity in sodium hydroxide, and she considered this as evidence that the entire material consisted of a single type of polysaccharide of uniform molecular weight. Since 90% of the weight of the material occurred in fractions having $[\alpha]_D$ values between -42 and -44° , it was assumed that the polysaccharide was a mannan, although no chemical analyses of it were given.

Later work on cellulose (15) and on a partially hydrolyzed cellulose acetate (16) showed that the efficiency of fractionation of a polysaccharide according to molecular weight depends greatly upon the choice of solvent and precipitants, for with some combinations no separation will result. Wethern (17) demonstrated that this was the case with a material obtained in similar manner to Huseman's--alkaline extraction of a holocellulose. He not only found that such a hemicellulose contained carbohydrates of a wide range of molecular weight, but that the original material and the various fractions that he could obtain from it were chemically heterogeneous.

In the light of this more recent evidence, it is assumed that Huseman's "mannan" was a chemically heterogeneous hemicellulose.

RESISTANT MANNANS IN WOOD

The observation has been made by a number of workers that sometimes a considerable percentage of the mannan in softwoods is not removed by dilute acid hydrolysis, caustic extraction, or the normal pulping and bleaching operations. The workers have already been cited who showed that boiling wood twice in 5% hydrochloric acid for 3.5 hours (as in Schorger's mannan determination) still left appreciable amounts of mannan in the residue.

Sherrard and Blanco (10) found that even Schorger's method indicated a presence of from 1 to 3% mannan in spruce sulfite (bleached and unbleached), soda, and kraft pulps. They also found mannan in the resistant alpha-cellulose, as well as in beta- and gamma-cellulose of sprucewood, and suggested that glucose and mannose might be chemically combined in the wood.

Wise and Ratliff (18) found that over half of the mannan originally present in slash pine and in spruce was retained in the alpha-cellulose prepared by successive extractions of a chlorite holocellulose with 5, 16, and 24% potassium hydroxide. By far the main portion of the extractable material had been removed after the 16% potassium hydroxide treatment, and this material from the slash pine was then given eight successive hydrolyses for one hour; each at 98-99° with fresh charges of 5% sulfuric acid. Approximately 40% of the mannan of the original wood still remained in the residue from these treatments. They considered this, also, was possible evidence for a chemical linkage between glucose and mannose.

In contrast to the above workers, Giertz (19, 20) has presented data

that he considers as evidence for the theory that there is a sharp line of demarcation between true cellulose and the hemicelluloses. He says that holocellulose and strong-pulp fibers are still structurally strong, and that this pertains particularly to the primary wall. He believes they are too strong to swell sufficiently and, as a consequence, not all the hemicelluloses--which he believes should be soluble in caustic soda--will diffuse out. In support of this view he claims that if the material is given a pretreatment with 2.5 N sulfuric acid at 100°C. for five minutes, an alpha-cellulose determination on a chlorite holocellulose or strong sulfite pulp from sprucewood yields a residue (40-43% on the basis of the wood) containing only 0.1 to 0.2% mannose and 0.1% xylose. By treating a strong spruce sulfite pulp with 5 N sulfuric acid at 100°C. for three hours, he obtained a residue of 40% of the original wood containing only 0.2-0.3% mannose and 0.1-0.2% xylose. In both cases the sugar determinations were made chromatographically, but no details were given.

Thus, by using a stronger acid and treating with the acid and then extracting with alkali he was able to obtain a product containing considerably less mannose than Wise obtained by the reverse treatment. However, this does not definitely prove his hypothesis that the resistant hemicelluloses are held within the fibers merely by physical means. For it is conceivable that if they are chemically combined with the cellulose, but occur mainly in the more amorphous regions, the acid treatment may hydrolyze them to such an extent that they are then soluble in alkali.

Leech (21) was the first to report the isolation, from wood, of a disaccharide that probably consists of glucose and mannose. Upon

acetolysis of a slash pine alpha-cellulose he was able to obtain such a disaccharide from the fraction (obtained by adsorption chromatography) that contained predominantly α -cellobiose octaacetate. After crystallization of the cellobiose octaacetate and deacetylation of the substances in the mother liquor, he obtained by paper chromatography a small amount of the disaccharide that on hydrolysis yielded chromatographic spots for glucose and mannose. He did not detect such a disaccharide upon subjecting a mixture of glucose, mannose, and cellobiose to the same acetolysis conditions. The work described in this report is a continuation of Leech's work.

Bradway (22), in his investigation of the haze in cellulose acetates made from wood pulps, found that all the xylose and mannose in the original acetate was associated with the haze fraction, which was 19% of the original acetates. The majority of the mannose was in the fraction of the haze that after deacetylation was soluble in 5% sodium hydroxide. Upon subjecting a particular portion of the haze to acetolysis conditions he was able to obtain, after deacetylation, four spots in the disaccharide region on a paper chromatogram. Three of them were tentatively identified as cellobiose, a mannoiose, and a glucosyl-mannose, respectively; the fourth was unidentified.

This was evidence that glucose and mannose were chemically combined in the wood, but it could not be proved that the linkage was not formed during the treatment or isolation.

In a study of the relationship between the properties of cellulose acetates made from wood pulps and the mannan content of these pulps,

Steinmann and White (23) observed that the acetylation process was not very effective in removing the mannan but was very effective in reducing the pentosan content of the pulps. They also found that there was a good correlation between the mannan content of the cellulose acetates and the abnormally high viscosity of the acetates that is sometimes observed in concentrated acetone solutions. They thought that these results might be explained by assuming the mannan existed in branch or cross chains connected to the rest of the cellulose.

EXPERIMENTAL PROCEDURES

ACETOLYSIS

The techniques used in isolating the desired disaccharides as degradation products from the alpha-cellulose involved an acetolysis, deacetylation, partial separation on a carbon column, and then complete separation on paper chromatograms.

The alpha-cellulose used in this work was prepared by Leech (21), and he has given the experimental procedures used in its preparation. Briefly, it was obtained from a slash pine (Pinus caribaea) chlorite holocellulose by giving it two successive extractions under nitrogen, with 5 and 24% potassium hydroxide, respectively. It assayed 10.4% mannan.

Leech's acetolysis conditions were used, but some slight changes were made. The new conditions are listed below:

1. A ratio of approximately 10 cc. of acetolyzing medium per gram of carbohydrate (ovendry basis) was used.
2. During the initial reaction period the temperature was maintained below 25°C. by means of an ice bath, and the room temperature of 18-23°C. was maintained subsequently.
3. After the first day only occasional stirring was employed since the solution then appeared to be homogenous.
4. A reaction time of either nine or eleven days was used.

The acetolyzing medium contained glacial acetic acid, acetic anhydride, and concentrated sulfuric acid in volume ratios of 10:10:1, respectively.

Since some difficulties were encountered in the recovery of the acetolyzates, the details have been given in a later section, on page 24.

DEACETYLATION

The acetolyzate was deacetylated by means of sodium methylate, which was originally used for this purpose by Zemplén (24). The alcohol used in its preparation was c.p. methanol that was further dried by distillation after the addition of fresh magnesium turnings and a small amount of iodine. The details given by Lund and Bjerrum (25) were followed except that less magnesium was used because the alcohol contained less than 0.5% water. This drying was done in order to reduce the small amount of sodium hydroxide that would otherwise be in the solution after the addition of sodium. This precautionary drying may not actually be necessary.

A solution that was 0.2 N in sodium methylate was then prepared by weighing out 4.6 - 5.0 g. of sodium per liter of methanol, momentarily dipping it in a small amount of methanol in order to clean it, and then dissolving it in the desired quantity of the dry methanol.

The acetates were then added to a glass-stoppered bottle that contained 15 cc. of this solution per gram of the added acetates. After shaking for one hour the solution was made slightly acidic to dampened litmus paper by the addition of 1:5 acetic acid. The insoluble material was filtered and washed with methanol. Most of the carbohydrates of about degree of polymerization four or above were insoluble, as shown by paper chromatograms.

By alternate additions of water followed by concentration at 50°C. in

vacuo, the methanol was replaced with water. The resulting solution was slightly cloudy but was clarified by filtration through Celite.

CARBON COLUMN

PREPARATION

The mixing of the carbon (Darco G-60)* and filter aid (Celite 535), in a ratio of 2:3 by weight, was carried out with 2000 to 3000 g. of mixture in a large pickle jar that was rotated horizontally about its long axis at about 8 r.p.m. Because of slipping of the contents against the side of the jar, the mixing was not very efficient, timewise, and required at least three days for completion. Subsequently, the mixture had a uniform appearance but still contained very small clumps of Celite, as evidenced by the fact that when some of the material was mashed on a glass plate with a spatula some white specks became visible. They were of such small size, however, that it would probably have taken a grinding action rather than a tumbling one to break them up.

The chromatographic tube was a commercial pyrex pipe 91 cm. in length. The top section of 78 cm. had an inside diameter of 10.0 cm., and it was joined by a 5.5 cm. tapered section to the bottom portion of the tube, which was 13 cm. in length and 7.5 cm. in inside diameter.

The column of carbon was supported in the tube on a pad of cotton four to five centimeters in thickness (when lightly compressed) which rested on a circular piece of nickel-chrome wire gauze. The wire gauze was firmly supported at the tapered part of the tube by a rubber-cushioned, brass cross piece. The carbon-Celite mixture was added to a depth of about an

*Obtained through the courtesy of the Atlas Powder Company.

inch or two at a time, and then in order to partially pack the contents the column was repeatedly tapped with a large rubber stopper on the end of a rod.

After nearly filling the tube, suction was applied at the bottom. In order to diminish any channeling through the column, it was tapped as when being filled. More carbon was then added to obtain the desired depth of packing of about 70 cm., and air was sucked through it. The total time during which air was pulled through was less than several minutes, since prolonged times decrease the flow rate at which the liquid passes through the column (26).

While applying suction at the bottom, water was added at the top, and it proceeded down the column in a uniform front. The flow rate of water through the column was 2-1/2 to 3 liters per hour upon application of 23 to 25 inches of mercury vacuum to the bottom. The flow rate decreased when alcohol was added to the water. The column contained 2,360 g. of the carbon-Celite, and the liquid capacity of the packing itself was approximately 4-1/2 liters.

OPERATION

During operation, a vacuum of approximately 25 inches of mercury was maintained in the receivers (10- or 20-liter bottles) used for collection of the fractions.

In the interest of safety, whenever a vacuum was maintained within the collecting bottles they were covered with a cloth and kept in a wire-mesh tube. The rubber tubes connected to the jugs were of the 3/16 by 3/32-

inch variety, and thus they did not collapse under vacuum but could easily be closed with a pinch clamp. This facilitated the quick transfer from one collecting bottle to another in order to maintain continuous operation. The new bottle was evacuated prior to its insertion into the system.

When the column was in operation and it was desired to change the solution being fed to it, the liquid level was allowed to drain almost down to the level of the carbon before adding the new solution.

After the column was started, it was operated continuously throughout a run, and in this way any tendency for the liquid to channel down the sides of the column seemed to be minimized.

CONCENTRATION OF FRACTIONS

The fractions of interest were concentrated to a volume of 1-1/2 liters in a large circulating-type evaporator operated at a solution temperature of 40-50°C. The evaporation rate was about eight liters in three hours. Concentration to 150 cc. was effected in a smaller circulation type of evaporator in about 1-1/2 hours while operating at 35°C. Final concentration was accomplished in an ordinary noncirculating type of concentrator heated by a 50° water bath. Most of the fractions were taken to a dry powder in the concentrator after successive additions of absolute ethanol.

In each case a slight grayish precipitate formed, or became evident, upon concentration and was removed by filtration through a small amount of Celite when the volume was about 50 cc. Possibly the precipitate was a

small amount of carbon and Celite that had passed through the layer of cotton. A small amount of a precipitate also developed when the dried fractions were later redissolved in water. It was flocculent in nature and was separated from the sugars by centrifuging. It was not noticeably soluble in alcohol or caustic soda, and it gave a negative Molisch test.

PAPER CHROMATOGRAPHY

FOR QUALITATIVE IDENTIFICATION

Procedure

The apparatus for paper chromatography consisted of a cylindrical glass tank 24 inches high and 12 inches is diameter that contained troughs for holding the chromatogram in contact with the solvent. A glass lid rested on top of the tank. The chromatograms were prepared by spotting or streaking the desired carbohydrate solution along a line 9 cm. from the top of a sheet of Whatman No. 1 filter paper that was 24 inches long and up to eight inches wide. After developing for the desired length of time, the sheets were dried and sprayed with a 0.1 M solution of aniline hydrogen phthalate in *n*-butanol. Spots appeared after heating for about five minutes at 110-120°C. The position of the unknown material was then compared with those of known sugars that had been placed on the same sheet.

Differences in Solvents

Nearly all the paper chromatographic separations were accomplished with two solvents, butanol-pyridine-water (6:4:3 by volume) and ethyl acetate-acetic acid-water (9:2:2). Initially, it was observed that the disaccharides moved approximately three times faster in the basic solvent

than in the acidic one. However, the resolution of monosaccharides from disaccharides was more favorable in the acidic solvent than in the basic one. Because of the mobility of the disaccharides in the basic solvent, it was used in determining what carbohydrates were in the various fractions obtained from the carbon column. A development time of 2-1/2 days was sufficient for distinguishing between the four disaccharide spots which were generally observed.

The acidic solvent had one advantage when chromatographing deacetylated acetolizates or other sugars that had been deacetylated using the sodium methylate procedure. The resulting solution of sugars and sodium acetate could be successfully chromatographed using the acidic solvent; whereas, in the case of the basic solvent the sodium acetate appeared as a spot below celotriose, and all sugar spots farther down the paper were badly streaked.

In the latter stages of this work it was also found that prolonged development (18 days) in the acidic solvent would partially separate disaccharides that could not be separated by the basic solvent even when the sugars traveled nearly the entire length of the sheet. It was also discovered that the disaccharides moved almost twice as fast in an ethyl acetate-acetic acid-formic acid-water (18:3:1:4) solvent than in the original acidic solvent. Also, a greater degree of separation may have been obtained.

This certainly emphasizes the need of using more than one solvent when chromatographically investigating carbohydrates of unknown character. If further work were to be done in this field it might be advisable to investigate the use of other solvents.

FOR SEPARATION IN QUANTITY

The materials from the desired fractions from the carbon column were dissolved in a small amount of water and centrifuged to remove the insoluble material, which was washed in the centrifuge. The final volume was one to two cubic centimeters, and the desired amount was streaked along the top of a sheet of Whatman 3 MM paper. Several superimposed streaks were required, with intermittent drying with the aid of a stream of air. The sheets were 61-cm. long, and the sugars were applied over the central 55 cm. of the 59-cm. width.

The chromatograms were run with butanol-pyridine-water (6:4:3) as solvent in a rectangular, stainless steel tank 30 inches long, 16 inches wide, and 30 inches deep. The entire tank was insulated on the outside with a 0.75-inch layer of hair felt in order to minimize temperature variations.

Very good separation among the disaccharides was obtained even when as much as 0.2 g. of total sugars was placed on each sheet. The time required for a given distance of travel of the sugars on the Whatman 3 MM paper was only $1/2$ to $2/3$ as long as when using Whatman No. 1 paper. Between two and three days were required for the faster-moving disaccharides to be near the bottom of the sheet.

After developing and drying, the chromatograms were streaked once or twice along their entire length with a permanganate-periodate reagent (27) applied by means of a capillary-tipped medicine dropper. After about five minutes standing at room temperature, the bands of carbohydrate material

showed up as yellowish-brown in contrast to the purple of the rest of the streak. After an additional 10 to 15 minutes the entire streak began to turn brown.

In some cases the bands of carbohydrates exhibited considerable waviness. In order to locate the bands more accurately, the cellobiose area was first established by additional streaks of the reagent. During this operation, the remainder of the chromatogram was carefully masked. Then the section containing only cellobiose was cut out, and the cellobiose area was specifically revealed with aniline hydrogen phthalate in the usual manner. Finally, the cellobiose band was used as a guide to locate the other sugar bands with maximum accuracy.

After removing the streaked areas (several mm. in width) the desired portions were eluted with water by the Dent (28) technique. Glass plates of about 3 by 3-1/4 inches were used as holders for the strips. All the sugars were removed, as shown by spraying and heating of the strips.

On taking the solutions to dryness in a desiccator a definite light-brown coloration was evident. It was only partially removed with a small amount of decolorizing carbon. If the colored matter pre-existed in the chromatographic paper, then prewashing the paper with water would possibly eliminate such impurities.

DETERMINATION OF CONSTITUENTS OF DISACCHARIDES

HYDROLYSIS

In order to determine what monosaccharides were combined to form the particular disaccharides, about 2 mg. of the material was hydrolyzed with

1 cc. of 0.5 N hydrochloric acid in a sealed tube in a steam bath for four hours. The hydrochloric acid was then replaced with acetic acid by adding about 3/4 cc. (1.5 meq.) of the acetate form of Amberlite IR-4B to the solution and stirring for several minutes. After filtering and washing, the solution was taken to dryness on a steam bath or in a vacuum desiccator.

OXIDATION AND HYDROLYSIS

If the disaccharide was found to consist of two different monosaccharides, a sample was oxidized to the aldonic acid and then hydrolyzed and chromatographed. Since the acid did not appear as a spot, the single spot that was obtained was for the monosaccharide that was on the non-reducing end of the disaccharide.

The oxidation was carried out in bromine water in the presence of barium carbonate as a buffer. The use of a sparingly soluble carbonate as a buffer in such a reaction was first made by Clowes and Tollens (29). At the end of the reaction period the ions were removed from the solution by methods similar to those of Hudson and Isbell (30).

Four milligrams of the disaccharide was added to 1 cc. of water containing about 1.3 λ (4.0 mg.) of bromine and 6.0 mg. of barium carbonate. The reaction was carried out in the dark at room temperature in a small glass container with a ground glass stopper. During the reaction time of two days, more bromine or barium carbonate was added if it appeared that they were about to be consumed.

At the end of the reaction period the excess bromine was removed with a stream of air. The solution was made acid to methyl red by adding one to ten λ of 5 N sulfuric acid. The precipitate was removed by centrifuging in a centrifuge tube of 1-cc. capacity. The other reactions and removal of precipitates were accomplished in such tubes.

The bromide ions were removed as silver bromide after the addition of enough silver carbonate to react with all the bromine, assuming it was all converted to bromide ions. The soluble silver was removed as the sulfide after passing hydrogen sulfide through the solution for several minutes. The excess hydrogen sulfide was removed with an air stream.

The solution (now about 0.7 cc.) was made 1 N in acid by the addition of 17 λ of concentrated sulfuric acid. After removal of precipitated calcium sulfate, hydrolysis was carried out in a sealed tube on a steam bath for three hours. After the acid was changed to acetic acid by means of Amberlite IR-4B, the solution was evaporated, and a portion of it was chromatographed.

PRESENTATION AND DISCUSSION OF DATA

OBTAINING THE DISACCHARIDES

ACETOLYSES

As previously mentioned, the starting material used in this work was the slash pine alpha-cellulose prepared by Leech (21). Since he obtained from an acetolysis of the alpha-cellulose a small amount of material that seemed to be a disaccharide of glucose and mannose, his acetolyzing procedures were used, with the exceptions given on page 14. There were two acetolyzates that were obtained and used in this work. In each case 93 g. of air-dry alpha-cellulose (92.3% oven-dry weight) were added to 840 cc. of acetolyzing medium. The first one was subjected to acetolysis conditions for eleven days, and the second one was for nine days. Although it could not be predicted in what length of time acetolysis would yield a maximum quantity of the desired disaccharides, it was thought that a longer time than Leech's seven days would be advisable. After one week he found that about 50% of the acetolyzate still had a degree of polymerization of four or more.

RECOVERY OF ACETOLYZATES

Acetolyzate I

After precipitation into three volumes of ice water containing 123 g. of sodium acetate, the first acetolyzate was made almost neutral by adding sodium bicarbonate. After standing overnight the pH was 6.3. About 1000 g. of the bicarbonate was used, and it was added slowly, with stirring, over a period of several hours in order to prevent losses because of foaming.

Leech (21) was able to obtain a reduction in the foam by adding small amounts of chloroform to the mixture, but in the larger volumes handled in this work the reduction was only a fleeting one. If the chloroform addition was continued, the precipitated acetates became sticky and hard to work with.

To extract the acetates, chloroform was added to the mixture, which was shaken thoroughly in separatory funnels. The shaking was too vigorous, for even after standing for a day there was still a large emulsion layer between the chloroform and water layers. A good portion of the chloroform was separated from the emulsion by centrifuging. The bottom chloroform layer was removed by means of a glass tube that had been drawn out almost to a capillary. Other attempts to break the emulsion were unsuccessful.

The total chloroform solution was concentrated to a brittle foam in vacuo in a water bath at 50°C. (Unless otherwise specified, other concentrations referred to in this report were accomplished under the same conditions.) The foam was dried further in a vacuum oven at 50°C. The yield was 81 g., or a little over half of what it would have been if all the acetolyzate had been recovered.

The acetolyzate was added to acetone, and after standing overnight the insoluble material (5 g.) was removed by centrifuging. The soluble materials were then chemically "stabilized" by passing the solution through a bed of 45 g. of Magnesol*-Celite (5:1 by weight) on sintered glass. The bed was washed with several volumes of acetone, and the resulting solution was concentrated to a brittle foam. The foam was dried for one more hour

* A synthetic, hydrated, magnesium acid silicate manufactured by the Westvaco Chlorine Products Company.

in vacuum ovens. However, 60 g. of the material were in an oven that had unknowingly been changed from 50 to 100°C. A light-tan syrup resulted. After drying, the darkest portions were discarded and the 50.5 g. remaining were redissolved in acetone, passed through Magnesol-Celite again, evaporated, dried (at 50°C.), and weighed; yield, 49.5 g.

Acetolyzate II

To avoid the difficulties of emulsion formation, the second acetolyzate was recovered by filtration rather than extraction with chloroform. It was precipitated as in the first case but was recovered by filtering in several stages through Whatman No. 1 paper on a large Büchner funnel. It was slurried in a small amount of water and filtered again in order to remove as much of the acid as possible. After slurrying with water again it was neutralized with sodium bicarbonate. After standing overnight the pH was 6.3.

The acetates were filtered, and after standing in the air for two days the drying was completed in 50°C. vacuum ovens in 14 hours. The yield was 134 g., but 30 g. were insoluble in acetone. The soluble material was stabilized, evaporated to a brittle foam, and dried in a vacuum desiccator.

Discussion

The effect that the excess heating had on acetolyzate I is not definitely known, but the recovered acetates produced chromatograms, upon deacetylation, identical with those obtained from the other acetolyzate. When a sample of Leech's acetolyzate was deacetylated, it also produced the same type of chromatogram. In particular, besides the major spot for cellobiose in the disaccharide region there were two spots below and one

spot above cellobiose in each case. Later work discussed in the section "Disaccharides 2-A, 2-B, and 4" showed that actually six disaccharides were present instead of just four, but the two new ones were also shown to be present in the portion that was not heated at 100°C.

In addition to the two acetolyzates mentioned above, a third one was obtained, but it was not actually used in this work. However, the yield of recovered material was significantly greater than in the other two cases, so the method of recovery deserves mention.

The acetolysis and neutralization were conducted as for the first acetolysis, but the acetates were extracted from the water by gently mixing with chloroform. A small amount of stable foam was produced even then. However, when it was observed that the emulsion was of the water-in-oil type it became evident that the chloroform could be recovered simply by washing the emulsion several times in the centrifuge with fresh chloroform. This was done, and the yield of chloroform-soluble substances was 150 g.

Therefore, the techniques were finally perfected to recover by far the major portion of the acetates.

In addition, the partial removal of the cellobiose octaacetate from the other acetates was obtained by fractional crystallization. Upon concentration of the acetone solution of the acetates to 500 cc. and then setting aside, crystals of crude cellobiose octaacetate developed. After allowing the solution to stand in the cold overnight, 22 g. of the crude crystals were separated by filtration. By further concentration of the acetone solution or by the use of a different solvent such as warm ethanol,

more of the cellobiose octaacetate could probably have been removed. If enough of the material could be separated in this way, then complete separation of the disaccharides on a carbon column might be possible. However, this type of separation was not made.

DEACETYLATION

The 49.5-g. portion of acetolyzate I was deacetylated by the methods already described. After the neutralization of the methanol solution, the insoluble material was removed and washed with 100 cc. of methanol on sintered glass. The insoluble material was soaked and partially washed on the filter with water. The final residue yielded 1.3 g. of airdry material, or less than six per cent of the total carbohydrate material--assuming about 50% yield of carbohydrates are obtained from the acetates. The residue was not investigated further.

The methanol-insoluble but water-soluble substances amounted to about 20% of the total carbohydrates, and a paper chromatogram indicated that a small proportion of them were mono- and disaccharides. This would indicate that inadequate washing with the methanol was obtained, but the loss of disaccharides was considered so small that it was ignored.

Acetolyzate II (100 g.) was deacetylated and washed with methanol in the same way as for acetolyzate I. With the thought that possibly the methanol-insoluble but water-soluble fraction might be investigated later, an attempt to separate it from the other insoluble material by successive water washes in the centrifuge was made. After the first wash the insoluble material was of such a character that it was impossible to centrifuge or

filter it even with the aid of Celite. The fractionation attempt was then abandoned.

The methanol-soluble portion gave a paper chromatogram that seemed to be the same in every respect as that given by acetolyzate I.

If this work were to be repeated, it would possibly be advantageous to wash the insoluble material not with dry methanol but with aqueous methanol. This would insure the removal of all the desired disaccharide materials. If another fraction containing the higher, but soluble, oligosaccharides were desired, a higher percentage of water could then be used in the washing liquid.

CARBON COLUMN

Preliminary Trials

In the preliminary attempts to find a method suitable for obtaining complete separation of the desired disaccharides from all other carbohydrate material, the deacetylated acetolyzate I was used after first deionizing with Amberlite IR-120 and Amberlite IR-4B. The general methods given by Samuelson (31) were used in the operation of the columns containing these resins.

Separation was first attempted on a cellulose powder column prepared and operated according to the directions of Hough, Jones, and Wadman (32). These workers were able to separate two disaccharides using 95% ethanol as developer, but in this case there was no complete separation of the individual disaccharides. Complete separation could possibly have been obtained if other developers had been tried, but in view of the small capacity of a cellulose column it was decided to try other methods first.

The data of Whistler and Durso (33, 34) indicated that a carbon column could be used to effect a separation, or partial separation, of different disaccharides from one another. Their data also showed that it would not be necessary to remove the sodium acetate that is in the solution after the deacetylation step. After a trial on a small column showed that the carbon gave as good results as were obtained on the cellulose column, a large column was prepared and operated by methods already described.

First Development

Operation

After packing the column, it was washed with 25 liters of water, the first part of which contained some fine material that was washed from the column. The carbohydrates obtained from acetolyzate II and the remaining ones obtained from acetolyzate I were combined (800 cc. of water solution) and added to the column.

The column was then washed with approximately 17 liters of water and 25 liters of 3% ethanol. Collection of fractions began as soon as the sodium acetate began to come through, as visually indicated by change in the index of refraction of the solution.

Fraction one was 2.5 liters; two, three, and four were 9.5 liters each; and five was about 7.0 liters. The change from water to ethanol came after the first liter of fraction three.

Each of the collected fractions had added to it a small amount of chloroform and enough toluene to cover the top surface. They were then stored at 70°C. About 100 cc. of solution from each of the fractions were

removed, concentrated on a steam bath, and spotted on paper chromatograms in order to determine what carbohydrates were in each fraction.

The main constituents (identified chromatographically only) in each fraction are listed in Table II in order of decreasing amounts. The total weight of each fraction after taking to dryness is also given. The disaccharides of unknown structure have been given numbers, by which they will be identified throughout the rest of this thesis. Disaccharide 1 has the greatest extent of travel of a paper chromatogram; disaccharide 2 has the next to greatest travel and occurs just below cellobiose; and disaccharide 3 has the least travel and occurs just above cellobiose. It was shown later that disaccharide 2 was actually two disaccharides (2-A and 2-B) and that another disaccharide (4), corresponding with cellobiose when the basic solvent was used, was present.

TABLE II

FRACTIONS OF FIRST CARBON COLUMN DEVELOPMENT

Fraction	Weight, g.	Constituents
2-C	-----	Monosaccharides
3-C	2.3	Glucose and mannose only
4-C	1.17	Cellobiose, disaccharides 3 and 2, glucose, and mannose
5-C	0.745	Disaccharide 3, glucose, mannose, and some cellobiose and disaccharide 2

Difficulties

The reason why only five fractions were collected was that during the collection of the fifth fraction air was accidentally pulled into the top of the column, and definite channeling of the liquid down the column was evident thereafter. It was then decided to repack the column and start over.

A good portion of the liquid in the column was removed and collected in fraction five by sucking air through the column for several minutes. The column was extruded, cut into thin slices, and air dried. The material was converted into a uniform powder again by working up with the hands after placing it in a Pliofilm bag, and was then repacked in the column and washed with 20 liters of 30% ethanol in order to recover the sugars that still remained on the column.

In the concentration of this sugar solution to remove the ethanol and reduce the volume, three complications developed. The first occurred while concentrating a minor portion of the solution in the small circulating evaporator; a part of it went to dryness and was subjected to 100°C. temperature for five to ten minutes. The resulting carbohydrates were chromatographed to see if any detectable change had occurred. None was detected, but the chromatogram was not developed long enough to be absolutely sure of the result. By the time this was known, the solution had already been combined with the major portion of the recovered sugars.

The second complication developed while concentrating the major portion of the recovered solution in the large circulating evaporator. It developed a crack, and up to one-third of the solution was lost. The third complication became evident when the remainder of this solution was being further concentrated in the smaller circulating evaporator. It developed a definite cloudy-pink coloration which was partially removed by extraction with chloroform and then completely removed by filtration through acid-washed asbestos. It is possible that microbiological action had started in the solution.

The solution was then added to the carbon column again.

Second Development

After placing the sugars on the column, it was washed with 76 liters of water. Solutions of increasing ethanol content were then used, and a total of thirty fractions of about 9.5 liters each were collected over a period of about eight days. Table III shows the percentage of ethanol in each fraction.

TABLE III

ETHANOL USED FOR DEVELOPMENT						
% Ethanol	3	5	5-1/2	6	8	15
Number of fractions	10	8-1/2	3-1/2	2-1/2	4-1/2	1
Fractions (D series) 1-10	11-18	1/2 of 19	23-24	1/2 of 25	30	
	&	&	&	&		
	1/2 of 19	20-22	1/2 of 25	26-29		

Except for traces of what were taken to be trisaccharides, fractions 17-26D produced spots corresponding only to cellobiose and disaccharide 1, and they yielded a combined weight of 0.755 g. of material. Fractions 4-16D yielded spots corresponding to cellobiose only.

The contents of fractions 1-3D were only tentatively identified on chromatograms as being the following materials:

Fraction 1D (0.057 g.)--a heavy spot corresponding to cellobiose (later shown to be mainly disaccharide 4) with traces of disaccharide 3 and a new disaccharide (5) that occurred in approximately the same place as disaccharide 1. However, it was not identical with disaccharide 1, since that disaccharide comes off the column much later.

Fraction 2D (0.231 g.)--about equal amounts of disaccharide 3 and disaccharide 5, with small amounts of cellobiose, disaccharide 2, and glucose.

Fraction 3D (0.925 g.)--predominantly cellobiose, along with a small amount of disaccharide 5 and a lesser amount of glucose.

Discussion

The results from both developments of the column show that it was operating normally. It should be noted that the order of removal of the disaccharides from the column was not the same as for a cellulose column or a paper chromatogram.

Evidence of the disaccharide 5, which was obtained from the second development of the column, was not obtained in the first development, in the operation of the small carbon column on which the preliminary tests were run, or in the operation of the cellulose column. Therefore, it was assumed that the treatment received by the carbohydrates between the first and second developments gave rise to this new disaccharide. More specifically, that either the 100°C. heating or the possible microbiological action (as evidenced by the development of the pink coloration) brought about the formation of the new disaccharide.

On hydrolysis of disaccharide 5, spots corresponding to glucose and to arabinose were obtained. On oxidation and then hydrolysis a single spot corresponding to glucose was obtained. It is not known how the monosaccharides were linked together, but if it were in a β , 1-3 fashion then this disaccharide could have been formed from cellobiose by removing one carbon atom from the reducing end of the molecule. Of the two mentioned treatments that may have brought about such an action it seems that microbiological action is the most plausible one. These possibilities are given with the greatest reservation.

Additional evidence that the disaccharide is an artifact is given by the comparatively large amount of material that was obtained. The intensity of the spots on the chromatogram indicated that at least 150 and maybe 300 mg. of material were present, although no significant amount of arabinose was reported by Leech to be in the original alpha-cellulose. In comparison with this, there were obtained only 1.06 gram of disaccharides that contained mannose, although the original alpha-cellulose assayed about 10% mannan. Also, of the three chromatographic spots for simple sugars that were obtained from the deacetylated acetolyzate in only very small amounts, the one corresponding to arabinose was the least of these. The spots for xylose and galactose were more intense. Certainly if disaccharide 5 were among the structural units of the alpha-cellulose, more significant quantities of arabinose would have appeared among the monosaccharides obtained from the acetolyses unless the disaccharide is unusually resistant to acetolysis.

Although it is concluded that the new disaccharide is an artifact, there can be no doubt that disaccharide 1, which was obtained from the same solution, was among the products of the acetolyses. This is true since the disaccharide was noted in the preliminary trials on the carbon and on the cellulose column.

SEPARATION ON PAPER CHROMATOGRAMS

Since the disaccharides of interest that were obtained from the carbon column were in each case accompanied by at least one other carbohydrate, it was necessary to obtain complete separation by an additional step. The materials used in this final step were large paper chromatograms.

Such chromatograms were considered to be the most convenient tool for the job, although a cellulose-powder column may have been adequate also.

Since the great majority of disaccharides 2 and 3 were in the fractions obtained from the first operation of the carbon column, their recovery was limited to these fractions, and thus the possibility of mixing them with an artifact was eliminated. Both disaccharides 2 and 3 were recovered from the same chromatograms. Some of the chromatograms were allowed to run too long, and about one-fourth of the quantity of disaccharide 2 was lost.

The amounts of each disaccharide, which were recovered as a dry syrup, are given in Table IV. The weight of original alpha-cellulose was 170 g., oven-dry basis.

TABLE IV

QUANTITY OF RECOVERED MATERIALS

Disaccharide	Amount, mg.	Percentage of Original Alpha-cellulose
1	290	0.17
2	170	0.10
3	600	0.35

Each of the disaccharides was checked and shown to be chromatographically pure when using the butanol-pyridine-water solvent, but acidic solvents later revealed that disaccharide 2 was actually a mixture of at least two disaccharides.

A calculated quantity of material that might have been obtained if all the acetolyzates had been recovered, the concentrator had not cracked, none of the material had run off the chromatograms, and all the disaccharides

had been recovered from each of the fractions, is estimated in Table V. Even at best, the estimated recovery would have been only 1.15%. It was subsequently shown that this total material contained more than 50% mannose, and therefore contained about 7% of the mannose that was present in the alpha-cellulose. If all of disaccharide 4 (see page 50) had also been recovered, then possibly 15% or more of the mannose would have been recovered in the disaccharides.

TABLE V

POSSIBLE QUANTITY OF RECOVERABLE MATERIALS

Disaccharide	Amount, mg.	Percentage of Original Alpha-cellulose
1	615	0.36
2	393	0.23
3	960	<u>0.56</u>
Total		1.15

CHARACTERIZATION OF DISACCHARIDES

DISACCHARIDE 1 (4-O-β-D-glucopyranosyl-D-mannose)

Upon hydrolysis of a small portion of the material, two spots of equal intensity were obtained on a paper chromatogram. They corresponded with known glucose and mannose. Upon hydrolysis after first oxidizing the reducing end of the molecule, only a single spot corresponding to glucose was obtained. This indicated that the disaccharide was a glucosyl-mannose.

Attempts were made to crystallize the free sugar by adding ethanol to the point of incipient turbidity to a concentrated solution of the sugar in water. Some small crystals developed, but attempts to make them

grow in size and quantity were failures. It was assumed that there were traces of interfering substances.

Acetylation

Since the attempts at crystallization of the free sugar did not yield a significant quantity of crystals, the material was acetylated and then crystallized. The methods of Thompson, et al. (35) were used.

Approximately 0.08 g. of freshly fused sodium acetate and 2 cc. of boiling acetic anhydride were added to 0.176 g. of disaccharide 1 in the form of a brittle foam. The mixture was boiled, with stirring, for several minutes, and then after cooling was poured into 10-15 cc. of ice water. After stirring for one-half hour to insure the hydrolysis of the acetic anhydride, the mixture was extracted twice with chloroform. Following concentration to a thick syrup, the material was left overnight in a vacuum desiccator over sodium hydroxide. The yield of dry material was 0.297 g., which is 85% of the theoretical value calculated on the basis of complete conversion of a disaccharide to an octaacetate.

The material was dissolved in 10 cc. of boiling 95% ethanol, from which long, needle-like crystals formed upon slowly cooling. After further cooling, the crystals were collected by filtration, washed with cold 95% ethanol, and air dried. The yield was 0.176 g.; m.p., 195-199°C.

After recrystallizing from 5 cc. of 95% ethanol the m.p. was 199-200°. Using Isbell's (36) recommendation for separation of the α and β isomers of the octaacetate of this disaccharide, two recrystallizations were made from 5 cc. of toluene.

The final crystals were dried for one-half hour at 60°C. in a vacuum oven and yielded 0.113 g. of material.

Properties of the Acetate

Melting Point and Specific Rotation

The melting point, determined in an oil bath that had a temperature rise of 2-3° per minute near 200°C., was 201.5-202.5°C. Under the same conditions both a known sample* of octaacetyl 4-O-β-D-glucopyranosyl-α-D-mannose, and a mixture of the two materials melted at 202-203°C.

In Table VI comparative data on the octaacetates of disaccharide 1 and on the known glucosyl-mannose are given.

TABLE VI

	M.P., °C.	$[\alpha]_D^{20}$ (in CHCl ₃)
Octaacetate of Disaccharide 1	201.5-202.5	36.0 ±0.8 (c,3;dm,2)
Data of Brauns (37)	202-203	36.2
Data of Isbell (38)	203	36.3 (c,7)
Data of Haskins, et al. (39) ¹	199-200	36.5 (c,0.8;dm,4)

¹ Haskins did not attempt to obtain a particularly pure material.

X-ray Powder Diagram

Powder x-ray diffraction diagrams of the acetate prepared from disaccharide 1 and of the known acetate were made by Joe Thomas under the direction of Professor George Clark (40) at the University of Illinois. The results are given in Table VII. In the column headings, d gives the interplanar spacings expressed in Angstrom units, and I/I₀ gives their relative

* Obtained from Dr. J. Green. Sample apparently originally came from the National Bureau of Standards.

intensities as expressed as a fraction of the strongest line of the diagram. It is apparent that the two materials are crystallographically identical.

TABLE VII

X-RAY DATA

Known Acetate		Octaacetate of Disaccharide 1	
d	I/I ₀	d	I/I ₀
10.04	1.00	9.95	1.00
8.07	.70	8.18	.70
5.65	very weak (.05)	--	--
4.93	.10	4.93	.20
4.55	.50	4.52	.50
4.18	.10	4.18	.20
3.94	.10	3.93	.20
3.61	.40	3.56	.10
3.31	.10	3.26	.05
3.06	diffuse	3.02	.05
2.83	.40	2.83	.05
2.63	very weak	--	--

Carbon and Hydrogen Analyses

Calculated for an octaacetate of a disaccharide, C₂₈H₃₈O₁₉: C, 49.56; H, 5.64. Found: C, 49.75 and 49.39; H, 5.74 and 5.61.

Conclusions

It is concluded that the acetate prepared from disaccharide 1 is identical with the known substance, octaacetyl 4-O-β-D-glucopyranosyl-~~α~~-D-mannose. The conclusion is based on the fact that the observed melting point, mixed melting point, specific rotation, x-ray powder diffraction pattern, and carbon and hydrogen content of disaccharide 1 all agree with those of the known substance.

DISACCHARIDE 3 (possibly 4-O- β -D-mannopyranosyl-D-glucose)

Several milligrams of the disaccharide were hydrolyzed, and spots of equal intensity corresponding to glucose and mannose were obtained on a chromatogram. Upon hydrolysis following oxidation of the reducing end of the molecule, a single spot corresponding to mannose was obtained. This is evidence that the disaccharide is a mannosyl-glucose.

Acetates

After an unsuccessful attempt to crystallize the free sugar from methanol-butanol and from ethanol-water, attempts were made to prepare a crystalline octaacetate. About 0.36 g. of the sugar in the form of a brittle foam was acetylated and recovered using the same procedures as for disaccharide 1. The chloroform solution of the acetate yielded 0.58 g. of dry material, which is 81% of the calculated value.

Attempts were made to obtain crystals of the acetate from 95% ethanol, toluene-ether, toluene-petroleum ether, ethyl ether, and acetone, but only oils or syrups resulted. In view of these difficulties, a portion of it was separated into several fractions by the use of a Magnesol column. Exactly 100 mg. of the acetate were separated using a packed number 2 chromatographic tube. The packing and operating procedures of Leech (21) were used.

After filling and packing the tube it was wetted with benzene. The acetate was added to the column after being dissolved in several cc. of benzene, and then the column was developed with 100 cc. of benzene-absolute ethanol (90:1 by volume). After the extruded column was streaked with a 1% solution of potassium permanganate in 10% aqueous sodium hydroxide, there

became evident one major band and two or three smaller and less-well-defined bands above the main one. The acetates were removed from the different segments of the column by soaking and washing with acetone. The main band yielded 40 mg. of material. After dissolving part of it in ethyl ether and adding petroleum ether until a slight turbidity was obtained, only an oil developed. When the acetate was dissolved in several cc. of 95% ethanol and then diluted to less than 40% ethanol a very small amount of flocculent precipitate developed after standing for several days. There was no evidence of crystals. This material was later accidentally spilled. No attempts were made to crystallize the other fractions, for reasons given below.

Attempt to Crystallize the Free Sugar

After receiving a sample and data on a mannosyl-glucose disaccharide isolated from Iles Mannan by Smith and Srivastava (41) of the University of Minnesota, investigations were again turned toward obtaining the crystalline free sugar. Smith believed his disaccharide to be 4-O- β -D-mannopyranosyl- α -D-glucose, but the actual proof of its structure was not completed at this writing.

Spots for his disaccharide and for disaccharide 3 had the same mobility on a paper chromatogram when either butanol-pyridine-water (6:4:3) or ethyl acetate-acetic acid-water (9:2:2) were used as solvent, even for prolonged periods.

The disaccharide 3 material (0.14 g.) that had not been acetylated was in the form of a clear, brittle solid, and a small amount of water was added to it and stirred. A slight amount of cloudiness was caused by very small particles whose shape could not be distinguished under a microscope.

In the hopes that they were crystal nuclei, and in order not to dissolve them, several drops of 95% ethanol were stirred into the solution, which was then left overnight in an atmosphere saturated with 95% ethanol. The syrup was then partly cloudy, and the polarizing microscope revealed that small crystals had developed. All attempts to make these crystals grow in size and number failed.

A small amount of the syrup was diluted with methanol, and the solid material was collected by filtration. It was brownish in color. Apparently the slight amount of coloring matter in the solution was concentrated on, or precipitated with, the crystals. Possibly the same impurities prevented the crystals from growing readily in the solution.

Another small amount of the syrup was transferred to another container and covered with 95% ethanol. After several days none of the syrup seemed to have been dissolved. It was stirred and set aside for several weeks. All the alcohol had then evaporated and five to ten large crystals remained. By this time crystals had been obtained more easily from the deacetylated acetate than from the original syrup.

Deacetylation and Crystallization

The 0.36 g. of the remaining acetates were developed with 500 cc. of benzene-absolute ethanol (90:1) on Magnesol in a number 3 chromatographic tube. Recovery was made in two fractions. Fraction 1 was the leading, and main, band and yielded 0.21 g. Fraction 2 was obtained from two or three remaining bands and yielded 0.12 g. Both fractions were decolorized with a little carbon while in an acetone solution. After concentration to a brittle foam both fractions were deacetylated with 0.2 N sodium methylate

solution, using the techniques employed in the case of the other acetates.

The fractions were filtered through a small bed of Celite and were then desalted by means of a small carbon column. The 15% ethanol solutions of the sugars recovered from the column were taken to dryness in vacuo after successive additions of absolute ethanol. After taking up in water they were filtered and taken down to a water-white syrup in a desiccator.

Enough water was added to Fraction 1 to give a fluid syrup, and 95% ethanol was added dropwise almost to the point of permanent turbidity. (Locally, an oil formed after the addition of each drop of ethanol.) After standing for several hours while loosely covered with a watchglass numerous very small crystals were evident. The dish was then left overnight in a closed vessel containing 85% ethanol. Most of the solution evaporated from the sugar and left a good crop of white crystals.

The crystal mass was broken up with a stirring rod, and several drops of 40% ethanol were added to dissolve any remaining syrup. This was immediately followed by the addition of a small amount of 85% ethanol, and the container was left overnight in an atmosphere saturated with 90% ethanol. Several cubic centimeters of 95% ethanol were then added to the crystals, the mixture was cooled, and the crystals separated on a micro, sintered-glass filter.

The yield of crystals after drying to constant weight in a vacuum oven at 55°C. was 51 mg. A higher yield would probably have been obtained if a higher percentage of ethanol had been used. The physical constants of the material are given in Table VIII, in comparison with those of Smith's disaccharide.

TABLE VIII

PHYSICAL CONSTANTS

	M.P. (uncorr.), °C		[α] _D ^{20°}	
			Initial	Equilibrium
Disaccharide 3	200-201	Smith's disaccharide		
Smith's disaccharide	199-200	(c, 1.0) (41)	+30°	+19°
Mixture	199-200	Disaccharide 3		
		(c, 1.59)	+37°	+19°

Melting Point and Specific Rotation

The melting points were determined simultaneously in an oil bath with a temperature increase of about 1-1/2 degrees per minute approaching the melting point. The melting-point tubes were placed in the bath at 195°C., for after a sample of disaccharide 3 was placed in the oil bath at 80°, it began to discolor at about 150° and was completely melted at 175°C.

Melting of all the samples was accompanied with partial decomposition, as evidenced by the discoloring of the material, the formation of bubbles in the melt, and the yielding of spots corresponding to glucose and mannose, as well as for the original disaccharide on a paper chromatogram of the melts. Smith reported (41) that the corrected melting point of his material was 203°C.

Although the equilibrium specific rotations of the two disaccharides are the same, there is a discrepancy in the values given for the initial rotations. This could possibly be explained by the fact that the points on the graph did not permit a very accurate extrapolation to zero time of the rotation in the case of disaccharide 3.

Carbon and Hydrogen Analyses

Calculated for a disaccharide, C₁₂H₂₂O₁₁: C, 42.10; H, 6.48. Found: C, 42.03 and 41.91; H, 6.59 and 6.26.

X-ray Powder Diagram

TABLE IX

X-RAY DATA

Smith's Disaccharide		Disaccharide 3	
d	I/I ₀	d	I/I ₀
15.54	.10	15.54	Very faint
-----	-----	10.85	1.00
-----	-----	9.95	1.00
7.61	.50	-----	-----
6.91	.50	-----	-----
5.24	faint	-----	-----
4.84	.05	4.98	faint
3.94	.70	3.97	.20
-----	-----	3.79	Very faint
3.60	1.00	3.63	.50
-----	-----	3.45	.40
3.31	.10	3.27	.30
3.16	.10	3.09	.20
2.83	weak, diffuse	2.81	diffuse, faint
2.58	weak, broad	2.63	diffuse, faint
2.41	weak, broad	2.41	?
2.23	weak, broad	-----	-----
2.04	weak, broad	-----	-----
1.88	weak, broad	-----	-----

X-ray powder diagrams of disaccharide 3 and of Smith's disaccharide were also obtained from the University of Illinois. They were found to be quite different, as can be seen from Table IX. Therefore, at least crystallographically the two materials are not identical. As for the possibility that the two materials are just polymorphic modifications of the same chemical compound, Professor Clark (40) stated: "In previous work in this field, we have found many cases of polymorphism for compounds of the same analysis and even the same properties in solution such as specific rotation." In addition, Mark (42) in a brief but general discussion of phase transitions in organic compounds stated that most organic compounds show polymorphism.

Therefore, it is possible that the two materials are identical chemically, but that they crystallized in two different patterns. If this is the case, then it is either mere coincidence that the two crystal types have the same melting point (actually 1° apart) or else one of the crystal forms is transformed into the other prior to fusion. This latter case is a definite possibility since neither of the substances was stable at temperatures near the melting point.

Conclusions

No definite conclusions can be drawn as to the structure of the mannosyl-glucose. The possibility exists that it is structurally the same as the one isolated by Smith, which he considered to be 4-O-β-D-mannopyranosyl-α-D-glucose. This is true because the two substances could not be separated chromatographically, they had the same melting point, and they gave the same equilibrium specific rotation in water. The fact that the two materials are crystallographically different casts some doubt about their being chemically identical, but it does not rule out the possibility.

DISACCHARIDES 2-A, 2-B, and 4

Disaccharides 2-A and 2-B

Upon hydrolysis of the material originally termed disaccharide 2, spots on a chromatogram corresponding to both glucose and mannose were obtained. The mannose spot predominated, however. The same results were obtained when the material was hydrolyzed after first oxidizing the reducing end of the molecules.

Although only one band on a paper chromatogram could be obtained from the original disaccharide material when butanol-pyridine-water (6:4:3) was

used as a solvent, there were definitely two bands that were at least partially separated upon prolonged development (two weeks) with ethyl acetate-acetic acid-water (9:2:2). The top band (disaccharide 2-B) was several times more intense than the bottom one (disaccharide 2-A).

After running a chromatogram in the 9:2:2 solvent using Whatman 3 MM paper, the top half of disaccharide 2-B and the bottom half of disaccharide 2-A were eluted and hydrolyzed. Disaccharide 2-B yielded a strong spot corresponding to mannose and a very weak one for glucose. Disaccharide 2-A yielded a strong spot for glucose and a very weak one for mannose.

Therefore, disaccharide 2 actually consists of at least two disaccharides, and on the basis of the chromatographic evidence it is possible that one of them (2-B) is a manno-*bio*se and the other (2-A) a glucobio-*se*. However, under the chromatographic conditions used, they apparently could not be completely separated from each other, and any conclusions must be tentative.

Because of its position below them on a paper chromatogram, disaccharide 2-A is neither cellobiose, gentiobiose, maltose, nor isomaltose.* Therefore, if the assumption is made that both molecules of glucose in the disaccharide are in the pyranose form, they must be linked together either in a 1-2 or in a 1-3 manner.

Leech (21) also obtained data that could be taken as evidence that besides cellobiose there was another disaccharide containing only glucose that was present in an acetolyzate of the alpha-cellulose. His Fraction 3 of the acetates occurred on a Magnesol column below α -cellotriose

* Sample obtained from Allene Jeanes, Northern Regional Research Laboratory.

hendecaacetate and above both α - and β -cellobiose octaacetate, and was therefore probably a disaccharide acetate. Upon deacetylation and hydrolysis he obtained essentially all glucose. This material could have been identical with disaccharide 2-A, but further work would be necessary to verify such an hypothesis. (If they were the same, then a Magnesol column might afford a convenient method of separating the acetate of disaccharide 2-A from the rest of an acetolyzate, including the acetate of disaccharide 2-B.)

Disaccharide 2-B (the mannobiose) is not 4-O- β -D-mannopyranosyl-D-mannopyranose*, for an authentic sample of this material was shown to be in a position slightly above the bands of disaccharide 2 on a chromatogram. No further work was done on establishing the structure of this disaccharide.

Since the chromatographic conditions in the early work were such that neither the second disaccharide of the disaccharide 2 substance nor disaccharide 4 (see Disaccharide 4) was evident, then there was still the question as to whether or not they might have arisen during the time the acetolyzate I was inadvertently heated at 100°C. To settle this question, a 6-g. portion of the acetolyzate I that had not been heated at 100°C. was deacetylated and worked up according to the procedures described for the major portion of the acetolyzates. Disaccharides 2-A, 2-B, and 4 were definitely present, and upon hydrolysis they produced the same monosaccharides as before. Therefore, these disaccharides were not produced during the heating.

* Sample obtained from Professor R. L. Whistler, Purdue University.
Cf. (43).

Disaccharide 4

In conducting the work on disaccharides 2-A and 2-B it was found that the butanol-pyridine-water solvent would separate neither maltose nor the known β , 1-4 manno-*bio*se from cellobiose. Therefore, either or both of these disaccharides could have been in the deacetylated acetolyzate, but they would not have been detected as such. Another solvent consisting of ethyl acetate-acetic acid-formic acid-water (18:3:1:4) did separate both of these disaccharides from cellobiose, however, and they occurred just below cellobiose and above disaccharide 2-B. They were not separated from each other.

On chromatographic reinvestigation of a sample of deacetylated acetolyzate I and of carbon-column fraction 1-D, they both produced a band of material (disaccharide 4) corresponding to maltose and the known manno-*bio*se. Disaccharide 4 seemed to be present in the total acetolyzate in at least as great a quantity as either disaccharide 1 or 3, and in fraction 1-D the band corresponding to cellobiose (butanol-pyridine-water solvent) was found to be predominantly disaccharide 4. It is possible that fractions 4-C and 5-C also contained disaccharide 4, but this could not be determined, since all of these fractions had been used up in the isolation of disaccharides 2 and 3.

The separation of disaccharide 4 for hydrolysis was accomplished in two steps. First, a chromatogram was developed with butanol-pyridine-water (6:4:3), which accomplished definite and complete separation between disaccharide 2 and cellobiose-disaccharide 4. Then the cellobiose-disaccharide 4 region was eluted and rechromatographed using ethyl acetate-acetic acid-formic acid-water (18:3:1:4).

The disaccharide 4 region was eluted, and after hydrolysis produced chromatographic spots indicating that the major portion of the hydrolyzate was mannose, but that appreciable amounts of glucose were also present. The fact that the mannose predominated is evidence that a manno-*bio*se was present. The glucose possibly came from a little overlapping cellobiose, but there is still a possibility that another disaccharide containing glucose (such as maltose) was also present.

TESTS FOR PRESENCE OF ARTIFACTS

SUBJECTING SIMPLE SUGARS TO ACETOLYSIS CONDITIONS

In order to determine whether these minor disaccharides obtained by the acetolysis procedures were formed by the methods of isolation or whether they existed in the original alpha-cellulose, two experiments were conducted. The first of these involved treating the simple sugars under the same acetylating conditions as those used on the alpha-cellulose, to see whether any disaccharide reversion products were formed.

A total of one gram of glucose and mannose (in a ratio of 3:1) was added to 9 cc. of the acetylating mixture as prepared for the alpha-cellulose and was shaken until dissolved. This ratio of glucose to mannose was approximately the same as the ratio of these monosaccharides which were present in acetolyzate I*. After standing at room temperature for nine days, the acetates were precipitated into ice water and neutralized to pH 6.3 with sodium bicarbonate. Extraction with chloroform yielded 2.11 g. of material, or 98% of the calculated amount.

* The ratio of the monosaccharides was obtained by a chromatographic procedure using a Recording Transmission Densitometer.

After deacetylation, a heavily loaded paper chromatogram of the sugars was run in the ethyl acetate-acetic acid-water solvent. The band for glucose was dark and up to eight centimeters in depth and was followed by a weaker mannose band, but there was no sign of any disaccharides. From this result it was concluded that none of the disaccharide spots evident upon chromatographing the deacetylated acetolyzate were caused by reversion of the simple sugars during the acetolysis.

TEST OF DEACETYLATION PROCEDURE

Since traces of sodium hydroxide were present in the sodium methylate solution during the deacetylation, it was also necessary to show that none of the minor disaccharides could have been produced by alkaline rearrangement of cellobiose. This was done by deacetylating a portion of the cellobiose octaacetate that was obtained from acetolyzate III, as previously described. After three recrystallizations from 95% ethanol this cellobiose octaacetate melted at 223-229°C., (229° is reported in the literature).

One-third of a gram of the recrystallized octaacetate was deacetylated using the techniques employed in the case of the other acetates and was chromatographed using ethyl acetate-acetic acid-water (9:2:2) as solvent. No disaccharide band other than for cellobiose was evident. (In the more heavily loaded portions, which yielded a band up to nine centimeters in depth, very faint monosaccharide spots considered to be glucose and mannose were present, but they possibly arose from monosaccharide acetate impurities in the starting material.)

Therefore, it was concluded that the procedures that were used could not have caused the formation of any of the disaccharides of interest, and that they existed as structural units in the original alpha-cellulose.

SIGNIFICANCE OF THE DISACCHARIDES

The isolation of a mannosyl-glucose disaccharide and a glucosyl-mannose disaccharide shows that glucose and mannose units were definitely linked together in the alpha-cellulose. Therefore, all the mannan in the alpha-cellulose could not have existed as a homopolymer. As to whether there were any mannan homopolymers present nothing can be stated conclusively. However, if an estimate of the recoverable amounts of each of the disaccharides in the acetolyzate can be used as a basis for judging the extent to which mannose and glucose were linked in the alpha-cellulose, then possibly one-fourth or more of them were so linked. This deduction requires the assumption that the mannose disaccharides and the mixed disaccharides were equally stable to further acetolysis. If this figure of one-fourth is of the right order of magnitude, then the probability that a large percentage of the mannan existed in polymers consisting solely of mannose units is not great.

Even though it has been shown that glucose and mannose units are chemically linked in the alpha-cellulose, there are still three possible reasons as to why the mannans in the alpha-cellulose are of the resistant type: (1) They exist in polymers that are rendered insoluble by physical occlusion in the insoluble cellulose of the fibers even though they are otherwise the same as the alkali-soluble hemicelluloses. (2) They are insoluble because they are in polymers having a higher degree of polymerization than the alkali-soluble hemicelluloses. (3) The polymers containing the mannan have branches or cross links that make them insoluble. The cross links could be between the mannan-containing polymers or between a mannan-containing polymer and an otherwise "true" cellulose polymer.

The statements of the preceding three possibilities has taken into account the work of Bradway (22) and of Giertz (19, 20) as discussed in the historical review. Their work indicated that essentially all the mannan in softwood alpha pulps was present in a relatively small number of the polymers.

The work described herein has no direct bearing on whether or not reasons 1 or 2 (on preceding page) are probable, but reason 3 appears to be a possibility. This is true since more than one type of linkage was present in the disaccharides that were isolated from the acetolyzates. Disaccharide 1 was definitely linked β , 1-4, disaccharide 3 was most probably linked in this fashion, and disaccharide 2-B was possibly so linked. By a process of elimination based on known compounds, the linkages within the two additional unknown disaccharides are probably not 1-4 bonds. The presence in the alpha-cellulose of linkages other than the predominant 1-4 type is a necessary but insufficient condition for having branches or cross links, since linear polymers could be linked by more than one type of bond also. There is also a good possibility that the disaccharide (2-A) that was not linked in a 1-4 manner was not associated with the resistant hemicelluloses at all, since it was composed solely of glucose and therefore could have been in the otherwise "true" cellulose.

Glucose has generally been found in hemicellulose hydrolyzates from conifers, but prior to the isolation and positive identification of a disaccharide containing both glucose and another sugar it was usually assumed that the glucose came from low-molecular-weight cellulose or cellulose fragments. If the classification of the mannan-containing polymers as resistant hemicelluloses is continued, it follows that glucose must now be considered as an integral part of at least some of the hemicellulose molecules.

POSSIBILITIES FOR FUTURE WORK

As yet the structures of disaccharides 2-A, 2-B, and 4 have not been determined; nor has the unequivocal identification of disaccharide 3 been completed. Future work could well be directed along these lines. One of the problems in such work would be in obtaining enough of these disaccharides. Three possible approaches to this problem are: (1) Investigation of different acetolysis conditions for greater yield of the disaccharides. (2) Running acetolyses on the hemicelluloses, or certain hemicellulose fractions, instead of the alpha-cellulose. (3) Using a dilute acid hydrolysis or enzymatic hydrolysis (or succession of such hydrolyses) on possibly a holocellulose in order to produce the disaccharides directly. In the latter case it would again be necessary to show that the disaccharides did not arise because of reversion.

If the acetolyzates of the carbohydrates are employed, then as discussed on page 51, Leech's method (21) of separation of the acetates should be investigated for the possibility of obtaining disaccharide 2-A.

This work has again raised, but not answered, the question as to whether branches or cross links exist in wood polysaccharides. Therefore, methylation of the polysaccharides followed by hydrolysis and identification of the hexoses containing two methyl groups would be of interest. If it could be shown that such sugars did not arise because of insufficient methylation of the original polysaccharide, then the presence of branches or cross links would be established.

SUMMARY OF RESULTS AND CONCLUSIONS

A summary of the results of the investigation of the disaccharide components of an acetolyzate of slash pine alpha-cellulose is given below:

1. A combination of acetolysis, deacetylation, carbon column separation, and paper chromatographic separation methods were adapted for use in the isolation of individual disaccharide degradation products of the alpha-cellulose. In addition to cellobiose, five disaccharides were obtained:

A. An amorphous form of 4-O-β-D-glucopyranosyl-D-mannose was identified by means of its crystalline octaacetate.

B. A crystalline mannosyl-glucose that was possibly 4-O-β-D-mannopyranosyl-D-glucose was isolated but was not conclusively identified; m.p., 200-201° (uncorrected); equilibrium $[\alpha]_{D}^{20} + 19^{\circ}$. The acetylated sugar was not crystallized.

C. A crude, amorphous substance that was considered to be a disaccharide corresponded chromatographically with known 4-O-β-D-mannopyranosyl-D-mannopyranose, and upon hydrolysis yielded mainly mannose by chromatographic methods.

D. An amorphous substance considered to be a disaccharide yielded chromatographic spots for only mannose.

E. An amorphous substance considered to be a disaccharide yielded chromatographic spots for only glucose, but it did not correspond chromatographically to the known disaccharides of glucose that are linked 1-4 or 1-6.

2. The disaccharides listed in parts 1A-1C were present in the acetolyzate to a greater extent than the other two (parts 1D and 1E),

and although the total amount of all these disaccharides was not recovered it is estimated that they existed in the acetolyzate to the extent of 1.5 to 2.0% of the original alpha-cellulose.

3. Upon subjecting a mixture of glucose and mannose to the same acetolysis conditions as used for the alpha-cellulose, no evidence of any disaccharide reversion products was obtained.

4. Upon deacetylation of cellobiose octaacetate, only a single chromatographic spot corresponding to cellobiose was obtained in the disaccharide region. This indicated that none of the disaccharides was formed by rearrangement of the cellobiose.

The conclusions that can be drawn from this work are as follows:

1. Glucose and mannose are definitely linked in the alpha-cellulose obtained from a slash pine holocellulose. This is the first time that a linkage between glucose and another sugar in the cell-wall polysaccharides of wood has been definitely established. Even though glucose has often been reported in hemicellulose preparation, either the glucose units must now be considered as integral parts of some of the hemicellulose molecules, or else the sharp line with which many investigators divide cellulose from the hemicelluloses must be considered to be nonexistent for this particular wood and possibly for the conifers in general.

2. The major portion of the disaccharides obtained from the resistant hemicelluloses seems to be linked in a β , 1-4 fashion.

3. Some disaccharides were obtained that were not linked in a 1-4 fashion. Such structural units may exist as variations in the pattern of a linear polymer or as branches or cross links in the polymers of the alpha-cellulose.

LITERATURE CITED

1. Whistler, Roy L., and Smart, Charles L. Polysaccharide chemistry. pp. 152-60. New York, Academic Press, 1953.
2. Schorger, A. W., Ind. Eng. Chem. 9:748-50(1917).
3. Nowotnowna, Anna, Biochem. J. 30:2177-83(1936).
4. Hägglund, E., and Klingstedt, F. W. Holzchemie. 1st ed. p. 78. Leipzig, Akad. Verlags, 1928.
5. Nishida, K., Hamashima, H., and Fukai, T., Cellulose Ind. (Tokyo) 11:330-5(1935); C.A. 30:3226.
6. Lenze, F., Pleus, B., and Müller, J., J. prakt. Chem. 101:213-64 (1921).
7. Heuser, Emil, and Dammel, Wilhelm, Cellulosechemie 5:45-53(1924).
8. Hägglund, Erik, and Bratt, L. C., Svensk. Kem. Tid. 48:125-31(1936).
9. Wise, Louis E., Ratliff, Evelyn K., and Browning, B. L., Anal. Chem. 20:825-8(1948).
10. Sherrard, E. C., and Blanco, G. W., J. Am. Chem. Soc. 45:1008-13 (1923).
11. Browne, C. A. Sugar analysis. p. 594. New York, John Wiley, 1912.
12. Hess, Kurt, and Lüdtke, Max, Ann. 466:18-26(1928).
13. Yundt, Albert P. The preparation, characterization, and hydrolysis of crystalline and amorphous xylan. Especially p. 103-09, 154-7. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry, 1949. 200 p.; Tappi 34:94-5(1951).
14. Huseman, E., J. prakt. Chem. 155:13-64(1940). Original not available; unpublished English translation by J. F. Smith consulted.
15. Battista, O. A., and Sisson, Wayne A., J. Am. Chem. Soc. 68:915(1946).
16. Morey, D. R., and Tambllyn, J. W., J. Phys. Chem. 50:12-22(1946).
17. Wethern, James D. A study of the molecular properties of the hemi-celluloses of black spruce. Doctor's Dissertation, Appleton, Wis., The Institute of Paper Chemistry, 1952. 69 p.; Tappi 35:267-71(1952).
18. Wise, Louis E., and Ratliff, Evelyn K., Arch. Biochem. 19:292-9(1948).

19. Algar, William H., Giertz, Hans W., and Gustafsson, Anne-Marie, Svensk. Papperstidn. 54:335-44(1951). English translation consulted.
20. Giertz, H. W., World's Paper Trade Rev. 138:1451-2, 1454, 1459-62, 1464(1952).
21. Leech, John G. Acetolysis products of slash pine alpha-cellulose studied by chromatographic methods. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry, 1952. 88 p.; Tappi 35: 249-53(1952).
22. Bradway, Keith E. An investigation of haze in cellulose acetates made from wood pulps. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry, 1953. 75 p.; Tappi 37:440-6(1954).
23. Steinmann, H. W., and White, B. B., Tappi 37:225-32(1954).
24. Zemplén, Geza, Ber. 59:1254-66(1926).
25. Lund, Hakon, and Bjerrum, Jannik, Ber. 64:210-18(1931); C.A. 25:3310.
26. Tu, C. C. Personal communication, 1954.
27. Lemieux, R. U., and Bauer, H. F., Anal. Chem. 26:920-1(1954).
28. Dent, C. E., Biochem. J. 41:240-53(1947).
29. Clowes, G. H. A., and Tollens, B., Ann. 310:166-70(1900).
30. Hudson, C. S., and Isbell, H. S., J. Am. Chem. Soc. 51:2225-9(1929).
31. Samuelson, Olaf. Ion exchangers in analytical chemistry. New York, John Wiley, 1953. 291 p.
32. Hough, L., Jones, J. K. N., and Wadman, W. H., J. Chem. Soc. 1949: 2511-16.
33. Whistler, Roy L., and Durso, Donald F., J. Am. Chem. Soc. 72:677-9 (1950).
34. Whistler, Roy L., and Durso, Donald F., J. Am. Chem. Soc. 73:4189-90 (1951).
35. Thompson, A., Anno, Kimiko, Wolfrom, M. L., and Inatome, M., J. Am. Chem. Soc. 76:1309-11(1954).
36. Isbell, Horace S., Bur. Standards J. Research 7:1115-31(1931).
37. Brauns, D. H., J. Am. Chem. Soc. 48:2776-88(1926).
38. Isbell, Horace S., Bur. Standards J. Research 5:1179-87(1930).

39. Haskins, W. T., Hann, Raymond M., and Hudson, C. S., J. Am. Chem. Soc. 63:1724-6(1941).
40. Clark, George L. Personal communication, 1955.
41. Smith, Fred, and Srivastava, H. Unpublished work, 1955.
42. Mark, H. Phase transitions in ordinary organic condensed systems. In Ott and Spurlin's Cellulose and cellulose derivatives. 2nd ed. Vol. 1 pp. 219-21. New York Interscience, 1954.
43. Whistler, Roy L., and Stein, Joan Z., J. Am. Chem. Soc. 73:4187-8 (1951).

ACKNOWLEDGMENT

In addition to the acknowledgments given in the body of the thesis, the author is indebted to the Analytical Department of the Institute for the carbon and hydrogen analyses reported herein.