AN ABSTRACT OF THE THESIS OF

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Title: DOUGLAS-FIR BARK; CARBOHYDRATES SOLUBILIZED BY THE ACIDIFIED SODIUM CHLORITE DELIGNIFICATION REACTION

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The inner bark of Douglas-fir was successively extracted with ethanol-water (4:1 v/v), benzene-ethanol (2:1, v/v), water, and 0.5% aqueous ammonium oxalate solution. The residue was reacted with acidified sodium chlorite, a commonly used reagent to separate lignin from carbohydrates. In general the reaction dissolves the lignin and leaves the carbohydrates as an insoluble "holocellulose" residue. However, a considerable amount of carbohydrates are often dissolved with the lignin. The primary objective of this work was to determine what carbohydrates were solubilized and how much was solubilized.

The solubilized materials were recovered as white solids by lyophilization. The solids were high in ash content (13.39%), but dialysis and re-precipitation did not lower the ash value.

The solubilized materials did not contain sulfur, phosphorus or halogens but did have some nitrogen (0.84%). The fraction was acid hydrolyzed and paper chromatography of the hydrolyzate showed ten amino acids.

The acid hydrolyzate also contained rhamnose, xylose, arabinose, mannose, galactose and glucose as shown by paper chromatography. The sugars were quantitatively analyzed by gas-liquid chromatography of their alditol acetates. The monosaccharide ratios were: glucose, 59.1; arabinose, 11.9; galactose, 3.9; mannose, 3.7; xylose, 1.0; rhamnose, 1.0. Rhamnose has not been previously reported in Douglas-fir bark.

Infrared spectroscopy, a carbazole-sulfuric acid color test, and paper chromatography showed the presence of uronic acids. However, the amount of uronic acids appeared less than in the starting material and it was concluded that the reaction did not result in significant oxidation of the primary hydroxyl groups on the polysaccharides to uronic acids.

The materials solubilized by the acidified sodium chlorite delignification reaction were methylated to block all of the free hydroxyl groups in the polysaccharides. The methylated material was hydrolyzed and identification of the monomers is expected to aid greatly in the elucidation of the polysaccharide structures.

Douglas-fir Bark; Carbohydrates Solubilized by the Acidified Sodium Chlorite Delignification Reaction

by

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VITA

Yu-chai Leslie Lai was born on April 25, 1947 in Tan-Tze, Taichung, Taiwan, Republic of China, the daughter of Chin-chih Lai and Fang-Zu Chang. She attended Tan-Tze Elementary School and First Girls' High School in Taichung. She went to National Taiwan University in 1965, and received a Bachelor of Science degree in June, 1969, from the Department of Forestry.

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DOUGLAS-FIR BARK; CARBOHYDRATES SOLUBILIZED BY THE ACIDIFIED SODIUM CHLORITE DELIGNIFICATION REACTION

I. INTRODUCTION

The chemical components which comprise the bark of Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] are not well understood. Two to three million tons of the bark are generated each year from forest commerce, but a relatively small portion of this natural raw material finds commercial outlets. Most of the bark is wasted. It is usually disposed of by burning which contributes to a general air pollution problem. Before new and more valuable uses of bark can be introduced, a better understanding of its chemical constituents and physical properties are needed.

Carbohydrates are the primary chemical components in bark and comprise 60-65% of Douglas-fir inner bark. However, little attention has been devoted to understanding the chemical and physical properties of these constituents. The carbohydrates of inner bark are grossly fractionated by the acidified sodium chlorite reaction commonly used to separate lignin material from carbohydrates. The reaction degrades and solubilizes the lignin constituents and leaves a carbohydrate fraction as an insoluble "holocellulose." However, a considerable quantity of carbohydrate materials are also solubilized. These materials are not well understood and little

or no effort has been made to recover them.

The work herein reported is a detailed chemical investigation of the carbohydrates solubilized by the acidified sodium chlorite delignification reaction. The study involves the use of modern techniques of separation, purification, analyses and characterization. The advent of these methods allowed a more comprehensive and complete understanding of bark materials than was previously possible.

II. HISTORICAL REVIEW

The work herein reported is concerned with Douglas-fir inner bark. This is a specific anatomical part of the bark and so a brief description of bark anatomy is included for purposes of definition.

For a detailed anatomical description of Douglas-fir bark, reference is made to Grillos (17), Grillos and Smith (18), Chang (9), and Ross and Krahmer (39). Briefly, however, bark can be considered to consist of inner bark and outer bark (Figure 1, page 4). The inner bark (phloem cells) is the portion from the vascular cambium to the cork cambium of the innermost cork layer. The outer bark (rhytidome) is everything to the outside of the innermost cork cambium (Figure 1, page 4).

The inner bark comes from the vascular cambium, that layer of living cells between the wood and bark which divide to form wood to the inside and bark to the outside. The inner bark is composed mainly of sieve cells, axial and ray parenchyma, and sclereids. Much of the inner bark is living in the living tree because many of the parenchyma and sieve cells remain alive as long as they are components of the inner bark.

Douglas-fir sclereids are short, sharply pointed, spindle-shaped fibers of a red brown color. They are often referred to as bast fibers. They are lignified cells and develop from axial

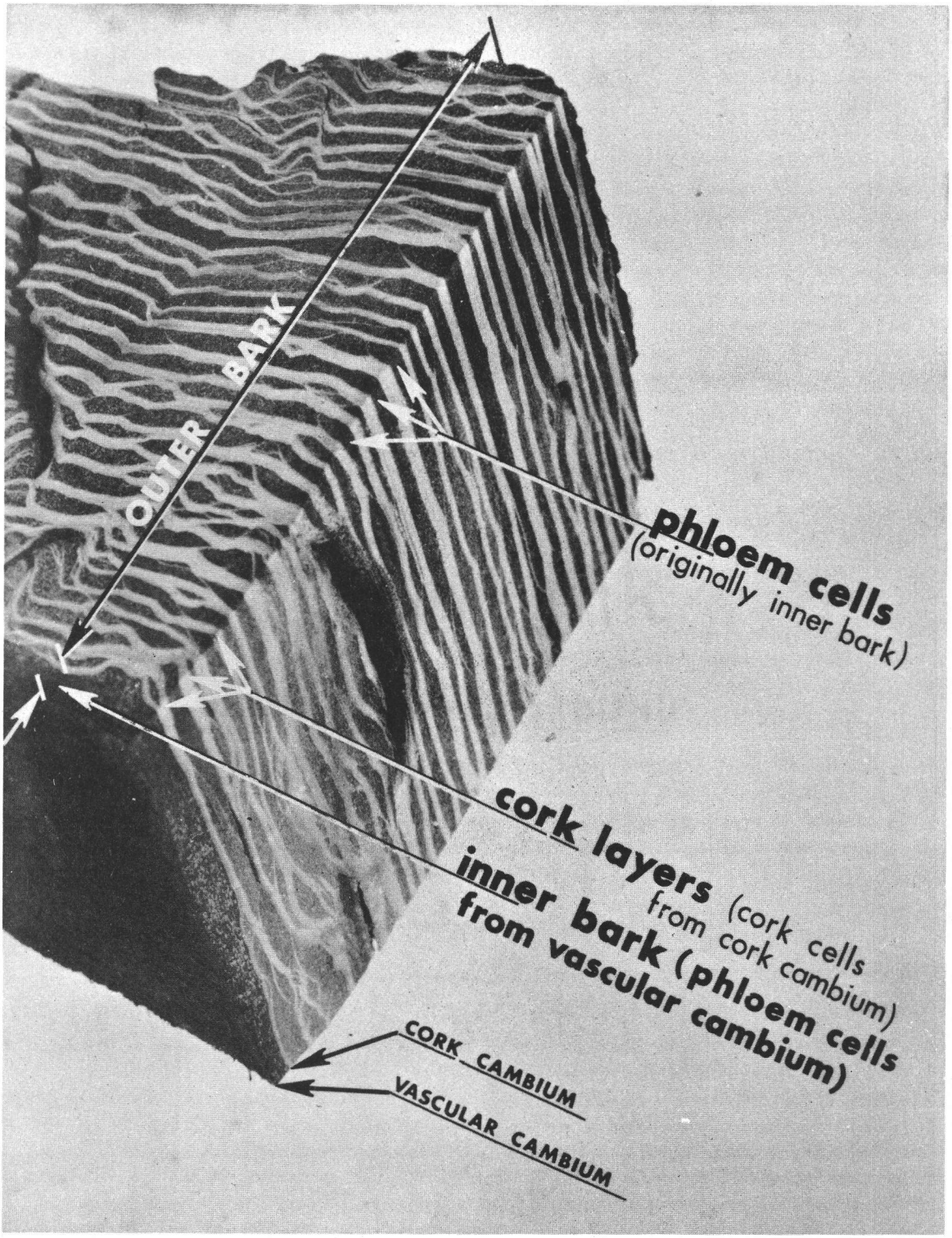


Figure 1. Anatomy of Douglas-fir bark.

parenchyma cells some distance from the vascular cambium. In becoming sclereids, axial parenchyma cells approximately 0.1 to 0.5 mm in length elongate to 1 to 2 mm by apical intrusive growth, and form thick walls. The sclereids are commonly straight and somewhate cigar-shaped. Kiefer and Kurth (25) and Ross and Krahmer (39) describe and illustrate the general appearance and position of the sclereids in Douglas-fir bark.

The outer bark of Douglas-fir consists of layers of cork in which growth layers are usually visible, as shown by Ross and Krahmer (39). Interspersed among the corky layers are areas of phloem tissue that contain the sclereids and other cell types found in the inner bark (Figure 1, page 4). The cork layers form from the cork cambium (Figure 1, page 4), which are living cells that were once living parenchyma cells of the inner bark. New cork cambia form in the inner bark and cut away part of the inner bark, which now becomes part of the outer bark. The cork cambium produces cork cells to the outside and a few storage cells to the inside. Cork cells have thin cellulose walls which are coated with suberin. Suberin is believed to consist largely of esters of hydroxy fatty acids (23, p. 633). The cork layers may also contain tannin and starch. All cells outside the innermost cork cells are dead because no food supply can pass through this layer of cork cells. This then results in an outer bark composed of cork cells and dead

phloem cells, which were once inner bark.

The studies on the anatomy of bark have shown it to be a complex physical material. The chemical composition of bark is equally complex. It is known, however, that the major constituents of bark are the carbohydrates, just as they are the major constituents in wood (28). However, little attention has been devoted to the carbohydrates present in the bark of trees. In contrast, the carbohydrates in the wood of trees have been extensively studied. Polysaccharides such as pectinic acids, galacturonogalactans, arabinogalactans, 4-O-methylglucuronoxylans, arabino-4-O-methylglucuronoxylans, glucomannans, galactoglucomannans, and cellulose have been isolated from the wood of numerous species of both gymnosperms and arborescent angiosperms and their chemical structures elucidated (55, p. 459; 3, 50, 19).

One reason for this has undoubtedly been the greater economic importance of wood as compared with bark. Another is probably to be found in the occurrence in bark of non-carbohydrate constituents such as suberin, tannins, phlobaphenes, and various phenolic compounds, all of which make the isolation of polysaccharides from bark difficult. Wood contains none or few of these components.

It has long been known that bark contains carbohydrates. Segall and Purves (43) reviewed the early literature to the extent of comparing the chemical composition of bark extractives as related to

species and age of the tree. Kurth (28) described the early work on bark in considerable detail. He reported that the barks studied contained hemicelluloses and celluloses and that the sugars associated with the hemicelluloses were glucose, galactose, mannose, arabinose, and xylose. Kiefer and Kurth (25) isolated a holocellulose fraction from the bast fibers, or sclereids, of Douglas-fir bark. Paper chromatographs of an acid hydrolyzate of the holocellulose showed the presence of glucose, galactose, mannose and xylose. The limited techniques of paper chromatography at that time did not resolve mannose and arabinose. Their fermentation procedures for quantitative analyses indicated an apparent absence of arabinose in Douglas-fir bark.

Painter and Purves (36) reported the first systematic attempt to classify the polysaccharides present in bark and to compare them with those in the wood of the same tree. They isolated at least six different carbohydrate fractions from the inner bark of white spruce and concluded that the bark contained (i) starch, mainly in the form of granules, (ii) much pectin material, consisting of pectinic acid, galactan, and arabinan, and (iii) hemicellulosic material, consisting of xylan and mannan components similar to those of wood.

Mian and Timell (33) studied the polysaccharides in white birch. They found that the bark contained a pectinic acid, an acidic xylan, and cellulose in addition to several other polysaccharides.

The carbohydrate composition of the original bark (Table 1, page 9) agreed closely with previously reported values for white birch bark (11).

In a separate study of the carbohydrates present in the phloem (inner bark) of white birch (Betula papyrifera Marsh.), Mian and Timell (51) succeeded in isolating several polysaccharides in high yields. However, the extraction gave evidence that only a few of the polysaccharides were obtained in a state pure enough to warrant further structural investigations. Hydrolysis of these polysaccharides yielded galacturonic acid, galactose, and arabinose in a ratio of 66:7:27 and also traces of glucose, xylose, and rhamnose units.

Timell (47) studied in detail the polysaccharides occurring in the bark of several gymnosperms each representing a different genus. After exhaustive extraction with ethanol-benzene, the products were analyzed by standard methods (46). The results are presented in Table 2, page 10. It should be noted that the procedure used for determination of the "lignin" content included not only the true lignin, known to be present in bark, but also most of the other non-carbohydrate components, such as tannins and phlobaphenes. The bark holocelluloses were prepared by the extraction sequence, hot water (51, 33), 0.5% aqueous ammonium oxalate, and acidified sodium chlorite (14). The holocelluloses were re-extracted with hot water, 0.5% ammonium oxalate, followed by 24% aqueous potassium

Table 1. Carbohydrate composition of bark and bark fractions.[a]

Component	Galacturonic Acid	Galactose	Glucose	Mannose	Arabinose	Xylose
Original bark	4	2	51	trace	5	38
Water extract	5	8	25	16	11	35
Pectin material	90	trace	nil	nil	10	nil
Glucuronoxylan	trace	nil	trace	nil	nil	87[b]
Alkali-borate extract	26	13	16	nil	45	nil
Residue (cellulose)	nil	nil	100	nil	nil	trace

[a] In percent of total reducing sugar content of each fraction.

[b] In addition to 13% of 4-O-methylglucuronic acid.

Table 2. Chemical composition of barks.[a, b]

Component	Fir	Spruce	Pine		Ginkgo	
			Inner	Outer	Inner	Outer
(Summative Data)						
Lignin	38.1	39.4	24.6	46.9	7.5	45.6
Ash	2.1	4.0	3.3	2.2	24.4	10.1
Acetyl	0.8	0.5	0.2	0.8	0.2	0.3
Uronic anhydride	5.6	8.0	9.9	7.7	11.5	9.4
Residues of:						
Galactose	1.6	2.4	4.3	4.2	4.5	1.7
Glucose	37.4	35.7	40.9	26.8	38.3	23.8
Mannose	8.0	2.9	2.5	2.5	3.4	2.1
Arabinose	3.2	3.3	10.6	5.5	6.2	3.5
Xylose	3.2	3.8	3.7	3.4	4.0	3.5
(Other Data)						
Pentosan	9.2	11.3	19.3	13.0	11.6	8.9
Material Soluble in Cold Water	4.8	9.0	13.7	5.4	19.5	8.7
Material Soluble in Hot Water	8.4	20.3	22.7	12.7	21.1	14.0
Material Soluble in 1% Sodium Hydroxide	35.4	51.6	----	----	47.0	56.5

[a] All values in percent of extractive-free bark.

Fir: Amabilis fir (Abies amabilis Forb.)

Spruce: Engelmann spruce (Picea engelmanni Parry)

Pine: Lodgepole pine (Pinus contorta Dougl.)

Ginkgo: (Ginkgo biloba L.)

[b] Timell, reference 47.

hydroxide, and finally with 17.5% sodium hydroxide containing 4% borate (24). The yields of these separations are summarized in Table 3.

Table 3. Yields[a] obtained on extraction of barks.

Component	Fir	Spruce	Pine	Ginkgo
Holocellulose	57.9	58.2	63.8	56.5
Water extract	2.1	2.6	4.3	0.7
Ammonium oxalate extract	7.0	11.2	11.9	12.5
Potassium hydroxide extract	7.4	9.4	14.2	3.8
Sodium hydroxide-borate extract	3.3	4.1	3.0	1.9
Residue	38.1	30.9	30.4	37.6

[a]All values in percent of extractive-free bark.

Timell (47) reported that the earlier difficulties in obtaining homogeneous polysaccharides from barks were probably due to the use of unsuitable extraction media. The major reason, however, appears to have resided in the fact that non-carbohydrate constituents often were not completely eliminated before a fractional extraction of the polysaccharides was attempted. The non-carbohydrate components in barks are largely aromatic in nature and therefore are chemically related to lignin. Meier (32) showed that even small amounts of residual lignin in a mixture of hemicelluloses is capable of impeding fractionation to an astonishing degree. Therefore, complete delignification of the bark is essential to the study of bark carbohydrates.

Numerous methods have been described for the delignification of plant materials with the aim of minimum alteration of the carbohydrates present. They can all be considered modifications of one of three principal procedures. The first was that of Ritter and Kurth (29, 38) who subjected wood to repeated chlorinations and subsequent extractions with a solution of pyridine in alcohol. The second method involved the in situ generation of chlorine dioxide by use of an aqueous solution of acetic acid and sodium chlorite (21). This in itself was a modification of the work of Schmidt and Graumann (42) who delignified wood with gaseous chlorine dioxide. However, chlorine dioxide is very hazardous and the in situ generation was a great improvement. The technique was further developed by Wise, Murphy and D'Addieco (56) and again modified by Whistler, Bachrach and Bowman (54). This is the procedure most often used today (55, p. 449).

The third procedure used for the preparation of wood holocelluloses involves peracetic acid oxidation followed by mild sodium borohydride reduction (31). Although this has been claimed to give a superior holocellulose it has not been generally applied.

The delignification reaction used in the present work was the acidified sodium chlorite method. It has advantages over the chlorination method of Ritter and Kurth (29, 38) because it bypasses the toxic gas chlorine and yields a better holocellulose. The peracetic acid method of Leopold (31) makes use of an uncommon reagent,

peracetic acid. The Leopold procedure also requires a reduction step with sodium borohydride which has not been studied well enough to be well evaluated.

The acidified sodium chlorite reaction is the one most commonly used to delignify wood and bark on a laboratory scale. The chemicals used are common reagents present in most laboratories and the reaction is very easy to run with no unusual precautions to be taken except that it should be performed in a good fume hood.

The holocellulose material remaining after complete delignification should, in theory, comprise the entire cellulose and hemicellulose portions of the wood. In practice, this goal is never reached and often not even approached (48). Timell (48) points out the considerable amount of work done on the degradation of the insoluble holocellulose remaining but little has actually been done of the carbohydrate components solubilized during the delignification reaction (21).

However, Bublitz (7) and Jayme and Hanke (21) isolated carbohydrate material from chlorite liquors and Thomas (45) observed polyuronide material in the wash waters from a chlorination method. The results of these workers showed the error in the previously held concept that the sodium chlorite delignification of wood resulted in complete recovery of the carbohydrates as an insoluble "holocellulose." The reaction did solubilize carbohydrates. Bublitz (7, 8)

isolated an ethanol insoluble carbohydrate fraction from the liquor following delignification of spruce wood with acidified sodium chlorite. The carbohydrate fraction was composed of mannose, galactose, arabinose, xylose and uronic acids. Later Browning and Bublitz (6) reported that the apparent increase in uronic acid content which occurred in the course of delignification may be caused not by oxidation of the carbohydrates but by oxidized lignin products.

Some of the reactions known to occur when carbohydrates are treated with a chlorine dioxide medium are outlined by Becker, Hamilton and Lucke (4) and by Theander (44). Two principal types of oxidation are known: oxidation of glycosidic functions, either directly or following hydrolysis; and oxidation of the 2, 3-glycol function to ketone groups with final ring opening to dicarboxylic units. A simplified scheme indicating possible pathways of chlorine dioxide oxidation of cellulose analogs is shown in Figure 2, page 15. However, little or no work of very recent date is recorded and none on polysaccharides. One of the objectives of this work was to determine some of the effects on the solubilized carbohydrate molecules.

The chemical behavior of sodium chlorite and chlorine dioxide on lignin model compounds (vanillin, vanillyl alcohol, α-methylvanillyl alcohol and syringyl alcohol) has been elucidated in a series of investigations by Sarkanen and his co-workers (10, p. 468; 13, 14, 40, 41). This showed that during the oxidation,

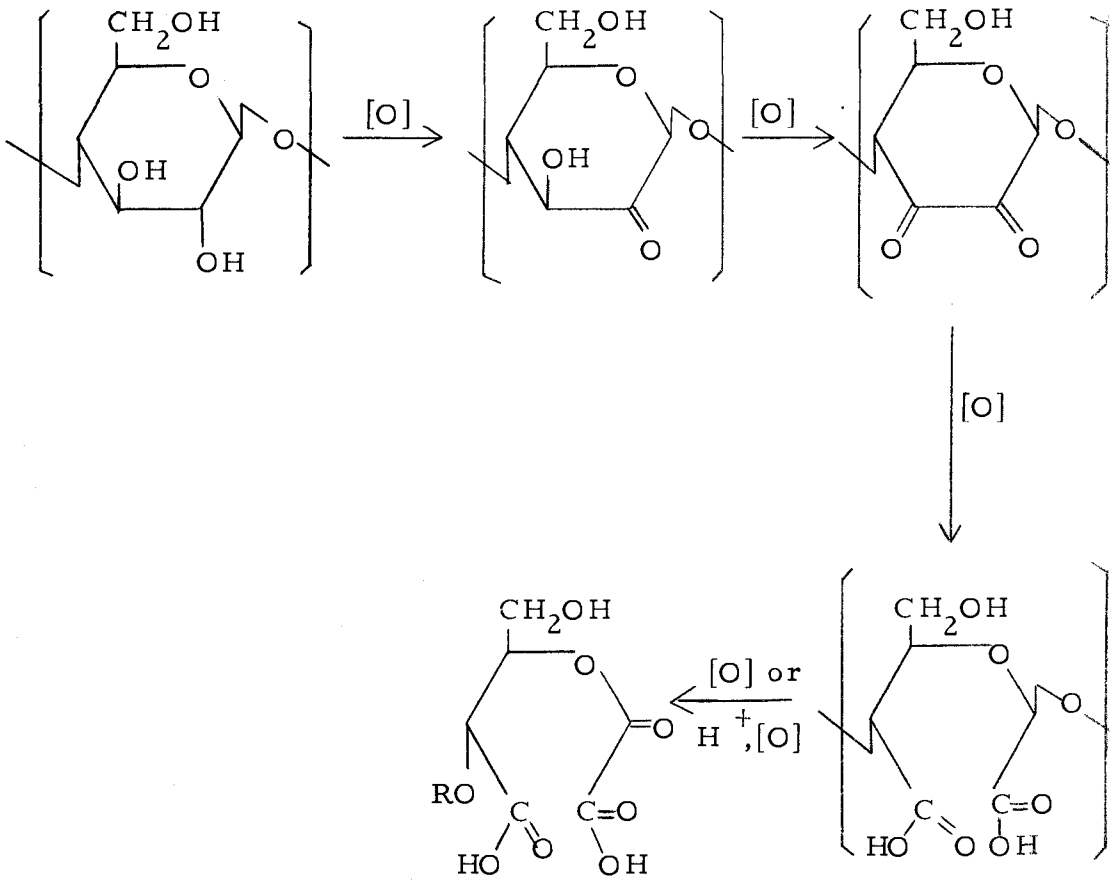


Figure 2. Simplified scheme indicating possible pathways of chlorine dioxide oxidation of cellulose analogs.

substituted p-benzoquinones are formed. They also reported that some of the aromatic rings, particularly the guaiacyl nuclei, suffer oxidative cleavage between the methoxyl and hydroxyl groups resulting in the formation of derivatives of muconic acid monomethyl esters. These reactions are illustrated in Figure 3, page 17.

The recent advances in chromatographic separations coupled with the physical techniques now available to characterize materials, allows for a thorough study of bark carbohydrates. It is possible to determine the amount of carbohydrates solubilized by the delignification reaction and to ascertain if the reaction resulted in significant alteration of the molecules. The experimental work reported here represents an effort to clarify many of these questions.

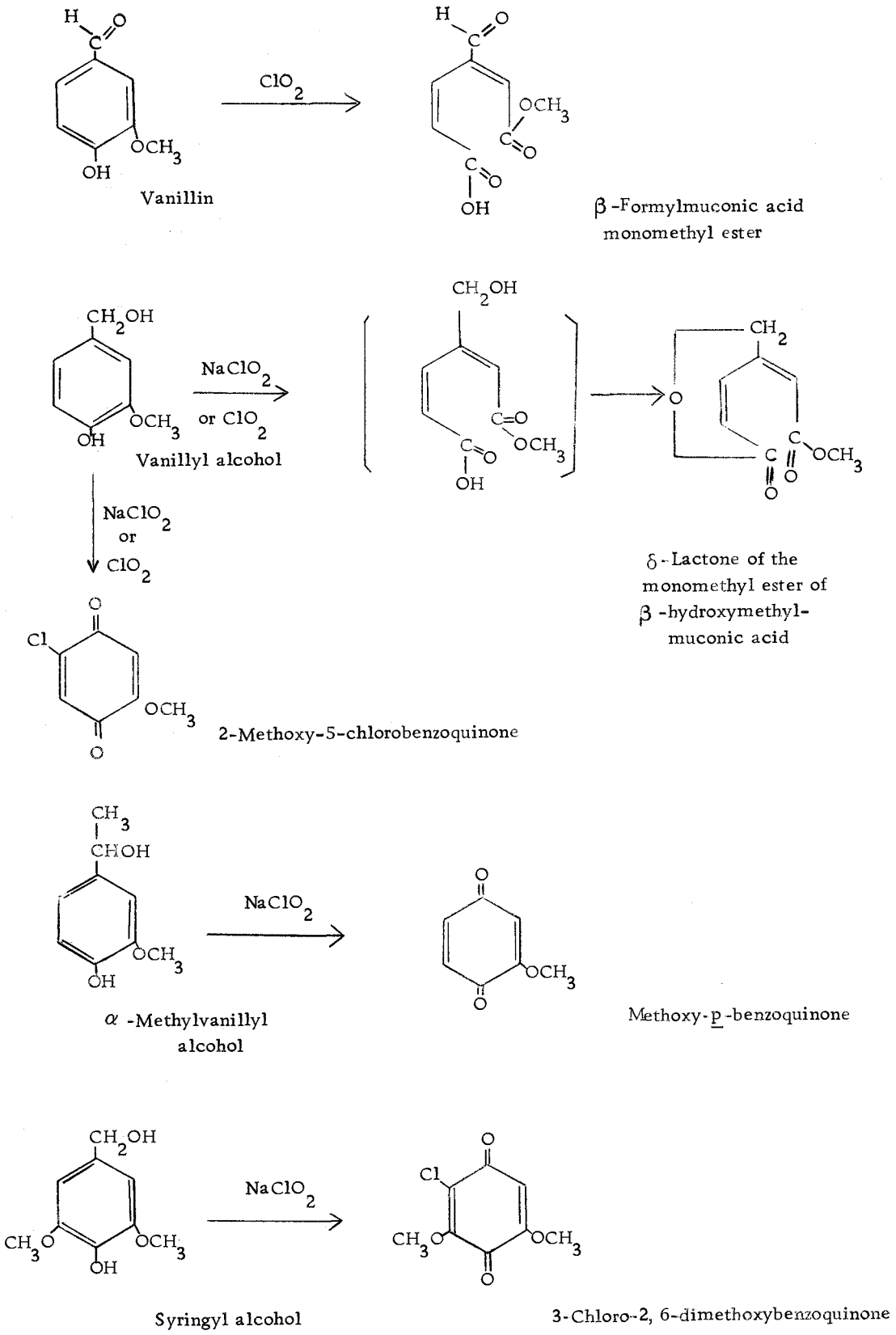


Figure 3. Reactions of lignin model compounds with sodium chlorite or chlorine dioxide.

III. EXPERIMENTAL

A. Collection of Bark Samples

On May 22, 1969, the outer bark was chipped from a standing Douglas-fir at Blackrock, Oregon. The inner bark plus cambium were then carefully stripped from the tree and immediately brought to the laboratory where the cambium layer was removed. The cambium-free inner bark (moisture content 44.9%, hot air oven at 110°) was immersed in 95% ethanol. Water was later added to adjust the solution to ethanol-water (4:1 v/v) with calculations for the moisture content.

The tree was later cut and by count of the annual rings was 130 years old.

B. Sample Preparation and Solvent Extraction

The inner bark, after standing in the ethanol-water (4:1 v/v) for 3 days, was recovered by decantation and air-dried. It was ground in a Wiley Mill and fractionated according to particle size by screening (The W. S. Tyler Company, Cleveland, Ohio). The fractions were recombined in the following amounts: material passed through a 60 mesh and retained on a 100 mesh screen, 1612.82 g; material passed through a 100 mesh and retained on a 150 mesh screen, 238.30 g. This provided a representative sample.

The bark sample (1632.32 g dry weight) was successively extracted with benzene-ethanol (2:1 v/v), hot water, and 0.5% ammonium oxalate as shown in Chart 1 (page 20). The residue from the ammonium oxalate extraction was washed well with water and lyophilized to yield a brown powder.

C. Acidified Sodium Chlorite Delignification (47, 54, 56)

An amount (1310.5 g. dry weight basis) of the above residue from the ammonium oxalate extraction was divided into six batches. Each batch was stirred into 3.0 liters of distilled water at 75-80° and the temperature was maintained throughout the reaction. A steady stream of nitrogen was bubbled through the mixture to assist in stirring and to prevent the accumulation of gases. Glacial acetic acid (20.0 ml) was added, followed by sodium chlorite (60.0 g). Fresh glacial acetic acid and sodium chlorite were added two more times at one hour intervals. At the end of four hours the yellow solids were recovered by filtration using a Büchner funnel. The filtrate was dialyzed for one week against running tap water and lyophilized to yield a white powder; weight 874.9 g. These materials which were solubilized by the acidified sodium chlorite delignification reaction were labeled Fraction A (Chart 1, page 20).

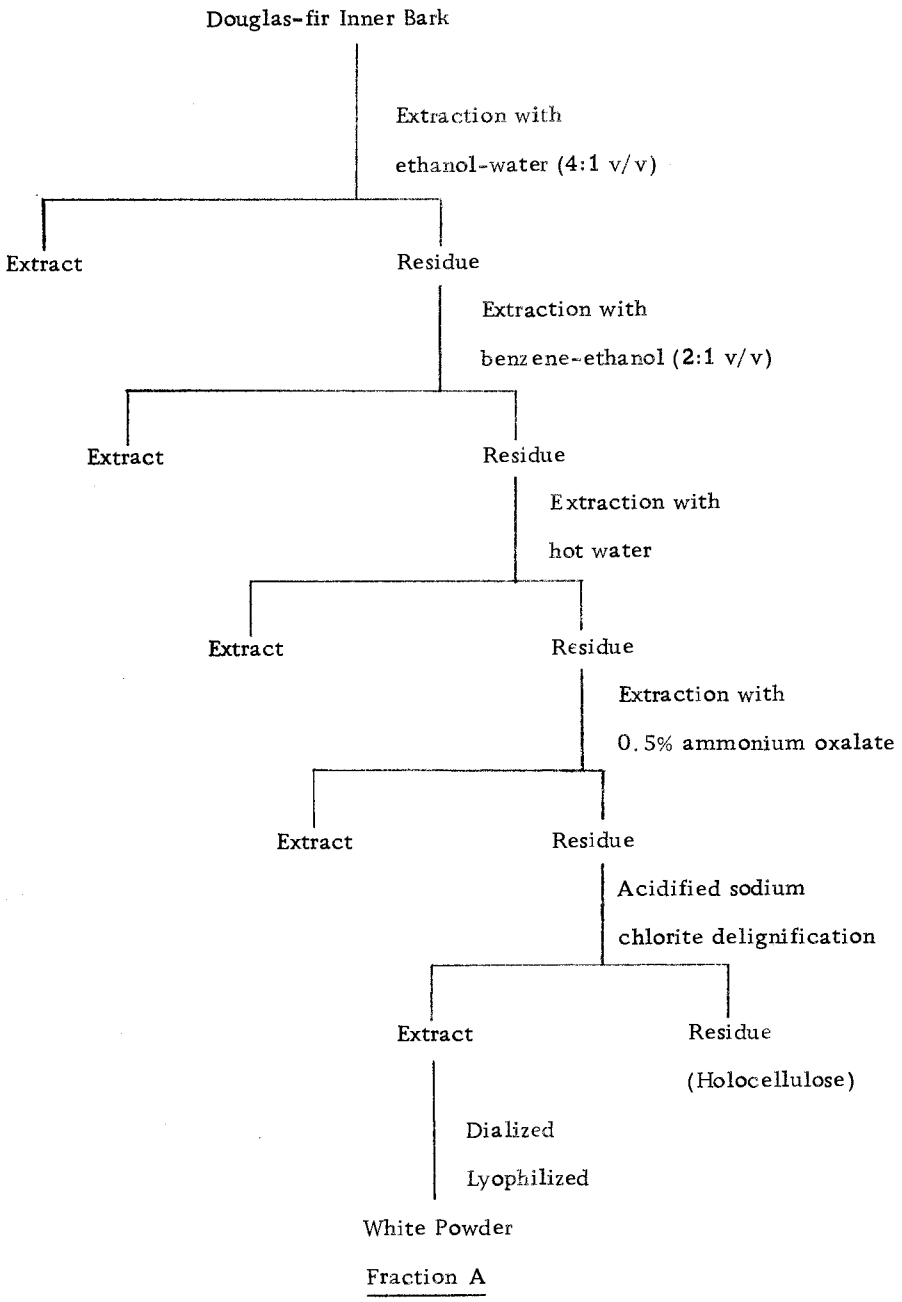


Chart 1. Isolation of the materials solubilized by the acidified sodium chlorite delignification reaction.

D. Characterization of the Carbohydrates Solubilized by the Acidified Sodium Chlorite Delignification Reaction

1. Ash Determination

The inorganic ash in Fraction A was determined by a modification of the procedure of Paech and Tracey (35).

Six samples of Fraction A (0.170 g to 0.180 g. dry weight) were weighed into silica dishes. The samples were saturated with concentrated sulfuric acid (0.2 ml). The mixtures were stirred thoroughly with glass rods and set aside for 1 1/2 hour. They were placed in a muffle furnace and heated gently (200°) until charring occurred. The temperature was increased to 300° to drive off the sulfuric acid (about 30 min). The temperature was increased to 500° and heating continued until ashing was complete (about 5 hours). The ashed samples were placed in a dessicator to cool and were weighed after 20 min. The ash content was 13.39±1.57%.

2. Dialysis and Precipitation with Ethanol

In an attempt to separate and purify the carbohydrates in Fraction A, it was dialyzed and re-precipitated (58).

An aliquot (22.2 g, dry weight) of Fraction A was dissolved in distilled water (500 ml) and dialyzed against distilled water. The water was changed each day for four days, concentrated to about

200 ml under reduced pressure on a rotary evaporator and lyophilized to yield a brown solid; weight 5.6 g or 22.4% of the starting material. An aliquot of the fraction was subjected to paper chromatography, under conditions to be described in detail later.

The solution in the dialysis bag was concentrated under reduced pressure on a rotary evaporator to a volume of 400 ml. The concentrate was added, with stirring, into 1200 ml of 95% ethanol to provide a 70-71% ethanol concentration resulting in the formation of a flocculent white precipitate. The mixture was allowed to stand at room temperature for several hours and then centrifuged. The decantate was concentrated and lyophilized to yield a fluffy white powder; weight 7.4 g or 33.3% of the starting material.

The white residue was lyophilized to yield a fluffy white powder; weight 7.7 g or 34.7% of the starting material; ash content 11.88 ± 2.83%.

Dialysis and precipitation did not lower the ash content of the original Fraction A beyond the degree of error of the ash determinations, and so all further work was done on the original Fraction A.

3. Elemental Analysis for Nitrogen, Sulfur, Phosphorus and the Halogens

The procedures used are slight modifications of the Lassaigne's sodium fusion method (53,p.1039). A small glass test-tube was supported

in a clamp and a small cube of sodium metal was added. The tube was gently heated in a flame until the sodium melted and the vapors rose 1-2 cm up the walls of the tube. A small amount of Fraction A was added directly onto the molten sodium. The tube was strongly heated over an open flame until the entire end was red hot. The heating was continued for one or two minutes and the tube was then plunged into an evaporating dish containing about 10 ml of distilled water so that the hot tube shattered. The mixture was heated to boiling, the insolubles removed by filtration, and the filtrate recovered for elemental analysis.

An aliquot (2-3 ml) of the filtrate was added to a test-tube containing about 0.1 g of powdered ferrous sulfate. The mixture was gently heated with shaking until it boiled. Without cooling, sufficient dilute sulfuric acid was added to dissolve the iron hydroxide and give the solution an acid reaction. A precipitate of Prussian blue formed, indicating the presence of nitrogen.

A small aliquot (15.0 mg dry weight) of Fraction A was quantitatively analyzed (Pascher and Pascher, 53 Bonn, Buschstrasse 54, West German); nitrogen content, 0.84%.

A second aliquot (2.0 ml) of the filtrate from the sodium fusion reaction was acidified with dilute acetic acid and a few drops of lead acetate was added. No reaction resulted, indicating that no sulfur was present.

A third aliquot (1.0 ml) of the filtrate from the sodium fusion reaction was acidified with 3.0 ml of concentrated nitric acid and boiled for one minute. The solution was cooled and an equal volume of ammonium molybdate reagent was added. The solution was warmed to 40-50° and allowed to stand but no yellow precipitate formed, indicating that no phosphorus was present.

A fourth aliquot (2.0 ml) of the filtrate from the sodium fusion reaction was acidified with dilute sulfuric acid and boiled gently until it had been reduced to about 1 ml to remove any hydrogen cyanide which might be present. A few drops of aqueous silver nitrate was added. No precipitate formed, indicating that no halogens were present.

4. Strong Acid Hydrolysis

An aliquot (177.6 mg dry weight) of Fraction A was dissolved in 77% sulfuric acid (3.00 g) and allowed to stand for 30 minutes at room temperature. Water (56.29 g) was slowly added with stirring to provide a 3.9% sulfuric acid solution. The solution was refluxed for 5 hr., cooled to room temperature, and neutralized to pH 5.0 by titration with a saturated aqueousl barium hydroxide solution. The resulting precipitate of barium sulfate was removed by centrifuge and washed well with water. The decantate plus washings were concentrated on a rotary evaporator to 50 ml.

5. Mild Acid Hydrolysis

A part (177.6 mg dry weight) of Fraction A was dissolved in 3% sulfuric acid (50 ml) and the solution was refluxed 5 hr. After cooling to room temperature the solution was titrated to pH 5.0, with a saturated solution of aqueous barium hydroxide. The resulting precipitate of barium sulfate was removed by centrifuge and washed well with water. The decantate plus washings were concentrated to 25 ml on a rotary evaporator.

6. Qualitative Amino Acid Analysis by Paper Chromatography

The hydrolyzates from the strong acid treatment and the mild acid treatment were subjected to two dimensional paper chromatography (Whatman No. 1 paper) using water-saturated phenol (beakers of 0.3% ammonium hydroxide were placed in the bottom of the developing tank) as developer in one direction and n-butanol-formic acid-water (20:6:5 v/v/v) as developer in the second direction. After air-drying, the papers were sprayed with ninhydrin spray reagent (0.02% ninhydrin in n-butanol) and heated at 100±5° in an oven for 5 minutes (12, pp. 93-96).

7. Qualitative Carbohydrate Analysis by Paper Chromatography

The hydrolyzates from the strong acid hydrolysis and the mild acid hydrolysis were subjected to paper chromatography using ethyl acetate-pyridine-water (8:2:1 v/v/v) as developer. The solvent was allowed to migrate almost to the bottom of the papers at which time they were removed from the tank and air-dried. The papers were returned to the tank and developed as before (repeated 3 times). The paper chromatograms were sprayed with o-aminodiphenyl reagent (0.4 g o-aminodiphenyl dissolved in a solution prepared from 100 ml glacial acetic acid and 20 ml distilled water) and heated at 100±2° in an oven for 5 min (49).

8. Quantitative Carbohydrate Analysis by Gas-Liquid Chromatography

The gas-chromatograph used was a Hewlett-Packard 5751B Research Chromatograph (Hewlett-Packard Company, Palo Alto, California) equipped with dual flame ionization detectors. The conditions were: column, 6.5% ECNSS-M on Gas Chrom Q 100/120 mesh, 6 ft. x 1/8 in. O.D. stainless steel; injection port 200°; detector 230°; column temp. 180° isothermal; helium flow 30 ml/min; range setting 10^2, attenuation setting 16.

Fraction A was hydrolyzed and the derivatives prepared for

injection into the gas chromatograph as follows. A portion of Fraction A (0.27219g, dry weight) was dissolved in 3% sulfuric acid (80 ml) and refluxed for 5 hours. After cooling, authentic myo-inositol (0.1004 g) was added. The solution was neutralized to pH 5.0 with a saturated aqueous solution of barium hydroxide. The resultant precipitate of barium sulfate was removed by centrifuge and the decantate plus washings were concentrated to about 25 ml and transferred to a 100 ml round-bottomed flask. Sodium borohydride (0.08 g) was added to the flask and allowed to react for 2 hr. at room temperature (1).

The excess sodium borohydride was decomposed by adding acetic acid until gas evolution ceased. The solution was concentrated to a sirup on a rotatory evaporator and methanol (10 ml) was added and re-evaporated. The addition and removal of methanol was repeated five times (2). The resulting sirup was dried in an oven at 105° for 15 min to ensure complete removal of water.

Acetic anhydride (7.5 ml) and concentrated sulfuric acid (0.5 ml) were added to the sirup and the solution was heated for 1 hour at 50-60° in a water bath. After cooling for 5 min the acetylation mixture was poured slowly with stirring into about 70 ml of ice-water. The mixture was transferred to a separatory funnel and the alditol acetates were extracted with three successive amounts of methylene chloride (25 ml, 15 ml, and 10 ml). The methylene

chloride extract was concentrated to dryness on a rotary evaporator at 75°. Water (1 ml) was added to the residue and re-evaporated. The alditol acetates were dissolved in 2 ml of methylene chloride and about 1.0 µl of the solution was injected into the gas chromatograph for quantitative analysis (5).

The areas under the peaks in the resulting spectrum were measured with a planimeter.

9. Qualitative Uronic Acid Analysis by Infrared Spectroscopy

A sample (0.5 mg) of dried Fraction A was carefully ground with potassium bromide (200 mg) and pressed into a pellet. A spectrum was taken of the pellet over the range of 4000 to 300 cm^{-1} (Beckman IR-20A). The residue from the 0.5% ammonium oxalate extraction (Chart 1, page 20) was also analyzed by infrared spectroscopy.

An infrared spectrum of Fraction A was also obtained in a Nujol mull prepared by grinding it to a thick paste in Nujol oil (paraffin oil (26, 52).

10. Qualitative Uronic Acid Analysis by Color Reactions

Concentrated sulfuric acid (6.0 ml) was added slowly to an aqueous solution (1.0 ml) of the 3% sulfuric acid hydrolyzate of

Fraction A and cooled under tap water. The reaction mixture was heated for 20 min in a boiling water bath and cooled. An aliquot (0.2 ml) of a 0.1% ethanolic solution of carbazole was added and the test sample allowed to stand for 2 hours at room temperature. The development of a purple color indicated that Fraction A contained a uronic acid. The solution was scanned in the ultraviolet range and showed maximum absorption at 535 nm indicating the presence of a uronic acid (15, p. 497).

An aliquot (177.6 g dry weight) of Fraction A was dissolved in 3% sulfuric acid (50 ml) and the solution was refluxed for 5 hr. The hydrolyzate was neutralized by passage through a column of Amberlite IRA-400 anion exchange resin in the -OH form. An amount (1.0 ml) of the neutralized solution was tested for uronic acids by the carbazole-sulfuric acid test described above. The results were positive.

A small aliquot of the 0.5% ammonium oxalate residue (Chart 1, page 20) was also tested with carbazole-sulfuric acid. The results showed a strong positive test. Color tests were also run on authentic galacturonic acid (positive test), oxalic acid and benzoic acid (negative tests).

11. Qualitative Uronic Acid Analysis by Paper Chromatography

The hydrolyzate prepared from the mild acid hydrolysis of Fraction A was subjected to paper chromatography. Whatman No. 1 filter paper was employed in conjunction with the following solvent systems:

1. water saturated n-butanol-absolute ethanol-water (10:9:1 v/v/v)
2. water saturated n-butanol-acetone-water (4:5:1 v/v/v)
3. ethyl acetate-pyridine-water (8:2:1 v/v/v)

Authentic galacturonic acid was chromatographed simultaneously with the unknown hydrolyzate.

For the first two solvent systems, the papers were first impregnated with a phosphate buffer solution prepared by titrating a 0.01 M solution of disodium hydrogen phosphate to pH 5.0 with a 0.1 M solution of phosphoric acid (22).

The chromatograms developed with solvent three were alternately irrigated and air-dried for a total of 6 times. They were thus in the solvent atmosphere for a total of about 30 hr.

After development, the paper chromatograms prepared from the first two solvent systems were sprayed with aniline hydrogen phthalate reagent (1.66 g of phthalic acid dissolved in 100 ml of water-saturated n-butanol containing 0.93 g of freshly distilled aniline (37). The chromatograms prepared from the third solvent

system were sprayed with the o-aminodiphenyl reagent. After spraying, all chromatograms were heated in an oven at 100±2° for 5 min.

12. Preparation of Methyl Ethers

An aliquot of Fraction A (1.461 g dry weight) was dissolved in 18% aqueous sodium hydroxide at 0° with stirring followed by the addition of 6.0 g of sodium hydroxide pellets. Sodium hydroxide (100 ml, 30%) and dimethyl sulfate (50 ml) were added simultaneously over a period of 3 hours while maintaining the temperature at 0°. Acetone (150 ml) was added to prevent foaming and the mixture was stirred for an additional 45 hr at room temperature. The solution was cooled to 0°, neutralized with 10% sulfuric acid, dialyzed against water (3 days), concentrated under reduced pressure (200 ml), lyophilized, and the entire methylation sequence repeated (57).

The product was further methylated by the method of Falconer and Adams (16) by solution in 150 ml of tetrahydrofuran followed by treatment over a period of 60 hours with seven 10 g portions of crushed sodium hydroxide, each followed by a 12 ml portion of dimethyl sulfate. The solution was stirred vigorously and additional solvent was added as needed to maintain fluidity. The product was recovered as described above for the first methylation, and the concentrated dialyzate (600 ml) was extracted with three 300 ml

portions of chloroform. The chloroform layer was concentrated under reduced pressure, yielding a light brown solid which was dissolved in acetone and filtered. The concentrated filtrate (50 ml) was poured into petroleum ether (b.p. 30-60°, 200 ml) and the white precipitate which formed was recovered by centrifuge (after the mixture was refrigerated for 6 days) and lyophilized, affording a white, fluffy powder; yield 80 mg.

13. Acid Hydrolysis of the Methyl Ethers

An aliquot (40 mg) of the methylated Fraction A prepared above was hydrolyzed for 3 hr at 97° with 88% formic acid (10 ml). The formic acid was removed by evaporation under reduced pressure followed by the addition and removal of water, then hydrolyzed with 0.5 N sulfuric acid (5 ml for 2.5 hr at 97°). Upon cooling the solution was neutralized with water-saturated barium hydroxide and the solids were removed by centrifugation. The decantate was concentrated to about 2 ml of sirup. The sirup was chromatographed on paper using the developer ethyl acetate-pyridine-water (8:2:1 v/v/v) (57).

IV. RESULTS AND DISCUSSION

A. Collection of Bark Samples

A sample of inner bark free from outer bark and cambium was desired because it provided a relatively homogeneous starting material. Outer bark contains considerable cork material which would interfere with experimental studies of carbohydrates. The cambium layer contains proteins as well as carbohydrates and the two are best separated at the outset.

In the spring of the year from April through June, the outer bark of Douglas-fir is easily separated from the inner bark by simply chipping it off. The inner bark for the present work was taken from a standing tree to reduce contamination from other sources. The cambium layer was then carefully separated from the inner bark and a relatively homogeneous sample resulted.

B. Sample Preparation and Solvent Extraction

It was desirable, before proceeding with polysaccharide separation, to remove as much as possible of the low-molecular weight material (simple sugars, organic acids, lipids, waxes, etc.) present in the bark. Some of these materials are readily oxidized and would interfere with the delignification reaction.

None of the extraction procedures shown in Chart 1 (page 20) accomplished a clear-cut separation of one type of material from another, but in general each step in the sequence performed a definite function. The ethanol-water (4:1 v/v) extraction served two purposes. It prevented possible enzyme action which might change the natural materials from their native state, and it also solubilized the simple sugars which could be identified to provide a more detailed investigation of the total carbohydrate function in the bark. The benzene-ethanol (2:1 v/v) azeotrope removed lipids and waxes (27) which would interfere with later reactions.

Having de-fatted the bark it was possible to carry out a water extraction and remove water-soluble polysaccharides, proteins and some pectins. However, not all of the pectic material of plants is extractable with water. The insoluble part, protopectin (calcium pectate) was removed by extraction with a 0.5% ammonium oxalate solution. Presumably a cation exchange occurred in which insoluble calcium oxalate was formed along with soluble ammonium pectate. The latter was extracted with the filtrate.

C. Acidified Sodium Chlorite Delignification

Through all of the extractions the bark retained its characteristic brown color. However, the strong oxidizing conditions of the acidified sodium chlorite reaction bleached the reaction mixture

to a pale yellow color. Fumes of yellow gases, undoubtedly chlorine and chlorine dioxide, were visible in the reaction vessel above the mixture. These gases emphasize the necessity of performing the delignification reaction in a fume hood and of bubbling nitrogen through the reaction to sweep away these toxic materials. Fresh sodium chlorite was added to the reaction at one hour intervals rather than all of it at the beginning to prevent an excess of gas formation and to give the reaction time to proceed.

The residue from the delignification, termed "holocellulose" (Chart 1, page 20), was pale yellow in color.

The filtrate was yellowish in color and was dialyzed to remove low-molecular weight impurities and the inorganic salts and ions which resulted from the reaction of the sodium chlorite. The solution which remained in the dialysis bag after several days was almost colorless and the solids which were recovered by lyophilization were white. These materials solubilized by the acidified sodium chlorite delignification reaction were called fraction A (Chart 1, page 20) for convenience of reporting.

D. Characterization of the Carbohydrates Solubilized by the Acidified Sodium Chlorite Delignification Reaction

The delignification reaction has been developed to solubilize the non-carbohydrate components of plants. Thus the insoluble residue (holocellulose) remaining is relatively pure carbohydrate material.

However, the reaction does dissolve some carbohydrates and it is these materials that are the subject of this study.

The solubilized solids of the present investigation (Fraction A, Chart 1, page 20) represented a complex mixture of carbohydrates, lignin-like compounds, tannins, proteins, and so forth. Therefore, as described below, a variety of approaches were used to characterize the carbohydrates in the mixture.

1. Ash Determination

The ash content of Fraction A was 13.39±1.57%, as sulfate. Although a considerable amount of inorganic sodium chlorite had been added to the delignification reaction, this was a high ash content considering that the fraction had been dialyzed. Although it would be of interest to know what elements were present in the ash, no effort was made to determine them in the present work.

2. Dialysis and Precipitation with Ethanol

In an effort to lower the ash content and remove more of the low-molecular weight materials, the acidified sodium chlorite soluble materials (Fraction A, page 20) were again dialyzed. More than 22.4% of Fraction A passed through the dialysis membrane. Paper chromatography of the dialyzate showed a relatively large spot for glucose, indicating that carbohydrate substances, probably

monosaccharides and short chain oligosaccharides, had passed through the dialysis membrane.

The solution retained in the dialysis bag was made to 70% ethanol, a method often employed for the purification of polysaccharides (55, p. 449), and resulted in the precipitation of a white, flocculent material.

However, the sulfated ash content of the precipitate was 11.88±2.83% indicating that dialysis and precipitation did not lower the ash content of the original Fraction A below the experimental error of the ash determination. A very high ash content for samples prepared in a similar manner was reported by Bublitz (7, page 52). He electrodialyzed sodium chlorite liquor from the delignification of spruce wood, and even after ethanol precipitation he found ash contents as high as 13.7%. The inorganic materials were either chemically attached to the polysaccharides, possibly as salts, or were physically wrapped in the polysaccharides so that diffusion through the dialysis bag was restricted.

Because of the fact that carbohydrates which might be important to the present investigation passed through the dialysis bag and might be lost, and that dialysis and precipitation did nothing to lower the ash content, it was concluded that simple purification of Fraction A was not feasible. Therefore, all additional investigations were done on the original Fraction A.

3. Elemental Analysis for Nitrogen, Sulfur, Phosphorus and the Halogens

Compounds isolated from natural sources often contain one or more of the above elements. Therefore, it is best to qualitatively determine if they are present.

Fraction A showed a positive test for nitrogen. However, quantitative analysis showed only 0.84% nitrogen, an amount not considered to represent an interfering contaminant and no special efforts were made to separate the nitrogenous material.

The tests for sulfur, phosphorus and the halogens were negative.

4. Strong Acid Hydrolysis

A fundamental aspect of polysaccharides is the component monosaccharides which are linked together to form the polymer chain. Often polysaccharides contain linkages which are resistant to acid cleavage. Thus, to ensure complete hydrolysis, Fraction A was treated with strong acid according to the procedure of Laver, Root, Shafizadeh and Lowe (30). The hydrolyzate was analyzed for amino acids and monosaccharides as described below.

5. Mild Acid Hydrolysis

If the polysaccharides under investigation are water soluble and

if hydrolysis can be accomplished under mild acid conditions, treatment with strong acids as described above is not desirable because of degradative side reactions. Since Fraction A was water soluble, a mild acid hydrolysis (3% sulfuric acid) was compared with the 77% sulfuric acid hydrolysis. The hydrolyzate was analyzed for amino acids and monosaccharides as described below.

6. Qualitative Amino Acid Analysis by Paper Chromatography

Elemental analysis showed the presence of nitrogen. It could possibly be a result of some residual ammonium oxalate from the ammonium oxalate extraction (Chart 1, page 20) or it could be part of protein material. The cambium layer of Douglas-fir bark has been shown to contain protein (20), and possibly some cambium layer remained with the inner bark in sample preparation.

The hydrolyzates from the strong acid hydrolysis (77% sulfuric acid) and the mild acid hydrolysis (3% sulfuric acid) were examined for amino acids by two-dimensional paper chromatography. It was found that the second solvent, n-butanol-formic acid-water (20:6:5 v/v/v), must be prepared just prior to use, otherwise gradual esterification occurred and altered its chromatographic usefulness.

Chromatograms of the hydrolyzate from the mild acid treatment did not show amino acids. However, chromatograms of the hydrolyzate from the strong acid treatment showed ten purple spots (Figure 4,

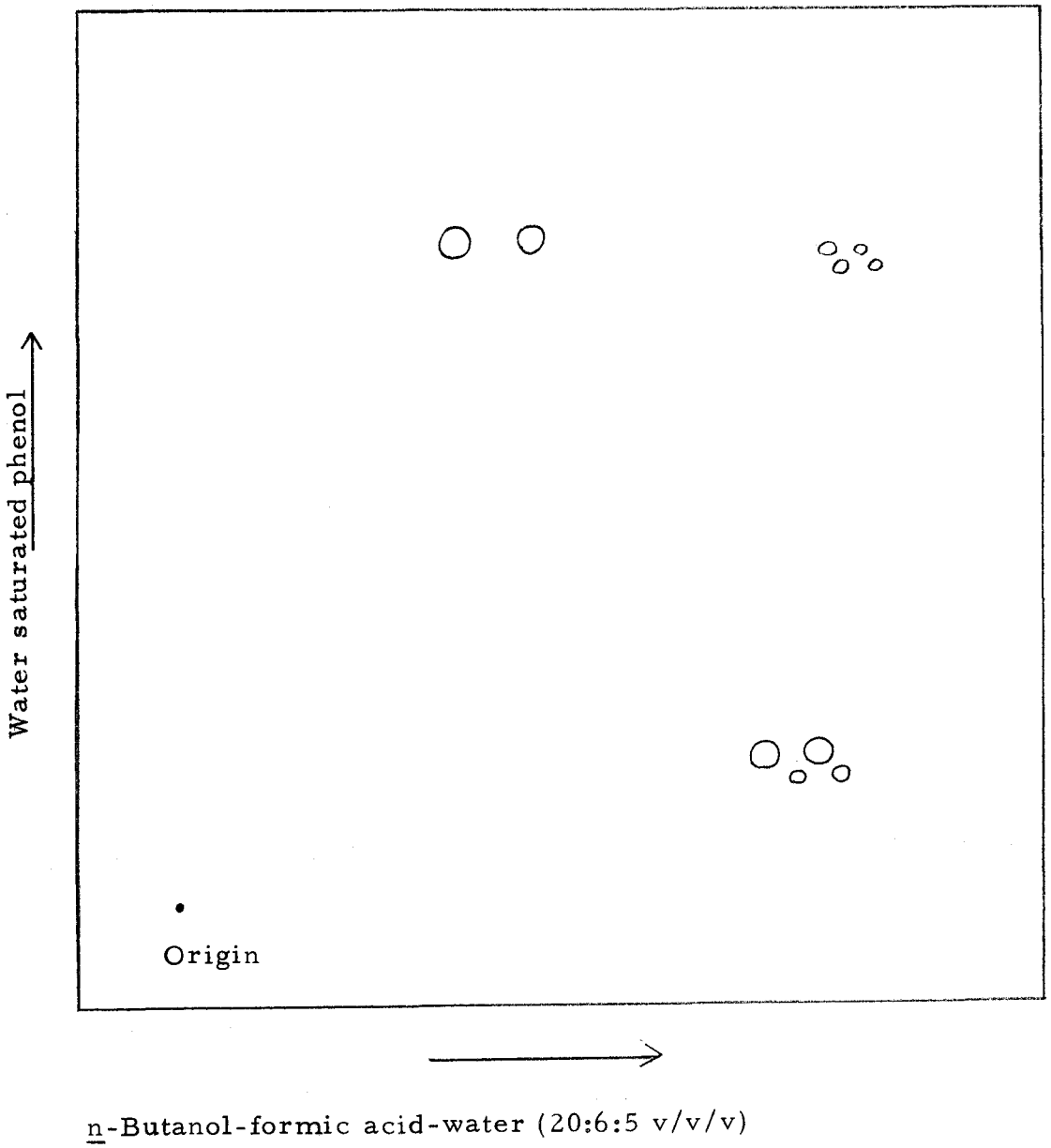


Figure 4. Two dimensional paper chromatogram of the amino acids in the acid hydrolyzate of Fraction A.

page 40). However, quantitative analysis showed only 0.84% nitrogen content indicating that the amount of protein material was not great. Therefore, no effort was made to identify the amino acids in Figure 4 (page 40).

7. Qualitative Carbohydrate Analysis by Paper Chromatography

The mixture of monosaccharide sugars resulting from acid hydrolysis of Fraction A was well resolved by paper chromatography. The chromatograms showed that the hydrolyzates contained a large proportion of glucose, a moderate amount of arabinose, slight amounts of mannose and galactose and trace amounts of xylose and rhamnose (Figure 5, page 42).

The hydrolyzates from both the strong acid hydrolysis and mild acid hydrolysis contained what appeared to be identical monosaccharides by paper chromatography. Therefore, strong acid was not necessary and to avoid degradation, the 3% sulfuric acid method was used in all subsequent hydrolysis.

The five major sugars, glucose, mannose, galactose, arabinose and xylose are those ordinarily found in wood and wood pulp (47). The trace amount of rhamnose represents the first time it has been found in Douglas-fir bark although it has been reported in other barks (51).

Since the monosaccharides resulting from the hydrolysis of bark are the same as those found in wood, it is expected that the

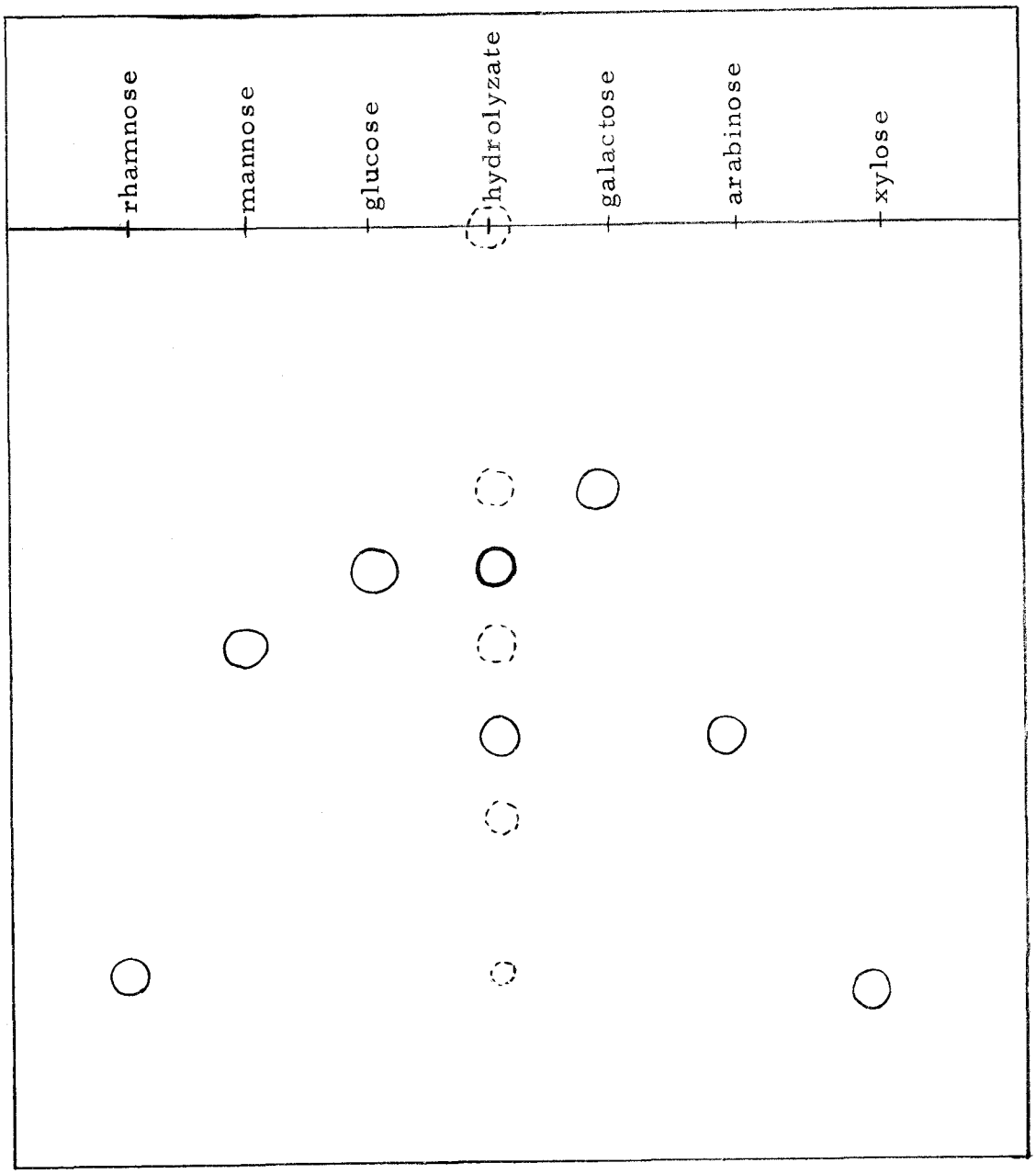


Figure 5. Paper chromatogram of the acid hydrolyzate of Fraction A to show monosaccharides. Solvent; ethyl acetate-pyridine-water (8:2:1 v/v/v).

polysaccharides of bark are similar to those in wood. If this is so then the major carbohydrate component is cellulose, but it also contains some hemicelluloses such as xylans, glucomannans, glucogalactomannans and so forth. The isolation of the cellulose fraction and an evaluation of its properties and its degree of polymerization will be of future interest.

8. Quantitative Carbohydrate Analysis by Gas-Liquid Chromatography

Carbohydrates are neither heat resistant nor volatile and so derivatives must be prepared which will volatilize without degradation in order to perform gas-liquid chromatographic analysis. There are numerous derivatives which have been tried but the ones most commonly used today are the "alditol acetates." In the preparation of the alditol acetates the monosaccharides are first treated with sodium borohydride to reduce the aldehyde function to the alcohol function. This has the result of preventing ring isomerization to the pyranose and furanose forms and so prevents the formation of alpha and beta forms of the sugars. The end result is only one form, the alditol, for each of the monosaccharide sugars rather than three, four or even five. The acetates are synthesized from the alditols to make a volatile, heat resistant derivative.

The gas chromatographic resolution of the alditol acetates

prepared from the acid hydrolyzate of Fraction A was excellent (Figure 6, page 45). The myo-inositol peak shown at the end of the spectrum resulted from the addition of an accurately measured amount of myo-inositol as an internal standard. The areas under the peaks of the other sugars in the spectrum are compared to the area under the peak of myo-inositol for quantitative results.

The sample of Fraction A taken (moisture-free, ash-free basis) yielded the following percentages of monosaccharides: glucose, 41.5%; arabinose, 8.3%; galactose, 2.7%; mannose, 2.6%; xylose, 0.7%; rhamnose, 0.7%. This total of 56.5% is less than 100% because Fraction A contains organic materials other than carbohydrates.

9. Qualitative Uronic Acid Analysis by Infrared Spectroscopy

The acidified sodium chlorite delignification reaction is an oxidation reaction. Therefore, it was anticipated that the primary hydroxyl groups in position six of the hexose containing polysaccharides would most likely be oxidized to uronic acids as demonstrated below. Therefore, several qualitative tests were applied to determine if uronic acids existed in Fraction A. Since uronic acids are known to be present in natural materials, all of the tests were run in direct comparison with the residue from the ammonium oxalate extraction, the material from which Fraction A was prepared (Chart 1, page 20).

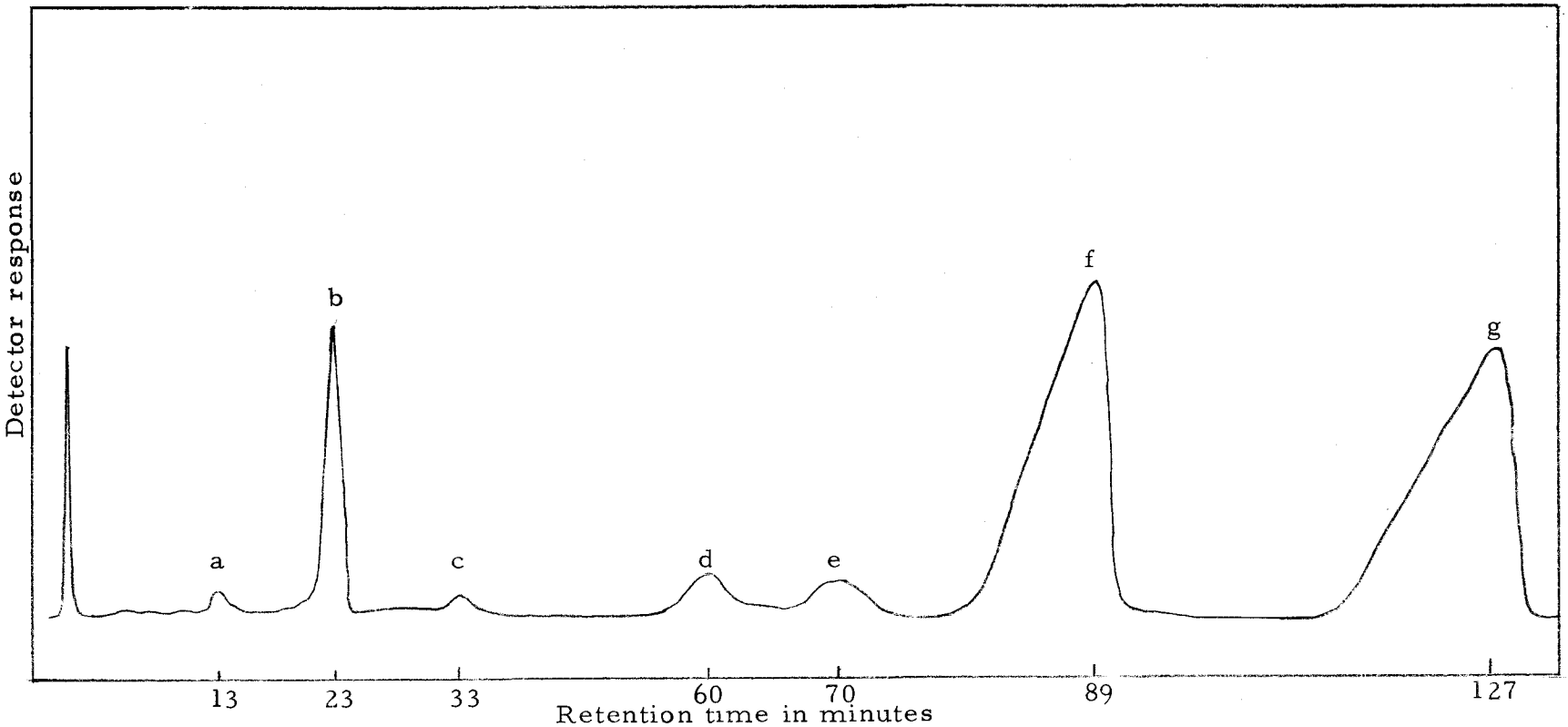
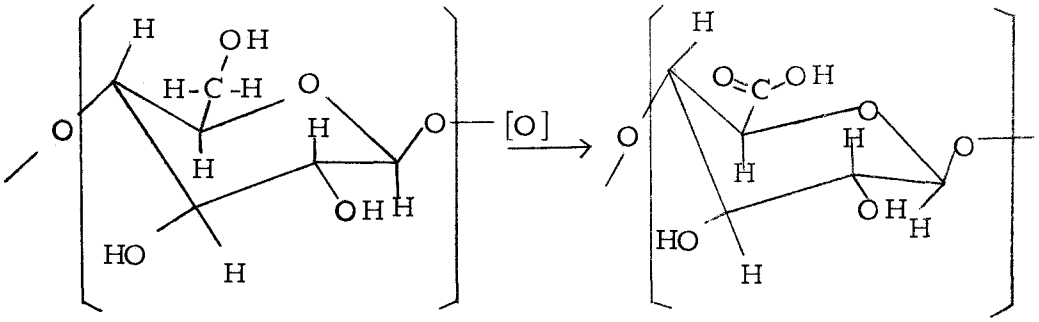


Figure 6. Gas-liquid chromatographic separation of the alditol acetates from the acid hydrolyzate of Fraction A. Peak "a" is from rhamnose, "b" from arabinose, "c" from xylose, "d" from mannose, "e" from galactose, "f" from glucose, and "g" from myo-inositol. Conditions: Column, 6.5% ECNSS-M on 100/120 mesh Gas Chrom Q, 6 ft x 1/8 in O.D. stainless steel; injection port 200°; detector 230°; column temp. 180° isothermal; helium flow 30 ml/min; range setting 10^2, attenuation setting 16.



Polysaccharide with primary hydroxyl in position six

Polysaccharide with carboxyl group in position six

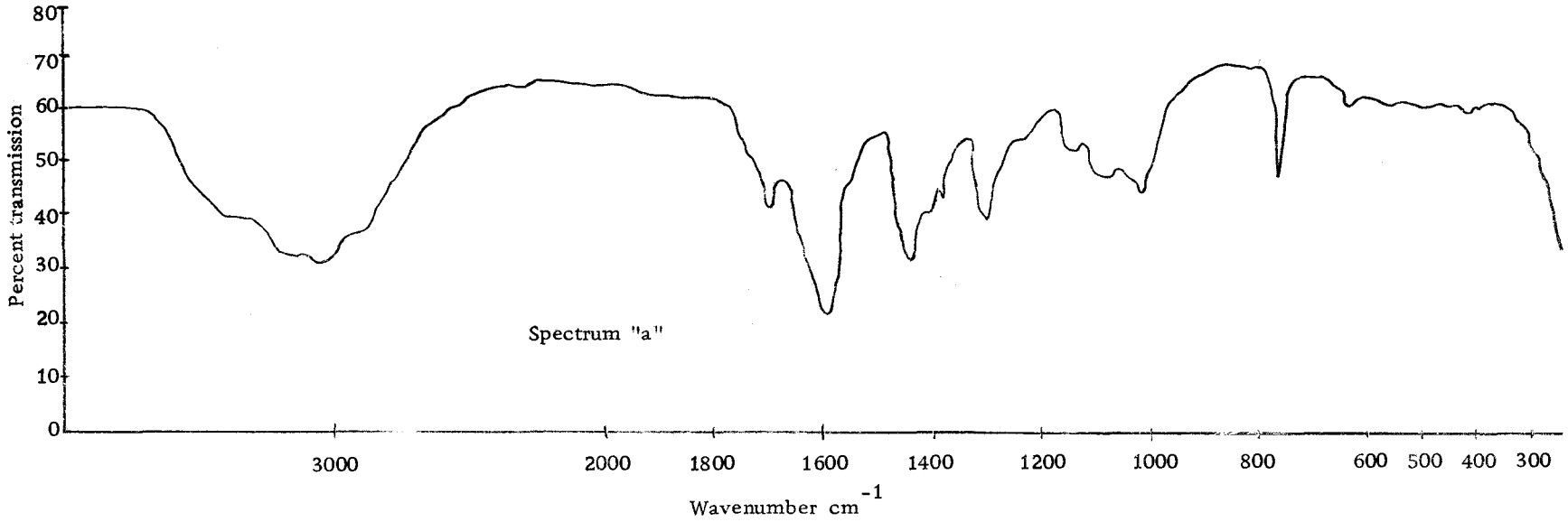
The infrared spectrum of the 0.5% ammonium oxalate insoluble material showed a strong absorption at 1590 cm^{-1} (Figure 7, page 47), corresponding to absorption by carboxylate ion (34, p. 44). Thus the starting material from which Fraction A was prepared contained uronic acids. Fraction A showed an absorption band at exactly the same place (Figure 7, page 47). However, the treatment with acidified sodium chlorite, although an oxidation reaction, did not appear to increase the uronic acid content, at least not as detected by infrared spectroscopy.

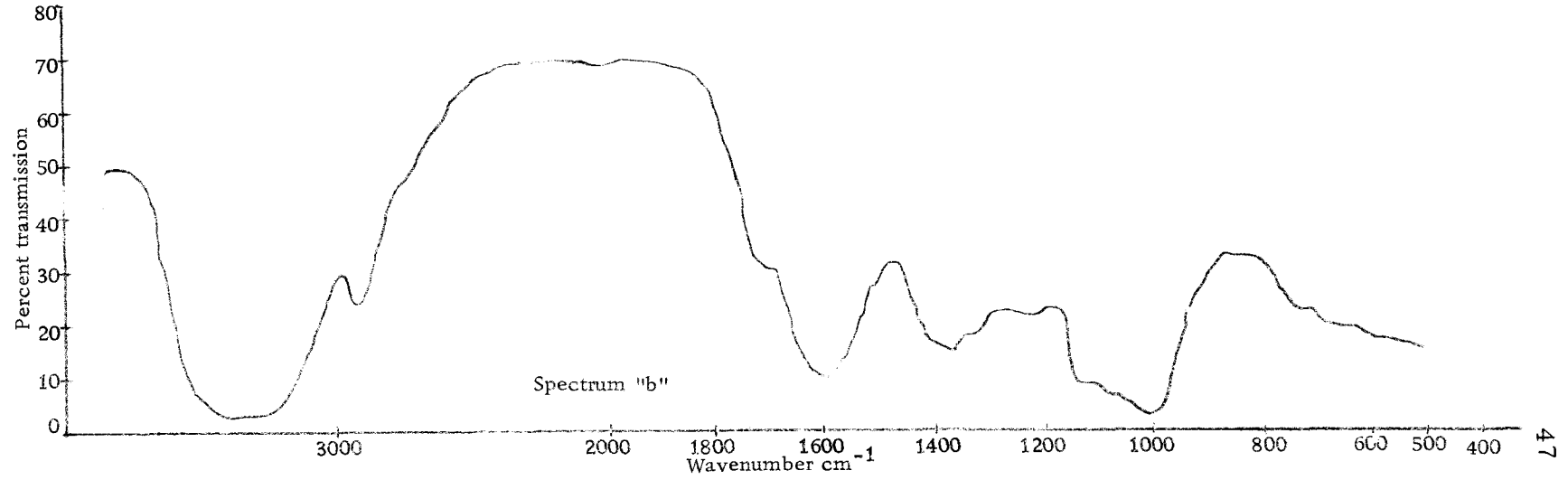
Becker, Hamilton and Lucke (4) showed that the 2, 3 glycols in polysaccharides were oxidized to carboxylic acids in model compounds (Figure 2, page 15). If these kinds of compounds were produced in the present reaction, they would be expected to show up as an increased carboxylate peak in the infrared spectrum of Fraction A.

Figure 7. Infrared spectra of uronic acids.

Spectrum "a" is from the 0.5% ammonium oxalate residue.

Spectrum "b" is from Fraction A.


Percent transmission
80
70
60
50
40
30
20
10
0
Spectrum "a"
3000
2000
1800
1600
1400
1200
1000
800
600
500
400
300
Wavenumber cm -1


Percent transmission
80
70
60
50
40
30
20
10
0
Spectrum "b"
3000
2000
1800
1600
1400
1200
1000
800
600
500
400
Wavenumber cm -1

However, the carboxylate peak was small (Figure 7, page 47). If these types of oxidation reactions did occur they possibly progressed to the point of hydrolysis with the release of small fragments which might have been lost through dialysis (Chart 1, page 20).

10. Qualitative Uronic Acid Analysis by Color Reactions

The carbazole-sulfuric acid color reaction is said (15, p. 497) to be reasonably specific for hexuronic acids. This was verified by testing monosaccharides and oxalic acid and benzoic acid. These compounds showed no reaction, indicating that the simple sugars and carboxylic acid functions other than those in uronic acids did not interfere with the color reaction.

The 3% sulfuric acid hydrolyzate of Fraction A showed a positive purple color, indicating the presence of hexuronic acids. The reaction showed a maximum absorption in the ultraviolet region at 535±1nm (Figure 8, page 49) as described for the test (15, p. 497).

The color was not particularly strong and it was considered possible that since the sulfuric acid had been neutralized with barium hydroxide that some of the uronic acids had formed barium salts and had precipitated with the barium sulfate. Therefore, a part of the acid hydrolyzate was neutralized with ion exchange resin but the results were the same.

The 0.5% ammonium oxalate insoluble material was also tested

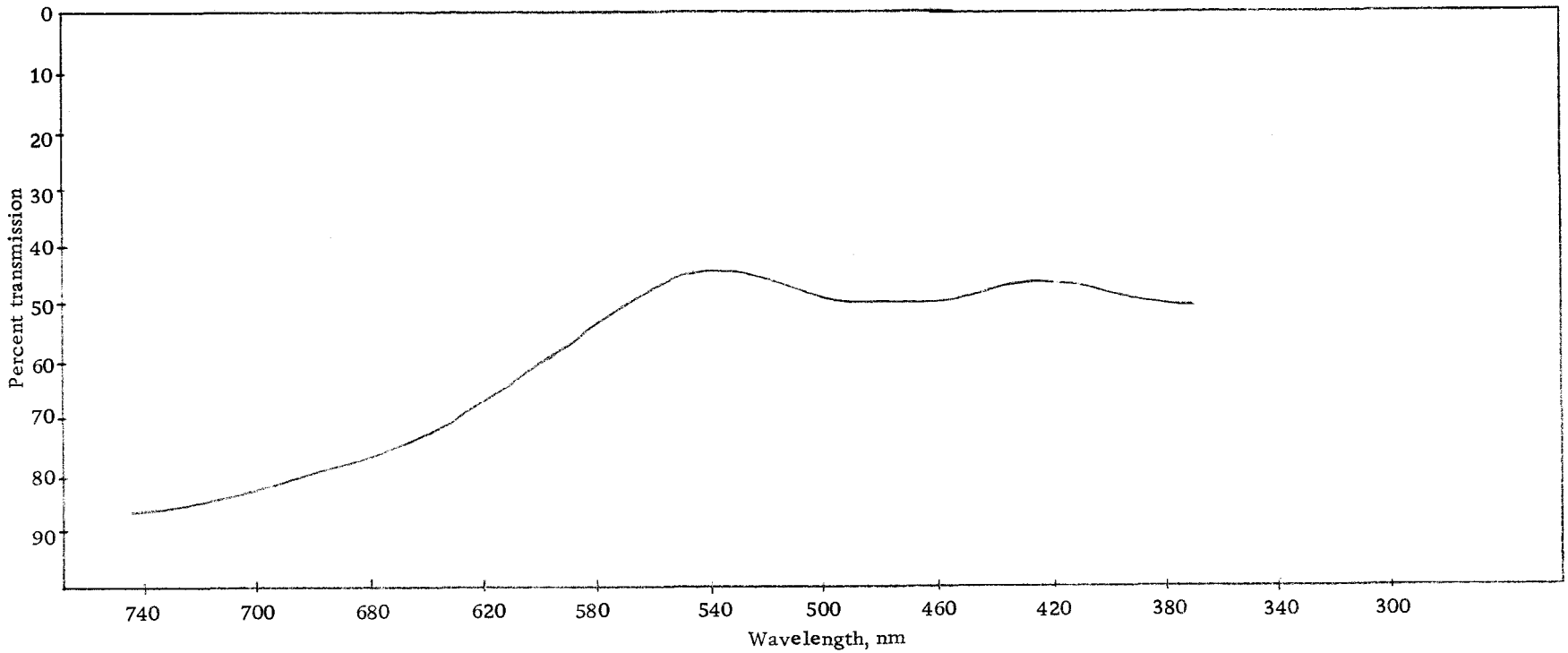


Figure 8. Ultraviolet spectrum of uronic acid from hydrolyzate of fraction A. Solvent: 87 % H_2SO_4.

for hexuronic acids without even hydrolysis to the respective monosaccharides. A strong positive test resulted.

These data support those from the infrared studies that the treatment with acidified sodium chlorite did not appear to oxidize the carbohydrates to hexuronic acids. Hexuronic acids were present in the material before oxidation with sodium chlorite and the reaction did not appear to oxidize this particular functional group.

11. Qualitative Uronic Acid Analysis by Paper Chromatography

Jayme and Knolle (22) have reported the separation and identification of selected hexuronic acids by paper chromatography. The rate of movement was very slow and when the technique was applied to the present study, poor resolution resulted using their solvents 1 and 2 (page 30). However, when the solvent system ethyl acetate-pyridine-water (8:2:1 v/v/v) was employed there were spots which migrated the same distance as authentic galacturonic acid (Figure 9, page 51). These results supported the infrared spectral data and the carbazole-sulfuric acid color test that Fraction A did contain uronic acids. However, since the infrared data and the color test results indicated that the acidified sodium chlorite delignification reaction did not produce more uronic acids than were in the starting material, no further effort was made to identify the uronic acids in Fraction A.

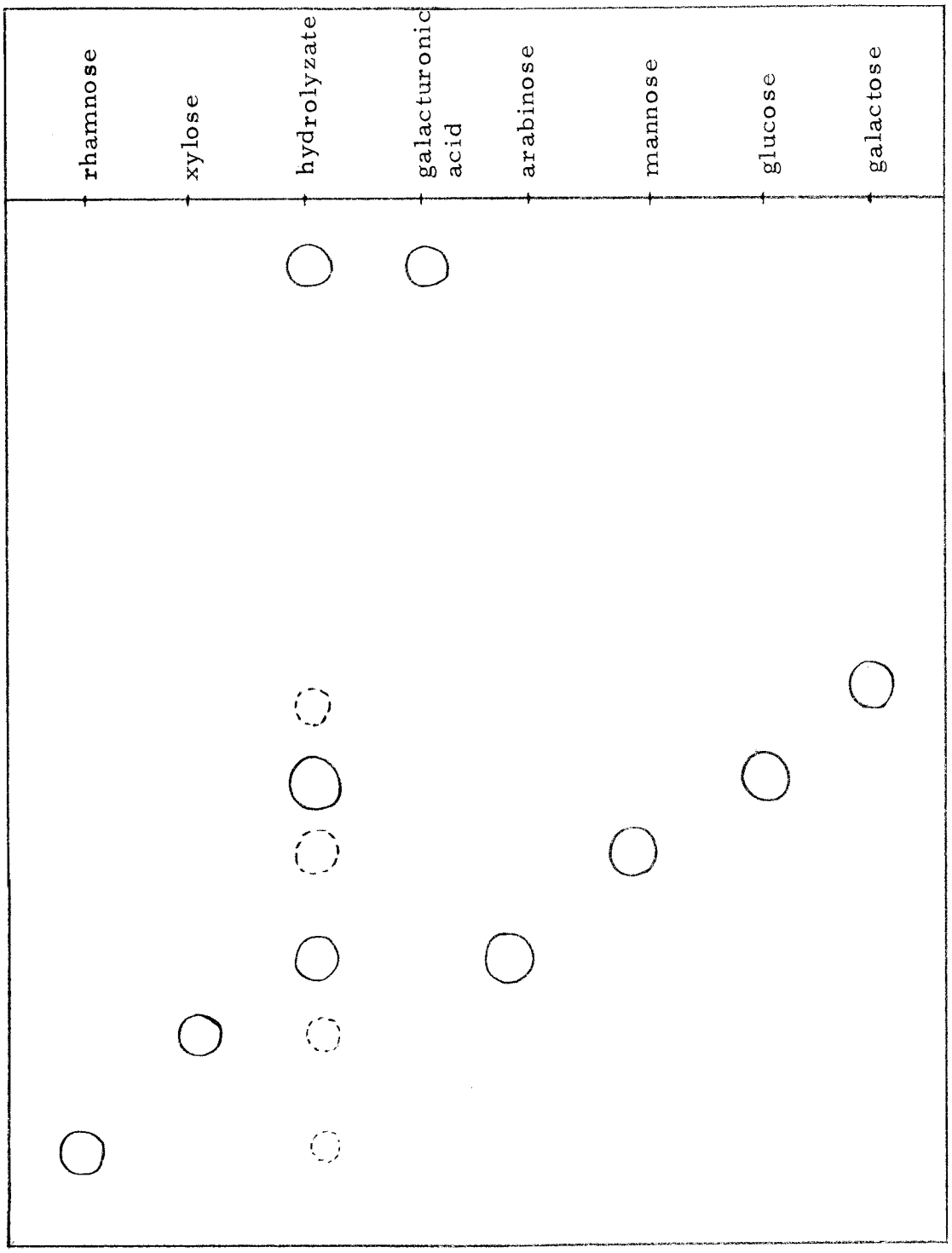


Figure 9. Paper chromatogram of the acid hydrolyzate of Fraction A to show uronic acids. Solvent; ethyl acetate-pyridine-water (8:2:1 v/v/v).

12. Preparation of the Polysaccharide Methyl Ethers

A standard method for the partial elucidation of polysaccharide structure is to block all of the hydroxyl groups in the parent polymer with methyl ether groups. The methylated polysaccharide is then hydrolyzed and th resulting methylated monosaccharides are identified. By this technique the positions of the hydroxyl groups freed by the hydrolysis can be determined. These hydroxyl groups must have been protected from methylation because they were a part of the polymer linkage. In this way the points of attachment for the monosaccharides can be located.

Preparing the methyl ethers of polysaccharides is a lengthy and tedious task. In the present work the methylation was monitored by infrared spectroscopy. When the absorption for the hydroxyl group had completely disappeared, it was concluded that the reaction was complete.

13. Acid Hydrolysis of the Polysaccharide Methyl Ethers

Since methylated polysaccharides are often quite insoluble in aqueous media, hydrolysis to monosaccharides is usually accomplished by strong formic acid. However, the hydroxyl groups freed by hydrolysis become formate esters. These esters can be hydrolyzed by dilute sulfuric acid because the monosaccharides are water soluble.

The resulting sirup therefore contains monosaccharides with some methyl ethers on the ring and some free hydroxyl groups which were involved in the linkage of the parent polysaccharide.

V. SUMMARY AND CONCLUSIONS

1. A successive extraction of Douglas-fir inner bark with ethanol-water (4:1 v/v), benzene-ethanol (2:1 v/v), water, and 0.5% ammonium oxalate solution effectively removed interfering low-molecular weight material (simple sugars, organic acids, lipids and waxes) prior to delignification with acidified sodium chlorite.
2. The acidified sodium chlorite delignification reaction bleached the brown-colored materials in the inner bark and produced a yellowish "holocellulose" residue and a yellow filtrate. After dialysis the solids in the filtrate were recovered as a white powder by lyophilization.
3. The ash content of the solubilized solids was 13.39%, an unusually high value. Dialysis and re-precipitation from ethanol did not significantly lower the ash content. The inorganic materials were either chemically attached to the solids, possibly as salts, or were physically wrapped in the polymers so that diffusion through the dialysis bag was restricted.
4. The materials solubilized by the acidified sodium chlorite delignification reaction did not contain sulfur, phosphorus or halogens. However, they contained 0.84% nitrogen.
5. The materials were equally well hydrolyzed by 3% sulfuric acid

as by 77% sulfuric acid indicating that there were no particularly acid resistant bonds present.

6. The acid hydrolyzate of the soluble materials contained ten amino acids as shown by paper chromatography. Therefore, the nitrogen content (0.84%) shown earlier was partially due to the presence of proteins. However, because of the small amount of nitrogen present, the amino acids were not further investigated.

7. Paper chromatography showed that the acid hydrolyzate of the soluble solids contained rhamnose, xylose, arabinose, mannose, galactose and glucose. Rhamnose has not been previously reported in the hydrolyzate of Douglas-fir bark.

8. The monosaccharides in the hydrolyzate were quantitatively analyzed by gas-liquid chromatography of their alditol acetates. The results showed the monosaccharides to be present in the following ratio: glucose, 59.1; arabinose, 11.9; galactose, 3.9; mannose, 3.7; xylose, 1.0; rhamnose, 1.0.

9. Since the monosaccharides identified in the hydrolyzate of bark were the same as those from wood, it is expected that the polysaccharides are similar. The quantitative analysis showed mostly glucose so the major polysaccharide must be a glucan, undoubtedly cellulose. However, the identification of other sugars showed the presence of hemicelluloses as well.

10. The presence of uronic acids in the soluble solids was demonstrated by infrared spectroscopy, a carbazole-sulfuric acid color test, and by paper chromatography. However, the amounts appeared less than in the starting material and it was concluded that although the acidified sodium chlorite delignification reaction was an oxidation reaction it did not significantly oxidize the primary hydroxyl groups of the polysaccharides to uronic acids.

11. The soluble material was methylated and infrared spectroscopy of the resulting product showed no absorption for hydroxyl groups. Therefore, the free hydroxyl groups on the polysaccharides were completely blocked.

12. The methylated material was acid hydrolyzed. Identification of the methylated monosaccharides is expected to greatly aid in the elucidation of the structure of the polysaccharides because those hydroxyl groups freed by acid hydrolysis were part of the polymer linkage. In this way the points of attachment of the monomers can be ascertained.

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