THE CONSTITUTION OF A GLUCOMANNAN FROM THE WOOD OF WHITE PINE (PINUS STROBUS L.)

by

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GENERAL INTRODUCTION

Wood is composed of three main components, namely cellulose, hemicellulose, and lignin. Cellulose is the chief constituent of the cell wall of all land plants and is composed of D-glucose residues linked by (1→4)-β-glycosidic bonds to linear macromolecules of considerable length (2.5 μ). Lignin is a three-dimensional polymer based on phenyl propane units, which are connected in a number of different ways and whose structure is still not known in detail. The hemicelluloses are the polysaccharides which accompany cellulose and which are easily hydrolyzed by dilute mineral acids. It is interesting to note that in the case of wood hemicellulose a relatively small group of sugars serve as building units. They are D-xylose, D-mannose, D-glucose and, to a much lesser extent, L-arabinose, D-galactose, L-rhamnose, L-fucose and 4-O-methyl-D-glucuronic acid. Hemicelluloses from hardwood present a rather consistent pattern, the chief constituent being a linear xylan containing side chains of 4-O-methyl-glucuronic acid. In coniferous wood, although xylans are also present, a diheteropolymer glucomannan is the predominant hemicellulose. Glucomannans are polymers containing glucose and mannose residues in a constant ratio which rarely varies from 1:3-4 and which are linked by (1→4)-β-glycosidic bonds.

The difference in average molecular weight between cellulose and hemicellulose is very large. Native wood cellulose seems to have a weight-average degree of polymerization of about 10,000, whereas the corresponding value for hardwood xylans is only 500.

Eastern white pine (Pinus strobus L.) is a light, soft, non-porous wood. It is grown mostly in the north-eastern United States and Canada where it is the largest native conifer and is the source of valuable softwood lumber. The trunk can attain a maximum height of 220 feet and a diameter of 6 feet. Large areas of the eastern part of this continent were originally covered by this tree but excessive logging has since destroyed most of the original pine forests. In recent years, this species has been extensively used for reforestation in the north east, especially on margin lands in New England. Eastern white pine is a fairly tolerant species which grows rapidly and has good seed production. It is sensitive to attack of a fungus which causes the "white pine blister" disease.

Wood glucomannans are of short chain length compared to cellulose, but a portion of them seems to be closely associated with cellulose, since part of the glucomannan is difficult to remove by alkaline extraction or by technical cooking processes. Possibly the glucomannan chain might be located on the surface of the microfibrils of the cellulose or in the paracrystalline areas of the hemicelluloses. Due to the similarity in constitution between glucomannans and cellulose, the former are probably firmly held to the latter by strong hydrogen bonding.

Earlier studies on the wood from loblolly pine and Scots pine have demonstrated the presence of glucomannan-containing glucose and mannose residues linked by $(1\rightarrow4)$-β-glycosidic bonds to linear, or possibly slightly branched, macromolecules. Galactose residues have also been observed to be associated with these glucomannans. The present study

is concerned with the isolation and properties of a glucomannan from eastern white pine (Pinus strobus). This investigation is parallel to another dealing with the xylan constituent of the wood of this important conifer.

HISTORICAL INTRODUCTION

A variety of polysaccharides occur in the cell walls of land plants besides cellulose. Many of them are closely associated with cellulose, but differ from it by being soluble in alkaline solutions. Schulze (1) in 1891 extracted plant materials with dilute alkali and obtained a group of polysaccharides which are more readily hydrolyzed than cellulose. He considered them to be structurally and chemically related to cellulose, and he designated them as hemicelluloses. This definition does not, however, fit all the polysaccharides which are found together with cellulose in wood, for it has been found that some of the non-cellulosic polysaccharides are no more easily attacked by dilute acid than cellulose itself (2). Various names have been designated such as wood polyoses (3) or cellulosans and polyuronides (4). Karrer (5) has suggested that a better system would be to classify all polysaccharides according to the sugars from which they are built up, for example mannans, galactans, glucomannans and arabogalactans. The term hemicellulose, however, is still in use and widely accepted nowadays.

The classical pioneering work carried out on wood hemicelluloses must be ascribed to Miss O'Dwyer (6). Her investigations were carried out at a time when most of the modern techniques of polysaccharide chemistry were not available. It is therefore noteworthy to find recurring structural building stones, in the oakwood hemicelluloses she studied, consisted of xylose units and methyl uronic acid units in a ratio of 6 to 1.

The species classified as softwoods (gymnosperms, conifers) generally show a higher mannan and a lower xylan content than do the species classified as hardwoods (woody angiosperms). Most mannans are based on β-D-mannopyranose units linked $1 \rightarrow 4$ linearly as the main structural unit and occur in woods and seeds of plants. A true mannan (i.e., polysaccharides with 95% or more of β-D-mannopyranose units) occurs in vegetable ivory (Phytelepha macrocarpa). Glucomannans contain linear chains of β- $1 \rightarrow 4$ glycosidic linked β-D-mannopyranose and β-D-glucopyranose residues as their main structural features.

One of the earliest attempts to isolate a pure mannan polysaccharide from wood was reported by Sherrard and Bianco (7). Attempts to precipitate the copper complex of the mannan from the alkali-soluble portion of wood by addition of Fehling's solution were unsuccessful. Hess and Ludtke (8) isolated a polysaccharide, which they considered was a pure mannan, by extraction of spruce sulphite pulp with alkali. The polysaccharide was purified by the formation of a copper complex on addition of Schweizer's reagent. They claimed that this polysaccharide and a similar material from ivory nut mannan were identical as to their specific rotations in cuprammonium solution (although no data were given), their specific rotations in aqueous sodium hydroxide and their X-ray diagrams. In addition, only mannose was detected in the hydrolyzate of the material, although no experimental figures are given to substantiate these claims.

As early as 1937 Brauns and Lewis (9) and Lewis, Brauns and Buchanan (10) isolated a polysaccharide from unbleached sulphite pulp by

extraction with cuprammonium hydroxide. This treatment caused the usual ballooning of the fibres and dissolved most of the cell wall but left small amounts of insoluble bands which retained some lignin as well as a copper complex, containing about 32% copper. This, when treated with dilute acid, gave rise to an interesting polysaccharide which was shown many years later to consist largely of mannose and glucose residues, present in a ratio of 7:2.

Yundt (11) next attempted to isolate a pure mannan from a hemicellulose fraction by a modification of the procedure of Hess and Ludtke. He found that even after repeated recrystallizations the product still contained only 50% "mannan". The crystals were similar to those which had been obtained from ivory nut mannan A (96% anhydromannose) and the specific rotation for the two materials in alkali agreed closely. However, the data failed to indicate the differences in chemical composition which existed between these two polysaccharides. Husemann (12) also isolated what was thought to be a pure mannan from spruce wood pulp, but evidence for the homogeneity of the product was largely based on rotational data.

It has been observed by a number of workers that sometimes a considerable percentage of the mannan in softwoods is not removed by acid hydrolysis. Wise and Ratliff (13) found that over half of the "mannan" originally present in slash pine and in spruce was retained in the respective alpha-cellulose prepared from the corresponding holocelluloses. Even after five successive treatments with 5% sulphuric acid on a steam bath, the slash pine alpha-cellulose still contained 40% of the

mannose residues present in the wood. This fact must be interpreted as evidence for a chemical link between glucose and mannose.

Leech (14) applying acetolysis and chromatographic techniques which had previously been used by Wolfrom and co-workers (15) for the purpose of isolating the acetates of oligosaccharides from cotton linters cellulose, was the first investigator to report the isolation from wood of a disaccharide that probably consisted of a D-glucose and a D-mannose residue. A slash pine alpha-cellulose was prepared from holocellulose by successive extractions with 5% and 24% potassium hydroxide. The residual alpha-cellulose contained 0.8% of lignin; 0.6% of ash; and 10.4% of mannose residues. No uronic acid or arabinose and only the slightest trace of xylose could be detected after hydrolysis and examination on the paper chromatogram. He obtained a disaccharide of mannose, and also a second disaccharide which, on hydrolysis, gave both D-glucose and D-mannose. It was concluded that the latter compound was an O-glucosyl-mannose, although the data obtained were not adequate for a definite proof of structure. This was the first evidence that a chemical linkage probably existed between D-mannose and D-glucose residues in a wood alpha-cellulose.

Acetolysis was also carried out to establish whether synthesis (reversion) had occurred during the hydrolysis. However, no glucosyl-mannose was observed.

Bradway (16) in an investigation of the haze in cellulose acetates from wood pulps found that all the xylose and mannose were associated with the haze fraction. After subjecting a portion of the material causing the haze to acetolysis, he obtained evidence for the presence of a disaccharide

containing only mannose, and for one containing both glucose and mannose.

Anthis (17), continuing the investigation initiated by Leech, applied a mild acetolysis to Leech's slash pine alpha-cellulose and by careful deacetylation of the products obtained six different disaccharides. One of the disaccharides was identified as 4-O-β-D-glucopyranosyl-D-mannose and another was probably 4-O-β-D-mannopyranosyl-D-glucose. Thus it became evident that certain glucose and mannose units were linked chemically through primary valencies.

Polysaccharide chemistry underwent an enormous advance with the development of new chromatographic techniques. It is now possible to resolve the smallest amounts of sugar mixtures into their components. The chief difficulties still are how to isolate the pure polysaccharides as their fractionation is a difficult and tedious procedure. Most fractionation procedures used involve aqueous solvent systems such as water, ammoniacal copper solutions, borax, N/10 solutions of N NaOH or 10% KOH, long-chain quaternary ammonium salts (e.g. Cetavlon) and lately aqueous barium hydroxide (18). Many of these form insoluble complexes with glucomannans and thus separate them from xylans. Ball, Jones, Nicholson and Painter (19) found that chloroform solutions of neutral glucomannan acetates and acidic xylan acetates when shaken with sodium carbonate solution formed two layers. A homogeneous layer which contained the soluble glucomannan acetate, and a stable emulsion layer which contained the insoluble xylan acetates. Both could be easily separated and on deacetylation gave back their respective polymers.

Sometimes when these methods fail a suitable derivative, such

as an acetate, is prepared and fractionally precipitated from suitable solvent systems. Analysis of the polymer at various stages of purification may give information as to the homogeneity of the material. Recently electrophoretic tests have been employed to determine the chemical uniformity of polysaccharides, but here again care must be exercised in the choice of a suitable electrolyte, and good preparative methods are lacking. Once the polysaccharide has been obtained, homogeneous in physical and chemical properties, the elucidation of it s structure is carried out.

The chief tools for the researcher are complete hydrolysis and characterization of component sugars, partial hydrolysis and identification of the oligosaccharide fragments, methylation studies, oxidation with periodate, osmometry, viscometry, ultracentrifuge, and light scattering measurements.

Although glucomannans also occur in the seeds of various land plants and tubers, only a few have so far been studied in detail, for example, those present in the tubers of Amorphophallus konjak (20,20(a)) and A. oncophyllus (21), the leaves of aloe vera (22), the roots of Gremastia variabilis, Bletilla striata, and bulbs of Narcissus tazetta (23). None of them appeared to contain more glucose than mannose, but since physical homogeneity was not established, they could possibly have been mixtures.

Andrew, Hough and Jones (24) studied a glucomannan obtained from the seeds of Iris ochroleuca and I. sibirica. The polysaccharides were isolated via their copper complexes, both contained D-glucopyranose and

D-mannopyranose residues in a ratio of 1:2. Methylation studies showed that the glucomannans were composed of D-glucopyranose and D-mannopyranose residues linked by 1→4-β-glycosidic bonds with a repeating chain length of 25 to 30 hexose units. Periodate oxidation showed that the polysaccharides were branched (with an average of 5 or 6 branches per molecule). Molecular weight determinations by means of isothermal distillation gave a value of 150 to 175 hexose units. The authors were of the opinion that the glucomannans may have been mixtures or contaminated with galactomannans as a small amount of D-galactopyranose residues were detected as a non-reducing end group in the Iris seed polysaccharide.

Andrew, Hough and Jones (25) studied a glucomannan isolated from bulbs of Lillium candidum, L. henryii, and L. umbellatim and found them similar to the glucomannans of Iris seeds. Both are constructed from units of D-glucopyranosyl and D-mannopyranosyl linked β-1→4 glycosidically in a ratio of 1:2. Structural studies by methylation methods showed that the L. umbellatum glucomannan had an average of about 27 hexose units per non-reducing end group, and that the chains were joined by a small number of D-glucopyranosyl units linked through C_1, C_3 and C_6. There were an average of about two branching points per molecule. L. henryii glucomannan contained an average chain length of about 75 hexose units per D-glucopyranosyl end group.

Smith and co-workers (26, 26(a)) studying the polysaccharides from Amorphophallus, the so-called Iles mannan, were the first to establish the complete structure of a glucomannan. Structurally it is a linear polymer consisting of D-glucopyranose and D-mannopyranose residues in a

ratio of 1:2, linked glycosidically by β-1→4 bonds. Methylation studies showed that the major fraction was a mixture of 2,3,6-tri-O-methyl-D-mannose and 2,3,6-tri-O-methyl-D-glucose not easily separated by any known chromatographic methods. Application of selective methyl furanoside formation in conjunction with partition chromatography on a hydrocellulose column gave two fractions, a faster-moving non-reducing portion which was 2,3,6-tri-O-methyl-D-glucose and a slower, reducing component which was 2,3,6-tri-O-methyl-D-mannose. The relative ratio of these two sugars was 1:2 as calculated from specific rotations. No tetramethyl or dimethyl hexoses were detected. Information regarding the sequence of the sugar residues in the glucomannan was obtained by partial acetolysis followed by deacetylation. The three oligosaccharides obtained in a crystalline form were shown to be 4-O-β-D-glucopyranosyl-α-D-mannopyranose, 4-O-β-D-glucopyranosyl-β-D-glucopyranose (cellobiose) and 4-O-β-D-mannopyranosyl-α-D-glucopyranose. The structure of the latter compound was proved by the methylation technique. The glucomannan could be represented as

$$4-\beta-D-Gp-(1\rightarrow4)-(\beta-D-Gp)-(1\rightarrow4)-(\beta-D-Manp)_4$$

where Gp = β-D-glucopyranose sugar

Manp = β-D-mannopyranose sugar

Smith and co-workers (27) have also isolated a glucomannan from wheat stem rust (Puccinia graminis tritici) urerdriopores with alkali and showed that it is composed of equal numbers of D-glucose and D-mannose residues. The authors claimed that as a result of methylation and periodate oxidation studies, the main structural features of the polymer are apparent, although the data do not permit a unique structure to be established. Of

the 114 sugar residues which comprise the average repeating unit of the glucomannan, 6 residues occupy terminal non-reducing portions, and of these, 3 are D-glucose and 3 D-mannose residues. The linear, doubly-linked non-terminal residues are composed entirely of D-mannose, half of them being joined by β-1⟶4 and the other half by β-1⟶3 glycosidic linkages. There are also 4 branching points probably consisting of D-glucose units joined through C_1, C_2 and C_6.

Smith and Srivastava (28) have investigated the structure of a glucomannan from konjak flour. Previous workers (29) claimed to have isolated a trisaccharide laevidulin ($[\alpha]_D$, -11.5°) and a similar trisaccharide laevidulinose $[\alpha]_D$, -15° composed of D-mannose (2 parts) and D-glucose (1 part). However, unequivocal proof of the structure was not established. Nishida and Hashima (30) isolated a trisaccharide ($[\alpha]_D$, -16°) by acetolysis of the konjak glucomannan, which was shown to be composed of mannose (2 moles) and glucose (1 mole). The structure of this oligosaccharide which appeared to be identical with laevidulinose was not established. Methylation and hydrolysis gave a mixture of 2,3,4-tri-O-methyl-D-mannose, 2,3,4-tri-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-mannose and a structure was formulated based on this evidence. Because the modern, more advanced techniques were not available to Nishida and Hashima, the structure of the glucomannan could not be completely established.

Methylation and periodate oxidation studies by Smith and Srivastava demonstrated that the glucomannan of konjak flour is composed of chains of β-glycosidically linked (1⟶4) residues of β-D-glucopyranose

and β-D-mannopyranose residues with an average repeating unit of 13 hexose units, whereas periodate oxidation data revealed a repeating unit of approximately 11 residues. The isolation of 2,3,4,6-tetra-O-methyl ethers of D-glucose and D-mannose indicated that the glucomannan possesses terminal non-reducing residues of D-glucopyranose and D-mannopyranose. Identification of 2,6-di-O-methyl-D-glucose as the 1,3,4-tris-p-phenylazobenzoate derivative and tentative characterization of 2,6-di-O-methyl-D-mannose suggested that the polymer is branched and that the D-glucose and D-mannose residues are involved. The branching at C_1, C_3 and C_4 was further corroborated by the observation that certain of the D-glucose and D-mannose residues were unattacked by periodate.

Glucomannans, as stated earlier, were also present in the wood of conifers. Jones and co-workers (31) found that partial hydrolysis with dilute sulphuric acid of a crude extract of loblolly pine sawdust gave two fractions, the neutral component being characterized as 4-O-β-D-glucopyranosyl-α-D-mannopyranose by identification of its octa-acetate and comparison of its X-ray diagram with an authentic sample.

Four main fractions were isolated from the hemicelluloses of loblolly pine with alkali. One of them formed an insoluble copper complex and gave glucose and mannose with minor amounts of galactose. Partial hydrolysis showed that four oligosaccharide fragments were isolated, namely mannosyl-glucose, glucosyl-mannose, mannobiose, and mannotriose, which were all characterized and identified. Cellobiose, and mannotetraose were not detected. From methylation and periodate oxidation studies, the authors inferred that the glucomannan from loblolly pine is structurally composed of linear β-(1→4)-linked chains of D-mannose and D-glucose

residues. The chains have an average of two non-reducing end groups for every 27-33 anhydrohexose units, one of which is a D-mannopyranose residue and the other a D-galactopyranose residue. Small amounts of di-O-methyl-hexoses were isolated which may probably have arisen from incomplete methylation of the polymer or demethylation during hydrolysis. The possibility that the chains contain a single branch point for every 27-33 anhydrohexose units cannot be excluded because four moles of formic acid were liberated for every 30 anhydrohexose residues.

Fractionation of glucomannans can be tedious and troublesome, as Hamilton and co-workers (32) found when attempting to prepare pure glucomannans for structural studies from the wood pulp of western hemlock (Tsuga heterophyllia). Aqueous solvent systems such as water, ammonia, copper solutions, borate, or dilute sodium or potassium hydroxide at best only removed small amounts of xylose and no significant fractionation was obtained. The mixture of glucomannans was acetylated and separated by fractional precipitation into fifteen fractions. Ten of these possessed homogeneous specific rotation, and acetyl content, but slight differences in intrinsic viscosities were noticed. Their chemical composition was similar, with a ratio of D-glucose to D-mannose of 1:3-4. Partial hydrolysis yielded five oligosaccharide fragments, namely mannosyl-glucose, glucosyl-mannose, mannobiose, cellobiose and mannotriose. Attempts were made to characterize mannotriose and mannosyl-glucose, but the sugars were not obtained crystalline. Methylation studies of the polymer indicated that it was a linear polymer composed of D-mannose and D-glucose residues linked β-(1$\longrightarrow$4) and it had a repeating chain length of 137 hexose units, terminated by 2,3,4,6-tetra-O-methyl-D-glucose and 2,3,4,6-tetra-O-methyl-

D-mannose in a ratio of 1:1. Two other unknown methylated sugars were detected and isolated. One of them had a chromatographic mobility corresponding to that of a tri-O-methylhexose, but it was neither 2,3,4-tri-O-methyl-D-mannose, nor 2,3,4-tri-O-methyl-D-glucose. The other unknown sugar was probably a di-O-methyl hexose, but did not exhibit any electrophoretic mobility in a borate buffer, thus indicating that position 2 was probably methylated. A possible structure for the glucomannan is formulated as

$4\text{-}\beta\text{-D-Gp-}(1\rightarrow4)\text{—}(\beta\text{-D-Manp})_3\text{-}(1\rightarrow4)\text{—}(\beta\text{-D-Gp})\text{-}(1\rightarrow4)\text{—}(\beta\text{-D-Manp})_4\text{-}(1\rightarrow4)\text{-}(\beta\text{-D-Gp})_2\text{-}(1\rightarrow4)\text{-}(\beta\text{-D-Manp})_2\text{-}$

Osmometric studies of the glucomannan acetate showed that it had a molecular weight of 38,000 corresponding to $\overline{DP}_n$ of 130. This work of Hamilton and his co-workers represented the first isolation and characterization of an authentic wood glucomannan.

Aspinall, Laidlaw and Rashbrook (33) investigated the structure of a glucomannan from Sitka spruce (Picea sitchensis). Structurally it is essentially a linear polymer of $\beta\text{-}(1\rightarrow4)$-linked residues of D-glucose and D-mannose in a ratio of 1:2.5. Partial hydrolysis was effected by treating the polysaccharide with a mixture of acetic anhydride, acetic acid, and concentrated sulphuric acid at 0° and deacetylating the product with barium methoxide. This gave monosaccharides, cellobiose, mannobiose, and a mannosyl-glucose, which were identified by paper chromatography only. Periodate and methylation studies suggested that the polysaccharide contained 45 repeating units. Molecular weight determinations by isothermal distillation gave a value of $10,000 \pm 500$ corresponding to a $\overline{DP}_n$ of 47-51.

One residue in 35 units was a non-reducing end group. The possibility that a small proportion of molecules contained a single branch point cannot therefore be excluded. It was not definitely proven whether the tetra-O-methyl hexose isolated was the 2,3,4,6-tetra-O-methyl-D-glucose or the corresponding mannose derivative but the latter alternative was stated to be the more probable.

At the same time as the above investigators, Dutton and Hunt (34) also tried to establish the structure of the glucomannan from Sitka spruce. Methylation studies showed that the polysaccharide contained a linear polymer of β-(1→4)-linked D-mannopyranose to D-glucopyranose residues in a (rather astonishing) ratio of 9:1. The repeating unit, which was apparently unbranched, contained 40-60 sugar residues. 2,3,4,6-Tetramethyl-O-methyl-D-galactose was isolated as an end group in one of the fractions examined. Perhaps osmometry studies, partial hydrolysis and periodate oxidation would strengthen their claim as to whether the polymer is branched or not and also afford some more information as to how the rest of the polymer is constructed.

Croon and Lindberg (35) extracted two glucomannan fractions from Norwegian spruce (Picea abies) holocellulose, previously swollen with dimethyl sulphoxide and subsequently extracted with hot water. Most of the xylan was removed from the holocellulose by extraction with 14% potassium hydroxide. Subsequent extractions of the residual holocellulose by potassium hydroxide-borate solutions by the method of Jones et al. (36) produced more glucomannans. After purification of the polysaccharides via their copper complexes, they had a constant ratio of glucose to mannose

of 1:3.5-4. Methylation, periodate oxidation and molecular weight determinations showed that the polymers had a $\overline{DP}_n$ of 68 and 100 respectively and are composed of β-(1⟶4)-linked D-glucopyranose and D-mannopyranose residues. High yields of 2,6-di-O-methyl hexoses and 2,3,4,6-tetra-O-methyl-D-mannose indicated that the polysaccharides are slightly branched and that the branches are located at C_3 of the glucose residues. The number of branches per macromolecule was 3-4.

Croon, Lindberg and Meier (37) found that the glucomannan in a holocellulose from Scots pine (Pinus silvestris) is partly bound to the remaining lignin, even when the holocellulose had a very low Klason lignin content. The $\overline{DP}_n$ value for a 3-4% lignin content was 97. Treatment of the crude glucomannan with gaseous chlorine and ethanolamine reduced the lignin content to 0.2%. The $\overline{DP}_n$ was now 93, thus substantiating the mild nature of the delignifying agent as originally found by Timell and Jahn (38).

Methylation and periodate oxidation indicated the presence of a chain of β-(1⟶4)-linked D-glucopyranose and D-mannopyranose residues. The glucomannan in this case is probably branched as inferred by the high percentages of tetra-O-methylhexoses isolated, indicating an average of 3 branches per 100 hexose residues. The 2,6-di-O-methylhexoses isolated could possibly be due to incomplete methylation. The authors claim, however, that the branch point is probably located at C_3 in the glucose residues.

Hamilton and Partlow (39) extracted with 18% sodium hydroxide a glucomannan from a chlorite holocellulose of western red cedar. The ratio

of glucose to mannose was 1:2:5 which is a ratio typical for most coniferous glucomannans. Methylation studies gave the usual 2,3,6-tri-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-mannose, and 2,3,4,6-tetra-O-methyl-D-glucose, in addition to trace amounts of di-O-methyl-D-galactose and 2,3,4,6-tetra-O-methyl-D-galactose, presumably from a contaminating galacto-glucomannan. Graded partial hydrolysis by dilute sulphuric acid indicated the presence of glucosyl-mannose, mannosyl-glucose, cellobiose, mannotriose and mannotetraose, all tentatively identified by paper chromatography. Structurally, this glucomannan can be written as

$$4\text{-}\beta\text{-D-Gp-}(1\rightarrow 4)\text{-}(\beta\text{-D-Manp})\text{-}(1\rightarrow 4)\text{-}(\beta\text{-D-Manp})\text{-}(1\rightarrow 4)\text{-}(\beta\text{-D-Gp})_2\text{-}(1\rightarrow 4)\text{-}(\beta\text{-D-Manp})_3\text{-}(1\rightarrow 4)\text{-}(\beta\text{-D-Gp})\text{-}$$

Timell and Tyminski (40) isolated a glucomannan from white spruce holocellulose via its copper complexes. The ratio between the anhydroglucose and mannose units was 1:3. The specific rotations of the purified material was -33.5°. The intrinsic viscosity in cupriethylenediamine was 0.24 dl./g. corresponding to a degree of polymerization of 35 to 40. Partial acid hydrolysis, methylation, periodate oxidation, osmometry and light-scattering studies are the basis of the structure proposed by the authors (40a). Mannotriose, mannosyl-glucose, glucosyl-mannose, mannotriose and mannotetraose were all obtained crystalline and found to be β-(1$\rightarrow$4)-linked. The main sugars obtained by hydrolysis of the fully methylated glucomannan were 2,3,6-tri-O-methyl-D-mannose and 2,3,6-tri-O-methyl-D-glucose. It could not be decided whether or not the polysaccharide was branched. Number and weight-average degrees of polymerization were 104 and 183 respectively.

Although most wood glucomannans isolated so far have been from

coniferous woods, there have been recent cases where woody angiosperms (hardwoods) have yielded similar glucomannans, for example, trembling aspen and white birch. Jones, Merler and Wise (41) isolated a mannose-rich hemicellulose fraction from aspen wood (Populus tremuloides) via its copper complex. Analysis showed that the ratio of glucose to D-mannose was 1:2, minor amounts of xylose also being present. Methylation studies indicated the presence of a linear heteropolymer of β-(1→4)-linked chains of D-glucopyranose and D-mannopyranose units, the non-reducing end groups being mainly mannose.

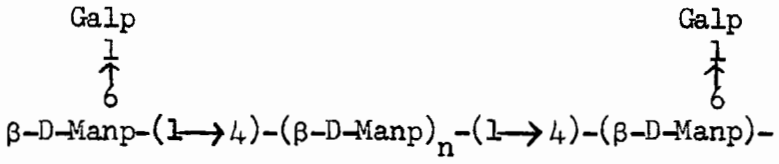
Recently Timell (42) has isolated a glucomannan from white birch (Betula papyriferea). The polysaccharide was electrophoretically homogeneous, and its composition specific rotation was -35°. The ratio of glucose to mannose residues was 1:1.1. Partial hydrolysis gave manno-biose, mannosyl-glucose, glucosyl-mannose, cellobiose, mannotriose, and mannotetrose and, in addition, several mixed trisaccharides. Except for the low ratio of glucose to mannose, this glucomannan is apparently very similar to the softwood glucomannans.

Very recently (43) a series of glucomannans have been isolated from several different hardwood species in this laboratory. The ratio of glucose to mannose is 1:1.5-2 except for the birches where it is close to 1:1. The detailed structure of the glucomannans from trembling aspen (Populus tremuloides) (43) and from red maple (Acer rubrum L.) (44) are presently being studied. The presence of a β-(1→4)-linked polysaccharide is strongly indicated in both cases.

Galactomannans are common constituents of the ungerminated seeds of leguminous plants, amounting in some cases to more than 40% of the total seed. They occur as mucilages in the endosperm of the seeds from which they may be isolated by extraction with water. Wise and Appling (45) have investigated the seeds of more than 1600 species of legumes and Anderson (46) has also found that many of them contain galactomannans. The amounts of polysaccharides present in the various seeds were found to vary considerably, the highest yield being found for carob seed (38% of the weight of the seed) and guar seed (35%). The composition of galactomannan is variable although all those examined contained less galactose than mannose. Invariably the molecular ratio of the sugars was 16:81 in sophara japonica while in guar seed the ratio was 38:58.5. Galactomannan derivatives from one species can also have a variable composition. The ratio of D-galactose to D-mannose in gum ghatto has been reported as 16:84 by Hirst and Jones (47), 20:80 by Smith (48) and Wise and Appling (45), 27:73 by Spada (49) and 18:82 by Lew and Gortner (50).

Fenugreek seed (51) had a D-galactose to D-mannose ratio of 48:52 and lucerne seed (52) is unique in that it contains more D-galactose than D-mannose, the ratio being 2:1. Most of the galactomannan is extracted from the milled seeds with hot water and is purified by complexing with copper. The structure of galactomannans has been determined by methylation and periodate oxidation studies. Hirst and Jones (53) identified 2,3,4,6-tetra-O-methyl-D-galactose (1 part), 2,3,6-tri-O-methyl-D-mannose (4 parts) and 2,3-di-O-methyl-D-mannose (1 part). Galactose was present in the pyranose form occupying a terminal position and was attached to a skeleton of mannose residues linked together by β-(1$\rightarrow$4)- and β-(1$\rightarrow$6)-linkages.

Guaran (45, 46, 54) has a similar structure. The recent isolation of two disaccharides from guaran by Whistler and co-workers (55, 55a), 4-(β-D-mannopyranosyl)-β-D-mannopyranose and 6-(α-D-galactopyranosyl)-β-D-mannose, form a structure in which the terminal galactopyranose units were joined by α-(1→6)-glycosidic links to a chain of mannopyranose units linked by units of the (1→4)-β type.



where Galp = D-galactopyranose

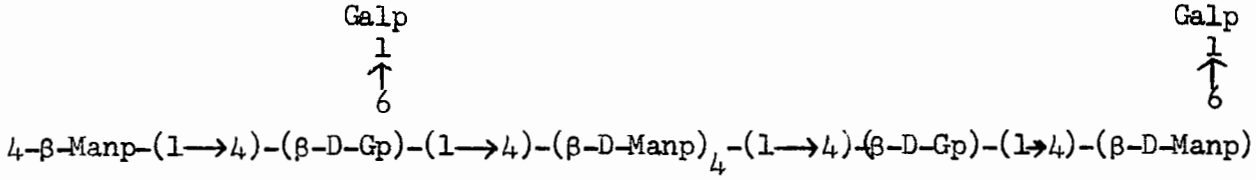
Manp = D-mannopyranose

The structure is in agreement with findings of Palmer and Ballantyne (56) who investigated guaran using X-ray diffraction methods.

Lucerne and clover galactomannans are highly branched because 40% of galactose is present as end groups. Coniferous and deciduous woods have both been shown to contain true glucomannans but the latter are not the only mannose-containing polysaccharides present in coniferous woods since mannose-containing polymers have been shown to be associated with galactose also. Adams (57) was able to separate the water-soluble extract of white spruce wood meal into what appeared to be an arabogalactan, and a galactoglucomannan. More recently the isolation of the first galactoglucomannan from wood has been reported by Hamilton, Partlow and Thompson (58).

They isolated a mixture of galactoglucomannan and an araboxylan by extraction with 5% sodium hydroxide of a wood cellulose produced from a 1:1 combination of slash pine (Pinus elliottii) and long leaf pine (Pinus

palustris) by the conventional kraft process. The polysaccharides were isolated as an acetone soluble galactoglucomannan acetate, which had a ratio of D-galactose, D-glucose, D-mannose of 1:1:3. Methylation and hydrolysis of the methylated polymer gave 2,3-di-O-methyl-D-mannose (10%), 2,3,-di-O-methyl-D-glucose (3.21%), 2,3,6-tri-O-methyl-O-mannose (55.0%), 2,3,6-tri-O-methyl-D-glucose (13.21%) and 2,3,4,6-tetra-O-methyl-D-mannose (3.21%) and 2,3,4,6-tetra-O-methyl-D-galactose (15.3%). These results indicate that the galactoglucomannan is a branched polymer, joined through positions 1 and 4, and occasionally 1 and 6 of the glucose and mannose units. The galactose residues all occur as non-reducing end groups, attached to the main framework, indicating a similar structure. The relatively slow oxidation of galactose may be explained by the bulky constituents attached at the C_6 atom of some glucose and mannose units. Further graded acid hydrolysis studies are essential before a definite structure can be assigned.



The above simplified structure was proposed by the authors.

RESULTS AND DISCUSSION

The hemicelluloses of conifers constitute approximately one-fifth of the weight of the wood, and glucomannan accounts for approximately two-thirds of the material. The relative ratios of the neutral sugars found in wood were determined according to the method of Timell and co-workers (59) and are given in Table I.

White pine wood (40-60) mesh was extracted first with ethanol-benzene to remove fats and waxes, and then with 0.5% ammonium oxalate to remove the pectin. A representative sample of holocellulose was prepared by treating the extractive-free wood with acid chlorite (pH 5) according to the method of Wise, Murphy and D'Addieco (60). Four treatments were sufficient to give a yield of holocellulose of 75.5%.

Further delignification was avoided since it has been shown by Timell and Jahn (38) that excessive treatment with chlorite causes both depolymerization and loss of carbohydrate.

A crude hemicellulose mixture was isolated from the holocellulose by extraction with 17.5% sodium hydroxide containing 4% boric acid according to Jones and co-workers (36). The hemicelluloses were precipitated in a mixture of ethanol and acetic acid, followed by solvent exchange through ethanol and drying in vacuo from diethyl ether. The yield corresponded to 39.3% of the holocellulose.

Chromatographic examination of a hydrolysate of the hemicellulose gave the results summarised in Table I. Preliminary attempts to resolve

TABLE I

Chemical Composition of White Pine Wood Derivatives

Component	Neutral sugars in wood	Hemicellulose	Glucomannan	
			Crude	Purified
Galactose	2.1%	3.3	6.4	1.8
Glucose	70.4%	13.3	17.5	20.6
Mannose	17.7%	41.5	70.3	77.6
Arabinose	3.0%	34.3	(trace)	-
Xylose	6.8%	7.1	5.8	(trace)

the hemicellulose into a glucomannan and a 4-O-methyl-glucuronoxylan met with little success. The crude mixture was dissolved in alkali and Fehling's solution (61) was added in an attempt to form an insoluble copper complex of the glucomannan component since it has been found that the vicinal cis-hydroxyl groups of the mannose residues have a tendency to form copper complexes with Fehling's solution whereas xylose will not do so. It was found, however, that the copper complex formed was unstable in water and reproducible results were difficult to obtain.

Two fractions, A and B, were isolated. Results obtained by

paper chromatography are given in Table II.

TABLE II

Qualitative Composition of Fractions A and B

Fraction (A)		Fraction (B)	
Neutral component	Acidic component	Neutral component	Acidic component
Glucose		Galactose	
Mannose		Glucose	
Arabinose (trace)		Mannose	
Xylose		Arabinose (trace)	
		Xylose (trace)	

Further fractionation of the hemicellulose mixture with barium hydroxide according to Meier (18) showed that this reagent forms an insoluble copper complex with glucomannans, while xylans remain in the alkaline solution. The reaction is probably due to the presence of vicinal cis-hydroxyl groups on carbon atoms 2 and 3 of the mannose residues. The method is quite effective, one or two treatments being sufficient for an almost

complete separation of the two polysaccharides.

One treatment with barium hydroxide followed by decomposition of the precipitate with acid and recovery of the polysaccharide in the usual way gave a product the composition of which is presented in Table I. Further barium hydroxide fractionation failed to remove the galactose residues. It has been noticed in previous investigations on glucomannans from Scots pine (Pinus silvestris) by Croon and Lindberg (35) that a residual lignin content of 3-4% might act as a linkage between a true glucomannan and a possible galactan, galactoglucomannan or galactomannans. It is desirable to remove this lignin before attempting to isolate pure hemicelluloses. An ultraviolet spectrum of the glucomannan showed a maximum at 280 mμ which was quite intense and the failure to remove galactose residues could possibly be due to the presence of this residual lignin. The purified glucomannan was accordingly delignified in a homogeneous medium with chlorine, followed by extraction with alcoholic ethanolamine after which it was fractionally precipitated as the insoluble copper complex. This method reduced the lignin content to a very low level, leaving the polysaccharide practically unchanged in chain length as indicated by results reported in (38) and (62).

Analysis of the glucomannan indicated the presence of glucose (20.6%), mannose (77.6%), xylose (traces) and galactose (1.8%).

Osmotic pressure measurements were carried out on the crude glucomannan, the purified and the fully methylated polymer with osmometers of the type designed by Zimm and Myerson (63) and later modified by Stabin and Immergut (64). The solvent employed was chloroform-ethanol (9:1 v/v)

freshly distilled before use. Tables V, VI and VII give the values obtained for w, the concentration in g./kg. solvent; h, the osmotic pressure height of solution in cm. of Hg and h/w, the reduced osmotic pressure. The latter, when plotted against the concentration and extrapolated to zero concentration, gave $(h/w)_o$. For the calculation of the molecular weight from osmotic pressure data, the following equation, which has been extensively verified, was used (88).

$$M = \frac{RT}{(\pi/c)_o} d,g = \frac{8.315 \times 10^7}{980.7} \times \frac{T}{(\pi/c)_o} d_1$$

In the equation d_1 is the density of the solvent, g is the acceleration due to gravity, R is the gas constant, T is the absolute temperature, π is osmotic pressure in $g./cm.^2$, c is concentration in g./cc. and the subscript zero indicates that the value of π/c must be taken at zero concentration. The determination of the density can be avoided if the solution is made up by weight. By using the concentration, w, in g./kg., of solution and the osmotic height, h, in centimeters, the equation becomes for 30

$$\bar{M}n = \frac{25,700}{(h/w)_o}$$

The values of $\bar{M}n$, the number molecular weight, and $\bar{P}n$, the number average degree of polymerisation were calculated for the glucomannan. The value for $(h/w)_o$ was 0.98, and thus for the crude glucomannan nitrate fraction

TABLE V

Osmometry Data for the Crude Nitrated Pine Glucomannan

	w*	h**	h/w
1.	5.004	5.136	1.026
2.	3.151	3.208	1.018
3.	2.225	2.205	0.991
4.	1.330	1.345	1.006
5.	0	-	0.98

* concentration in g./kg. solvent

** osmotic height in cm. solution.

TABLE VI

Osmometry Data for the Delignified Nitrated Pine Glucomannan

	w*	h**	h/w
1.	4.429	5.208	1.176
2.	3.323	3.790	1.140
3.	2.218	2.580	1.163
4.	2.198	2.504	1.139
5.	1.121	1.344	1.108
6.	1.113	1.253	1.125
7.	0	-	1.11

* concentration in g./kg. solvent

** osmotic height in cm. solution.

TABLE VII

Osmometry Data for the Methylated Pine Glucomannan

	w*	h**	h/w
1.	2.511	3.627	1.444
2.	1.952	2.661	1.363
3.	1.399	1.880	1.343
4.	0.823	1.098	1.334
5.	0	0	1.31

* concentration in g./kg. solvent

** osmotic height in cm. solution.

$$\bar{M}n = \frac{25,700}{0.98} = 26,500$$

and $$\bar{P}n = \frac{26,500}{285} = 93$$

whereas for the purified glucomannan nitrate $\bar{M}n = \frac{25,700}{1.11} = 23,200$ and $\bar{P}n = \frac{23,200}{285} = 82$.

The corresponding value of $\bar{M}n$ and $\bar{P}n$ for the methylated white pine glucomannan was 19,600 and 96. A plot of reduced osmotic pressure against concentration for the crude nitrated glucomannan fraction and the methylated glucomannan are shown in Fig. 1.

After fractionation of the crude delignified glucomannan by the copper complex method, the final purified glucomannan was a pale bluish white powder containing small quantities of galactose, and xylose residues. The ratio of glucose to mannose remained unchanged at a ratio of 1:3-4, thus indicating the homogeneous nature of the product.

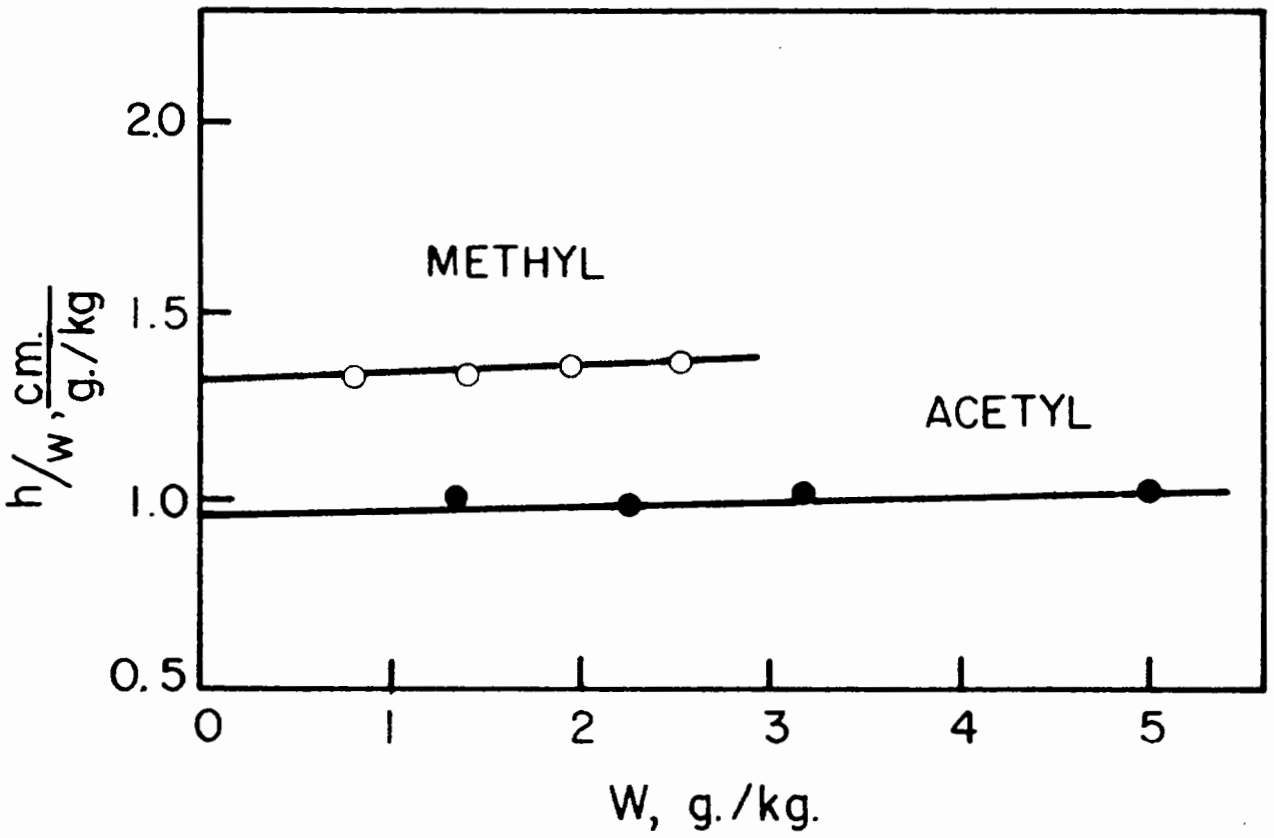
Several conifers have been reported to contain a galacto-glucomannan in addition to glucomannans (31,58). Fractional extraction after delignification of the crude glucomannan failed to produce evidence for the presence of a galactoglucomannan in white pine.

The negative rotation $\left[\alpha\right]_D^{22}$, -31.6° of the glucomannan suggested that the polysaccharide was predominantly a β-linked heteropolymer.

Information regarding the sequence of the sugar residues in the glucomannan was obtained by partial hydrolysis of the glucomannan. The mixture of oligosaccharides obtained was resolved on a charcoal-Celite

FIGURE 1

Variation of Osmotic Pressure of Glucomannan Derivatives with Concentration


2.0
1.5
1.0
0.5
METHYL
ACETYL
h/w, cm./g./kg
0
1
2
3
4
5
W, g./kg.

column (65) by gradient elution with ethanol and the results are tabulated in Table III.

Twelve oligosaccharides were isolated of which five were obtained crystalline and fully characterised. These include four disaccharides namely 4-O-β-D-mannopyranosyl-D-mannose (mannobiose), 4-O-β-D-mannopyranosyl-D-glucose (mannosyl-glucose), which was chromatographically indistinguishable from a sample from white spruce (40a) but could not be induced to crystallise, and 4-O-β-D-glucopyranosyl-D-mannose (glucosyl-mannose) and 4-O-β-D-glucopyranosyl-β-D-glucose (cellobiose). A mannotriose, O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-D-mannose, and one mannotetraose O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-D-mannose also crystallised.

The first three components were isolated previously from several coniferous woods (14,17,31,32,33,39,66). Mannotriose was obtained crystalline from loblolly pine wood (31) and white spruce (40a) and was probably present also in the glucomannan hydrolysate from western hemlock (32), red cedar (39) and a spruce sulfite pulp (66). The mannotetraose was previously isolated from the mannan of vegetable ivory (67) and from a white spruce glucomannan (40a). The remaining six oligosaccharides were presumably trisaccharides but attempts to crystallise them were unsuccessful and they could not be fully identified.

One of the six oligosaccharides was partially identified by acid hydrolysis as glucose (1 part) and mannose (2 parts). Partial

TABLE III

Sugars Obtained on Partial Hydrolysis of the Glucomannan

Sugar components	Weight in grams
Total amount added	7.50
Monosaccharides	
Glucose, Mannose, Xylose and Galactose	2.8888
Oligosaccharides	
Mannosyl glucose	0.0725
Glucosyl mannose	0.3717
Mannobiose	1.0148
Cellobiose	0.0233
Mannotriose	0.2620
Trisaccharide A	0.2846
Mixture of trisaccharides	0.9411
Mannotetraose	0.1573
Mixture of higher oligosaccharides	0.8768
Total =	6.8929

hydrolysis with formic acid followed by paper chromatography indicated glucosyl-mannose and mannobiose. The structure of this oligosaccharide as glucosyl-mannosyl-mannose was supported by borohydride reduction followed by acid hydrolysis and paper chromatography which gave glucose (1 part) and mannose (1 part). The identification of these two monosaccharides indicated that mannose was the reducing end group. The DP of 3 as given by the method of Peat, Whelan and Roberts (80) corroborated the structure as glucosyl-mannosyl-mannose, but the mode and position of the linkages could not be determined.

The classical methylation technique was employed for further structural studies of the glucomannan. A sample was subjected to two methylations with dimethyl sulfate and 40% sodium hydroxide according to the method of Haworth (68) followed by two methylations in dimethyl formamide solution using the method of Kuhn (69). A final methylation by the method of Purdie (70) was used to attain the degree of methylation theoretically required for a glucomannan. The product had a methoxyl content of 45.0% indicating complete substitution. Acid hydrolysis of the fully methylated glucomannan yielded a mixture of methylated monosaccharides which was resolved on a charcoal-Celite column by gradient elution with aqueous ethanol, and the results are tabulated in Table IV. A mixture of di-O-methyl hexoses was obtained constituting 1.9% of the total hydrolysate. Paper electrophoresis indicated that the predominant component was 2,6-di-O-methyl-D-glucose together with minor amounts of 3,6-di-O-methyl-glucose and traces of 2,4-di-O-methyl-glucose. The presence of these sugars may be due to branching in the polysaccharide. However, they could also be a result of incomplete methylation or demethyl-

TABLE IV

Separation of Mixture of Methylated Sugars on a Charcoal-Celite Column

Tube No.	Component	Yield mg.
1-51	Monosaccharides and impurities	27.2
54-66	Mixture of two di-O-methyl-hexoses	21.0
66-96	Mixture of di-O-methyl-hexoses, and 2,3,6-tri-O-methyl-D-mannose	436.4
99-200	2,3,6-tri-O-Methyl-D-mannose (negative rotation)	2060.7
200-259	2,3,6-Tri-O-methyl-D-glucose (positive rotation)	814.3
259-271	2,3,4,6-Tetra-O-methyl-D-mannose and possibly 2,3,4,6-tetra-O-methyl-D-glucose	43.7

ation during methanolysis or hydrolysis of methylated polysaccharides. A similar demethylation was recently reported by Glaudemans and Timell (62).

Elution with 20-40% of ethanol gave pure, sirupy 2,3,6-tri-O-methyl-D-mannose, identified through its crystalline di-p-nitrobenzoate ester and its aniline derivative. 2,3,6-Tri-O-methyl-D-mannose, and the corresponding glucose derivative are usually difficult to separate. Their rates of movement on the paper chromatograms are almost identical and various other techniques have therefore been developed for their separation, e.g., selective lactone formation (25) or selective formation of the glucofuranoside (26). It was originally observed by Lindberg and coworkers (35) however that these sugars can be separated by gradient elution from charcoal, albeit with some overlapping. In the present study, 2,3,6-tri-O-methyl-D-glucose was accordingly obtained directly from the charcoal-Celite column, immediately following the 2,3,6-tri-O-methyl-D-mannose. The sugar crystallized and was identified through its di-p-nitrobenzoate derivative.

A mixture of tetra-O-methyl hexoses was finally obtained. Demethylation of a sample indicated the presence of mannose together with small amounts of glucose. An infrared spectrograph of the sirup showed the characteristic peaks for a 2,3,4,6-tetra-O-methyl-D-mannose.

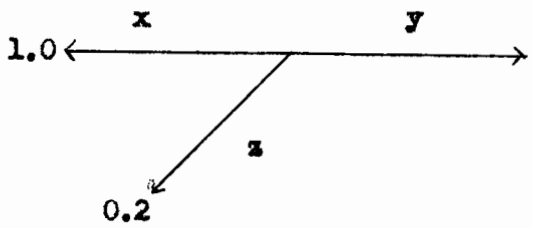
The aniline derivative of the sirup, on repeated recrystallization, had the melting point of 2,3,4,6-tetra-O-methyl-N-phenyl-D-mannosylamine. The tetramethyl-hexose fraction was therefore predominantly 2,3,4,6-tetra-O-methyl-D-mannose.

From the above evidence a tentative structure can be suggested for the glucomannan present in the wood of white pine. The isolation of a large percentage by weight of 2,3,6-tri-O-methyl-D-mannose and 2,3,6-tri-O-methyl-D-glucose from the methylated hemicelluloses proved that the polysaccharide was mainly composed of mannose and glucose residues linked by -β(1→4) glycosidic bonds. This conclusion was further corroborated by the consumption of 1.108 moles of sodium metaperiodate per anhydro hexose unit during oxidation of the glucomannan. The negative rotations indicated that the hexose residues are present in the β-configurations. These conclusions are further corroborated by the isolation and identification of the hexose oligosaccharides, all of which were (1→4)-β-linked. The preponderance of mannose-containing oligosaccharides indicated that large segments of each macromolecule must be composed of mannose residues only, with the glucose residues probably distributed at random along the chains. The low yield of cellobiose and the absence of any cellotriose suggested that some of these glucose residues are contiguous.

The presence of 1.5 part of di-O-methyl-hexoses, 77 parts of tri-O-methyl-hexoses and 1 part of tetra-O-methyl-hexoses in the methylated polysaccharides indicated that there was one non-reducing end group present per 80 hexose residues. Most of these end groups appeared to be mannose but the possibility that glucose occasionally occurred as a non-reducing end group could not be excluded.

The osmotic pressure measurements suggested a number-average degree of polymerisation of 96 for the methylated glucomannan. Each

molecule therefore contained on the average $\frac{96}{80} = 1.2$ non-reducing end groups. Since one of these end groups originated from the hexose framework this



$$x + y + z = 96$$

would correspond to 0.2 branch points, that is, one average molecule in every four would be branched, and the remainder would be linear. The presence of only 0.2 end groups which would approximate to a branch point in the polymer is not conclusive evidence for the exact nature of the polysaccharide since tetra-O-methyl-hexose might have been lost in the resolution of the sugar mixture. On the basis of the present evidence, however, it is tentatively suggested that the glucomannan is essentially linear, the possibility of a slight branching not being excluded. The presence of 1.5 parts of di-O-methyl-hexose in the hydrolyzate from the methylated hemicellulose might arise also from demethylation, but on the other hand supports a low degree of branching of the macromolecules. The consumption of 1.1 mole of sodium metaperiodate per anhydrohexose unit during oxidation of the glucomannan is also consistent with a linear structure for the hexosan.

As mentioned in the introduction, hemicelluloses of the glucomannan type have been isolated from several other softwoods, namely western hemlock (32), white spruce (40a), Norway spruce (35) and western red cedar (39). The main contributors to the chemistry of the glucomannans were

the following: Aspinall (33), Dutton (34), Hamilton and co-workers (32, 39), Lindberg (35,37), Wise (10,13,21,36,41) and, recently, the workers in this laboratory (40,40a,42,43,44).

The number-average degree of polymerisation found for the methylated ($\bar{P}n$ = 96) and nitrated ($\bar{P}n$ = 82) glucomannan from white pine by the author is in the same range as the values reported for the glucomannan triacetate of western hemlock ($\bar{P}n$ = 130), and the glucomannan nitrate esters of Scots pine ($\bar{P}n$ = 70-115) and Norway spruce ($\bar{P}n$ = 68-100).

Difficulties were encountered by Lindberg (37) and Tyminski (40) during the isolation and preparation of several wood glucomannans. When the choice of the copper or the barium complex for purification was determined by small-scale tests, the method giving the best yield together with the removal of the xylose was used. The elimination of galactose residues (35) was previously found to require additional delignification of the crude hemicellulose. From the chemical and physical evidence reported, a tentative and simplified structure for the glucomannan present in the wood of white pine is presented in Fig. 2.

4-β-D-Manp-(1⟶4)-(β-D-Manp)-(1⟶4)-(β-D-Gp)-(1⟶4)-

(β-D-Manp)-(1⟶4)-(β-D-Gp)-(1⟶4)-(β-D-Manp)-. . . .

Manp = mannopyranose

Gp = glucopyranose

FIGURE 2

Simplified Linear Structure of the White Pine Glucomannan

EXPERIMENTAL

Analytical Methods and Reagents

Paper Chromatography

Chromatographic separations of sugars were carried out on Whatman No. 1 (or for preparative purposes) No. 3 M.M. filter papers by the descending technique. The systems used were (A) ethyl acetate-acetic acid-water (9:2:2, v/v); (B) 1-butanol-pyridine-water (10:3:3, v/v); (C) 1-butanol-pyridine-water (6:4:4, v/v), for disaccharides; (D) butan-1-one-water (89:11, v/v), for methylated sugars.

The spray reagent was made up of 3.0 g. of o-amino-diphenyl dissolved in acetic acid (100 ml.) to which was added 85% reagent grade phosphoric acid (1.3 ml.) (59). This reagent was used for detecting monosaccharides, disaccharides, uronic acids, and methylated sugars on paper chromatograms. Aldohexoses gave dark brown, aldopentoses, bright red, and hexuronic acid, orange-coloured spots. Quantitative paper chromatography was carried out by the spectrophotometric method for determination of sugars by Timell, Glaudemans and Currie (59). The indicator employed was o-amino-diphenyl and relative densities were determined with a Beckman Model DU spectrophotometer. Chromatographic papers employed were Whatman No.1 previously washed to remove water-soluble polysaccharides and other impurities.

Electrophoresis: Electrophoretic separations were carried out on What-

man No. 1 filter paper in a 0.5M borate buffer at pH 10 at 750 volts for 3-5 hr. The spray reagent was o-amino-diphenyl. For polysaccharides, glass fiber paper was used in conjunction with a spray reagent consisting of 2 g. of α-naphthol dissolved in 100 ml. of butan-1-ol containing 5 ml. of concentrated sulfuric acid (72).

Ion Exchange Resin Column

Amberlite IR-120 Cation Exchange Resin (20-50 mesh)

This ion exchange resin (chloride cycle) was added to a large column and 20% ammonium hydroxide solution was allowed to percolate after which the resin was washed with water to remove excess base. The resin was generated with 20% hydrochloric acid solution and then washed with water until all acid had been removed. Unless otherwise specified, the resin was used in the hydrogen cycle.

Dowex 1-X4 Ion Exchange Resin (50-100 mesh) (chloride cycle)

The resin was washed with 2N sodium hydroxide solution until all chloride was removed (the eluate was tested with silver nitrate solution for chloride). Elution was continued with the water until the eluate did not contain any chloride ion, after which the resin was washed with 8N acetic acid, followed by water until the eluant was neutral.

Anhydrous Methanol

Dry methanol was prepared according to the magnesium procedure given in

Vogel, A.I. "Practical Organic Chemistry", Longmans, 3rd edition, page 169.

Anhydrous Dimethyl Formamide

The reagent grade product was refluxed over barium oxide and then distilled shortly before use.

Darco G-60 Charcoal

The product was washed with 10% hydrochloric acid, water, 50% aqueous ethanol, until acid free, and finally with water.

Silver Oxide

Silver oxide was prepared by the method of Helferich and Klein (73) immediately prior to use.

Silver nitrate (340 g.) was dissolved in 2 l. of water and the solution was heated to 80°C. A solution containing 80 g. of sodium hydroxide which was also heated to 80°C was added with stirring to the above solution. The resulting solution was decanted from the brown precipitated silver oxide which was washed eight times by decantation with 3 l. portions of distilled water each time. The silver oxide was suspended in 125 ml. of absolute ethanol, the ethanol was decanted and the silver oxide washed with ethanol. After suction filtration on a hardened filter paper and air drying, the silver oxide was dried *in vacuo* over phosphorus pentoxide.

Methoxyl Analysis

The procedure described by Viebock and Schwappach (74) and modified by Timell and Purves (75) was used.

Molisch Test

One or two drops of a 1% ethanol solution of α-naphthol were added to one ml. of sugar. Concentrated sulfuric acid was carefully added to form a second layer beneath the above solution. A purple ring between the two liquid layers indicated the presence of sugars (76).

Analysis and Composition of White Pine Wood

Alpha-cellulose, lignin, pentosan, and uronic anhydride were determined by standard Tappi methods (77).

For estimation of the sugar content of the wood, 1 g. samples of the latter were hydrolyzed to the corresponding sugar mixtures according to the procedure of Saeman and co-workers (78). The sugars were separated on Whatman No. 1 filter paper with both ethyl acetate-acetic acid-water (9:2:2), and butanol-pyridine-water (10:3:3) as solvent systems. Guide strips were employed in the usual way for locating the sugars, and the latter were quantitatively determined in triplicate analyses by the spectrophotometric o-amino-diphenyl method of Timell, Glaudemans and Currie (59). Found: galactose (2.1%); glucose (70.4%); mannose (17.7%); arabinose (3.0%); xylose (6.8%) (see also Table I).

Preparation of White Pine Wood

Wood from a 20 year old tree of white pine (cut in the Fall of 1957) was converted into wood meal. The 40-60 mesh fraction was exhaustively extracted in a Soxhlet apparatus with ethanol-benzene (1:2, v/v) for 24 hr. After drying in the air, the wood was extracted for 2 days with cold water, washed with water and alcohol, and dried in the air. Found: α-Cellulose (43.0%); lignin (29.3%); pentosan (9.8%); acetyl (1.23%); ash (0.24%); uronic anhydride (3.98%); galactose (1.40%); glucose (46.0%); mannose (11.7%); arabinose (2.0%); xylose (4.5%) (based on extractive-free, oven-dry wood).

Preparation of White Pine Holocellulose

Holocellulose was prepared from extractive-free white pine wood meal (40-60 mesh) by the method of Wise, Murphy and D'Addieco (60). Wood meal (200 g.) was suspended in 5 l. of water in a 6 l. Erlenmeyer flask and the temperature was raised to 75-80° with stirring. Acetic acid (20 ml.) and technical sodium chlorite (60 g.) were added and the reaction was allowed to proceed at 75-80° for 1 hr., after which the same amounts of acid and chlorite were added. After 4 treatments, the flask was removed, and the solids were recovered by filtration and washed with distilled water until free from chlorine. About 2 l. of ethanol was slowly allowed to drain through the holocellulose, which was then dried in air overnight. Yield 157 g. (75.5%).

Preparation of Crude Hemicellulose from White Pine Holocellulose

White pine holocellulose (300 g.) was suspended in a 17.5%

sodium hydroxide solution (4 l.) containing boric acid (40 g. per l.) in a stoppered 6 l. Erlenmeyer flask (36). The air was replaced by nitrogen, and the flask was shaken continuously for 2-3 hr. The mixture was cooled to room temperature, and the solid residue was removed by filtration on a sintered-glass filter funnel. It was further washed with 17.5% sodium hydroxide solution (3 l.) and was then washed with 50% aqueous acetic acid, water and ethanol. The product was dried in the air to yield 109 g. (dry) of α-cellulose. The aqueous lemon-coloured filtrate from above was slowly poured with stirring into a jar containing ice-cold ethanol (9 l.) and glacial acetic acid (2 l.). The precipitated hemicellulose was collected on a sintered glass, washed 3 times with 80% ethanol, 3 times with 100% ethanol, and finally 3 times with diethyl ether. The product was dried over phosphorus pentoxide in a vacuum dessicator under high vacuum, yielding crude hemicellulose (118 g.). Analysis for constituent sugars gave the following results. Glucose (13.3%); mannose (41.5%); arabinose (7.4%); and xylose (34.8%). (See Table I).

Fractionation of the Hemicellulose Mixture

The hemicellulose (10 g.) was shaken for 2 hr. in an Erlenmeyer flask containing 5% sodium hydroxide solution (1 l.) in a nitrogen atmosphere. The flask contents were then centrifuged and the centrifugate added to a centrifuged solution of Fehling's solution, stirring continually (61). A pale blue gelatinous copper complex precipitated. A slight excess of Fehling's solution was added, and the mixture centrifuged. The precipitated copper complex was washed once with water, and

then decomposed with ice-cold aqueous acetic acid (1:1). The solution formed was centrifuged and the clear centrifugate was poured into 2 vols. of ice-cold ethanol with stirring. A white precipitate was obtained, which was washed twice with 80% ethanol, 3 times with 100% ethanol, and finally 3 times with diethyl ether after which it was dried over phosphorus pentoxide in vacuo yielding fraction A (4 g.). The centrifugate obtained on removal of the copper complex was neutralised with ice-cold acetic acid (1:1) and then poured into 2 volumes of ethanol. The reprecipitated polysaccharide was recovered on the centrifuge and was successively washed 3 times each with 80% ethanol, 100% ethanol, and finally diethyl ether. Drying in vacuo yielded fraction B (4 g.).

Fraction (A) and Fraction (B) (1 g.) were separately hydrolysed for qualitative chromatographic examination of constituent sugars. The results are tabulated in Table II.

Fractionation of the Crude Hemicellulose with Barium Hydroxide

Hemicellulose (100 g.) was suspended in water (3 l.) and shaken on a mechanical shaker for 1 hr. Ten per cent sodium hydroxide solution (2 l.) was then added and the mixture shaken in an atmosphere of nitrogen for 2 hr. The solution was centrifuged and the small amounts of residual precipitate were discarded. To the pale yellow centrifugate an equal volume of saturated aqueous barium hydroxide (18) was added dropwise with constant stirring. The barium complex, which settled was centrifuged off, washed twice with 3-4% alkali, suspended in ice-cold water, stirred and decomposed with aqueous acetic acid (1:1) to pH 7. The

solution was poured with stirring into ethanol (2 volumes), which precipitated the polysaccharide. The latter was collected on the centrifuge, washed 3 times each with 80% ethanol, 100% ethanol, and diethyl ether and dried over phosphorus pentoxide in vacuo, yielding a white solid (68 g.). Analysis, according to the method of Saeman and co-workers (78) and by the spectrophotometric method of Timell and co-workers (59), showed the presence of galactose (6.4%); glucose (17.5%); mannose (70.3%); arabinose (trace); and xylose (5.8%). Further fractionation with barium hydroxide or Fehling's solution failed to remove or alter the high ratio of galactose to glucose. The ultraviolet spectrum of the polysaccharide suggested the presence of residual lignin.

Delignification of the Glucomannan

Crude glucomannan (35 g.) was dissolved in water (3 l.). Chlorine gas was bubbled into the solution for 5 min. at a temperature of 6°C, a small quantity of ethanol being added to prevent foaming. The polysaccharide was precipitated by pouring into vigorously stirred ethanol (12 l.), washed twice with 80% aqueous ethanol and once with ethanol. The polysaccharide was suspended by stirring in a 3% solution of alcoholic ethanolamine (3 l.) and was added under stirring and heated for 5 min. The polysaccharide was then recovered by centrifuging and washed 3 times with ethanol. The precipitate was added to water (3 l.) and most of the ethanol adhering to the polysaccharide was evaporated. The chlorine treatment was repeated 3 times. The polysaccharide was treated twice with Fehling's solution to give 18 g. (50%) of a purified product.

Analysis of the glucomannan showed the presence of glucose (20.6%); mannose (77.6%); xylose (trace); and galactose 1.8%). (See Table I).

Partial Hydrolysis of the Glucomannan

The purified glucomannan (2 g.) was dissolved in 90% formic acid (50 ml.) and diluted with water to 100 ml. The solution $[\alpha]_{22}$, -27.4° was heated at 97 ± 1°C and the change in specific rotation was followed.

Time (hr.)	0.00,	1.0,	2.0,	3.0,	4.0,	5.0,	6.0,	7.0,
$[\alpha]_D$	-27.4,	- 6.0,	+ 7.8,	+14.9,	+26.8,	+32.6,	+38.8,	+38.8 (constant)

Small portions were each time repeatedly evaporated on a water-bath with water to remove formyl esters and spotted on paper chromatograms. Visual examinations of the chromatograms showed that maximum yield of oligosaccharides was attained after 3 hr. A large sample (10 g.) was accordingly hydrolysed for 3 hr. under similar conditions. The cooled solution was concentrated to yield a sirup, which was added to 0.5N sulfuric acid (320 ml.) and the solution obtained heated at 100°C for 10 min., cooled, neutralised with barium carbonate and deionized on a mixed bed resin of Amberlite IR-120 (H^+) and Dowex 1-X4 (acetate). Chromatography (systems (A) and (C)) indicated the presence of monosaccharides and oligosaccharides. The residual glucomannan was again hydrolysed with formic acid, and the extracts were combined. A portion of the hydrolysate (7.5 g.) was added to the top of a charcoal-Celite (1:1 w/w) column (6 x 64 cm.) and fraction-

ated by gradient elution (65) with air-free aqueous ethanol. The eluant was collected in 20 ml. fractions on an automatic fraction collector at intervals of 30 min. Every third fraction (1 ml. portion) was examined by paper chromatography (systems (A) and (C). Chromatographically identical fractions were combined and concentrated to sirups. The gradient elution was carried out as indicated below.

0	2%	aqueous	ethanol	3 l.
2	10%	"	"	6 l.
10	20%	"	"	4 l.
20	40%	"	"	2 l.

Where necessary, the sugars were fractionated on paper chromatograms (19 x 57 cm.) to give pure compounds (See Table III).

Identification of Sugars

1. Monosaccharides

A mixture of glucose, mannose, and xylose (2.889 g.) was first obtained. The sugars were tentatively characterized by paper chromatography (systems (A) and (B)) and were not further examined.

2. Oligosaccharides

Fraction A - 4-O-β-D-mannopyranosyl-D-mannose (mannobiose): This disaccharide (1.015 g.) moved on the paper chromatogram at the same rate as an authentic sample of (1→4)-β-mannobiose (79) and chromatographic examination of the hydrolyzate indicated the presence of mannose only.

The disaccharide, after recrystallization from methanol, had m.p. and mixed m.p. 209 - 210° $[\alpha]_D$, +32°→-8° (c = 3.5 in water) at equilibrium. The literature quotes m.p. 193.5 - 194° (89); 204° (31); 208 - 209° (32); 203-204° (40a). $[\alpha]_D^{23}$ -7.7→-2.2° (89); -5→-8° (31); -8.13→-7.42° (32); -7° (40a) (H_2O). The X-ray diffraction pattern and the infrared absorption spectrograph were identical with those from an authentic specimen.

Fraction B - 4-O-β-D-mannopyranosyl-D-glucose (mannosyl-glucose): This sirupy disaccharide (72.5 mg.) had $[\alpha]_D^{22}$ + 28.8° (c = 2.4 in water) and was chromatographically identical with a sample of authentic (1→4)-β-mannosyl-glucose (79). Hydrolysis of the disaccharide and paper chromatographic analysis indicated the presence of equal amounts of glucose and mannose. Sodium borohydride reduction prior to hydrolysis left mannose as the only reducing sugar, showing that the disaccharide was a mannosyl-glucose. Repeated attempts to crystallize the material failed. The identification of the disaccharide as 4-O-β-D-mannopyranosyl-D-glucose, therefore, rests upon its hydrolytic products before and after reduction. The literature quotes m.p. 202-203° (26a); 203° (31); 201-202° (40a); $[\alpha]_D^{25}$ + 30°→+ 19° (26a); + 20° (31); + 35°→+ 18° (31); +18° (40a).

Fraction C - 4-O-β-D-glucopyranosyl-D-glucose (cellobiose): This disaccharide (107 mg.) was obtained as a sirup mixed with mannotetraose. Chromatographic analysis showed 2 spots, one running at the same rate as mannotetraose, the other at the same rate as an authentic sample of cellobiose. The sugars were separated on a large sheet of Whatman No. 1 paper

in solvent system (C). On recrystallization of the cellobiose (23.3 mg.) from aqueous methanol, the crystals had m.p. and mixed m.p. 234-235° and $[\alpha]_D^{22}$ + 25.8°⟶35.5° (c = 1 in water). The literature quotes m.p. 225° (90); 233-234° (32) $[\alpha]_D$ + 14.2°⟶+ 34.6° (90); + 26.7°⟶+ 35.1° (32).

Fraction D - O-β-D-mannopyranosyl-(1⟶4)-O-β-D-mannopyranosyl-(1⟶4)-D-mannose (mannotriose): This trisaccharide (262 mg.) was chromatographically indistinguishable from an authentic specimen of a (1⟶4)-linked mannotriose (79). Complete hydrolysis yielded mannose, while partial hydrolysis yielded both mannose and mannobiose.

A molecular weight determination using the method of Peat, Whelan and Roberts (80) gave a value of 2.7 ± 0.2. After seeding with an authentic crystal of (1⟶4)-linked mannotriose the product crystallized from ethanol, having m.p. and mixed m.p. 164-168° $[\alpha]_D$ - 21.9°⟶25.4° (c = 2.4 in water) at equilibrium. The literature quotes m.p. 155-165° (31); 165-169° (40a); 164-168° (66); 134.5-135.5° (67); 137-137.5° (92) $[\alpha]_D$ - 22°⟶- 25° (31); - 25° (40a); - 22°⟶-26° (66); - 15.7°⟶- 20.2° (67); - 24.7°⟶- 23.3° (92).

Fraction E - 4-O-β-D-glucopyranosyl-D-mannose (glucosyl-mannose): The disaccharide (373 mg.) was chromatographically identical with an authentic specimen of (1⟶4)-β-linked glucosyl-mannose (79). Equal amounts of mannose and glucose were obtained by hydrolysis. The disaccharide crystallized from ethanol having m.p. and mixed m.p. 134-137° $[\alpha]_D$ + 10.5 5.8° (equilibrium) (c = 4.0 in water). The literature quotes m.p. 134-139° (26a); 135-138° (31); 137° (91) $[\alpha]_D$ + 4.7° (26a); + 6° (31); + 14.5° ⟶5.9° (91).

For the preparation of the crystalline octaacetate (26) glucosyl-mannose (100 mg.) was heated with acetic anhydride (12 ml.) and anhydrous sodium acetate (0.4 g.) on a water-bath for 6-8 hr. The crude product was poured into ice-cold water and neutralized with saturated sodium bicarbonate until effervescence ceased. The mixture was extracted with chloroform (3 x 25 ml.) and washed 3 times with water (25 ml.), dried over sodium sulfate and evaporated to dryness. Recrystallization from methanol gave the octaacetate (146 mg.) $[\alpha]_D$ +34.3° (c = 1.0 in chloroform), having m.p. 201° which was not depressed on admixture with the authentic octaacetate.

Oligosaccharide A was a sirup (285 mg.) $[\alpha]_D^{22}$ -9.13°→-7.9° (c = 6.57 in water). A portion (5 mg.) when hydrolyzed with N sulfuric acid (1 ml.) in a sealed tube followed by chromatographic separation, gave glucose and mannose in an approximate ratio of 1:2. The sirup (10 mg.) dissolved in 5 ml. of water was added gradually to a solution of sodium borohydride (0.1 g.) in water (3 ml.). After standing overnight at room temperature the product no longer reduced Fehling's solution. An excess of dilute acetic acid was added, followed by an excess of methanol and the solvent was removed in vacuo. The residue was dissolved in a small volume of water and passed through a small column of Amberlite IR-120 (H^+) and Amberlite IR-45 (OH^-) resins. The eluate was concentrated in vacuo to a small volume and hydrolyzed with N sulfuric acid in the usual manner. The acid was neutralized with barium carbonate, and the solution analyzed chromatographically using system (A). The mixture contained equal parts of mannose and glucose, indicating that the trisaccharide had a mannose residue as the reducing end group. The tri-

saccharide had a D.P. of 2.9 as estimated by the sodium borohydride-anthrone method of Peat, Whelan and Roberts (80). The sirup (5 mg.) was partially hydrolyzed with 50% formic acid for 2-3 hr. on a water-bath in a sealed tube. The mixture was repeatedly evaporated from water for removal of formyl esters, after which it was neutralized by passing through small columns containing Amberlite IR-120 (H^+) and IR-45 (OH^-) resins. The residue after concentration was analyzed chromatographically using system (C). The mixture contained manno-biose, glucosyl-mannose, glucose and mannose. This evidence suggested that the trisaccharide might possibly be a (1→4)-β-linked glucosyl-mannosyl-mannose.

A fraction (0.9411 g.) was obtained which was a mixture of trisaccharides. Partial acid hydrolysis by standard methods indicated the presence of glucose, mannose and traces of xylose. The nature of the trisaccharides was not further determined.

Fraction F - O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-β-D-mannose (mannotetraose): This fraction (1.034 g.), on chromatographic analysis, showed spots for manno-tetraose, and higher oligosaccharides. The mixture was resolved on three sheets of Whatman No. 1 chromatographic paper for three days using system (C). The area corresponding to mannotetraose was excised and eluted with water and aqueous ethanol to yield the mannotetraoses (113.6 mg.). Complete hydrolysis of a portion of the sample indicated the presence of mannose only, while partial hydrolysis gave mannose, manno-biose, and mannotriose which were identified on a paper chromatogram.

The tetraose crystallized from methanol-water, m.p. 214°, which was raised to m.p. and mixed m.p. 232-234°, after recrystallization from aqueous ethanol $[\alpha]_D^{22}$ -31° (c = 1.0 in water). The literature quotes m.p. 232-234° (40a); 231.5-232° (67); $[\alpha]_D$ -31° (40a); -31.6°⟶-28.7° (67) (H_2O).

Methylation of the Glucomannan

The glucomannan (19 g.) was suspended by stirring in water (100 ml.) overnight in a 2 l. flask. Forty per cent (w/w) sodium hydroxide (300 ml.) was added in an atmosphere of nitrogen, and dimethyl sulfate (200 ml.) was added dropwise at a rate of 1 drop per 5-10 sec. at 0°C to avoid excessive foaming. This treatment was repeated twice, water being added whenever necessary for efficient stirring. The reaction mixture was heated to 90°C for 2 min. and allowed to cool to room temperature. Sulfuric acid (6N) (520 ml.) was added for neutralization of the alkali. The mixture was warmed slowly on a small flame when a brown precipitate appeared on the surface. Since it was not possible to recover all the material from the solution in this way, the whole mixture was concentrated to 1 l. and dialyzed against running tap water for 1 week until free from sulfate ions. The contents of the dialysis bags were concentrated (200 ml.) and freeze-dried for 2 days, yielding a crisp, dry, partially methylated glucomannan (18 g.). Found: (OCH_3, 26.8%). The partially methylated product was dissolved in dimethyl formamide (300 ml.) according to the method of Kuhn (69). Silver oxide (50 g.) and methyl iodide (50 ml.) were added to the mixture which was shaken at room temperature on a mechanical shaker for 20 hr. Another

portion of silver oxide (50 g.) and methyl iodide (50 ml.) were added and the mixture was again shaken for 20 hr. The insoluble material was washed on the centrifuge with chloroform. The chloroform extract (1.5 l.) was concentrated (500 ml.) and the excess dimethyl formamide removed by co-distillation with water. The chloroform solution was extracted with 5% aqueous potassium cyanide (3 x 300 ml.) and subsequently with water (3 x 500 ml.). After drying over anhydrous sodium sulfate, the chloroform solution was concentrated (200 ml.) and poured with stirring into petroleum ether (2 l.). Yield: 14 g. (77%).

Another treatment by Kuhn's method was carried out as above. The material was finally methylated according to Purdie (70). Methyl iodide (200 ml.) was added and the mixture was shaken for 4 hr. Silver oxide (20 g.) was added and the mixture shaken for 20 hr. Two more 20 g. portions of silver oxide were added, followed by a third portion of 40 g. The methylated product was centrifuged to remove insoluble material, and extracted with chloroform. The chloroform extracts were concentrated, dried over anhydrous sodium sulfate and the fully methylated glucomannan was precipitated by pouring the chloroform extract into petroleum ether. Yield: 7.5 g. (75%). OCH_3, 45% (calculated for a fully methylated glucomannan 45.3%). $[\alpha]_D^{22}$ - 21° (c = 1 in chloroform).

Methanolysis of the Glucomannan

A portion of the fully methylated polysaccharide (4 g.) was dissolved in methanol (120 ml.) containing 2% hydrogen chloride and refluxed for 12 hr. Most of the methanol was evaporated and N sulfuric

acid (120 ml.) was added and the methyl glycosides hydrolyzed at 100°C for 7 hr. The solution was neutralized with silver carbonate and filtered through Celite. Hydrogen sulfide was introduced to remove silver ions. Since sulfide could not be completely removed, the solution was evaporated to a small volume and hot acetone (80 ml.) was added and the solution again filtered through Celite. The washings and filtrate were concentrated to a sirup (3.6 g.). Yield: 90%. Chromatographic examination of the hydrolyzate (system (D)) revealed the presence of di-, tri-, and tetra-O-methyl-hexoses. The optical rotation of the sirup changed from $[\alpha]_D^{22}$ -70.0°; initial→+ 11.8° (48 hr.) (c = 1.53 in water).

Separation of the methylated sugars on a charcoal-Celite column

A portion of the sirup containing the methylated sugars (3.4 g.) was added to the top of a charcoal-Celite column (56 x 5 cm.), which was then eluted with the following solvents using the gradient elution technique (65).

5	20%	aqueous ethanol	4,000 ml.
20	40%	" "	4,000 ml.

The eluate was divided into fractions (20 ml.) which were examined on paper chromatograms (system (D)). Those fractions which were chromatographically indistinguishable were combined and evaporated to dryness. The results are presented in Table IV.

Mixture of Dihexoses

Fraction 1: Three components (56 mg.) were isolated by paper electrophoresis. The principal component on demethylation gave glucose only. This component was chromatographically and electrophoretically indistinguishable from authentic 2,6-di-O-methyl-D-glucose.

The other two components were minor amounts of 2,4-di-O-methyl-D-glucose, and 3,6-di-O-methyl-D-glucose and behaved in a manner similar to authentic samples on paper electrophoretograms.

Identification of 2,3,6-Tri-O-methyl-D-glucose

Fraction 2: The 2,3,6-tri-O-methyl-D-glucose component (814 mg.) was recrystallized from ether having m.p. and mixed m.p. 120-121° $[\alpha]_D^{22}$ + 70.5° (c=1.0 in water). The literature quotes m.p. 123° $[\alpha]_D^{22}$ + 70° (water) (84).

A portion of the 2,3,6-tri-O-methyl-D-glucose was treated with pyridine (2 ml.) and p-nitrobenzoyl chloride (142 mg.) using the method of Smith and co-workers (26). The 1,4-di-O-p-nitrobenzoate of the 2,3,6-tri-O-methyl-D-glucose, after recrystallization from methanol, had m.p. 189-190° $[\alpha]_D^{22}$ - 34° (c = 0.7 in chloroform).

Identification of 2,3,6-Tri-O-methyl-D-mannose

Fraction 3: The 2,3,6-tri-O-methyl-D-mannose component (206 mg.) was separated from a mixture of di-O-methyl-hexoses and tri-O-methyl-hexoses (Fraction 1) by paper chromatography using system (D). The main

fraction (2.06 g.) was also obtained as a sirup which had $[\alpha]_D^{22}$ - 12.6° (c = 2.6 in water). The literature quotes $[\alpha]_D$ - 11.6° (26).

The 2,3,6-tri-O-methyl-D-mannose (100 mg.) was dissolved in dry pyridine (6 ml.) and treated with p-nitro-benzoyl chloride (426 mg.) for 30 min. at 65-75° and then left overnight at room temperature. A saturated solution of sodium bicarbonate was added dropwise to the reaction mixture until no further effervescence occurred. Water (70 ml.) was added after which the mixture was extracted with chloroform (3 x 25 ml.). The extract was dried over sodium sulfate and evaporated in vacuo to 2-3 ml. Addition of petroleum ether induced crystallization of the di-O-nitrobenzoate of 2,3,6-tri-O-methyl-D-mannose (80 mg.), m.p. 187-188° $[\alpha]_D^{22}$ + 33 ± 3° (c = 0.6 in chloroform) after recrystallization from ethanol. The compound could also be recrystallized from ethyl acetate in fine hair-like needles, m.p. 187-188°. 2,3,6-Tri-O-methyl-D-mannose (200 mg.) was boiled under reflux with a solution of aniline (100 mg., freshly distilled) in ethanol, (6 ml.), on a water-bath for 6 hr. After evaporating off the ethanol and removing aniline under vacuum, 2,3,6-trimethyl-N-phenyl-D-mannopyranosylamine ether was obtained on addition of ether. Recrystallization from ether - light petroleum ether gave m.p. 125° $[\alpha]_D^{22}$ - 151.6° ⟶ - 40.0° (c = 0.6 in methanol) (25).

Identification of 2,3,4,6-Tetra-O-methyl-D-mannose

Fraction 4: A mixture of 2,3,4,6-tetra-O-methyl-D-glucose, and 2,3,4,6-tetra-O-methyl-D-mannose was obtained as a sirup (43.7 mg.). It had $[\alpha]_D^{22}$ + 4.8° (c = 4.4 in water) and $[\alpha]_D^{22}$ + 22.3° (c = 2.33 in methanol

close to the reported value $[\alpha]_D^{22}$ + 27.3° (81). A small portion of the sirup (52 mg.) was demethylated with 48% hydrobromic acid (1 ml.) (82). Paper chromatography indicated the presence of glucose and mannose. An infrared spectrograph of the sirup contained maxima at 790, 835, 875-880, 2,050 $cm.^{-1}$ in close agreement with an authentic sample of 2,3,4,6-tetra-O-methyl-D-mannose sirup, which had maxima at 794, 845, 875-880 and 2050 $cm.^{-1}$ A sample of the sirup when chromatographed on Whatman No. 3 MM paper in butanone-water azeotrope system travelled 34.4 cm., and 30.5 cm., as measured from origin to the leading and trailing edges of the spot. The values were 35.8 and 31.2 for an authentic sample of 2,3,4,6-tetra-O-methyl-D-mannose (sirup) while 2,3,4,6-tetra-O-methyl-D-glucose on the same chromatogram gave 23 cm. and 28.5 cm. respectively. The solvent front travelled 39 cm. The sirup (8 mg.) was boiled under reflux with aniline (5 mg.) and ethanol (1 ml.) for 6 hr. The ethanol was evaporated and the last traces of aniline were removed in vacuo. Addition of ethyl ether caused crystallization. The 2,3,46-tetra-O-methyl-N-phenyl-D-mannosylamine had m.p. 145-146° after 3 recrystallizations from ethyl ether. The literature quotes 144-145° (83).

Periodate Oxidation of the Glucomannan

Samples (150-200 mg.) were oxidized for various lengths of time in the dark at 30° with 0.05M sodium metaperiodate (50 ml.), the consumption of periodate being measured by standard procedures (85). The formic acid formed was determined in separate experiments by the iodometric method. The molar consumption of periodate per hexose residue was .9458 (24 hr.), 1.04 (48 hr.), 1.08 (72 hr.), remaining constant after

72 hr. The number of hexose residues producing 3 moles of formic acid was 34 (24 hr.).

Examination of Sugar Residues Unoxidized by Periodate

Glucomannan (5 g.) was treated with aqueous 0.4M sodium meta-periodate (250 ml.) and shaken for 1 day and then kept with occasional shaking at + 4°C for 4 weeks in the dark. The filtrate was dialyzed against running tap water for 2 days and concentrated to a sirup (3.77 g.).

A portion of the sirup (500 mg.) was hydrolyzed with 72% sulfuric acid in the usual way. The constituent sugars were separated by paper chromatography with system (A) and showed the presence of approximately equal amounts of glucose and mannose.

Preparation of the Nitrate Derivative of the Glucomannan

Glucomannan (1 g.) was treated with a mixture of nitric acid, phosphoric acid, and phosphorus pentoxide (86) at + 17°C for 1 hr. The reaction mixture was poured into aqueous acetic acid (1:1) and cooled to - 16°C. The nitrate was recovered by filtration and washed with ice-water until the washings were neutral. Yield (90%), nitrogen content (13.555) (87). A duplicate experiment gave a product with the same nitrogen content, and in the same yield.

Determination of the Number-Average Molecular Weight of Glucomannan Nitrate

The osmometers used were of the Zimm and Myerson type (63) as modified by Stabin and Immergut (64). Hypodermic syringes with long needles were used for filling and mercury was employed for closing the instrument completely during measurements in addition to the stainless steel rods. Gel cellophane membranes, 5 cm. in diameter, which had never been allowed to dry, were used and there was no leakage or diffusion. The solvent employed was chloroform-ethanol (9:1 v/v) and the temperature was kept constant at 30° ± 0.01°C. The osmotic pressure was determined by the static method at seven different concentrations (Tables V, VI and VII). The (h/w) was plotted against the concentration (w) and the value $(h/w)_{c=o}$ was obtained from the graph by extrapolating the resulting straight line to zero concentration. The number-average molecular weight was $\bar{M}n = 26,500$, corresponding to a $\bar{P}n$ value of 93, for the crude nitrated glucomannan fraction. The corresponding values of $\bar{M}n = 23,200$ and $\bar{P}n = 82$ were for the delignified nitrate of glucomannan; and $\bar{M}n = 19,600$ and $\bar{P}n = 96$ for the methylated pine glucomannan (See also Fig. 1).

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SUMMARY AND CLAIMS TO ORIGINAL RESEARCH

1. The wood of eastern white pine (Pinus strobus) has been found to contain cellulose (43%), lignin (29.3%), pentosan (9.83%), acetyl (1.23%), ash (0.24%), uronic anhydride (3.98%), galactose (1.40%), glucose (46.0%), mannose (11.7%), arabinose (2.0%), and xylose (4.5%).

2. Preliminary extraction and delignification of white pine wood with acid chlorite gave holocellulose in 75.5% yield.

3. Extraction of the holocellulose with alkaline borate solution gave a crude hemicellulose mixture in 39.3% yield consisting of an arabino-4-O-methyl-glucuronoxylan and a glucomannan.

4. Fractionation of the crude hemicellulose mixture with saturated aqueous barium hydroxide gave a crude glucomannan which contained galactose (6.4%), glucose (17.5%), mannose (70.3%), arabinose (traces) and xylose (5.8%) residues.

5. Further delignification with gaseous chlorine of the crude glucomannan yielded a purified product which contained glucose (20.6%), mannose (77.6%) and galactose (1.83%) and xylose (traces) residues.

6. Partial hydrolysis of the glucomannan with 50% aqueous formic acid gave monosaccharides as well as a mixture of oligosaccharides which were resolved on a charcoal-Celite column by step-gradient elution with

aqueous ethanol of increasing concentrations.

7. The first fraction was a mixture of mannosaccharides consisting of glucose, mannose, xylose and galactose.

8. Fraction (A) was 4-O-β-D-mannopyranosyl-D-mannose (mannobiose).

9. Fraction (B) was 4-O-β-D-mannopyranosyl-D-glucose (mannosyl-glucose).

10. Fraction (C) was a 4-O-β-D-glucopyranosyl-D-glucose (cellobiose).

11. Fraction (D) was O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-D-mannose (mannotriose).

12. Fraction (E) was 4-O-β-D-glucopyranosyl-D-mannose (glucosyl-mannose).

13. Oligosaccharide A was partially identified as glucosyl-mannosyl-mannose.

14. Fraction (F) was O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→4)-β-D-mannose (mannotetraose).

15. The glucomannan was methylated to composition by the methods of Haworth, Kuhn and Purdie.

16. Hydrolysis of the fully methylated glucomannan afforded a mixture of sugars which could be resolved on a charcoal-Celite column by gradient elution with aqueous ethanol.

17. Fraction 1 was a mixture of di-O-methyl hexoses containing 2,6-di-O-methyl-D-glucose as its principal component with minor amounts of 2,4- and 3,6-di-O-methyl-D-hexoses.

18. Fraction 2 was 2,3,6-tri-O-methyl-D-glucose which crystallized and was also identified through its di-p-nitrobenzoate derivative.

19. Fraction 3 was 2,3,6-tri-O-methyl-D-mannose identified through its aniline and di-p-nitrobenzoate derivatives.

20. Fraction 4 consisted of 2,3,4,6-tetra-O-methyl-D-mannose, and possibly together with 2,3,4,6-tetra-O-methyl-D-glucose. It was identified through its infrared diagram and the crystalline aniline derivative of the tetra-O-methyl-D-mannose.

21. Periodate oxidations of the glucomannan indicated that 1.08 mols of oxidant were consumed per hexose residue.

22. The formic acid formation on periodate oxidation corresponded to 34 hexose residues per average molecule.

23. Examination of the sugar residue unoxidized by periodate showed

equal amounts of glucose and mannose thus indicating that the polymer might contain some branched residues.

24. Osmotic pressure measurements on the methylated glucomannan suggested a degree of polymerization of 96. From the methylation data it can be concluded that the average molecule contains 1.2 non-reducing end groups, corresponding to 0.2 branch points per macromolecule. The polysaccharide is accordingly branched, albeit only slightly.

25. Osmotic pressure measurements on the acetate derivative of the original glucomannan gave a degree of polymerization of 82. Since the polysaccharide had been subjected to treatment with acid chlorite, this would probably not represent the molecular magnitude of the native polymer but was only a minimum value.

26. It was concluded that the predominant hemicellulose in white pine wood is a (1→4)-β-linked diheteropolymer containing glucose and mannose residues in a ratio of 1:3-4. The average macromolecule contains a minimum number of 93 hexose residues, with few contiguous glucose residues. The polysaccharide is slightly branched.