120°. They were combined and acetylated to yield 3.2 g. of β-isomaltose octaacetate, m.p. 145–146°.

The residues from the last six 100-ml. fractions above, which showed specific rotations in water in the range 80–105°, were combined and acetylated. Fractional crystallization from ethanol then yielded 50 mg. of β-gentiobiose octaacetate, m.p. and m.m.p. 194–195°, and 240 mg. of β-isomaltose octaacetate, m.p. 144–145°; $[\alpha]^{25}D$ 98° (c 1 in chloroform). The sirupy residue from these crystallizations, in two 40-mg. portions, then was chromatographed on 200 × 24 mm. columns of Magnesol–Celite, using 200 ml. of benzene–ethanol (250:1, v./v.) as developer. Streaking of the extruded columns with alkaline permanganate showed two well-separated zones at 1–55 and 80–105 mm., respectively, from the top. Precisely similar zones were observed when a mixture of known β-isomaltose octaacetate and 5-O-β-D-glucopyranosyl-D-glucose octaacetate prepared from hydrol were chromatographed in the same manner. The combined top zones, after elution with acetone and concentration, provided 53 mg. of β-isomaltose octaacetate, m.p. 145–146°. The combined lower zones yielded 21 mg. of sirupy 5-O-β-D-glucopyranosyl-D-glucose octaacetate, $[\alpha]^{25}D$ −29 ± 1° (c 0.8 in chloroform).

Gentiobiose.—The "gentiobiose fraction" above (3.4 g.) yielded, by direct crystallization from methyl cellosolve, 1.0 g. of gentiobiose, m.p. 192–193°; $[\alpha]^{25}D$ 10.2° (c 0.5, final in water); octaacetate, m.p. 194–195°.

Cellobiose.—The residue (7.9 g.) of the 15% ethanol effluent from the preliminary fractionation was rechromatographed on a 750 × 40 mm. carbon–Celite column with 8% ethanol. The first 1.8 l. of effluent was concentrated to an amorphous residue weighing 160 mg. Acetylation yielded 80 mg. of β-cellobiose octaacetate, m.p. and m.m.p. 200–201°.

An additional 7.5 g. of carbohydrate material was desorbed from this column with 3 l. of 15% ethanol. Acetylation of this fraction yielded no crystalline material.

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[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

The Constitution of the Hemicellulose of Western Hemlock (Tsuga heterophylla). I. Determination of Composition and Identification of 2-O-(4-O-Methyl-D-glucopyranosiduronic acid)-D-xylose[1]

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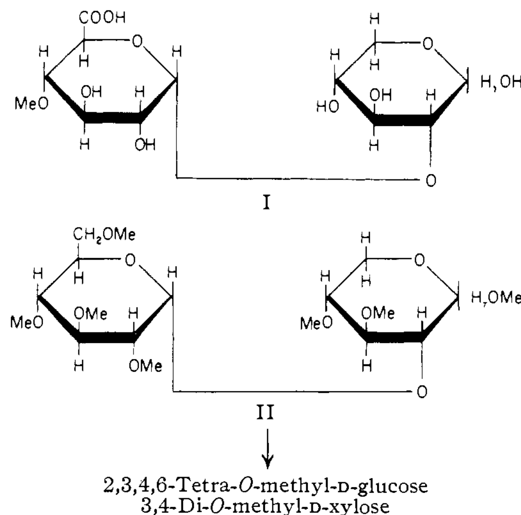
The hemicellulose of Western Hemlock (*Tsuga heterophylla*) gave upon hydrolysis arabinose, xylose, galactose, glucose, mannose and an aldobiouronic acid (I). The aldobiouronic acid (I) has been identified as 2-O-(4-O-methyl-α-D-glucuronosyl)-D-xylose.

Western Hemlock hemicellulose obtained from delignified sawdust by alkaline extraction gave upon hydrolysis a mixture of an aldobiouronic acid (I), arabinose, xylose, galactose, glucose and mannose. The acidic component I, which forms the subject of this communication, was separated from the hydrolysate by the use of an anion-exchange resin. The methoxyl content of I and its equivalent weight indicated that it was composed of a methoxyuronic acid and a pentose sugar, a deduction further substantiated by the observation that vigorous hydrolysis furnished D-xylose and 4-O-methyl-D-glucuronic acid.

Cleavage of the aldobiouronic acid (I) with 8% methanolic hydrogen chloride at 105° for 10 hr., followed by treatment of the cleavage products with ammonia, yielded the crystalline amide of methyl 4-O-methyl-α-D-glucopyranosiduronic acid.[2]

After removal of this uronic acid derivative hydrolysis of the neutral sugar glycoside gave crystalline D-xylose. The point of attachment of the 4-O-methyl-D-glucuronic acid unit to the D-xylose residue was determined from a study of the fully methylated disaccharide II obtained by lithium aluminum hydride reduction[3,4] and methylation of the aldobiouronic ester. Hydrolysis of II and chromatographic separation of the components on a cellulose–hydrocellulose column[5] using methyl ethyl ketone–water azeotrope as the irrigating solvent[6] yielded 3,4-di-O-methyl-D-xylose[7] and 2,3,4,6-tetra-O-methyl-D-glucose.[8] The former was identified as the crystalline 3,4-di-O-methyl-D-xylono-δ-lactone[7] and the latter both as the crystalline sugar[8] and as the crystalline anilide.[9]



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The above evidence shows that in the aldobiouronic acid (I) the 4-*O*-methyl-D-glucuronic acid moiety is glycosidically linked to position 2 of the xylose residue. The fully methylated disaccharide II is therefore 2-*O*-(2,3,4-tri-*O*-methyl-D-glucopyranosyl)-3,4-di-*O*-methyl-D-xylose, whence it follows that the aldobiouronic acid (I) is 2-*O*-(4-*O*-methyl-D-glucopyranosiduronic acid)-D-xylose. Further confirmation of this deduction is provided by the isolation of 2-*O*-(2,3,4-tri-*O*-methyl-D-glucopyranosiduronic acid)-3-*O*-methyl-D-xylose by the graded hydrolysis of the fully methylated hemicellulose.[10] This acid I is identical with that obtained by the graded hydrolysis of the hemicellulose of aspen,[11] beech,[12] birch,[13] Scots pine,[13] black spruce,[14] flax[15] and "hemicellulose-B" of corncobs.[16]

The work of Bishop indicates that the biose linkage is of the α-type.[17]

Experimental

(All evaporations were carried out under reduced pressure at a bath temperature not exceeding 40°).

Isolation of Western Hemlock Hemicellulose.—A log of Western Hemlock (*Tsuga heterophylla*), as received from Rayonier Incorporated, Shelton, Washington, was reduced to sawdust which was ground in a Wiley mill to pass a 40 mesh sieve. The sawdust was delignified in 100 g. batches by the chlorite method[18]; the yield of air-dried holocellulose was 72–78% of the air-dried sawdust. Holocellulose (*ca.* 75 g.) was extracted at room temperature with 5% potassium hydroxide (1500 ml.) for 4 hr., and after filtration the residue was re-extracted with a further 500 ml. of alkali and washed with water. To the combined alkaline extracts and washings (total volume, 2 l. approx.) was added Fehling solution (200 ml.)[19] when a gelatinous precipitate slowly formed. Preliminary experiments have shown that this volume of Fehling solution gave adequate precipitation of the polysaccharide but that a 1:1 ratio of alkaline extract to Fehling solution gave only a slight precipitate. The copper complex was washed with 5% potassium hydroxide and then with 50% aqueous alcohol after which it was decomposed by stirring with methanol containing a little dilute hydrochloric acid. The hemicellulose (5.4 g.) thus obtained as a cream colored powder, was washed successively with methanol, diethyl ether, petroleum ether and dried. An electrodialyzed sample had $[\alpha]^{22}D$ −36.9° (*c* 1.8 in water). Found: OMe, 3.0; equiv. wt., 1100 (by direct titration to *p*H 7 using a *p*H meter).

Hydrolysis of the hemicellulose with *N* sulfuric acid and estimation of the neutral sugars by the phenol–sulfuric acid method[20] indicated the presence of xylose (37%), arabinose (8%), mannose (42%), glucose and galactose (14%, calculated as glucose).

Determination of the Acid Component in Western Hemlock Hemicellulose.—A solution of the hemicellulose (100 mg.) in *N* sulfuric acid (5 ml.) was heated in a sealed tube at 100° for 12 hr. A small amount (7.2 mg.) of brown residue was centrifuged off and the clear solution neutralized with barium carbonate. Barium sulfate was removed by centrifuging and the yellow solution passed through Amberlite resin IR 120.[21] The eluate had a *p*H of 3.2. The acidic components were selectively adsorbed by passage through a column of Duolite A4 resin[22] and the column washed until the washings gave a negative Molisch test. The acidic components were eluted with *N* sodium hydroxide (5 ml.) and the free acids liberated by passage through Amberlite IR 120. The eluate (*p*H 3.1) was concentrated and examined chromatographically using 1-butanol:acetic acid:water (2:1:1) as the irrigating solvent. After 60 hr., spraying with *p*-anisidinetrichloroacetic acid in water[23] revealed a bright pink spot in a position corresponding with that given by an authentic sample of 4-*O*-methyl-D-glucuronic acid.[2]

Separation of the Acidic Component of Western Hemlock Hemicellulose.—The hemicellulose (10 g.), isolated from the copper complex, was heated for 9 hr. with *N* sulfuric acid (500 ml.) until the rotation became constant. The hydrolyzate was neutralized with barium carbonate and the filtrate passed through a column of Amberlite IR 120 resin to remove barium ions. The strongly acid eluate was passed through a column of Duolite A 4 resin which was washed with water until the washings gave a negative Molisch test. The acid component was eluted from the resin with *N* sodium hydroxide solution (25 ml.), the alkaline solution passed through Amberlite IR 120 and the eluate therefrom concentrated to dryness, giving a glassy solid (1.096 g.).

Anal. Calcd. for $C_{12}H_{19}O_{11}$: OMe, 9.1; equiv. wt., 339. Found: OMe, 7.0; equiv. wt., 318.

Methanolysis of 2-*O*-(4-*O*-Methyl-D-glucopyranosiduronic acid)-D-xylose.—The aldobiouronic acid (300 mg.) was dissolved in methanol (15 ml.) containing hydrogen chloride (8%) and heated in a sealed tube at 105° for 10 hr. The solution was diluted with methanol (25 ml.), neutralized with silver carbonate, centrifuged and the supernatant filtered. The residual silver salts were extracted with methanol (3 × 40 ml.) and the combined methanol solutions evaporated to a brown sirup (330 mg.).

In order to ascertain whether methanolysis was complete, a small portion of the sirup (*ca.* 15 mg.) was hydrolyzed with *N* hydrochloric acid (1 ml.) under reflux for 6 hr. A portion of the resulting solution was chromatographed, using 1-butanol:acetic acid:water (2:1:1), and sprayed with *p*-anisidine solution. No spot was observed corresponding to the original biouronic acid, but there was a faster-moving spot having the same R_f value as xylose.

Identification of Methyl 4-*O*-Methyl-α-D-glucopyranosiduronic acid.—The sirup obtained from methanolysis of the aldobiouronic acid was dissolved in methanol saturated with ammonia at 0° (25 ml.) and kept at 5° for 24 hr. After a further period of 4 hr. at room temperature, the solvent and excess ammonia were removed by evaporation. On seeding the resulting sirup with methyl 4-*O*-methyl-α-D-glucopyranosiduronamide, partial crystallization occurred. After several days recrystallization of the product from ethanol afforded methyl 4-*O*-methyl-α-D-glucopyranosiduronamide, m.p. and mixed m.p. 235–236° and $[\alpha]^{23}D$ +145° (*c* 0.1 in water). When mixed with a sample of the β-anomer (m.p. 231°),[2] the m.p. was 223–227°.

Identification of D-Xylose.—The mother liquor from which the amide had been separated was dissolved in barium hydroxide solution (10 ml., saturated at room temperature) and the solution heated at 80° under reflux for 4 hr. in a stream of nitrogen when ammonia was no longer evolved (tested with litmus paper). The solution was neutralized with carbon dioxide, filtered and evaporated to a pale yellow sirup which was heated for 10 hr. at 100° in a sealed tube with *N* sulfuric acid (2 ml.). Filtration and passage through a column of Duolite A 4 resin yielded a neutral solution which on a paper chromatogram showed a strong spot for xylose and three faint spots attributed to arabinose, mannose and glucose. Concentration of the solution and seeding with D-xylose gave crystals melting at 142–143°. Recrystallization from methanol afforded pure D-xylose, m.p. and mixed m.p. 142–143°, $[\alpha]^{25}D$ +17.8° (*c* 1 in water).

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Preparation of Methyl 2-*O*-(2,3,4,6-Tetra-*O*-methyl-α-D-glucopyranosyl)-3,4-di-*O*-methyl-D-xylopyranoside.—A portion of the aldobiouronic acid (350 mg.) was dissolved in methanol (16 ml.) containing hydrogen chloride (3%) and refluxed for 7 hr., by which time the optical rotation was constant. Neutralization (Ag_2CO_3) and evaporation gave a thick sirup (410 mg.) which did not reduce Fehling solution. The sirup was dissolved in acetone (5 ml.) and methyl iodide (6 ml.) added. The addition of the latter caused some precipitation, and the solution was cleared by the addition of methanol (5 ml.) when the remainder of the methyl iodide (6 ml.) was added. Silver oxide was added in portions and the solution, containing a little Drierite, was refluxed for 24 hr. Recovery of the excess methyl iodide and extraction of the residue with methanol gave a pale yellow sirup (440 mg.). This sirup was dissolved in acetone (10 ml.) and methyl iodide (10 ml.) and was remethylated as above. The sirup, isolated from the second methylation as before, was freely soluble in ether.

Finely crushed lithium aluminum hydride (400 mg.) was added to ether (20 ml.), dried by distillation from lithium aluminum hydride and the solution refluxed for 0.5 hr. The hydride solution was cooled to room temperature and a solution of the partly methylated aldobiouronic ester in dry ether (10 ml.) was added dropwise with swirling. A vigorous reaction occurred and the reduction was completed by refluxing for 0.5 hr. To the cooled solution ethereal ethyl acetate was added to decompose the excess hydride followed by a small excess of dilute acetic acid. The reaction mixture was evaporated to dryness and the reduced product isolated by acetylation in the following way.

Anhydrous sodium acetate (500 mg.) and acetic anhydride (15 ml.) were added to the mixture of organic material and dry salts and the whole heated on a steam-bath for 3 hr. After removal of the excess anhydride by distillation, dilute hydrochloric acid was added to dissolve the salts. The aqueous solution was extracted with chloroform (50, 50, 25 ml.) and the combined extracts washed with water until free of chloride ion. Removal of the chloroform gave a thin sirup (1.2 g.) which was dissolved in ethanol (10 ml.) and deacetylated by heating on the steam-bath for 1 hr. with *N* sodium hydroxide (10 ml.). Cations were removed by passage through a column of Amberlite IR 120 resin and any unreduced aldobiouronic acid adsorbed by passage through a column of Duolite A4 resin. The effluent, which was now colorless, was evaporated to give the partly methylated disaccharide as a sirup (350 mg.).

The partly methylated disaccharide was dissolved in methyl iodide (10 ml.) containing acetone (2 ml.) and methylated with silver oxide and methyl iodide as before. Two further methylations without the addition of acetone gave a sirup (320 mg.) which was distilled giving methyl 2-*O*-(2,3,4,6-tetra-*O*-methyl-α-D-glucopyranosyl)-3,4-di-*O*-methyl-D-xylopyranoside, b.p. (bath temp.) 150–170° (0.005 mm.), as a yellow, mobile sirup (270 mg.) having $[\alpha]^{24}D$ +76° (*c* 4.7 in methanol).

Anal. Calcd. for $C_{18}H_{34}O_{10}$: OMe, 52.9. Found: OMe, 51.0.

Hydrolysis of Methyl 2-*O*-(2,3,4,6-Tetra-*O*-methyl-α-D-glucopyranosyl)-3,4-di-*O*-methyl-D-xylopyranoside.—A solution of the methylated disaccharide (220 mg.) in *N* sulfuric acid (5 ml.) was refluxed for 10 hr. The optical rotation could not be followed owing to darkening of the solution but at the end of 10 hr. the solution had $[\alpha]^{24}D$ +51° (*c* 5 in water). It may be noted that this value agrees well with that calculated for a mixture of equimolecular amounts of 2,3,4,6-tetra-*O*-methyl-D-glucose, $[\alpha]D$ +84°, and 3,4-di-*O*-methyl-D-xylose, $[\alpha]D$ +20.5°.

Removal of the acid by passage through a column of Duolite A4 resin and concentration of the eluate gave a colorless sirup (176 mg.). Examination of this on a paper chromatogram irrigated with methyl ethyl ketone–water azeotrope showed the presence of four spots. A strong spot with R_f 0.84 corresponded with 2,3,4,6-tetra-*O*-methyl-D-glucose and a second strong spot, with R_f 0.57, corresponded with 3,4-di-*O*-methyl-D-xylose. Although 2,3-di-*O*-methyl-D-xylose has almost the same R_f value it gives a dark red spot when sprayed with *p*-anisidine, whereas the 3,4-isomer gives a buff-colored spot. Two weaker spots, with R_f values 0.71 and 0.20, were attributed to demethylation products.

Identification of 2,3,4,6-Tetra-*O*-methyl-D-glucose and 3,4-Di-*O*-methyl-D-xylose.—The sirup (176 mg.) from the hydrolysis was added to the top of a hydrocellulose–cellulose column and separated in the usual way with methyl ethyl ketone:water azeotrope, fractions (6 ml.) being collected at ten-minute intervals.[5] No dye was used to mark the front, but the collection of fractions was started 4.5 hr. after the addition of the sirup to the column. Examination of the fractions in the usual manner showed that the 2,3,4,6-tetra-*O*-methyl-D-glucose was in tubes 10–21 and the 3,4-di-*O*-methyl-D-xylose in tubes 46–70.

Concentration of the contents of tubes 10–21 yielded a sirup (75 mg.) which crystallized on seeding with 2,3,4,6-tetra-*O*-methyl-D-glucose and after recrystallization from ether–petroleum ether it had m.p. and mixed m.p. 92–94°.[8] The anilide had m.p. and mixed m.p. 136–138°.[9]

3,4-Di-*O*-methyl-D-xylose was isolated as a sirup (35 mg.), $[\alpha]^{24}D$ +25° (*c* 0.8 in methanol), by combining the contents of appropriate tubes. The literature gives $[\alpha]D$ +22.1° in methanol for 3,4-di-*O*-methyl-D-xylose.[15]

The sirup (35 mg.) was dissolved in water (2 ml.) and bromine (10 drops) added. The reaction was kept at room temperature in the dark for 53 hr., by which time the oxidation of the sugar was complete, as judged by paper chromatography. The solution was diluted with water (10 ml.) and excess bromine removed by aeration. Acid was neutralized by the addition of silver carbonate and the solution filtered. Silver ions were removed by passage of hydrogen sulfide and filtration. Evaporation of the filtrate gave a sirup (25 mg.) which gave 3,4-di-*O*-methyl-D-xylono-δ-lactone upon distillation, b.p. (bath temp.) 120–140° (0.01 mm.). Nucleation caused immediate crystallization and after crystallization from ether the 3,4-di-*O*-methyl-D-xylono-δ-lactone had m.p. and mixed m.p. 62–63°.[7]

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