134. *The Hemicelluloses of Loblolly Pine* (Pinus taèda) *Wood. Part I. The Isolation of Five Oligosaccharide Fragments.*

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Controlled acid hydrolysis of the extractive-free wood of loblolly pine has led to the isolation of 2-*O*-(4-*O*-methyl-α-D-glucuronosyl)-D-xylose (A) and 4-*O*-β-D-glucopyranosyl-α-D-mannopyranose (B). Alkaline extraction of the wood holocellulose afforded a mannose-rich hemicellulose fraction, which on partial hydrolysis gave (B), 4-*O*-β-D-mannopyranosyl-α-D-mannopyranose (C), 4-*O*-β-D-mannopyranosyl-α-D-glucopyranose (D), and *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-α-D-mannopyranose (E).

A study is being made of the polysaccharides which constitute the hemicellulosic fraction of loblolly pine (*Pinus taèda* Linnaeus) wood.[1] As part of the programme, the isolation is being attempted of oligosaccharides, formed by partial acid hydrolysis of the hemicelluloses. In the present work, partial hydrolysis was carried out on the crude wood, and also on a hemicellulose fraction which was prepared from the wood.

In the first series of experiments, the crude, extractive-free sawdust was partially hydrolysed with dilute sulphuric acid. Chromatography of the products indicated that the major monosaccharide components were glucose, mannose, galactose, arabinose, xylose, 4-*O*-methylglucuronic acid, and galacturonic (and/or glucuronic) acid. No other sugars were detected when further hydrolysates of the wood residue were examined on chromatograms. It is therefore probable that residues of the above monosaccharides are the only major structural units in the hemicellulosic fraction of the wood.

In addition to the monosaccharides, at least five acidic oligosaccharides and three neutral oligosaccharides were detected in the hydrolysate. The acidic and neutral sugars were separated on an anion-exchange resin,[2] and were further fractionated by charcoal-adsorption chromatography and by paper chromatography.

The major component of the acidic oligosaccharide fraction was 2-*O*-(4-*O*-methyl-α-D-glucuronosyl)-D-xylose (A). This aldobiuronic acid has also been isolated from the hydrolysates of aspen [2], beech [3], birch [4], Scots pine [5], black spruce [5], flax [6], maize-cob [7], and western hemlock [8] hemicelluloses. It was characterised according to the general procedure of Jones and Wise.[2] The major component of the neutral oligosaccharide fraction was 4-*O*-β-D-glucopyranosyl-α-D-mannopyranose (B); it was characterised by comparison of the sugar and its octa-acetate with authentic specimens. Control experiments indicated that this sugar was not an artefact.

Loblolly pine holocellulose, prepared by the chlorite procedure of Wise, Murphy, and D'Addieco,[9] was extracted with aqueous potassium hydroxide containing boric acid.[10] Addition of Fehling's solution to the extract yielded an insoluble copper complex, from which, after acidification, a copper-free hemicellulose fraction (I) was obtained. Chromatography of the hydrolysed material indicated the presence of glucose and mannose, together with minor amounts of galactose, xylose, and 4-*O*-methylglucuronic acid.

The fraction (I) was completely soluble in aqueous formic acid, and the change in optical rotation of a heated solution was followed. It became constant after 10 hr. When hydrolysis under the conditions chosen was stopped after 4 hr., a mixture

[1] A report on some aspects of this work appeared in *TAPPI*, 1956, **39**, 438.
[2] Jones and Wise, *J.*, 1952, 2750, 3389.
[3] Aspinall, Hirst, and Mahomed, *J.*, 1954, 1734.
[4] Saarnio, Wathèn, and Gustafsson, *Acta Chem. Scand.*, 1954, **8**, 825.
[5] Gorrod and Jones, *J.*, 1954, 2252.
[6] Geerdes and Smith, *J. Amer. Chem. Soc.*, 1955, **77**, 3569.
[7] Whistler, Conrad, and Hough, *ibid.*, 1954, **76**, 1668.
[8] Dutton and Smith, *ibid.*, 1956, **78**, 2505.
[9] Wise, Murphy, and D'Addieco, *Paper Trade J.*, 1946, **122**, No. 2, 35.
[10] Jones, Wise, and Jappe, *TAPPI*, 1956, **39**, 139.

containing at least four oligosaccharides was obtained; the sugars were detected chromatographically. After separation of the sugars by charcoal chromatography,[11] all four oligosaccharides were obtained crystalline. They were the glucosidomannose (B), 4-*O*-β-D-mannopyranosyl-α-D-mannopyranose (C), 4-*O*-β-D-mannopyranosyl-α-D-glucopyranose (D), and *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-α-D-mannopyranose (E). They were characterised by comparison of the sugars with authentic specimens. Control experiments indicated that these oligosaccharides were not reversion products.

The isolation of the disaccharides (B) and (D) from the hemicellulose fraction (I) establishes that, in loblolly pine, glucose and mannose residues are linked together by β-(1→4) glycosidic bonds in a polysaccharide (or group of polysaccharides) which is distinct and separable from the residual "α-cellulose" of the wood. Leech [12] has obtained a glucosidomannose by acetolysis of slash pine holocellulose, and Hamilton, Kircher, and Thomspon [13] have isolated a mannoglucan from western hemlock.

It is significant that no cellobiose, and no trisaccharides other than mannotriose (E), were detected in the present work. The apparent absence of cellobiose suggests that the loblolly pine mannoglucan is dissimilar to that of the Iles mannan studied by Smith and his co-workers.[14, 15] The gross preponderance of mannotriose over other trisaccharides is probably due to the choice of hydrolytic conditions, and to the fact that mannose residues predominate in fraction (I) (unpublished results). However, it might further signify that the parent polysaccharide contains relatively long chains of contiguous mannose residues. If this is the case, fraction (I) either contains a single mannoglucan, in which mannose and glucose residues are unevenly distributed, or it contains a mixture of a mannoglucan and one or more other mannans. At least one of the mannans in fraction (I) is believed to be associated with galactose residues.[1]

A hemicellulose fraction similar to (I) has subsequently been resolved into a fraction (II) containing mannose, glucose, and galactose residues only, and a fraction (III) containing xylose and 4-*O*-methylglucuronic acid residues only. Thus (II) appears to have been the source of fragments (B), (C), (D), and (E), and (III) is considered to be the source of fragment (A) in the original wood. Details of this, and the results of methylation studies on fractions (II) and (III) will be published later.

Experimental

Paper chromatography was carried out by the descending method [16] on Whatman No. 1 filter paper, the following solvent systems being used: (*a*) ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4), (*b*) butan-1-ol–ethanol–water (40 : 11 : 19), (*c*) butan-1-ol–pyridine–water (10 : 3 : 3) (all v/v). The positions of the sugars on the chromatograms were determined by spraying with either silver nitrate in acetone, followed by sodium hydroxide in ethanol,[17] or with *p*-anisidine hydrochloride in butan-1-ol.[18] The rates of movement of the sugars are quoted relative to those of galactose (R_{gal}) or xylose (R_{xyl}) on the same chromatogram. Optical rotations were determined at 23° ± 3° (unless otherwise stated, solution was in water, and the figures quoted are equilibrium values). Solvents were removed under reduced pressure.

Partial Acid Hydrolysis of the Crude Wood.—The sawdust was set aside for 24 hr. in benzene–ethanol (2 : 1 v/v) at room temperature, then separated, washed with ethanol, and air-dried. The extractive-free sawdust (*ca.* 300 g.) was stirred with N-sulphuric acid (2·5 l.) at 70° (60 hr.). The mixture was cooled and filtered, and the filtrate and washings were brought to pH 2 (barium hydroxide). The filtered solution was passed through Amberlite resin IR-120, and then through a column (40 × 4 cm.) of Amberlite resin IR-4B (acetate form).

[11] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[12] Leech, *TAPPI*, 1952, **35**, 249.
[13] Hamilton, Kircher, and Thompson, *J. Amer. Chem. Soc.*, 1956, **78**, 2508.
[14] Rebers and Smith, *ibid.*, 1954, **76**, 6097.
[15] Smith and Srivastava, *ibid.*, 1956, **78**, 1404.
[16] Partridge, *Biochem. J.*, 1948, **42**, 238.
[17] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[18] Hough, Jones, and Wadman, *J.*, 1950, 1702.

The neutral effluent and washings were concentrated (500 ml.). Chromatography of the concentrate indicated the presence of glucose, mannose, galactose, xylose, arabinose, and at least three oligosaccharides, the last having R_{gal} 0·62, 0·51, and 0·44 respectively (solvent *a*). The mixture was adsorbed on a charcoal (Darco G60)–Celite (1 : 1; w/w) column (50 × 5 cm.). Elution with water (3 l.) removed monosaccharides only (chromatographic detection). Subsequent elution with aqueous ethanol (15% v/v; 3 l.) displaced the oligosaccharides; concentration of the effluent yielded a syrup (i) (1 g.).

The acidic sugars were displaced from the IR-4B resin column with 0·5N-formic acid (2 l.). Concentration of the effluent yielded a syrup (ii) (4 g.), chromatography of which indicated the presence of galacturonic (and/or glucuronic) acid, 4-*O*-methylglucuronic acid, and five oligosaccharides, the last having R_{xyl} 0·73, 0·40, 0·27, and 0·07 respectively (solvent *a*).

Isolation and Identification of 4-O-β-D-*Glucopyranosyl*-α-D-*mannopyranose*.—Syrup (i) (100 mg.) was separated on a paper chromatogram (60 × 50 cm.) (solvent *b*). The fastest-moving component (R_{gal} 0·62, solvent *a*) had $[\alpha]_D$ +6° (*c* 1·0), and, on chromatograms irrigated with solvent *a*, *b*, or *c*, it ran at the same rate as an authentic sample of 4-*O*-β-D-glucopyranosyl-α-D-mannopyranose (supplied by Dr. N. K. Richtmyer). The syrup (10 mg.) was hydrolysed with N-sulphuric acid (2 ml.) at 100° (12 hr.), neutralised with barium carbonate, filtered, concentrated, and examined on chromatograms (solvents *b* and *c*). Glucose and mannose only were detected; the spots on the treated chromatograms appeared to be of equal intensity. When a concentrated solution of the sugar in moist methanol was allowed to evaporate slowly in air, crystals were formed; they had m. p. 135—138°, undepressed on admixture with the authentic glucosidomannose monohydrate, $[\alpha]_D$ +18°→+15° (12 hr.; *c* 1·0 in MeOH).

The crystals (8 mg.) were treated with acetic anhydride (5 ml.) containing anhydrous zinc chloride (2% w/w) at 80° (1 hr.). Excess of anhydride was removed by distillation, and methanol (5 ml.) was added. Dropwise addition of water initiated crystallisation of the octa-*O*-acetyl-glucosidomannose; after recrystallisation from methanol–water, it had m. p. and mixed m. p. 203—204°, $[\alpha]_D$ +36° (*c* 1·0 in $CHCl_3$). *X*-Ray powder diffraction patterns of the octa-acetate and of an authentic compound were identical.

The possibility that the glucosidomannose was a reversion product was investigated. A solution of D-glucose (1·0 g.) and D-mannose (0·5 g.) in N-sulphuric acid (10 ml.) was heated at 70° for 60 hr. The solution was cooled, neutralised with barium carbonate, filtered, concentrated, and placed on a charcoal column. Elution with water removed monosaccharides, and aqueous ethanol (10% v/v) eluted the oligosaccharides (80 mg.). Chromatography (solvent *a*) on the same sheet as syrup (i) indicated that the reversion product had three major components, having R_{gal} 0·53, 0·46, and 0·35, which were distinct from those of syrup (i).

Isolation and Identification of 2-O-(4-O-*Methyl*-α-D-*glucuronosyl*)-D-*xylose*.—Syrup (ii) (4 g.) was adsorbed on a charcoal (Darco G60)–Celite (1 : 1, w/w) column (40 × 3 cm.). Elution with water until the effluent gave a negative Molisch test removed galacturonic (and/or glucuronic) acid and 4-*O*-methylglucuronic acid (chromatographic detection, solvent *a*). Subsequent elution was by the gradient technique of Bacon and Bell,[19] with ethanol–water as the solvent system. The major component of the mixture (R_{xyl} 0·73, solvent *a*) was eluted in the range 2—4% v/v ethanol. The effluent (2 l.) was concentrated to a syrup (1·5 g.), $[\alpha]_D$ +83° (*c*, 3·5) [Found : OMe, 9·0%; equiv. (by titration), 348. Calc. for $C_{12}H_{20}O_{11}$: OMe, 9·12%; equiv., 340].

The acid (100 mg.) was boiled with N-sulphuric acid (10 ml.) (24 hr.), neutralised with barium carbonate, and filtered, and the filtrate examined chromatographically (solvent *a*). Xylose, 4-*O*-methyl-glucuronic acid, and the original disaccharide were detected. The filtrate was concentrated, and the residue was separated on a paper chromatogram (60 × 50 cm.) (solvent *b*). The acidic fragment had $[\alpha]_D$ +82° (*c*, 1·0), and was characterised by conversion into α-methyl 4-*O*-methyl-D-glucuronamide,[2] m. p. and mixed m. p. 235°. The neutral fragment had $[\alpha]_D$ +18°, and was characterised by conversion into D-xylose dibenzylidene dimethyl acetal,[20] m. p. and mixed m. p. 210°.

The disaccharide (1·1 g.) was methylated with methyl sulphate and sodium hydroxide, and the partly methylated product was isolated as described by Jones and Wise.[2] The acid was further methylated with Purdie's reagents, and the product (800 mg.), $[\alpha]_D$ +88° (*c*, 15·0; $CHCl_3$) (Found : OMe, 49·8. Calc. for $C_{18}H_{32}O_{11}$: OMe, 51·2%), was reduced with lithium aluminium hydride in ether. The methylated disaccharide was isolated in the usual manner,

[19] Bacon and Bell, *J.*, 1953, 2528.
[20] Breddy and Jones, *J.*, 1945, 738.

and re-methylated twice with Purdie's reagents, to give a product (400 mg.), which had n_D^{18} 1·4625, $[\alpha]_D$ +108° (*c* 1·0 in $CHCl_3$) (Found: OMe, 52·7. Calc. for $C_{18}H_{34}O_{10}$: OMe, 52·9%). This product was hydrolysed with aqueous formic acid (20% v/v) at 80° until the optical rotation of the solution became constant {$[\alpha]_D$ +86° (*c* 2·0) (10 hr.)}. The solution was then evaporated to dryness; the residue was boiled with water (5 min.) and again evaporated to dryness. This latter procedure was repeated twice to ensure hydrolysis of the formyl esters. The resulting syrup contained two sugars, which were indistinguishable on paper chromatography with solvent *a*, *b*, or *c*, from 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose and 3 : 4-di-*O*-methyl-D-xylose. These sugars were separated on a paper chromatogram (60 × 50 cm.) (solvent *b*), and identified as: (i) 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose, m. p. 88—90°, $[\alpha]_D$ +86° in $CHCl_3$ {the derived *N*-phenylglucosylamine 2 : 3 : 4 : 6-tetra-*O*-methyl ether had m. p. and mixed m. p. 137°, $[\alpha]_D$ +250° in acetone}, and (ii) 3 : 4-di-*O*-methyl-D-xylose, $[\alpha]_D$ +12° in MeOH, converted by bromine-water oxidation into 3 : 4-di-*O*-methyl-D-xylonolactone, m. p. and mixed m. p. 64°, $[\alpha]_D$ −50°→−23° (3 days).

Preparation of Mannose-rich Hemicellulose Fraction (I).—The extractive-free wood (1·4 kg.) was delignified by the chlorite procedure of Wise, Murphy, and D'Addieco.[9] The resulting holocellulose was washed well with water, and the excess of water was removed by filtration. The residue was then stirred with aqueous potassium hydroxide (24% w/v) containing boric acid (4% w/v) (4 l.) at room temperature (24 hr.); the mixture was filtered, and the filtrate and washings were treated with Fehling's solution (*ca.* 600 ml.), which was added slowly and with vigorous stirring, until no further precipitation occurred. The insoluble copper complex was collected (centrifuge), and washed by repeated dispersion in water followed by centrifugation (6 times). It was finally dispersed by means of a macerator in ice-cold water (1·5 l.), acidified with acetic acid, and poured into ethanol (3 l.). The hemicellulosic material was filtered off, washed with acetone acidified with hydrochloric acid until copper-free (colourless washings), then with ethanol, followed by ether, and air-dried. The yield was *ca.* 70 g. Chromatography of a sample hydrolysed by 2N-sulphuric acid at 100° for 24 hr. indicated the presence of glucose, mannose, galactose, xylose, and 4-*O*-methylglucuronic acid.

Partial Acid Hydrolysis of Hemicellulose Fraction (I).—The hemicellulose (2·00 g.) was dissolved in 90% formic acid (50 ml.) and diluted with water to 100 ml. The solution {$[\alpha]_D$ −21°} was heated at 97°, and the change in specific rotation was followed:

Time (hr.)	0·00	0·50	1·75	2·75	3·75	4·75	6·75	10·00
$[\alpha]_D^{20}$	−21°	−12°	0°	4°	6·5°	10°	12°	13·5° (const.)

A further sample (8 g.) was hydrolysed for 4 hr. under similar conditions. The cooled solution was concentrated to yield a syrup, then 0·5N-sulphuric acid (250 ml.) was added. The resultant solution was heated at 100° for 10 min., cooled, neutralised with barium carbonate, and de-ionised on a mixed-bed resin (Amberlite MB3). Chromatography (solvent *a*) indicated the presence of the above monosaccharides and of four oligosaccharides, having R_{gal} 0·62, 0·48, 0·41, and 0·14 (hereinafter termed B, C, D, and E respectively). The latter materials, on visual examination of the treated chromatogram, were considered to comprise about 30% of the total mixture. The hydrolysate (7 g.) was placed on a charcoal (Darco G60)–Celite (1 : 1 w/w) column (30 × 3·5 cm.) and fractionated by stepwise elution [11] with water and with aqueous ethanol. The effluent was collected in 500 ml. fractions; each was examined on a chromatogram, and chromatographically identical fractions were combined and concentrated to syrups. The water displaced first the monosaccharides (4·5 g. in 1 l. of effluent), and then component (C) (0·45 g. in 12 l. of effluent). Aqueous ethanol (2% v/v) displaced component (D) (0·10 g. in 3 l. of effluent), followed by component (E) (0·25 g. in 3 l. of effluent). Elution with aqueous ethanol (5% v/v) displaced component (B) (0·50 g. in 3 l. of effluent). Where necessary, the sugars were further fractionated on paper chromatograms (60 × 50 cm.) (solvent *b*), to give pure specimens of each sugar.

Identification of Component (*C*).—The syrup (95% pure) had $[\alpha]_D$ −5° (*c* 6·7) and was indistinguishable on paper chromatograms, run in solvent *a*, *b*, or *c*, from authentic 4-*O*-β-D-mannopyranosyl-α-D-mannopyranose [prepared from the mannogalactan of fenugreek seed, and characterised by Dr. L. Hough (unpublished results)]. Chromatography of the hydrolysed sugar indicated the presence of mannose only. The equivalent weight of the sugar was 182 (by iodometric titration). The disaccharide was dissolved in hot moist ethanol and, after cooling, the solution was seeded with a crystal of the authentic mannobiose. Tufts of fine needles

separated, having m. p. and mixed m. p. 204°, $[\alpha]_D$ −5°→−8° (1·5 hr.; *c*, 1·8). *X*-Ray powder diffraction patterns of component (C) and of the authentic mannobiose were identical.

Identification of Component (*D*).—The pure syrup had $[\alpha]_D$ +20° (*c*, 1·0) and was indistinguishable on paper chromatograms, run in solvent *a*, *b*, or *c*, from authentic 4-*O*-β-D-mannopyranosyl-α-D-glucopyranose (supplied by Professor Fred Smith [15]). Determination of the degree of polymerisation by the method of Peat, Whelan, and Roberts [21] showed that the sugar was a disaccharide. Chromatography of the hydrolysed sugar indicated the presence of glucose and mannose only. The spots on the treated chromatogram appeared to be of equal intensity. A solution of the disaccharide in moist ethanol yielded crystals when seeded with a crystal of the authentic mannosidoglucose; they had m. p. and mixed m. p. 203°, $[\alpha]_D$ +35°→+18° (±5°) (40 min.; *c* 0·2). *X*-Ray powder diffraction patterns of component (D) and of the authentic sugar were identical.

Identification of Component (*E*).—The pure syrup had $[\alpha]_D$ −22° (*c*, 4·4), and indicated mannose only on chromatograms after hydrolysis. It was indistinguishable on chromatograms, run in solvent *a*, *b*, or *c*, from authentic *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-α-D-mannopyranose (prepared by Dr. L. Hough). Determination of the degree of polymerisation by the method of Peat *et al.*[21] showed that the sugar was a trisaccharide. When a seeded solution of the trisaccharide in ethanol–water was allowed to evaporate in a desiccator, flat plates of the mannotriose trihydrate separated, having m. p. and mixed m. p. 155—165° (decomp.), $[\alpha]_D$ −22°→−25° (*ca.* 3 days; *c* 2·4). An *X*-ray powder diffraction pattern of the sugar was identical with that of the authentic mannotriose trihydrate.

A solution of the trisaccharide (20·5 mg.) in water (5 ml.) was added dropwise to a solution of potassium borohydride (100 mg.) in water (3 ml.) with stirring. The solution was set aside overnight, after which time a portion acidified with acetic acid did not reduce Fehling's solution. The solution was then de-ionised (Amberlite resin MB3), and concentrated to a syrup (20 mg.), $[\alpha]_D$ −30° (*c* 0·7). [The syrup ran as a single spot on a chromatogram (silver nitrate spray).] The syrup (10 mg.) was acetylated with acetic anhydride and zinc chloride as described above, and the crystalline product was recrystallised from methanol–water, to yield the dodeca-*O*-acetylmannotriitol (20 mg.), m. p. 112°, $[\alpha]_D$ −20° ± 5° (*c* 1·0 in $CHCl_3$). These figures are in close agreement with those reported by Whistler and Smith for this compound.[22]

Identification of Component (*B*).—The syrup crystallised on being seeded with 4-*O*-β-D-glucopyranosyl-α-D-mannopyranose monohydrate. After recrystallisation from ethanol–water, the sugar had m. p. 136—138°, undepressed on admixture with authentic glucosidomannose monohydrate, $[\alpha]_D$ +18°→+15° (*c* 1·0 in MeOH).

Control Experiment.—A solution of D-mannose (0·3 g.), D-glucose (0·1 g.), and D-galactose (0·05 g.) in aqueous (50% v/v) formic acid (20 ml.) was heated at 100° for 4 hr. The solution was evaporated to dryness; 0·5N-sulphuric acid (10 ml.) was added, and the solution was heated at 100° for 10 min. It was then neutralised with barium carbonate, filtered, and concentrated to a syrup. Chromatography of the syrup (solvent *a*) showed the presence of traces of oligosaccharides only. Since the oligosaccharides in the partial hydrolysate of fraction (I) were present in substantial amounts, this result is taken as strong evidence that they were not reversion products. A detailed study of the acid reversion of D-mannose is now in progress.

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[21] Peat, Whelan, and Roberts, *J.*, 1956, 2258.
[22] Whistler and Smith, *J. Amer. Chem. Soc.*, 1952, **74**, 3795.