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ISOLATION OF TWO PURE POLYSACCHARIDES FROM THE HEMICELLULOSE OF SLASH PINE (*Pinus elliottii*)

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ABSTRACT

Two pure, acidic polysaccharides have been isolated from the hemicellulose of slash pine in yields of 1–2% and 4–5%. Their properties are compared, and the structure of one of them has been investigated by methylation analysis The results indicate that the glycan is a β-D-(1 → 4)-linked xylan chain with many branch points 4-*O*-Methyl-D-glucopyranosyluronic acid, L-arabinofuranose, and D-xylopyranose residues occur as non-reducing end groups The uronic acid occurs as single-unit attachments to the main chain Some of the D-xylose residues in the polysaccharide are doubly branched The total hemicellulose components of the wood probably represent a complex mixture of chemical types, from which the two pure fractions described above may be separated fortuitously by careful, fractional precipitation.

INTRODUCTION

Slash pine, an industrially important wood in the Southern United States and Australia, has previously been extracted with various concentrations of alkali[1] and has been shown to contain a glucomannan[2] and another type of polysaccharide, wherein the 4-*O*-methyl-D-glucuronic acid residues are joined to the chain preponderantly as illustrated in the isolated 2-*O*-(4-*O*-methyl-D-glucopyranosyluronic acid)-α-D-xylose[3].

We have applied fractional precipitation to slash pine hemicellulose, in order to investigate further the potential of this already recognised[4] method of fractionation on a polysaccharide mixture that appears to be particularly complex Other methods of fractionation of hemicelluloses were recently investigated[5], and Timell has emphasised[6] the need for development of purification methods for such mixtures

Holocellulose, prepared by chlorite delignification[4], was extracted with oxygen-free, 16% aqueous potassium hydroxide, which is known[1] to effect maximum extraction The hemicellulose so obtained was subjected to a preliminary fractional precipita-

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tion from cold water at pH 2 with ethanol, and two of the crude fractions (**1** and **2**) were ultimately purified by reprecipitation at a suitable pH The criteria of purity used were a single inflection in the precipitation curve and a single peak on electrophoresis in borate buffer Final yields of 1 2 and 4 5%, respectively, for the two principal hemicellulose fractions are undoubtedly lower than the amounts present in the wood The absence of excessive amounts of arabinose in the precipitation liquors precludes the occurrence of any extensive hydrolysis of L-arabinofuranose linkages during the precipitation

The pure fractions are strongly acidic and have similar properties (Table I), with the exception that fraction **1** shows a marked tendency to separate as a gel from acidic solution It is probable that both fractions consist mainly of a β-D-(1→4)-linked xylan skeleton with varying degrees of substitution and branching The other components of the hemicellulose mixture showed no inflection in the precipitation curve at pH 2 and were not investigated further They probably consist of a complex mixture of many similar polysaccharides, which would be difficult to separate by fractional precipitation[6,7].

TABLE I

ANALYSIS AND PROPERTIES OF PURE HEMICELLULOSE FRACTIONS

	Fraction **1c**	*Fraction* **2a**
D-Xylose	20	8 9
L-Arabinose	2	1 0
D-Mannose	1	0
D-Glucose+D-galactose	1	0
Ash (as sulphate, %)	1 0	1 2
Equivalent weight	1150	1110
$[\alpha]_D^{25}$ (*c* 1, M KOH)	−58°	−58°
Intrinsic viscosity	0 69	0 85
Electrophoretic mobility	$6\,4\times10^{-5}$	$3\,9\times10^{-5}$ (descending)
($cm^2\ volt^{-1}\ sec^{-1}$)	$7\,9\times10^{-5}$	$5\,0\times10^{-5}$ (ascending)
Methoxyl content (%)	3 15	3 25

The structure of fraction **2** was investigated by methylation analysis Methylation by the Haworth procedure, and then with silver oxide and methyl iodide, yielded a product (OMe, 38 8%) which showed no i r. absorption for free hydroxyl groups Following methanolysis and subsequent hydrolysis of the methylated polymer, the products were separated into neutral and acidic fractions, the latter being mostly aldobiouronic acids Reduction and further hydrolysis demonstrated that the acidic fraction was a mixture of 2,3,4-tri-*O*-methyl-D-glucuronic acid linked to 3-*O*-methyl-D-xylose and to D-xylose. A trace of 2,3-di-*O*-methyl-D-xylose, probably arising from an aldotriouronic acid (see Table II), was also tentatively identified The neutral, methylated sugars were separated by preparative paper chromatography, and their identity and relative molar yields are shown in Table II. The ratio (1.9.8) of the

relative, total molar yields of arabinose to xylose derivatives isolated after methylation is similar to the corresponding ratio (1 8 9) in the original polysaccharide Table II also shows a good correlation between the non-reducing end groups and products derived from branch points

TABLE II

PRODUCTS FROM METHYLATION AND HYDROLYSIS OF FRACTION 2a

	Molar ratio
2,3,4-Tri-*O*-methyl-D-glucose[a]	8
3-*O*-Methyl-D-xylose[a,b]	7
D-Xylose[a,b]	2
2,3-Di-*O*-methyl-D-xylose[b]	35
2-*O*-Methyl-D-xylose[b]	3
2,3,4-Tri-*O*-methyl-D-xylose[b]	2
2,3,5-Tri-*O*-methyl-L-arabinose[b]	5

[a]Isolated from the acidic fraction of the hydrolysate after reduction of the uronic acid groups
[b]Isolated from the neutral fraction of the hydrolysate

A polysaccharide which corresponds to these results is a β-D-(1 $\rightarrow$ 4)-linked xylan containing both D-xylopyranose and L-arabinofuranose end groups, with the latter preponderating 4-*O*-Methyl-D-glucuronic acid occurs as a substituent on C-2 of the main chain, present on about one in seven of the xylose residues Branch points other than the uronic acid occur at both C-2 and C-3 of the D-xylopyranose residues, and some units are doubly substituted with the uronic acid at C-2 and another group such as L-arabinofuranose at C-3 Since the methylated polymer readily forms a strong film, the xylan is probably essentially linear, with single-unit side chains to account for the end groups enumerated above.

The evidence from methylation and hydrolysis does not preclude the possibility that a part of the acidity attributed to 4-*O*-methyl-D-glucuronic acid may be due to D-glucuronic acid, but earlier results[3], together with the relation of the methoxyl content of the pure fraction **2** to its equivalent weight, suggest that any contribution from D-glucuronic acid must be small.

The overall structure of fraction **2** therefore is similar to those derived for arabino-(4-*O*-methylglucurono)xylans of other soft woods[6] Fraction **1** also appears to be a chemically homogeneous polysaccharide on the basis of fractional precipitation at two different pH values and of electrophoresis in borate buffer, and if, in fact, the composition shown in Table I does represent a single molecular type, then this is an unusually complex hemicellulose The two "pure" hemicelluloses probably represent chemically homogeneous components which are fortuitously separated by fractional precipitation from a continuous range of chemical types and molecular sizes in the total hemicellulose fraction

MATERIALS AND METHODS

Paper-chromatographic separations were made on Whatman No. 1 (analytical) and 3MM (preparative) papers, using the following irrigants (*A*) ethyl acetate–pyridine–water (40 11 6); (*B*) butanone–water azeotrope Detection was effected with sprays (*A*) aniline hydrogen phthalate[8] and (*B*) *p*-anisidine hydrochloride[9]. R_X and R_G are the rates of movement relative to D-xylose and 2,3,4,6-tetra-*O*-methyl-D-glucose, respectively

For paper electrophoresis, the procedure described by Foster[10] was used, with Whatman 3MM filter paper and borate buffer (pH 10) at 900 volts M_G is the rate of movement relative to D-glucose, corrected for endosmotic flow

The composition of polysaccharides was determined by heating a 2% solution in 0 5M sulphuric acid solution at 100° for 6 h, until the optical rotation became constant Solutions were then neutralised with barium carbonate and analysed for component sugars by paper chromatography.

Equivalent weights of the polysaccharides were determined by potentiometric titration of 0 1% aqueous solutions with 0 01M potassium hydroxide solution to pH 7 2 in an atmosphere of nitrogen.

Electrophoretic mobilities were determined at 0° with 2% solutions of polysaccharide in borate buffer (pH 10) of 0 1M ionic strength, using a Tiselius cell in a Perkin–Elmer apparatus[11] Viscosities were measured in M potassium hydroxide, at concentrations below 0 25%, in an Ostwald–Cannon–Fenske viscometer at 25° Specific rotations of polysaccharides were determined at 1% concentration in M potassium hydroxide

Isolation of crude hemicellulose. — A freshly cut, 20-year-old log of slash pine (*Pinus elliottii*) was freed from bark and planed with exclusion of heart-wood, and the planings were passed through a Wiley mill The ground wood was immediately extracted in a Soxhlet apparatus with benzene–ethanol (2 1) continuously for 24 h, and the resulting, extractive-free, air-dried wood was exhaustively extracted with water at 51° The water extracts, when added to 3 vol of ethanol gave a pale-brown precipitate (0 12% by weight of wood), which was re-dissolved in water and subjected to fractional precipitation with ethanol The fraction (0 04% by weight of wood) precipitating between 37 5 and 44% ethanol was collected and, when hydrolysed and analysed by paper chromatography, was found to contain galactose, arabinose, and xylose in the molar proportions 74 10 1. This arabinogalactan had an intrinsic viscosity of 0 10 and an OAc content of 3 0%. An attempt to measure electrophoretic mobility was unsuccessful because the product formed an insoluble gel in sodium borate solution

The extracted wood was delignified by treatment with sodium chlorite[4], and the holocellulose was washed with acetone and air-dried; yield, 72% of the original wood A 750-g portion of the holocellulose was de-gassed and flushed repeatedly with nitrogen in a 9-l bottle; 6 l of oxygen-free, 16% aqueous potassium hydroxide

were then added under nitrogen, and the mixture was agitated gently at 10° for 15 h. The resulting mixture was quickly filtered through cloth, and the solids were washed with 1.5 l of 5% aqueous potassium hydroxide and pressed dry The combined filtrate and washings were immediately cooled with ice, the pH was adjusted to 5 with glacial acetic acid, and the mixture was kept at 5° for 20 h Centrifugation of the mixture removed 3 g of a brown solid which appeared to consist mostly of fine particles of undissolved holocellulose On hydrolysis, this gave mainly D-glucose The clear, neutral solution was added with vigorous stirring to 3 volumes of ethanol, and the resulting precipitate was washed several times by centrifugation with ethanol and then dried over calcium chloride. The product (125 g) was a beige powder. Hydrolysis in M sulphuric acid at 100° for 4 h produced a small amount of an insoluble precipitate, and a solution which contained (paper chromatography, irrigant *A* and spray *A*) xylose, arabinose (R_X 0 81), acidic material (R_X 0), mannose (R_X 0 67), and galactose (R_X 0 44).

Preliminary fractionation of the crude hemicellulose — The foregoing preparation of hemicellulose yielded a faintly opalescent, aqueous solution, which was readily clarified by centrifugation; $[\alpha]_D^{25}$ −20 4° ±1° (*c*, 1 water) Clarified, 2% solutions prepared with (*a*) 0 3M sodium borate (pH 10), (*b*) 0 3M potassium acetate adjusted to pH 10 with potassium hydroxide, and (*c*) dilute sulphuric acid (pH 2) were each precipitated by the gradual addition of ethanol, at 25° for (*a*) and (*b*), and at 10° for (*c*) The precipitation was determined by optical rotation as described earlier[12] (see Fig 1) The precipitation at pH 2 gave the most clear-cut inflections in

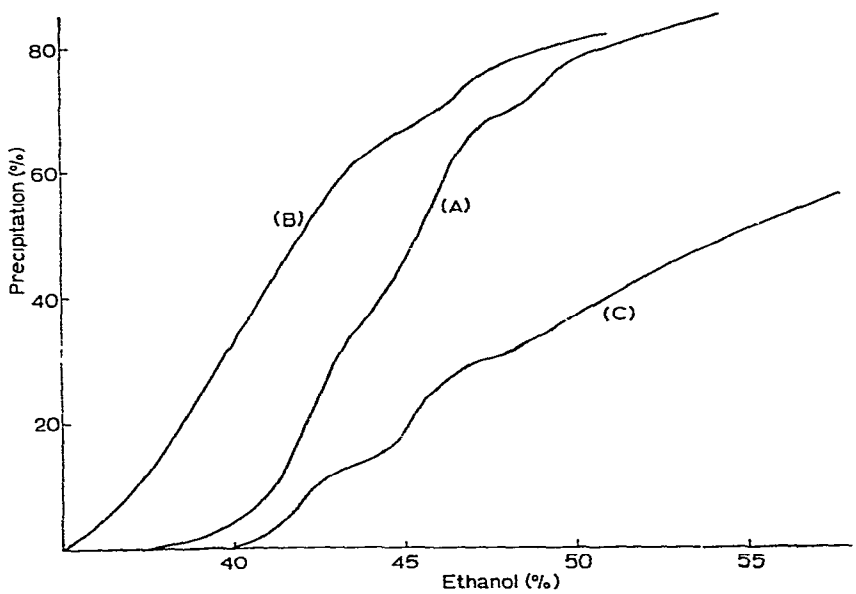

Fig 1 Fractional precipitation of the crude hemicellulose (*a*) from 0 3M sodium borate (pH 10), (*b*) from 0 3M potassium acetate (pH 10), (*c*) from sulphuric acid (pH 2)

the precipitation curve, at ethanol concentrations between 40–42% and 42–49%, respectively.

A solution of 100 g of crude hemicellulose in 1 liter of water was centrifuged for clarification, diluted to 4.8 l with water, cooled to 10°, and adjusted to pH 2 with 200 ml of 0 5M sulphuric acid Ethanol (3 7 l) was added with vigorous stirring, and the resulting suspension was passed through a Sharples supercentrifuge at 10° as quickly as possible, to give precipitate **1** The clear centrifugate was treated with an additional 400 ml of ethanol and then centrifuged as before, and the precipitate was discarded Another 800 ml of ethanol was finally added and the resulting precipitate (**2**) was separated in the same manner Some evaporation of ethanol occurred during the centrifugation A portion of the final, clear solution was immediately neutralised with barium carbonate, concentrated, and shown (paper chromatography, irrigant *A* and spray *A*) to contain only traces of glucose, xylose, galactose, mannose, and arabinose.

The precipitates **1** and **2** were each dissolved immediately in 300 ml of 0 05M potassium hydroxide, the solutions were acidified (pH 2) with sulphuric acid, and each was poured into 1 liter of ethanol The resulting precipitates were washed several times with ethanol and dried over calcium chloride Average yields from several such fractionations were as follows. **1**, 10 2 g, $[\alpha]_D^{25}$ −37 5° ±0 5° (*c* 2, water), (**2**) 13 8 g, $[\alpha]_D^{25}$ −40 6° ±0 3° (*c* 2, water)

Purification of hemicellulose Fraction **1** — The general procedure for purification of fractions **1** and **2** consisted of a preliminary, small-scale, gradient fractionation, in which the relative amounts of precipitates were determined by optical rota-

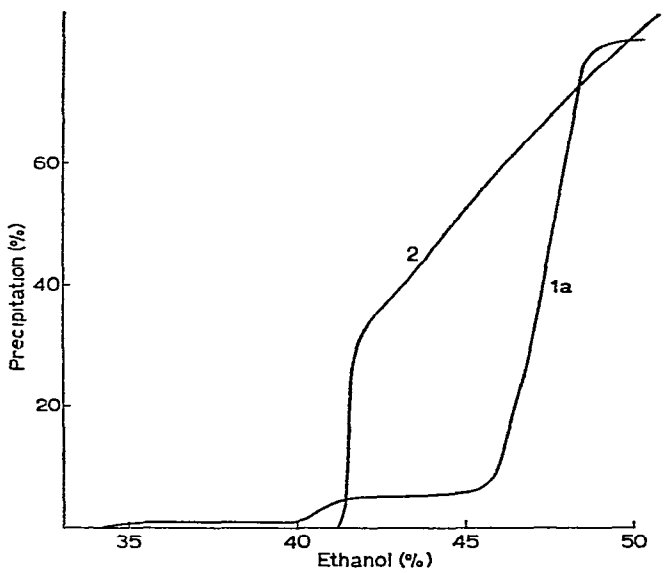

Fig 2 Re-precipitation of hemicellulose fractions **1a**, from 0 1M potassium hydroxide (pH 9), **2**, from hydrochloric acid (pH 2)

tion (Fig. 2), followed by a large-scale, gradient precipitation to collect the material which separated at the point of inflection of the precipitation curve Sub-fractions were tested for homogeneity by electrophoresis in borate buffer.

A solution of 9 095 g of fraction **1** in 530 ml of water was adjusted to pH 2 with 10 ml of M hydrochloric acid and precipitated by gradual addition of ethanol at 25° The portion precipitating between 35 and 38% ethanol was collected, washed with ethanol, and dried over calcium chloride (**1a**, 3 447 g) Electrophoresis showed two components, and further re-fractionation from acidic solution was abandoned because of the formation of stable gels which made the complete removal of supernatant liquors difficult A solution of 2 865 g of fraction **1a** in 376 ml of 0 1M potassium hydroxide was next adjusted to pH 9 with 24 ml of M hydrochloric acid and precipitated by gradual addition of ethanol at 25°. The fraction precipitating between 45 and 49% ethanol was collected, washed successively with 55% ethanol, 75% ethanol containing 5% of acetic acid, 75% ethanol, and then several times with absolute ethanol, and dried over calcium chloride (**1b**, 1 47 g) Electrophoresis showed a preponderant, single component with a trace impurity. After re-fractionation of 1 425 g of **1b** at pH 9 as described above, and collecting between 46 and 48% ethanol, a fraction (**1c**, 0 882 g, representing 1 2% of the crude hemicellulose) was obtained which gave only a single peak on electrophoresis and showed the properties recorded in Table I

Purification of hemicellulose Fraction **2** — A solution of 6 582 g of fraction **2** in 393.5 ml of water was adjusted to pH 2 with 6 5 ml of M hydrochloric acid and then precipitated by gradual addition of ethanol at 25° The fraction (**2a**) precipitating between 41 0 and 41 9% ethanol was collected, washed with ethanol, and dried over calcium chloride, yield 2 144 g, representing 4 5% of the crude hemicellulose Electrophoresis showed an extremely sharp, single peak The properties of this fraction are recorded in Table I Further quantities were prepared by repetition of the fractionation

Methylation analysis of Fraction **2a**. — Fraction **2a** (22 0 g) was methylated first with potassium hydroxide and methyl sulphate, and then with methyl iodide and silver oxide in *N*,*N*-dimethylformamide. The final product was purified by fractional precipitation from chloroform solution with hexane, and the fraction (14 g) which separated between 70–78% of hexane was used for further study (Found ash, 0 0; OCH_3, 38 8%), $[\alpha]_D^{25}$ −49 8° (*c* 2, chloroform) A pliable film was readily cast from chloroform solution and showed no i r. absorption at ~3,500 cm^{-1}.

The methylation product was hydrolysed with hydrochloric acid in methanol, followed by aqueous hydrochloric acid, then neutralised with silver carbonate, and passed successively through columns of Amberlite IR-120(H^+) and IRA-400(CO_3^{2-}) resins The eluate, on evaporation, yielded the neutral methyl sugars, *N* (4.83 g) The resin carbonate was eluted with 50 ml of 0 5M sulphuric acid, and the eluate and washings were neutralised with barium carbonate, filtered, passed through 25 ml of Amberlite IR-120(H^+) resin, and evaporated to dryness at 25° to yield the acidic products as a pale-yellow syrup *A* (1 375 g)

Analysis of the neutral fraction N. — A 2 26-g portion of the syrup *N* from the preceding experiment was separated by chromatography (irrigant *B*) on eight sheets (46 × 57 cm) of Whatman 3MM paper. Eluates (95% ethanol) from the relevant zones were evaporated to dryness, the residues re-dissolved in ethanol, and the solutions filtered and again evaporated to dryness The following fractions were obtained, each being examined by paper chromatography, paper electrophoresis, and demethylation with hydriodic acid[9].

(*1*) Xylose (15 mg), M_G 1 00 This was converted into the dibenzylidene dimethyl acetal[13], m p 209–210° alone or in admixture with the authentic D-xylose derivative

(*2*) A mixture (0 193 g) of 2-*O*-methyl-D-xylose and 3-*O*-methyl-D-xylose in approximately equal proportions; R_G 0 24 (solvent *B*), M_G 0 31 and 0 71 with a trace of unidentified component of M_G 0 59 Hydriodic acid treatment gave only xylose A solution of 0 180 g of this mixture in 10 ml of water was shaken with 2 ml of Amberlite IRA-400(borate) resin[14] at room temperature for 1 h, and then filtered The filtrate was evaporated to 0 100 g of colourless syrup, showing mainly M_G 0 31 on paper electrophoresis The syrup crystallised slowly when seeded with authentic 2-*O*-methyl-D-xylose. When recrystallised from ethanol, the product showed m p 130–132°, and the X-ray powder diffraction pattern was identical with that given by authentic 2-*O*-methyl-D-xylose The borate resin was eluted with 50 ml of saturated, aqueous boric acid, and the eluate was evaporated to dryness. Methanol was repeatedly distilled from the residue to remove boric acid, and the final residue (70 mg) was mainly 3-*O*-methyl-D-xylose (M_G 0 71) with small proportions of 2-*O*-methyl-D-xylose (M_G 0 31) and an unidentified substance (M_G 0 59). The syrup crystallised when seeded, and the product, when recrystallised from ethanol, showed m p 90–95°. The X-ray powder diffraction pattern was identical with that of authentic 3-*O*-methyl-D-xylose.

(*3*) 2,3-Di-*O*-methyl-D-xylose (1 307 g), $[\alpha]_D^{25}$ +21 2° (*c* 2, water), R_G 0 66 (solvent *B*), M_G 0, which yielded only D-xylose on demethylation with hydriodic acid. Part of the syrup was treated with aniline in the usual manner, and the product, when recrystallised from ethyl acetate–hexane, showed m p 123–125°. The X-ray powder diffraction pattern was identical with that given by authentic 2,3-di-*O*-methyl-*N*-phenyl-D-glucosylamine

(*4*) A mixture of 2,3,4-tri-*O*-methyl-D-xylose and 2,3,5-tri-*O*-methyl-L-arabinose (0 305 g) M_G 0, giving two overlapping spots, R_G 0 99 and R_G 1 02 (solvent *B*) Demethylation with hydriodic acid yielded arabinose and xylose, with the former predominating A portion (0 180 g) of the syrup was re-chromatographed on two sheets (46 × 57 cm) of Whatman 3MM paper, using a "wick" of Whatman No 52 paper, solvent *B*, and spray *B* Almost complete separation of the two zones was indicated by guide strips, and they were excised, without leaving an intermediate zone, and eluted as described above 2,3,4-Tri-*O*-methyl-D-xylose (32 mg), obtained in this way, was a syrup which crystallised slowly and, when recrystallised from ether–hexane, showed m p and m m p 87–88°. The X-ray powder diffraction pattern was identical with that of an authentic sample 2,3,5-Tri-*O*-methyl-L-arabinose was

obtained as a colourless syrup (85 mg) which was converted into 2,3,5-tri-*O*-methyl-L-arabinonamide in the usual manner The product, when recrystallised from acetone–hexane, had m p. and m m p 136–137°. The X-ray powder diffraction pattern was identical with that of an authentic sample

Analysis of the acidic fraction A — A portion (1 360 g) of fraction *A* was boiled under reflux for 4 h with 50 ml of 5% methanolic hydrochloric acid The solution was neutralised with silver carbonate, filtered, and evaporated to dryness The syrupy residue was reduced[15] with lithium aluminium hydride in ether and yielded the reduction product as a colourless syrup (1 058 g) This was hydrolysed with 0.5M sulphuric acid for 4 h at 100° to yield a pale-yellow syrup (1 082 g), a portion (0 660 g) of which was fractionated by paper chromatography as described above The following fractions were obtained

(*1*) Xylose (50 mg), M_G 1 00, which was converted into the dibenzylidene dimethyl acetal, m p 210–211° alone or in admixture with the authentic D-xylose derivative

(*2*) Mainly 3-*O*-methyl-D-xylose (0 114 g), R_G 0 28 (elongated spot; solvent *B*), M_G 0 72 with an unidentified impurity of M_G 0 61 Demethylation with hydriodic acid yielded xylose The syrup crystallised slowly and the crystals, m p 91–95°, gave an X-ray powder diffraction pattern identical with that of 3-*O*-methyl-D-xylose

(*3*) 2,3,4-Tri-*O*-methyl-D-glucose (0 292 g), R_G 0 79 (solvent *B*), probably containing a trace of 2,3-di-*O*-methyl-D-xylose (R_G 0 69) Paper electrophoresis showed M_G 0 only Demethylation with hydriodic acid yielded glucose and a trace of xylose A portion of the syrup was converted into 2,3,4-tri-*O*-methyl-*N*-*p*-nitrophenyl-D-glucosylamine[16], m p and mixed m p 223–225° The X-ray powder diffraction pattern was identical with that of an authentic sample kindly supplied by Dr J W Van Cleve

REFERENCES

1 L E Wise and E K Ratcliff, *Anal Chem*, 19 (1947) 459
2 A F Anthis, *Tappi*, 39 (1956) 401
3 R L Whistler and G N Richards, *J Amer Chem Soc*, 80 (1958) 4888
4 R L Whistler, J Bachrach, and D R. Bowman, *Arch Biochem Biophys*, 19 (1948) 25
5 J D Blake and G N Richards, *Carbohyd Res*, 17 (1971) 253
6 T. E Timell, *Advan Carbohyd Chem*, 20 (1965) 433
7 J K N Jones, E Merler, and L E Wise, *Can J Chem*, 35 (1957) 634
8 S M Partridge, *Nature (London)*, 164 (1949) 443
9 L Hough, J K N Jones, and W H Wadman, *J Chem Soc*, (1950) 1702
10 A B Foster, *Chem. Ind (London)*, (1952) 828
11 D H Northcote, *Biochem J*, 58 (1954) 353
12 R L Whistler and G E Lauterbach, *Arch Biochem Biophys*, 77 (1958) 62
13 L E Wise and E K Ratcliff, *Anal Chem*, 19 (1947) 691
14 M V Lock and G N Richards, *J Chem Soc*, (1955) 3024
15 R L Whistler, H E Conrad, and L Hough, *J Amer Chem Soc*, 76 (1954) 1668
16 J W Van Cleve, W C Schaefer, and C E Rist, *J Amer Chem Soc*, 78 (1956) 443