[CONTRIBUTION FROM THE OLYMPIC RESEARCH DIVISION, RAYONIER, INCORPORATED]

The Nature of the Hemicelluloses Associated with Wood Cellulose from Western Hemlock (Tsuga heterophylla)[1,2]

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Two distinct types of hemicellulosic material have been obtained by an alkaline extraction of a wood cellulose produced from western hemlock by the sulfite process. The principal constituent was found to be a glucomannan in which the predominant anhydro unit was D-mannose. The remaining material was found to be a mixture of polysaccharides composed of D-glucose, D-mannose, D-xylose and 4-*O*-methyl-D-glucuronic acid. Fractionation led to the isolation of xylan polyuronides contaminated with small amounts of D-glucose and D-mannose. The general physicochemical properties of these two types of hemicellulose are discussed.

The hemicelluloses, found universally in nature, are mainly associated with cellulose in the cell walls of plants. They rank second to cellulose itself as the most abundant naturally occurring organic material.

This paper is concerned with the isolation, physicochemical properties and preliminary constitutional studies of the hemicelluloses associated with wood cellulose produced from western hemlock by the sulfite process.

The most widely distributed hemicelluloses are those in which the main building unit is anhydro D-xylose. Some hemicelluloses of this type when highly purified are composed of this sugar only and are classified as true xylans (*i.e.*, esparto grass xylan).[3] More frequently, however, uronic acids are found in association with this type of polysaccharide, forming a class of compounds known as xylan polyuronides.[4] The pentose L-arabinose is also often found as an integral part of these materials giving rise to an arabo-glucurono-xylan of a highly branched nature.[5,6]

The classical studies of O'Dwyer,[7] Anderson,[8] and Haworth[9] as well as the more recent studies of numerous other investigators have shown that the existence of xylan polyuronides is much more universal than heretofore realized.[10-17]

In most cases these polyuronides give rise to relatively large amounts of aldobiuronic and aldotriuronic acid upon hydrolysis, and in only a few instances is the uronic acid isolated in good yields under the normal conditions of hydrolysis.[18] The uronic acid residues that have been isolated from these materials by prolonged and severe hydrolysis are D-glucuronic acid, D-galacturonic acid, 4-*O*-methyl-D-glucuronic acid and more recently, 3-*O*-methyl-D-glucuronic acid.[15]

In addition to the xylan polyuronides associated with wood cellulose there occurs a group of polysaccharides termed cellulosans.[4] In the case of the softwoods these constitute a large percentage of the non-cellulosic carbohydrates. Previously it has been shown that hydrolysis of crude hemicellulose extracts from certain softwoods led to the isolation of D-mannose.[19] Hess and Ludtke,[20] and later Husemann,[21] using spruce sulfite pulp and spruce holocellulose, respectively, reported the isolation of a mannan. Unfortunately these workers reported no quantitative data and thus it was impossible to tell whether or not the materials were pure mannans. Rotational data and X-ray patterns were very similar to those obtained from Mannan-A of ivory nuts, which is composed predominantly of anhydro D-mannose units.[22] The fact that the techniques of chromatography were not available to these investigators makes it seem probable that the presence of other sugars, such as glucose, galactose, xylose, arabinose or rhamnose as well as uronic acids might have been overlooked, especially if they were present in small amounts.

Paper partition chromatographic studies on the acetolysate of a potassium hydroxide extracted holocellulose from a slash pine have shown the presence of a disaccharide composed of glucose and mannose.[23] More recently the isolation of a polysaccharide from Sitka spruce has been reported which appears to contain both glucose and mannose in approximately equal amounts.[24]

In this Laboratory two distinct types of hemicellulosic material have been obtained by an extraction with 18.5% sodium hydroxide of wood cellulose produced from western hemlock by the sulfite process. The extract was neutralized with glacial acetic acid and the resulting insoluble material removed by filtration. This material was found to be a glucomannan in which the ratio of D-mannose

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(2) Presented at the 128th meeting of the A.C.S., Minneapolis, Minnesota, September, 1955.

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(15) P. C. Das Gupta and P. B. Sarkar, *Text. Res. J.*, **24**, 1073 (1954).

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(17) R. L. Whistler, H. E. Conrad and L. Hough, *ibid.*, **76**, 1668 (1954).

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(19) G. Bertrand, *Compt. rend.*, **129**, 1025 (1899).

(20) K. Hess and M. Ludtke, *Ann.*, **466**, 18 (1928).

(21) E. Husemann, *J. prakt. Chem.*, **155**, 13 (1940).

(22) G. O. Aspinall, E. L. Hirst, E. G. V. Percival and I. R. Williamson, *J. Chem. Soc.*, 3184 (1953).

(23) J. G. Leech, *Tappi*, **35**, 249 (1952).

(24) Rep. For. Prod. Res. Board., 1953, p. 43.

to D-glucose is approximately 4 to 1 and showed $[\alpha]^{25}D$ −37.8° in 8% sodium hydroxide. A trace of xylose was observed (less than 1%), however when the material was purified *via* Fehling solution, no xylose was noted.

The filtrate was dialyzed, and after removal of additional glucomannan which had precipitated during dialysis, was concentrated under diminished pressure to $^1/_4$, $^1/_6$ and $^1/_{12}$ of its volume. At each step the precipitate which had formed during the concentration was removed by centrifugation. In this manner several mixtures of polysaccharides composed of D-glucose, D-mannose, D-xylose and 4-*O*-methyl-D-glucuronic acid units were obtained. The amount of the latter two substances increased as the concentration continued. The final water-soluble polysaccharides (predominantly xylan polyuronides) were precipitated by the addition of methanol.

Further fractionations were carried out in an effort to obtain polysaccharides which were homogeneous with respect to chain length and composition. In this manner two relatively pure xylan polyuronides were obtained which differed markedly in physical properties and in quantitative analysis. One showed $[\alpha]^{25}D$ −66.1° (0.1 *N* sodium hydroxide) and had an intrinsic viscosity in cupriethylenediamine hydroxide (cuene) of 0.39 and analyzed as follows: D-xylose, 7.0 moles; D-glucose, 0.3 mole; D-mannose, 0.5 mole, 4-*O*-methyl-D-glucuronic acid, 1 mole. The other showed $[\alpha]^{25}D$ −40.6° (0.1 *N* sodium hydroxide) and had an intrinsic viscosity of 0.22 (cuene) and analyzed as follows: D-xylose, 4.6 moles; D-glucose, 0.6 mole, D-mannose, 2.0 moles; 4-*O*-methyl-D-glucuronic acid, 1 mole.

Under ordinary hydrolytic conditions the polyuronides were readily hydrolyzed to their constituent monomeric sugars and 4-*O*-methyl-D-glucuronic acid. It is interesting to note that very little aldobiuronic acid was obtained even when extremely mild conditions of hydrolysis were employed.

Various methods were employed in an effort to obtain a pure xylan polyuronide completely devoid of D-glucose and D-mannose. The best separation was effected with Fehling solution. A xylan polyuronide composed only of D-xylose and 4-*O*-methyl-D-glucuronic acid was isolated in poor yield from a hemicellulose fraction which was obtained from another wood cellulose produced from western hemlock by the sulfite process (see Experimental).

Several attempts were made to separate the glucose–mannose containing polysaccharide into a glucan and a mannan. These procedures included the use of a solution of cuprammonium hydroxide of carefully adjusted *p*H,[20] the Salkowski copper complex method employing Fehling solution,[25] solution in cupriethylenediamine hydroxide followed by an adjustment of the *p*H, fractionation employing borax solutions of varying concentrations,[26] and finally the use of water to obtain soluble and insoluble polysaccharides. None of the above methods was successful in markedly changing the ratio of glucose to mannose in the cellulosan. The water soluble portion had an intrinsic viscosity of 0.24 (cuene) while that of the water-insoluble portion was 0.26. The proximity of these two values to one another indicates a uniformity of chain length. In all of the purification methods the trace of xylose noted in the original polysaccharide was found mainly associated with the soluble fraction.

Periodate oxidation studies have been carried out on these two types of hemicellulose. These data in conjunction with the specific rotations before and after hydrolysis give some indication of the mode of union and type of glycosidic linkage involved. The indications in the case of the polyuronides are that that these polysaccharides are β-linked and joined predominantly by 1→4 - glycosidic bonds. The hemicellulose polyaldehyde resulting from prolonged periodate oxidation, after reduction using sodium borohydride, followed by acid hydrolysis of the polyalcohol, gave a hydrolysate which was shown by chromatographic analysis to contain D-xylose.[27,28] The polyuronide thus appeared from the periodate studies to have a slightly branched structure. The number of anhydro units (av. weight 140) per mole of formic acid produced was found to be 14 to 16 with one fraction giving a value of 8.

Similar studies on the glucomannan and its various fractions indicate that these polysaccharides are β-linked and joined predominantly by 1→4-glycosidic bonds. The glucomannan polyaldehyde, resulting from prolonged periodate oxidation, after reduction using sodium borohydride followed by acid hydrolysis of the polyalcohol, gave a hydrolysate which was shown by chromatographic analysis to contain D-glucose and perhaps a trace of D-mannose.[27,28] The glucomannan thus appeared from periodate studies to have a slightly branched structure. The number of anhydro hexose units per mole of formic acid produced was found to be 9 to 12.

Graded acid hydrolysis on each of the two types of hemicelluloses substantiated the preliminary findings. In the case of the glucomannan, disaccharides composed of mannose, mannose and glucose, and glucose (not cellobiose or maltose) have been identified chromatographically. Similar studies on a xylan polyuronide have led to the chromatographic identification of a series of neutral and acidic oligosaccharides, ranging from xylobiose to xylopentaose and from the aldobiuronic to the aldopentauronic acid, respectively.

The constitution of these hemicelluloses will have to await the isolation and characterization of the methylated sugar residues obtained from the hydrolysis of the fully methylated polysaccharides as well as the identification of the oligosaccharides obtained by graded acid hydrolysis. This work is now in progress.

Experimental[29]

The wood cellulose employed in this study contained 90% α-, 2% β- and 8% γ-cellulose and was produced from western

(25) E. Salkowski, *Z. physiol. Chem.*, **34**, 162 (1902).

(26) H. Deuel, H. Neukom and F. Weber, *Nature*, **161**, 96 (1948).

(27) M. Abdel-Akher, J. K. Hamilton and F. Smith, THIS JOURNAL, **73**, 4691 (1951).

(28) M. Abdel-Akher, J. K. Hamilton, R. Montgomery and F. Smith, *ibid.*, **74**, 4970 (1952).

(29) All m.p.'s corrected and all rotations are calculated on a moisture free basis. The ash content of all polysaccharides was less than 1% and hence was neglected in calculations.

hemlock by the sulfite process. It had a moisture content of 15%.

Isolation of the Hemicelluloses.—The wood cellulose (12.3 kg. in batches of 2.9, 4.7 and 4.7 kg., respectively) was extracted with 18.5% sodium hydroxide at 25° and 16% consistency for 20 minutes. The pooled alkaline extract (approximately 9 liters) was made slightly acidic with glacial acetic acid (about 3 liters were required) and after standing overnight the insoluble material was removed by filtration. This material was designated β. The filtrate was dialyzed against running water for a week. During dialysis an insoluble fraction was obtained which was removed by filtration and designated γ_1. The filtrate was concentrated under diminished pressure using a cyclone evaporator to $^1/_4$ and $^1/_6$ of its volume. At each stage the precipitate which had formed during the concentration was removed by centrifugation. After further concentration to $^1/_{12}$ the original volume, the evaporated solution was poured into 3 volumes of methanol. The different fractions obtained during the concentration were designated γ_2 and γ_3 and the final methanol precipitated fraction was designated γ_4.

The β fraction was redissolved in sodium hydroxide (9–10%) and filtered through a sintered glass filter to remove undissolved cellulose fibers and was reprecipitated by acidification with glacial acetic acid. The precipitate was dialyzed until salt free, filtered, washed thoroughly with water and dried by solvent exchange using methanol, acetone, ether and finally *in vacuo* over phosphorus pentoxide. It is interesting to note that this fraction, although soluble in 10% sodium hydroxide, is only slightly soluble in 10% potassium hydroxide.

The total weight of hemicellulosic material was 439 g. (4.2% based on bone dry weight of starting material). Table I indicates the yield and some of the physical properties of these polysaccharides.

TABLE I

PROPERTIES AND COMPOSITION OF HEMICELLULOSE FRACTIONS

Fraction	Yield, g.	Cuene[a] I.V.	OCH_3, %	$[\alpha]^{22}D$ [b]	Qualitative chromatographic anal.[c,d] Glucose	Mannose	Xylose	Uronic acid
β	220	0.23	0.0	−37.8°	M	L	T	..
γ_1	24	.28	0.3	−52.5°	M	L	S	T
γ_2	47	.24	1.2	−62.5°	M	L	S	T
γ_3	5	.22	1.6	−40.0°	M	L	S	T
γ_4	137	.32	2.7	−54.6°	S	S	L	S

[a] Intrinsic viscosity. [b] c 4.0, in 8% sodium hydroxide. [c] L = large, M = medium, S = small, T = trace. [d] The irrigating solvents employed were: 1-butanol–ethanol–water (4:1:5), ethyl acetate–acetic acid–water (3:1:3), (9:2:2), 1-butanol–pyridine–water (10:3:3), 1-butanol saturated with water–formic acid (20:1), and ethyl acetate–acetic acid–formic acid–water (18:3:1:4). All made up on a volume to volume basis.

In another experiment a xylan polyuronide designated Hemicellulose B (in the O'Dwyer sense) was isolated from a 96% alpha wood cellulose produced from western hemlock by the sulfite process. This fraction was isolated by an extraction of the wood cellulose with 10% sodium hydroxide. The extract was neutralized with glacial acetic acid and poured into ethanol (4 volumes). The precipitated polysaccharide mixture was extracted with 5% sodium hydroxide and the insoluble material removed by filtration. The filtrate was acidified by the addition of glacial acetic acid and allowed to stand overnight. A very small precipitate (a few mg.) was removed by centrifugation and discarded. The acidified supernatant was poured into ethanol (4 volumes) in order to precipitate the polysaccharide. The Hemicellulose B was dried by solvent exchange using methanol, acetone and ethyl ether. Typical physical and chemical properties of this polysaccharide are included in this paper in order to illustrate the similarity in properties between it and the xylan polyuronides isolated using the more concentrated sodium hydroxide solution.

In a third experiment a water-soluble xylan polyuronide, designated X-P, was isolated from a 96% alpha wood cellulose produced from western hemlock by the sulfite process. It was contaminated with water soluble glucose–mannose containing polymers, but was purified by means of Fehling solution to give practically pure xylan polyuronide. Typical physical and chemical properties of this polysaccharide are included in this paper.

Hydrolysis of the Hemicellulose Fractions.—The polysaccharides were hydrolyzed by dissolving them in 72% sulfuric acid (25°) followed by dilution with water to approximately 3% sulfuric acid and immediately heating the solution on a boiling water-bath for 8 hours. The cooled solutions were neutralized using barium carbonate and deionized using a cation-exchange resin (Amberlite I.R.-120), filtered and evaporated under diminished pressure to a sirup

Qualitative Chromatographic Analysis of the Hemicellulose Fractions.—The sirups from the various hydrolysates were dissolved in a small volume of aqueous ethanol and analyzed for their constituent sugars and uronic acids by paper partition chromatographic techniques. The studies showed the presence of substances, the R_f values of which corresponded to glucose, mannose, xylose and 4-*O*-methylglucuronic acid. The qualitative distribution of these sugars and uronic acid is shown in Table I.

Identification of D-Glucose and D-Mannose from the β-Hydrolysate.—The sirup showed $[\alpha]^{25}D$ +21.2° (c 5.8, water) which is in good agreement for a mixture of D-glucose and D-mannose in the ratio of 1 to 4 which shows $[\alpha]D$ +21.7°.

(a) Treatment of a portion of the hydrolysate in water containing a few drops of glacial acetic acid with phenylhydrazine and allowing the reaction mixture to stand for 30 minutes at 10–12° yielded D-mannose phenylhydrazone, m.p. and mixed m.p. 187°.

(b) A sample of the hydrolysate was separated into its two constituent sugars on a cellulose column using an irrigating solvent of ethyl acetate–acetic acid–water (6:3:2) v./v.[30] The glucose fraction was freed from solvent by evaporation under diminished pressure. The sirupy product was treated with *p*-nitroaniline (1.1 moles) and 3.5 ml. of methanol containing a trace of hydrochloric acid (0.15 ml. of concd. hydrochloric acid added to 200 ml. of methanol) following the procedure of Weygand.[31] The reaction mixture yielded crystalline D-glucose-*p*-nitroanilide, m.p. and mixed m.p. 181°.

Alternative Hydrolysis Procedure for the Xylan Polyuronide Fractions.—The polyuronide γ_4 (0.2 g.) was added to a solution of 0.1 N sodium hydroxide (10 ml.) and shaken mechanically overnight to effect solution. A trace of insoluble material (~1%) was removed by centrifugation and the clear solution or suitable aliquots thereof were employed for the determination of optical rotations, equivalent weights and qualitative analysis. The sodium ions were removed from the hemicellulose solution by slurrying with Amberlite IR-120 cation-exchange resin. Sulfuric acid was added until the concentration was 2 N and the solution hydrolyzed for 7 hours on a boiling water-bath. Barium carbonate was added to neutralize the solution and after filtering the barium sulfate the filtrate was shaken with Amberlite IR-120 resin and evaporated under diminished pressure to a sirup which showed $[\alpha]^{25}D$ +28° (c 0.3, water). Upon removal of the uronic acid by anion-exchange resin (Duolite A_4), the sirup from the sugars showed $[\alpha]^{25}D$ +21.0° (c 1.4, water) which is in good agreement for a mixture of D-glucose, D-mannose and D-xylose in the ratio of 0.8, 1.7 to 5.7. The 4-*O*-methyl-D-glucuronic acid from the above showed $[\alpha]^{25}D$ + 85 ± 2° (c 0.3, water). Thus in the molar ratio of 1.0 uronic acid, 5.7 D-xylose, 0.8 D-glucose, 1.7 D-mannose the rotation for the sirup $[\alpha]^{25}D$ +28° agrees very well with the calculated value of $[\alpha]D$ +28°.

Identification of Glucose, Mannose and Xylose.—The mixture of sugars and uronic acid from the hydrolysis of 0.5 g. of polyuronide was separated using sheet chromatography (5 sheets of Whatman No. 3 filter paper) and an irrigating solvent of ethyl acetate–acetic acid–water (3:1:3) v./v. The areas containing these sugars were extracted with water, filtered and the filtrates concentrated under diminished pressure to sirups. Each sugar was rechromatogrammed on a single sheet of Whatman No. 1 filter paper using the same irrigating solvent, eluted and concentrated as above to a sirup. Crystalline D-xylose was obtained, m.p. and mixed m.p. 169° and the dibenzylidene dimethyl acetal of D-xylose

(30) D. A. Durso and W. A. Mueller, paper presented at A.C.S. meeting, Minneapolis, 1955.

(31) F. Weygand, W. Perkow and P. Kuhner, *Ber.*, **84**, 594 (1951).

had m.p. and mixed m.p. 211°.[32] The sirups of the D-mannose and D-glucose were converted to the corresponding *p*-nitroanilides following the procedure of Weygand.[31] The D-mannose-*p*-nitroanilide had m.p. and mixed m.p. of 218° while the D-glucose-*p*-nitroanilide had m.p. and mixed m.p. of 183–184°.

Identification of 4-*O*-Methyl-D-glucuronic Acid.—The original wood cellulose (1 kg.) was suspended in 1 *N* sulfuric acid (8 liters) for 90 hours at room temperature followed by refluxing for 8 hours according to the procedure of Jones and Wise.[33] The suspension was filtered, washed with water (8 liters) and the combined filtrate and washings neutralized with barium carbonate. The precipitate of barium sulfate was filtered and the filtrate slurried with Amberlite IR-120 cation-exchange resins. The solution was concentrated under diminished pressure to 4 liters and passed slowly through a Duolite A_4 anion-exchange column. After thorough washing to remove neutral sugars the resin was resuspended in sulfuric acid (1 *N*) for 24 hours, filtered and washed free of acid. The acidic solution was neutralized with barium carbonate, filtered and the filtrate stripped of cations by passage down an Amberlite IR-120 cation-exchange column. Evaporation under reduced pressure at 40° gave a dark sirup (3.6 g.). Paper partition chromatographic analysis using irrigating solvents of ethyl acetate–acetic acid–water (3:1:3) and (9:2:2) and a *p*-anisidine spray revealed the presence of a red heart shaped spot which corresponded in color, characteristic shape and R_f value to that of an authentic specimen of 4-*O*-methyl-D-glucuronic acid and to the uronic acid present in the xylan polyuronides isolated from the wood cellulose.

The amide of methyl 4-*O*-methyl-D-glucuronoside was prepared according to the procedures of Smith.[34] The methyl ester methyl glycoside of the acid was distilled under high vacuum (0.08 mm. at a bath temp. of 190°) and the distillate treated with methanolic ammonia. The mixture of α- and β-amide glycosides was separated by recrystallization. The α-form had m.p. and mixed m.p. 236° and showed $[\alpha]^{25}D$ +148° (*c* 0.95, water).

Water Fractionation of the Hemicelluloses.—Small scale experiments on 200 mg. of the fractions showed the feasibility of a water fractionation for the separation of the xylan polyuronide from the glucomannan. All of the large scale extractions were carried out in the following manner. The hemicellulose fraction was stirred for 24 hours with 20 times its weight of distilled water. The water-insoluble material was spun down with a centrifuge, and after decantation of the supernatant was washed with a fresh portion of distilled water. It was then dried by solvent exchange using methanol, acetone and ether. The aqueous solutions of the water-soluble portions were concentrated *in vacuo* to a small volume and poured into methanol. The precipitated polysaccharides were dried by solvent exchange as mentioned above.

TABLE II

ANALYTICAL DATA ON HEMICELLULOSES FROM WATER FRACTIONATION

Fraction	% H_2O sol.[a]	$[\alpha]^{25}D$ [b]	Cuene I.V.	D-Glucose[c]	D-Mannose	D-Xylose	Uronic acid[d]
β	34	−37.8	0.23	1	3.8	0.04	0
β-H_2O sol.		−36.6	.24	1	4.3	Trace	0
β-H_2O insol.		−37.8	.26	1	4.7	Trace	0
β-F[e]		−36.6	..	1	3.8	0	0
γ_1	22.5	−43.5	.28	1	3.1	0.6	0
γ_1 H_2O sol.		−53.6	.26	1	1.1	3.6	Trace
γ_1 H_2O insol.		−42.7	.25	1	3.7	0	0
γ_2	19.2	−42.8	.24	1	5.2	0.3	0
γ_2 H_2O sol.		−46.3	.28	1	2.5	1.2	Trace
γ_2 H_2O insol.		−42.2	.25	1	3.7	0	0
γ_4	93.7	−54.6	.32	1	2.1	7.1	1.25
γ_4 H_2O insol.		−40.5	.22	1	3.4	0	0

[a] Calculated from water-insoluble portion. [b] All rotations carried out in 8% sodium hydroxide at a concentration of 4% except in case of γ_4, in which case the solvent was 0.1 *N* sodium hydroxide. [c] Expressed as molar ratios. [d] 4-*O*-Methyl-D-glucuronic acid. [e] β, Fehling purified.

(32) L. J. Breddy and J. K. N. Jones, *J. Chem. Soc.*, 2750 (1952).
(33) J. K. N. Jones and L. E. Wise, *ibid.*, 2750 (1952).
(34) F. Smith, *ibid.*, 2646 (1951).

An exception to the above procedure was the β-fraction, which was extracted with a much larger quantity of water than the γ-fractions in order to insure complete removal of the water soluble material. In all cases the recoveries were greater than 90 per cent. The amount of water-soluble material in the main fractions expressed as per cent. is included in Table II.

Treatment of the β-Fraction with Cuprammonium Hydroxide.—The β-fraction (1 g.) was mixed with copper hydroxide (1 g.) and to this was added concd. ammonium hydroxide (100 ml.). The flask was stoppered and mechanically shaken for 48 hours at 25° in the dark. The solution was filtered to remove traces of insoluble material and sodium hydroxide was added to the filtrate until the concentration was 0.2 *N* with respect to the sodium hydroxide. This resulted in the formation of a large gelatinous precipitate which was isolated by centrifugation. The precipitate was dissolved in water and decomposed by the addition of acetic acid (50%) keeping the reaction mixture cool. The glucomannan was precipitated from the solution by the addition of methanol (2 volumes). The precipitated polysaccharide was washed thoroughly with methanol containing 5% acetic acid, methanol, acetone, ether and dried *in vacuo*. The yield, specific rotation and qualitative analytical data are shown in Table III.

TABLE III

TREATMENT OF β-FRACTION WITH VARIOUS REAGENTS TO ESTABLISH HOMOGENEITY[a]

Treatment	$[\alpha]^{25}D$	Yield, %
Cuprammonium hydroxide	−36.8°	66
Fehling solution	−36.6°	75
Cupriethylenediamine hydroxide	−35.6°	67
Original β-fraction	−37.8°	..

[a] All the above fractions showed the same ratio of glucose to mannose by qualitative paper partition chromatographic analysis as the original. All rotations were determined in 8% sodium hydroxide.

Treatment of the β-Fraction with Fehling Solution.—Two grams of the β-fraction was dissolved in 8% sodium hydroxide (100 ml.) and to this was added an equal volume of freshly prepared Fehling solution resulting in the formation of a large gelatinous precipitate. This was isolated by centrifugation and washed three times with a solution of the same composition as that which resulted in the precipitation. The insoluble copper complex was suspended in water and placed in an ice-bath and cold hydrochloric acid (2 *N*) was added carefully with stirring to destroy the complex. Upon acidification, the solution turned from deep blue to light green and was centrifuged to remove a slight trace of insoluble material. Acetone (3 volumes) was added to the supernatant and a finely divided white precipitate was obtained. This was washed successively with acetone/water 60/40 containing 2% acetic acid, acetone, ether and dried *in vacuo*. The results are shown in Table III.

Treatment of the β-Fraction with Cupriethylenediamine Hydroxide.—To this fraction (1 g.) was added 1 *M* cuene solution (100 ml.) and the mixture stoppered and mechanically shaken for 16 hours at room temperature. The solution was acidified with glacial acetic acid whereupon a white precipitate was obtained which was isolated by centrifugation. The precipitated polysaccharide was washed with methanol containing 5% acetic acid, methanol, acetone, ether and finally dried *in vacuo*. The results are shown in Table III.

Attempted Fractionation of a Glucomannan with Borax Solutions.—Commercial borax was recrystallized from distilled water and a saturated solution of the salt prepared at 25°. This solution contained 2.15 g. of $Na_2B_4O_7$ per 100 ml. water.[35] Aliquots of the saturated solution were diluted with varying quantities of distilled water to obtain solutions of different degrees of saturation. The *p*H of all solutions was between 9 and 9.3.

The water-insoluble β-fraction (500 mg.) was weighed into each of six tared centrifuge cones and shaken for 24 hours with 20 ml. of the appropriate borax solution. The

(35) A. Seidell, "Solubilities of Inorganic and Organic Compounds," D. Van Nostrand Co., New York, N. Y., 2nd. Ed., p. 629.

insoluble portion was centrifuged down, washed with distilled water (2 ml.) and with a large quantity of methanol to remove borax. Following solvent exchange through acetone and ether the samples were dried *in vacuo* and from their weights the quantity of glucomannan which dissolved in each concentration of borax was calculated. The aqueous supernatants were dialyzed, concentrated and the borax soluble portions dried as above. Where enough material was available the optical rotations were obtained. The soluble and insoluble portions were hydrolyzed to their component sugars with sulfuric acid and the hydrolysates examined by qualitative paper chromatography. In no case did a significant difference appear between any of the fractions or between them and the starting material. The results of this study are shown in Table IV.

TABLE IV

SOLUBILITY OF β-WATER INSOLUBLE FRACTION IN BORAX SOLUTIONS[a]

Borax soln. % satd.	% β-Insol. sol. in borax soln.	Fraction	$[\alpha]^{25}D$
5	38	Soluble	.....
		Insoluble	−35.5°
10	48.5	Soluble	−34.1°
		Insoluble	−35.1°
15	58.5	Soluble	.....
		Insoluble	−34.0°
20	65.3	Soluble	−37.5°
		Insoluble	−32.5°
45	75.9	Soluble	−35.9°
		Insoluble	.....
75	82.9	Soluble	−37.3°

[a] The $[\alpha]^{25}D$ of β-water insoluble fraction in 8% NaOH was −37.8°. All rotations were determined in 8% sodium hydroxide.

material are included in Table V under the designation X-P.

Dilute Sulfuric Acid Fractionation of γ_4.—During small scale experiments on the dilute acid solubility of the original fractions it was observed that the polysaccharides were about one-half as soluble in 0.5 *N* sulfuric acid as they were in water. In all cases but one the use of acid rather than water led to no apparent advantage. The water solubility of γ_4 was 93.7%, leaving as an insoluble residue a fraction composed of glucose and mannose. The solubility of γ_4 in 0.5 *N* sulfuric acid was 43.8% leaving an insoluble residue composed almost entirely of xylose and uronic acid and containing very little glucose and mannose. The weighted intrinsic viscosities of the acid-soluble and -insoluble fractions showed that little or no degradation had taken place during the acid extraction. In order to obtain a fairly pure xylan polyuronide the following procedure was followed.

The γ_4-water soluble fraction (104 g.) was pulverized in a mortar and divided into 12 equal parts. Each portion (8.6 g.) was placed in a 250-ml. centrifuge cup with 167 ml. of sulfuric acid (0.5 *N*), and the mixtures were shaken on a Burrell shaker for 24 hours at room temperature. The mixture was centrifuged, the supernatant liquid decanted and the residual solid shaken with a fresh 100 ml. of 0.5 *N* sulfuric acid for 2 hours. After centrifugation and decantation the insoluble polysaccharide was washed with methanol containing a small amount of pyridine, methanol, acetone, ether and finally dried *in vacuo* over P_2O_5. It was found to be composed chiefly of xylose and uronic acid and yielded only very small quantities of glucose and mannose upon acid hydrolysis. It was designated γ_4-water soluble, sulfuric acid insoluble.

The aqueous supernatants from the extraction were combined and neutralized by the slow addition of a solution of 1.65 equivalents of sodium hydroxide in 300 ml. of water (1.60 equivalents of sulfuric acid were used for the extraction). External cooling and vigorous stirring were carried out during the neutralization, and when completed, a small excess of glacial acetic acid was added to make the solution very slightly acidic. The solution (3.3 liters) was concentrated under diminished pressure to one liter, which was

TABLE V

PROPERTIES OF VARIOUS XYLAN POLYURONIDE FRACTIONS

Fraction	Wt., g.	Cuene I.V.	$[\alpha]^{25}D$[a]	OMe, %	Equiv. wt., g.	Quant. chromatographic anal.[b] U. acid[c]	Xylose	Glucose	Mannose
γ_4	....	0.32	−54.6°	2.7	1200	1	5.7	0.8	1.7
γ_4-H_2O sol.	104	.33	....	..	..	S[d]	L	S	S
γ_4-H_2O sol.-H_2SO_4 insol.	62.7	.39	−66.1°	2.6	1300	1	7.1	0.3	0.5
γ_4-H_2O sol.-H_2SO_4 sol.	21.7	.22	−40.6°	2.7	1200	1	4.6	0.6	2.0
Hemi B	....	.22	−53.5°	2.2	1400	1	7.2	1.1	2.0
X-P	19.50	.16	−55.5°	2.2	..	T	3.9	1.0	0.5
X-P Fehling pptd.	1.78	.17	−49.2°	..	..	T	2.3	1.0	2.1
X-P Fehling sol.	7.20	.23	−61.0°	2.4	900	1	5.5	0.1	0

[a] 0.1 *N* sodium hydroxide. [b] Expressed as molar ratios. [c] 4-*O*-Methyl-D-glucuronic acid. [d] See Table I, note *c*.

Purification of a Xylan Polyuronide with Fehling Solution.—A water-soluble xylan polyuronide (designated X-P) was obtained from a different wood cellulose prepared from western hemlock by the sulfite process. It was contaminated with water soluble glucose–mannose containing polymers. The material was purified in the following manner.

A solution of the xylan polyuronide (19.50 g.) in water (500 ml.) was filtered and added to freshly prepared Fehling solution (500 ml.). A precipitate was formed immediately and after 2 hours the mixture was filtered through a coarse sintered glass filter and then through a medium sintered glass filter. The filtrations were slow, but after the second filtration a brilliantly clear dark blue filtrate was obtained. The precipitate remaining on the filters was dissolved in water and acidified with glacial acetic acid. The filtrate was also acidified with glacial acetic acid. Both solutions were concentrated *in vacuo* to 200 ml. and dialyzed for three weeks to remove copper salts and excess acetic acid. The dialyzed solutions were treated with cation-exchange resin, Amberlite IR-120 (H^+) to remove the last traces of cupric ion. The solutions were concentrated under diminished pressure to a small volume and poured into methanol. The precipitated polysaccharides were dried by solvent exchange using methanol, acetone, ether and finally over P_2O_5. The analytical properties of the subfractions and the original dialyzed for two weeks against running water. The dialysate was concentrated *in vacuo* to 200 ml. and poured into methanol (3.5 liters) with rapid stirring to precipitate the polysaccharide. This was dried by solvent exchange and finally *in vacuo* over P_2O_5. Upon hydrolysis it yielded mainly xylose and uronic acid with small amounts of glucose and mannose and was designated γ_4-water soluble, sulfuric acid soluble. Table V gives the properties of the starting material and subfractions.

Quantitative Analysis of a Typical Glucomannan.[35a]—The β-fraction (900 mg.) was dissolved in 72% sulfuric acid (9 ml.), the solution diluted to 100 ml. and heated on a boiling water-bath for 12 hours. After neutralization with barium carbonate, removal of insoluble barium salts and deionization with a mixture of Amberlite IR-120 (H^+) cation resin and Duolite A_4 anion resin, the sugar solution was evaporated to dryness under diminished pressure in a tared flask. A qualitative chromatogram was carried out at this stage to make sure no oligosaccharides were present. The sirup (0.83 g.) was dissolved in 60 ml. of methanol–water 1 to 1 and an aliquot of this solution (0.048 ml.) was accurately transferred with a micropipet to a sheet of (7″ × 22 1/8″)

(35a) M. Dubois, K. Gillis, J. K. Hamilton, P. A. Rebers and F. Smith, *Nature*, **168**, 167(1951); *Anal. Chem.*, **28**, 350 (1956).

Whatman No. 1 filter paper. A drop of solution containing glucose, mannose and xylose was also put on the starting line in the marginal strips 3/4" from either edge of the chromatogram (halfway between the band of sugars to be analyzed and the edge of the paper). The chromatogram was developed (descending technique) for 48 hours using ethyl acetate–acetic acid–water 3:1:3 v./v. as the irrigating solvent. The chromatogram was dried in air, the marginal strips cut off and the free sugars located by spraying with *p*-anisidine. The chromatogram was reassembled and those areas of the central unsprayed portion containing the component sugars were cut out and extracted separately with water. The sugars (glucose, mannose and xylose) were eluted with 25, 60 and 10 ml. of water, respectively, and the eluates filtered through glass wool. In triplicate determinations 2 ml. of each extract was mixed with an aqueous 5% phenol solution (1 ml.) followed by concentrated sulfuric acid (5 ml.). The absorbance of the orange-yellow color was determined using a Spectronic 20 spectrophotometer at a wave length of 490 mμ for the hexoses and 480 mμ for the pentose.

A blank experiment was carried out on a sheet of filter paper irrigated in the same way as the chromatogram in order to correct for trace amounts of carbohydrates extracted from the filter paper that would otherwise affect the results.

By reference to standard curves, the relative amounts of the sugars in the original sample were calculated. The results, expressed as molar ratios, are included in Table II.

Quantitative Analysis of a Typical Xylan Polyuronide.—The procedure followed was identical to that employed for the glucomannan with the exception that the quantity of 4-*O*-methyl-D-glucuronic acid was not determined spectrophotometrically. The hydrolysates were checked chromatographically to make sure there were no oligosaccharides present. The quantitative estimation of the uronic acid was determined as the average of the equivalent weight per carboxyl group, the average of the equivalent weight per methoxyl group, and gravimetrically by the amount of material not determined as glucose, mannose or xylose. The results are shown in Table VI.

TABLE VI

EQUIVALENT WEIGHT OF POLYURONIDES AND THEIR SUBFRACTIONS

Fraction	Carboxyl[a]	Methoxyl[b]	Residue[c]
γ_4	1200	1200	1100
γ_4-H_2O insol.	1300	1200	1200
γ_4-H_2O sol.	1200	1300	1100
Hemicellulose B	1400	1400	1300

[a] Material completely deionized and titrated to neutrality using phenolphthalein as indicator. [b] Standard Zeisel. [c] The amount of carbohydrate not determined in the quantitative analysis for glucose, mannose and xylose expressed as equivalent weight, by assuming it arises from 4-*O*-methyl-D-glucuronic acid.

Oxidation of the β-Fraction and Subfractions with Sodium Periodate.—The glucomannan (0.527 g.) was added to water (100 ml.) and allowed to stand with occasional shaking for 6 hours at room temperature in order to become swollen. To this suspension 0.4 *M* sodium periodate (50 ml.) was added and the volume quickly adjusted to 250 ml. This gave a final sodium periodate concentration of 0.08 *N*. The reaction mixture was placed in a refrigerator at 2–4°. At suitable intervals an aliquot was removed, treated with excess ethylene glycol and the formic acid titrated with 0.0205 *N* sodium hydroxide using phenolphthalein as the indicator. An appropriate blank determination was also carried out. The periodate consumption was determined at the same time on another sample by the usual arsenite method[36] using 0.0100 *N* iodine for the back titration. The reaction was allowed to proceed for 18 days although the consumption was constant after 9 days. The formic acid production was essentially constant after 24 hours although it rose very slightly and continuously thereafter. Similar experiments were carried out on the water soluble and water insoluble β-fractions. The results are shown in Table VII.

The reaction mixtures were removed from the refrigerator and ethylene glycol (1 ml.) was added to each to destroy the excess periodate. The samples were shaken and allowed to stand for two hours at room temperature and then dialyzed against running water for two weeks. The dialyzed solutions were concentrated to a small volume and acetone (7 to 8 volumes) added. Only in the case of the water insoluble fraction was any precipitate obtained. Apparently the polyaldehydes diffused out of the dialysis casings.

The polyaldehyde from the β-water insoluble fraction was dissolved in 72% sulfuric acid and the solution diluted to 5% with respect to this acid and hydrolyzed on a boiling water-bath for 8 hours. The solution was neutralized with barium carbonate, filtered and concentrated under diminished pressure to a sirup. This was analyzed for unoxidized sugars using paper chromatography with an irrigating solvent of ethyl acetate–acetic acid–water (3:1:3). A spot corresponding in R_f to that of glucose was noted.

In another experiment the water-insoluble glucomannan was oxidized with sodium periodate under conditions similar to those described above. The excess periodate was converted to iodate by the addition of ethylene glycol. The addition of barium acetate caused the precipitation of the insoluble barium iodate which was removed by filtration. The solution was concentrated to a small volume and sodium borohydride (100 mg.) was added and the reaction stirred and allowed to stand for 3 hours. A second portion of sodium borohydride (75 mg.) was added and the reaction allowed to stand overnight. The solution was acidified with glacial acetic acid and concentrated hydrochloric acid added to obtain an acid concentration of 1 *N*. The solution was hydrolyzed on a boiling water-bath for 6 hours followed by deionization and neutralization by passage down a cation-exchange column of Amberlite IR-120 and an anion-exchange column of Duolite A_4, respectively. The neutral solution was concentrated under diminished pressure to a sirup and analyzed by paper chromatography. The results showed the presence of a small amount of unoxidized glucose, a large spot was noted corresponding to erythritol and a small spot for glycerol. The irrigating solvents employed were ethyl acetate–acetic acid–water (3:1:3) and (9:2:2) and 1-butanol–ethanol–water (3:2:1). The presence of unoxidized mannose was questionable. The results are shown in Table VII.

TABLE VII

PERIODATE OXIDATION OF THE GLUCOMANNAN AND XYLAN POLYURONIDE AND THEIR SUBFRACTIONS

Fraction	Moles periodate consumed/ anhydro unit	No. anhydro units giving 1 mole HCOOH
β	1.03	9
β-H_2O sol.	1.12	8
β-H_2O insol.	1.01	11
	1.03	12
γ_4	0.74	..
γ_4-H_2O sol.-H_2SO_4 sol.	.91	8
γ_4-H_2O sol.-H_2SO_4 insol.	.77	14
Hemicellulose B	.75	16

Oxidation of the Xylan Polyuronide with Sodium Periodate.—A typical xylan polyuronide (γ_4-water soluble–sulfuric acid insoluble) was treated in the following manner. The polysaccharide (580 mg.) was dissolved in sodium hydroxide (50 ml. of 0.1 *N*) and was centrifuged to remove a small amount of insoluble material (less than 1%). The cations were removed by slurrying the solution with Amberlite IR-120 cation-exchange resin. The solution was then titrated to neutrality using phenol red as indicator. To the above neutral solution sodium periodate (125 ml. of 0.2 *N*) was added and the entire solution made up to 250 ml. in a volumetric flask. All solutions were at 10° at the time of mixing and the final solution was maintained at this temperature in the dark. At suitable intervals an aliquot was removed, treated with excess ethylene glycol and the formic acid titrated with 0.0205 *N* sodium hydroxide using phenol red as the indicator. The periodate consumption was determined at the same time on another aliquot by the standard arsenite method of Fleury and Lange[36] using 0.0100 *N* iodine. The formic acid production was essentially constant after 24 hours although the consumption of periodate did not level off to a constant value for several days. Similar experiments were carried out on the water soluble γ_4,

(36) P. Fleury and J. Lange, *J. Pharm. Chem.*, [8] **17**, 107 (1933).

the water soluble–sulfuric acid soluble γ_4 subfraction and on hemicellulose B. These results are shown in Table VII.

The samples were removed from the 10° bath and were allowed to remain at room temperature (24°) in the dark for 2 months. At the end of this time the periodate consumption was well in excess of 1 mole consumed per mole anhydro sugar unit. The excess periodate was destroyed with ethylene glycol and the solution of the polyaldehyde dialyzed until free of iodate. This solution was concentrated under diminished pressure to a small volume and the polyaldehyde reduced to the corresponding polyalcohol by sodium borohydride (200% excess). The polyalcohol solution was adjusted so that the concentration of sulfuric acid was 1 *N* and the solution was hydrolyzed for 6 hours on a boiling water-bath. The hydrolysate was neutralized with barium carbonate and the excess barium ions removed by slurrying with a portion of Amberlite IR-120 cation-exchange resin. The deionized solution was concentrated *in vacuo* to a sirup and dry methanol was added and removed by vacuum distillation (this procedure was repeated several times) in order to remove the last trace of borates as the highly volatile methyl borate. Similar studies were carried out on the original water soluble γ_4, the sulfuric acid soluble γ_4 subfraction and on hemicellulose B. Paper partition chromatographic analyses in all cases showed the presence of a small amount of unoxidized xylose indicating the presence of a branch in the polysaccharides. The irrigating solvents employed were ethyl acetate–acetic acid–water (3:1:3) and (9:2:2). In the experiment with the latter solvent a check was made for the presence of glycerol and this was shown to be present in large amounts as expected.

Acetylation of the Glucomannan.—The β-fraction (5 g.) containing 8.3% moisture was dispersed in water (15 ml.) in a 250-ml. centrifuge cup and allowed to stand for 16 hours at room temperature. The mixture was flooded with ethanol (10–12 volumes), spun down and the precipitated polysaccharide washed with ethanol (170 ml.). The alcohol wet hemicellulose was transferred with pyridine (150 ml.); acetic anhydride (100 ml.) was added to the mixture and it was stirred and heated on a boiling water-bath for 20 hours under a nitrogen atmosphere. During this time the reaction mixture had turned quite dark and only a small quantity of the glucomannan remained undissolved. The glucomannan acetate was isolated by filtering the reaction mixture through sintered glass and pouring the filtrate into water. The product was isolated by filtration, and dried (yield 7 g. or 86%). The acetyl analysis was 47.5% and the product showed $[\alpha]^{25}D$ −24.2° (*c* 5.0, tetrachloroethane–ethanol 9:1). The theoretical acetyl content of a fully acetylated hexosan of infinite chain length is 44.8%. The intrinsic viscosity of the glucomannan acetate in tetrachloroethane–ethanol 9:1 was found to be 0.21.

Acetylation of a Xylan Polyuronide.—The γ_4-water soluble–sulfuric acid insoluble fraction (5 g.) was acetylated by a similar method to that employed for the glucomannan. The notable difference in the two acetylations was that the polyuronide acetate was insoluble in the acetylation medium and hence the latter was not filtered but was isolated by pouring directly into water. The yield of the acetate was 7.35 g. (90.5% of theoretical), the acetyl content was 40.8% and it showed $[\alpha]^{25}D$ −72.5° (*c* 5.0, tetrachloroethane–ethanol 9:1). The theoretical acetyl content for a fully acetylated pentosan of infinite chain length is 39.8%. The intrinsic viscosity of the acetate in tetrachloroethane–ethanol 9:1 was found to be 0.31.

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SHELTON, WASHINGTON

[CONTRIBUTION FROM THE NORTHERN UTILIZATION RESEARCH BRANCH[1]]

The Preparation, Properties and Structure of the Disaccharide Leucrose

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Leucrose has been prepared from sucrose in 7.9% yield by the action of dextransucrase derived from the bacterium *Leuconostoc mesenteroides* (NRRL B-512F). The disaccharide gave fructose and glucose on hydrolysis and showed a low order of reactivity with hypoiodite. Complete methylation of leucrose phenylosotriazole produced a hepta-*O*-methyl derivative which on hydrolysis with acid gave 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4,6-tri-*O*-methyl-D-*arabino*-hexose phenylosotriazole. The disaccharide was shown to belong to the α-glucosyl series. On the basis of these data the structure of leucrose has been established as 5-*O*-α-D-glucopyranosyl-D-fructose (Fig. 1 in text).

In a preliminary note[2] we reported the isolation of a new disaccharide, leucrose, which was produced as a by-product in the synthesis of dextran from sucrose by the bacterium *Leuconostoc mesenteroides* (NRRL B-512F). In the present paper a method is described for the preparation of this sugar in quantity; in addition, the chemical and physical properties of the disaccharide are summarized and a complete structure proof is presented which shows that leucrose is 5-*O*-α-D-glucopyranosyl-D-fructose.

A study of the factors influencing leucrose formation showed that the best yields could be obtained at high sucrose concentration. The sucrose solution was treated with partially purified dextransucrase for six days, the dextran was removed by alcohol precipitation and the fructose by a yeast fermentation. Crystalline leucrose was obtained directly from the remaining sugars.

Leucrose, which crystallizes as a monohydrate melting at 156–158°, shows slight mutarotation, going from a value of $[\alpha]^{25}D$ −8.2° at 7 minutes to a constant value of −7.6° in less than one hour. It is not readily hydrolyzed by acids which is in conformance with the finding of Wise, *et al.*,[3] that leucrose shows only 19% fructose by their anthrone method under conditions which gave 100% yields of fructose with sucrose, melezitose and raffinose.

Leucrose forms a phenylosazone (m.p. 186–188°) which crystallizes as yellow needles from ethyl ace-

(1) One of the Branches of the Agricultural Research Service, U. S. Department of Agriculture.

(2) F. H. Stodola, H. J. Koepsell and E. S. Sharpe, THIS JOURNAL, **74**, 3202 (1952).

(3) C. S. Wise, R. J. Dimler, H. A. Davis and C. E. Rist, *Anal. Chem.*, **27**, 33 (1955).