Studies on Mannan in Wood Pulp

Part IV. Isolation and Purification of Glucomannan

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Glucomannan was isolated from holocellulose pulp of pine (*Pinus densiflora* Sieb. et Zucc.) using dimethylsulfoxide and aqueous alkali. It was found that there were present also, in the pulp, separate two glucomannans as the possible contaminants. Purification of the glucomannan by extraction with dimethylsulfoxide, the following acetylation and fractionation resulted essentially homogeneous glucomannan triacetate $[\alpha]_D -30.7^\circ$ contained mannose and glucose only in the ratio 36:10. Measurements of intrinsic viscosity in the chloroform solution gave $[\eta]$ 0.776 and 0.291 for high and low molecular parts of the acetate respectively. The glucomannan derived its acetate showed $[\alpha]_D -36.5^\circ$ and 78.5% mannose content.

Recently it has been found that coniferous woods and their pulp contained glucomannans which have been studied by Aspinall¹⁾, Dutton²⁾, Hamilton^{3,8)}, Lindberg⁴⁾, Timell⁵⁾, Wise⁶⁾, Jones^{7,10)} and their co-workers. In spite of their important contributions in this field, the structural feature of glucomannan remains unclear in many points. Jones⁷⁾ reported the presence of galactose residues in the glucomannan but he also considered it as originated from galactomannan. On the other hand, Hamilton³⁾ thought that the galactose residue is attributed to a separate galactoglucomannan. Further, there are divergences of opinions on the ratio of mannose to glucose, the presence of cellobiose fragment as a constituent of the glucomannan

or the configuration and size of the molecule. It may be considered that these questions are all owing to the difficulties of removing the contaminants from glucomannan molecule. In the previous paper⁹⁾, the presence of glucomannan in dissolving pulp prepared from pine was recognized by isolation of octaacetyl 4-O- β -D-glucopyranosyl- α -D-mannose from the acetolysis product. This paper is concerned with separation of the glucomannan from the contaminants and characterization of the pure specimen. This also includes the detection of the contaminating hexosans. Polysaccharides constructing hemi-cellulose were isolated by successive extractions with dimethylsulfoxide and aqueous alkali from holocellulose pulp of pine which was free from pectin and water-soluble polysaccharides. First extraction was effected with dimethylsulfoxide-acetic acid mixture which gave a polysaccharide mixture (I), $[\alpha]_D -39^\circ$, composed of 4-O-methylglucurono-araboxylan¹¹⁾ and a new type of glucomannan (II). Dimethylsulfoxide-boric acid was then used as extractant and the extracted material was purified by adsorption to Amberlite

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2) G. G. S. Dutton and K. Hunt, *J. Am. Chem. Soc.*, **80**, 5697 (1958).

3) J. K. Hamilton and H. W. Kircher, *J. Am. Chem. Soc.*, **80**, 4703 (1958).

4) B. Lindberg and H. Meier, *Svensk Papperstidn.*, **60**, 785 (1957).

5) T. E. Timell and A. Tyminski, *TAPPI*, **40**, 519 (1957).

6) E. Merler and L. E. Wise, *TAPPI*, **41**, 80 (1958).

7) J. K. N. Jones and T. J. Painter, *J. Chem. Soc.*, **1959**, 573.

8) J. K. Hamilton and E. V. Partlow, *J. Am. Chem. Soc.*, **80**, 4880 (1958).

9) T. Koshijima and I. Tachi, *This Bulletin*, **22**, 11 (1958).

10) J. K. N. Jones and T. J. Painter, *J. Chem. Soc.*, **1957**, 669.

11) J. K. Hamilton, E. V. Partlow and N. S. Thompson, *TAPPI*, **41**, 803 (1958); *ibid.*, **41**, 811 (1958).

IR-A 400. The material eluted from the resin with 1 N ammonium carbonate solution was a new type of glucomannan (II), $[\alpha]_D^{25} - 8^\circ$ (water) contained 3.8 parts of mannose and one part of glucose. The constitution of the glucomannan is under experiments. A large part of 4-O-methylglucurono-araboxylan (III), $[\alpha]_D - 42^\circ$ was obtained from the residual pulp by the extraction with 1% sodium hydroxide and the usual glucomannan eluted for the first time by the following 10% sodium hydroxide extractions. It was purified using dimethylsulfoxide which dissolved only 4-O-methylglucurono-araboxylan (B), $[\alpha]_D - 49^\circ$ and left glucomannan (IV), $[\alpha]_D - 39^\circ$. Mannose and glucose were present, respectively, in a ratio 3.6:1 with trace of xylose and galactose in (IV). The glucomannan (IV) precipitated with methanol from its water solution was made free from water by washing with methanol and the methanol-wet material was acetylated twice with excess of acetic anhydride and pyridine. The fully acetylated (IV) was then fractionally precipitated from the chloroform solution with petroleum ether and the pure glucomannan triacetate fractions were combined, $[\alpha]_D - 30.7^\circ$ (chloroform) (see Table I). Deacetylation of the acetate with sodium methoxide resulted glucomannan $[\alpha]_D - 36.5^\circ$ (10% NaOH) contained 78.5% mannose residue. Paper chromatography showed the presence of mannose and glucose only. The intrinsic viscosity of a solution of fraction (22) and (31) in chloroform was 0.776 and 0.291 100 ml/g respectively. These correspond to average D.P. of 146 and 55 when Staudinger and Daumiller's constant for cellulose triacetate, $K_m \cdot 10^4 = 5.3$ is applied to the glucomannan triacetate. A part of the acetylated glucomannan was fractionated by Jones and Painter's procedure⁷⁷ and divided into fraction (GM) and (X), the latter being fractionally precipitated in the same way as above described (Table II). Fraction (GM) was the glucomannan triacetate $[\alpha]_D - 30.6^\circ$ containing a small amount of galactose which, however, had also a tendency to distribute over fraction (X). This result indicates that galactose residues may be origi-

nated from the other polysaccharides.

A part of the extract contained (IV) precipitated upon neutralization with acetic acid. The precipitate purified with alkali was a separate glucomannan (V) indicating a specific optical rotation of $[\alpha]_D - 32^\circ$ and consisted of four parts of glucose and one part of mannose. The results of the fractionation (Table I) indicates that the glucomannan acetate prepared is composed of two or three polysaccharides. It is probable that the acetate of this type of glucomannan $[\alpha]_D - 19^\circ$ ¹²⁾ contaminates the higher molecular part of the present glucomannan acetate.

EXPERIMENTAL

Paper Chromatography

Aniline hydrogen phthalate was used as spray reagent throughout the experiment. For the separation of neutral sugars solvent (a), ethylacetate-pyridine-water 10:4:3, and for uronic acid fragments solvent (b), ethylacetate-acetic acid-formic acid-water 18:3:1:4 were used respectively. Measurement of the sugar-ratio in the fractionated glucomannan acetate was made on the revealed spots which were cut off from the paper, and weighed after conditioning in desiccator for twenty-four hours. In each of the cases, five series of the chromatogram of the hydrolyzate were developed two times with solvent (a) under the same conditions and the ratios of spot-weights of mannose to glucose were calculated from five specimens. From the average value of the ratios expressed in logarithm, the ratio of the amount of mannose to glucose in the hydrolyzate of the deacetylated glucomannan was determined immediately using the calibration curve I in Fig. 1 which showed that the ratios of spot-weights were proportioned to the ratios of sugar-amounts adsorbed to the paper in a fairly range when both of the ratios were calculated on the corresponding values of glucose and expressed with logarithms. In this way, the ratios of xylose to glucose and galactose to glucose were determined by the curve II and III, respectively.

Preparation of Holocellulose Pulp

Delignification of acetone-extracted wood meal (360 g) was effected by four times chloriting¹³⁾ followed by one treatment with chlorine and monoethanolamine¹⁴⁾. The holocellulose (240 g) contained 4.26% Klason-lignin was

12) T. Koshijima, *J. Japan Wood Res. Soc.*, in press.

13) T. Koshijima, *ibid* in press.

14) T. E. Timell and E. C. Jahn, *Svensk Papperstidn.*, **54**, 831 (1951).

FIG. 1.

For xylose and galactose, abscissa is graduated in one-tenth of the scale designed for mannose.

- I. Mannose: glucose.
- II. Xylose: glucose
- III. Galactose: glucose
- R: Ratio of spot-wt.

extracted with 0.5% ammonium oxalate (pH, 6.3) for twenty hours and the water suspension was then beaten in a laboratory beater. This treatment resulted very swollen holocellulose pulp which was soaked in water (10 l) over-night and filtered, water-soluble polysaccharide (A) (3.5 g) being obtained from the filtrate as usual manner. Fraction (A) showed $[\alpha]_D^{20} - 37^\circ$ (10% NaOH, c, 1.0) and contained mannose (26.4%), glucose, xylose, arabinose, galactose and 4-O-methylglucuronic acid. The chloriting of the residual lignin in the pulp was repeated more three times using the same reagent and one-half of the reaction time. The resultant pulp (197 g) was washed with water, methanol, benzene and air-dried: Klason-lignin, 1.88; mannose residue, 13.8; Ac, 1.46%.

Isolation of Polysaccharides Constructing Hemicellulose

Air-dried pulp (157 g) was extracted with dimethylsulfoxide-acetic acid and then dimethylsulfoxide-boric acid as described in the previous report¹³. Dimethylsulfoxide-acetic acid extraction yielded a polysaccharide mixture (I) (2.16 g); $[\alpha]_D^{20} - 39^\circ$ (1% NaOH, c, 1.0); Ac, 2.41; mannose residues, 22.4%. Paper chromatography (solvent a and b) showed the presence of xylose, arabinose, mannose, glucose, galactose and 4-O-methylglucuronic acid in the hydrolyzate. Dimethylsulfoxide-boric acid extract was composed of a glucomannan (1.28 g) as main product which contained 4.43% Ac. and mannose, glucose, galactose and xylose as component sugars (solvent a). It was treated with Amberlite IR-120 and IR A-400 (carbonate form) resin with stirring. The anion-exchanger was washed with water until negative for anthrone and then soaked in 1 N ammonium carbonate

solution for one month. The resin was separated from the solution which was evaporated to a small volume in vacuo and precipitated with 10 volumes of ethanol. The resultant white powder (II) was easily soluble in water and was composed of mannose and glucose in a ratio 3.8:1 with trace amounts of xylose, yield 1.21 g, $[\alpha]_D^{22} - 8^\circ$ (water, c, 1.0), -4° (1% NaOH, c, 1.0). Uronic acid fragments in the hydrolyzate showed the presence of methyl-glucuronic acid and an unknown spot neighboring start line (solvent b).

Dimethylsulfoxide-extracted pulp (240 g) was mixed with 1% sodium hydroxide solution (3 l) and allowed to stand for thirty minutes. The filtrate and washings acidified with acetic acid were combined and the precipitate produced after three days was separated. The clear, light yellow extracts were made precipitate by addition of 3 volumes of methanol, removed by centrifugation, resuspended in methanol and dried, yield 14 g. The bulky white powder (III) showed an optical rotation of $[\alpha]_D^{15} - 42^\circ$ (10% NaOH, c, 0.9) and the paper chromatography followed by hydrolysis of (III) indicated the presence of a large amount of xylose, arabinose and 4-O-methylglucuronic acid fragments (solvent a and b).

Ten percent sodium hydroxide solution (2.5 l) was added to the residual pulp without drying and nitrogen gas bubbled through the suspension for one hour. Extraction was continued for twenty-four hours and then the extract was filtered and washed with this solution through cloth into the vessel containing acetic acid with external cooling. The acidic extract was turbid and, after five days, the precipitate (Va) produced removed by centrifugation. The residual pulp was extracted again with 10% sodium hydroxide and the centrifuged liquor of two extracts were combined and dialyzed through gel cellophane for two weeks. The water solution was evaporated to one-fifth and precipitated with methanol, the precipitate being removed by filtration, resuspended in methanol, filtered and washed with methanol, yield 34 g (IVa). Paper chromatography of the hydrolyzate showed the presence of a large amount of mannose, glucose, galactose and xylose (solvent a).

The precipitate (Va) was washed with water by centrifugation, resuspended in methanol-water (1:3) and the supernatant liquor discarded. This treatment was repeated six times and the final precipitate recovered from a methanol suspension. The methanol-wet precipitate was dissolved in 1% sodium hydroxide, neutralized with acetic acid and the resultant precipitate was centrifuged. It was dissolved again in 10% sodium hydroxide and made precipitate as above. This was repeated once

more and the final precipitate was washed with water, 50% methanol and methanol. The product (V) (1.3 g) contained 19.8% mannose residue, indicated $[\alpha]_D^{18} - 32^\circ$ (10% NaOH, c, 0.3). On hydrolysis and paper chromatography, the only sugars present were glucose and mannose (solvent a).

Purification of the Glucomannan

Polysaccharide (IVa) was suspended in dimethylsulfoxide (800 ml) which was stirred vigorously for three hours and allowed to stand overnight. The insoluble part was removed by centrifugation and treated again with dimethylsulfoxide as described above. The light brown insoluble part was centrifuged, washed with this reagent, resuspended in methanol, filtered through cloth, washed with methanol and dried, yield 24 g (IV). The glucomannan (IV) indicated a specific optical rotation of $[\alpha]_D^{18} - 39^\circ$ (10% NaOH, c, 0.9) and contained mannose and glucose in the ratio 3.6:1, together with a trace amount of xylose and galactose. Dimethylsulfoxide-soluble part (B) produced a bulky precipitate by pouring into methanol. This was separated as usual manner and the resultant white powder (9.8 g) containing xylose, arabinose and 4-O-methylglucuronic acid as component sugars, showed $[\alpha]_D^{15} - 49^\circ$ (1% NaOH, c, 1.0).

Acetylation of the Glucomannan

The glucomannan (IV) (9.0 g) was dissolved in warm water (2 l, 70°C) with stirring and standing for 2 days at 0°, the insoluble material being separated by centrifugation. The centrifugate was concentrated in vacuo and poured into 5 volumes of methanol. The white precipitate separated was resuspended in methanol, filtered through cloth, and methanol was removed as much as possible by evaporation under reduced pressure. The methanol-wet glucomannan was dispersed in dry pyridine (100 ml) and then there was added dropwise 130 ml of acetic anhydride with vigorous stirring and ice-cooling. The temperature of the reaction mixture was maintained to 30° during this time, after which stirring was continued at room temperature for twenty-four hours. Portions of pyridine and acetic anhydride were added over five days until a total of 210 ml of pyridine and 290 ml of acetic anhydride had been added. After addition of these reagent, the temperature of the liquid was raised to 40° which was maintained for twenty-four hours with continuous stirring. The resultant light brown solution was poured into ice-water (3 l), whereupon the acetylated glucomannan appeared as a flocculent precipitate. The suspension was thoroughly extracted with chloroform but a large portion of the glucomannan acetate formed emulsion, which was ex-

tracted with chloroform several times, the clear chloroform extracts being combined and used for the preparation of fraction (GM I) and (X I). Partly acetylated glucomannan (Ac, 30.5%, 11 g) was recovered from the emulsion and acetylated again using 130 ml of dry pyridine and 113 ml of acetic anhydride. The reaction was complete for three hours at 40° and then the resultant brown solution poured into ice-water containing hydrochloric acid (30 g). The fibrous precipitate produced was filtered, washed with water and dried over anhydrous calcium chloride and then sodium hydroxide, yield 11.5 g, Ac, 43.9%.

Fractional Precipitation of the Glucomannan Acetate

The acetate (4.0 g) was dissolved in chloroform (300 ml) and precipitated in the usual way by the gradual addition of equal volume of petroleum ether (b.p. 30~50°). The precipitate was dissolved in chloroform and precipitated again as a white powder by pouring this solution into 10 volumes of petroleum ether. It was recovered and divided into three portions by fractional precipitation of its chloroform solution, each portions being refractionated further into two or three. The individual fractions were treated as described above, and the resultant white powders washed with petroleum ether and dried in vacuo. A soluble part in chloroform-petroleum ether (1:1) was made precipitate by concentration and addition of petroleum ether and the last fraction obtained. The specific optical rotation and sugar composition of each fraction indicate that fraction (22) to (32) inclusive are essentially homogeneous as given in Table I. They were combined and characterized, $[\alpha]_D^{20} - 30.7^\circ$ (chloroform, c, 1.0). *Anal.* Found: COCH₃, 44.5. Calcd. for C₁₂H₁₆O₈: COCH₃, 44.8%.

Deacetylation of the Glucomannan Triacetate

To a solution of the glucomannan triacetate (180 mg) in chloroform (18 ml), 0.2 N sodium methoxide in abs. methanol (9 ml) was added with shaking and the mixture was allowed to stand over-night at room temperature. After addition of methanol (27 ml), acetic acid and water, the resultant white precipitate was removed by filtration and washed with 75% ethanol, ethanol and dried, yield 90 mg, $[\alpha]_D^{22} - 36.5^\circ$ (10% NaOH, c, 1.3). Determination of mannose content in the glucomannan was carried out as described in the previous report¹⁵⁾ which was modified the Haggglund's method¹⁶⁾. To make complete the precipitation of mannose phenylhydrazone, however, the hydrolyzed solution was kept to forty-eight hours

15) T. Koshijima and I. Tachi, *This Bulletin*, **21**, 253 (1957).

16) E. Haggglund and L. C. Bratt, *Sevensk Kem. Tidskr.*, **48**, 125 (1936).

TABLE I. FRACTIONAL PRECIPITATION OF THE GLUCOMANNAN ACETATE

Fraction	Acetyl, %	Yield, % ^{a)}	$[\alpha]_D^{18}$ in Chloroform	Xylose	Mannose	Glucose	Galactose
1	43.2	46.3	—	0.11	2.5	1	0.15
11		22.1	—	0.07	1.4	1	0.12
12		11.8	-21.8	0.03	1.0	1	0.13
13		3.8	-19.4	Trace	1.4	1	Trace
2	44.8	27.5	-30.0	Trace	3.6	1	None
21		1.8	-44.5	Trace	4.6	1	None
22		14.0	-30.6	None	3.7	1	None
23		6.8	-30.8	None	3.5	1	None
3	44.3	25.8	-30.2	None	3.6	1	None
31		18.2	-30.7	None	3.6	1	None
32		3.2	-30.6	None	3.7	1	None
4		0.5	-20.5	None	2.1	1	None

a) Expressed on the original acetate.

TABLE II. FRACTIONATION OF THE GLUCOMANNAN ACETATE BY JONES' PROCEDURE

Fraction	Acetyl, %	Yield, % ^{a)}	$[\alpha]_D^{18}$ in Chloroform	Xylose	Mannose	Glucose	Galactose
GMI	44.2	0.5	-30.8	Trace	3.5	1	0.03
GMII	44.5	31.3	-30.6	None	3.6	1	0.03
XI		0.8	-41.0	0.23	2.7	1	0.20
XII		28.0	—	—	—	—	—
E1		5.0	-33.7	0.20	2.1	1	0.05
2		11.0	-31.1	0.03	3.6	1	None
3		3.0	-28.6	Trace	3.4	1	None
4		Trace	—	Trace	2.0	1	None
R		9.1	—	0.20	1.4	1	0.06

a) Expressed on the original acetate.

at 0° and washings of the precipitate were effected successively by two of 1 ml of cold water, two of 1 ml of cold ethanol and 2 ml of ether for 224 mg of the hydrazone. The hydrazone derived from the glucomannan (67.0 mg) was 82.0 mg: this corresponds to 78.5% mannose residue.

Fractionation of Glucomannan Acetate by the Jones' Procedure

To the chloroform solution of the glucomannan acetate (3.5 g in 500 ml), saturated sodium bicarbonate solution was added and shaken. The chloroform layer which separated was removed and again treated with this reagent further two times. It was combined to the washings of emulsion, washed with water, dried over anhydrous sodium sulfate, concentrated to 150 ml and poured into petroleum ether. The glucomannan triacetate (GM II) was recovered as white yellow powder, yield 0.94 g $[\alpha]_D^{18}$ -30.6° (chloroform, c, 1.5).

The emulsion was washed with chloroform, acidified

with ice-cold 1 N hydrochloric acid and shaken with chloroform, whereupon chloroform layer did not separate from the emulsion. Equal volume of water was added and chloroform layer separated. The dried chloroform solution (X II) was fractionally precipitated as before and fraction (E1) to (E4) obtained. The emulsion remaining after the repeated extraction with chloroform was recovered by pouring into methanol (ER). The partly acetylated glucomannan was treated in the same way as described here and small amount of glucomannan triacetate (GM I) was obtained, $[\alpha]_D^{18}$ -30.8° (chloroform, c, 1.0), as well as fraction (X I) (see Table II).

Determination of Intrinsic Viscosity

The intrinsic viscosity was measured on the chloroform solution of fraction (22) and (31) at $20 \pm 0.01^\circ$ using an Ubbelohde capillary viscometer. In each of the cases, the reduced viscosity was determined at 3 different concentrations and linearly extrapolated to zero concentration. Fraction (22), η_{sp}/c , 1.141, 1.048, 0.961; $[\eta]$, 0.776.

Fraction (31), $\eta_{sp}/c.$, 0.538, 0.478, 0.420; $[\eta]$, 0.291.

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Studies on the Conjugated Lipids

Part XIV*. Fatty Acid Constituents of Sphingomyelin from Horse Spinal Cord**

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The chemical nature of fatty acids was studied on sphingomyelin which was prepared from horse spinal cord. Nervonic, palmitic, stearic and behenic acids were isolated from the hydrolysate of the sphingomyelin preparation. The presence of behenic acid in sphingomyelin has in this paper been reported for the first time.

It is known that several kinds of fatty acid can attach to the amino group of the shingosine moiety of shingomyelin, the structure of which is shown in Fig. 1. However, an assortment of fatty

R: Fatty alkyl group

FIG. 1. Structure of Sphingomyelin

acids in shingomyelin from a certain animal tissue seems to be almost settled, although some confusion and inconsistencies on this point are seen in the literature. This may be unavoidable

because sphingomyelin prepared by the older methods might often have been contaminated with other lipids. Nowadays, it is generally believed that sphingomyelin from brain contains lignoceric, stearic and nervonic acids as the component fatty acids¹⁻³, and that sphingomyelin from lung or spleen contains lignoceric and palmitic acids^{3,4}. The present authors undertook to elucidate the fatty acid constituents of sphingomyelin from spinal cord, since the tissue has never been studied in respect to this problem.

A pure sample of sphingomyelin was prepared from horse spinal cord, and hydrolyzed with methanolic sulfuric acid to produce methyl

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** A preliminary communication of this work has been published in the *Nature*, 184, 817 (1959).

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