[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE OHIO STATE UNIVERSITY]

Carbohydrates of the Coffee Bean. II. Isolation and Characterization of a Mannan[1]

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A mannan has been isolated from the green coffee bean in 5% yield. Evidence is presented that the polysaccharide is homogeneous, although containing 2% galactose, and consists of a linear chain of β-D-(1 → 4)-linked mannose units with a weight average degree of polymerization of 45.

The observation by Hamilton, Kircher, and Thompson[3] that mannans are more soluble in sodium hydroxide than in potassium hydroxide, was utilized by Dutton and Hunt[4] in obtaining the mannan of Sitka spruce. When the fraction of holocellulose insoluble in 10% potassium hydroxide (herein-after called holocellulose A) and consisting of 58% D-mannose was extracted with 18% sodium hydroxide, 21% of the starting material was solubilized. Upon acidification (*p*H 5) of the soluble portion, a white precipitate was formed in a 61% yield. Upon hydrolysis of this material with formic acid a yield of 94% D-mannose and 2% galactose was obtained. The galactose units could not be removed by reprecipitation from sodium hydroxide or by complexing with Fehling solution. The product was considered to be a true mannan by Aspinall's[5] definition of a mannan as being a "polysaccharide containing 95% or more of D-mannose residues."

The mannan was completely methylated (45.4% methoxyl) and hydrolyzed. Paper chromatography showed the primary repeating unit to be a tri-*O*-methyl sugar. The homogeneity of the tri-*O*-methyl sugar was established by two chromatographic solvent systems. One of the possible linkages, β-D-(1→2), which would result in 3,4,6-tri-*O*-methyl-D-mannopyranose was eliminated because this sugar failed to complex with borate during paper electrophoresis. The major spot was not resolved on development with butanone-water azeotrope (which produces R_f differences of 0.05 between each of the three possible remaining tri-*O*-methyl sugars). The amounts of di-*O*-methyl and tetra-*O*-methyl sugars indicate an essentially linear chain with a fairly high degree of polymerization.

The tri-*O*-methyl sugar was isolated as a sirup by elution from paper chromatograms. It was shown to be 2,3,6-tri-*O*-methyl-D-mannopyranose by the preparation of two crystalline derivatives, 2,3,6-tri-*O*-methyl-D-mannono-1,4-lactone and 2,3,6-tri-*O*-methyl-D-mannopyranose 1,4-bis(*p*-nitrobenzoate). The negative rotation of the polymer indicated that the glycosidic linkage was β-D, and hence the primary linkage was shown to be β-D-(1→4).

Molecular weight determination by the sedimentation equilibrium method established an average degree of polymerization of 45 (mol. wt. 7300), with a narrow distribution about this value, establishing this mannan as having a chain length intermediate between those of mannans A and B from the vegetable ivory nut (Table I).

The described polysaccharide is one of the few mannans found in land plants; it constitutes 5% of the dry weight of the green coffee bean. Both the coffee bean and ivory nut are extremely hard seeds and neither contains much cellulose. In his review of mannans Aspinall[5] concludes that there are no essential differences in chemical structure between mannans A and B of the ivory nut and that they vary distinctly only in molecular size. The present authors conclude, through a comparison of rotations and linkages (Table I), that the only distinct difference between the mannans of the ivory nut, coffee bean, salep, seaweed, and probably other sources, is one of chain length. Indeed the occurrence of D-mannose linked β-D-(1→4) in these seeds and sea plants can be compared with the more common occurrence of cellulose in plants. The possible occurrence of a small degree of branching in these mannans is not excluded by the present data (Table I).

EXPERIMENTAL

Isolation of a mannan. Freshly lyophilized holocellulose A (the 10% potassium hydroxide insoluble material) (68.5 g.), isolated by the method of Wolfrom, Plunkett, and Laver,[1] was extracted for 8 hr. at 25° with 18% aqueous sodium hydroxide (1 l.) in the presence of nitrogen. The white solid was recovered and washed with water until neutral. The combined filtrate and washings (clear and colorless) were acidified to *p*H 5 with acetic acid, causing immediate precipitation of a white solid. The entire mixture was refrigerated for 1 week, dialyzed, and the solids recovered

(1) Previous communication: M. L. Wolfrom, R. A. Plunkett, and M. L. Laver, *J. Agr. Food Chem.*, **8**, 58 (1960). A preliminary report of this work has appeared in *Abstracts Papers Am. Chem. Soc.*, **138**, 23D (1960).

(2) Westreco Co. Fellow of The Ohio State University Research Foundation (Projects 530 and 878).

(3) J. K. Hamilton, H. W. Kircher, and N. S. Thompson, *J. Am. Chem. Soc.*, **78**, 2508 (1956).

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

(5) G. O. Aspinall, *Advances in Carbohydrate Chem.*, **14**, 448 (1959).

TABLE I

PHYSICAL CONSTANTS OF β-D-(1 → 4)-MANNANS

Source		$[\alpha]_D^{15-20}$, c 1, Anhyd. Formic Acid	$[\alpha]_D^{15-20}$, c 1, N Sodium Hydroxide	Degree of Polymerization	Reference
Plant	Common name				
Phytelephas macrocarpa	Ivory nut mannan A	−28	−46	10–13	[a]
		—	—	17–21	[b]
	Ivory nut mannan B	−20	—	38–40	[a]
		—	—	80	[b]
		−26	−48	—	[c]
Porphrya umbilicalis	Red alga (edible)	−22	—	—	[d]
Orchis and *Eulophia*	Salep	—	−44	1340	[e,f]
Coffea arabica	Green coffee	−22	—	45	This work

[a] G. O. Aspinall, E. L. Hirst, E. G. V. Percival, and I. R. Williamson, *J. Chem. Soc.*, 3184 (1953). [b] H. Meier, *Biochim. et Biophys. Acta*, **28**, 229 (1958). [c] G. O. Aspinall, R. B. Rashbrook, and G. Kessler, *J. Chem. Soc.*, 215 (1958). [d] J. K. N. Jones, *J. Chem. Soc.*, 3292 (1950). [e] E. Husemann, *J. prakt. Chem.*, **155**, 241 (1940). [f] F. Klages and R. Niemann, *Ann.*, **523**, 224 (1936).

by centrifugation were lyophilized and dried under reduced pressure over phosphorus pentaoxide for 5 days (analytical sample dried at 78° and <1 mm.); yield of white amorphous solid, 8.8 g., 12.8% of holocellulose A or 5% (dry wt.) of the green coffee bean. This substance was slowly soluble in 90–100% formic acid and 25–30% sodium hydroxide; $[\alpha]_D^{22}$ −22° (*c* 1, anhydrous or 90% formic acid, first reading at 4 hr., unchanged at 24 hr.).

Anal. Calcd. for $C_6H_{10}O_5$: C, 44.50; H, 6.22. Found (after correction for 2.6% ash): C, 44.64; H, 6.50.

Hydrolysis of the mannan. The above material (0.5 g.) was hydrolyzed to constant rotation (3 hr.) at 97° with 90% formic acid (50 ml.); $[\alpha]_D^{22}$ +18° (*c* 1, formic acid). The formic acid was removed by evaporation under reduced pressure followed by the addition and removal of water. The resultant sirup was subjected to paper chromatography using ethyl acetate, acetic acid, and water (9:2:2 v./v.) (developer A) and aniline phthalate as indicator to show the presence of formate esters which were hydrolyzed with 0.5*N* sulfuric acid (25 ml., for 2.5 hr. at 97°). Upon cooling, the solution was neutralized with barium hydroxide, and the solids were removed by centrifugation. The remaining inorganic salts were removed by refluxing the concentrated sirup with absolute methanol, followed by filtration (repeated four times) and removal of solvent.

The sirup so obtained was chromatographed on paper using developer A followed by 1-butanol, pyridine, and water (10:3:3 v./v.) (developer B) according to the procedure of Quick.[6] Aniline phthalate indicator showed spots corresponding to mannose, galactose, and oligosaccharide (very faint and slow moving). The mannose:galactose ratio was quantitatively determined by densitometry closely following the method of McFarren, Brand, and Rutkowski[7] as modified by Wolfrom, Plunkett, and Laver.[1]

Anal. Found: mannose, 94%; galactose, 2%.

The polymer was tested for homogeneity by reprecipitation from 18% sodium hydroxide on acidification to *p*H 5 with 50% acetic acid and by complexing it with Fehling solution (after solvation in 20% sodium hydroxide). The gelatinous precipitate obtained with Fehling solution was separated and treated with 2*N* hydrochloric acid. The white solid so formed was recovered, washed with water and precipitated again from acetone. Both purifications did not lower the galactose content.

Isolation of D-*mannose phenylhydrazone.* To an aliquot of the above hydrolyzate (20 ml.), 0.2 ml. of phenylhydrazine, and 0.2 ml. of glacial acetic acid were added. After refrigeration for 12 hr., crystalline D-mannose phenylhydrazone was obtained; yield 95%, m.p. 187–190° unchanged on admixture with authentic material $[\alpha]_D^{26}$ +25°, x-ray powder diffraction data[8] (identical with that of authentic material): 11.94m, 5.60m, 5.23s, 4.43vs(1), 3.96vs(3), 4.38vw, 3.30vs(2), 3.10vw, 2.99w, 2.79vw, 2.68vw, 2.41m.

Methylation of the mannan. To the polysaccharide (1.4 g.) was added 50 ml. of 18% sodium hydroxide at 0° with stirring followed by the addition of 6 g. of sodium hydroxide. Sodium hydroxide (100 ml., 30%) and 50 ml. of dimethyl sulfate was added simultaneously over a period of 3 hr. while maintaining the temperature at 0°. Acetone (150 ml.) was added to prevent foaming and the mixture was stirred for an additional 45 hr. at room temperature. The solution was cooled to 0°, neutralized with 10% sulfuric acid, dialyzed against water (3 days), concentrated under reduced pressure (200 ml.), lyophilized, and the entire methylation sequence repeated. The product was further methylated by the method of Falconer and Adams,[9] by solution in 150 ml. of tetrahydrofuran followed by treatment over a period of 60 hr., with seven 10-g. portions of crushed sodium hydroxide, each followed by a 12-ml. portion of dimethyl sulfate. The solution was stirred vigorously and additional solvent added as needed to maintain fluidity. The product was recovered as described above for the first methylation, and the concentrated dialyzate (600 ml.) was extracted with three 300-ml. portions of chloroform. The chloroform layer was concentrated under reduced pressure, yielding a light brown solid which was dissolved in acetone and filtered. The concentrated filtrate (50 ml.) was poured into petroleum ether (b.p. 30–60°, 200 ml.) and the white precipitate which formed was recovered (after the mixture was refrigerated for 6 days) and lyophilized, affording a white, fluffy powder; yield 0.6 g., $[\alpha]_D^{25}$ −70° (*c* 0.6, 90% formic acid). A Nujol suspension of the material showed no infrared hydroxyl absorption.

Anal. Calcd. for $C_6H_7O_2(OCH_3)_3$: OCH_3, 45.58. Found[10]: 45.4.

Hydrolysis of the methylated mannan. The methylated mannan (222 mg.), was hydrolyzed with 35 ml. of formic acid and processed as described above for the unmethylated mannan. The product was obtained as a sirup; yield 98%

(6) R. H. Quick, *Anal. Chem.*, **28**, 1439 (1957).

(7) E. F. McFarren, Kathleen Brand, and H. R. Rutkowski, *Anal. Chem.*, **23**, 1146 (1951).

(8) Interplanar spacing, Å, CuK_α radiation; relative intensity, estimated visually: s, strong; m, medium; w, weak; v, very. First three strongest lines are numbered (1 strongest).

(9) E. L. Falconer and G. A. Adams, *Can. J. Chem.*, **34**, 338 (1956).

(10) D. O. Hoffman and M. L. Wolfrom, *Anal. Chem.*, 19, 225 (1947).

(based on total solids as determined[11] on an aliquot in dilute aqueous solution).

A descending paper chromatogram of a dilute aqueous solution of the hydrolyzate sirup, as developed with developer A and indicated by the aniline phthalate reagent, showed five spots of the following R_G values: 0.98 (trace), 0.92 (trace), 0.82 (very strong), 0.59 (trace), 0.18 (trace). The main spot did not separate when obtained by development with the butanone-water azeotrope[12] and indicated by aniline phthalate. Paper electrophoresis[13] in borate of *p*H 9.2 showed no migration.

*2,3,6-Tri-O-methyl-*D*-mannono-1,4-lactone.* The tri-*O*-methyl-D-mannopyranose sirup was obtained by developing the methylated mannan hydrolyzate chromatographically (Whatman No. 3 filter paper and solvent A), excising the areas corresponding to the tri-*O*-methyl sugar, eluting with water, and concentrating. To the sirup (100 mg.) was added water (7 ml.) and bromine (2.5 ml.) followed by vigorous shaking for 4 hr.[14] The solution was stored for 10 days in the dark at 25°, at which time chromatographic investigation failed to indicate a reducing sugar. The solution was aerated, neutralized with silver carbonate, filtered, and the filtrate and washings concentrated to 100 ml. A crystal of lead tetraacetate was added (to prevent colloidal silver sulfide formation) and the solution was saturated with hydrogen sulfide. The silver sulfide was removed by filtration, the clear solution concentrated, passed through Amberlite IR-120, and evaporated under reduced pressure to dryness. The residue was dissolved in 50 ml. of ether and upon slow evaporation a yellow precipitate was recovered. The filtrate was concentrated to dryness, the residue dissolved in benzene and hexane (petroleum ether, b.p. 60–68°) added. Nucleation caused immediate crystallization affording 2,3,6-tri-*O*-methyl-D-mannono-1,4-lactone; yield 17 mg., m.p. 79.5–81° unchanged after one recrystallization (accepted[15] m.p. 84–85°), x-ray powder diffraction data[8] (identical with those of authentic[16] 2,3,6-tri-*O*-methyl-D-mannono-1,4-lactone): 10.98s(3), 8.36m, 7.15vs(1), 6.63m, 5.45m, 5.10w, 4.60vs(2), 4.35s, 4.16m, 3.96s, 3.85s, 3.52s, 3.30s, 3.26s, 2.88m, 2.69m, 2.36m, 2.25m, 1.96w.

*X-Ray powder diffraction data for 2,3,4-tri-O-methyl-*D*-mannono-1,5-lactone.* For comparative purposes an authentic[17] specimen of this substance was measured[8]: 11.86vs(2), 9.36vs(1), 7.73s, 6.15w, 5.84w, 5.10m, 7.83m, 4.57s, 4.56s, 4.18s, 3.87m, 3.60vw, 3.46w, 3.40vs(3), 3.21s, 3.03s, 2.91w, 2.80w, 2.72w, 2.51w, 2.47w, 2.36w.

*2,3,6-Tri-O-methyl-*D*-mannopyranose 1,4-bis(p-nitrobenzoate).* A portion (150 mg.) of the isolated tri-*O*-methyl-D-mannopyranose was dissolved in pyridine (10 ml.) and *p*-nitrobenzoyl chloride (700 mg.) was added.[18] The reaction mixture was kept at 60–70° for 30 min. and was then maintained overnight at room temperature. It was neutralized with saturated sodium bicarbonate solution until effervescence ceased. After further dilution with 10 ml. of water, the solution was extracted with three consecutive 20-ml. portions of chloroform, dried with magnesium sulfate, and evaporated under reduced pressure to dryness. Crystallization occurred spontaneously. The material was recrystallized twice from methanol, washed with cold methanol, and dried at 50° for 3 hr.; yield 202 mg., m.p. 185–186°. Pure material was obtained on two more recrystallizations from methanol; m.p. 190–191°, $[\alpha]_D^{24}$ +30° (*c* 1.53, chloroform) (reported[18] m.p. 190–191° and +33°), x-ray powder diffraction data[8] (identical with authentic material): 11.19w, 8.45m, 6.56w, 6.19vs(1), 5.80w, 5.31s, 5.10s, 6.27m, 4.21w, 4.08w, 3.94s, 3.77vw, 3.65w, 3.45vs(2), 3.34m, 3.10s(3), 2.97vw, 2.86vw, 2.78vw, 2.63vw.

Anal. Calcd. for $C_{23}H_{24}N_2O_{12}$: C, 53.08; H, 4.63; OCH_3, 17.9. Found: C, 53.34; H, 5.01; OCH_3, 18.2.

Molecular weight determination.[19] The molecular weight was estimated by the method of Archibald applying the equations of Klainer and Kegeles.[20] A Spinco Model E ultracentrifuge equipped with the schlieren optical system was used. The concentration of the mannan was 2.54 g./l. in 90% formic acid. The density of the solution was assumed to be that of the formic acid, 1.1938. The specific volume, $\bar{V}$, was assumed to be that of D-glucose, 0.621.[21] The results indicated a molecular weight average of 7300 (degree of polymerization 45), with the polymer being narrowly distributed about this value.

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(16) The authentic specimen was kindly furnished by Professor F. Smith.

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