633. *The Polysaccharides of the Green Seaweed* Codium fragile. *Part III.*[1] *A* β-1,4-*Linked Mannan.**

By Jim Love and Elizabeth Percival.

A mannan, $[\alpha]_D$ −41°, containing *ca.* 5% of glucose residues, was isolated as the copper complex from an alkaline extract of the weed. Enzymic hydrolysis and separation on thick paper gave crystalline β-1,4-linked mannobiose and mannotriose, and syrupy mannotetraose, together with a small quantity of a second syrupy disaccharide containing mannose and glucose. Acetolysis gave similar oligosaccharides together with a third disaccharide which was tentatively identified as a 1,4-mannosylglucose.

Periodate oxidation and methylation studies confirmed that this was an essentially linear β-1,4-linked mannan.

Polysaccharides containing 95% or more of mannose are thought to be rare. The only authenticated mannans from higher plants are those from Ivory nut (*Phytelephas macrocarpa*)[2] and from coffee bean.[3] These are characterised by their highly insoluble nature and a backbone of β-1,4-linked mannose residues. In seaweeds, the mannan from *Porphyra umbilicalis*[4] is the only polysaccharide of this type reported from the Rhodophyceae.

* A brief account of part of this investigation has appeared in *Biochem. J.*, 1962, **84**, 29P.

[1] Part II, preceding Paper.

[2] Aspinall, Hirst, Percival, and Williamson, *J.*, 1953, **3184**; Aspinall, Rashbrook, and, in part, Kessler, *J.*, 1958, 215.

[3] Wolfrom, Laver, and Patin, *J. Org. Chem.*, 1961, **26**, 4533.

[4] Jones, *J.*, 1950, 3292.

A preliminary investigation by Iriki and Miwa [5] of the cell walls of species of the green seaweeds *Codium*, *Acetabularia*, and *Halicoryne* revealed mannose to be the major structural unit. On the basis of periodate oxidation and the negative rotation of these polysaccharides extracted with 50% zinc chloride, these authors tentatively proposed that they were β-1,4-linked mannans.

In the present studies it was found that similar extraction of *C. fragile* gave polysaccharide material largely composed of mannose units, but which was highly contaminated with non-carbohydrate and inorganic material (probably silica). Mild chlorite treatment [6] of the weed residue remaining after aqueous extraction, followed by extraction with dilute sodium hydroxide under nitrogen at room temperature, again gave polysaccharide material mainly comprising a mannan [1] with a high ash content. Neither of these extracts was examined further. Extraction of the residual weed under nitrogen with 20% sodium hydroxide and isolation of the polysaccharide from this extract through its copper complex yielded a mannan comprising *ca.* 95% of mannose and *ca.* 5% of glucose residues. It had $[\alpha]_D$ −41° (*c* 0·38 in 95% formic acid) (Found: ash, 2·3%; moisture, 4%), and was now insoluble in 20% alkali. It was exceedingly insoluble and difficult to hydrolyse and the " apparent " 87% purity determined [7] by sequential hydrolyses with 2N-acid is obviously a patently low figure, degradation of the monosaccharides inevitably occurring during the hydrolyses. The reduction of periodate [0·87 mole per C_6 anhydro-unit (87 hr.)] is low for a 1,4-linked polysaccharide, but this may be due to the high insolubility of the mannan hindering the access of the periodate. Chromatographic examination of a hydrolysate of the oxidised polysaccharide revealed a trace of uncleaved mannose. It is difficult to decide whether this was due to branch points, anomalous linkages in the polysaccharide, or under-oxidation.

Where more than a single linkage is present in a polysaccharide, oligosaccharides obtained by enzymic hydrolysis are often different from those obtained by acidic hydrolysis.[8] It is possible to use continuous dialysis during enzymic and polystyrenesulphonic acid hydrolysis, and, since the oligosaccharides produced are removed in this way from the mixture before they are degraded, higher yields are obtainable by this technique.

In an attempt to characterise the linkages in the present mannan, enzymic, acidic, and polystyrenesulphonic acid hydrolyses, and acetolysis studies were used. The first of these was carried out in a dialysis sac [9] and the total dialysate was collected. From this, mannose, 4-*O*-β-D-mannopyranosyl-D-mannose (mannobiose), and *O*-β-D-mannopyranosyl(1→4)-*O*-β-D-mannopyranosyl(1→4)-D-mannopyranose (mannotriose) were separated and characterised. In addition a small quantity of a second disaccharide (X) containing mannose and glucose was separated. It had a chromatographic mobility between that of mannobiose and mannotriose, but the quantity was insufficient for complete characterisation. Chromatographic evidence for the presence of mannotetraose and mannopentaose in the hydrolysate was obtained.

Hydrolysis with dilute acid for varying periods of time failed to yield any oligosaccharides, mannose and glucose being the sole products. In contrast 0·05N-polystyrenesulphonic acid at 80° [10] had no action on the mannan.

Acetolysis gave rise to the oligosaccharides obtained from the enzymic hydrolysis (paper chromatography) togther with a third disaccharide (Y). After repeated separation on paper a small quantity of this material was separated. It gave mannose and glucose on hydrolysis, and had a degree of polymerisation (DP) of 2·3.[11] This slightly high figure may be due to contamination with a small quantity of mannotriose. After reduction

[5] Iriki and Miwa, *Nature*, 1960, **185**, 178.
[6] Wise, *Ind. Eng. Chem., Analyt.*, 1946, 290.
[7] Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.
[8] Araki and Arai, *Bull. Chem. Soc. Japan*, 1957, **30**, 287.
[9] Bishop and Perila, *Canad. J. Chem.*, 1961, **37**, 815.
[10] Painter, *Chem. and Ind.*, 1960, **63**, 1214.
[11] Timell, *Svensk Papperstidn.*, 1960, **63**, 668; Peat, Whelan, and Roberts, *J.*, 1956, 2258.

and hydrolysis, disaccharide (Y) gave a single spot on a paper chromatogram with the mobility of mannose, indicating that in this disaccharide glucose is the reducing moiety. Gas chromatography [12] of the glycosides derived after methylation and methanolysis gave rather a confused picture. Apart from a small proportion of tetra-*O*-methyl-mannose and -glucose, both 2,3,6-tri-*O*-methylglucose and 2,3,6-tri-*O*-methylmannose were present. This again could be explained by the presence of contaminating mannotriose. Since the 2,3,6-derivative was the only tri-*O*-methylmannose revealed on the gas chromatogram, the presence in material (Y) of a second mannobiose with linkages other than 1,4, mixed with a glucose disaccharide, appears to be unlikely. The overall evidence indicates that material (Y) is 1,4-mannosylglucose. However, since it failed to crystallise this can only be regarded as tentative proof that it is a single disaccharide and that the gluose residues are linked to the mannan and are not present in a contaminating glucan.

Complete methylation of the mannan proved difficult. After ten Haworth and five Purdie methylations a portion of highly methylated (OMe, 42%) material was extracted with chloroform. Methanolysis and gas chromatography [12] of the product gave peaks with the retention times of methyl 2,3,4,6-tetra-*O*-methyl-, 2,3,6-tri-*O*-methyl-, and di-*O*-methyl-mannosides. After hydrolysis with formic acid, syrupy tetra-, tri-, and di-*O*-methylmannoses were separated. The first of these was identified by its chromatographic mobility and the fact that it gave only mannose on demethylation. The tri-*O*-methyl derivative was characterised by the formation of the crystalline di-*p*-nitrobenzoate. The di-*O*-methyl fraction also gave only mannose on demethylation and was not characterised further. No evidence for the presence of methylated glucose derivatives was obtained in this highly methylated fraction of the mannan.

These results are in keeping with the preliminary studies of Iriki and Miwa and show that the mannan synthesised by *C. fragile* is a β-1,4-linked mannan containing possibly a small proportion of 1,4-linked glucose units.

Experimental

The analytical methods used have been described by Percival and McKinnell.[13] R_c = mobility relative to cellobiose.

Extraction of the Mannan.—(*a*) The residual weed (*ca.* 20 g.), after decolourisation and exhaustive aqueous extraction, was stirred with 50% zinc chloride solution at room temperature overnight. After removal of residual weed the polysaccharide (3·6 g.) was isolated from the solution by precipitation with acetone after dialysis and concentration (Found: ash 31·5%). Attempts to remove the ash with hydrofluoric acid [14] were only partly successful and gave poor yields of polysaccharide.

(*b*) The residual weed (250 g. initially), after removal of colouring matter and exhaustive aqueous extraction, was stirred with water (350 ml.) containing glacial acetic acid (1 ml.) at 70°, and sodium chlorite (3 × 5 g.) was added at hourly intervals [6] during 3 hr. The residual weed, after washing with water, was extracted with 4% sodium hydroxide solution under nitrogen with stirring for 4 hr. Removal of the weed and neutralisation of the solution with acetic acid was followed by precipitation with acetone. The precipitate (5·01 g.), which had a low carbohydrate [7] and high ash content and was contaminated with protein (paper chromatogram of a hydrolysate sprayed with ninhydrin), was discarded. The residual weed was stirred with 20% sodium hydroxide solution (800 ml.) at 80° for 5 hr. under nitrogen. After cooling and removal of the residual weed, Fehling's solution was added until the polysaccharide was completely precipitated. The complex was washed with water (2 × 100 ml.) and decomposed by stirring with 1% ethanolic hydrogen chloride (200 ml.) until the precipitate was white. It was washed alternately with ethanol and acetone until the washings were colourless, dried with ethanol–ether, and the last traces of solvent were removed in vacuo. The product, hereinafter known as the mannan (5·6 g.), $[\alpha]_D$ −41° (*c* 0·28 in 95% formic acid) (Found: ash 2·3; H_2O 4·0%), was insoluble in 20% alkali. It contained mannose and glucose (paper

[12] Bishop and Cooper, *Canad. J. Chem.*, 1960, **38**, 388; Aspinall, *J.*, 1963, 1676.
[13] Percival and McKinnell, *J.*, 1962, 3141.
[14] Jones, Dimler, and Wise, *Analyt. Chem.*, 1956, **28**, 1352.

chromatography of a hydrolysate). Determination of the proportion of glucose by separation of the mannose and glucose in a hydrolysate on a paper chromatogram (butanol saturated with water) and estimation by the phenol–sulphuric acid method [15] gave a value of *ca.* 5%. Three sequential hydrolyses of an aliquot with 2N-sulphuric acid for 6 hr. and determination of the carbohydrate content (as mannose) of the combined supernatant liquors by the method of Somogyi [7] gave a value of 87%. The mannose solution had to be heated with reagent for 45 min. at 100° to obtain maximum reduction (cf. 20 min. for glucose and 30 min. for maltose).

Periodate Oxidation.—The mannan (48·3 mg.) was oxidised with 0·015M-sodium periodate (50 ml.) at 30° in the dark. Aliquots were removed at intervals and the amount of periodate reduced was determined (see Table).[16]

	Moles of periodate reduced * per C_6 anhydro-unit at 30°.					
Time (hr.)	8	19	25	43	67	91
Mole	0·25	0·47	0·66	0·74	0·80	0·87

* Calculated on the basis of 87% purity.

The reaction was stopped after 91 hr. by the addition of ethylene glycol (1 ml.). Potassium borohydride (10 mg.) was added and the mixture allowed to stand overnight. After dialysis (3 days) the polyalcohol was isolated by freeze-drying. Chromatography of a hydrolysate of this material indicated a trace of mannose.

Enzymic Hydrolysis of the Mannan.—The mannan (580 mg.), suspended by continuous stirring in water (100 ml.) in a dialysis sac,[9] was dialysed against distilled water for 2 days and the dialysate discarded. Commercial hemicellulase (57 mg.) (Light and Co.), shown to be free from oligosaccharides, was added to the suspension of polysaccharide in the dialysis sac and the whole maintained at 30°. Distilled water was circulated round the sac and the dialysate was collected at the rate of 1 ml./min. The reaction was allowed to proceed until the dialysate gave a negative reaction [15] for carbohydrate (7 days). Concentration of the total dialysate gave a syrup (200 mg.). Paper chromatography indicated the presence of mannose, manno-biose-triose, and tetraose, and higher oligosaccharides. The mixture (200 mg.) was applied to a number of Whatman 3MM papers (20 × 40 cm.) and the papers eluted in solvent 2 for 24 hr. The bands were located in the normal way and the following fractions were eluted with water.

Fraction 1. Syrupy D-mannose (34 mg.), chromatographically identical with D-mannose run as control (solvents 2 and 3), $[\alpha]_D$ +12° (*c* 0·34). The derived phenylhydrazone [17] had m. p. and mixed m. p. 187—188°.

Fraction 2. Crystalline 4-*O*-β-D-mannopyranosyl-β-mannose (44 mg.), $[\alpha]_D$ −11° (*c* 0·4), chromatographically identical with the authentic material run as control insolvents 2 and 3, R_c 0·92 in solvent 2. It had m. p. and mixed m. p. 193—196° (from methanol). An *X*-ray powder photograph was identical with that of the authentic material.

Fraction 3. Oligosaccharide (X) (3 mg.), R_c 0·59 in solvent 2. Chromatography of a hydrolysate of a portion revealed mannose and glucose. It had DP [11] = 2·01.

Fraction 4. Crystalline *O*-β-D-mannopyranosyl(1→4)-*O*-β-D-mannopyranosyl(1→4)-β-D-mannose (17 mg.) had $[\alpha]_D$ −23° (*c* 0·2), m. p. and mixed m. p. 178—180° (from ethanol). It was chromatographically identical with the authentic material run as control in solvents 2 and 3, and had R_c 0·50 in solvent 2. An *X*-ray powder photograph was identical with that of authentic material.

Fraction 5. Impure syrupy β-(1→4)-mannotetraose (12 mg.), R_c 0·19 in solvent 2.

Fraction 6. Syrup (35 mg.) containing higher oligosaccharides.

Acid Hydrolysis of the Mannan.—(*a*) Hydrolysis of the mannan (5 mg.) with 0·1M-hydrochloric acid at 100° for 4, 6, 7, and 16 hr. failed to yield any oligosaccharides. Mannose and glucose were the sole products apart from unhydrolysed material.

(*b*) Replacement of hemicellulase by 0·05N-polystyrenesulphonic acid [10] (kindly given by Dr. T. Painter) in the dialysis sac and elevation of the temperature to 80° failed to have any appreciable hydrolytic action on a fresh sample of the mannan.

Acetolysis of the Mannan.—The mannan (2·5 g.) was added slowly with stirring to a mixture of acetic anhydride (32 ml.), glacial acetic acid (32 ml.), and concentrated sulphuric acid (3 ml.) at 0°. The mixture was kept at room temperature for 72 hr., and the mannan, apart from a

[15] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.
[16] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 1216.
[17] Hirst, Jones, and Woods, *J.*, 1947, 1048.

negligile amount of dark coloured solid, dissolved after *ca.* 24 hr. The solid was removed by filtration and the filtrate was poured on to ice–water. The precipitated acetate was filtered off and dissolved in chloroform. The filtrate was brought to pH 3—4 with sodium hydrogen carbonate and extracted with chloroform (3 × 40 ml.). The combined extracts were washed with sodium hydrogen carbonate solution (2 × 50 ml.), and dried (Na_2SO_4) overnight. The residue obtained on removal of the chloroform was dissolved in chloroform (12 ml.) and methanol (24 ml.) at 0°, and to the solution barium methoxide (*ca.* 0·55 g.) in methanol (5 ml.) was added. The mixture was shaken for 1 hr. and set aside overnight at 2°. The cloudy viscous solution was exactly neutralised with sulphuric acid, and water (10 ml.) added. The aqueous layer, after filtration through a pad of acid-washed Celite, gave, on concentration, a cream friable solid (1·8 g.). Chromatography of this material in solvent 2 revealed spots with the mobility of mannose, glucose, mannobiose, mannotriose, and mannotetraose, in addition to a new spot with R_c 0·76 and a very faint spot, R_c 0·59, corresponding to disaccharide (X).

Separation of a New Disaccharide from the Acetolysate.—The foregoing mixture (0·5 g.) was applied to a number of Whatman 3MM papers (20 × 40 cm.) and the papers eluted in solvent 2 for 24 hr. The band containing the material of R_c 0·76 was detected in the usual manner and, after two re-separations on Whatman No. 1 paper, this oligosaccharide (Y) (2 mg.) [15] was isolated as a chromatographically pure syrup, R_c 0·76, M_G 0·58 in borate buffer pH 10,750 v. It had DP[11] 2·3 and gave, on hydrolysis, equal proportions of mannose and glucose [paper chromatogram eluted for 4 days with butanol saturated with water, spray *b*, visual examination]. After reduction of an aliquot with sodium borohydride in boric acid buffer, the product gave, on hydrolysis, a single spot on a paper chromatogram (eluted for 4 days with butanol saturated with water) corresponding to mannose (spray *a*). A similar chromatogram developed with spray *b* gave two spots corresponding to mannose and sorbitol–mannitol.

The remainder of (Y), after drying with alcohol and benzene, was methylated in freshly distilled dimethylformamide (0·2 ml.) with freshly distilled methyl iodide (0·2 ml.) and dry silver oxide (200 mg.) in the dark with shaking for 18 hr. The mixture was diluted with freshly dried chloroform (5 ml.) and filtered. The silver salts were washed with chloroform (3 × 5 ml.) and the filtrate and washings dried over anhydrous calcium sulphate overnight. The chloroform was removed under reduced pressure and the methylated disaccharide dried by the addition of benzene and evaporation, and refluxed with dry 3% methanolic hydrogen chloride for 4 hr. After neutralisation with silver carbonate and filtration, the silver salts were washed with dry methanol and the combined filtrate and washings concentrated to dryness. The derived syrup was subjected to gas chromatography [12] on a Pye Argon Chromatogram using argon as the mobile gas phase. The stationary liquid phase was supported on Celite and consisted of (a) apiezon M, (b) butanediol–succinate polyester, and (c) polyphenyl ether. The operating temperatures used were 150° for (a), 175° for (b), and 200° for (c). Peaks with the retention times of methyl 2,3,4,6-tetra-*O*-methylmannose–glucose, methyl 2,3,6-tri-*O*-methylmannose, and 2,3,6-tri-*O*-methylglucose were revealed on the gas chromatogram.

Methylation of the Mannan.—Mannan (1·5 g.) was dispersed in 12% sodium hydroxide (25 ml.) under nitrogen and, after the addition of potassium borohydride (0·5 g.), was set aside overnight. Sodium hydroxide (60 ml.; 30%) and dimethyl sulphate (20 ml.) were added with vigorous stirring during 6 hr. at room temperature under nitrogen, and the stirring was continued overnight. After repeating the methylation 5 times the mixture was brought to pH 8 with sulphuric acid and then dialysed for 6 days. The polysaccharide solution was concentrated to 25 ml., re-methylated 5 more times as before, and the product isolated (1·5 g.) by freeze-drying after neutralisation and dialysis. It was subjected to 5 Purdie methylations. After concentration of the filtrate and washings to dryness the residue was extracted (Soxhlet) with dry chloroform (200 ml.) for 12 hr. The chloroform was extracted with aqueous potassium cyanide (3 × 50 ml.) and water (2 × 50 ml.); during these extractions emulsions formed at the interface between the two layers. These, combined with the aqueous solutions, were dialysed and freeze-dried (0·77 g.). The dried ($CaSO_4$) chloroform solution was evaporated to dryness, the residue dissolved in acetone (30 ml.) and filtered, and the methylated mannan precipitated with light petroleum (b. p. 60—80°) (yield 120 mg.) (Found: OMe, 42; Calc. for $C_9H_{16}O_5$: OMe, 45·6%).

Examination of the Methylated Mannan.—(*a*) A portion (5 mg.) was methanolysed with 3% methanolic hydrogen chloride and the derived syrupy glycosides analysed by gas chromatography [12] as for the methylated disaccharide (Y). Peaks with the retention times of methyl

2,3,4,6-tetra-*O*-methyl- and 2,3,6-tri-*O*-methyl-mannoside were obtained, together with small peaks corresponding to two methyl di-*O*-methylglycosides.

(*b*) A portion of the partly methylated mannan (120 mg.) was hydrolysed for 8 hr. at 100° with 90% formic acid (10 ml.). Formic acid was removed by repeated addition of water, and the derived syrup hydrolysed for 2 hr. with N-sulphuric acid (20 ml.) at 100°. Neutralisation with Duolite A_4 resin, concentration to a syrup (92 mg.), and separation on Whatman 3MM paper gave:

Fraction 1. Syrupy 2,3,4,6-tetra-*O*-methylmannose (5 mg.) chromatographically identical with authentic material run as a control and which gave only mannose on demethylation.[18]

Fraction 2. Syrupy 2,3,6-tri-*O*-methylmannose (67 mg.). The 1,4-bis-*p*-nitrobenzoate had m. p. and mixed m. p. 189—190°.

Fraction 3. Syrup (3 mg.) with the chromatographic mobility of a di-*O*-methylmannose. Demethylation gave only mannose.

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CHEMISTRY DEPARTMENT, UNIVERSITY OF EDINBURGH.
CHEMISTRY DEPARTMENT, ROYAL HOLLOWAY COLLEGE,
UNIVERSITY OF LONDON.

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[18] Bonner, Bourne, and McNally, *J.*, 1960, 2929.