1299. *Carbohydrates of* Phaeodactylum tricornutum. *Part II.* A Sulphated Glucuronomannan*

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The material extracted with hot alkali from this organism comprises mannose, together with 27% of glucuronic acid and 7·5% of ester sulphate. It has been characterised, by acid hydrolysis, methylation, periodate oxidation, and Smith degradation, as a β-1,3-linked mannan with *O*-D-glucopyranosyluronic acid(1 → 3)-*O*-D-mannopyranosyl(1 → 2)-*O*-D-mannopyranose side-chains. Larger side-chains of similar constitution await complete characterisation. Most of the sulphate appears to be linked to mannose; no evidence for sulphated uronic acid was obtained.

The mannan isolated from the *Phaeodactylum* organism in 5% yield, although it comprised mainly mannose, was readily soluble in water, in contrast to the highly insoluble β-1,4-linked mannans of ivory nut,[1] coffee bean,[2] and *Codium fragile*.[3] The polysaccharide was found to have a carbohydrate content [4] of 70%, and equivalent of 418, a uronic acid content of 27%,[5] ester sulphate of 7·5%, and to be devoid of nitrogen. Such a uronic acid and sulphate content correspond to an equivalent of 434, a figure within 4% of the found equivalent. Both the acid and sulphate are present as sodium salts; allowing for this, for the 7·5% of sulphate and *ca.* 10% of moisture a 70% carbohydrate content is a reasonable figure.

Attempted fractionation of this glucuronomannan on a DEAE-cellulose column gave 33% recovery of polysaccharide which had the same chemical composition as the starting material. No copper complex was formed with Fehling's solution, another point of difference from β-1,4-linked mannans which complex strongly with copper.

Partial hydrolysis gave mannose, glucuronic acid, and three acidic materials of lower chromatographic mobility. Two of these were characterised by standard techniques as *O*-D-glucopyranosyluronic acid(1 → 3)-D-mannopyranose and *O*-D-glucopyranosyluronic acid(1 → 3)-*O*-D-mannopyranosyl(1 → 2)-D-mannopyranose, respectively. The over-oxidation of periodate by the former of these is in keeping with the ready over-oxidation of end-group glucuronic acid,[6] although this was much less pronounced in the trisaccharide. The chromatographic mobility of the third acidic fragment indicated *ca.* 6 units, and further hydrolysis gave mannose, glucuronic acid, some glucose, and small amounts of the above aldobio- and trio-uronic acids, indicating the essential similarity of this larger fragment to the other oligouronic acids.

The application of Smith degradation [7] (periodate oxidation, reduction, and mild acid hydrolysis) to this polysaccharide led to the recovery of 25% of a degraded polymer which comprised only mannose residues and *ca.* 10% of sulphate. It was proved that the latter was not due to contamination from sulphur dioxide used to destroy the excess periodate. But in view of the reported ability of carbohydrate sulphate to prevent precipitation of barium sulphate [8] it is not certain that all the sulphate is bound in ester linkage. Methylation and periodate oxidation showed that this degraded polymer comprises chains of *ca.* 15 1,3-linked mannopyranose units with occasional residues carrying sulphate groups. It appears to constitute the back-bone of the glucuronomannan. In the native polymer

* Part I, C. W. Ford and E. Percival, preceding Paper.

[1] G. O. Aspinall, E. L. Hirst, E. G. V. Percival, and I. R. Williamson, *J.*, 1953, 3184; G. O. Aspinall, R. B. Rashbrook, and (in part) G. Kessler, *J.*, 1958, 215.
[2] M. L. Wolfrom, M. L. Laver, and D. L. Patin, *J. Org. Chem.*, 1961, **26**, 4533.
[3] J. Love and E. Percival, *J.*, 1964, 3345.
[4] M. DuBois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Analyt. Chem.*, 1956, **28**, 350.
[5] Axel Johansson, B. Lindberg, and O. Theander, *Svensk Papperstidn.*, 1954, **51**, 41.
[6] R. A. Edington, E. L. Hirst, and E. Percival, *J.*, 1955, 2281.
[7] G. G. S. Dutton and A. M. Unrau, *Canad. J. Chem.*, 1962, **40**, 348.
[8] J. R. Helbert and M. A. Marini, *Biochim. Biophys. Acta*, 1964, **83**, 122.

it is considered that side-chains of the aldotriouronic acid, *O*-D-glucopyranosyluronic acid (1 → 3)-*O*-D-mannopyranosyl(1 → 2)-D-mannopyranose, and of the material constituting the third oligouronic acid, are attached by, as yet, undetermined linkages to this back-bone.

During the Smith degradation the 1,2-linked mannose units and the glucuronic acid residues are oxidised by the periodate, and on mild hydrolysis these are cleaved together with the unoxidised 1,3-linked mannose and glucose units present in the side-chains and are, respectively, responsible for the glycerol, glyceric acid, mannose, and traces of glucose found in the supernatant. The significance of the glucose in the overall structure of the molecule is difficult to assess. The amount present in the whole polysaccharide must be very small since all attempts to identify it with certainty in a total hydrolysate of the polysaccharide were unsuccessful.

The reduction of 0·45 mole of periodate per anhydro unit by the polysaccharide is in keeping with the proposed structure, since apart from the end-residues the main-chain should be immune to periodate, and the triouronic acid side-chains should reduce 3 moles for every three units. Since partial hydrolysis of the other side-chains indicates something of the same type of structure, any differences are probably too small to affect the overall oxidation picture. The increased reduction to 0·95 mole at room temperature is explained by the additional reduction at the reducing end of the main chains and the over-oxidation of the end-group glucuronic acid. The relatively high proportion of formic acid (0·39 mole per C_6 anhydro-unit) released on complete periodate oxidation supports the branched structure of the macromolecule.

Sulphate ester groups which are adjacent and *trans* to a free hydroxyl group in a polysaccharide are labile to alkali.[9] Any such groups present in the native glucuronomannan would have been lost during its extraction. Treatment of such sulphate groups with sodium methoxide causes their cleavage and the intermediate formation of epoxide rings.[9] Attack by the methoxide ion on either side and *trans* to the epoxide oxygen can then occur with the formation of monomethyl sugars. However, application of this procedure [9] to *Phaeodactylum tricornutum* residue (after reduction of the reducing group with borohydride to prevent degradation) before extraction with alkali failed to yield any methylated sugars on hydrolysis, from which it may be concluded that all the ester sulphate is still linked to the extracted glucuronomannan and is stable to alkali. In the absence of 1,3-linked glucuronic acid it also follows that the sulphate must be linked to mannose since any linked to glucuronic acid would be alkali-labile. On acid hydrolysis the rate of sulphate release [10] corresponded to a half-life of 1·51.

Experimental

The analytical methods were described in Part I.

Properties of the Glucuronomannan.—The glucuronomann, $[\alpha]_D$ +34° (450 mg.) isolated in Part I was completely soluble in water (Found: uronic anhydride,[5] 27; free SO_4^{2-}, nil; ester sulphate,[11] 7·5%; N_2, nil). Chromatography of a hydrolysate (solvents A, B, and Fischer and Dorfel [12]) revealed spots with the mobility of mannose (major constituent), glucuronic acid (sprays 1, 3, and 5), and three other acidic substances $R_{mannose}$ 0·4, 0·2, and 0·05 (solvent A). The proportion of carbohydrate [4] based on 73% of mannose and 27% of glucuronic acid as standard was 70%. The polysaccharide was converted into the free acid by repeated elution of an aqueous solution through a column of Amberlite IR-120 (H^+) resin. Potentiometric titration with carbon dioxide-free 0·01N-sodium hydroxide gave an equivalent of 418.

Characterisation of the Monosaccharides and Oligosaccharides Produced on Hydrolysis of the

[9] E. G. V. Percival, *Quart. Rev.*, 1949, **3**, 369; E. Percival and J. K. Wold, *J.*, 1963, 5459.
[10] D. A. Rees, *Biochem. J.*, 1963, **88**, 843.
[11] S. A. Jones and D. S. Letham, *Chem. and Ind.*, 1954, 662.
[12] F. G. Fischer and H. Dorfel, *Z. physiol. Chem.*, 1955, **301**, 224; **302**, 186,

Glucuronomannan.—The polysaccharide (1·8 g.) was hydrolysed with N-sulphuric acid at 100° for 1 hr. Ethanol (10 vol.) was added to the cooled solution and the precipitated polysaccharide rehydrolysed under the same conditions. This procedure of hydrolysis and alcohol precipitation was repeated until no alcohol-insoluble material remained. The combined supernatant solutions were neutralised and the derived syrup (1·4 g.) on chromatography (solvent A, spray 1) revealed spots with R_{mannose} 1·0, 0·4, 0·2, and 0·05, together with a trace of material with the speed of glucuronic acid. The syrup was separated on a cellulose column into four mixed fractions with solvent E. These were re-separated on 3MM paper and gave:

Fraction 1. A syrup which gave a single spot chromatographically and ionophoretically (pH 10), identical with that of mannose. The derived phenylhydrazone had m. p. and mixed m. p. with D-mannose phenylhydrazone, 197°.

Fraction 2. It was necessary to hydrolyse a fresh sample of polysaccharide for 4 hr. in order to obtain sufficient of this fraction for complete characterisation. It crystallised, and the crystals gave two spots on a paper chromatogram identical with glucuronic acid and its lactone. After recrystallisation from glacial acetic acid it had m. p. and mixed m. p. with D-glucurone 175°. An *X*-ray powder photograph was identical with that of authentic D-glucurone.

Fractions 3 *and* 4. Syrups (*ca.* 40 mg. of each) with R_{mannose} 0·4 and 0·2 (solvent A, spray 1) and M_{GA} 0·7 and 0·51, respectively. Separate acid hydrolyses of an aliquot of each and paper chromatography of the hydrolysates revealed mannose and a fainter spot corresponding to glucuronic acid from each fraction.

A dried aliquot of fraction 3 (3 mg.) suspended in dried tetrahydrofuran was reduced with lithium aluminium hydride in tetrahydrofuran which was added in small portions during 1·5 hr. with the mixture under reflux. After destruction of the excess lithium aluminium hydride and deionisation, the reduced product in dimethylformamide was methylated with methyl iodide and silver oxide.[13] The methylated material was methanolysed, and gas chromatography of the glycosides revealed methyl 2,3,4,6-tetra-*O*-methylglucosides and a number of unidentified peaks. Formation of the methyl ester methyl glycosides of aliquots of fraction 3 (15 mg.) and fraction 4 (15 mg.) and reduction with borohydride was followed by the hydrolysis of a portion (3 mg.) of fraction 4. Chromatography showed mannose and glucose. Incubation of the hydrolysate with the enzyme glucose oxidase followed by chromatography revealed the complete removal of material with the speed of glucose. The spot with the mobility of mannose was still present, and in addition a substance with the mobility of gluconic acid, run as control, was detected (spray 3). The remainder of the reduced methyl ester methyl glycosides were methylated and methanolysed. Gas chromatography gave peaks with the retention times, from fraction 3, of methyl 2,3,4,6-tetra-*O*-methylglucosides and 2,4,6-tri-*O*-methylmannosides. From fraction 4, in addition to the peaks given by the products from fraction 3, peaks with the retention times of methyl 3,4,6-tri-*O*-methylmannosides were also present. The products from each of these fractions were separately hydrolysed with N-sulphuric acid. Paper chromatography (solvent C, spray 1) showed spots corresponding to tetra-*O*-methylglucose and tri-*O*-methylmannose in both cases (2,4,6-tri-*O*-methylmannose and 3,4,6-tri-*O*-methylmannose have R_{G} 0·88). Separation of the methylated sugars gave:

From fraction 3. *Fraction* (i). A chromatographically and ionophoretically pure syrup identical with tetra-*O*-methylglucose, and which gave only glucose on demethylation.[14] *Fraction* (ii). A chromatographically and ionophoretically pure syrup, R_{G} 0·88, M_{G} (pH 10) 0·0. *From fraction* 4. *Fraction* (i). Identical with fraction (i) above. *Fraction* (ii). A syrup which on paper chromatography had the mobility of tri-*O*-methylmannose, but the spot appeared to comprise both pink (2,4,6-tri-*O*-methyl) and brown (3,4,6-tri-*O*-methyl) (spray 1). It gave only mannose on demethylation.[14] Ionophoresis (pH 10, spray 3) gave two spots, M_{G} 0·33 and 0·0. The former is characteristic of 3,4,6-tri-*O*-methylmannose and the latter of 2,4,6-tri-*O*-methylmannose.

The degree of polymerisation (D.P.) of each of these fractions was determined by a modification of the method of Timmell,[15] based on the comparison of the optical densities given by solutions treated with phenol–sulphuric acid reagents, using the reduced and unreduced fractions and mixtures of glucuronic acid and mannose in different proportions. The only structures

[13] O. Perila and C. T. Bishop, *Canad. J. Chem.*, 1961, **39**, 815.
[14] T. G. Bonner, E. J. Bourne, and S. McNally, *J.*, 1960, 2929.
[15] T. E. Timell, *Svensk Papperstidn.*, 1960, **63**, 668; C. W. Ford, Ph.D. Thesis (London), 1964.

which give the same values for the fractions and for the standards are that fraction 3 is glucuronosylmannose, and fraction 4 is an aldotriouronic acid comprising a glucuronosyl residue and two mannose residues.

Periodate oxidation of Fractions 3 *and* 4. Each fraction (7·0 and 13·3 mg., respectively) was converted into the ester–glycoside and oxidised with 0·01M-sodium metaperiodate in acetate buffer (pH 4·5). The reduction of periodate was measured [16] on aliquots withdrawn at intervals.

TABLE 1

Moles of periodate reduced per C_6 anhydro unit

Time (hr.)	12	24	92	132	144	214	312
Fraction 2	0·99	1·92	—	2·12	—	2·12	2·12
Fraction 3	1·07	1·07	1·25	—	1·25		

Fraction 5. A syrup (*ca.* 60 mg.) had $R_{mannose}$ 0·05, M_{GA} 0·2. Chromatography of a hydrolysate (N-H_2SO_4, 3 hr., 100°) revealed spots with $R_{mannose}$ 0·05 (unchanged material), 0·02 (aldotriouronic acid), 0·40 (aldobiouronic acid, faint), 0·82 (glucuronic acid/glucose) and 1·0 (mannose) (solvent A, spray 1); 0·0 (unchanged material), 0·05—0·1 (acidic material, strong), 0·61 (faint), 0·82 (glucose, medium), and 1·0 (mannose, strong) (solvent B, spray 3); M_{GA} 0·00 (mannose/glucose), 0·20 (unchanged material), 0·51 (triouronic acid), and 1·0 (glucuronic acid). The glucose was confirmed by conversion into gluconic acid by incubation with glucose oxidase.

Periodate Oxidation of the Glucuronomannan.—Two portions of polysaccharide (17·1 and 12·5 mg.) were oxidised, respectively, with 0·015M-periodate (20 ml.) at 2° and 0·1M-sodium periodate (5 ml.) at room temperature. Both oxidations proceeded in the dark, and aliquots were withdrawn at intervals and the reduction of periodate was measured.[16]

TABLE 2

Moles of periodate reduced per C_6 anhydro residue

Time (hr.)	3	6	18	30	48	66	96	120
At 2°	—	0·14	0·20	0·32	0·35	0·38	0·45	0·45
Room temp.	0·57	0·63	0·94	0·94	0·94			

Excess periodate was destroyed with sulphur dioxide, and the oxopolysaccharide precipitated with ethanol and reduced with borohydride. The polyalcohol after hydrolysis with N-sulphuric acid at 100° gave reducing spots with the mobility of mannose (strong), glucose (trace), and a trace of a non-reducing substance (spray 3) with the mobility of glycerol (paper chromatogram). Ionophoresis showed the complete absence of glucuronic acid and the presence of a trace of material M_{GA} 1·72 tentatively identified as glyceric acid.

A third portion of the glucuronomannan (123 mg.) in water (35 ml.) was brought to pH 5·8 and then oxidised with 0·1M-sodium periodate at 2° in the dark. After 4 days an aliquot (10 ml.) was removed and the excess periodate destroyed with ethylene glycol (0·5 ml.). The solution saturated with nitrogen for 5 min. was then titrated potentiometrically with carbon dioxide-free 0·005N-sodium hydroxide to a final pH 5·8. The amount of formic acid released was 0·39 mole per C_6 anhydro unit. The addition of ethanol (10 vol.) to the residual oxidation solution precipitated oxopolysaccharide (80 mg.). This was hydrolysed with N-sulphuric acid for 4 hr. at 100° and the derived sugars were reduced with sodium borohydride. After removal of excess borate and concentration, crystals separated. These had m. p. and mixed m. p. with mannitol 166°. The mother-liquor gave strong spots with the mobilities of mannitol and glycerol (solvent B, spray 3).

Smith Degradation of the Polysaccharide.—The polysaccharide (340 mg.) in acetate buffer (pH 4·9) was oxidised with 0·1M-sodium periodate (50 ml.). The reduction of periodate was measured [16] on aliquots withdrawn at intervals, and found to be complete after 60 hr. A portion (10 ml.) of the residual solution was treated with ethylene glycol to destroy excess periodate, and then dialysed. After concentration, the oxopolysaccharide was recovered by freeze-drying. The excess oxidant in the residual oxidation solution (39·5 ml.) was destroyed with sulphur dioxide, and the oxopolysaccharide recovered by ethanol (10 vol.) precipitation. Both oxopolysaccharides were reduced with borohydride and hydrolysed with N-sulphuric acid at

[16] G. O. Aspinall and R. J. Ferrier, *Chem. and Ind.*, 1957, 1216.

room temperature for 2 days. Addition of ethanol (10 vol.) to each solution precipitated a degraded polysaccharide (22·4 and 89·7 mg., respectively). These were removed and the combined supernatant solution on concentration gave a syrup. Chromatographic examination (solvent A, spray 2) revealed spots with the mobility of glucose and glycerol, together with several slower spots which streaked badly. After further hydrolysis of the syrup, material with the mobility of glucose, mannose, and glycerol, and considerably less of the material of lower mobility was detected (paper chromatography). After incubation with glucose oxidase the glucose had disappeared and a spot with the mobility of gluconic acid was revealed.

Each of the degraded polysaccharides gave only mannose on hydrolysis and each had an ester sulphate content of *ca.* 10%. They were combined, and an aliquot (16·3 mg.) was re-oxidised with 0·017M-sodium periodate at 2° in the dark.

TABLE 3

Moles of periodate reduced per C_6 anhydro unit

Time (hr.)	4	7	26	50
Degraded polysaccharide	0	0·102	0·195	0·195

The remaining degraded polysaccharide (*ca.* 85 mg.) was methylated in dimethyl sulphoxide (7 ml.) and dimethylformamide (4 ml.) with sodium hydroxide (5 g.), and dimethyl sulphate (4 ml.) added with stirring during 8 hr. in an atmosphere of nitrogen. The stirring was continued for 18 hr. and then the mixture was heated for 30 min. to decompose the residual dimethyl sulphate. Water (10 ml.) was added to dissolve solid sodium hydroxide, and, after cooling to 5°, the mixture was neutralised with 10N-sulphuric acid. After dialysis and concentration to small volume it was subjected to five Haworth methylations with 30% sodium hydroxide (15 ml.) and dimethyl sulphate (6 ml.) in each methylation. The partly methylated material isolated after dialysis by freeze-drying was extracted with dry chloroform. The small amount of chloroform-soluble material was subjected to three Purdie methylations. It was then methanolysed and examined by gas chromatography. The major peaks had the retention times of methyl 2,4,6-tri-*O*-methylmannosides, and smaller peaks with the retention times of methyl 2,3,4,6-tetra-*O*-methyl and 4,6-di-*O*-methylmannosides were observed. Paper chromatography of an acid hydrolysate showed spots with the mobilities and colours of tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl-, and di-*O*-methyl-mannoses.

Treatment of the Phaeodactylum *residue prior to Alkali Extraction with Sodium Methoxide.*—The material (1 g., 31% carbohydrate; 4% ester sulphate) remaining after aqueous extraction and chlorite treatment was suspended in water (40 ml.). To this a solution of sodium borohydride was added and the mixture set aside for 48 hr. It was then dialysed, concentrated, and freeze-dried. The derived material, after drying over phosphorus pentoxide, was treated with 4% sodium methoxide under reflux for 48 hr. A hydrolysate of the derived material gave a chromatogram identical with that of a hydrolysate of the initial material. No spot with the mobility of a methylated sugar could be detected.

Rate of Sulphate Hydrolysis.[10]—Samples of the polysaccharide in 0·25N-hydrochloric acid (0·25 ml.; containing 50 μg. of sulphate ester) were sealed in Pyrex tubes (3 × $\frac{3}{8}$ cm.) and heated at 100° for times ranging from 15 min. to 7 hr., and the quantity of sulphate liberated was determined with aminochlorobiphenyl reagent.[11]

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