480. *Polysaccharides from the Green Seaweed* Caulerpa filiformis. *Part II.* A Glucan of Amylopectin Type.*

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Addition of cetyltrimethylammonium hydroxide to the borate complexes of the mixture of water-soluble polysaccharides isolated from *Caulerpa filiformis* led to the separation of a pure glucan. Methylation and oxidation by periodate provided evidence that this glucan contains α-1,4′-linked glucose units with branches from $C_{(6)}$ and has an average chain length of *ca.* 24. The results of enzymic investigations are in harmony with this structure.

A starch-type polysaccharide, the so-called Floridean starch,[1] has been isolated from the red seaweed, *Dilsea edulis*, and an amylopectin-type polysaccharide has been obtained from the blue-green fresh-water alga, *Oscillatoria*.[2] However, although it is often accepted that the reserve carbohydrate of green seaweeds is a starch, there is no record of the isolation and characterisation of a starch-type polysaccharide. Hitherto fractionation of the complex mixture of water-soluble polysaccharides isolated from these seaweeds has not been achieved. O'Donnell and Percival[3] reported the separation of a glucose-rich fraction from the mixture of acetylated polysaccharides extracted from *Acrosiphonia centralis*, and characterised it as a starch-like polymer containing α-1,4′-linked glucose units with branches at $C_{(6)}$. The present paper describes an investigation of a glucan fractionated from the mixture of polysaccharides extracted by dilute acid from *Caulerpa filiformis*.

The water-soluble sulphated polysaccharides, $[\alpha]_D$ +120°, extracted from *Caulerpa filiformis* (see Part I) were treated as their borate complexes with an aqueous solution of cetyltrimethylammonium hydroxide. This precipitated sulphated polymeric material and left in the supernatant liquid a pure glucan which was precipitated as an amorphous powder by ethanol. It contained 98% of glucose and 0·9% of ash and had a limiting viscosity number $[\eta]$, measured in 0·1M-sodium chloride solution, of 15. An aqueous solution, which had $[\alpha]_D$ +154°, gave a purple colour with iodine, exhibiting maximum absorption at 540 mμ. On treatment with salivary α-amylase it gave an apparent percentage conversion into maltose of 90. The β-amylolysis limit was 57 and this was increased to 83 after treatment with isoamylase. Attempted fractionation by the thymol method[4] in the absence of oxygen failed to separate any amylose. Consumption of periodate by this polysaccharide corresponded to *ca.* 1 mole for every anhydro-sugar residue, and the oxo-polysaccharide, isolated after dialysis, was devoid of unattacked glucose units. This provides qualitative evidence that 1,2- and 1,3-glucosidic linkages are absent and that the polysaccharide contains 1,4′-linked glucose units with possible branch points at $C_{(6)}$. The production of formic acid on oxidation at room temperature with potassium meta-periodate[5] corresponded to an average chain length of 21.

The properties of this polysaccharide are compared with those of other branched 1,4′-linked glucans in the annexed Table. This reveals that the glucan from *C. filiformis* and the amylopectin component of the starch of land plants have many properties in common.

Methylation of the glucan was carried out with sodium and methyl iodide in liquid ammonia.[7] The low yield (*ca.* 50%) of partially methylated glucan isolated after a single

* Part I, Mackie and Percival, *J.*, 1959, 1151.

[1] Fleming, Hirst, and Manners, *J.*, 1956, 2831 and references cited therein.
[2] Hough, Jones, and Wadman, *J.*, 1952, 3393.
[3] O'Donnell and Percival, *J.*, 1959, 2168.
[4] Haworth, Peat, and Sagrott, *Nature*, 1946, **157**, 19.
[5] Hirst, Jones, and Roudier, *J.*, 1948, 1779; Bell and Manners, *J.*, 1954, 1891.
[6] Fleming, Hirst, and Manners, *J.*, 1956, 2831; Manners and Wright, unpublished work.
[7] Hodge, Karjala, and Hilbert, *J. Amer. Chem. Soc.*, 1951, **73**, 3312.

methylation is probably due to loss of shorter-chain material during dialysis of the methylated material. The loss of staining power to iodine caused by methylation of the glucan is further proof of an amylopectin-type structure since Hirst, Jones, and Roudier [5] record that methylated amylopectins give no appreciable colour with iodine.

Comparison of the properties of amylopectin, Floridean starch, glycogen, and the amylopectin-type glucan.

Property	Amylopectin [6]	Floridean starch [6]	Glycogen [6]	Glucan
$[\alpha]_D$ in H_2O	+212°	+176°	+196°	+154°
Iodine coloration	Purple	Deep red-dish-brown	Reddish-brown	Purple
$\lambda_{max.}$ of iodine complex	540	500	460	540
Optical density at $\lambda_{max.}$	1·06	0·84	0·34	0·68
β-Amylolysis limit	54	46	45	57
Iso- and β-amylolysis limit	76	54	65	83
α-Amylolysis (% P_M*)	88	65	70	90
Reduction of IO_4 (moles/anhydroglucose unit)	1·04	1·05	1·08	0·95
Average chain length	20	9	12	21
Limiting viscosity number	*ca.* 150	—	10	15

* P_M = Apparent conversion into maltose.

The tri-*O*-methyl derivative, $[\alpha]_D$ +200°, had a number-average molecular weight of 15,120, corresponding to 76 anhydroglucose units, when measured by the isothermal distillation method.[8] Although this value is small for an amylopectin-type polysaccharide, the method of extraction [dilute acid (pH 3—4) at 70° for 6 hours, followed by removal of protein with trichloroacetic acid] would degrade the polysaccharide considerably, and there is no doubt that in the native state the glucan has a much larger molecule.

The tri-*O*-methyl derivative was heated with methanolic hydrogen chloride, and the methylated methyl glucosides so obtained were hydrolysed with aqueous hydrochloric acid. The mixture of reducing sugars was separated on a column of powdered cellulose into 2,3,4,6-tetra-*O*-methylglucose (*ca.* 1 part), 2,3,6-tri-*O*-methylglucose (*ca.* 22 parts), 2,3-di-*O*-methylglucose (*ca.* 1 part), and a mixture (*ca.* 1 part) of 2,6- and 3,6-di-*O*-methylglucose which probably owed their origin to demethylation during hydrolysis. A trace of monomethylglucose, but no free glucose, was found. These results confirm the presence of α-1,4′-linked glucose residues with probable branch points at $C_{(6)}$; the α-linkage being inferred from the high positive rotations of the glucan and its trimethyl ether. The molecular proportion of tetra-*O*-methylglucose isolated corresponds to the presence of one non-reducing terminal glucose residue to every 28 glucose units in the molecule. This value, which is higher than the value of 21 found by periodate oxidation of the unmethylated material, is in agreement with the postulated loss of shorter-chain material during dialysis of the partially methylated polysaccharide.

Experimental

The analytical methods used have been described by O'Donnell and Percival,[3] the solvent system number (6) being used for paper partition chromatography.

Separation of the Glucan.—The water-soluble polysaccharide material (12 g.), isolated from *Caulerpa filiformis* (Part I), was dissolved in water (1 l.), and 0·6M-boric acid (400 c.c.) was added with stirring. Further addition of an aqueous solution of 0·1N-cetyltrimethylammonium hydroxide (180 c.c.) and 0·5N-sodium hydroxide [9] (4·0 c.c.) produced a flocculent precipitate (A). After removal of (A), further addition of the quaternary hydroxide yielded a negligible amount of precipitate. Addition of ethanol to the supernatant liquid gave a gelatinous precipitate (B).

Properties of the Glucan.—Adhering reagent was removed from material (B) by thorough washing with ethanol and ether. The resulting amorphous powder (3·9 g.) was soluble in

[8] Gee, *Trans. Faraday Soc.*, 1940, **36**, 1164; W. N. Broatch, Ph.D. thesis, Edinburgh, 1956.
[9] Bouveng and Lindberg, *Acta Chem. Scand.*, 1958, **12**, 1977.

cold water, and had $[\alpha]_D$ +154° (*c* 1·0) (Found: Ash, 0·9%; SO_4^{2-}, 0; N,0). An acid-hydrolysate (N-sulphuric acid at 100° for 4 hr.) contained only glucose (paper chromatography) (Found: glucose, by cuprimetric titration 98·0%) which was separated in 77% yield as crystals, m. p. and mixed m. p. with glucose hydrate 82°, $[\alpha]_D$ +52·0 (*c* 1·0). The polysaccharide gave a purple colour with iodine, exhibiting maximum absorption at 540 mμ.

Viscosity Determination (*with* Mr. A. Wright).—The specific viscosity (η_{sp}) of 0·1M-sodium chloride solutions of the polysaccharide was determined at several concentrations at 25°, and the limiting viscosity number $[\eta]$ determined graphically [10] from the relation $[\eta] = \lim_{c \to} \eta_{sp}/c$. Solvent time = 647·33 (T_0). Final concentrations (*c*) are expressed as g. per ml. of solution. Results were as tabulated.

	T (sec.)	$(T - T_0)/T$	$\eta_{sp.}/c$	*c*
15 ml.	738·0	0·1401	15·66	0·00895
,, + 5 ml. 0·1M-NaCl	714·18	0·1032	15·38	0·00671
,, + ,, ,,	700·14	0·0816	15·20	0·00537
,, + ,, ,,	691·50	0·0683	15·30	0·00447

From graph of $\eta_{sp.}/c$ against 10^3c we find $[\eta]$ = 15.

Enzymic Degradation.—(a) *Salivary α-amylase.* Polysaccharide (26·2 mg.), sodium chloride (5 mg.), and freeze-dried salivary α-amylase [11] (5 mg.; kindly supplied by Dr. D. J. Manners) in a total volume of 50 c.c. was incubated at 35° for 48 hr. The P_M value was 90. In a control experiment, potato amylopectin (25·8 mg.) gave a P_M value of 88.

(b) *Soya-bean β-amylase* (*with* Dr. D. J. Manners *and* Mr. A. Wright). Polysaccharide (12·8 mg.) was incubated with 0·2M-acetate buffer (pH 4·6; 3 c.c.) containing soya-bean β-amylase solution (0·05 c.c.; 1000 units) in a total volume of 25 c.c. After 48 hr. the β-amylolysis limit was 57%.

(c) *Isoamylase and β-amylase* (*with* Dr. D. J. Manners *and* Mr. A. Wright). Polysaccharide (20 mg.) in acetate buffer (pH 5·9; 6 c.c.) and water (5 c.c.) was treated with isoamylase solution (50 mg.) at room temperature for 65 hr. (The isoamylase was extracted from brewer's yeast by Dr. Zeenat H. Gunja.) After inactivation of the isoamylase by heat, denatured protein was centrifuged off. To the supernatant solution (10 c.c.), 0·2M-acetate buffer (pH 4·6; 5 c.c.), β-amylase (20 units per mg. of polysaccharide), and water (to 25·0 c.c.) were added.

Periodate Oxidation.—(i) *Uptake of periodate.* The dry glucan (447 mg.) was oxidised with 3% aqueous sodium periodate, and the reduction of periodate was measured at intervals: [12]

Time of oxidn. (hr.)	1	3·5	4·5	24	76	96	120
Moles of $NaIO_4$ consumed/$C_6H_{10}O_5$...	0·739	0·848	0·864	0·933	0·957	0·960	0·980

Chromatographic examination of the resulting solution of the oxopolysaccharide (isolated after 120 hours' oxidation) showed the absence of glucose.

(ii) *Oxidation by potassium metaperiodate.* Glucan (159 mg.), dissolved in 3% potassium chloride solution (80 c.c.), was oxidised with 4% sodium metaperiodate solution (20 c.c.) at room temperature in the dark.[5] Portions (10 c.c.) were analysed at intervals:

Time of oxidn. (hr.)	16	64	88	160	208	256
10^2 Moles of $H{\cdot}CO_2H/C_6H_{10}O_5$	0·91	3·1	3·8	4·2	4·7	4·7
Apparent chain-length (glucose residues)						21

Methylation in Liquid Ammonia.—The dried glucan (3·0 g.) was methylated in liquid ammonia with sodium and methyl iodide under the conditions used by Mackie and Percival (Part I). After addition of water the aqueous mixture was dialysed until free from inorganic ions. The partially methylated polysaccharide (1·75 g.; OMe, 18·6%) was isolated by freeze-drying and subjected to two further methylations under the same conditions. The product (1·6 g.) had $[\alpha]_D^{17}$ +200° (*c* 1·0 in $CHCl_3$) (Found: OMe, 45·3. Calc. for a tri-*O*-methylglucan: OMe, 45·6%), was soluble in water, ethanol, chloroform, and benzene, and gave no colour with iodine.

Determination of the Degree of Polymerisation by Isothermal Distillation.[7]—The methylated glucan (73·4 mg.) (after drying to constant weight; 24 hr. at 80°/12 mm. over P_2O_5) was dissolved in dried " AnalaR " benzene (6·953 g.), the solution having a concentration of 1·05%.

[10] Greenwood and Robertson, *J.*, 1954, 3769.
[11] Liddle and Manners, *J.*, 1957, 3432.
[12] Halsall, Hirst, and Jones, *J.*, 1947, 1399, 1427.

The changes in level of solution and solvent, in an apparatus kindly lent by Dr. T. G. Greenwood, were measured over a period of 100 hr. The graph of change in level with time was a straight line, and the slope of the graph was the rate of distillation, which is proportional to the solute mole fraction. The apparatus constant K ($2{\cdot}2 \times 10^{-3}$) was determined by measuring the rate of distillation for benzene with mole fractions of triolein (M, 885·4). The value of K was the same whether calculated for the change in solvent level or for the change in solution level. The slope of the graph was 0·237/95 mm. hr.$^{-1}$, and the calculated number-average molecular weight 15,120.

Hydrolysis of the Methylated Polysaccharide.—The material (1·1 g.) was refluxed with methanolic 3% hydrogen chloride (50 c.c.) until the rotation was constant (7 hr.). Water (150 c.c.) was added and, after removal of methanol under reduced pressure, the mixture was heated at 100° until the rotation was again constant (6 hr.). After neutralisation by silver carbonate, de-ionisation with hydrogen sulphide and Amberlite resins, and concentration, the resulting syrup (0·9 g.) was separated into its constituents on a cellulose column under the conditions used for the methylated xylan hydrolysate (Part I). The fractions were weighed after filtration through "Filter Cel," concentration to dryness, dissolution in methanol, filtration, and concentration.

Fraction I. Crystalline material (95·7 mg.), R_G 1·0, which had m. p. 40°, $[\alpha]_D$ −16°, was apparently a mixture of tetra-*O*-methylglucose and methyl 2,3,6-tri-*O*-methylglucoside. It was therefore heated with N-hydrochloric acid (5 c.c.) at 100° until the rotation was constant (14 hr.). Neutralisation, de-ionisation, and concentration gave a syrup which was separated into fractions I*a* and I*b* on a cellulose column (30 × 1·5 cm.).

Fraction I*a*. Crystalline 2,3,4,6-tetra-*O*-methylglucose (25 mg.), R_G 1·0, m. p. and mixed m. p. 84° after recrystallisation from ether, $[\alpha]_D$ +80° (*c* 2·4). The derived aniline compound had m. p. and mixed m. p. 135°.

Fraction I*b*. Crystalline 2,3,6-tri-*O*-methylglucose (70 mg.), R_G 0·83, had m. p. and mixed m. p. 115° after recrystallisation from ether (Found: OMe, 41·0. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction II. Syrupy methyl 2,3,6-tri-*O*-methylglucoside, after being heated with N-hydrochloric acid (5 c.c.) at 100° for 14 hr. and neutralised, gave syrupy 2,3,6-tri-*O*-methylglucose (228 mg.), R_G 0·83, $[\alpha]_D$ +70° (*c* 2·3).

Fraction III. Crystalline 2,3,6-tri-*O*-methylglucose (259 mg.), R_G 0·83, had m. p. and mixed m. p. 115° after recrystallisation from ether, $[\alpha]_D$ +98° (initial) ⟶ +70° (const.) (*c* 2·5), $[\alpha]_D$ +70° (initial), dropping to −35° (after 10 hr. in 1% HCl–MeOH at 18°; *c* 1·0) (Found: OMe, 41·1%). Total yield of 2,3,6-tri-*O*-methylglucose, 557 mg.

Fraction IV. Syrup (19·5 mg.), M_G 0·10, R_G 0·54 and 0·83 (trace), $[\alpha]_D$ +53° (*c* 2·0).

Fraction V. Syrupy 2,3-di-*O*-methylglucose (8·0 mg.). The M_G (0·10) and R_G (0·54) values were identical with those of 2,3-di-*O*-methylglucose, $[\alpha]_D$ +48°. The derived aniline compound had m. p. and mixed m. p. 132°.

Fraction VI. Syrup (34 mg.), $[\alpha]_D$ +42°, R_G 0·51. The M_G (0·05 and 0·65) values were identical with those of 2,6- and 3,6-di-*O*-methylglucose respectively.

Fraction VII. Syrup (8·3 mg.), R_G 0·25, corresponding to mono-*O*-methylglucose, M_G 0·85 identical with that of 3-*O*-methylglucose run as a control. Aqueous washing of the column failed to yield any further carbohydrate.

Examination of the Precipitated Sulphated Polysaccharide.—To regenerate the polysaccharides, the precipitate (A) (see above), after thorough washing with ethanol, was dissolved in warm M-sodium chloride, and the mixture was poured into ethanol. The precipitate (C) was obtained as a white powder after filtration and drying. Hydrolysis with N-sulphuric acid at 100° for 7 hr. and chromatography of the resulting syrup showed the presence of galactose, glucose, mannose, xylose, and rhamnose. Incubation of the polysaccharides (C) with salivary α-amylase, followed by dialysis and precipitation with ethanol, gave material which was devoid of glucose. Fractionation of this glucose-free sulphated material is in progress and will form the subject of a future communication.

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