577. *Polysaccharides from the Green Seaweeds of* Caulerpa *spp. Part III.*[1] *Detailed Study of the Water-soluble Polysaccharides of* C. filiformis: *Comparison with the Polysaccharides Synthesised by* C. racemosa *and* C. sertularioides.

By I. M. Mackie and Elizabeth Percival.

Arabinose, galactose, xylose, and mannose have been separated and characterised from a hydrolysate of a water-soluble extract of *C. filiformis* harvested in November. Fractionation provides evidence for the presence of more than a single polysaccharide in this extract.

Preliminary studies on *C. racemosa* and *C. sertularioides* have established the essential similarity of their polysaccharides with those of *C. filiformis*, though the two former species appear to be devoid of arabinose-containing polysaccharide, and the proportion of starch-type polysaccharide is considerably less than in *C. filiformis*.

A third sample of *C. filiformis* was collected in November in S. Africa from the same site as the two earlier batches of weed which had been harvested in February.[2] The properties of the water-soluble extract (D), from this third batch of weed, and that of the water-soluble polysaccharide (C) [1] separated from the first two samples of weed, are compared in Table 1 (all the figures refer to extracts after removal of the amylopectin-type glucan with salivary α-amylase).

Table 1.

	Ash (%)	Sulphate (%)	$[\alpha]_D$	Nitrogen (%)	Approx. molar propns. of sugars: Galactose	Mannose	Xylose	Arabinose
Polysaccharide (C)	14·0	16·4	+10·7°	3	5	2	1	0
Polysaccharide (D)	15·2	17·6	+12·8	2·9	5	2	2	1

No trace of ketose could be found in the hydrolysates of either polysaccharide, but both gave positive tests for 3,6-anhydro-hexoses. It is seen that polysaccharide (D)

[1] Part II, Mackie and Percival, *J.*, 1960, 2381.
[2] Mackie and Percival, *J.*, 1959, 1151.

differs from polysaccharide (C) in that it contains arabinose units. The third sample of weed from which (D) was isolated had been extracted under milder conditions than the earlier samples, and the arabinose might have been lost during extraction of the first two samples though treatment of extract (D) under the conditions used in the earlier extractions failed to remove the arabinose. The individual sugars present in the hydrolysate of polysaccharide (D) were separated on a cellulose column and fully characterised. (The proportions of sugars isolated differed from those determined colorimetrically by Wilson's method, but this is attributed to the difficulty of separating on a cellulose column pure samples of xylose, arabinose, and mannose from mixtures containing these three sugars. At the same time it should be mentioned that the recorded yields are those obtained after extensive purification.) In addition to the sugars given in Table 1 traces of L-rhamnose, ribose, L-fucose, and glucose were separated and tentatively identified. The ribose is considered to have arisen from contaminating nucleic acids.

Application of the standard method devised by O'Neill [3] for the determination of 3,6-anhydro-hexose gave a value of about 1% for this polysaccharide extract.

Fractional precipitation of polysaccharides (D) and (C) with ethanol, acetone, Fehling's solution,[4] cupric acetate,[5] cetyltrimethylammonium hydroxide at various pHs,[6] and cetylpyridinium chloride [6] all yielded precipitates which contained the same proportions of sugars as the original materials. Treatment of an aqueous solution either with potassium chloride solution [7] or with Amberlite IR-400 (OH) resin failed to induce any fractionation. No precipitate was deposited with the former and no absorption of polysaccharide occurred with the latter. Partial fractionation of the free acid polysaccharide (D) was achieved with saturated barium hydroxide.[8] By this means three fractions were obtained (see Table 2). Complete fractionation was not achieved although it seems probable from the partial fractionation that more than a single polysaccharide is present in this extract.

Partial hydrolysis with acid or with taka-diastase failed to yield any oligosaccharides. Although this mixture of enzymes was able to degrade the polysaccharide extract to each of the constituent monosaccharides degradation was incomplete, and the residual polysaccharide material still contained the original mixture of sugar residues and the same proportion of sulphate groups. Evidence that at least some of the sulphate residues are located on the galactose units was provided by the separation of a small quantity of a galactose monosulphate from a partial acid hydrolysate.

The relatively small reduction of periodate by polysaccharides (D) and the presence of each of the sugars in the hydrolysate of the oxopolysaccharide, with possibly a slightly

TABLE 2. *Fractionation by barium hydroxide.*

	Weight (mg.)	Sulphate (%)	$[\alpha]_D$	Sugars (mol. %) Galactose	Arabinose	Xylose	Mannose
Polysaccharide (D) ...	500	17·6	+12·8°	49·4	9·2	18·9	22·5
Fraction 1	128	17·4	+12·6	46·1	6·9	21·5	25·5
,, 2	93	18·1	+12·7	61·3	12·0	13·6	13·1
,, 3	222	18·3	+13·2	62·2	12·1	13·2	12·5

increased proportion of glucose and rhamnose, confirm the complexity of this material. Either it has a highly branched structure or the majority of the units are 1,3'-linked. Indeed a 1,3'-linked xylan and glucan have already been isolated from *C. filiformis* [2] and it is very probable that the xylose- and glucose-containing polysaccharides in extract (D) are the same as those characterised earlier in an alkaline extract. Similarly, by analogy

[3] O'Neill, *J. Amer. Chem. Soc.*, 1955, **77**, 2837.
[4] Chanda, Hirst, and Percival, *J.*, 1951, 1240.
[5] Erskine and Jones, *Canad. J. Chem.*, 1956, **34**, 821.
[6] Scott, *Chem. and Ind.*, 1955, 168; Barker, Stacey, and Zweifel, *ibid.*, 1957, 330.
[7] Smith and Cook, *Arch. Biochim. Biophys.*, 1953, **45**, 232.
[8] Meier, *Acta Chem. Scand.*, 1958, **12**, 144.

with the sulphated galactans separated from red seaweeds,[9] it is likely that the present galactan consists of 1,3′-linked units carrying sulphate groups at position 4. Mannans hitherto extracted from algæ[10] appear to consist of unsulphated 1,4′-linked residues. From the periodate-oxidation results the present mannan must be different and the majority of the mannose units are either linked to sulphate or are themselves 1,3′-linked. The rotation of the polysaccharides is low ($[\alpha]_D$ +12·8°), indicating that the majority of the glycosidic linkages are β.

Earlier studies on *C. filiformis* revealed that the major polysaccharide synthesised by this green seaweed is a β-1,3′-linked xylan,[2] a result that has been substantiated by Iriki *et al.*[10b] for *C. anceps*. In addition, a water-soluble mixture of polysaccharides was extracted from *C. filiformis* and fractionated into an amylopectin-type glucan and a sulphated mixture containing galactose, mannose, and xylose.[1] Similar extractions of *C. racemosa* and *C. sertularioides* have shown that these two species also synthesise mainly xylans which in the case of *C. racemosa* have been shown from periodate studies to be mainly 1,3′-linked. The water-soluble extracts of *C. racemosa* and *C. sertularioides*, however, contain a much lower proportion of glucose-containing polysaccharide than *C. filiformis*, and they, like the first two samples of *C. filiformis*, are devoid of arabinose-containing polysaccharides. Both these may be seasonal and environmental variations. However, until arabinose-containing polysaccharides have been found in other samples of *Caulerpa* spp. they cannot be regarded as characteristic of this genus of green seaweeds.

Comparison of the polysaccharides synthesised by *Caulerpa* spp. with those of other green algæ reveals some similarities and some major differences. In all cases aqueous extraction has yielded a complex mixture of water-soluble sulphated polysaccharides which have resisted fractionation to homopolysaccharides. *Acrosiphonia centralis*,[11] *Ulva lactuca*,[12] and *Enteromorpha* spp.[13] all yielded aqueous sulphated extracts comprising mainly glucose, xylose, rhamnose, and uronic acid. In contrast there is no indubitable evidence for the presence of uronic acid units in the polysaccharides of *Cladophora rupestris*;[14] and its water-soluble extract consists of L-arabinose, D-galactose, D-xylose, and smaller amounts of D-glucose and L-rhamnose. While *Caulerpa* spp. more closely resemble *Cladophora* spp. in the polysaccharide content of the aqueous extracts they differ from all the other genera of green algæ so far examined in the presence of the β-1,3′-linked xylan as the main polysaccharide constituent. Indeed, even after exhaustive alkaline extraction xylose residues are the major carbohydrate units present in the residual weed. It appears that this polysaccharide is closely integrated into the cell wall of the seaweed and replaces cellulose as the structural polysaccharide, a feature which is in accord with the *X*-ray studies of *Ulva lactuca* and *Enteromorpha* spp. by Cronshaw *et al.*[15]

Experimental

The analytical methods used have been described by O'Donnell and Percival.[11]

Two fresh samples of *Caulerpa filiformis* were collected from the same site as the original sample,[2] the first in February 1958 and the second in November 1959. After removal of most of the colouring matter and mono- and di-saccharides by extraction with 80% aqueous ethanol a second supply of the water-soluble sulphated polysaccharide material (C) was extracted from the weed collected in February 1958 by dilute acid at 70° [2] (yield, 2·7% of dry weight of weed). In contrast, weed collected in November 1959, after aqueous-alcholic extraction, was extracted repeatedly with cold water for 24 hr. periods and the extracts were combined and concentrated (D). Both extracts (C and D) were separately digested twice with salivary α-amylase for 24 hr. to remove all the starch-type glucan [1] and then deproteinised with trichloroacetic acid.[14]

[9] Hirst, *Proc. Chem. Soc.*, 1958, 177.
[10] (*a*) Jones, *J.*, 1950, 3293; (*b*) Iriki, Suzuki, Nisizawa, and Miwa, *Nature*, 1960, **187**, 82.
[11] O'Donnell and Percival, *J.*, 1959, 2168.
[12] Brading, Georg-Plant, and Hardy, *J.*, 1954, 319.
[13] Lowe, Ph.D. Thesis, London, 1956.
[14] Fisher and Percival, *J.*, 1957, 2666.
[15] Cronshaw, Myers, and Preston, *Biochim. Biophys. Acta*, 1958, **27**, 89.

Ionic material was removed from the derived solutions by dialysis, and the polysaccharides (C) (yield 2%) and (D) (yield 0·7%) were precipitated with ethanol as off-white powders. The proportions of sugars present in comparable hydrolysates (N-sulphuric acid for 7 hr. at 100°) were determined by Wilson's method [16] after elution with solvent (1) on a paper chromatogram for 60 hr. These and the properties of the two extracts are given in Table 1.

Quantitative determination of the 3,6-anhydro-hexose in polysaccharide (D) [3] (28·0 mg., 18·6 mg.) gave percentage absorbances at 285 mμ of 0·050 and 0·045 respectively. These correspond to 0·9% and 0·8% of 5-hydroxymethyl-2-furfuraldehyde and 1·2 and 1·1% of anhydro-hexose.

Separation and Characterisation of the Sugars Present in Polysaccharide (D).—The polysaccharide was hydrolysed with N-sulphuric acid at 100° for 8 hr. After neutralisation of the cooled solution with barium carbonate and deionisation with resins the derived syrup (0·52 g.) was separated on a cellulose column which was eluted with butan-1-ol half saturated with water, and the following fractions were collected (R_F values refer to solvent 1 of ref. 11):

Fraction I. A syrup (8·2 mg.), R_F 0·55, $[\alpha]_D$ +7·8°, chromatographically identical with L-rhamnose.

Fraction II. A syrup (3·6 mg.), R_F 0·47, chromatographically indistinguishable from ribose.

Fraction III. A syrup (2·9 mg.), R_F 0·45, with a negative rotation and chromatographically identical with L-fucose.

Fraction IV. Crystalline D-xylose (36·3 mg.), m. p. and mixed m. p. 143°, R_F 0·42, $[\alpha]_D$ +19·6° (*c* 1·0). The derived dibenzylidene dimethyl acetal [17] had m. p. 210° undepressed by admixture with a sample prepared from authentic D-xylose.

Fraction V. A syrup (71 mg.), R_F 0·36, was contaminated with fractions (IV) and (VI). Separation on Whatman No. 3 paper gave a syrup (17·2 mg.) chromatographically indistinguishable from L-arabinose. It had $[\alpha]_D$ +105° (*c* 1·7). The derived benzoylhydrazone had m. p. and mixed m. p. 189°.[18]

Fraction VI. A syrup (72 mg.), R_F 0·36, indistinguishable from D-mannose, had $[\alpha]_D$ +14·2° (*c* 1·2). The derived phenylhydrazone [19] had m. p. and mixed m. p. 196°.

Fraction VII. A syrup (6·5 mg.), R_F 0·31, identical with glucose.

Fraction VIII. Crystalline D-galactose (231·2 mg.), m. p. and mixed m. p. 167°, R_F 0·25, $[\alpha]_D$ +75·5° (*c* 1·1). The derived diethyl mercaptal [20] had m. p. and mixed m. p. 140°.

Fractionation of Polysaccharide (D) *with Saturated Barium Hydroxide.*[8]—Polysaccharide (D) (500 mg.) in water (100 c.c.) was re-cycled six times through a column of Amberlite IR-120 (H) resin (80 c.c.). To the derived acid eluate (pH 3·5), diluted to 1 l. with water, was added dropwise a saturated solution of barium hydroxide until precipitation was complete (precipitate 1). Addition of ethanol (3 vol.) to the dialysed concentrated supernatant solution precipitated the residual polysaccharide. This was redissolved in water (500 c.c.), re-converted into the free-acid, and treated with saturated barium hydroxide. The precipitated polysaccharide (precipitate 2) was removed, and addition of alcohol to the dialysed concentrated supernatant liquid deposited precipitate 3. All three fractions were washed with water to remove contaminating barium hydroxide and vigorously stirred in 6% acetic acid solution. The regenerated polysaccharides were precipitated with ethanol, and the precipitates washed free from acid with ethanol and dried with ether. The properties and relative proportions of the sugars present in each of these fractions are given in Table 2.

Separation of a Galactose Monosulphate from a Partial Acid Hydrolysate.—The polysaccharide (D) (987 mg.) was hydrolysed with 0·25N-sulphuric acid for 1 hr. at 100°. Unhydrolysed polysaccharide and sulphate ions were precipitated from the cooled solution by the addition of barium hydroxide until pH 7·5 was attained. The supernatant solution was passed down a column of IR-4B(OH) resin (200 c.c.) at the rate of about 200 c.c. an hour. The column was washed free from neutral sugars with water (2 l.), and the sugar sulphate eluted from the resin with 2N-ammonia (200 c.c.). Concentration of the eluate at room temperature gave a white solid (4·4 mg.). Chromatography (solvent 3) gave a single spot $R_{Gal.}$ 0·32, dark blue

[16] Wilson, *Analyt. Chem.*, 1959, **31**, 1199.
[17] Breddy and Jones, *J.*, 1945, 738.
[18] Hirst, Jones, and Woods, *J.*, 1947, 1048.
[19] Butler and Cretcher, *J. Amer. Chem. Soc.*, 1931, **53**, 4358.
[20] Wolfrom, *J. Amer. Chem. Soc.*, 1930, **52**, 2466.

with Toluidine Blue spray [21] and dark brown with aniline hydrogen phthalate spray. No spot for free galactose could be detected. Hydrolysis of a portion by N-sulphuric acid for 4 hr. at 100° and chromatography (solvent 3) of the neutralised hydrolysate gave a single spot identical in speed and colour with galactose run as a control.

Enzymic Hydrolysis with "Taka-diastase." [22]—Commercial "taka-diastase," freed from contaminating mono- and oligo-saccharides by dialysis, was incubated at 37° with polysaccharide (D) (199 mg.) in water (250 c.c.) in a dialysis sac in a closed system. Chromatographic examination, after 24 hr., of the concentrated dialysate revealed the presence of arabinose with smaller amounts of galactose, mannose, and xylose. No indication for the presence of oligosaccharides was obtained. After digestion for 7 days, during which the water surrounding the dialysis sac was changed frequently, the residual polysaccharide (104 mg.) was isolated (Found: SO_4^{2-}, 17·9%). The combined dialysates yielded on concentration a syrup (52 mg.) consisting of arabinose, galactose, mannose, and xylose. A hydrolysate of the residual polysaccharide contained all four sugars in the same proportions as the hydrolysate from polysaccharide (D) (paper chromatography, visual examination).

Periodate Oxidation of Polysaccharide (D).—The polysaccharide (0·175 g.) was dissolved in 0·1030M-sodium periodate (50 c.c.). The reduction of periodate was measured on samples (1 c.c.) withdrawn at intervals: [23]

Time (hr.)	0	6	24	48	96
Moles of periodate reduced per $C_6H_{10}O_5$ unit	0	0·145	0·189	0·227	0·236

After 96 hr. 1 mole of periodate was reduced by 686 g. of polysaccharide (it being assumed that the contaminating nitrogen-containing material had no action on the periodate). The oxidation was stopped by addition of an excess of ethylene glycol (10 c.c.). After dialysis, freeze-drying afforded the oxopolysaccharide (108 mg.). Chromatography of the hydrolysate revealed the presence of galactose, mannose, arabinose, xylose, glucose, and rhamnose. The first four of these sugars appeared to be present in the same, and the last two in slightly increased, proportions compared with those of the original polysaccharide. A similar oxopolysaccharide was isolated after oxidation for 14 days.

Preliminary Examination of the Polysaccharides of C. racemosa *and of* C. sertularioides.—Samples of *C. racemosa* (Forsk) Weber-van Bosse var. *clarifera* (Turn.) Weber-van Bosse (29 g.) and *C. sertularioides* (Gmal.) Harv. var. *farlowii* Weber-van Bosse (26 g.) collected in October from dead coral in strong surf on the Gata Islands, have been investigated. Both weeds, after removal of colouring matter with 80% aqueous ethanol, were exhaustively extracted with cold water (500 c.c.) in an atmosphere of nitrogen. Partial removal of contaminating protein with trichloroacetic acid by the method used on the *C. filiformis* extracts was followed by precipitation of the polysaccharides with ethanol (4 vol.). After being washed with ethanol and ether these were dried to cream-coloured powders (0·72 g., 2·5%; 0·91 g., 3·5% yield, respectively). From the residual weeds xylans were extracted with alkali.[2] It was necessary to add ethanol to the acidified alkaline extract from *C. sertularioides* to precipitate the xylan. The yield of xylan from *C. racemosa* was 1 g., *i.e.*, 3·3%, and from *C. sertularioides* was 0·50 g., *ca.* 2·0%.

Both water-soluble extracts had a high sulphate content (*ca.* 20·0%). Chromatography of hydrolysates (N-sulphuric acid, 7 hr. at 100°) revealed the presence of the following sugars:

	Galactose	Glucose	Mannose	Xylose	Rhamnose
C. racemosa	× × × × ×	×	× × ×	× × ×	×
C. sertularioides	× × × × ×	×	× × ×	× × ×	×

× = trace

Hydrolysis of the xylans and chromatographic analysis revealed in *C. racemosa* a trace of glucose and much xylose, and in *C. sertularioides* about half as much glucose as xylose.

Examination of the hydrolysates of the residual weeds after removal of the xylans showed the presence of a large proportion of xylose units together with a small quantity of glucose.

[21] Ricketts, Saddington, and Walton, *Biochem. J.*, 1954, **58**, 532.
[22] Courtois, Kada, and Petek, *Bull. Soc. Chim. biol.*, 1958, **40**, 2031.
[23] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 196.

Oxidation of the xylan (323·6 mg.) from *C. racemosa* with 0·103M-sodium periodate (50 c.c.) was carried out at room temperature:

Time (hr.)	0	24	48
Moles of periodate reduced per $C_5H_8O_4$ unit	0	0·120	0·126

The oxidation was complete in 24 hr. and corresponded to the reduction of one mole of periodate for every 8·5 anhydro-xylose units. Slow oxidation subsequently occurred, possibly due to the contaminating protein.[24]

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DEPARTMENT OF CHEMISTRY, UNIVERSITY OF EDINBURGH. [*Received, February 6th*, 1961.]

[24] Bragg and Hough, *Biochem. J.*, 1961, **78**, 11.