345. *The Water-soluble Polysaccharides of* Cladophora rupestris. *Part II.*[1] *Barry Degradation and Methylation of the Degraded Polysaccharide.*

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Repeated successive treatment of the water-soluble polysaccharide, cladophoran, with periodate and with phenylhydrazine gave a 25% yield of a degraded polymer containing L-arabinose, D-galactose, and L-rhamnose and 15% of sulphate groups. Evidence is presented that sulphate groups are linked to sugar residues other than galactose. Methylation studies established the linkages present in the degraded material. The significance of these results in relation to the whole polymer is discussed.

The water-soluble extract from the green seaweed, *Cladophora rupestris*, is a heteropolysaccharide material, which failed to separate into simple polymers when subjected to the usual fractionation techniques. The presence of 20% of organically bound sulphate made complete methylation difficult. However, separation and characterisation of a number of the sugars in the hydrolysate of the methylated material revealed some of the linkages present in the original polysaccharide, although it was not possible to advance any general structure for the polysaccharide or to allocate the sulphate residues to any particular sugar.[1]

Repeated degradation by Barry's method [2] appeared to offer a means of obtaining information on the structure of the inner part of this molecule. A single Barry degradation involves oxidation of the polysaccharide with periodate, followed by treatment of the oxo-polysaccharide with phenylhydrazine. This treatment removes all the residues in the original molecule which contained α-glycol groups and new vicinal hydroxyl groups are exposed in the degraded polymer. The process can then be repeated, and in this way successive layers of residues are removed from the periphery of the molecule. In the present experiments the reduction of periodate and the production of formic acid were measured for each oxidation. At the same time the oxo- and degraded polymers were

[1] Part I, Fisher and Percival, *J.*, 1957, 2666.
[2] Barry and McCormick, *J.*, 1957, 2777; Finan and O'Colla, *Chem. and Ind.*, 1958, 493, 1404.

isolated at each stage and their constituent sugars and sulphate and nitrogen contents were determined (see Tables 1 and 2).

TABLE 1.

	IO_4 reduced (moles/kg.)	Formic acid (moles/kg.)	Yield g.	Yield % †	N (%)
O_1 *	2·82	1·8	18·2	89	—
O_1D_1	—	—	13·7	63	3·5
D_1O_2	1·97	0·9	9·9	80	2·7
O_2D_2	—	—	6·3	66	4·2
D_2O_3	2·52	1·0	4·8	73	2·4

* O and D refer to the respective oxo- and degraded polysaccharides isolated. The second letter indicates the nature of the last treatment and the subscripts correspond to the number of such treatments.

† In calculating percentage yields of oxo-polysaccharide, allowance is made for material consumed during measurements.

Analysis of the hydrolysate of the oxo-polysaccharide O_1 confirmed the cleavage of all the xylose and a considerable proportion of the galactose units. For these sugars to be attacked by periodate they must be present as end-group or linked only through positions 1 and 4 (and/or 6 in the case of galactose) in the original polysaccharide. Glyoxal bisphenylhydrazone was isolated from the ethereal extract of the degraded material O_1D_1. This is in keeping with the cleavage of 1 : 4-linked xylose and galactose residues. No

TABLE 2.

	Molar proportions of sugars [3]					Ash (%)	SO_4 (%)	N (%)
	Arabinose	Galactose	Glucose	Xylose	Rhamnose			
Cladophoran	4·0	3·0	0·23	1·1	0·5	15·7	18·3	1·7
O_1	4·0	0·88	0·35	—	0·67	14·9	19·2	1·5
O_1D_1 *	—	—	—	—	—	15·6	16·6	3·5
D_1O_2	4·0	0·89	—	—	0·65	12·3	17·5	2·7
O_2D_2 *	—	—	—	—	—	12·8	16·9	4·2
D_2O_3	1·0	1·04	—	—	0·56	13·4	15·2	2·4

* A sample hydrolysate of the degraded material was chromatographically indistinguishable from that of the oxo-polysaccharide (visual examination).

other phenylhydrazone fragments were detected in this extract, although the presence of a large amount of *N*-acetylphenylhydrazine may be responsible for the failure to detect glycerosazone which has very similar solubility and chromatographic properties.

The second oxidation consumed one mole of periodate for every three sugar residues. Apparently all the glucose residues (*ca.* 5% of the molecule) were attacked, since this sugar was absent from the hydrolysate of the oxo-polysaccharide D_1O_2. The relative proportions of the other sugars were unchanged, and they must therefore have been attacked to approximately the same extent in this second oxidation. The disappearance of glucose, which was verified by a second experiment, is difficult to understand. Earlier work on cladophoran had indicated that at least part of the glucose was present as a separate 1 : 3-linked glucan. This would be immune to periodate attack, except at the ends of the chains. It may be that the glucan is a small, highly branched molecule with other linkages also present and that two oxidations are necessary to remove the branches and leave a linear molecule of dialysable proportions.

During the third oxidation, a large proportion of the arabinose residues were attacked, and an oxo-polysaccharide D_2O_3 containing arabinose : galactose : rhamnose in the molar proportions of 1 : 1 : 0·56 and representing *ca.* 25% of the original material was isolated.

Degradation removes those units from the polymer that have previously been attacked by periodate. The calculated yields of O_1D_1 and of O_2D_2, based on this loss, are in good

[3] Pridham, *Analyt. Chem.*, 1956, **28**, 1967.

agreement with the actual yields of the respective degraded polymers (see Table 1). Although the nature of the nitrogen-containing groups in the degraded material is uncertain, they are apparently attacked by periodate since the nitrogen content is reduced on oxidation.

Complete methylation of the degraded polymer D_2O_3 was carried out by the method of Fear and Menzies.[4] After hydrolysis of the methylated material with hydrochloric acid, and neutralisation with silver carbonate, the mixture of neutral methylated sugars and inorganic salts was evaporated to dryness. Exhaustive extraction of the residue with water was efficacious in leaving brown nitrogenous material adsorbed on the precipitated silver salts. The neutral methylated sugars were separated on a cellulose column, and the remainder of the contaminating nitrogenous material was removed in the first fraction as a fast-flowing mixture which was discarded. 2 : 4-Di-*O*-methylrhamnose (1 part), 2 : 4-di-*O*-methylarabinose (4 parts), 2-*O*-methylarabinose (1 part), D-galactose (1 part), 2 : 4 : 6-tri-*O*-methylgalactose (3 parts), 6-*O*-methylgalactose (1 part) and L-rhamnose (3 parts) were separated and characterised. None of these sugars has methoxyl groups on adjacent carbon atoms. This is in keeping with the immunity of the material D_2O_3 to further attack by periodate. With the exception of the proportion of rhamnose, which is somewhat higher in the hydrolysate of the methylated material, the relative proportions of the methylated derivatives are in good agreement with those of the sugars in the degraded material D_2O_3 (Table 2).

Earlier studies [1] revealed the presence of galactofuranose residues in cladophoran. While it is recognised that the present experiments have not eliminated the possibility of the presence of arabo- and rhamno-furanose residues, the resistance of these units in the polysaccharide to mild acid-hydrolysis is in favour of a pyranose structure. Therefore, all the available evidence being borne in mind, the linkages which may be present in the centre of the cladophoran molecule are:

..3Rl..	...3Al..	..3Al.. 4	..3Gal.. 4 6	..3Rl.. 4	3Gal.. 4	..3Gal..
(1)	(4)	(1)	(1)	(3)	(1)	(3)

R = rhamnose, A = arabinose, Ga = galactose.

Comparison of these methylation results with those from the whole polymer show that the first four sugars given above are common to the hydrolysates of both methylated materials. Of the remaining derivatives isolated from the methylated degraded hydrolysate all except the L-rhamnose could have been formed, from lesser methylated derivatives present in the undegraded hydrolysate, by methylation of hydroxyl groups set free during the degradation. The failure to separate any free rhamnose from the hydrolysate of the methylated whole cladophoran is not very surprising when it is remembered that the total rhamnose content corresponded to *ca.* 5%, and that some seventeen different sugar derivatives were shown to be present in this hydrolysate including three different methylated rhamnoses.

The sulphate content of the various degraded products remained approximately constant, indicating that the sulphate ester groupings are spread relatively evenly throughout the molecule. The sulphate content of the degraded cladophoran D_2O_3 is equivalent to about one sulphate group per 3 or 4 sugar residues, and earlier work [1] has shown that this is not reduced on methylation. The 1 : 3-linked galactose is unlikely to be present as end group carrying sulphate on $C_{(3)}$, as such sulphate would be labile in the alkaline conditions of methylation and give rise to 3 : 6-anhydrogalactose.[5] If the sulphate groups are linked to galactose at all then, since they are not labile to alkali, they are probably located at position 4. Only one residue in seven of the sugars present in the hydrolysate of the methylated degraded material is a galactose unit with $O_{(4)}$ available for linkage.

[4] Fear and Menzies, *J.*, 1926, 937.
[5] Percival, *Quart. Rev.*, 1949, **3**, 369.

At least some of the sulphate groups must therefore be carried by either arabinose and/or rhamnose. This is the first evidence of sulphate groups linked to residues other than galactose in seaweed polysaccharides. The large amount of quadruply linked rhamnose in the polymer makes this sugar a very likely site for these residues, but a definite answer to this question must await other methods of investigation.

Certain broad conclusions can be drawn from these experiments: the material has a highly branched structure, with xylose and galactose units at the ends of the branches, whereas galactose, arabinose, and rhamnose residues comprise the centre of the molecule. Sulphate groups are linked to residues both on the outer branches and in the centre of the molecule. Finally, after three oxidations and two treatments with phenylhydrazine the molecule is still sufficiently large to be retained by a dialysis sac.

Experimental

Paper partition chromatography was carried out on Whatman No. 1 filter paper. Paper ionophoresis [6] was done in borate buffer (pH 10) at 750 v for 5 hr. Evaporations were carried out at 40°/15 mm. Specific rotations were measured in water at 18°.

The water-soluble polysaccharide, cladophoran (26·3 g.), $[\alpha]_D$ +69° (ash, 13·7%), was alternately oxidised with periodate and degraded with phenylhydrazine three times. In a typical experiment the polysaccharide (4% concentration) was treated in the dark at room temperature with 0·1M-sodium metaperiodate and the course of the reaction followed by measuring the periodate reduced [7] and the formic acid released [8] on samples (1 c.c.) withdrawn at intervals (see Table 1). After 50 hr. the reaction had reached completion, and the excess of periodate was removed with sulphur dioxide and the mixture dialysed until free from inorganic ions. An aliquot part was removed and hydrolysed with N-sulphuric acid at 100° for 6 hr. The molar proportions of the sugars in the resulting syrup were determined by Pridham's method [3] (see Table 2). For this method of estimation a synthetic mixture of the sugars present in the hydrolysate was made by dissolving millimolar quantities of these sugars in 25 c.c. of water. Measured quantities of this mixture (0·02, 0·04, 0·06, 0·08 c.c.) were applied from an Agla microburette at intervals along the starting line of a paper chromatogram (20 × 40 cm.). Also spotted on the paper were samples of the hydrolysate. After elution the chromatogram was sprayed with a 1% solution of freshly prepared *p*-anisidine hydrochloride and heated at 130° for 10 min. Each of the coloured spots which developed, together with suitable blanks, were cut out and left for 10 min. in 3 c.c. of methanolic stannous chloride (1 g. of stannous chloride dissolved in 5 c.c. of water, 90 c.c. of methanol added, and the mixture filtered). The density of the resulting solution was then measured in a Unicam spectrophotometer at the wavelength of maximum absorbance for the particular sugar being examined. When the readings for the standard solutions of the respective sugars were plotted against concentration a straight-line graph was obtained in each case, and these were used to determine the unknown materials. A complete duplication of the experiment ensured accuracy within ±5%.

The oxo-polysaccharide in the remainder of the solution was isolated by freeze-drying. The second oxidation was not complete until after 70 hr. and the third oxidation after 86 hr.

The oxo-polysaccharide was degraded by heating a 4% aqueous solution at 100° for 2 hr. with 7% acetic acid and 3% phenylhydrazine. After exhaustive extraction with ether and dialysis the degraded polysaccharide was isolated, by freeze-drying, as a light yellow powder. Further extraction with ether failed to reduce the nitrogen content.

The ethereal extracts after evaporation to dryness and extraction with glacial acetic acid afforded a brown powder. Dissolution in ether and addition of light petroleum afforded yellow crystals of glyoxal bisphenylhydrazone (0·9 g.), m. p. and mixed m. p. 167°. Removal of the acetic acid yielded crystalline *N*-acetylphenylhydrazine, m. p. and mixed m. p. 124°.

Methylation of the Degraded Cladophoran D_2O_3.—Thallium hydroxide (6 g.) and an aqueous solution of cladophoran D_2O_3 (3·6 g.; 30 c.c.) were freeze-dried and the product was refluxed overnight with methyl iodide (25 c.c.).[4] After evaporation to dryness the residue was exhaustively extracted with methanol (3 × 25 c.c.), hot 50% aqueous methanol (3 × 25 c.c.),

[6] Foster, *Chem. and Ind.*, 1952, 1050.
[7] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107.
[8] Chanda, Hirst, Jones, and Percival, *J.*, 1950, 1289.

and hot water (3 × 25 c.c.). The combined extracts were re-treated with thallium hydroxide and methyl iodide. After four methylations in all, the final residue was exhaustively extracted with chloroform. Removal of the chloroform from the combined extracts gave a brown powder (1·88 g.) (Found: OMe, 26·7%). Several methylations with Purdie reagents failed to raise the methoxyl content above 26·9%.

Hydrolysis of the Methylated Polysaccharide and Characterisation of the Methylated Sugars.—The above powder (1·80 g.) was hydrolysed under reflux with N-methanolic hydrogen chloride–water (9 : 1 by vol.) for 7 hr. Following neutralisation with silver carbonate and evaporation to dryness, the residue was thoroughly extracted with water. Removal of the water from the aqueous extracts and chloroform-extraction of the residue gave on evaporation an amorphous hydrolysate (0·96 g.). This was separated into its constituents on a cellulose column (55 × 2·3 cm.). After elution of five fractions with a water-saturated mixture of light petroleum (b. p. 60—80°)–butan-1-ol (8 : 2), the proportions were changed to 7 : 3. R_G values are recorded for paper chromatograms developed with butan-1-ol–ethanol–water (4 : 1 : 5). The products of demethylation [1] were detected by paper chromatography. The following fractions were collected:

Fraction 1, a yellow syrup (213 mg.) of R_G 1·05 (Found: N, 14·3%).

Fraction 2, syrupy 2 : 4-di-*O*-methylrhamnose (40 mg.), R_G 0·83, $[\alpha]_D$ −17° (*c* 0·3) (Found: OMe, 33·6. Calc. for $C_8H_{16}O_5$: OMe, 32·3%); demethylation gave only rhamnose; nucleation with an authentic specimen afforded needles of 2 : 4-di-*O*-methylrhamnose [9] with m. p. and mixed m. p. 82—83°.

Fraction 3, syrupy 2 : 4-di-*O*-methylarabinose (162 mg.), R_G 0·67, $[\alpha]_D$ +28° (*c* 2·0) (Found: OMe, 34·9. Calc. for $C_7H_{14}O_5$: OMe, 34·8%); demethylation of a portion of the syrup gave only arabinose; the derived anilide [10] had m. p. 126° and gave an *X*-ray powder photograph identical with that of 2 : 4-di-*O*-methyl-*N*-phenyl-L-arabinosylamine.

Fraction 4, syrupy 2 : 4 : 6-tri-*O*-methylgalactose (143 mg.), R_G 0·64, which crystallised from methanol; the crystals had m. p. 116°, $[\alpha]_D$ +88° (*c* 0·92) [11] (Found: OMe, 41·7. Calc. for $C_9H_{18}O_6$: OMe, 41·9%); demethylation gave only galactose.

Fraction 5, syrupy 2-*O*-methylarabinose (37 mg.), R_G 0·43, $[\alpha]_D$ +74° (Found: OMe, 18·0. Calc. for $C_6H_{12}O_5$: OMe, 17·8%); this was chromatographically and ionophoretically identical with authentic 2-*O*-methylarabinose and in keeping with this structure failed to give a red spot on spraying of a paper chromatogram with triphenyltetrazolium hydroxide; [12] the derived phenylhydrazone had m. p. 115°.

Fraction 6, crystalline L-rhamnose (89 mg.), R_G 0·30, $[\alpha]_D$ +8·2 (const.), m. p. and mixed m. p. 68°.

Fraction 7, syrupy 6-*O*-methylgalactose (23 mg.), R_G 0·23, $[\alpha]_D$ +74° (*c* 1·1) (Found: OMe, 15·1. Calc. for $C_7H_{14}O_6$: OMe, 16·0%); demethylation gave only galactose; the derived phenylhydrazone had m. p. 117°.[13]

Fraction 8, crystalline D-galactose (46 mg.), R_G 0·10, $[\alpha]_D$ +80° (const.), m. p. and mixed m. p. 162—164°.

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[9] Charalambous and Elizabeth Percival, *J.*, 1954, 2443.
[10] Andrews, Ball, and Jones, *J.*, 1953, 4090.
[11] Bell and Williamson, *J.*, 1938, 1196.
[12] Wallenfels, *Naturwiss.*, 1950, **37**, 491.
[13] Pacsu and Trister, *J. Amer. Chem. Soc.*, 1940, **62**, 2301.