632. *The Polysaccharides of the Green Seaweed* Codium fragile. *Part II.*[1] *The Water-soluble Sulphated Polysaccharides.*

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Exhaustive extraction of *Codium fragile* has led to the separation of water-soluble sulphated polysaccharides, a starch-type glucan,[1] and a mannan. The sulphated material comprised mainly D-galactose and L-arabinose, with smaller amounts of D-xylose and traces of uronic acid and L-rhamnose. Partial fractionation on diethylaminoethylcellulose indicated the heterogeneity of this material. Mild hydrolysis led to the isolation and characterisation of all the neutral monosaccharides, of 3-*O*-β-L-arabinopyranosyl-L-arabinose, 3-*O*-β-D-galactopyranosyl-D-galactose, and of galactose 4- and 6-monosulphates. The results of periodate oxidation and alkali treatment of the sulphated polysaccharides are discussed in the light of their overall structures.

All the green seaweeds investigated so far have been shown to synthesise water-soluble sulphated heteropolysaccharides which contain a variety of sugar residues. Attempts to fractionate these materials into homopolysaccharides have so far been unsuccessful. The sulphated polysaccharides from *Ulva*,[2] *Acrosiphonia*,[3] and *Enteromorpha* [4] comprise mainly L-rhamnose, D-xylose, and D-glucuronic acid, with smaller proportions of D-glucose and trace amounts of other sugars. In contrast, species of *Cladophora* [5] and of *Caulerpa* [6] are devoid of uronic acid and contain sulphated polysaccharides comprising mainly arabinose and galactose together with, in the case of *Cladophora*, lesser amounts of D-xylose and trace amounts of D-glucose and rhamnose, and in *Caulerpa* some D-xylose and D-mannose.

The isolation of glucuronosylrhamnose from the partial acid hydrolysates of the polysaccharides from *Ulva*,[7] *Enteromorpha*,[4] and *Acrosiphonia* [2] provides evidence that these two sugars are present in a single polysaccharide, and for the similarity of the polysaccharides in these three genera. Periodate oxidation studies on these materials have confirmed this and indicate a high proportion of 1,3-linked units and/or highly branched molecules. That the majority of the sulphate groups are linked to the rhamnose units in both the *Enteromorpha* [4] and the *Ulva* [8] polysaccharides was shown by desulphation with methanolic hydrogen chloride and periodate oxidation of the polysaccharide before and after desulphation. Proof that C-2 of some of the xylose units in the material from *Ulva* also carries sulphate groups was obtained from the action of alkali and sodium methoxide on the polysaccharide.[8]

The present Paper describes the exhaustive extraction of the green seaweed *Codium fragile* and structural studies on the isolated water-soluble sulphated polysaccharides. A starch-type polysaccharide [1] and a mannan were also separated from the weed and a structural investigation of the latter forms the subject of the next Paper.

Both the cold-[2(i)] and hot-water-extracted material [2(ii)] comprised mainly galactose and arabinose, with lesser amounts of glucose, xylose, mannose, and rhamnose (see Table 1), although the latter contained a higher proportion of glucose than the former, and gave the typical blue strain of a starch-type polysaccharide with dilute iodine solution. It was found possible to separate the majority of the glucose-containing polysaccharide from this hot-water extract as the starch–iodine complex and the residual polysaccharide was very similar in rotation and sugar content to the cold-water-extracted material. The two

[1] Love, W. Mackie, McKinnell, and Percival, *J.*, 1963, 4177, is considered to be Part I.
[2] Brading, Georg-Plant, and Hardy, *J.*, 1954, 319.
[3] O'Donnell and Percival, *J.*, 1959, 2168.
[4] McKinnell and Percival, *J.*, 1962, 3141.
[5] Fisher and Percival, *J.*, 1957, 2666.
[6] I. M. Mackie and Percival, *J.*, 1959, 1151; 1961, 3010.
[7] McKinnell and Percival, *J.*, 1962, 2082.
[8] Percival and Wold, *J.*, 1963, 5459.

materials were therefore combined and designated polysaccharide (A). This had $[\alpha]_D$ +46° and contained, in addition to the above sugars, 17% of sulphate (as SO_3) and 6·2% (by decarboxylation) of uronic anhydride. It was also contaminated with *ca.* 25% protein. Since pentoses give rise to carbon dioxide on treatment with hot hydrochloric acid it is

TABLE 1.

Monosaccharides extracted from *Codium fragile.*

Extract *	Yield (% dry wt. weed)	Glucose	Galactose	Mannose	Arabinose	Xylose	Rhamnose
2 (i)	3·0	xx	xxxxx	x	xxxx	xx	x
2 (ii)	5·0	xxx	xxxxx	xx	xxxx	xx	x
3	1·5	x	xx	xxxxx	—	—	—
4	0·6	—	—	xxx	—	—	—
5	1·2	x	x	xxxxx	—	—	—
6	2·3	x	—	xxxxx	—	—	—
Residual material		x	—	xxxxx	—	—	—

xxxxx = major constituent, x = trace.

* For details of extraction see Experimental section.

probable that the actual uronic acid content is considerably less than 6%. Although chromatographic evidence for the presence of uronic acid in hydrolysates of polysaccharide (A) was obtained, all attempts to isolate and characterise it were unsuccessful.

The small proportion of glucose present in this material was due to incomplete removal of starch and could be completely eliminated by the action of salivary α-amylase. The mannose present in this extract is also considered to be due to contamination, in this case by a small proportion of the β-1,4-linked mannan (probably low-molecular-weight material) which was subsequently isolated as a pure mannan on alkaline extraction of the residual weed.[1] Supporting evidence for these conclusions was derived from periodate-oxidation studies on polysaccharide (A); all the glucose and the mannose, as would be expected for 1,4-linked units, being cleaved by the periodate.

Fractionation experiments on diethylammoniumethyl(D.E.A.E.)—cellulose of polysaccharide (A) were partly successful. The major fraction (B) from a preliminary fractionation was further separated into four fractions (Table 2) with the constitutions shown in Table 3. Fractions 2, 3, and 4 had increasing sulphate contents and the latter two fractions contained *ca.* 3·5% of anhydro-sugar (Table 2), and had infrared bands characteristic of ester sulphate (1240 cm.$^{-1}$), secondary axial sulphate (850 cm.$^{-1}$), and primary sulphate (820 cm.$^{-1}$).

TABLE 2.

Fractionation of polysaccharide (A).

	Wt. mg.	$[\alpha]_D$	% SO_3^{2-}	Anhydro-sugar (%)
Polysaccharide (B)	350	37·5°	12·7	0·74
Fraction 1, tubes 10—25	17	—	—	—
,, 2, ,, 26—48	46	—	11·0	—
,, 3, ,, 49—70	115	+45	18·9	3·5
,, 4, ,, 71—110	64	+55	29·3	3·5

TABLE 3.

Composition of the fractions derived from polysaccharide (A).

Fraction	Glucose	Galactose	Mannose	Arabinose	Xylose	Rhamnose
B	xx	xxxxx	xx	xxx	xx	x
1 (7%)	xxx	—	xxx	—	xxx	x
2 (18%)	x	xxx	x	—	x	—
3 (47%)	x	xxxxx	—	xxx	x	—
4 (27%)	—	xxxx	—	xxxx	—	—

xxxx = major component; x = trace.

TABLE 4.

Moles of periodate reduced per C_6-anhydro-unit.

Time (hr.)	21	51	72	96	120
Fraction 2	0·246	0·269	0·280	0·301	0·301
Fraction 3	0·162	0·162	0·178	0·198	0·199
Fraction 4	0·240	0·260	0·260	0·285	0·287

Few conclusions regarding the difference in structure of fractions 2, 3, and 4 could be deduced from periodate oxidation studies (see Table 4). They reduced 0·3, 0·2, and 0·3 moles of periodate per anhydro-sugar, respectively. Since fraction 4 has a much higher sulphate content (29%) than fraction 2 (11%) it is possible that some of the units mono-sulphated and immune to periodate in fraction 2 are disulphated in fraction 4. From these fractionation studies the heterogeneity of the material is apparent. In addition to a separate glucan and mannan, it appears probable that this species of seaweed synthesises a xylan, a sulphated galactan, and a more highly sulphated arabinan and/or arabinogalactan.

Earlier work [8] on the sulphated polysaccharide from *Ulva lactuca* revealed that alkali caused the removal of a small proportion of sulpate groups from the xylose units and conversion of the latter into arabinose. That the sulphate had been located on C-2 was proved by the isolation of 2-*O*-methyl-D-arabinose from a hydrolysate of the polysaccharide after treatment with sodium methoxide. In the present experiments trace quantities of xylose and of a methylpentose were detected on a paper chromatogram after similar treatment of the arabinogalactan fraction 4. Repetition of these experiments on a larger scale with unfractionated polysaccharides (B) and (A) revealed that the alkaline treatment of (B) reduced the sulphate from 12·7 to 8·9% with an increase of anhydro-sugar from 0·7 to 2·7% and a 90% recovery of polysaccharide. The relative proportions of galactose and arabinose remained virtually unchanged by this treatment. These facts indicate that the removal of the sulphate was partly from galactose and partly from arabinose units and that the former were converted into anhydrogalactose and the latter into a comparable amount of xylose units during the sulphate removal. The quantity of mono-*O*-methylpentose produced by the action of sodium methoxide on polysaccharides (A) was, even in this larger-scale experiment, too small for isolation.

Since sulphate groups are only labile to alkali if attached to carbon atoms with an adjacent free *trans*-hydroxyl group it follows that the residues removed from arabinose must have been located on C-2 or C-3. Unfortunately, the inability to characterise the methylated sugar formed by the action of sodium methoxide precludes a decision between these two positions. Anhydrogalactose arises from units sulphated at either C-3 or C-6 and, in view of the subsequent isolation of galactose 6-sulphate from a partial hydrolysate, it is very probable that the anhydro-sugar formed on alkaline treatment was derived from 6-sulphated units.

Unlike the experiments on polysaccharides which contain uronic acid, such as that from *Ulva*,[8] no information about the site of the sulphate groups could be deduced from the action of methanolic hydrogen chloride on polysaccharides (B). Although the sulphate was again reduced from 12·7 to 8·6%, the relative proportions of galactose and arabinose were little changed in the partially desulphated material (Y) which was recovered in *ca.* 56% yield.

In view of the difficulty of separating more than milligram quantities of polysaccharide on D.E.A.E.-cellulose, the mixed polysaccharide (A) was used for subsequent studies. From the neutral fraction of a partial acid hydrolysate of this mixture D-galactose, D-glucose, D-mannose, L-arabinose, and D-xylose were separated and characterised as crystalline materials or as crystalline derivatives. In addition, the disaccharides, 3-*O*-β-L-arabinopyranosyl-L-arabinose and 3-*O*-β-D-galactopyranosyl-D-galactose, were separated as chromatographically pure materials. The former was characterised by its chromatographic mobility. It gave only arabinose on hydrolysis, had a degree of polymerisation

(DP) [9] of 2, and gas chromatography of the methanolysed methylated material revealed the presence of methyl 2,3,4-tri-*O*-methyl- and 2,4-di-*O*-methyl-arabinoside. The crystalline galactose disaccharide was identical (mixed m. p. and *X*-ray powder photograph) with authentic 3-*O*-β-D-galactopyranosyl-D-galactose. It gave only galactose on hydrolysis, and methyl 2,3,4,6-tetra-*O*-methyl- and 2,4,6-tri-*O*-methyl-galactoside on a gas chromatogram after methylation followed by methanolysis. The isolation of these two disaccharides indicates that a high proportion of the arabinose and galactose units in the polysaccharide are β-1,3-linked. Supporting evidence for this was supplied by the presence of these two sugars in the hydrolysate of the oxo-polysaccharide obtained by the periodate oxidation of polysaccharide (A).

After fractionation of the partial acid hydrolysate of polysaccharide (A) on anion-exchange resin, charcoal, and finally 3MM paper, two monosaccharide monosulphates (P and Q) were separated. They were shown by chromatography of their hydrolysates to be derivatives of galactose. Their DP was shown [9] to be 1 and their galactose : sulphate molar ratio supported a monosaccharide monosulphate structure. Material (P) had $[\alpha]_D$ +64° and (Q) +49°, values which compare with those of authentic galactose 4-sulphate ($[\alpha]_D$ +59°) and 6-sulphate ($[\alpha]_D$ +48°).[10] Methylation, followed by methanolysis and gas chromatography of the derived glycosides, confirmed their structures as the 4- and 6-sulphates, respectively.

The rates of hydrolysis of axial, equatorial, and primary ester sulphate groups in galactose have been studied and values for their half-lives recorded.[11] When these measurements were carried out on authentic glucose 3-sulphate (equatorial) and the above monosaccharide sulphates the rate of hydrolysis sequence was found to be glucose 3-sulphate > (P) > (Q); this is the expected order if (P) is galactose 4-sulphate and (Q) the 6-sulphate. The half-lives of these two sulphates, 1·17 and 1·43 hr., respectively, are, however, somewhat lower than the recorded figures,[11] 1·45 and 1·55.

Galactose 4-sulphate should reduce 3 moles of sodium periodate fairly rapidly with the liberation of one mole of formaldehyde. If " over-oxidation " occurs, there is the possibility of another 3 moles of periodate being reduced.[12] Compound (P) behaved as would be expected for galactose 4-sulphate, 3 moles of periodate being reduced fairly rapidly (Table 5). However only 0·3 mole instead of 1 mole of formaldehyde was liberated.

TABLE 5.

Moles of periodate reduced per mole of sugar.

Time (hr.)	2·5	8	22	46	70	147
Galactose 4-sulphate	1·6	2·6	2·6	3·0	3·3	3·7
Galactose 6-sulphate	5·1	5·8	6·0	6·1	6·2	6·4
	4·9	5·6	5·8	6·0	6·2	6·3

Gibbons and O'Dea [13] found that when malondialdehyde-type structures are formed as intermediates during periodate oxidation any liberated formaldehyde is liable to react with the active hydrogen present in such a structure. This may be the reason for the low yield of formaldehyde from the present monosulphate since it is envisaged [12] that a sulphated malondialdehyde will be formed after the reduction of 3 moles of periodate. In contrast, compound (Q) reduced more than 6 moles of periodate (see Table 4) instead of the maximum of 4 expected for galactose 6-sulphate. In spite of apparent " over-oxidation " no free formaldehyde could be detected. This excessive reduction of periodate, which was determined several times, cannot be explained since, in all other respects, compound (Q) had the properties of galactose 6-sulphate.

[9] Timmell, *Svensk Papperstidn.*, 1960, **63**, 668; Peat, Whelan, and Roberts, *J.*, 1956, 2558.
[10] Turvey and Williams, *J.*, 1962, 2119.
[11] Rees, *J.*, 1963, 1821.
[12] Turvey, Clancy, and Williams, *J.*, 1961, 1692.
[13] Gibbons and O'Dea, *Chem. and Ind.*, 1953, 1338.

The present study has shown that the water-soluble sulphated extract from *C. fragile* contains a number of polysaccharides. Like those from *Cladophora rupestris* [5] and from various species of *Caulerpa* [6] its major water-soluble polysaccharide(s) comprises galactose and arabinose units. Whilst periodate oxidation studies on the materials from all three genera have indicated a high proportion of 1,3-linkages, definite proof of this linkage in the case of *C. fragile* has been obtained by the isolation of the 1,3-linked galactose and arabinose disaccharides from a partial acid hydrolysate. The isolation of two galactose monosulphates from the same hydrolysate provided the first direct evidence of sulphated galactose in a green seaweed polysaccharide. One of these had all the properties of the 4-sulphate whilst the other, apart from its reaction with periodate, corresponded to authentic galactose 6-sulphate. Some of the arabinose units also appear to carry sulphate groups but unequivocal proof of this still awaits further investigation.

Experimental

The analytical methods used have been described by McKinnell and Percival.[4] Ionophoresis of the sugar sulphates was carried out (300v) in 0·01M-acetic acid (brought to pH 7 with pyridine) for 1 hr.; M_{gs} is the ionophoretic mobility relative to glucose 3-sulphate.

Extraction of the Weed.—Three samples of dried *C. fragile* weed were investigated, two from Capetown, South Africa [(*a*) 100 g. collected in February 1958; (*b*) 250 g. collected in October 1960], and a third sample from near Biarritz, France, collected in September 1961. There was no apparent difference in the polysaccharides synthesised by the three samples, although a strict quantitative measurement was not carried out. Each of the samples was sequentially extracted as follows: (1) with acetone until all the colouring matter was removed, (2) exhaustively with (i) cold and then (ii) hot water (90°) under nitrogen, (3) 50% zinc chloride at room temperature overnight, (4) after thorough washing with distilled water the residual weed 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 [14] during 3 hr., (5) the residual weed after washing with water was extracted with 4% sodium hydroxide at room temperature under nitrogen, and (6) the residual material was treated with 20% sodium hydroxide at 80° in an atmosphere of nitrogen and the polysaccharide isolated from the alkaline solution as its complex with Fehling's solution.[15] After washing free from alkali the residue (34 g. from 170 g. of dried weed), which contained 84% of carbohydrate, was washed free from alkali, kept with 36% sulphuric acid for 36 hr., and hydrolysed with N-sulphuric acid at 100° for 12 hr. Except in the case of (6), the polysaccharides were isolated by freeze-drying the solution obtained after dialysis and concentration. Visual examination of paper chromatograms eluted with solvents 2 and 3 of hydrolysates of the various extracts indicated the sugars shown in Table 1.

After removal of starch from the hot-water extract as the starch–iodine complex,[4] the residual material was essentially similar to the cold-water extract.

Examination of the Water-soluble Material.—The combined cold- and hot-water extracts, after the removal of starch from the latter, hereinafter known as polysaccharide (A), had $[\alpha]_D$ +46° [Found: N, 4·0; ash, 13·5; SO_3^{2-}, 17·0; uronic anhydride (by decarboxylation), 6·2%]. Visual examination of a paper chromatogram of a hydrolysate (N-sulphuric acid, 6 hr., 100°) revealed the presence of galactose and arabinose as the major sugars with lesser, approximately equal amounts of glucose, mannose, and xylose, and a trace of rhamnose. Sequential incubation of a portion (28 mg.) in water (10 ml.) at 35° with salivary α-amylase (12 mg. and 10 mg.), followed by dialysis and freeze-drying, gave polysaccharide which was almost devoid of glucose (paper chromatography of a hydrolysate).

Attempted Fractionation of Polysaccharide (A).—Polysaccharide (A) (1 g.) in water (50 ml.) was allowed to drain into a column (45 × 4 cm) of DEAE-cellulose in the chloride form. The column was then subjected to gradient elution with potassium chloride (0·025—1·5M; 1·5 l. of each), then with 1·5M-potassium chloride–0·3M-potassium hydroxide (1 l. of each), and finally with 0·3M-potassium hydroxide (1·5 l.). Fractions (25 ml.) were collected every 30 min. and analysed for their carbohydrate content. The major fraction (B) (350 mg.) was eluted in the first 1250 ml. and isolated by freeze-drying after dialysis. The contents of the remaining

[14] Wise, *Ind. Eng. Chem., Analyt.*, 1946, 290.
[15] Jones, *J.*, 1950, 3292.

tubes were combined, dialysed, and isolated as a white solid (C) (212 mg.) by freeze-drying. Materials (B) and (C) after hydrolysis were shown to contain the same relative proportions of sugars as (A) (paper chromatography after hydrolysis). A fresh column (45 × 4 cm.) of DEAE-cellulose in the chloride form was prepared and polysaccharide (B) (350 mg.) was re-fractionated under the same conditions as before and four fractions were separated by elution with potassium chloride (0·025—1·5M; 1·5 l. of each) (see Table 2).

Examination of the Four Fractions.—A portion of each fraction was hydrolysed (N-sulphuric acid, 100°, 6 hr.) and paper chromatography revealed the sugars (visual examination) shown in Table 3.

The infrared spectra of fractions 3 and 4 were very similar, both having the characteristic broad band at 1240 $cm.^{-1}$, a sharp peak at 850 $cm.^{-1}$, and a broad weak peak at 820 $cm.^{-1}$.[16] The infrared spectrum of fraction 2 showed only slight indications for the presence of sulphate.

Samples of fractions 2, 3, and 4, as their ammonium salts, were oxidised with 0·015M-sodium periodate at room temperature in the dark and the reduction of periodate measured on aliquots.[17]

Paper chromatography of the oxopolysaccharides from fractions 3 and 4, after reduction and hydrolysis, indicated the same proportions of galactose and arabinose as were present before oxidation.

The Action of Alkali on Fraction 4 and on Polysaccharide (B).—Treatment of fraction 4 (10 mg.) with sodium borohydride then alkali,[8] followed by dialysis, concentration, and freeze-drying, gave polysaccharide which, after hydrolysis, gave arabinose, galactose, and a faint spot with the mobility of xylose.

Similar treatment of polysaccharide (B) (112 mg.) gave polysaccharide (X) (98 mg.) (Found: SO_3^{2-}, 8·9; anhydro-sugar, 2·7%).[18] Paper chromatography of a hydrolysate revealed the same sugars as were present in polysaccharide (B).

Treatment of Fraction 4 and of Polysaccharide (A) with Sodium Methoxide.—Fraction 4 (15 mg.) and sodium borohydride (4 mg.) in water (2 ml.) were set aside at room temperature for 18 hr., then freeze-dried. The product was added to a solution of sodium borohydride (4 mg.) and sodium (120 mg.) in dry methanol (5 ml.) and the mixture refluxed for 48 hr. The polysaccharide was removed by filtration, washed with dry methanol, dissolved in water, and freeze-dried. Chromatography of a hydrolysate gave a faint spot with the mobility of a mono-*O*-methylpentose.

Similar treatment of polysaccharide (A) (1 g.) gave a product (615 mg.) which only revealed a trace amount of a mono-*O*-methylpentose on paper-chromatographic examination of a hydrolysate.

Treatment of Polysaccharide (B) with Methanolic Hydrogen Chloride.—Polysaccharide (B) (112 mg.) was shaken with dry 0·015M-methanolic hydrogen chloride (150 ml.) for 48 hr. at room temperature. After washing with methanol (2 × 100 ml.) the polysaccharide was dissolved in water, dialysed, and the solution concentrated and freeze-dried. The recovered polysaccharide (Y) (61 mg.) contained SO_3^{2-}, 8·6%.

Quantitative determination[19] of the relative proportions of galactose to arabinose in polysaccharides (B), (X), and (Y) gave 3·2 : 1, 3·1 : 1, and 2·9 : 1, respectively.

Partial Acid Hydrolysis of Polysaccharide (A).—Polysaccharide (A) (5 g.) was hydrolysed with N-sulphuric acid (200 ml.) at 100° for 1 hr. After neutralisation with barium carbonate, deionisation with Amberlite IR-120 H^+ resin, and concentration, the derived solution (100 ml.) was applied to a column (9 × 2 cm.) of Amberlite IR-400 resin in the acetate form. The column was washed with water (250 ml.) until the effluent gave a negative test for carbohydrate.[20] The aqueous effluent which contained the neutral fragments was concentrated to a syrup (D) (*ca.* 1 g.). The column was then eluted with N-sulphuric acid (1·5 l.) until the effluent was almost free from carbohydrate.[20] The acidic effluent was neutralised with barium carbonate and passed through a column (20 × 2 cm.) of Amberlite IR-120 H^+ resin. The effluent was freed from acetic acid by repeated addition of water and concentration. The neutral solution, reduced to 50 ml., was shaken twice with a 5% solution of di-n-octylamine in chloroform and finally washed with chloroform. After neutralisation with ammonia it was concentrated to a syrup (E).

[16] Lloyd, Dodgson, Price, and Rose, *Biochem. Biophys. Acta*, 1961, **46**, 108.
[17] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 1216.
[18] Yaphe, *Analyt. Chem.*, 1960, **32**, 327.
[19] Wilson, *Analyt. Chem.*, 1959, **31**, 199.
[20] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.

Characterisation of the Neutral Fragments.—The neutral syrup (D) (*ca.* 1 g.) was separated into a monosaccharide and an oligosaccharide fraction on Whatman 3MM chromatography paper by elution with ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4) (solvent 9) for 30 hr. Pure monosaccharides and two disaccharides were separated from the respective fractions by repeated chromatography on 3MM paper with solvent 2 for 16 hr. and solvent 9 for 30 hr. for the monosaccharides and 30 and 48 hr., respectively, for the disaccharides. The fractions were examined as follows:

D-Galactose (53 mg.) was crystalline and had m. p. and mixed m. p. 166—168° (from ethanol), $[\alpha]_D$ +71° (*c* 1·5). The derived methylphenylhydrazone [21] had m. p. and mixed m. p. 203—204°. Syrupy D-glucose (13·5 mg.) had $[\alpha]_D$ +50° (*c* 0·6). Incubation of an aliquot with glucose oxidase converted the sugar completely into gluconic acid (paper chromatography, solvent 9, sprayed with Bromocresol Green). Syrupy D-mannose (17 mg.) had $[\alpha]_D$ +14° (*c* 0·8). The phenylhydrazone [21] had m. p. and mixed m. p. 187—188°. L-Arabinose (21 mg.) was crystalline, m. p. and mixed m. p. 159—160°, $[\alpha]_D$ +107° (*c* 1·1). The derived benzoylhydrazone [21] had m. p. and mixed m. p. 190—192°. Syrupy D-xylose (12 mg.) had $[\alpha]_D$ +20° (*c* 0·6). The dibenzylidene dimethyl acetal [22] had m. p. and mixed m. p. 210—212°.

3-O-β-L-*Arabinopyranosyl*-L-*arabinose*. The syrupy sugar (2·6 mg.) had $[\alpha]_D$ +238° (*c* 0·13). Chromatographically identical with authentic material in solvents 2 and 9. Chromatographic examination of a hydrolysate revealed a single spot with the mobility of arabinose. Estimation of the DP [9] indicated a disaccharide. A portion (1·3 mg.) was thoroughly dried and shaken with redistilled methyl iodide (0·2 ml.), redistilled dimethylformamide (0·2 ml.), and dry silver oxide (200 mg.) at room temperature in the dark for 18 hr. The mixture was filtered and the salts were washed with dry chloroform (3 × 5 ml.). Concentration of the combined filtrate and washings, after drying ($CaSO_4$), gave a syrup. This was methanolysed with 3% methanolic hydrogen chloride and the derived methylglycosides were examined by gas chromatography.[23] Peaks with the retention times of methyl 2,3,4-tri-*O*-methyl- and 2,4-di-*O*-methyl-arabinoside were obtained.

3-O-β-D-*Galactopyranosyl*-D-*galactose*. The crystalline sugar (20 mg.) $[\alpha]_D$ +75° (*c* 1·0) had m. p. and mixed m. p. 155—157° (from aqueous acetone) and gave only galactose on hydrolysis. Its *X*-ray powder photograph was identical with authentic material. A sample (3 mg.) was converted into the methylated glycosides as for the above arabinose disaccharide and these, on gas-chromatography [23] had the retention times of methyl 2,3,4,6-tetra-*O*-methyl- and 2,4,6-tri-*O*-methyl-galactoside.

Characterisation of the Acidic Fragments.—From the acidic fraction (E) galactose 4- and 6-sulphate (P and Q, respectively) were separated by fractionation on a charcoal column, followed by separation on Whatman 3MM paper with solvent 9 and elution for 36 hr. A second separation on 3MM paper was necessary to obtain pure monosaccharides. Before concentration of the respective fractions, ammonia was added to the solutions to bring the pH to 8, and the monosaccharide sulphates were isolated as their ammonium salts.

Galactose 4-sulphate (10·6 mg.[20]) had $[\alpha]_D$ +64° (*c* 0·53) and galactose 6-sulphate (6·6 mg.[20]) had $[\alpha]_D$ +49° (*c* 0·33). The two materials were characterised as follows. (a) Both had a DP [9] of 1, (b) on hydrolysis they each gave a single spot with the mobility of galactose (paper chromatography), (c) the molar proportion of ester sulphate [24] to galactose [20] present in the 4-sulphate was found to be 1·15 : 1 and in the 6-sulphate 1·04 : 1, (d) a portion of each (*ca.* 3 mg.) was methylated separately under the conditions used for 3-*O*-β-L-arabinopyranosyl-L-arabinose. After methanolysis, gas chromatography [23] of the derived glycosides revealed methyl 2,3,6-tri-*O*-methyl- and 2,3,4-tri-*O*-methyl-galactosides as the major peaks from the 4- and the 6-sulphate, respectively.

Rate of Sulphate Hydrolysis.[11]—Samples of each of the sulphates in 0·25N-hydrochloric acid (0·25 ml.; containing 50 μg. of sulphate ester) were sealed in Pyrex tubes (3 × ⅜ cm.) and heated at 100° for varying times. After cooling, centrifuging, and opening, the contents of each tube were neutralised with 2·5N-sodium hydroxide (0·025 ml.). Aminochlorobiphenyl reagent (0·25 ml.) and a trace of cetyltrimethylammonium bromide were added to each and the mixture allowed to stand overnight. After centrifugation, a portion of the supernatant liquor (0·1 ml.)

[21] Hirst, Jones, and Woods, *J.*, 1947, 1048.
[22] Breddy and Jones, *J.*, 1945, 738.
[23] Aspinall, *J.*, 1963, 1676.
[24] Jones and Letham, *Chem. and Ind.*, 1954, 662.

was diluted to 10 ml. with 0·1N-hydrochloric acid and the optical density of the solution read at 254 mμ. The graph of log $a/a - x$ against time was plotted in each case; log $a/a - x = E_{blank} - E_{24}/E_t - E_{24}$, where E_{24} = optical density after 24 hr. and E_t = optical density after t hr. The half-life ($t_{\frac{1}{2}}$) was found in each case using the expression $t_{\frac{1}{2}} = \log 2/\text{slope}$. The half-lives for the sulphate hydrolysis in the two galactose sulphates and for glucose 3-sulphate, run as control, are galactose 4-sulphate, 1·17 hr. (axial sulphate); galactose 6-sulphate, 1·43 hr. (primary sulphate); and glucose 3-sulphate, 0·43 hr. (equatorial sulphate).

Periodate Oxidation of the Monosulphates.—Samples of galactose 4-sulphate (100 μl.) and 6-sulphate (100 μl.) were oxidised separately with 0·01M-sodium periodate (1 ml.) at room temperature in the dark. Aliquots (25 μl.) were taken to determine the amount of periodate reduced [17] (see Table 5) and the formaldehyde released.[25] Galactose 4-sulphate released 0·2 mole formaldehyde after 46 hr. and 0·3 mole in 147 hr. and no formaldehyde was released from the galactose 6-sulphate.

Periodate Oxidation of Polysaccharide (A).—Polysaccharide (A) (26 mg.) in 0·1M-sodium periodate (10 ml.) was set aside at 30° for 48 hr. After removal of the excess of periodate by the addition of ethylene glycol (1 ml.), potassium borohydride (30 mg.) was added and the mixture set aside overnight. The oxopolysaccharide, isolated from this solution by freeze-drying after dialysis, was devoid of glucose and mannose but contained approximately the same relative proportions of galactose, arabinose, xylose, and rhamnose as the original material.

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[25] Frisell, Meech, and McKenzie, *J. Biol. Chem.*, 1954, **207**, 709.