517. *The Water-soluble Polysaccharides of* Cladophora rupestris.*

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Water-soluble sulphated polysaccharides containing mainly arabinose, galactose, and xylose and small amounts of rhamnose and glucose have been extracted from *Cladophora rupestris*. Each of the sugars has been isolated as crystals. Chloroform-extraction of the acetylated material separated a small quantity of a glucose-rich fraction resembling laminarin. Methylation studies on the glucose-free residue have revealed a highly branched structure and established the presence of some 1 : 3-linked arabinose, galactose, and rhamnose, and some 1 : 4-linked xylose. Partial hydrolysis and periodate experiments support these results.

The polysaccharide material extracted from the green seaweed, *Cladophora rupestris*, by boiling water or by dilute hydrochloric acid (pH 3—4) had a nitrogen content of 3—4%. Neither ammonium salts nor amino-sugar units could be detected in it, but amino-acids were present in acid hydrolysates. This indicated contamination with protein to the extent of 20—25% and the usual methods for removal of protein were investigated.[1-7] None proved entirely satisfactory but the best and most economical was partial precipitation of the protein with 4% trichloroacetic acid,[8] followed by precipitation of the polysaccharide by addition of ethanol to the clarified, dialysed, concentrated filtrate. Polysaccharide containing 2·0 and 1·8% of nitrogen was precipitated from solutions containing 50 and 70% of ethanol respectively. After removal of these two fractions addition of ethanol to a concentration of about 95% precipitated an off-white powder (A) (N, 1·26%; yield 6·5% from the cleaned dried weed), $[\alpha]_D$ +69°. Examination of the different fractions precipitated from aqueous solution by different concentrations of alcohol showed them to be essentially similar in sugar content and each contained the same amount of sulphate. Although several treatments of material (A) by the Sevag method[9] effected a further slight reduction in nitrogen content (1·26 ⟶ 1·05%) the recovery was very low. Hot N-alkali reduced the nitrogen content of material (A) to 0·06% but caused considerable degradation of the carbohydrate material. Consequently all subsequent investigations were carried out on material (A).

Similar results have been obtained with the sulphated polysaccharide from the green seaweed, *Ulva lactuca*;[10] the original extracts contained 25% of protein and although repeated treatment with chloroform–butanol[9] reduced the nitrogen content to 1·01% the recovery of polysaccharide was very poor.

Paper chromatography of the hydrolysate from material (A) showed the presence of arabinose, galactose, xylose, rhamnose, and glucose in the molar proportions 3·7 : 2·8 : 1·0 : 0·4 : 0·2. Concordant estimations of rhamnose were difficult to obtain, the figure quoted being an average. It should be emphasised that the hydrolysates of all the fractions and products from the different purification procedures contained all these five sugars. Column chromatography of the hydrolysate gave L-rhamnose, α-D-xylose,

* A preliminary account of some of this work was given at the Second International Seaweed Symposium at Trondheim, 1955.

[1] Fugita and Iwatake, *Biochem. Z.*, 1931, **242**, 43; Arni and Percival, *J.*, 1951, 1826.
[2] Percival and Ross, *J.*, 1950, 717.
[3] Engstrøm and Jakus, *Nature*, 1948, **161**, 168.
[4] Hough, Jones, and Wadman, *J.*, 1952, 3393.
[5] Chanda, Hirst, Jones, and Percival, *J.*, 1950, 1289.
[6] Somogyi, *J. Biol. Chem.*, 1945, **160**, 69.
[7] Stumpf, *ibid.*, 1948, **176**, 240.
[8] Kunitz and Northrop, *J. Gen. Physiol.*, 1936, **19**, 991.
[9] Sevag, Lackmann, and Smollens, *J. Biol. Chem.*, 1938, **124**, 425.
[10] Brading, Georg-Plant, and Hardy, *J.*, 1954, 319.

L-arabinose, D-glucose, and α-L-galactose, all as crystals which, with the exception of glucose, were further characterised as crystalline derivatives. Glucose was characterised by oxidation to gluconic acid by the specific enzyme D-glucose oxidase. D-Glucose,[11] D-xylose,[12] and D-galactose [13] are fairly common constituents of algal polysaccharides, and L-rhamnose has been reported in *Ulva lactuca*,[10] but the only other reported occurrence of arabinose in an algal polysaccharide is that from the blue-green weed, *Anabaena cylindrica* [14] where it is present to the extent of about 6%.

Addition of potassium chloride to an aqueous solution of material (A) failed to yield a "κ"-fraction [15] similar to that given by carrageenin. Extraction of acetylated (A) by chloroform removed all the glucose (4%) as a glucose-rich fraction which was practically sulphate-free. Further, addition of cetyltrimethylammonium bromide [16] to an aqueous solution of material (A) gave a precipitate which was devoid of glucose. Material (A) gave a schlieren electrophoretic diagram with a symmetrical peak, indicating a monodispersed polysaccharide; it appears that the detection of relatively small amounts of contaminating carbohydrate is beyond the scope and sensitivity of this procedure. Electrophoresis on paper indicated a single sulphated polysaccharide.

Sulphate in the polysaccharide is undoubtedly ethereally linked. It is not reduced on prolonged dialysis and, whereas ions such as calcium could be precipitated from solution, no sulphate could be detected until after hydrolysis. The sulphate content, 19·6%, and that calculated from the sulphated ash, 10·0%, agrees with the ratio 2 : 1 characteristic of polysaccharide ethereal sulphates.[13] After allowance of 8% for protein and 4% for unsulphated glucose-rich fraction the sulphate content of glucose-free material corresponds to seven $SO_3Ca_{\frac{1}{2}}$ radicals to every seventeen sugar residues. The sulphate was extremely stable to alkali, even under conditions which degraded the carbohydrate. Methylation with alkali, under conditions not expected to degrade the polysaccharide, gave a methylated material which contained 16·2% of sulphate. From the alkali-stability of these groups it appears [17] that the galactose units carry the sulphate at position 4, but, as far as the authors are aware, no work has been done on pentose sulphates and it is impossible at this stage to assign the sulphate group to any particular carbon atom of the pentose sugar molecules.

It is well known that it is difficult to methylate sulphated polysaccharides; [18] all attempts to prepare a methylated material from the glucose-free acetate with a methoxyl content higher than 25·1% proved unsuccessful. This methylated material was subjected to methanolysis, the glycosides formed were then hydrolysed by aqueous acid, and the resulting sugars analysed on a cellulose column and on paper. A large number of components, some of which chromatographed at closely similar rates, were detected; this made separation and identification of some of the derivatives exceedingly difficult.

The following sugars were separated and identified: 2 : 3 : 4 : 6-tetra-*O*-methyl-D-galactose, 2 : 3 : 5-tri-*O*-methyl-D-galactose, and 2-*O*-methyl-D-galactose by chromatography, rotation, methoxyl content, and demethylation to the free sugar, and the last two derivatives were further characterised by the rotation of the derived lactone. 2 : 4-Di-*O*-methyl-D-galactose and free galactose were isolated as crystals. 2 : 4-Di-*O*-methyl-L-arabinose, 2-*O*-methyl-L-arabinose, and 3-*O*-methyl-L-arabinose were detected by chromatographic mobility, rotation, demethylation and rotation of the derived lactone. Crystalline L-arabinose was also isolated. 2 : 3 : 4-Tri-*O*-methyl-D-xylose and 2 : 3-di-*O*-methyl-D-xylose were shown to be present by chromatography and rotation and the latter

[11] Connel, Hirst, and Percival, *J.*, 1950, 3500.
[12] Chanda and Percival, *Nature*, 1950, **166**, 787.
[13] Buchanan, Percival, and Percival, *J.*, 1943, 51.
[14] Adams, Bishop, and Hughes, *Canad. J. Chem.*, 1954, **32**, 999.
[15] Cook and Smith, *Arch. Biochim. Biophys.*, 1953, **45**, 232; Cook, Neal, and Smith, *ibid.*, 1954, **53**, 192.
[16] Bera, Foster, and Stacey, *J.*, 1955, 3788.
[17] Percival, *Quart. Rev.*, 1949, **3**, 369.
[18] Buchanan and Percival, *Nature*, 1940, **145**, 1020.

also by demethylation. 4-*O*-Methyl-L-rhamnose was isolated as crystals with the correct melting point and rotation, and a mixture of 2 : 4- and 3 : 4-di-*O*-methyl-L-rhamnoses was also obtained.

The isolation of tetra-*O*-methylgalactose shows that some of the galactose functions as end-units, while the 2 : 3 : 5-tri-*O*-methyl derivative reveals the presence of galactofuranose residues linked through positions 1 and 6 in the polysaccharide. The monomethyl derivative and the free galactose could arise from galactose residues situated in a main chain and at branch points, as such residues, if they carried sulphate groups, would have at the most one hydroxyl group free for methylation. The occurrence of 2 : 4-di-*O*-methyl-L-arabinose shows that some of the arabinose occurs in the pyranose form with 1 : 3-linkages. The relatively large amounts of monomethyl and free arabinose isolated suggests that this sugar originates mainly from inner chains or at branch points. Trimethylxylose must arise from xylose that functions as end units, and the 2 : 3-di-*O*-methyl derivative may arise from 1 : 4-linked units of the branch chains or from end units which carry a sulphate residue on $C_{(4)}$. Apart from the presence of the relatively small quantity of the 3 : 4-di-*O*-methylrhamnose the other derivatives isolated indicate that this sugar is joined in the main by $C_{(1)}$ and $C_{(3)}$ linkages in the polysaccharide.

It may be that some of the hydroxyl groups of the monomethyl and free sugars isolated from the hydrolysate of the methylated polysaccharide escaped methylation because of the shielding effect of the sulphate groups. Furthermore the polysaccharide is bound up with protein or peptide material and this too may hinder complete methylation of the sugar residues. On the other hand the methylation results are supported by partial hydrolysis and periodate studies.

Proof that arabinose, xylose, and galactose occur as adjacent units in a single polysaccharide was obtained by the separation, after partial hydrolysis of material (A), of di- and tri-saccharides containing these three sugars. In addition the separation from this hydrolysate of oligosaccharides containing solely arabinose indicates the presence of several contiguous arabinose residues in the polysaccharide materials. Evidence that these oligosaccharides were not artefacts was obtained by the lack of formation of oligosaccharides when a synthetic mixture of the same sugars was subjected to identical hydrolysis (a small quantity of sulphuric acid being added comparable to that liberated from the ethereal sulphates) and separation procedures.

Oxidation of material (A) by sodium metaperiodate for 48 hr. gave an oxopolysaccharide in high yield. Quantitative analysis of the sugars in the hydrolysate from this material showed the almost entire elimination of xylose and the removal of about two-thirds of the galactose, indicating that these units contain two vicinal hydroxyl groups. That this is possible for 1 : 4-linked xylose and for galactose and xylose residues occurring as end units in the polysaccharide molecule is further support for the results of methylation. Arabinose, glucose, and rhamnose showed a relative increase in quantity in the oxopolysaccharide hydrolysate. This is in agreement with the methylation results; two contiguous methylated hydroxyls were not found in any of the methylated arabinoses and only a trace quantity of such a rhamnose derivative, namely, the 3 : 4-dimethyl compound, was isolated.

Subjection of the oxopolysaccharide from material (A) to mild acid hydrolysis followed by dialysis gave a residual polymer (X; SO_4, 19·95%) which revealed a single spot on an electrophoretogram when sprayed with toluidine-blue. Chromatographic analysis of the hydrolysate of the polymer (X) revealed the presence of mainly arabinose together with some galactose. The high sulphate content makes it clear that at least some of the arabinose must be sulphated, while the presence of arabinose and galactose is a further indication that they constitute the inner portion of the molecule. The hydrolysate from material (X) was also contaminated with glucose from the unremoved glucose-rich fraction.

All the results indicate that the glucose-free material has a highly branched structure consisting mainly of arabinose and galactose residues, the ends of the branches being

terminated by galactose and xylose units. However the failure to separate this material into more than a single polysaccharide does not, in the authors' opinion, constitute definite proof of its homogeneity. It is probable that differentiation of a mixture of sulphated polysaccharides into single entities is impossible with the techniques that are available at present. For this reason no attempt has been made to advance a unique structure for this material.

The small glucose-rich fraction which was separated was devoid of nitrogen, contained less than 3% of sulphate, and gave no colour with iodine. It was essentially resistant to periodate attack since glucose was found in the oxopolysaccharide hydrolysate in relatively increased amount (6·6 compared with 3%). Partial hydrolysis of material (A) gave two glucose-containing oligosaccharides: one (R_{gal} 0·81) was chromatographically and electrophoretically identical with laminaribiose, while the other moved at a rate (R_{gal} 0·69) suggestive of a trisaccharide. No oligosaccharide containing glucose joined to any other sugar was isolated. On treatment of the extract (A) with β-glucosidase, an enzyme specific for β-glucosidic linkages, glucose was the only sugar liberated. Taken as a whole the evidence strongly suggests that the glucose-containing fraction is the β-1 : 3-linked glucan, laminarin. That this polysaccharide might be present in green seaweeds is not unlikely in view of its wide occurrence in the brown weeds.[19]

Experimental

Evaporations were carried out at 40° under reduced pressure. Paper-partition chromatography was done on Whatman No. 1 filter paper with the upper layers of the following solvent systems (v/v): (1) butan-1-ol–ethanol–water (5 : 4 : 1), (2) butan-1-ol–acetic acid–water (4 : 1 : 5), (3) ethyl acetate–pyridine–water (10 : 4 : 3), (4) benzene–butan-1-ol–pyridine–water (1 : 5 : 3 : 3). Electroionophoresis was carried out on a water-cooled apparatus [20] with Whatman No. 1 paper in an 0·1N-borate buffer at pH 10, 1 hr. being allowed for equilibration. After 4 hours' running (500 V; 12·5 mA) the paper was air-dried and the sugars were located by aniline oxalate–5% acetic acid spray. Demethylations were carried out by hydrobromic acid (*d* 1·46—1·49) in a sealed tube at 100° for 5—10 min., followed by addition of ethanol, neutralisation (silver carbonate), concentration to dryness, and extraction with ethanol; the demethylated sugars were identified by paper chromatograms.

Preparation of the Polysaccharide.—The seaweed was kindly collected, during July and November 1953, by the Institute of Seaweed Research, Inveresk, from rock pools midway between high and low tide near Dunbar. The whole weed (500 g.) was freed from extraneous matter by washing with cold running water for 12 hr., and, after removal of some of the colouring matter by treatment with cold alcohol (2·5 l.), was dried in a vacuum-desiccator for 3 days. Two extractions of a sample of this dried material (43 g.) with boiling water (6·5 l.) or with dilute hydrochloric acid (pH 3—4; 6 l.) for 4 hr. at 70° and concentration of the filtered extract to small volume gave a light green viscous solution. Adding this slowly, with stirring, to excess of ethanol precipitated a cream-coloured powder. This material, dried over phosphoric oxide at 50°/15 mm. to constant weight (4·9 g., *i.e.*, 11·4% of the cleaned dry weed), contained 3·4% of nitrogen. Direct Kjeldahl distillation, without prior digestion, showed the absence of salts of volatile bases. Acid hydrolysis followed by paper chromatography showed the absence of amino-sugars, but the presence of a number of amino-acids was detected (ninhydrin spray). Preferential removal of either protein or polysaccharide from the mixture with cadmium hydroxide,[1] with basic lead acetate,[2] with picric, phosphotungstic,[3] or phosphomolybdic acids, with debasing resin (Zeo-Karb 215), with copper chloride,[4] with Fehling's solution,[5] with zinc sulphate–sodium hydroxide,[6] and with ammonium sulphate [7] were investigated. All these attempts gave low recovery of polysaccharide with a nitrogen content of at least 2%. An attempt to reduce the nitrogen content by the action of the proteolytic enzymes papain and pepsin was unsuccessful. It was found that a 4% solution of trichloroacetic acid [8] constituted the most economical and efficient protein precipitant. Various scales of working were examined,

[19] Kylin, *Kgl. Fysiograf. Sällskap, Lund, Forh.*, 1944, **14**, No. 18.

and the following procedure was finally adopted. To an aqueous solution of the crude extract (1—1·5% w/v) was added a concentrated solution of trichloroacetic acid sufficient to give a final concentration of 4% (w/v). After several days at room temperature the fine precipitate was removed by centrifugation and the clear solution dialysed to neutrality (3 weeks). The polysaccharide was isolated (60—65% yield from the crude extract) by freeze-drying or by alcohol precipitation. The nitrogen content varied between 1·4 and 1·6%, and was not further reduced either by a second treatment with acid at 4% concentration or at various other concentrations (4—10%). Subsequent treatment with basic lead acetate or with chloroform and butan-1-ol [9] gave a very poor recovery of polysaccharide and failed to reduce the nitrogen content below 1%. This was, however, lowered with reasonable yield of material by fractional ethanolic precipitation of the polysaccharide from aqueous solution. After removal of the first fractions (50%, 70% alcohol; N, 2·0, 1·8% respectively) the supernatant liquor was evaporated to small volume and the polysaccharide (A) (2·8 g. from 43 g. of cleaned dried weed) precipitated as an off-white powder by pouring into excess of alcohol. It had $[\alpha]_D^{20}$ +69° (*c*, 1·0 in H_2O) [Found: N, 1·26; Ac, 0; OMe, 0; ash (direct), 13·7; (as sulphate) 16·1; Ca, 3·5; Fe, 0·7; Na, 0·6; K, trace; insoluble residue (Al_2O_3 + SiO_2), 0·9; SO_4 in ash, 10·0; total SO_4, 19·6%]. The sulphate content was not reduced by prolonged dialysis of an aqueous solution of material (A) against running water. While cations such as Ca^{2+} could be precipitated, no SO_4^{2-} could be detected in solutions of the polysaccharide until after hydrolysis. An aqueous solution of material (A) was non-reducing to Fehling's solution, gave a faintly reddish colour on treatment with iodine solution and a red colour only after treatment overnight with Selivanoff reagent. No evidence for the presence of ketose or anhydro-sugar residues was given with anthrone reagent.[21]

Hydrolysis of the polysaccharide at 100° with (*a*) N-sulphuric acid for 4 hr. or (*b*) 4N-sulphuric acid for 5 hr. followed by neutralisation with barium carbonate and paper chromatography (solvents 1 and 4) gave spots corresponding to galactose, glucose, arabinose, xylose, and rhamnose (aniline oxalate spray). Spraying with urea oxalate and with anthrone reagent [21] gave no indication of ketose or anhydro-sugars. Examination of the acid hydrolysate with solvent 2 indicated absence of uronic acids * (naphtharesorcinol spray). Each of the extracts (including a cold-water extract) and fractions of the extracts and each of the products from the various purification procedures gave identical chromatograms. Spots corresponding to arabinose, galactose, xylose, rhamnose, and glucose were obtained in every sample examined in, as far as could be judged visually, the same relative proportions. Quantitative analysis of the hydrolysate with sodium metaperiodate [22] (solvent 4) gave arabinose 42, galactose 38, xylose 11·5, rhamnose 3·3—7·7, and glucose 3·0%. Concordant results were obtained in duplicate determinations, but rhamnose gave variable results within the range shown. Arabinose and galactose determined by the Nelson–Somogyi method [23] were 41 and 38% respectively. Material (A) (9 g.) was hydrolysed by 0·5N-oxalic acid (400 ml.) for 6 hr. at 100° in an atmosphere of carbon dioxide. The cooled, neutralised (calcium carbonate), filtered solution was evaporated to dryness in a current of carbon dioxide. The dried (P_2O_5) residue was extracted with ethanol, the extracts were evaporated to dryness, and the residue was re-extracted until the syrup (2·94 g.; $[\alpha]_D^{20}$ +59°, *c* 1·7 in H_2O; *N*, 0) was free from inorganic ions. A portion (2·01 g.) of this syrup was separated on a cellulose column [24] with butan-1-ol two thirds saturated with water as eluant. After 9·5 l. had been collected the eluant was changed to butanol saturated with water +5% of ethanol and after 13 l. had been collected the column was washed with water (6 l.). Each fraction was evaporated to dryness and the resulting syrup purified with charcoal–Celite. The following substances were separated: 1, The syrup (58·2 mg.), R_F 0·78 (pink spot), $[\alpha]_D^{20}$ +20·8° (*c* 0·9 in H_2O), n_D 1·4875, gave a positive Selivanoff test. 2, Crystallised (81·2 mg.), $[\alpha]_D^{19}$ +8·8° (*c* 1·2 in H_2O); the derived benzoylhydrazone, m. p. and mixed m. p. 179—181° (decomp.), characterised this fraction as L-rhamnose. 3, The syrup (40·7 mg.), R_F 0·39, gave a positive Selivanoff test. 4, The syrup (232 mg.) crystallised from methanol had m. p. and

* Since the completion of this work, decarboxylation of material A with 19% w/v hydrochloric acid by Dr. D. M. W. Anderson has indicated a uronic acid content of 4%.

[20] Foster, *Chem. and Ind.*, 1952, 1050.
[21] Johanson, *Nature*, 1953, **172**, 956.
[22] Hirst and Jones, *J.*, 1949, 1659.
[23] Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.
[24] Hough, Jones, and Wadman, *J.*, 1949, 2511.

mixed m. p. with α-D-xylose 144—144·5°, $[\alpha]_D^{19}$ +41° ⟶ +16° (*c* 0·9 in H_2O). The derived dibenzylidene dimethyl acetal,[25] after recrystallisation from methanol, had m. p. and mixed m. p. 210—211°. 5, The crystals (585·5 mg.) had m. p. 154—154·5°, $[\alpha]_D^{20}$ +156° ⟶ +107° (*c* 1·3 in H_2O; 30 min.) (Found: C, 39·9; H, 6·5. Calc. for $C_5H_{10}O_5$: C, 40·0; H, 6·7%); this fraction was further characterised as essentially β-L-arabinose by conversion into the benzoylhydrazone,[26] m. p. and mixed m. p. 199—203° (decomp.). 6, Crystallised from methanol (75·7 mg.), m. p. 148—150°, $[\alpha]_D^{18}$ +55·5° (*c*, 0·8 in H_2O); its identity as D-glucose was established by aerobic oxidation to gluconic acid by the specific enzyme D-glucose oxidase (notatin),[27] the rate of oxygen uptake being measured in a Warburg apparatus. 7, The syrup (513·6 mg.), crystallised from methanol, had m. p. 165—166°, $[\alpha]_D^{19}$ +138 ⟶ +83·5° (*c* 1·2 in H_2O); its identity as α-D-galactose was further established by the preparation of the diethyl mercaptal,[28] m. p. and mixed m. p. 140—141° (Found: C, 41·8; H, 7·6; S, 21·9. Calc. for $C_{10}H_{22}O_5S_2$: C, 41·8; H, 7·7; S, 22·4%). 8, The syrup (143 mg.) consisted of a mixture of oligosaccharides (41·6 mg. were obtained as a syrup from overlap of fractions). Total recovery from the column was 93·5%.

Attempted Fractionation of the Extract (A).—Polysaccharide fractions precipitated from aqueous solution at different concentrations of alcohol (50, 70, 85, and 95%) gave identical chromatograms on hydrolysis and had sulphate contents of 19·1, 19·8, 19·2, and 19·9% respectively.

A 1% solution of material (A) in borate buffer (pH 10) gave a schlieren diagram with a symmetrical peak (Dr. C. T. Greenwood).

Electroionophoresis of material (A) on paper [20] followed by spraying with toluidine-blue,[29] a reagent specific for sulphate residues, gave a single discrete spot.

A portion of material (A), after conversion into the sodium salt,[15] was dissolved in water (200 ml.; 0·24% solution) and treated with stirring at room temperature with sufficient solid potassium chloride to give a 0·25M-solution.[15] No precipitation occurred immediately, after 4 days, or on repetition of the experiment at 0·15, 0·50, and 0·66M-concentrations of potassium chloride.

A dilute aqueous solution of material (A) in a sodium acetate–acetic acid buffer (pH 5·0) was incubated at 35° with a β-glucosidase preparation from *Cladophora rupestris*. Samples of the solution were spotted on a chromatogram initially and after 0·5 hr., 1 hr., 1·5 hr., and then at 2-hourly intervals, an enzyme control being run concurrently. The papers were irrigated with solvent (3) and sprayed with ammoniacal silver nitrate.[30] A single spot corresponding to glucose appeared after 2 hr. The enzyme control gave no spots on the chromatogram.

Treatment of an aqueous solution of material (A) with cetyltrimethylammonium bromide [16] gave an insoluble complex which on hydrolysis showed arabinose, galactose, xylose, and rhamnose (paper chromatography). Concentration and hydrolysis of the supernatant liquor gave a syrup which consisted mainly of glucose with minute quantities of the other four sugars (paper chromatography).

Periodate Oxidation and Investigation of the Oxopolysaccharide.—An aqueous solution of the sodium salt of material (A) was treated with 0·1M-sodium metaperiodate, and the periodate uptake was determined at intervals by the method of Fleury and Lange.[31] When this had virtually ceased (36 hr.) one mole of periodate had been consumed by 347 g. of polysaccharide. After 48 hours' treatment the oxopolysaccharide was isolated by (*a*) dialysis against running water (3—4 weeks) and freeze-drying, and (*b*) reduction of the periodate and iodate with gaseous sulphur dioxide followed by precipitation with ethanol. The precipitate was dissolved in water and reprecipitated in ethanol until free from inorganic contaminants. Recovery from both methods was 80—87% (Found: SO_4, 20·2; N, 1·3%). Hydrolysis and quantitative estimation of the relative proportions of sugars present gave arabinose 61·4, galactose 16·6, xylose trace, rhamnose 10·2—15·4, and glucose 6·6%.

Alkaline Degradation of the Polysaccharide and of the Oxopolysaccharide.—Material (A) (520 mg.) was heated at 100° with N-sodium hydroxide (100 ml.). Samples were withdrawn at

[25] Breddy and Jones, *J.*, 1945, 738.
[26] Hirst, Jones, and Woods, *J.*, 1947, 1048.
[27] Keilin and Hartree, *Biochem. J.*, 1948, **42**, 230.
[28] Wolfrom, *J. Amer. Chem. Soc.*, 1930, **52**, 2466.
[29] Rickets, Saddington, and Walton, *Biochem. J.*, 1954, **58**, 532.
[30] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.
[31] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 196.

intervals and the liberated sulphate was estimated as the barium salt. The percentage hydrolysis was calculated on an initial sulphate content of 19·6% :

Time of hydrolysis (hr.)	0·5	4	6	8
Hydrolysis of sulphate (%)	12·0	58·5	65·0	68·0

Material (A) (1·123 g.) and its derived oxopolysaccharide (1·820 g.) were treated with N-potassium hydroxide solution (100 ml.) at 95° in an atmosphere of nitrogen. Samples were withdrawn at intervals, dialysed free from extraneous ions against running water, evaporated to small bulk, and freeze-dried :

	Time of hydrolysis (hr.)	Recovery (%)	SO_4 content (%)	N content (%)
Material (A)	4	54	15·1	0·69
	8	51	13·2	0·60
Oxopolysaccharide	1	43	17·6	0·90
	3	37	14·1	0·50

As far as could be judged visually from chromatograms of the hydrolysates alkali-degradation effected no change in the relative proportions of the sugars in material (A) or in the oxopolysaccharide.

Partial Hydrolysis of the Polysaccharide and Oxopolysaccharide with Acid.—Material (A) (400 mg.) was kept at room temperature with 0·1N-oxalic acid solution (100 ml.). After one month a small quantity of galactose (paper chromatography) had been liberated. The solution was then heated at 50° in an atmosphere of carbon dioxide and samples were removed at intervals, neutralised (calcium carbonate) and analysed chromatographically (Table 1).

TABLE 1.

Duration of hydrolysis (hr.)	1	2	3	5	9
Galactose	××	××	××	××	××
Arabinose	—	—	×	××	××
Xylose	—	×	××	××	××
Rhamnose and glucose	—	—	—	—	××
Oligosaccharides	—	—	×	×	××

× = trace.

Material (A) and oxopolysaccharide therefrom (each *ca.* 350 mg.) were hydrolysed with 0·04N-sulphuric acid for 3 hr. at 90°. The solutions were neutralised and dialysed. Chromatography of the dialysates showed galactose, xylose, and a trace of arabinose from material (A), but the oxopolysaccharide dialysate showed general streaking down the paper. After further hydrolysis with N-sulphuric acid (2 hr. at 100°) clean spots for arabinose, rhamnose, and galactose were obtained. Chromatograms sprayed with toluidine-blue showed no sign of sulphated sugars.

The residual polysaccharides in the dialysis bags were dialysed against running water and isolated by freeze-drying. The polysaccharide (59 mg., 24%) on hydrolysis gave the original five sugars (paper chromatography). The oxopolysaccharide (X, 43 mg.; SO_4, 19·95%) gave a single spot (toluidine-blue) on the electrophoretogram, and on hydrolysis was shown to contain principally arabinose with much smaller amounts of galactose and glucose.

The acidity of a solution of material (A) (200 mg.), when treated with 0·014N-sulphuric acid (20 ml.) at 100°, increased to 0·021N during 7 hr.

Isolation of Oligosaccharides.—Material (A) (12 g.) was hydrolysed with 0·5N-oxalic acid (400 ml.) at 95° for 3 hr. The dialysate, after neutralisation with calcium carbonate, de-ionisation with Amberlite resin IR-120H, 4-OHB, and evaporation, gave a syrup (B) (10 g.). A portion of this was separated into two fractions on a charcoal–Celite column (50 : 50).[32] Fraction I (4 g.), eluted with water, contained monosaccharides and two fast-moving oligosaccharides. Fraction II (521 mg.), eluted with 15% aqueous ethanol, consisted of a series of oligosaccharides. Fraction I was separated on a cellulose column. After elution of the monosaccharides (butan-1-ol-saturated with water) the two oligosaccharides were eluted with ethanol. They both gave pink spots with aniline oxalate, had R_{gal} values (rate of travel relative

[32] Bacon and Bell, *J.*, 1953, 2528.

to galactose) of 0·87 (main component) and 0·97 [cf. 3-*O*-β-L-arabopyranosyl-L-arabinose, R_{gal} 0·78 which was run on the same paper (solvent 3)]. Both oligosaccharides gave only arabinose on further hydrolysis.

Fraction II was separated on a second charcoal–Celite column (3 × 40 cm.) at a constant temperature of 20°. The initial eluant was water containing 1% of ethanol at 30 ml./hr. The ethanol concentration was increased by 1% at 24 hr. intervals. Considerable overlapping of the constituents occurred. The oligosaccharides were separated finally by paper chromatography (solvent 3), as shown in Table 2.

TABLE 2.

Fraction	Wt. (mg.)	Colour of spot, aniline oxalate spray	R_{gal} (solvent 3)	Sugars on hydrolysis *
1	4·0	Pink	0·58	Arabinose
2	4·7	,,	0·68	Arabinose, xylose (1 : 1)
3	8·9	Brown	0·38	Arabinose, galactose, xylose (2 : 1 : 1)
4	5·7	*a*, ,,	0·29	Galactose
		b, ,,	0·69	Glucose
5	6·5	,,	0·58	Galactose, arabinose, xylose (1 : 1 : 1)
6	6·0	Pink	0·28	Arabinose
7	3·4	Brown	0·81	Glucose

* The proportions of sugars were estimated visually. Fraction 7 was chromatographically and electroionophoretically [20] identical with laminaribiose (solvents 1 and 4), and moved at the same rate as an authentic specimen of this disaccharide in the form of their *N*-benzylglycosylamines.[33]

A synthetic mixture of sugars (galactose 3·8 g., arabinose 4·2 g., xylose 1·2 g., rhamnose 0·5 g.) was treated with 0·5N-oxalic acid (400 ml.) and N-sulphuric acid (2·5 ml.) as in the preparation of syrup B (yield 8·7 g.). An aqueous solution of this syrup was allowed to percolate down the charcoal column used in the oligosaccharide separation. After the column had been left overnight at 20° graded elution, as before, gave monosaccharides (8·3 g.) from the aqueous eluant and (0·2 g.) from 10% and 50% aqueous-ethanolic eluants. No evidence of oligosaccharides was obtained.

Acetylation of the Polysaccharide.—The polysaccharide (19 g.), dispersed in formamide (150 ml.) in an " Ato-Mix " mixer at room temperature, was kept with pyridine (1 l.) and acetic anhydride (500 ml.) for 2 weeks at room temperature. The clear brown solution was diluted with ice-water and dialysed for 2—3 weeks. Concentration and freeze-drying gave a white powder (18·0 g.; Ac, 17·2%) which on dispersion in pyridine (70 ml.) at room temperature in the " Ato-Mix " mixer gave a clear brown jelly. This was kept with acetic anhydride (60 ml.) at room temperature for a week. Dialysis and freeze-drying gave the acetylated polysaccharide (17·8 g.; Ac, 19·4%). A third treatment with pyridine (50 ml.) and acetic anhydride (40 ml.) gave a recovery of 17·8 g. (Ac, 20·6; N, 1·4%). Repeated acetylation at room temperature and at higher temperatures failed to increase the acetyl content of the product. Precipitation by alcohol or acetone–ether (50 : 50) gave an acetylated polysaccharide difficult to purify. The acetylated material was extracted (Soxhlet) with chloroform, the extracts were evaporated to dryness, and the residue was washed with hot water and extracted with cold chloroform (3 × 10 ml.). The latter extracts, after evaporation to dryness, were saponified by warm dilute aqueous alkali. The solution was dialysed to neutrality, concentrated, and freeze-dried. The product (51·2 mg.), on acid hydrolysis, showed glucose with traces of galactose, arabinose, and xylose (paper chromatography). This glucan (SO_4 <3%; N, 0) gave no colour with iodine and had a periodate uptake [31] of 1 mole for 420 g. of glucan. The chloroform-insoluble acetate (16·5 g.) contained arabinose, galactose, xylose, and rhamnose in their usual proportions (chromatography of the hydrolysate) but was devoid of glucose.

Methylation of the Acetylated Polysaccharide.—The glucose-free acetylated polysaccharide (16·5 g.; Ac, 20·6%) was dispersed in water (400 ml.). Dimethyl sulphate (110 ml.) and 40% aqueous sodium hydroxide (120 ml.) were then added simultaneously, with stirring, during 6 hr. at <10° in nitrogen. Further quantities of dimethyl sulphate (110 ml.) and 40% alkali (120 ml.) were added dropwise daily for 5 days, and the mixture, after dialysis for 10 days against running water, was evaporated to small bulk. The methylation was repeated twice at room temperature. After a final dialysis for 3 weeks the polysaccharide was isolated, by freeze-drying, as an

[33] Bayly and Bourne, *Nature*, 1953, **171**, 385.

off-white powder (9·9 g.; OMe, 19·6%). Two further methylations during 6 hr. at 45° followed by 2 hr. at 70° gave, after neutralisation with acetic acid, dialysis for 10 days, and freeze-drying, a white hygroscopic solid (6·9 g.) (Found: OMe, 25·1; N, 0·6; SO_4^{2-}, 16·2; ash as sulphate, 8·3%), $[\alpha]_D^{21}$ +92° (*c* 4·0 in H_2O). This material was soluble in hot water and to a slight extent in chloroform. A hydrolysate of the chloroform extract (OMe, 26·1%) was chromatographically indistinguishable from that of the chloroform-insoluble residue (OMe, 24·8%).

Attempts to raise the methoxyl content by methylation with Purdie reagents, with thallous hydroxide and methyl iodide,[34] and by dispersion in dimethylformamide [35] were unsuccessful.

Hydrolysis of the Methylated Polysaccharide.—The methylated polysaccharide (6·0 g.) was heated with 5% methanolic hydrogen chloride (200 ml.) for 6 hr. The solution which was too dark for polarimetric readings was neutralised with silver carbonate and concentrated to a syrup. Hydrolysis with 0·5N-hydrochloric acid (200 ml.) for 3 hr. at 95°, neutralisation as before, and deionisation [hydrogen sulphide, then Amberlite resins IR-100(H) and IR-4B(OH)] gave a syrup which was thrice purified by dissolution in alcohol (40 ml.), filtration, and evaporation (yield 4·18 g.). This syrup was separated on a cellulose column (85 × 2·7 cm.) with light petroleum (b. p. 100—120°)–butan-1-ol (7 : 3 v/v) saturated with water. After 10 l. had been collected the ratio of the solvents was gradually changed first to 1 : 1 (v/v) and then, after another 6 l. had passed, to butan-1-ol saturated with water. Twenty-three fractions were separated, nine of which were mixtures due to overlap. The remaining fractions required purification and many of them were re-separated on paper. The overall recovery from the column was 85% but the weights recorded for the individual fractions are those obtained after repeated purification by treatment with activated charcoal in boiling alcohol or hot water, followed by alternate extraction of the resulting syrup with alcohol and water and concentration after each extraction.

Fraction 1. The syrup (75 mg.), R_G 0·94 [Solvent (1) for all methylated sugars], was a mixture of 2 : 3 : 5-tri-*O*-methylarabinose (trace) and 2 : 3 : 4-tri-*O*-methylxylose. Separation on paper [benzene–ethanol–water (169 : 47 : 15)] and purification gave a syrup (14 mg.), $[\alpha]_D^{18}$ +13° (*c* 0·8 in H_2O) (cf. 2 : 3 : 4-tri-*O*-methylxylose, $[\alpha]_D$ +18°).[36]

Fraction 2. The syrup (30·9 mg.) had $[\alpha]_D^{19}$ +106° (*c* 0·3 in H_2O), R_G 0·88 (Found: OMe, 50·8. Calc. for a tetramethylhexose: OMe, 52·5%), and was chromatographically identical with 2 : 3 : 4 : 6-tetra-*O*-methylgalactose. Demethylation gave galactose.

Fraction 3. The syrup (24 mg.) had $[\alpha]_D^{18}$ +8° (*c* 0·6 in H_2O), R_G 0·88, and corresponded to a dimethylrhamnose (chromatography). Electroionophoresis showed two components one moving at the same rate as 3 : 4-di-*O*-methylrhamnose, and the other stationary. Demethylation gave rhamnose. (Cf. 2 : 4-di-*O*-methyl-L-rhamnose, $[\alpha]_D$ −19°;[37] 3 : 4-di-*O*-methyl-L-rhamnose, $[\alpha]_D$ +18·6°;[30] 2 : 3-di-*O*-methyl-L-rhamnose, $[\alpha]_D$ +42·5°.[39])

Fraction 4. The syrup (60·2 mg.) had $[\alpha]_D^{18}$ −3·8° (*c* 0·9 in H_2O), R_G 0·86 (Found: OMe, 40·9. Calc. for $C_9H_{18}O_6$: OMe, 41·9%), and gave galactose on demethylation. Conversion into the lactone gave a syrup (13 mg.), $[\alpha]_D^{18}$ −29° (*c* 0·6 in H_2O) (cf. 2 : 3 : 5-tri-*O*-methyl-D-galactose, $[\alpha]_D$ −5°;[40] 3 : 4 : 6-tri-*O*-methyl-D-galactose, $[\alpha]_D$ −4·3°;[41] 2 : 3 : 5-tri-*O*-methyl-D-galactonolactone, $[\alpha]_D$ −32°;[40] 3 : 4 : 6-tri-*O*-methylgalactonolactone, $[\alpha]_D$ +46·8°[41]).

Fraction 5. The syrup (41 mg.) had $[\alpha]_D^{18}$ +20° (*c* 1·1 in H_2O), crystallising very slowly. It was chromatographically and ionophoretically identical with 2 : 3-di-*O*-methylxylose[42] ($[\alpha]_D$ +23°) and gave xylose on demethylation.

Fraction 6. The syrup (235 mg.) had $[\alpha]_D^{18}$ +100° (*c* 1·3 in H_2O), R_G, 0·63. Chromatography (solvents 1, 3, and 4) and electroionophoresis gave a single spot corresponding to 2 : 3 : 4-tri-*O*-methylgalactose, but demethylation gave galactose and arabinose (Found: OMe, 38·6. Calc. for $C_9H_{18}O_6$: OMe, 41·9. Calc. for $C_7H_{14}O_5$: OMe, 34·8%) (cf. 2 : 3 : 4-tri-*O*-methylgalactose,[43] $[\alpha]_D$ +121°; 2 : 3-di-*O*-methyl-L-arabinose,[44] $[\alpha]_D$ +107°).

[34] Hirst and Jones, *J.*, 1938, 502.
[35] Kuhn, Löw, and Frischmann, *Angew. Chem.*, 1955, **67**, 32; *Chem. Ber.*, 1955, **88**, 1492.
[36] Hirst, Percival, and Wylam, *J.*, 1954, 195.
[37] E. E. Percival and Charalambous, *J.*, 1954, 2443.
[38] Laidlaw and Percival, *J.*, 1949, 1600.
[39] Brown, Hough, and Jones, *J.*, 1950, 1125.
[40] Luckett and Smith, *J.*, 1940, 1116.
[41] Levene and Meyer, *J. Biol. Chem.*, 1931, **92**, 257.
[42] Chanda, Percival, and Percival, *J.*, 1952, 260.
[43] Levene and Kreider, *J. Biol. Chem.*, 1937, **121**, 155.
[44] Hirst and Jones, *J.*, 1948, 2311.

Fraction 7. The syrup (27 mg.) crystallised and had $[\alpha]_D^{18}$ +15° (*c* 1·4 in H_2O),[45] R_G 0·57, m. p. and mixed m. p. with 4-*O*-methylrhamnose ($[\alpha]_D$ +13°) 122°.

Fraction 8. The syrup (33 mg.) had $[\alpha]_D^{18}$ +101° (*c* 1·6 in H_2O), R_G 0·54, and gave arabinose on demethylation. The derived lactone had $[\alpha]_D$ +88° ⟶ +40° (*c* 0·4 in H_2O) (cf. 2 : 4-di-*O*-methyl-L-arabinose,[46] $[\alpha]_D$ +118°; 2 : 4-di-*O*-methyl-L-arabonolactone,[47] $[\alpha]_D$ +99° ⟶ +39°).

Fraction 9. The syrup (539 mg.) had $[\alpha]_D^{18}$ +101° (*c* 2·0 in H_2O), R_G 0·38, and was chromatographically and electroionophoretically identical with 2-*O*-methylarabinose (Found : OMe, 18·0. Calc. for $C_6H_{12}O_5$: OMe, 18·9%). Demethylation gave arabinose. The derived lactone had $[\alpha]_D$ −38° (*c* 1·1 in H_2O) (cf. 2-*O*-methyl-L-arabinose,[48] $[\alpha]_D$ +100°; 2-*O*-methyl-L-arabonolactone,[44] $[\alpha]_D$ −44° ⟶ −40°).

Fraction 10. The syrup (32 mg.), R_G 0·38, was chromatographically and ionophoretically identical with 2 : 4-di-*O*-methylgalactose, and crystallised. After recrystallisation from ethyl acetate it had m. p. and mixed m. p. 80° (the recorded m. p. for this derivative [49] is 98—99° but authentic specimens in our possession all melted at 78—80°).

Fraction 11. The syrup (51·5 mg.) had $[\alpha]_D^{18}$ +112° (*c* 1·5 in H_2O), R_G 0·28, and was chromatographically and electroionophoretically identical with 3-*O*-methyl-L-arabinose. Demethylation gave arabinose. The derived lactone had $[\alpha]_D^{18}$ −65° (*c* 0·6 in H_2O) (cf. 3-*O*-methyl-L-arabinose, $[\alpha]_D$ +110°; derived lactone [50] −74°).

Fraction 12. The syrup (113 mg.) had $[\alpha]_D^{17}$ +78° (*c* 1·5 in H_2O), R_G 0·21, was chromatographically and electroionophoretically identical with 2-*O*-methylgalactose and gave galactose on demethylation. The derived lactone had $[\alpha]_D^{18}$ −18° (*c* 0·5 in H_2O) (cf. 2-*O*-methylgalactose, $[\alpha]_D$ +80°; derived lactone [51] $[\alpha]_D$ −27 ⟶ −24°).

Fraction 13. The syrup (303 mg.), R_G 0·14, chromatographically identical with arabinose, crystallised from methanol, then having m. p. and mixed m. p. with β-L-arabinose 154°, $[\alpha]_D^{18}$ +195° ⟶ +106° (*c* 1·2 in H_2O).

Fraction 14. The syrup (498 mg.), R_G 0·08, chromatographically identical with galactose, crystallised from methanol, then having m. p. and mixed m. p. with α-D-galactose 164—166°, $[\alpha]_D^{19}$ +130 ⟶ +81° (*c* 1·4 in H_2O).

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[45] Levene and Compton, *J. Biol. Chem.*, 1936, **114**, 9.
[46] Andrews, Ball, and Jones, *J.*, 1953, 4090.
[47] Jones, *J.*, 1953, 1672.
[48] Jones, Kent, and Stacey, *J.*, 1947, 1341.
[49] Andrews, Hough, and Jones, *J.*, 1954, 806.
[50] Hirst, Jones, and Williams, *J.*, 1947, 1062.
[51] Hirst and Jones, *J.*, 1946, 506.