The Polysaccharide from the Alga Ulva lactuca. *Purification, Hydrolysis, and Methylation of the Polysaccharide.*

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The isolation of sulphated polysaccharide material from *Ulva lactuca* is described. Preliminary evidence of structure is adduced in the detection and estimation of residues of D-xylose, L-rhamnose, D-glucose, and D-glucuronic acid, and in the identification of 2 : 3 : 4-tri-*O*-methyl- and 2 : 3-di-*O*-methyl-L-rhamnose and 2 : 3 : 6-tri-*O*-methyl-D-glucose after hydrolysis of the methylated polysaccharide. The sugar derivatives present in a less fully methylated residue have been investigated by paper chromatography and include 2 : 3 : 4-tri-*O*-methyl- and 2 : 3-di-*O*-methylxylose, 2 : 3 : 4-tri-*O*-methyl- and 2 : 3-di-*O*-methylrhamnose, and unmethylated xylose and rhamnose. Oxidation of the carbohydrate by periodate, before and after attempted desulphation, has been investigated.

The main object of the work described is to obtain information concerning the polysaccharides of green algæ, detailed work in this field having been previously concerned with similar products isolated from red or brown seaweeds. In the latter, residues of galactose, fucose, and uronic acid commonly occur as structural units and many of these are present as sulphate esters.

The polysaccharide from *Ulva lactuca* is structurally complex, containing sulphate ester and uronic acid groups as well as xylose, rhamnose, and glucose residues. The first investigations were carried out on material obtained from the Sussex coast in early July. Owing to cessation of work during the war a preliminary account was published (Plant and Johnson, *Nature*, 1941, **147**, 390) wherein the sulphate ester nature of the polysaccharide, and the isolation of a crystalline derivative of L-rhamnose, from its hydrolysate were reported. The available evidence for a uronic acid component was not considered adequate. For the continuation of the work, material was obtained, in August 1946 and 1947, from the Kyles of Bute, through the kind assistance of the Director of the Scottish Marine Biological Association Laboratory, Millport. The method of extraction was similar to that used previously but purification has been carried further, yielding a product with 0·8% of ash and 4% of nitrogen. Purified material, in the form of its sodium salt, was used for methanolysis and hydrolysis experiments.

During these studies, amino-acids and peptides were detected among the breakdown products, indicating contamination by protein, even though the common protein colour reactions were not noticeable when applied to the polysaccharide. The Sevag process (*Biochem. Z.*, 1934, **273**, 419) proved the most satisfactory method for removing this protein, giving material with a nitrogen content of 1%, but the purification involved much loss of carbohydrate. No evidence for the presence of an amino-sugar was obtained.

It was extremely difficult to remove the sulphate groups (cf. Percival and Ross, *J.*, 1950, 717; Percival and Johnston, *J.*, 1950, 1994). There was only a small loss of sulphate on treatment with barium hydroxide under conditions which caused extensive loss of polysaccharide. The periodate oxidation of the polysaccharide, before and after treatment with barium hydroxide, was investigated, sodium metaperiodate at 3° being used (Potter and Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3488). The rate of periodate uptake fell to a small steady value after about 16 hours and the values obtained by extrapolation, namely, 1 mole absorbed by 960 g. of polysaccharide and liberation of 1 mole of formic acid from 4300 g., indicate that there are few contiguous free hydroxyl groups in the structure. Reaction with sodium hypoiodite showed one oxidisable group in 3450 g. of the polysaccharide (SO_4, 15·9%). Parallel results on the partly desulphated material (SO_4, 13·8%) were: 1 mole of periodate absorbed by 520 g., with liberation of 1 mole of formic acid from 1780 g., the hypoiodite reducing power remaining virtually unchanged. These results suggest that ·CH(OH)·CH(O·SO_3Na)·CH(OH)· groups may be present.

One mole of uronic acid is present in 930 g. The equivalent weight, determined by titration, is in agreement with that calculated from the uronic and sulphate content. There was no reduction in the uronic acid content of material which had been treated with barium hydroxide.

In the earlier stages of the work (Plant and Johnson, *loc. cit.*), partial hydrolysis of the polysaccharide was effected by 0·2N-sulphuric acid. After conversion of the products into methyl glycosides, the mixture was methylated and fractionated. Methyl tri-*O*-methylrhamnoside was isolated, this being the first instance of the occurrence of rhamnose in an algal polysaccharide. In this work, methanolysis was preferred as a method of breakdown, since the loss of uronic acid was less serious: the uronic acid, with other acidic material, was separated as its barium salt, and shown to be D-glucuronic acid both by reduction of its methyl ester to glucose by lithium aluminium hydride and by complete methylation to methyl (methyl 2 : 3 : 4-tri-*O*-methyl-D-glucosid)uronate. The glycosides were partly fractionated by solvent extraction, then hydrolysed and examined on the paper chromatogram. In addition to rhamnose, this showed the presence of xylose and glucose, the identities of which were established respectively by the preparation crystalline dibenzylidene dimethyl acetal of D-xylose and 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose. Glucose hydrazone or osazone derivatives could not be isolated.

The free sugars liberated on acid hydrolysis of the polysaccharide were estimated by periodate oxidation (Hirst and Jones, *J.*, 1949, 1659) after separation on the paper chromatogram. The composition was found to be xylose 9·4%, rhamnose 31%, glucose 7·7%, these being minimal values, since a small amount of material which moved more slowly than these sugars on the paper chromatograms was present. By distillation with hydrochloric acid, the glucuronic acid was shown to form 19·2% of the polysaccharide.

The polysaccharide could not be acetylated, as it formed a hard insoluble product in pyridine, but it was methylated by repeated treatments with methyl sulphate and alkali. The product was then fractionated by extraction with chloroform and the soluble portion (approx. 17%) was treated with Purdie reagents until a maximum methoxyl content (31%) was attained. This product contained 3% of organically bound sulphate; the material which was not soluble in chloroform contained 13% of sulphate. In addition, the insoluble material did not appear to contain glucose, so that some fractionation into different methylated polysaccharides took place on treatment with chloroform, although attempts to fractionate the starting material by precipitation in ethanol at various pH's did not effect any separation. After breakdown of the fully methylated product with methanolic hydrogen chloride and removal of acids as barium salts, 2 : 3 : 4-tri-*O*-methylrhamnose was isolated after hydrolysis and was the only product indicative of an end group detected in

this fraction. The presence of 2 : 3-di-*O*-methyl- and 2 : 3 : 6-tri-*O*-methyl-glucose was also established. The latter shows the presence of 1 : 4-linked glucose residues, and the possibility that some of these are present in cellulosic material cannot be excluded definitely. Methylated cellulose is soluble in chloroform, and after hydrolysis of the insoluble fraction and examination of the resultant sugar derivatives by paper chromatography, no partially methylated or free glucose was detected. The paper chromatograms showed the presence of 2 : 3 : 4-tri-*O*-methyl- and 2 : 3-di-*O*-methyl-xylose, 2 : 3 : 4-tri-*O*-methyl- and 2 : 3-di-*O*-methyl-rhamnose, and unmethylated rhamnose and xylose. A monomethyl-rhamnose and -xylose were also present. Thus it appears that the insoluble fraction is either very resistant to methylation, possibly owing to the presence of sulphate groups, or that it is a highly branched polysaccharide.

No evidence is yet available for fixing the position of the sulphate groups except that they must be linked to xylose or rhamnose, for organically bound sulphate remains in the chloroform-insoluble methylated material and this does not contain glucose.

Experimental

Isolation of the Polysaccharide.—*Ulva lactuca* was obtained, washed and dried, from the Marine Biological Station, Millport. Pigments were extracted from the fronds with cold 85% acetone. The product (100 g.) was heated with 0·5% and 0·25% sodium carbonate solution on a boiling-water bath. The combined extracts were concentrated, at 85°, to about one-third volume (10 l.) and decanted from a residue of calcium salts, and hydrochloric acid was added during further concentration to maintain the pH between 7 and 8. The neutralised extract (600 ml.) was dialysed against 0·02N-acetic acid, followed by distilled water. The remaining inorganic impurity (ash 6%, Ca^{2+}, Fe^{3+}, SO_4^{2-}) was removed from a 1% aqueous solution of the carbohydrate by " Zeocarb 215(H)," followed by dialysis. This solution, containing free acid polysaccharide, was used for rotation, ash, and equivalent determinations, but it cannot be satisfactorily concentrated. The sodium salt was prepared by neutralisation with sodium hydroxide, concentration to 400 ml., and precipitation in ethanol (4 l.). The fibrous product was converted into a white hygroscopic powder (~20 g.) by trituration with ethanol and ether; this had $[\alpha]_{5461}^{20}$ −84° (free acid, *c*, 0·2 in H_2O), $[\alpha]_D^{21}$ −47° (Na salt, *c*, 0·35 in H_2O) [Found : C, 40·4; H, 5·34; N, 3·9; OMe, 2·2; ash, 0·8, 19·4 (Na salt); total SO_4, 15·9; SO_4 from ash of sodium salt, 12·7%; equiv. (by alkali titration), 386]. Oxidation of the sodium salt with alkaline iodine in a carbonate–bicarbonate buffer (Auerbach and Bodländer, *Z. angew. Chem.*, 1923, **36**, 602) showed one oxidisable group to be present in 3450 g. of (acid) polysaccharide. Estimation of uronic acid by determination of the carbon dioxide liberated on distillation with 13·15% hydrochloric acid (Dickson, *J. Amer. Chem. Soc.*, 1930, **52**, 775) showed one uronic acid residue to be present in 913 g.

Unless otherwise stated, all figures are given as a percentage of ash-free acid polysaccharide.

Removal of Protein.—The sodium salt (10 g.) was dissolved in water (300 ml.), chloroform (225 ml.) and amyl alcohol (22 ml.) were added, and the whole was shaken for 6 hr. The aqueous phase was separated, by centrifugation, from the white, gelatinous lower layer and given four more similar treatments, giving the purified polysaccharide (3 g.) (Found : C, 36·4; H, 5·82; N, 1·08; ash of Na salt, 19·0; SO_4, 17·5%). The chloroform layers were washed with water and the solvent distilled off, leaving 2 g. of residue (Found : ash, 11·5; SO_4, 5·3%; biuret and xanthoproteic tests, positive).

Attempted Removal of Sulphate from the Polysaccharide.—Treatment with barium hydroxide was best effected at >60°. The sodium salt of the polysaccharide was treated with N-barium hydroxide for 30 hr. in nitrogen. The solution was neutralised with sulphuric acid, then centrifuged, and organic material was removed from the barium sulphate by dilute sodium hydroxide at pH 8. The combined aqueous material was concentrated and worked up by dialysis and treatment with resin, as described for the *Ulva* polysaccharide. Approx. 25% of the organic starting material passed through the Cellophane in the first dialysis. Examination by paper chromatography showed that this material contained very little carbohydrate and at least eight amino-acids. The recovery of polysaccharide was 13% (Found : C, 40·2; H, 5·49; N, 0·63; OMe, 2·95; ash, 1·4; SO_4, 13·8%; equiv., 450). One g.-equiv. of alkaline iodine solution reacts with 3400 g. One uronic acid residue is present in 770 g.

Periodate Oxidation.—An approx. 1% solution of the polysaccharide (4% of N) was cooled to 3° and 0·2M-sodium metaperiodate was added (10 ml. to 100 ml. of solution). A series of 10-ml. samples were withdrawn and kept at 3°; at intervals up to 36 hr. they were analysed seriatim

for periodate content. After 16 hr., the rate of periodate uptake fell to a small steady value, and a back-extrapolation of the plot of periodate uptake against time gave the periodate consumption by the polysaccharide (1 mole was required for the oxidation of 960 g. of the polysaccharide and 520 g. of the material which had been treated with barium hydroxide). The production of formic acid was estimated after 50 hours' oxidation (1 mole of formic acid was liberated from 4300 g. of carbohydrate and from 1780 g. of material treated with barium hydroxide). A trace of ammonia was liberated during the oxidation.

Methanolysis of the Polysaccharide.—A concentrated aqueous solution of the sodium salt of the polysaccharide was precipitated in ethanol containing concentrated hydrochloric acid. The product (56 g.; ash, 7·2%) was triturated with methanol, suspended in 3% methanolic hydrogen chloride (560 ml.), and refluxed for 72 hr. with two further additions of gaseous acid. After aeration, a solid residue was filtered off (6 g.), and the solution neutralised with silver carbonate. The methanol extract and a suspension of the silver salts in methanol were each treated with hydrogen sulphide to liberate organic acids. The syrup obtained on removal of the methanol from these solutions {50·4 g.; $[\alpha]_{5461}^{15}$ +2° (in MeOH); OMe, 15%} was treated with 500 ml. of N-barium hydroxide, in nitrogen, for 30 hr. at 60°; at this stage precipitation of barium sulphate appeared complete. Excess of barium hydroxide was removed by carbon dioxide; the filtrate and washings were concentrated at 40° to a stiff syrup (43·7 g.; SO_4, 3·2%).

The syrup was fractionated, first by extraction with acetone, giving fraction A (1·8 g.). The residue was extracted with ethanol containing 1% of ether, giving fraction B (22·9 g.). The remaining barium salts, fraction C (15·2 g.), were subdivided by twice esterifying them with 2% methanolic hydrogen chloride in the usual way and extracting the resultant syrup with acetone. The combined acetone extracts, fraction C_1 (9·1 g.), contained the esterified uronic constituents. The residue was extracted with 96% ethanol to separate the barium salts of organic sulphates from inorganic salts, to give fraction C_2 (2·3 g.). Fraction B contained organic sulphate, but further cross-extractions of fractions B and C did not improve the separation.

After removal of glycosidic groups, the fractions were examined by paper chromatography (Hough, Jones, and Wadman, *J.*, 1950, 1702), with the upper phases of the following solvent mixtures: *n*-butanol (4), ethanol (1), and water (5) containing 1% of ammonia; ethyl acetate (2), pyridine (1), and water (2); and ethyl acetate (3), acetic acid (1), and water (3); with aniline hydrogen phthalate and naphtharesorcinol sprays. Constituents were recognised by their moving identically with authentic samples in all three solvent media.

p-Dimethylaminobenzaldehyde, with or without the prior addition of acetylacetone, did not give the cherry-red colour, characteristic of amino-sugars, on any of the fractions from methanolysis, or on chromatography papers. But the original polysaccharide and fraction B slowly developed an orange-red colour, without acetylacetone. No acetyl groups are present and this colour is probably due to protein. No 2-hydroxy-1-naphthaldehyde derivative of an amino-sugar could be obtained.

Fraction A gave negative tests for sulphate and uronic acid. After hydrolysis it was shown by chromatography to contain xylose, rhamnose, and glucose. Following Breddy and Jones's method (*J.*, 1945, 738), the product (0·40 g.) was treated with benzaldehyde in 0·4 N-methanolic hydrogen chloride (0·4 ml. in 1·6 ml.) and left for 7 days. The crystalline product was washed with water and methanol, and dried (0·08 g.; m. p. 202—205°). After recrystallisation from chloroform–light petroleum, it had m. p. 206° (not depressed by admixture of an authentic specimen of di-*O*-benzylidene-D-xylose dimethyl acetal), $[\alpha]_D^{17}$ −8° (*c*, 1·0 in $CHCl_3$) (cf. Breddy and Jones, m. p. 211°, $[\alpha]_D^{20}$ −9°) (Found: C, 66·5; H, 6·2. Calc. for $C_{21}H_{24}O_6$: C, 67·7; H, 6·5%).

Fraction B contained nitrogen and sulphate (3·2%). After hydrolysis, chromatography showed the presence of xylose, rhamnose, glucose, a uronic acid, and two other slow-moving constituents. It was not possible to isolate glucosazone from fraction A, after hydrolysis and removal of xylose, nor from fraction B after hydrolysis, although the latter was fermented by *Saccharomyces cerevisiae*.

An ethanol (95%) extract of fraction B was methylated twice with methyl sulphate and alkali, followed by Purdie reagents in the usual way (3·26 g.; OMe, 41·5%). Some fully methylated xylose and rhamnose (1·14 g.) were removed at 135—140°/18 mm., and the syrup was then distilled at 0·4 mm., giving fractions (*a*) (0·217 g.), b. p. (bath) 97—100°, (*b*) (1·086 g.), b. p. 105—110°, and (*c*) (0·293 g.), b. p. 110—145°. Fraction (*b*) (0·19 g.), $[\alpha]_D^{20}$ +79°, n_D^{25} 1·4418 (OMe, 56·5%), was hydrolysed for 8 hr. in N-sulphuric acid (10 ml.) at 100°, the rotation becoming constant ($[\alpha]_D^{25}$ +67°). After removal of the acid with "Deacidite," paper chromatography in butanol showed the presence of two constituents; the faster was tetra-*O*-methylglucose, the

slower (R_G 0·89) appeared to be a di-*O*-methylrhamnose. This mixture was quickly extracted with a little light petroleum and when kept deposited 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose (0·10 g.); when recrystallised from light petroleum, this had m. p. 88—90°, $[\alpha]_D^{21}$ +84°, const. (*c*, 0·8 in H_2O) (Found: C, 50·7; H, 8·3; OMe, 51·7. Calc. for $C_{10}H_{20}O_6$: C, 50·9; H, 8·5; OMe, 52·6%).

Fraction C_1 (SO_4, 1·8; OMe, 15·2%), on examination by paper chromatography after hydrolysis, was shown to contain only traces of xylose, rhamnose, and glucose, with much uronic acid. The Dische colorimetric test for glucuronic acid (*J. Biol. Chem.*, 1947, **171**, 725) gave no conclusive evidence for the presence of this acid. The Ehrlich basic lead acetate reaction gave a yellow precipitate, indicative of glucuronic or mannuronic acid.

Fraction C_1 (0·2 g.) in boiling dioxan (15 ml.) was reduced by lithium aluminium hydride for 4 hr. (Lythgoe and Trippett, *J.*, 1950, 1983). The mixture was cooled to 0° and neutralised with N-sulphuric acid, aluminium hydroxide was filtered off, and the filtrate and washings were concentrated. After acidification and hydrolysis of glycosidic groups, the solution was de-ionised by barium carbonate and resins, and concentrated, and the residue (0·16 g.) was examined on the paper chromatogram together with samples of fraction C_1. There was a marked increase in the amount of glucose, relative to the xylose and rhamnose, and only a trace of uronic acid remaining in the reduced sample.

Fraction C_1 (0·3 g.) was methylated by five treatments with Purdie reagents. The product (0·23 g.; OMe, 58·4%) was hydrolysed by barium hydroxide, in the usual way, for separation of the acidic constituents as barium salts. The salts were re-esterified by 3% methanolic hydrogen chloride, and the product was distilled in a small still at 90—95°, leaving a little residue of less volatile material and affording methyl 2 : 3 : 4-tri-*O*-methyl-D-glucuronosidate, $[\alpha]_D^{19}$ +146° (Found: OMe, 58·1%). Treatment of this with methanolic ammonia at 0° and two recrystallisations from ethanol–light petroleum gave 2 : 3 : 4-tri-*O*-methyl-α-D-glucuronosidamide, m. p. 186°, $[\alpha]_D^{20}$ +145° (Smith, *J.*, 1951, 2649, gives m. p. 188—189°, $[\alpha]_D^{25}$ +149°) (Found: C, 48·0; H, 7·6; N, 5·9; OMe, 49·5. Calc. for $C_{10}H_{19}O_6N$: C, 48·2; H, 7·7; N, 5·6; OMe, 49·8%).

Estimation of the Sugars Liberated on Hydrolysis.—It was found that a higher value for the xylose content of the polysaccharide was obtained if hydrolysis was effected in two stages. Accordingly, the polysaccharide (314 mg.) was first hydrolysed with 0·3N-sulphuric acid (7 ml.) in a sealed tube at 100° for 12 hr.; the product was neutralised with barium carbonate, concentrated, and extracted with 98% ethanol for removal of monosaccharides. The remaining organic material was extracted with water, and the extract was concentrated and hydrolysed with N-sulphuric acid for 15 hr. This solution, the ethanol-soluble syrup, and mannose (84 mg.) were combined and the whole de-ionised by Amberlite IR-4B and IR-400. The solution was concentrated to approx. 1·5 ml. and samples were run on the paper chromatogram in butanol solvent (Hough, Jones, and Wadman, *J.*, 1950, 1702). The sugars were located, extracted from the paper with water, and estimated by periodate oxidation (Hirst and Jones, *loc. cit.*). Assuming that mannose is quantitatively assayed gave the results: xylose 3·26, rhamnose 10·64, glucose 2·66, and mannose 8·18 mg., showing the polysaccharide to contain 9·4% of xylose, 31% of rhamnose, and 7·7% of glucose (percentages of anhydro-sugars). Slower-moving constituents on the chromatograms were not estimated.

Methylation of the Polysaccharide.—The sodium salt of the polysaccharide (15 g. of organic material), which had been precipitated in alcohol and triturated with water, was treated with 30% aqueous sodium hydroxide (670 ml.) and methyl sulphate (250 ml.) for 10 hr. in nitrogen at room temperature. After dialysis, the solution was concentrated at 45°. Six more methylations were carried out at 40°, with reduced quantities of reagents. The average yield was 85%. This material, $[\alpha]_D^{18}$ −11° (*c*, 2 in water) (OMe, 24%), was separated by chloroform extraction into a soluble fraction, $[\alpha]_D^{20}$ −27° (*c*, 0·6 in $CHCl_3$), and an insoluble residue (75% of the methylated material).

The soluble fraction (9 g.; ash, 4%) was repeatedly methylated with methyl iodide (40 ml.) and silver oxide (20 g.). After four treatments no further increase in methoxyl content was obtained. The product (7·2 g.) was a white powder; $[\alpha]_D^{20}$ −30° (*c*, 0·4 in $CHCl_3$) (Found: C, 50·5; H, 7·6; N, 2·4; OMe, 31; ash, 4; organically bound sulphate, 2·6%). On distillation with 13·15% hydrochloric acid, 44 g. of carbon dioxide were liberated from 2150 g. This material was dispersed in 4% methanolic hydrogen chloride and refluxed for 60 hr. with five further additions of acid gas. After aeration and treatment with silver carbonate, the organic material (80%) was separated into an ether-soluble fraction (50%), $[\alpha]_D^{21}$ +20° (OMe, 42%), and an insoluble fraction (30%; OMe, 32%). The soluble portion was treated with barium

hydroxide, in the usual way, giving fraction M (33%; OMe, 43%). The barium salts were re-esterified with 1·5% methanolic hydrogen chloride to give an ester fraction (10%; OMe, 37%). Yields and methoxyl figures are average values.

The insoluble fraction, after dialysis against 0·02N-acetic acid and distilled water, was neutralised with sodium hydroxide solution and worked up in the usual way, yielding a greenish solid, fraction N (OMe, 17·2; ash, 11·4; organically bound sulphate, 13%; 44 g. of carbon dioxide liberated from 1010 g.).

Fraction M was distilled at 2×10^{-3} mm., to give fractions: (i) (233 mg.) b. p. (bath) 106—108°, n_D^{25} 1·4503 (OMe, 49·1%); (ii) (53 mg.) b. p. 110—115°; (iii) (619 mg.) b. p. 122°, n_D^{25} 1·4562 (OMe, 45·3%); (iv) (199 mg.) b. p. 144—148°, n_D^{18} 1·4633 (OMe, 51·3%); (v) (43 mg.) b. p. 158—168°; (vi) (100 mg.) b. p. 170—175° (OMe, 44·7%); and a residue (1·094 g.). Positive Schiff and Seliwanoff reactions were given by fractions (iii) and (iv).

Fraction (i) was hydrolysed by 0·1N- followed by N-hydrochloric acid, $[\alpha]_D^{20}$ +35·4° (½ hr.) changing to +41·6° (5 hours, const.). Acid was removed by "Deacidite," and the free sugars were extracted with ether to remove traces of material from the resin (93%), giving material having $[\alpha]_D^{25}$ +46·2° (OMe, 42·0%). Paper chromatography showed the presence of 2 : 3 : 4-tri-*O*-methylrhamnose (major part), 2 : 3 : 6-tri-*O*-methylglucose, and an unidentified constituent (R_G 0·86—0·87). Extracting the reducing sugars with cold light petroleum and refluxing the extract (72 mg.) with alcoholic aniline gave the aniline derivative (32 mg.) of tri-*O*-methylrhamnose; after recrystallisation from ethanol–ether, this had m. p. and mixed m. p. 110°, $[\alpha]_D^{19}$ +130° (after 14 hr.; *c*, 0·3 in $COMe_2$) (Found: C, 64·8; H, 8·1; N, 5·15; OMe, 32·6. Calc. for $C_{15}H_{23}O_4N$: C, 64·1; H, 8·2; N, 5·0; OMe, 33·1%). The sugars remaining after petroleum extraction deposited crystals of 2 : 3 : 6-tri-*O*-methylglucose; drained on a porous tile and recrystallised from ethanol–ether, this had m. p. and mixed m. p. 120°, $[\alpha]_D^{19}$ +69° (*c*, 1·5 in H_2O). The crude solid (55 mg.) was oxidised with bromine in the usual way, and the product was distilled, giving 2 : 3 : 6-tri-*O*-methylgluconolactone, $[\alpha]_D^{20}$ +57° ⟶ +38° (2 days, *c*, 1·1 in H_2O containing a trace of acid). This was converted into 2 : 3 : 6-tri-*O*-methylgluconophenylhydrazide, m. p. 143—144° (from ethyl acetate) (Found: C, 54·7; H, 7·4; N, 8·4; OMe, 28·0. Calc. for $C_{15}H_{24}O_6N_2$: C, 54·9; H, 7·3; N, 8·6; OMe, 28·4%).

After hydrolysis, nucleation, and storage, fraction (ii) deposited crystals of 2 : 3 : 6-tri-*O*-methylglucose, m. p. 119°, and was not further investigated.

Fraction (iii) was hydrolysed by 0·1N-sulphuric acid, $[\alpha]_D$ +34° ⟶ +48° (8 hr., const.); after its neutralisation with barium carbonate, reducing sugars were recovered in 60% yield; this product was hydrolysed with N-acid {$[\alpha]_D^{18}$ +46° (const. after 3 hr.)} and recovered in 90% yield, having $[\alpha]_D^{18}$ +45° (*c*, 1·0 in H_2O) (OMe, 33·1%). The loss is probably connected with the presence of a labile constituent, responsible for the colour reactions, and in a similar experiment lævulic acid (dinitrophenylhydrazone, m. p. 205°) was obtained in small yield from the residue of barium salts.

Examination by paper chromatography showed that the reducing sugars consisted mainly of 2 : 3-di-*O*-methylrhamnose with some 2 : 3 : 6-tri-*O*-methylglucose. Treatment with ethanolic aniline gave the aniline derivative of 2 : 3-di-*O*-methylrhamnose (*J.*, 1950, 690), which had m. p. 138°, not depressed by an authentic specimen kindly provided by (the late) Dr. E. G. V. Percival (Found: C, 62·5; H, 7·8; N, 4·9; OMe, 22·6. Calc. for $C_{14}H_{21}O_4N$: C, 62·9; H, 7·9; N, 5·2; OMe, 23·2%).

Fraction N (2 g.) was hydrolysed by N-sulphuric acid for 24 hr. and the insoluble material which remained was hydrolysed by N-sulphuric acid for a further 48 hr. Inorganic material was removed from the combined hydrolysates by resins, and the syrup obtained (0·9 g.) was examined by paper chromatography, with aniline oxalate, *p*-anisidine hydrochloride, and naphtharesorcinol sprays. Constituents of the syrup were shown to behave identically with the following sugars: 2 : 3 : 4-tri-*O*-methylxylose, 2 : 3 : 4-tri-*O*-methylrhamnose, 2 : 3-di-*O*-methylrhamnose, xylose, and rhamnose.

2 : 3-Di-*O*-methyl- and 2-*O*-methyl-xylose, 4-*O*-methylrhamnose, and methylated uronic acids were also probably present. No glucose or methylated glucoses were detected.

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