535. *Properties of Periodate-oxidised Polysaccharides. Part VI.* The Mucilage from* Dilsea edulis.

By VINCENT C. BARRY and JOAN E. MCCORMICK.

The mucilage extracted by dilute hydrochloric acid from the red alga, *Dilsea edulis*, has been shown to yield, on acid hydrolysis, D-galactose, xylose, glucurone, sulphuric acid, and traces of 3 : 6-anhydro-D-galactose and hydroxymethylfurfuraldehyde. By successive applications of the method of periodate oxidation followed by phenylhydrazine degradation results have been obtained on the basis of which the structures of the mucilage and its degradation products are discussed.

IN a previous report [1] Barry and Dillon suggested that the main structural unit of this mucilage was a repeating unit of five D-galactopyranose residues, the first four of which were linked 1 : 3, while the fifth, which was linked 1 : 6, also carried a sulphuric acid residue on $C_{(4)}$. This suggestion was based on the results of periodate oxidation and on the difficulty of removing the sulphuric acid by alkaline hydrolysis. Subsequently, Dillon and McKenna [2] described acetylated and methylated derivatives of the mucilage and also showed that a uronic acid was present to the extent of about 10%. The uronic acid appeared to be present in the lactone form since determinations of combined sulphate in the mucilage by titration and by gravimetric methods gave approximately the same

* Part V, *J.*, 1955, 222.

[1] Barry and Dillon, *Proc. Roy. Irish Acad.*, 1945, **50**, *B*, 349.
[2] Dillon and McKenna, *ibid.*, 1950, **53**, *B*, 45.

result. From a study of the methylated sugars resulting from the hydrolysis of the methylated mucilage these authors agreed that galactose was the main sugar present and that it was mostly linked 1 : 3. They did not, however, confirm the presence of 1 : 6-glycosidic linkages since they failed to isolate 2 : 3 : 4-tri-*O*-methylgalactose from the hydrolytic products of a methylated, sulphur-free, though degraded galactan.

The present findings confirm the presence of 10% of a uronic acid which has been identified as glucurone and reveal new constituents, xylose (*ca.* 7%) and 3 : 6-anhydro-D-galactose (trace). The mucilage appears to have a highly ramified structure in which the D-galactopyranose is linked, for the most part, 1 : 3. The other constituents are present in branches which are removed by degradation. These branches also contain galactose units linked 1 : 3 and 1 : 4. Some of the galactose units are esterified with sulphuric acid at $C_{(6)}$ and these appear to be present, mainly, in the outer parts of the macromolecule.

Acid hydrolysis of the mucilage was shown by chromatographic examination to yield, in addition to galactose and sulphuric acid, small amounts of xylose (*ca.* 7%) and glucurone (estimated as 10·0%) and traces of 3 : 6-anhydro-D-galactose and hydroxymethylfurfuraldehyde. The presence of the last compound together with 3 : 6-anhydrogalactose is not unexpected (cf. O'Neill [3]) and it has been shown to be formed from the anhydro-sugar under the conditions of hydrolysis. Examination of the behaviour of D-glucurone under these conditions eliminated the possibility that such treatment could have produced from the lactone the amount of xylose present in the hydrolysate.

The neutralised hydrolysate gave a mixture of osazones which was shown by paper chromatography to contain galactosazone, xylosazone, and, surprisingly, a substantial amount of 3 : 6-anhydro-D-galactosazone. The identity of the last was confirmed after isolation from an alumina column. The increase in the proportion of the anhydro-sugar, after treatment with phenylhydrazine, was thought to be due to elimination of combined sulphuric acid from barium galactose 3(or 6)-sulphate remaining in the hydrolysate. Configurational considerations rule out such an elimination if the sulphate groups are located at $C_{(2)}$ or $C_{(4)}$ of galactose residues (see Peat [4] and Percival [5] for reviews on anhydro-sugars and carbohydrate sulphates respectively). Subsequent experiments showed that barium galactose 6-sulphate on treatment for osazone formation is converted in part into 3 : 6-anhydrogalactosazone. Similar treatment of galactose failed to yield the anhydro-sugar. The behaviour of 3- and 6-sulphated sugars towards 0·5N-sulphuric acid at 90° is such that only in the case of the latter is there unhydrolysed sulphate remaining after 8 hr. Since combined sulphate was found in the mucilage hydrolysate, the indications are that the sulphate groups in the polysaccharide are located at $C_{(6)}$ of galactose residues.

Oxidation of the mucilage with sodium periodate, as described by Barry and Dillon,[1] yielded a product which, after acid hydrolysis, was shown chromatographically to contain galactose, xylose, and uronic acid. When a portion of the product was condensed with *iso*nicotinhydrazide (cf. Part III [6]) the nitrogen content of the material formed indicated that 31·5% of the sugar units present in the mucilage have α-glycol groupings. The oxidised mucilage was degraded by phenylhydrazine in acetic acid solution as described in Part IV.[7] Glyoxal and erythrose were isolated as osazones, and paper chromatography indicated the presence also of galactosazone and xylosazone. In addition, a polysaccharide material was recovered which had a higher sulphur content than the original mucilage and which was shown, after acid hydrolysis, to consist virtually of galactose. This material was in turn oxidised and degraded, yielding a small amount of glyoxalbisphenylhydrazone together with a polysaccharide substance having a lower sulphur content. A third, similar treatment gave material, still polysaccharide in character, in which the sulphur

[3] O'Neill, *J. Amer. Chem. Soc.*, 1955, **77**, 2837.
[4] Peat, *Adv. Carbohydrate Chem.*, 1946, **2**, 37.
[5] Percival, *Quart. Rev.*, 1949, **3**, 369.
[6] Barry, McCormick, and Mitchell, *J.*, 1954, 3692.
[7] Barry and Mitchell, *J.*, 1954, 4020.

content was further reduced. In view of the small amount of material remaining and its very high ash content, a fourth oxidation and degradation were not attempted. Purification of the degradation products could not be effected by dialysis as all the organic material passed through the membrane (cf. Barry and Dillon [1]).

We are here attempting to throw light on the structure of a complex polysaccharide without having recourse to the classical methods of methylation. The persistence after three periodate oxidations and phenylhydrazine degradations of a material, polysaccharide in character and containing galactose as the sole sugar present, indicates that the mucilage contains a back-bone or core of 1 : 3-linked galactopyranose units. The once degraded material consists virtually of galactose and has a sulphur content of 5·9%. Degradation reduces this to 3·8% and another oxidation and phenylhydrazine treatment brings about a further reduction to 2·4%. Reasons have already been given for assigning the sulphate group to $C_{(6)}$ of galactose units and the reductions in sulphur content can be explained by assuming that the core of 1 : 3-linked galactose units is flanked by 6-sulphated 1 : 3-linked residues which are removed stepwise by oxidation followed by degradation. This idea is illustrated by the structural repeating units (requiring S, 5·2, 4·0, and 2·3% respectively) put forward for the three degradation products (Figs. 1—3). It should be emphasised that the formulæ (Figs. 1—5) are designed merely to explain the results obtained, and are not advanced as firm structures for the mucilage and its degradation products (they are intended, partly, to be an aid in following the present discussion). The once oxidised mucilage contains xylose and glucurone. The xylose is therefore linked 1 : 3 and the glucurone, as a 6 ⟶ 3-lactone, is invulnerable to periodate. The degradation of this oxidised material removes all the glucurone and all but a trace of the pentose and these are probably present in peripheral side-chains. Further, the isolation of glyoxalbisphenylhydrazone and erythrosazone from the degradation products confirms previous assertions [1,2] that some of the galactopyranose residues are linked 1 : 4 because, of the constituents of the mucilage, only galactose can yield erythrosazone on periodate oxidation and degradation. The xylose in the side-chains is visualised as consisting of individual units joined on either side to periodate-vulnerable galactopyranose. The evidence for this is the presence of xylosazone among the degradation products, our experience being that phenylhydrazine only cleaves linkages contiguous to carbonyl groups and then only when cleavage will result in osazone formation. Similar considerations point to the presence in the side-chains of single 1 : 3-linked galactose residues attached on each side to periodate-vulnerable sugar units. The above conclusions, together with the approximate, relative proportions of the constituent sugars in the original material, are taken into account in the proposed structure of the outer side-chains, represented in Fig. 4, four of which must be associated

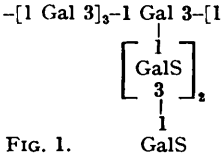

FIG. 1.

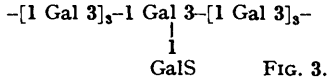

FIG. 3.

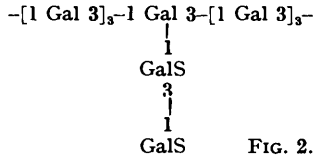

FIG. 2.

-1 Gal 4-1 Gal 3-1 Gal 4-1 Xyl 3-1 Gal 4-1 GalS 3-1 GA

FIG. 4.

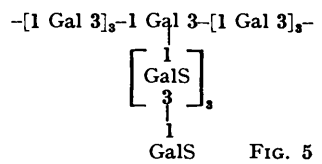

FIG. 5

(Gal = D-galactopyranose; GalS = D-galactopyranose sulphated at $C_{(6)}$; Xyl = xylose; GA = Glucurone.)

in the mucilage with each repeating unit of seven galactose and four sulphated galactose residues (Fig. 5). We have no information regarding the point of attachment of these

side-chains. However they are joined, oxidation followed by phenylhydrazine treatment will result in the formation of the once degraded material whose structure is given in Fig. 1. We have placed the glucurone as a terminal unit in the side-chain for the following reasons: Galactopyranose residues in this position would be oxidised by periodate and would yield glycerosazone on degradation. Again, the fact of the small amount (*ca.* 7%) of xylose in the mucilage together with the evidence of the presence of xylosazone in the products obtained after the first degradation make it unlikely that the outer side-chains are terminated by xylose residues. It is possible that some of the side-chains are terminated by 3 : 6-anhydrogalactose as this would also be invulnerable to periodate. The amount of this present is, however, too small to be considered as structurally significant.

Experimental

The mucilage was isolated from fronds of *Dilsea edulis* by Barry and Dillon's method [1] as a white, fibrous material. It was dissolved in water and precipitated by the addition of ethanol, then further purified by dialysis in 2% hydrochloric acid against distilled water. The product was a white, granular powder, $[\alpha]_D^{20}$ +65·7° (*c* 0·35 in H_2O) (Found: ash, 14·7; uronic anhydride, estimated by the method of Dickson, Otterson, and Link,[8] 10·0%).

The sulphur content of polysaccharide material was calculated from gravimetric estimations of barium sulphate derived from the material and from its ash, the assumption being made that the sulphate present in the ash was entirely inorganic in origin.

In the paper chromatography, the sugar mixtures were separated by elution with butan-1-ol–pyridine–water–benzene (5 : 3 : 3 : 1 v/v; top layer) [9] (*a*) or ethyl methyl ketone–acetic acid–water (6 : 1 : 1 v/v) [10] (*b*), and the osazone mixtures with benzene–95% ethanol (9 : 1 v/v) [7] (*c*). The sugars were located by spraying the paper with a solution of aniline hydrogen phthalate [11] or *p*-anisidine hydrochloride,[12] and the osazones by viewing the paper in ultra-violet light.

Aqueous solutions were concentrated at 40° under reduced pressure. Brockmann-standardised Merck alumina was used throughout for chromatography of the osazones.

Acid Hydrolysis.—The mucilage was heated with 0·5N-sulphuric acid at 90° for 8 hr. After neutralisation with barium carbonate and filtration the concentrated hydrolysate was shown by paper chromatography, using solvent (*a*), to contain galactose (mainly), xylose, and uronic acid, and traces of 3 : 6-anhydrogalactose and hydroxymethylfurfuraldehyde. By visual comparison of the intensity of the galactose and xylose spots with that of the spots from various galactose–xylose mixtures the ratio pentose : hexose was estimated as approximately 1 : 10.

Another portion of the polysaccharide was heated with N-sulphuric acid at 90° for 13 hr. After neutralisation with silver carbonate and filtration, excess of silver was precipitated by hydrogen sulphide. The acid filtrate, after concentration, was shown by paper chromatography, using solvent (*b*), to contain glucurone.

Treatment of D-glucurone with 0·5N-sulphuric acid at 90° for 8 hr. was shown on a paper chromatogram, using solvent (*a*), to cause virtually no decarboxylation to xylose.

A sample of the acid (0·5N) hydrolysate was freed quantitatively from sulphuric acid by means of barium chloride, and sufficient sodium hydroxide was added to make a 10% solution which was refluxed for 90 min. A white precipitate of barium sulphate was formed.

Another portion of the same hydrolysate, after neutralisation with barium carbonate, was treated with sufficient glacial acetic acid to make a 30% solution. To this was added phenylhydrazine and the resulting mixture was heated at 95° for 20 min. After cooling, dilution with water gave a precipitate which was shown on a circular paper chromatogram, using solvent (*c*), to contain galactosazone (mainly) together with xylosazone and 3 : 6-anhydrogalactosazone in approximately equal amounts. Xylosazone was further identified in the mixture of osazones by its characteristic, microscopic, crystalline appearance.

[8] Dickson, Otterson, and Link, *J. Amer. Chem. Soc.*, 1930, **52**, 775.
[9] Albon and Gross, *Analyst*, 1950, **75**, 454.
[10] Irwin and Leaver, *Nature*, 1956, **177**, 1126.
[11] Partridge, *ibid.*, 1949, **164**, 443.
[12] Hough, Jones, and Wadman, *J.*, 1950, 1702.

Action of 0·5N-*Sulphuric Acid on* 3 : 6-*Anhydro*-D-*galactose*.—An authentic sample [13] of the anhydro-sugar was heated with 0·5N-sulphuric acid at 90° for 8 hr. Examination of the product, after neutralisation with barium carbonate and filtration, on a paper chromatogram, using solvent (*a*), indicated a partial conversion into hydroxymethylfurfuraldehyde.

Isolation of 3 : 6-*Anhydro*-D-*galactosazone*.—The mucilage (2·7 g.; dry, ash-free wt.) was heated with 0·5N-sulphuric acid (140 c.c.) at 90° for 8 hr. The hydrolysate, after neutralisation with barium carbonate and filtration, was concentrated to 50 c.c. and treated with phenylhydrazine (15 c.c.), glacial acetic acid (19 c.c.), and ethanol (100 c.c.). The resulting solution was refluxed for 2 hr., the ethanol removed under reduced pressure, and the solution diluted with water to give a precipitate (1·26 g.) which was dissolved in methanol (20 c.c.) and adsorbed on alumina (40 g.). Fractions 1—10 (1·04 g.), eluted by methanol (520 c.c.), yielded, after concentration, material, m. p. 185—189°, which was shown by a circular paper chromatogram, using solvent (*c*), to be substantially galactosazone. The mother-liquors from this were evaporated to dryness giving a residue (0·62 g.) which was dissolved in ether containing a small amount of methanol and re-adsorbed on alumina (20 g.). Fractions 1—2 (28 mg.), eluted by ether (100 c.c.), gave an oil. Fraction 3 (66 mg.), eluted by 9 : 1 ether–methanol (50 c.c.), consisted of 3 : 6-anhydro-D-galactosazone which crystallised from methanol as yellow needles m. p. 200—201°, undepressed on admixture with authentic material.[14] Light absorption: Max. (in 95% aqueous ethanol), 257, 311, 395 mμ ($E^{1\%}_{1cm.}$ 558, 294, 592 respectively). The molecular weight (determined from the value of $E^{1\%}_{1cm.}$ at 395 mμ, cf. Part V) was 344 (Calc. for $C_{18}H_{20}O_3N_4$: *M*, 340).

Hydrolysis of Barium Galactose 6-*Sulphate and Barium Glucose* 3-*Sulphate*.—The two sulphuric esters [15,16] were, separately, heated at 90° with 0·5N-sulphuric acid. At intervals, portions of the hydrolysates were neutralised with barium carbonate, filtered, concentrated, and examined on a paper chromatogram using solvent (*a*). It was thus shown that after 90 min. barium glucose 3-sulphate had been completely hydrolysed to glucose whereas, after 8 hr., some barium galactose 6-sulphate remained unhydrolysed.

Action of Phenylhydrazine in Acetic Acid Solution on Barium Galactose 6-*Sulphate*.—The galactose sulphuric ester [15] (3·7 g.) was refluxed for 2½ hr. with phenylhydrazine (14 c.c.), glacial acetic acid (17 c.c.), water (75 c.c.), and ethanol (150 c.c.). Removal of ethanol at reduced pressure followed by dilution with water gave a precipitate (1·5 g.) which was isolated in the centrifuge. Examination of this on a circular paper chromatogram, using solvent (*c*), showed it to contain galactosazone and 3 : 6-anhydrogalactosazone in addition to a large amount of material which did not move from the starting line. The methanol-soluble portion (0·9 g.) of the precipitate was dissolved in methanol (5 c.c.) and adsorbed on alumina (30 g.). Fractions 1—2 (170 mg.), eluted by methanol (40 c.c.), were dissolved in ether containing a small amount of methanol and re-adsorbed on alumina (10 g.). Fraction 1 (90 mg.), eluted by ether (40 c.c.), was an oil. Fraction 2 (62 mg.), eluted by 4 : 1 ether–methanol (25 c.c.), consisted of 3 : 6-anhydro-D-galactosazone, m. p. 200—202°.

First Periodate Oxidation.—Acid hydrolysis of oxidised polysaccharide. The oxidation product, B, obtained by the method of Barry and Dillon [1] from the mucilage, A, was a white powder (Found: ash, 40·2%). It was heated with 0·5N-sulphuric acid at 90° for 1½ hr. The hydrolysate, after neutralisation with barium carbonate, filtration, and concentration, was shown on a paper chromatogram, using solvent (*a*), to contain galactose, xylose, and uronic acid.

A sample of the acid (0·5N) hydrolysate, after neutralisation with barium carbonate and filtration, was treated with phenylhydrazine and sufficient glacial acetic acid to make a 30% solution and warmed at 95° for 20 min. After cooling, dilution with water gave a precipitate which was shown on a circular paper chromatogram, using solvent (*c*), to contain galactosazone, xylosazone, and glyoxal bisphenylhydrazone together with erythrosazone and/or 3 : 6-anhydrogalactosazone. (The last two osazones have the same R_F values in this solvent system.)

Isoniazid derivative. The oxidised polysaccharide, B (0·14 g.; dry, ash-free wt.), was treated in water (5 c.c.) with a solution of *iso*nicotinhydrazide (isoniazid) (0·5 g.) in water (3 c.c.). After neutralisation with saturated sodium hydrogen carbonate solution, the addition of a large volume of ethanol precipitated a polymeric *substance* which coagulated on being

[13] Haworth, Jackson, and Smith, *J.*, 1940, 620.
[14] Percival, *J.*, 1945, 783.
[15] Percival and Soutar, *J.*, 1940, 1475.
[16] Percival, *J.*, 1945, 119.

shaken. The product (0·27 g.) was filtered off and washed successively with ethanol and ether (Found : ash, 38·2; N, in ash-free material, 6·1%).

First Degradation.—Isolation of small fragments. The oxidised polysaccharide, B (6 g.; dry, ash-free wt.), was refluxed for 4 hr. with phenylhydrazine (30 c.c.), glacial acetic acid (36 c.c.), water (120 c.c.), and ethanol (240 c.c.). Removal of ethanol at reduced pressure followed by dilution with water gave a precipitate which was removed by filtration. Addition of water to the filtrate yielded an oil which was extracted with difficulty with ether. The extract was washed with dilute acetic acid and water and yielded an oil which was combined with the solid product. Examination on a circular paper chromatogram, using solvent (*c*), showed the combined material to contain galactosazone, xylosazone, and glyoxal bisphenylhydrazone together with erythrosazone and/or 3 : 6-anhydrogalactosazone. The material (6·1 g.) was dissolved in benzene (50 c.c.) and adsorbed on alumina (180 g.). Fractions 4—11 [1·15 g., eluted by benzene (200 c.c.); 0·85 g., eluted by 1 : 1 benzene–ether (200 c.c.)] consisted of glyoxal bisphenylhydrazone, m. p. 159—161°, undepressed on admixture with authentic material. Fraction 27 (0·47 g.), eluted by 1 : 1 ether–ethanol (50 c.c.), was *N*-acetylphenylhydrazine. Fractions 34—38 (0·11 g.), eluted by 9 : 1 ethanol–water (250 c.c.), consisted of erythrosazone which crystallised from benzene as yellow needles, m. p. 153—155°, undepressed on admixture with material obtained from the degradation of oxidised starch (cf. Part IV [7]). Fractions 45—47 (0·23 g.), eluted by 6 : 4 ethanol–water (150 c.c.), were shown by a circular paper chromatogram, using solvent (*c*), to consist mainly of xylosazone. This material did not crystallise well.

Once-degraded polysaccharide, A′. The oxidised mucilage, B (4·6 g.; dry, ash-free wt.), was degraded with phenylhydrazine in acetic acid solution, the same proportions being used as above. A large volume of ethanol precipitated a pale-yellow material, A′ (3·4 g.) (Found : ash, 47·4; S, in ash-free material, 5·9%). This was heated for 8 hr. at 90° with 0·5N-sulphuric acid, and the resulting hydrolysate, after neutralisation with barium carbonate, filtration, and concentration, was shown by paper chromatography, using solvent (*a*), to contain galactose and a trace of xylose. The neutral hydrolysate was treated with phenylhydrazine and sufficient glacial acetic acid to make a 30% solution and the mixture was heated at 95° for 20 min. After cooling, dilution with water gave a precipitate shown on a circular paper chromatogram, using solvent (*c*), to contain galactosazone and 3 : 6-anhydrogalactosazone. Another sample of the once degraded material, A′, was heated with N-sulphuric acid at 90° for 13 hr. After neutralisation with silver carbonate and filtration, excess of silver was removed by hydrogen sulphide. The acid filtrate, after concentration, was examined on a paper chromatogram, with solvent (*b*). No evidence was found of the presence of glucurone.

Second Oxidation and Degradation.—The once degraded material, A′ (1·8 g.; dry, ash-free wt.), was kept with a solution of sodium metaperiodate (5·0 g.) in water (20 c.c.) for 36 hr. The solution was cooled in ice–salt, and the periodate and iodate were reduced by a slow stream of sulphur dioxide. Extraneous material was removed by centrifugation, and addition of ethanol precipitated a pale, buff-coloured material, B′ (4·3 g.) (Found : ash, 59·3%). This was refluxed for 3 hr. with phenylhydrazine (6 c.c.), glacial acetic acid (7 c.c.), water (24 c.c.), and ethanol (48 c.c.). Ethanol was removed from a portion of the mixture and dilution with water produced an opalescence in the solution which deposited, overnight, a small amount of glyoxal bisphenylhydrazone. The main bulk of the mixture was then treated with ethanol to precipitate a yellow material, A″ (1·9 g.) (Found : ash, 50·2; S, in ash-free material, 3·8%). Hydrolysis of this material with 0·5N-sulphuric acid at 90° for 8 hr., followed by neutralisation with barium carbonate and concentration, was shown by paper chromatography, using solvent (*a*), to yield galactose. The neutral hydrolysate was treated with phenylhydrazine and sufficient glacial acetic acid to make a 30% solution which was then heated at 95° for 20 min. After cooling, dilution with water gave a precipitate which was shown on a circular paper chromatogram, using solvent (*c*), to contain galactosazone and 3 : 6-anhydrogalactosazone.

Third Oxidation and Degradation.—The twice degraded material, A″ (0·57 g.; dry, ash-free wt.), was kept for 43 hr. with sodium metaperiodate (1·66 g.) in water (6 c.c.). The product, B″ (96 mg.) (Found : ash, 57·7%), isolated in the same manner as the twice oxidised material, B′, was degraded by being refluxed for 3¾ hr. with phenylhydrazine (2 c.c.), glacial acetic acid (2·3 c.c.), water (8 c.c.), and ethanol (16 c.c.). This mixture was treated with a large volume of ethanol and ether to precipitate a yellow material, A‴ (0·54 g.) (Found : ash, 75·1; S, in ash-free material, 2·4%). Hydrolysis of this material with 0·5N-sulphuric acid at 90° for 8 hr.

and treatment of the neutralised (barium carbonate) hydrolysate with phenylhydrazine and acetic acid for osazone formation gave a product, shown by a circular paper chromatogram, using solvent (*c*), to contain galactosazone and 3 : 6-anhydrogalactosazone.

One of us (J. E. McC.) is a Lasdon Foundation Research Fellow of University College, Dublin.

LABORATORIES OF THE MEDICAL RESEARCH COUNCIL OF IRELAND,
TRINITY COLLEGE, DUBLIN. [*Received, November 21st*, 1956.]