102. *Red-seaweed Polysaccharides. Part III.* Polysaccharide from* Hypnea specifera.

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A polysaccharide was isolated from *Hypnea specifera*, which was precipitated from aqueous solution by potassium chloride and yielded D-galactose and 3 : 6-anhydro-D-galactose and sulphate on hydrolysis. Partial methanolysis followed by hydrolysis and reduction afforded 3 : 6-anhydro-4-*O*-β-D-galactopyranosyl-D-galactitol. Methylation indicated that the galactose was joined to other residues through positions 1 and 3 with the sulphate probably on $C_{(4)}$. It appears to be very similar to κ-carrageenin.

Hypnea specifera is one of the most widely distributed seaweeds of the South African coastline but is of greater importance ecologically in warmer waters. It is believed to be of potential economic importance.[1]

Extraction of fresh, wet *Hypnea specifera* with hot water, followed by filtration and precipitation by ethanol, afforded a crude sulphated polysaccharide in 16% yield (dry weight). This polysaccharide was precipitated by potassium chloride (cf. Smith *et al.*[3]). The material remaining in solution was chiefly protein associated with an insignificant amount of polysaccharide. Polysaccharide, fractionated in this way, was completely converted into the sodium form by suitable ion-exchange resins, precipitated in ethanol, washed, dried, and used as the analytical sample. It had a specific rotation of +66·5° and contained 21·2% of sulphate (calc. as $NaSO_3^-$ on a protein-free basis). Chromatography of the hydrolysate of the polysaccharide showed galactose, a trace of xylose, and a pronounced streak indicating the probable presence of anhydro-sugar.

The galactose was isolated and identified in the usual way by comparison with an authentic specimen. The anhydro-sugar was isolated as its dimethyl acetal by chromatography from the methanolysis products of the polysaccharide, and it was characterised as 3 : 6-anhydro-D-galactose by conversion into the *iso*propylidene derivative of the free

* Part II, *J.*, 1957, 1094.

[1] Isaac and Hewitt, *J. S. African Botany*, 1953, **19**, 73; Fox and Stephens, *S. African J. Sci.*, 1943, **39**, 147.

[2] O'Neill, *J. Amer. Chem. Soc.*, 1955, **77**, 6324.

[3] Smith and Cook, *Arch. Biochem. Biophys.*, 1953, **45**, 232; Smith, Cook, and Neal, *ibid.*, 1954, **53**, 192; Smith, O'Neill, and Perlin, *Canad. J. Chem.*, 1955, **33**, 1353.

sugar. The yield of galactose was estimated by the Somogyi micro-method,[4] after hydrolysis and separation by paper chromatography,[5] and found to be 47·4% (calc. as $C_6H_{10}O_5$). The content of 3 : 6-anhydro-D-galactose (calc. as $C_6H_8O_4$) was 31·4% by difference. The molecular ratio of galactose : 3 : 6-anhydrogalactose : $NaSO_3$ is thus 1·4 : 1·1 : 1·0.

Partial methanolysis of the polysaccharide and fractionation of the products on cellulose yielded 3 : 6-anhydro-D-galactose dimethyl acetal, a mixture of methyl galactosides, and an amorphous disaccharide dimethyl acetal. The last material failed to crystallise, as did its acetate, and it was hydrolysed with dilute oxalic acid to the free disaccharide, which streaked on paper chromatograms similarly to agarobiose.[6] This sugar was reduced with sodium borohydride, and the product fractionated on cellulose, to yield a crystalline disaccharide glycitol, m. p. 174°, $[\alpha]_D$ +15°. It depressed the m. p. of the agar disaccharide glycitol,[6] m. p. 174°, $[\alpha]_D$ −15°. Hydrolysis of the compound and separation of the products on cellulose afforded D-galactose and a syrup chromatographically identical with 1 : 4-anhydro-L-galactitol (= 3 : 6-anhydro-D-galactitol). The compound formed a crystalline hepta-*O*-acetate. During periodate oxidation 3 mol. of periodate were consumed and 1 mol. of formic acid and 1 mol. of formaldehyde were produced. These facts are consistent with the structure 3 : 6-anhydro-4-*O*-β-D-galactopyranosyl-D-galactitol. The β-configuration was assigned because of the low specific rotation of the compound and its acetate as well as its upward mutarotation on acid hydrolysis. The infrared spectrum of this disaccharide glycitol and of agar disaccharide glycitol[6] displayed the presence of type 2b absorption[7] (bands at 887 and 890 $cm.^{-1}$ respectively for the two compounds) and the absence of type 2a absorption (bands at 844 ± 8 $cm.^{-1}$). This is strong evidence for the presence of a β-glycosidic link.[7] The 1→5 glycosidic linkage was eliminated on stereochemical grounds[8] and the 1→2 linkage would not yield formaldehyde on periodate oxidation.

Methylation of the polysaccharide proved difficult, probably owing to the presence of sulphate groups[9] in the molecule. The final product still contained sulphate (18·3%). Hydrolysis of this product and fractionation of the mixture on cellulose yielded 2 : 6-di-*O*-methyl-D-galactose as the main product. Very small amounts of mono-, tri-, and tetra-*O*-methylgalactose were detected on paper. The methylated anhydrogalactoses were, of course, destroyed by the hydrolysis. The retention of sulphate during methylation suggests that it is not attached to position 3 of the D-galactose residue, as it would easily have been removed by alkaline hydrolysis.[8]

In another experiment the polysaccharide, after methylation with methyl sulphate, was partially hydrolysed with methanolic hydrogen chloride. The resultant mixture was then fully methylated with Purdie's reagents and hydrolysed. Separation of the products on cellulose afforded tetra-*O*-methyl-D-galactose, 2 : 4 : 6-tri-*O*-methyl-D-galactose and 2 : 6-di-*O*-methyl-D-galactose.

The precipitation of the polysaccharide by potassium chloride and the isolation of a disaccharide in high yield seem to indicate that a single polysaccharide is involved. The high yield of disaccharide also indicates that the molecule is largely built up of this unit. Since it seems unlikely that much of the sulphate is attached to position 3 of the galactose,[8] the isolation of 2 : 4 : 6-tri-*O*-methyl-D-galactose after methylation and hydrolysis of partially degraded methylated material, and of 2 : 6-di-*O*-methyl-D-galactose from the hydrolysis of fully methylated material, shows that the galactose is chiefly linked through positions 1 and 3 with half ester sulphate on $C_{(4)}$. Evidence from the structure of the disaccharide glycitol shows that galactose is linked to 3 : 6-anhydro-D-galactopyranose by

[4] Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.
[5] Flood, Hirst, and Jones, *J.*, 1948, 1679.
[6] Clingman, Nunn, and Stephen, *J.*, 1957, 197.
[7] Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171, 3468.
[8] Peat, *Adv. Carbohydrate Chem.*, 1946, **2**, 38.
[9] Foster and Huggard, *Adv. Carbohydrate Chem.*, 1955, **10**, 356.

a 1→4 link. Although it is impossible to be certain, it appears that the molecule is mainly linear. The difficulties associated with methylation of a sulphated polysaccharide make it impossible to draw any more conclusions at this stage.

κ-Carrageenin [3] from *Chondrus crispus* is precipitated with potassium chloride and gives the same disaccharide on hydrolysis,[2] and appears to be very similar to *Hypnea* polysaccharide. Araki and Hirase [10] have also isolated this disaccharide from *Chondrus ocellatus*, Holmes.

Experimental

Concentration of solutions was carried out at 40°/20 mm., and specific rotations were measured for aqueous solution unless otherwise stated. Paper chromatograms (Whatman No. 1 paper) were run in (*a*) butanol–ethanol–water (40 : 11 : 19) or (*b*) ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4). *p*-Anisidine hydrochloride spray was used to detect reducing sugars and a periodate–starch spray [11] to detect non-reducing carbohydrates.

Extraction of the Polysaccharide.—Fresh, wet *Hypnea specifera*, collected near Gordons Bay, Cape, was suspended in water and brought to pH 4 with acetic acid, and steam was passed in for 1 hr. The hot solution, after filtration through a cotton cloth, was centrifuged twice and then poured into ethanol (5 vol.) to precipitate the polysaccharide, which was filtered off, washed with ethanol, acetone, and ether, and dried in a vacuum-desiccator. In another experiment oven-dried seaweed (50 g.) was extracted as described above, to give 8 g. polysaccharide.

An analytical specimen of the polysaccharide was prepared by fractionation with potassium chloride as follows: M-Potassium chloride was added dropwise to a stirred solution of the sodium salt of the polysaccharide (1·55 g.) in water (540 c.c.) until the solution was 0·2M with respect to potassium chloride. The precipitate (1·3 g.) was removed by centrifugation and washed several times with 80% ethanol, twice with absolute ethanol, then with ether, and dried at 60°/20 mm. The material in the supernatant solution was precipitated with alcohol (3 vols.), centrifuged, washed as above, and dried at 60°/20 mm. (0·17 g.) (Found: ash, 40·9; N, 1·4%). It was not further investigated.

The potassium chloride-precipitated polysaccharide (1·3 g.) was dissolved in hot water (200 c.c.) and passed through a column (2·4 × 0·15 cm.) of Amberlite IR-120 resin in the sodium form. Ethanol (3 vols.) was added to the eluate to precipitate the polysaccharide, which was separated by centrifugation. After being washed several times with 80% ethanol, absolute ethanol, and ether it was dried at 60°/20 mm. for 2 days to give a product (1·0 g.), $[\alpha]_D^{17}$ +66·5° (*c* 0·3 in H_2O) (Found: $NaSO_3^-$, 20·4; N, 0·6%).

Isolation and Estimation of D-*Galactose.*—The polysaccharide (1 g.) was hydrolysed with N-sulphuric acid at 100° for 16 hr., and spotted on Whatman No. 3 MM papers which were developed overnight in solvent (*b*). The portions of the papers containing galactose were cut out and extracted (Soxhlet) with methanol. The methanolic solution was concentrated, to give a crystalline product which, after one recrystallisation from methanol, had m. p. and mixed m. p. 166·5—167·5° (with D-galactose), $[\alpha]_D^{18}$ +86° (*c* 1·0).

The polysaccharide was hydrolysed with N-sulphuric acid at 100° for 24 hr. A known amount of maltose was added to the hydrolysate [12] which was neutralised with barium carbonate and chromatographed on paper in solvent (*b*). The strips containing galactose and maltose were cut out and macerated in water; after filtration the sugars were estimated by the Somogyi micro-method [4] [Found: galactose (calc. as $C_6H_{10}O_5$), 47·4% on a moisture-free and protein-free basis].

Methanolysis.—The polysaccharide (21 g.) was refluxed with 2% methanolic hydrogen chloride (210 c.c.) for 20 min. and the mixture was filtered. The insoluble residue (17 g.) was again refluxed in 2% methanolic hydrogen chloride (170 c.c.) for 20 min., and the mixture was filtered. This process was repeated another four times. The filtrates were neutralised with silver carbonate, combined, and concentrated to a syrup (10·2 g.).

Paper chromatograms run in solvent (*a*) disclosed spots of R_R (relative to rhamnose) 0·79, 1·3, and 1·7. The syrup (10 g.) was fractionated on a cellulose column (33 × 5 cm.) with butanol–water (9 : 1). The fractions were sorted by paper chromatography, and like ones combined and concentrated. The first fraction, a syrup (0·96 g.), was chromatographically

[10] Araki and Hirase, *Bull. Chem. Soc. Japan*, 1956, **29**, 770.
[11] Metzenberg and Mitchell, *J. Amer. Chem. Soc.*, 1954, **76**, 4187.
[12] Hough, Jones, and Wadman, *J.*, 1949, 2511.

identical with 3 : 6-anhydro-D-galactose dimethyl acetal, R_R 1·7 in solvent (*a*), $[\alpha]_D^{17}$ +29° (*c* 1·0), $[\alpha]_D^{17}$ +33° (*c* 1·1 in MeOH): Haworth, Jackson, and Smith [13] report $[\alpha]_D^{18}$ +36·5° (*c* 0·9) for 3 : 6-anhydro-D-galactose dimethyl acetal. It gave a positive Selivanoff reaction.

The syrup (0·8 g.) was hydrolysed in 0·1N-sulphuric acid at 20° for 2 days, neutralised with barium carbonate, and concentrated to dryness. The residue was shaken with acetone (50 c.c.) containing concentrated sulphuric acid (2 c.c.) and anhydrous copper sulphate (5 g.) for 5 hr. The mixture was neutralised with anhydrous potassium carbonate, filtered, boiled with charcoal, and filtered again. The filtrate was concentrated to dryness. The residue in water was extracted with ether. Evaporation of the ethereal solution afforded a solid which, crystallised from light petroleum (b. p. 60—80°), had m. p. and mixed m. p. 90—91° with 1 : 2-*O*-*iso*propylidene-3 : 6-anhydro-D-galactose, $[\alpha]_D^{18}$ +20° (*c* 1·0 in $CHCl_3$) (Araki and Arai [14] report m. p. 92°) (Found: C, 53·4; H, 7·15. Calc. for $C_9H_{14}O_5$: C, 53·5; H, 7·0%).

The next fraction (0·14 g.) off the column, R_R 1·3 in solvent (*a*), probably methyl galactofuranoside, was not investigated.

This was followed by an amorphous substance (3·88 g.), $[\alpha]_D^{16}$ +20° (*c* 1·1); it did not reduce Fehling's solution and gave a positive Selivanoff test. It showed up on paper chromatograms sprayed with *p*-anisidine hydrochloride and heated in an oven (110°) for 15 min. as a yellow spot with the same R_R 0·80 as agarobiose dimethyl acetal. The acetate prepared in the usual way with acetic anhydride and pyridine failed to crystallise. Hence the material (2·8 g.) was hydrolysed in 0·01N-oxalic acid (100 c.c.) at 100° for 3 hr., $[\alpha]_D^{18}$ +20° ⟶ +27° (constant value). The solution was neutralised (Amberlite IR-4B resin) and concentrated to dryness. The residue [long streaky spot of R_R 0·44 in solvent (*a*)] in methanol (100 c.c.) was treated with sodium borohydride (0·35 g.) in methanol (3·5 c.c.) and left overnight. The solution was neutralised (Amberlite IR-120) and concentrated to dryness. The residue was eluted from a cellulose column (27·5 × 3·0 cm.) with butanol–formic acid–water (45 : 1 : 4). The main fraction (2·1 g.), R_R 0·51 in solvent (*a*), was collected and concentrated. A small amount of dulcitol was also obtained, m. p. 188°.

Disaccharide Glycitol.—This substance, crystallised from methanol, had m. p. 173—174°, mixed m. p. with agar disaccharide glycitol [6] (of m. p. 173—174°) 161—163°, $[\alpha]_D^{21}$ +15° (*c* 1·0) (Found: C, 44·2; H, 7·4. Calc. for $C_{12}H_{22}O_{10}$: C, 44·2; H, 6·8%).

Disaccharide glycitol (0·326 g.), in 2N-sulphuric acid (25 c.c.) at 100° for 24 hr., had $[\alpha]_D^{20}$ +14 ⟶ +45·5° (constant value), the final value corresponding to the specific rotation of an equimolar mixture of D-galactose and 1 : 4-anhydro-L-galactitol (= 3 : 6-anhydro-D-galactitol). The hydrolysate was neutralised with barium carbonate and concentrated. Chromatography on papers in solvent (*a*) disclosed two spots having R_{Ga} 1·0 and 2·2 (relative to galactose). The mixture was separated by chromatography on large sheets of paper in the usual way. The one fraction afforded D-galactose (0·080 g., 44·4%), having m. p. and mixed m. p. 166—167° and $[\alpha]_D^{22}$ +82° (*c* 0·8 in H_2O) after crystallisation from methanol. The aglycone was a syrup, (0·077 g., 47·0%), $[\alpha]_D^{22}$ +12° (*c* 1·0), identical with 1 : 4-anhydro-L-galactitol on paper chromatograms. Hockett, Conley, Yusem, and Mason [15] report $[\alpha]_D$ +16·1° for 1 : 4-anhydro-L-galactitol. The disaccharide glycitol (0·1524 g.) was hydrolysed as above and the galactose estimated by the Somogyi micro-method,[4] maltose being used as a standard [12] (Found: 1·11, 1·04 mol. of galactose per mol. of disaccharide glycitol).

Disaccharide glycitol (0·3 g.) in pyridine (5 c.c.) was treated with acetic anhydride (5 c.c.) in pyridine (3 c.c.) at 5°. After 2 days at 5° and 2 days at room temperature the mixture was poured into ice-water (200 c.c.) with stirring. The acetate was filtered off and recrystallised from 96% ethanol; it had m. p. 144—145°, $[\alpha]_D^{22}$ −7·6° (*c* 1·05 in $CHCl_3$) (Found: C, 50·8; H, 6·4; Ac, 49·9. Calc. for $C_{26}H_{36}O_{17}$: C, 50·3; H, 5·9; Ac, 48·5%).

Periodate Oxidation of Disaccharide Glycitol.—To the disaccharide glycitol (0·1234 g.) in water (25 c.c.) was added 0·4M-sodium metaperiodate (5·0 c.c.), and the solution made up to 50 c.c. The progress of the reaction, which was complete in 24 hr. was followed by titration (0·01N-sodium hydroxide) of aliquot parts at regular intervals (Bromocresol Purple) [16] after destruction of excess of periodate with ethylene glycol. The periodate consumed was then determined in the usual way, and the formaldehyde was estimated on an aliquot portion as its

[13] Haworth, Jackson, and Smith, *J.*, 1940, 620.
[14] Araki and Arai, *J. Chem. Soc. Japan*, 1942, **63**, 1720; *Chem. Abs.*, 1947, **41**, 3765.
[15] Hockett, Conley, Yusem, and Mason, *J. Amer. Chem. Soc.*, 1946, **68**, 922.
[16] Hartman, *J. Appl. Chem.*, 1953, **3**, 308.

dimedone derivative,[17] m. p. and mixed m. p. 188—189° (Found: 0·95 mol. of formic acid, 0·75 mol. of formaldehyde produced, and 2·98 mol. of periodate consumed).

Methylated Polysaccharide.—The polysaccharide (15 g.) in hot water (150 c.c.) was treated with methyl sulphate (225 c.c.), and 30% sodium hydroxide solution (675 c.c.) was added during 7 hr. A little acetone was added to the mixture during methylation to help dissolve the methylated material. The mixture was heated at 80° for 30 min. and then neutralised with 50% sulphuric acid. The solution was filtered free from precipitated sodium sulphate, concentrated, and freed from more sodium sulphate by the addition of 60% ethanol. The methylation and concentration procedure was repeated three times.

The final product, in water, was dialysed against running tap-water for 10 days, then concentrated to a residue (2 g.). Those sodium sulphate precipitates which gave a positive Molisch test were also dissolved in water and dialysed against running tap-water for 10 days. The dialysate was concentrated to give a product (0·8 g.) which was combined with the main batch of methylated material (Found: OMe, 20·9; SO_4^{2-}, 18·3%).

The methylated polysaccharide (2·3 g.) in N-sulphuric acid (23 c.c.) was hydrolysed at 100° for 20 hr., neutralised with barium carbonate, and concentrated to a syrup (1·34 g.). Chromatography on paper in solvent (*a*) revealed the presence of 2 : 6-di-*O*-methylgalactose, R_{TMG} (relative to tetramethylglucose) 0·64, together with small amounts of mono-*O*-methylgalactose, R_{TMG} 0·41, tri-*O*-methylgalactose, R_{TMG} 0·77, and tetra-*O*-methylgalactose, R_{TMG} 0·90, and also a furfuraldehyde derivative just behind the solvent front. The syrup (1·3 g.) on a cellulose column (35 × 3·5 cm.) irrigated with butanol–water (9 : 1) yielded as main fraction a syrup (0·7 g.), R_{TMG} 0·64. This syrup crystallised from ethyl acetate, then having m. p. and mixed m. p. 128·5—130° with synthetic[18] 2 : 6-di-*O*-methyl-D-galactose, $[\alpha]_D$ +45° ⟶ +88° (*c* 0·8) (Found: C, 46·4; H, 7·9; OMe, 28·9. Calc. for $C_8H_{16}O_6$: C, 46·2; H, 7·75; OMe, 29·8%).

Methylation of Partially Degraded Methylated Polysaccharide.—The polysaccharide (11 g.) in water (70 c.c.) was methylated with methyl sulphate (150 c.c.) and 30% sodium hydroxide solution (450 c.c.) in the usual way. The reaction mixture was freed from excess of sodium sulphate, and the methylation procedure repeated a further six times. The final solution was dialysed against running water and concentrated, to yield a residue (6·6 g.) (Found: OMe, 21·4; SO_4^{2-}, 16·0%). This residue (6·2 g.) was shaken at 30° for 16 hr. with 1% methanolic hydrogen chloride (300 c.c.). After centrifugation to remove a trace of undissolved material, the clear solution was neutralised with silver carbonate and concentrated to a syrup (5·4 g.) (Found: OMe, 32·0; SO_4^{2-}, 7·7%). The syrup (5·0 g.) in dry methanol (10 c.c.) was twice methylated according to Purdie's method[19] with Drierite (1 g.), methyl iodide (30 c.c.), and silver oxide (30 g.) each time, to yield a syrup (3·4 g.), $[\alpha]_D^{28}$ +7° (*c* 1·2) (Found: OMe, 35·8; SO_4^{2-}, 7·4%).

The syrup (2·9 g.) was hydrolysed in N-sulphuric acid at 95° for 16 hr. and the solution neutralised with barium carbonate and concentrated to a syrup (2·1 g.). The latter was fractionated on a cellulose column (35 × 3·5 cm.) with butanol–light petroleum[12] (b. p. 100—120°) (2 : 3) to yield two fractions.

Fraction I (0·4 g.), a syrup, identical with tetra-*O*-methylgalactose on chromatograms, was treated with aniline to yield a derivative, m. p. and mixed m. p. 189—190° with 2 : 3 : 4 : 6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine.

Fraction II (0·35 g.), a syrup, was a mixture of tri- and tetra-*O*-methylgalactose (paper chromatograms). These were resolved by chromatography on large sheets of paper (Whatman No. 20) in solvent (*a*). The tri-*O*-methylgalactose fraction (0·2 g.) was heated with aniline in the usual way, to yield a product, m. p. 170·5—171·5° after recrystallisation from ethanol, $[\alpha]_D^{20}$ −80° ⟶ +38·2° (*c* 1·1 in acetone). Hirst and Jones[20] reported m. p. 179° (sometimes 169°), $[\alpha]_D$ −92° ⟶ +38° (in acetone), for 2 : 4 : 6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine. None of this was available for comparison, but our specimen depressed the m. p. of 2 : 3 : 4-tri-*O*-methyl-*N*-phenyl-D-galactosylamine (m. p. 163—164°) supplied by Dr. A. M. Stephen.

After fraction II had been removed the column was eluted with ethanol, the solution concentrated, and the resulting syrup resolved on a large paper chromatogram in solvent (*a*). Apart from some further tri-*O*-methyl-D-galactose this yielded 2 : 6-di-*O*-methyl-D-galactose

[17] Reeves, *J. Amer. Chem. Soc.*, 1941, **63**, 1477.
[18] Bell, *J.*, 1945, 692.
[19] Purdie and Irvine, *J.*, 1903, 1021.
[20] Hirst and Jones, *J.*, 1939, 1482.

(0·09 g.) which, crystallised from ethyl acetate, had m. p. and mixed m. p. 128·5—130°. A very small quantity of a mono-*O*-methylgalactose, R_{TMG} 0·36, was also obtained.

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